Caudal-related Homeobox (Cdx) Protein-dependent Integration of Canonical Wnt Signaling on Paired-box 3 (Pax3) Neural Crest Enhancer*□

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Background: Cdx transcription factors are known to convey the posteriorizing signals from the canonical Wnt pathway.

Results: Cdx proteins integrate canonical Wnt signals on a Pax3 neural crest enhancer.

Significance: Our data suggest that Cdx proteins are important novel players within the neural crest gene regulatory network.

One of the earliest events in neural crest development takes place at the neural plate border and consists in the induction of Pax3 expression by posteriorizing Wnt/β-catenin signaling. The molecular mechanism of this regulation is not well understood, but several observations suggest a role for posteriorizing Cdx transcription factors (Cdx1/2/4) in this process. Cdx genes are known as integrators of posteriorizing signals from Wnt, retinoic acid, and FGF pathways. In this work, we report that Wnt-mediated regulation of murine Pax3 expression is indirect and involves Cdx proteins as intermediates. We show that Pax3 transcripts co-localize with Cdx proteins in the posterior neuroectoderm and that neural Pax3 expression is reduced in Cdx1-null embryos. Using Wnt3a-treated P19 cells and neural crest-derived Neuro2a cells, we demonstrate that Pax3 expression is induced by the Wnt-Cdx pathway. Co-transfection analyses, electrophoretic mobility shift assays, chromatin immunoprecipitation, and transgenic studies further indicate that Cdx proteins operate via direct binding to an evolutionarily conserved neural crest enhancer of the Pax3 proximal promoter. Taken together, these results suggest a novel neural function for Cdx proteins within the gene regulatory network controlling neural crest development.

Pax3 is a paired-box homeodomain transcription factor essential for normal neural crest (NC), 5 neural tube (NT) and skeletal muscle development. In mice, homozygous Pax3 loss of function, as seen in Splotch (Sp) mutants, leads to early embryonic lethality due to its role in NC cells (NCC). Although Pax3+/−/Sp mice only display pigmentation anomalies (white belly spot), most Pax3+/−/Sp embryos die around embryonic day 14.0 (e14.0) due to a severe NC defect leading to lack of outflow tract septation and heart failure (1). Pax3+/−/Sp mice also display other severe anomalies, including spinal ganglia malformations, intestinal aganglionosis, and posterior NT defects (spina bifida) (2). At the molecular level, Pax3 plays a critical role in the gene regulatory network controlling NCC development downstream of canonical Wnt signals. Together with members of the Msx, Dlx, and Zic families, Pax3 specifies the neural plate border and promotes induction of NCC (3–5). At later stages, Pax3 also controls survival of dorsal NT progenitors through stimulation of p53 degradation (6, 7). In humans, heterozygous Pax3 mutations have been associated with Waardenburg syndrome, which is characterized by NC defects such as cranio-facial and pigimentary anomalies (8–10).

Neural Pax3 expression begins at the early somite stage (around e8.25) prior to initiation of NT closure. At this stage, Pax3 transcripts are detected on the lateral borders of both the anterior neural plate and posterior neural plate (PNP) (11). Following neural plate bending in a closed NT (from e8.5 onward), Pax3 transcripts are then detected in the dorsal neuroectoderm, including pre-migratory NCC, in an almost continuous manner along the anterior-posterior (AP) axis. Indeed, strong Pax3 expression is detected in two large domains extending 1) from the forebrain down to rhombomere 4 and 2) from rhombomere 6 down to the rostral half of the PNP. Although detectable, Pax3 expression is clearly much weaker in rhombomere 5. At later stages, caudal Pax3 expression follows progression of posterior elongation and is maintained in the dorsal half of the closed neural tube until e14.5. Pax3 expression is also detected in a subset of migratory NCC contributing to the cardiac outflow tract, peripheral, and enteric nervous systems as well as in melanocytes but is generally down-regulated as NCC differentiate.

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5 The abbreviations used are: NC, neural crest; NT, neural tube; NCC, NC cell(s); PNP, posterior neural plate; NCE, neural crest enhancer; RA, retinoic acid; CdxBS, Cdx binding site; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; CHX, cycloheximide; W3a, Wnt3a-conditioned medium; Ctrl, control-conditioned medium; en, embryonic day n.
Regulation of Pax3 Expression by the Wnt-Cdx Pathway

Regulatory sequences sufficient to mediate induction and dorsal restriction of Pax3 expression in the hindbrain and trunk are contained in the proximal 1.6-kb promoter (12). Deletion analysis of this promoter has revealed that a block of 674 bp containing two evolutionarily conserved regions of ∼250 bp, called neural crest enhancer 1 and 2 (NCE1 and -2), is sufficient to drive expression in the dorsal NT and NCC (13). NCE1 bears Pbx binding sites (activated by Pbx1-containing transcriptional complexes), which appear to be specifically required to control Pax3 expression in the hindbrain (14, 15). NCE2 contains a Tead binding site that was shown to be required for the activity of the whole 674-bp NCE in e10.5 transgenic embryos (13). In addition, both NCE1 and NCE2 contain a binding site for Pou class III members, and mutation of these sites leads to reduced NT activity of the 1.6-kb promoter in e9.5 transgenic embryos (15). On the other hand, Pax3 expression is induced by posteriorizing Wnt signals and dorsally restricted in response to dorso-ventral patterning signals, such as Sonic Hedgehog (Shh) (5, 16–20). However, how the canonical Wnt and Shh signals are integrated at the transcriptional level is still unclear.

