Development 136, 4099-4110 (2009) doi:10.1242/dev.041582

Cdx2 regulation of posterior development through non-Hox targets

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The homeodomain transcription factors Cdx1, Cdx2 and Cdx4 play essential roles in anteroposterior vertebral patterning through regulation of Hox gene expression. Cdx2 is also expressed in the trophectoderm commencing at E3.5 and plays an essential role in implantation, thus precluding assessment of the cognate-null phenotype at later stages. *Cdx2* homozygous null embryos generated by tetraploid aggregation exhibit an axial truncation indicative of a role for Cdx2 in elaborating the posterior embryo through unknown mechanisms. To better understand such roles, we developed a conditional *Cdx2* floxed allele in mice and effected temporal inactivation at post-implantation stages using a tamoxifen-inducible Cre. This approach yielded embryos that were devoid of detectable Cdx2 protein and exhibited the axial truncation phenotype predicted from previous studies. This phenotype was associated with attenuated expression of genes encoding several key players in axial elongation, including *Fgf8*, *T*, *Wnt3a* and *Cyp26a1*, and we present data suggesting that *T*, *Wnt3a* and *Cyp26a1* are direct Cdx2 targets. We propose a model wherein Cdx2 functions as an integrator of caudalizing information by coordinating axial elongation and somite patterning through Hox-independent and -dependent pathways, respectively.

KEY WORDS: Cdx, Vertebral patterning, Axis elongation, Brachyury, Wnt3a, Retinoic acid, CYP26a1, Mouse

INTRODUCTION

The vertebrate Cdx family, *Cdx1*, *Cdx2* and *Cdx4*, encodes homeodomain transcription factors related to the *Drosophila* gene *caudal* (*cad*). Beginning at the late primitive streak stage [embryonic day 7.5 (E7.5)], Cdx genes are sequentially activated leading to a nested expression set in all germ layers of the caudal embryo. *Cdx1* transcripts are first observed at E7.5 in the primitive streak region, in the ectoderm and the nascent mesoderm with an anterior limit in the posterior hindbrain (Meyer and Gruss, 1993). *Cdx2* exhibits an early onset of expression in the extra-embryonic trophectoderm at E3.5, with expression persisting in the placenta through to at least E12.5 (Beck et al., 1995; Strumpf et al., 2005). The expression of *Cdx2* in the embryo proper initiates at E8.5 in all germ layers of the posterior embryo, extending caudally into the base of the allantois and rostrally into the posterior neural plate, hindgut endoderm and unsegmented paraxial mesoderm. The expression of *Cdx2* continues into the tail bud, the posterior neural plate and the endoderm, and is eventually confined to the hindgut endoderm located posterior to the foregut/midgut junction from E12.5 onwards, and perdures in the adult intestinal epithelium (Beck et al., 1995). *Cdx4* is initially detected at E7.5 in the allantois and posterior tip of the primitive streak and subsequently in the paraxial mesoderm, with a rostral limit posterior to the most recently formed somite. *Cdx4* is also found in the neural ectoderm, with expression slightly more rostral than its expression in the paraxial mesoderm, and is extinguished by E10.5 (Gamer and Wright, 1993).

In *Drosophila*, *cad* is involved in the patterning of the early embryo as well as in the specification of posterior embryonic structures (Mlodzik et al., 1985; Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987; Kispert et al., 1994). Analysis of Cdx loss-of-function mutant mice reveals that, like *cad*, murine Cdx gene products are involved in anteroposterior (AP) patterning (Subramanian et al., 1995; van den Akker et al., 2002; Chawengsaksophak et al., 2004; van Nes et al., 2006). *Cdx1*$^{-/-}$ mice are viable and exhibit homeosis of cervical and anterior thoracic vertebrae (Subramanian et al., 1995). *Cdx2*$^{-/-}$ mutants die at E3.5 owing to implantation failure, whereas *Cdx2* heterozygous offspring exhibit homeosis affecting the posterior cervical and thoracic vertebral elements, consistent with its later onset of expression relative to *Cdx1* (Chawengsaksophak et al., 1997). *Cdx2* heterozygotes also exhibit defects that impact on the elongation of the AP axis, manifested as a foreshortened tail (Chawengsaksophak et al., 1997), a phenotype that is exacerbated in *Cdx2*-null embryos generated by tetraploid aggregation (Chawengsaksophak et al., 2004). Finally, *Cdx4*-null mutants are phenotypically normal; however, loss of Cdx4 function synergizes with loss of either Cdx1 or Cdx2 (van Nes et al., 2006). This latter finding is suggestive of functional overlap, consistent with the observation of increased vertebral homeosis and axial foreshortening in *Cdx1*$^{-/-}$ *Cdx2*$^{+/-}$ offspring (van den Akker et al., 2002), and the finding that Cdx2 can fully compensate for loss of Cdx1 in vertebral patterning (Savory et al., 2009).