The vertebrate Cdx genes (Cdx1, Cdx2, and Cdx4) are related to Drosophila caudal (cad) (21), and their gene products have conserved the ancestral ability to specify the posterior embryo and pattern the AP axis. Murine Cdx genes are sequentially activated in ectodermal and mesodermal cells of the primitive streak around e7.25, with Cdx1 activated first and Cdx4 activated last (22–24). At e8.5, all Cdx genes are expressed in the caudal embryo and form a nested set along the AP axis. Whereas Cdx1 and Cdx2 exhibit an almost perfect overlap in expression around the hindbrain/spinal cord boundary, Cdx4 is expressed slightly more posterior at this stage. The most anterior domain of Cdx expression appears to be restricted to the dorsal NT, with Cdx1 protein expressed in NCC emigrating from this domain (23, 25). This anterior limit of expression regresses concomitantly with axial elongation, persisting in the caudal embryo until e10.5 for Cdx1 and Cdx4 and until e12.5 for Cdx2. All three Cdx genes are also expressed in the developing hindgut epithelium with Cdx1 and Cdx2 expression maintained postnatally (26).

Cdx gene expression is regulated by posteriorizing signals from Wnt, retinoic acid (RA), and fibroblast growth factor (FGF) pathways in multiple species (27–38). Among these posteriorizing signals, the evolutionarily conserved role of the canonical Wnt pathway in Cdx regulation is the best characterized. Indeed, both Cdx1 and Cdx4 have been clearly identified as direct targets of the Wnt-β-catenin pathway (27, 29, 30). Moreover, other data suggest that Cdx2 is also responsive to canonical Wnt signals, although evidence for a direct regulation is sparse (39–45). In addition, Cdx proteins can interact with the Lef1-β-catenin transcriptional effector of the canonical Wnt pathway (46).

Our understanding of Cdx function has long been hampered by the functional redundancy between Cdx members and the vital role of Cdx2 during implantation (47, 48). Recent development of a conditional Cdx2 allele (49, 50) and analysis of Cdx double mutants (51–54) has demonstrated important regulatory roles for Cdx proteins in several processes during mouse embryogenesis. In the mesoderm, Cdx proteins regulate axial patterning, axial elongation, and somitogenesis in addition to placentogenesis and hematopoiesis. An important part of the Cdx function is executed through direct regulation of diverse Hox genes (28, 51–53, 55, 56). However, recent work has shown that a significant proportion of the Cdx function is also fulfilled via direct regulation of several non-Hox targets (49, 57). In the endoderm, Cdx proteins are involved in intestinal patterning and cell differentiation via Hox-dependent and -independent mechanisms (50, 58–61).

The function of Cdx members in the neur Crest is less well understood. As in the mesoderm and endoderm, Cdx proteins control neur ectoderm AP patterning via Hox-dependent and -independent mechanisms (37, 43, 62–64). Multiple gain- or loss-of-function mouse models have also demonstrated that Cdx proteins are important for proper formation of the NT and NC-derived spinal ganglia, but whether they do so in a tissue-autonomous or -non-autonomous (via the mesoderm) manner remains an open question (53, 54, 65–67). Nevertheless, analysis of neur ectoderm–specific Cdx loss of function in ascidian embryos demonstrates that Cdx proteins may impact NT and NC formation in a tissue-autonomous manner. Indeed, such mutant embryos display NT defects as well as an absence of pigment cells, which are derived from NC-like cells (68, 69).

In this study, we investigated the possibility that Cdx proteins might convey the posteriorizing Wnt signals to Pax3. We found that murine Pax3 is in fact an indirect Wnt target and that Cdx proteins can directly activate neural Pax3 expression at least via the previously described NCE2 (13). Altogether, our data strengthen the idea that Cdx members are involved in both NT and NC development in a tissue-autonomous manner downstream of Wnt signals.

EXPERIMENTAL PROCEDURES

Ethics Statement—Experiments involving mice were performed following Canadian Council of Animal Care guidelines for the care and manipulation of animals used in medical research. Protocols involving the manipulation of animals were approved by the institutional ethics committee of the University of Quebec at Montreal (Comité Institutionnel de Protection des Animaux; reference number 0511-R2-648-0512).

Generation and Analysis of Mice—Cdx1−/− mutant mice (56) were kindly provided by D. Lohnes (University of Ottawa, Canada). Pax3+/Sp mice were obtained from Jackson Laboratories (Bar Harbor, ME). Cdx1+−/Pax3+−/Sp mutants were generated by Pax3+/Sp and Cdx1−/− intercrosses. Cdx1−/−Pax3+/Sp mutants were then generated by crossing Cdx1+−/Pax3+/Sp and Cdx1−/− mice.

Transgenes carrying the lacZ gene under control of the Hsp68 minimal promoter (70) and either a wild type or Cdx binding site (CdxBS)-mutated NCE2 were prepared, and transgenic embryos were generated from injected B6C3 oocytes according to standard techniques (71). In order to facilitate identification of transgenic embryos and provide a positive control for transgene expression, a previously described Gata4p-GFP transgene (72) was co-injected with each lacZ construct. Nine days after microinjection, foster mothers were sacrificed, and embryos were collected and individually analyzed for GFP and β-galactosidase activity. For X-gal staining,
embryos were fixed in 2% paraformaldehyde for 15 min, washed twice with PBS, and incubated overnight in staining solution (5 mm K₃Fe(CN)₆, 5 mm K₄Fe(CN)₆, 2 mm MgCl₂, 0.01% Nonidet P-40, 0.01% sodium deoxycholate in PBS) containing 1 mg/ml X-gal. Staining reactions were carried out overnight at 37 °C.

Whole-mount in situ hybridization and immunohistochemistry were performed as previously described (29, 48). Mice were mated overnight, and noon of the day of vaginal plug detection was considered as e0.5. Embryos to be compared were stage-matched according to established criteria (73) and processed in parallel. The probe for in situ hybridization of Pax3 mRNA was generated from a previously described plasmid (11), kindly provided by J. Epstein. Immunohistochemistry was performed using polyclonal antisera to Cdx1 and Cdx2 (48). All embryo images were taken with a Leica DFC 495 camera mounted on a Leica M205 FA stereomicroscope (Leica Microsystems Canada).

Chromatin Immunoprecipitation (ChIP) Analysis—ChIP assays in Neuro2a (N2a) cells were performed as described previously (29) using anti-FLAG M2 antibody (Sigma). PCR amplifications were performed with GoTaq DNA polymerase (Promega) and consisted of 30 cycles of 30 s at 96 °C, 30 s at 60 °C, and 30 s at 72 °C. The primers used for this study were Pax3 NCE2 Forward (5’-GGCACAATGTTACCTTCTCTAAGG) and Reverse (5’-AAGCTTCCCTTGAGAAAGCGGGGACTTTAAA) and Pax3 exon 7 Forward (5’-CCGTGTACGATCCAGTAGC) and Reverse (5’-CTGAGGTGAAAGGCC-ATTGCCG). PCR products were resolved on a 1.5% agarose gel.

Electrophoretic Mobility Shift Assays (EMSA)—Pax3 NCE2 was scanned by using eight double-stranded end-labeled oligonucleotides and tested for Cdx binding by EMA. Five micromolars of nuclear extracts from mock- or FLAG-Cdx1-transfected COS7 cells were used in each reaction as described previously (29). Supershifts were performed using 1 μg of anti-FLAG M2 antibody (Sigma). Specificity of binding was assessed by competition with a 100-fold excess of unlabeled WT or mutated cold probes. The oligonucleotides comprising mutated CdxBS1 and CdxBS3 sequences were as described for site-directed mutagenesis. The upper strands of each wild type or mutated double-stranded probe as well as of a probe harboring a consensus Cdx binding site (as positive control) are summarized in supplemental Table S1.

Plasmid Constructs—For generation of the Engrailed-Cdx1 (EnRCdx1) fusion construct, the coding sequence of the Drosohila Engrailed repressor domain (74) was subcloned upstream of a PCR product corresponding to the DNA binding domain (homeodomain) of Cdx1 ended by a Stop codon (see supplemental Fig. S1). FLAG-tagged Cdx1 expression vector has been described previously (46). FLAG-tagged EnRCdx1 and EnR constructs were generated by subcloning the relevant sequences into a modified pCEP4 plasmid (Invitrogen) (46). Cdx1, Cdx2, Cdx4, EnR, and EnRCdx1 bicistronic expression vectors were generated by subcloning the respective cDNA into the pIRES2-EGFP vector (Clontech).

Pax3 proximal promoter sequences were obtained by PCR amplification in accordance with previous work (1, 13). Oligonucleotide sequences are available upon request. These PCR products were cloned in pGEM-T vector and validated by sequencing. Luciferase reporter constructs bearing the Pax3 minimal promoter with or without various lengths of the 1.6-kb 5’ upstream sequences were generated by subcloning these sequences into pXP2 (75).

Point mutations were introduced into each of the three CdxBS of NCE2 by using the QuikChange multisite-directed mutagenesis kit (Stratagene), according to the manufacturer’s instructions. Sequences of the oligonucleotides used for site-directed mutagenesis were as follows: CdxBS1, 5’-CAG-CAGTITTAGCTGAAATGCCCATTCAATCTCTCAGGA; CdxBS2, 5’-CTAGCAAGACGTGTTCTCTCTTTATTTTGACATTTTA; CdxBS3, 5’-AAGGACAGACATCTCAGA CAAACATCTGGGCCTCATATCC (point mutations are denoted in lowercase letters).

Cell Culture and Transfection Analysis—P19 cells were propagated in AMEM supplemented with 7.5% FBS and 2.5% NCS, N2a cells were propagated in MEM supplemented with 10% FBS, and COS7 cells were propagated in DMEM supplemented with 10% FBS. All transfections were performed using Genejuice reagents (Novagen) in accordance with the manufacturer’s instructions.