The correlation between the loss of functional Cdx alleles and the severity of vertebral homeosis suggests that Cdx proteins participate in a common pathway, and it is now established that Cdx proteins affect AP patterning through the regulation of cohorts of Hox genes (Shashikant et al., 1995; Subramanian et al., 1995; Epstein et al., 1997; Isaacs et al., 1998; van den Akker et al., 2002; Houle et al., 2003; Chawengsaksophak et al., 2004; Tabaries et al., 2005; Pilon et al., 2007). The mechanism by which Cdx members affect axial elongation is, however, less well understood, and a better understanding of this is hampered by the peri-implantation lethality of *Cdx2*-null mutants. To circumvent this, we used the Cre-loxP

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Accepted 29 September 2009

system to derive a conditional null allele. In agreement with previous studies (Chawengsaksophak et al., 2004), loss of Cdx2 in the post-implantation embryo resulted in axial truncation posterior to the forelimb. This axial truncation, together with the nature of many of the genes impacted by Cdx2 loss, suggested that precocious cessation of the generation of presomitic mesoderm (PSM) is the primary basis for this phenotype. To further define the role of Cdx2 and to elucidate the mechanistic basis for this phenotype, we sought to determine whether any of the affected genes were direct Cdx2 targets. Chromatin immunoprecipitation (ChIP) demonstrated occupancy of the *Wnt3a*, *Cyp26a1* and *T* promoters by Cdx2 in vivo. Moreover, all of these promoters harbor functional Cdx response elements (CDREs) as determined by transfection analysis or transgenic assays. Thus, Cdx2 directly regulates the expression of multiple players essential for the development of the posterior embryo. Taken together with previous work, these findings suggest that Cdx2 is required to couple the generation of paraxial mesoderm through multiple Hox-independent mechanisms with Hox-dependent AP vertebral patterning.

MATERIALS AND METHODS

Gene targeting and the generation of *Cdx2*$^{-/-}$ mutants

A 5 kb fragment of genomic *Cdx2* sequence encompassing the first intron through to the 3′ UTR was subcloned into pBluescript II KS^{+}. A floxed thymidine kinase/neomycin resistance cassette (*loxPGK-TK-Neolox*) (Iulianella and Lohnes, 2002) was cloned into the *Bgl*II site in intron 1, and a single loxP site was inserted into the *Nru*I site in intron 2, generating the targeting vector (see Fig. S1A in the supplementary material). R1 embryonic stem cells were electroporated with 30 μg linearized targeting vector and selected with G418 (180 μg/ml) for 10 days. Surviving clones were isolated and assessed for homologous recombination by genomic Southern blot using probes 5′ or 3′ to the targeting sequences (see Fig. S1 in the supplementary material). Two positive ES clones were transiently transfected with a *Cre* expression vector to remove the *loxPGK-TK-Neolox* sequences and recombined clones, identified by PCR and Southern blot analyses (see Fig. S1 in the supplementary material; data not shown) were used to generate germline chimeras by injection into C57BL/6 blastocysts. F1 *Cdx2*$^{+/f}$ offspring were subsequently intercrossed to generate the *Cdx2*$^{f/f}$ line, or crossed with the *CMV*-β-*actin-Cre-ERT2* (*Cre-ERT2*) transgenic line, as described previously (Santagati et al., 2005), to yield *Cdx2*$^{+/f}$*; Cre-ERT2* offspring. *Cdx2*$^{f/f}$*; Cre-ERT2* males, derived from the appropriate backcross, were subsequently mated with *Cdx2*$^{f/f}$ females, and pregnant females were dosed with 2 mg tamoxifen by oral gavage at E5.5. Embryos were subsequently harvested at E8.5-E10.5 for analysis.

Embryo collection and analysis

Animals were mated overnight, and noon on the day of detection of a vaginal plug was considered as E0.5. Pregnant females were treated with tamoxifen as described above, embryos were dissected in PBS, yolk sacs were collected for genotyping and embryos were fixed overnight in 4% paraformaldehyde at 4°C. Wholemount in situ hybridization was performed as described previously (Houle et al., 2000; Pilon et al., 2007). Probes for in situ hybridization were generated from previously described plasmids: *Wnt3a* (Takada et al., 1994), *T* (Wilkinson et al., 1990), *Fgf8* (Crossley and Martin, 1995), *Tbx6* (Chapman et al., 1996), *Sox2* (Kamachi et al., 1998), *Mox1* (ATCC EST, IMAGE: 3984366; *Meox1* – Mouse Genome Informatics), *Paraxis* (ATCC EST, IMAGE: 5143248), *Raldh2* (Mic et al., 2002), *Wnt5a* (Gavin et al., 1990), *Uncx4.1* (Mansouri et al., 1997), *Dll1* (Hrabe de Angelis et al., 1997) and *Cyp26a1* (Abu-Abed et al., 1998). A probe against mesogenin 1 was derived by RT-PCR using the primers described in Table S1 in the supplementary material.

Wholemount immunohistochemistry was performed as previously described (Savory et al., 2009). For in situ hybridization and immunohistochemistry, embryos to be compared were processed in parallel to control for variations in signal intensity and were stage-matched according to established criteria.

Retinoic acid signaling

Mice were crossed to the *RARE-lacZ* reporter (*Rare1* – Mouse Genome Informatics) (Rossant et al., 1991) and wholemount X-Gal staining was performed by standard methods.

Skeletal preparation

Pregnant females from matings between *Cdx2*$^{+/-}$ and wild-type mice were treated with a single dose of RA (10 or 100 mg RA/kg body weight) by oral gavage at E8.5. Wholemount skeletal preparations were subsequently prepared from E18.5 fetuses as previously described (Allan et al., 2001).

Identification of putative CDREs

Sequences were analyzed for potential Cdx response elements (CDREs) using the Transcription Element Search System (TESS; http://www.cbil.upenn.edu/cbi-bin/tess/tess).

Chromatin immunoprecipitation (ChIP) analysis

ChIP assays on embryos were performed as described previously using polyclonal antisera to Cdx2 (Pilon et al., 2006). Rabbit pre-immune serum or an anti-Gal4 antibody (Santa Cruz) were employed as controls. PCR was performed using primers (see Table S1 in the supplementary material) flanking the putative CDREs.

Electrophoretic mobility shift assay (EMSA)

GST or GST-Cdx2 was used to assess binding to putative CDREs as previously described (Houle et al., 2000; Pilon et al., 2006). Sequences of the upper strands of the wild-type and mutant double-stranded probes are listed in Table S1 in the supplementary material.

Cell culture and transfection analysis

Cdx expression vectors have been described previously (Béland et al., 2004). The 1.1 and 2 kb 5′ flanking sequences from *Wnt3a* and *Cyp26a1*, respectively, were PCR amplified from mouse genomic DNA, verified by sequencing and subcloned into pXP2 (Nordeen, 1988) to generate luciferase-based reporter constructs. Putative CDREs were mutagenized using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's directions.

Transfection analyses were performed as previously described (Pilon et al., 2006). DNA mixes comprised 1.0 μg luciferase reporter construct, 0.25 μg Cdx expression vector (where appropriate) and/or empty expression vector to a total of 2 μg of DNA per transfection.