For time course analysis, P19 cells were seeded in 6-well plates (2 × 10⁵ cells/well), and the following day, standard culture medium was replaced by a Wnt3a- or a control-conditioned medium (29). Cells were harvested before treatment (t = 0 h) and 2, 4, 8, 12, or 18 h post-treatment; snap frozen; and stored at −80 °C prior to RT-PCR analysis. To assess the requirement for de novo protein synthesis, P19 cells were pretreated for 30 min with 30 μg/ml cycloheximide (CHX) or with the vehicle (DMSO) alone and then treated for 24 h with Wnt3a- or control-conditioned medium in the presence of 1 μg/ml CHX. Afterward, cells were harvested, snap frozen, and stored as described above prior to RT-PCR analysis. To inhibit Cdx function, P19 cells were seeded in 100-mm plates (2 × 10⁶ cells/plate) and transiently transfected with 6 μg of EnRCdx1-IRES-GFP or the negative control EnR-IRES-GFP expression vector. Thirty hours after transfection, cells were treated with Wnt3a- or control-conditioned medium and cultured for another 20 h. GFP-positive cells were then recovered by FACs and analyzed by RT-PCR.

To modulate Cdx activity in N2a cells, transient transfections with 6 μg of EnRCdx1-IRES-GFP, Cdx1-IRES-GFP, Cdx2-IRES-GFP, or Cdx4-IRES-GFP expression vectors were performed in 100-mm tissue culture plates (2 × 10⁶ cells/plate) and transiently transfected with 6 μg of EnRCdx1-IRES-GFP or the negative control EnR-IRES-GFP expression vector. Thirty hours after transfection, GFP-positive cells were recovered by FACs and analyzed by RT-PCR.

For luciferase reporter assays, N2a (8 × 10⁴ cells/well) and/or P19 (3 × 10⁴ cells/well) cells were transfected in 24-well plates with 100 ng of Pax3p-luciferase reporter construct alone or with increasing amounts (1.25–5 ng) of Cdx expression vectors or with the maximum amount of Cdx expression vector and increasing amounts (2.5–20 ng) of EnRCdx1 expression vector. When required, an empty expression vector was also included to ensure a total of 125 ng of DNA/well. For analysis of deleted or mutated Pax3p-luciferase reporter constructs, a fixed concentration (5 ng) of Cdx2 expression vector was used. All transfections were performed at least three times in triplicate. Forty-
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FIGURE 1. Cdx members and Pax3 are co-expressed in the caudal neurectoderm during the early steps of NT and NCC formation. A and B, whole-mount immunohistochemistry showing Cdx1 (A) and Cdx2 (B) protein distribution in e8.5 embryos. Note the overlap in the PNP and NT. C and D, higher magnification view of Cdx1 protein distribution and comparison with Pax3 gene expression domain as detected by whole-mount in situ hybridization. Note the overlap at the site of initiation of NT closure (arrow) and at the lateral PNP (arrowhead). E–H, comparison of Cdx2 (E and G) protein distribution with Pax3 (F and H) gene expression domain in the caudal end of e9.5 embryos (lateral views). For comparative purposes, the location of the last formed somite is indicated by an asterisk. The black arrowhead in E indicates the anterior limit of Cdx2 expression; note the overlap with the posterior domain of Pax3 gene expression (white arrowhead in F). The dotted lines in E and F indicate the level at which the transverse sections shown in G and H were cut. The Cdx2 protein is widely detected in the tailbud region (E), but this pattern becomes more restricted anteriorly, with notable strong detection in the whole NT (G). Note the overlapping detection of Cdx2 protein (G) and Pax3 mRNA (H) in the posterior NT.

eight hours after transfection, cells were disrupted in 100 μl of lysis buffer (0.1 M Tris (pH 8.0), 1% Igepal, 1 mM dithiothreitol) and assessed for luciferase activity with a Berthold LB9507 luminometer (Berthold Technologies).

RNA Extraction and RT-PCR Analysis—Total RNA was isolated from frozen cell pellets by using the RNeasy kit (Qiagen), and cDNA was synthesized using a poly(dT) oligonucleotide and Superscript II reverse transcriptase (Invitrogen) in accordance with manufacturer’s protocols. PCR amplifications were then performed with Platinum Taq DNA polymerase (Invitrogen) and consisted of 25–35 cycles of 30 s at 96 °C, 30 s at 60 °C, and 45 s at 68 °C. Amplified bands were size-fractioned on a 2% agarose gel. The primers used in this study were Cdx1 (forward, 5'-GCAAGTCCGAGCTGGCTGCTA; reverse, 5'-GGGTAG-AACTCTCTCCTTGACG); Cdx2 (forward, 5'-CCACACCTTG-GGCTCTCCGAGA; reverse, 5'-GGGTCACTGGGTGAC-AAGTTGAC); Cdx4 (forward, 5'-AGTTAACCTGGGCTTCCTT-GTA; reverse, 5'-ATTCAGAAACTATGACCTGCTGT); Pax3 (forward, 5'-CTGCGTACATCGGCTCTG; reverse, 5'-CTGAGGTGAGGAGGCATTGCCG); Gapdh (forward, 5'-TCCCGACCAACACTCTGCTAGG; reverse, 5'-AGTGTCACACCCCTGTGTCGA). All oligonucleotides were designed to encompass an intron allowing the detection of contaminating genomic DNA by the presence of a larger band. As an additional control, PCR was also performed with RNA that had not been reverse-transcribed. Statistical analysis was carried out using GraphPad Prism software version 5.0. For the comparison of groups, a paired Student’s t test was used (two-tailed p value, α = 0.05). Differences between means were classed as not significant (p > 0.05) or significant (*, p < 0.05).

RESULTS

Cdx Members and Pax3 Are Co-expressed in Caudal Neurectoderm—To validate our hypothesis that Cdx proteins are good candidates to induce Pax3 expression in the caudal neurectoderm, we compared the expression pattern of Cdx proteins and Pax3 mRNA. This analysis revealed extensive Cdx-Pax3 overlap in the caudal neurectoderm of e8.5 and e9.5 embryos (Fig. 1), in accordance with the well documented Cdx and Pax3 expression patterns (11, 22, 23). In e8.5 embryos, Pax3 transcripts are detected at both the PNP border and the site of initiation of NT closure (around the level of the fifth somite), where Cdx proteins are strongly expressed (Fig. 1, C and D). In e9.5 embryos, Pax3 and Cdx overlapping expression domains are displaced caudally in parallel with progression of axial elongation, NCC induction, and NT closure (Fig. 1, E–H). In brief, Cdx spatio-temporal expression patterns are consistent with a role in the induction of Pax3 expression in the caudal neurectoderm.

Pax3 Neural Expression Is Regulated by Cdx Proteins—To determine whether Pax3 is a Cdx target gene, we evaluated its expression in e8.5 Cdx1-null embryos. This analysis revealed that Pax3 expression is slightly reduced in the dorsal NT of 4–6-somite stage Cdx1−/− embryos (Fig. 2A). Our data also
We generated a FLAG-tagged dominant negative Cdx protein for expression of the Cdx neural target worthy that a similar transient effect has been recently reported in embryos (62). Taken together, this suggests that all Cdx proteins as well as Cdx2 and Cdx4 are known direct targets of Wnt signaling (77, 78). On the other hand, P19 cells have been used previously to study the regulation of the endogenous Pax3 promoter by Cdx because they co-express all three Cdx members as well as Pax3 (Fig. 2B). N2a cells were transiently transfected with expression constructs encoding Cdx1, Cdx2, Cdx4, or the dominant negative EnRCdx1, and endogenous Pax3 expression was evaluated by semiquantitative RT-PCR. As shown in Fig. 2C, Pax3 expression levels are significantly altered by modulation of Cdx activity in N2a cells. Overexpression of any of the three Cdx members results in a robust increase, whereas overexpression of EnRCdx1 leads to a complete knockdown of Pax3 expression. Therefore, these data indicate that Pax3 is a Cdx target gene in N2a cells.

Pax3 Is Induced by Wnt-Cdx Pathway in Undifferentiated P19 Cells—Previous studies have reported that neural Pax3 expression is induced by canonical Wnt signals (5, 16, 19, 20). To evaluate the potential contribution of Cdx proteins in this process, we performed a series of RT-PCR analyses in P19 embryocarcinoma cells cultured in the presence or absence of Wnt3a-conditioned medium, which is known for activating the canonical pathway (29, 76). The N2a cell line could not be used for this assay because the canonical Wnt pathway is constitutively activated in these cells due to Wnt7a autocrine regulation (77, 78). On the other hand, P19 cells have been used previously to study the regulation of Pax3 expression in the context of neural differentiation (12, 13, 15), and activation of the Wnt/β-catenin pathway (79). As shown in Fig. 3A, a time course analysis first revealed that expression of each Cdx member is rapidly induced (2–4 h) following treatment with Wnt3a-conditioned medium. Under the same conditions, Pax3 expression is also induced but considerably delayed (18 h). This delay being suggestive of an indirect regulation, we then directly assessed the necessity for an intermediary factor by inhibiting β-catenin signaling (79). As shown in Fig. 3B, expression of Cdx1 and Cdx4 induction by Wnt3a-conditioned medium is not affected by inhibition of protein synthesis (Fig. 3B). Interestingly, Cdx2 induction was also found to be independent of de novo protein synthesis, suggesting that Cdx2 is a direct target of Wnt-β-catenin signaling in this model. In marked contrast, Pax3 induction is blocked by treatment with CHX, demonstrating that a protein intermediary is needed to convey canonical Wnt signals to the Pax3 promoter (Fig. 3B). Of note, such an outcome is also supported by transient co-transfection assays indicating that Lef1-β-catenin complexes are very weak activators of the proximal 1.6-kb promoter of Pax3 in N2a or P19 cells (1.6-fold; data not shown).

To determine whether Cdx members act as intermediaries between canonical Wnt signals and Pax3, we knocked down the Cdx function in P19 cells using the EnRCdx1 dominant nega-
Regulation of Pax3 Expression by the Wnt-Cdx Pathway

Identification of Cdx Binding Sites in Proximal Pax3 Promoter—Previous studies have demonstrated that the proximal 1.6-kb promoter of Pax3 recapitulates endogenous posterior expression of Pax3 in the dorsal NT and NCC (12, 13). In order to understand the mechanism of Cdx-mediated regulation of Pax3 expression, we performed a series of co-transfection assays in N2a cells. We asked whether Cdx proteins transactivate the proximal 1.6-kb Pax3 promoter, we generated a luciferase reporter construct driven by these regulatory elements (Fig. 5A). Although each Cdx is equally expressed, Cdx2 elicits the strongest response (32-fold), followed by Cdx1 (11-fold), and Cdx4 is not.