Semi-quantitative RT-PCR

Semi quantitative RT-PCR was performed as previously described (Savory et al., 2009) using the primers listed in Table S1 in the supplementary material.

Generation of transgenic embryos

Sequences encompassing the 5′ proximal ~500 bp region of the mouse T promoter, previously shown to recapitulate *T* expression in nascent mesoderm (Clements et al., 1996; Yamaguchi et al., 1999b), were cloned into p610ZAL (Kothary et al., 1992) to generate a *lacZ* reporter. The three putative CDREs were mutagenized using the QuikChange Site-Directed Mutagenesis Kit. Transgenes were purified by agarose gel electrophroresis after digestion with *Not*I and pronuclear microinjection was performed according to established procedures (Nagy et al., 2003). Embryos were harvested at E8.5, then fixed and stained for β-galactosidose by standard means. The yolk sacs were subjected to PCR analysis using primers against *lacZ* to identify transgenic progeny.

RESULTS

Generation of a *Cdx2* conditional null allele

Cdx2 homozygous null mutants die around E4.5 owing to implantation failure (Chawengsaksophak et al., 1997; Tamai et al., 1999). To circumvent this early lethality, we generated a conditional allele in which exon 2 of the *Cdx2* locus (which encodes most of the DNA binding homeodomain) was flanked by loxP sites (see Fig. S1 in the supplementary material). Targeted clones were used to generate a *Cdx2* floxed line, which was then crossed to the *CMV*-β-

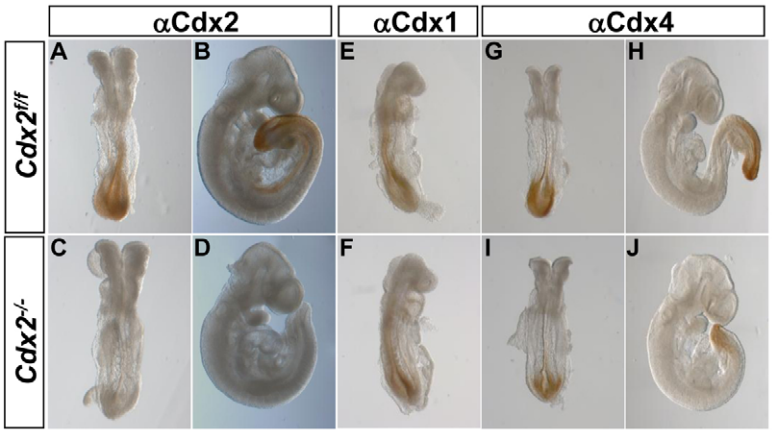

Fig. 1. Cdx protein in *Cdx2* conditional null mutants. (**A-J**) Wholemount immunohistochemistry of control and mutant embryos using Cdx1, Cdx2 or Cdx4 antibodies. E8.5 (A,C,E-G,I) and E9.5 (B,D,H,J) wild-type (A,B,E,G,H) and *Cdx2*$^{-/-}$ (C,D,F,I,J) embryos stained with either Cdx1 (E,F), Cdx2 (A-D) or Cdx4 (G-J) antibody. Loss of Cdx2 was observed in the conditional null mutant at E8.5 (A,B compared with C,D), and there was a significant reduction in Cdx4 levels at E8.5 (G,H compared with I,J). Axial truncation was evident in E9.5 *Cdx2*-null embryos (D,J).

actin-Cre-ERT2 (*Cre-ERT2*) transgenic line. *Cre-ERT2* expresses an inducible Cre recombinase driven by a *CMV*-β-*actin* promoter cassette (Feil et al., 1997) and elicits efficient, tamoxifen-dependent recombination of floxed targets in a near-ubiquitous manner (Santagati et al., 2005). *Cre-ERT2-Cdx2*$^{f/f}$ × *Cdx2*$^{f/f}$ matings were established and a single 2 mg dose of tamoxifen was administered by oral gavage to pregnant females at E5.5. *Cdx2* conditional mutants generated in this manner were recovered in a Mendelian ratio from E8.5 to E10.5 (data not shown), and typically exhibited no detectable Cdx2 protein (Fig. 1C,D). The antibody used for this analysis was raised against N-terminal Cdx2 sequences (Savory et al., 2009), and any truncated Cdx protein that could theoretically arise from the targeting strategy was undetectable. This suggests that any such N-terminal protein is unstable and therefore unlikely to confound interpretation of the results.