To determine whether Cdx binding sites identified by EMSA are present on the endogenous Pax3 promoter, we performed ChIP-PCR analysis showing alteration of endogenous Pax3 expression levels were normalized against Gapdh expression. n.s., not significant.

EMSA was then used to verify whether putative Cdx binding sites (named CdxBS1, CdxBS2, and CdxBS3) contained in NCE2 can be directly bound by a Cdx protein. In order to rule out the possibility that other unpredicted Cdx binding sites might also exist, we first scanned the whole NCE2 sequences with eight overlapping double-stranded oligonucleotide probes (supplemental Fig. S2 and supplemental Table S1). Incubation of these probes with nuclear extracts from COS7 cells expressing FLAG-Cdx1 revealed that Cdx1 preferentially binds to probes bearing either CdxBS1 or CdxBS3. Cdx1 binding to the probe bearing CdxBS2 was very weak, whereas no binding was observed for the remaining probes. The presence of FLAG-Cdx1 in the complex formed with probes containing CdxBS1 or CdxBS3 was demonstrated by supershift with an anti-FLAG antibody (Fig. 5B). Moreover, specificity of Cdx binding to CdxBS1 and CdxBS3 was confirmed by the absence of competition with cold probes bearing point mutations in the predicted elements (Fig. 5B).

Cdx Binding Sites Are Essential for Pax3 NCE2 Activity—To determine whether Cdx binding sites identified by EMSA are described NCE1 and NCE2. To better define the sequence elements that mediate Cdx transactivation, a promoter deletion analysis was carried out in N2a cells, and Cdx2 was used to assay Cdx responsiveness. This assay revealed that the NCE2 region, which contains three putative Cdx binding sites, mediates most of Cdx2 transactivation (Fig. 4D). Accordingly, ChIP-PCR assays performed in N2a cells indicate that both FLAG-tagged Cdx1 and EnRCdx1 proteins are present on the endogenous Pax3 NCE2 (Fig. 4E).
functionally important for Pax3 NCE2 activity, we generated a series of NCE2-luciferase reporter constructs containing the mutated elements (alone or in combination) and evaluated their responsiveness to Cdx2 in co-transfection assays. In P19 cells, single and double mutations of CdxBS reduce Cdx2-mediated transactivation of the NCE2 reporter, whereas simultaneous mutation of all three CdxBS results in complete loss of Cdx2-mediated transactivation (Fig. 5C). In accordance with our EMSA data, this analysis also revealed that the contribution of CdxBS1 and CdxBS3 to NCE2 activity is more important than that of CdxBS2. Similar results were obtained in N2a cells, although it can be noted that mutation of CdxBS1 does not considerably inhibit the Cdx2 responsiveness of Pax3 NCE2 in these cells. Interestingly, it can also be noted that the order of transactivation of the wild type NCE2 reporter is higher in N2a (~14-fold) than in P19 cells (~6-fold) and that the triple mutant construct remains slightly more active in N2a than in P19 cells. These observations suggest that Cdx proteins, in addition to directly binding the Pax3 NCE2, might also be recruited to and/or stabilized on this enhancer via an interaction with a neuroectoderm-specific factor also required for strong activation.

To verify the importance of all three Cdx binding sites of Pax3 NCE2 in vivo, we generated lacZ reporter constructs driven by either wild type or mutated Pax3 NCE2 upstream of the Hsp68 minimal promoter and evaluated β-galactosidase activity in transient e9.5 transgenic embryos (Fig. 6). This analysis first revealed that this short enhancer of 245 bp is sufficient to recapitulate Pax3 expression in the dorsal NT and premigratory NCC of the caudal embryo (Fig. 6A; compare with Fig. 1H).

Importantly, we also found that point mutations in all three Cdx binding sites abolish NT and NCC expression of the NCE2 reporter (Fig. 6B). Of note, this lack of expression of the mutated NCE2 reporter is not due to transgene integration in a repressive chromatin region because expression of a co-injected Gata4p-GFP transgene is not affected (Fig. 6). Therefore, these results demonstrate that intact Cdx binding sites in Pax3 NCE2 are required to recapitulate caudal and dorsal neuroectoderm-specific expression of Pax3 in transgenic mice.

**DISCUSSION**

We presented data indicating that the dorsal NT/NCC marker Pax3 is a direct target of the Cdx proteins downstream of canonical Wnt signals. Cdx proteins convey canonical Wnt signals to the proximal Pax3 promoter through direct binding to Cdx binding sites located in the evolutionarily conserved NCE2. These Cdx binding sites are essential both for Cdx-mediated transactivation of NCE2 in cell culture experiments and for expression of a NCE2 reporter in the dorsal NT and NCC of e9.5 transgenic embryos, supporting the existence of the Wnt-Cdx-Pax3 pathway in vivo.