As Cdx members exhibit both auto- and cross-regulation (Lorentz et al., 1997; Trinh et al., 1999; Prinos et al., 2001; Béland et al., 2004; Chawengsaksophak et al., 2004), we assessed whether the expression of Cdx1 or Cdx4 was impacted by the loss of Cdx2. *Cdx1* mRNA (data not shown) and protein (Fig. 1E,F) were unaffected in *Cdx2* mutants at E8.5. By contrast, Cdx4 expression was markedly attenuated in the *Cdx2* conditional null embryos beginning at E8.5 (Fig. 1I,J), in agreement with previous studies (Chawengsaksophak et al., 2004). As Cdx members exhibit functional redundancy as regards both AP vertebral patterning and axial elongation (van den Akker et al., 2002; van Nes et al., 2006; Savory et al., 2009), this reduction in Cdx4 might contribute to certain aspects of the *Cdx2* mutant phenotype described below.

Cdx2 mutant embryos exhibit axial elongation defects

Cdx2 conditional mutants (referred to hereafter as *Cdx2*$^{-/-}$), generated as described above, died in utero around E11.5. Mutant embryos gastrulated and displayed normal cranial development (Fig. 2D,E); however, by E8.5 their AP axis was shortened relative to that of control littermates and they failed to develop hindlimb buds at later stages. In situ hybridization with the somite markers *Mox1* (Fig. 2) and *Paraxis* (*Tcf15* – Mouse Genome Informatics; see Fig. S2 in the supplementary material) revealed that Cdx2 was dispensable for somite formation. Somite polarity was likewise unaffected as evidenced by *Uncx4.1* and *Paraxis* expression (see Fig. S2 in the supplementary material). However, although the first 5-7 somites of the *Cdx2* mutant were indistinguishable from wild-type littermates, subsequent somites were progressively smaller (Fig. 2E,F), in agreement with previous observations (Chawengsaksophak et al., 2004). By E9.5, *Cdx2* mutants had fewer somites (an average of 17) than control littermates (an average of 22) and the somites extended to almost the caudal extremity of the mutant embryo (Fig. 2A,B, arc) owing to the lack of PSM. By E10.5, when wild-type embryos had developed ~35 somites, the *Cdx2* mutant somite number remained at 17, indicating that somitogenesis and axial extension ceased prematurely (Fig. 2C,F).

Cdx2 conditional null mutants and *Cdx2*-null aggregation chimeras died at mid-gestation, presumably owing to failure of chorio-allantoic fusion. It is also possible that this could impact on PSM generation, and thus the precocious cessation of axial elongation could be a secondary outcome. To address this, pregnant dams were treated with tamoxifen at E8.5, which allowed for development to term. Wholemount skeletal analysis of E18.5 mutant fetuses generated in this manner revealed an axial truncation at the level of the sacral vertebrae (Fig. 3A,B), whereas more-anterior segments appeared normal (Fig. 3C-F). These observations indicate that Cdx2 is essential for axis elongation independently of its impact on chorio-allantoic fusion.