Wnt-mediated Induction of Pax3 Expression at Neural Plate Border—Although several studies have reported that Pax3 is a posterior Wnt-induced gene, very little is known regarding the possible mechanism of this regulation (5, 16, 19, 20). Our data now indicate that induction of murine Pax3 expression by canonical Wnt signals is indirect, involving Cdx proteins as intermediaries. In this regard, it is interesting to note that all three Cdx genes are direct targets of Wnt-β-catenin signaling in
FIGURE 5. Identification and characterization of Cdx binding sites in Pax3
NCE2. A, sequence comparisons of all three putative Cdx-binding sites
(CdxBS1, CdxBS2, and CdxBS3) relative to a consensus CdxBS sequence. Mis-
matches are denoted by lowercase letters. B, analysis of Cdx1 binding to
CdxBS1 and CdxBS3 via electrophoretic mobility shift assay. All in vitro bind-
ing reactions were performed in parallel under identical conditions. M, mock.
The presence of Cdx1 in the shifted bands was confirmed by the addition of 1
μg of anti-FLAG antibody to the binding reaction mix. Cdx1 binding and
anti-FLAG supershifts (SS) are indicated by arrows. A probe containing a con-
sensus CdxBS present in the Hoxb8 promoter was used as a positive control.
Specificity of Cdx1 binding was assessed by preincubation of FLAG-Cdx1-
containing nuclear extracts with a 100-fold excess of wild type (wt) or
mutated (mut) cold probes. Note that preincubation with wild type unlabeled
probes leads to inhibition of Cdx1 binding to the radiolabeled probes, whereas preincubation with mutated probes did not. C, impact of CdxBS
mutation on Cdx2-mediated activation of the NCE2 reporter in cell culture.
Wild type or CdxBS mutant versions of a Pax3NCE2-luciferase reporter were
generated and assessed for Cdx2-mediated transactivation in P19 and N2a
cells. Cells were transiently transfected with the NCE2-Luc reporter alone or
with a Cdx2 expression vector. The results (mean ± S.E. (error bars) of seven or
eight independent experiments performed in triplicate) are expressed as
fold induction compared with the relevant reporter vector alone. m1, m2,
and m3, Pax3NCE2-luciferase reporter constructs containing point mutations
in the CdxBS1, CdxBS2, and CdxBS3, respectively; m1,2, m1,3, and m2,3,
double mutations of CdxBS; m1,2,3, triple mutation. Note that concomitant muta-
tion of all three CdxBS is required to almost completely abolish the transacti-
vation of Pax3 NCE2 by Cdx2.

P19 cells. Although this was expected for Cdx1 and Cdx4 (27–30), such an outcome was somehow surprising for Cdx2, given a previous report indicating that exogenous Wnt3a can specifically induce Cdx1 but not Cdx2 in embryo culture (27). However, this result is in agreement with more recent work suggest-
ing that Cdx2 is also a direct target of canonical Wnt signals (39–42, 44).

Our data challenge a recent report showing that Pax3 expression in the dorsal NT can also be regulated by another evolutionarily conserved enhancer located in intron 4 (named ECR2) and described as containing multiple putative Lef/Tcf binding sites (81). Indeed, it was reported that mutation of these putative binding sites abrogates ECR2 activity in transgenic zebra-
fishes. However, this mutated transgene was not assayed in mice, and these putative binding sites were not shown to be bound by Lef/Tcf proteins. Because the consensus Lef/Tcf binding site exhibits rather low binding specificity for HMG-
box proteins, this raises the possibility that putative Lef/Tcf binding sites identified in Pax3 ECR2 are not bound by Lef/Tcf proteins but rather by other HMG-box proteins, such as Sox members (82–85). Alternatively, it is also possible that Pax3 is a direct Wnt target in zebrafish and not in mice. Regardless of the mechanism operating in other organisms, our CHX experiments indicate that murine Pax3 is an indirect Wnt tar-

FIGURE 6. Cdx binding sites are required for the activity of a Pax3NCE2-
lacZ reporter in transgenic embryos. A and B, whole mount β-galactosidase
staining performed in e9.5 transgenic embryos generated from the wild type
(Pax3NCE2-lacZ) (A) or mutated (Pax3NCE2mut-lacZ) transgene (B), the latter
carrying specific point mutations in all three CdxBS of NCE2. The dotted line in
A indicates where the transverse section shown in the inset was cut. C and D,
direct GFP fluorescence detection of a Gata4p-GFP transgene co-injected
with the wild type (C) or mutated transgene (D). Note that staining is detected
in the dorsal NT (arrow) of transgenic embryos generated from the wild type
construct (A). Mutation of all CdxBS abrogates β-galactosidase activity in the
dorsal NT (B), whereas detection of Gata4p-GFP is not affected (D). E, table indicating the total number of transgenic embryos obtained for each con-
struct as well as the number of embryos exhibiting β-galactosidase activity in the
dorsal NT.
Regulation of Pax3 Expression by the Wnt-Cdx Pathway

**Regulation of Pax3 Expression via NCE2**—We have shown for the first time that the Pax3 NCE2 alone is sufficient to recapitulate induction as well as dorsal restriction of Pax3 expression in the caudal NT. Taken together with previous work, this suggests that NCE2 is involved in the posterior whereas NCE1 is rather involved in the anterior expression of Pax3 (13–15). As summarized in Fig. 7, our *in vitro* and *in vivo* data further indicate that the activity of NCE2 is regulated by the posteriorizing Wnt-Cdx pathway. Given the broad distribution of Cdx proteins in the posterior neurectoderm, it is currently unclear how NCE2 exhibits dorsally restricted activity. As described previously for Pax3 expression, this could first be ensured by a repressive mechanism involving Shh signals emerging from the node, notochord, and floor plate (17), implying that Shh-responsive regulatory sequences are contained within NCE2. Such restricted activity of NCE2 might also be due to an interaction between Cdx proteins and a neural plate border co-factor. In this regard, our transfection assays in N2a cells have suggested that Cdx-mediated transactivation of NCE2 might rely in part on the presence of a neural factor. The identity of such co-factor is currently unknown, and it is most likely not a transcription factor previously reported to act on NCE2 (Tead2 and Brn1/2) (13, 15). Indeed, although both Tead2 and Brn1/2 have been implicated in the regulation of Pax3 expression, their expression pattern is not consistent with a role in the induction of Pax3 expression *in vivo*. On one hand, Tead2 and its co-factor YAP65 are almost ubiquitously expressed at e8.5–e9.5, becoming restricted to neural tissues only after e10.0 (89, 90). On the other hand, the proneural factors Brn1 and Brn2, as well as other Pou class III members Brn4 and Tst1/Oct6, are not expressed in the PNP (91–96). Thus, these observations strongly suggest that Tead2 and Brn1/2 are involved in the maintenance rather than induction of Pax3 expression. More work will obviously be required to better understand the regulatory mechanisms involved in the dorsal restriction of Pax3 expression, and our data indicate that at least some of them are operating via NCE2.

**Novel Function for Cdx Proteins in Caudal Neurectoderm Development**—Strong Cdx expression in the caudal neurectoderm is highly conserved across chordates. However, Cdx function in this lineage is poorly understood because of functional redundancy. Until recently, Cdx proteins were mostly known for their evolutionarily conserved role in the control of neural AP patterning via Hox-dependent mechanisms (37, 64, 67, 97). Cdx proteins are now also known to regulate neural AP patterning via Hox-independent mechanisms in different species (62, 63). More recently, Cdx1-Cdx2 double knock-out mice were generated and revealed a novel redundant role for Cdx members in the control of NT closure (54). This work showed that Cdx proteins regulate the planar cell polarity gene Ptk7 and further suggested that Cdx members are involved in the regulation of convergent extension movements in the caudal embryo. This analysis involved a conditional mutagenesis approach to circumvent the peri-implantation lethality associated with the Cdx2 null mutation, via a CMV-β-actin-CreERT2 transgene and a floxed allele of Cdx2. Thus, the Cdx function was lost in all three germ layers, and it is uncertain whether this novel Cdx role in neurulation is tissue-autonomous, -non-autonomous, or a combination of both. Ptk7 loss of function in *Xenopus* appears to affect convergent extension of the neuractoderm (98), but this has not been reported in mice. Indeed, although Ptk7−/− mouse mutants have been shown to have defective convergent extension movements in the mesoderm, an analysis of the neurectoderm has not been reported (99). Therefore, more detailed analysis of Cdx1-Cdx2 double knock-out animals and conditional approaches involving tissue-specific loss of function will be required to clarify the Cdx-dependent processes involved in NT formation.

On the other hand, our work now suggests that Cdx proteins may impact caudal neurectoderm development in a tissue-autonomous manner.
Regulation of Pax3 Expression by the Wnt-Cdx Pathway

tonomous manner, at least via the regulation of Pax3 expression. As evidenced by the severe NC and NT defects observed in Pax3<sup>Sp/Sp</sup> mutants, Pax3 plays a crucial role in the neuroectoderm (1, 2). Pax3 is important for NCC induction, and analysis of Pax3-deficient embryos has indicated that NC defects are due to a marked reduction in the number of NCC that emigrate from the NT at cranial levels and a progressive complete loss of NCC at more caudal levels (100–102). This progressive increase in the severity of NC defects along the AP axis reflects the cranial co-expression of the functionally redundant Pax7 (103). Pax3 is also important for NT closure, being required for the survival of dorsal progenitors via down-regulation of p53 activity (6, 7). Thus, by acting upstream of Pax3, the canonical Wnt-Cdx pathway might control cell specification and maintenance of progenitor populations required for proper NC and NT development.

A role for Cdx proteins in NC development has not been formally reported in any species. This is most likely because such a role is masked by functional redundancy and/or the presence of very severe posterior truncation phenotypes in Cdx compound mutants. However, several observations are in agreement with a role for Cdx proteins in NC development as well as the conservation of this role through evolution. In mice, an analysis of NCC in Cdx1-Cdx2 double knockouts has not been reported, but Cdx<sup>1−/−Cdx2<sup>+/−</sup> mutants are known to display abnormal and fused dorsal root ganglia (53). In zebrafish, Cdx loss of function leads to a reduced number of Rohon-Beard cells (which share a common precursor with NCC) and the absence of NC-derived spinal nerve roots (63, 104). In ascidian, Cdx loss of function results in the absence of pigment cells, which are derived from NC-like cells (68, 69). Future work focusing on a more detailed characterization of compound mutants or tissue-specific loss-of-function studies should help validate these observations and confirm a role for Cdx proteins in NC development.

In conclusion, our work suggests that Cdx proteins occupy a strategic position between canonical Wnt signals and Pax3 at the beginning of the gene regulatory cascade controlling NCC development. Because Cdx genes are not expressed in the anterior neuroectoderm, the Wnt-Cdx pathway cannot impact cranial NC induction. Therefore, our data are in accordance with the general idea that NCC are intrinsically different along the AP axis (105–108) and strongly suggest that these differences are already in place during induction of NCC.

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