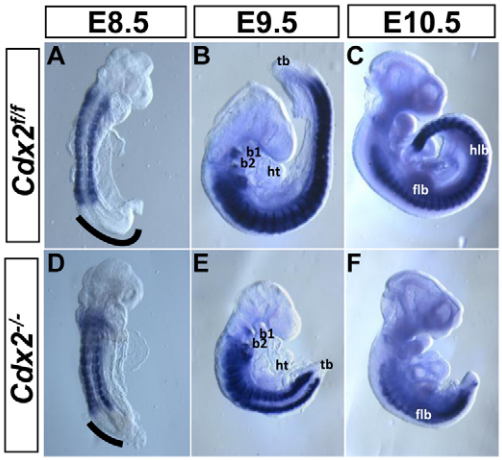

Fig. 2. Reduced presomitic mesoderm in *Cdx2*-null embryos. (**A-F**) In situ hybridization for *Mox1* in E8.5 (A,D), E9.5 (B,E) and E10.5 (C,F) control (A-C) and *Cdx2*$^{-/-}$ mutant (D-F) embryos. Reduced unsegmented mesoderm, demarcated by the absence of *Mox1* expression, was observed in Cdx2$^{-/-}$ embryos at E8.5 (A,D, compare arcs). The caudal-most somites in the mutant were smaller and deposited more posteriorly relative to wild-type controls at E9.5 and E10.5 (B,C compared with E,F).

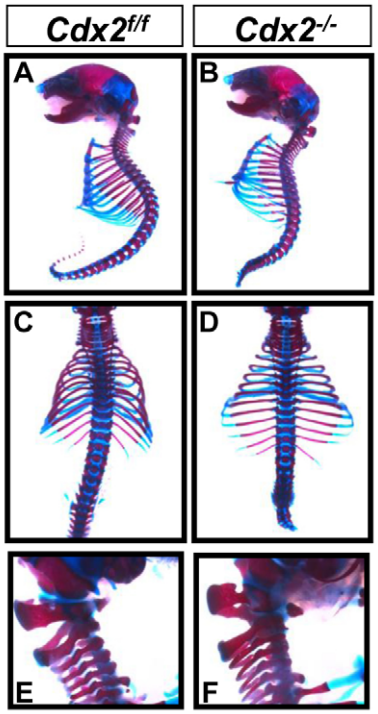

Fig. 3. Skeletal analysis of *Cdx2*-null offspring. (**A-F**) Wholemount skeletal analysis illustrating lateral (A,B,E,F) and dorsal (C,D) views of representative control (A,C,E) and *Cdx2* mutant (B,D,F) preparations generated by Cre-mediated recombination at E8.5. Vertebral truncation at the sacral level was observed in the mutants.

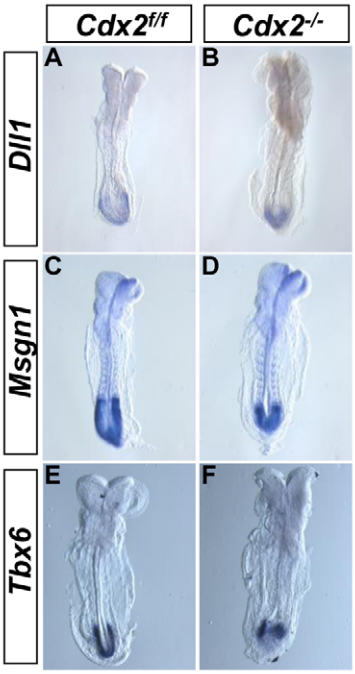

Fig. 4. Loss of posterior mesoderm in *Cdx2*-null embryos. (**A-F**) In situ hybridization analysis of *Dll1* (A,B), *Msgn1* (C,D) and *Tbx6* (E,F) expression in control (A,C,E) and *Cdx2*$^{-/-}$ mutants (B,D,F).

Cdx2 is required for PSM generation

Somites form by coalescence and segmentation of paraxial mesoderm from the anterior end of the PSM, which is replenished by the addition of new cells provided initially by the primitive streak and later the tail bud, until cessation of axial elongation. PSM generation and patterning are controlled by a network of factors including *Fgf8*, *Tbx6*, *Cyp26a1* and *Wnt3a*, with the latter contributing to expression of additional players such as *Dll1* and mesogenin 1. As morphological analysis suggested that the loss of Cdx2 impacted on PSM generation, we performed wholemount in situ hybridization analysis to determine whether any of these genes were implicated in this phenotype.

The Notch1 ligand *Dll1* is an early mesodermal marker necessary for somite formation (Hrabe de Angelis et al., 1997) and is directly regulated in the PSM by the synergistic action of Tbx6 and Wnta3a (Hrabe de Angelis et al., 1997; Hofmann et al., 2004). *Msgn1* is specifically expressed in the PSM and is necessary for PSM maturation; *Msgn1*-null mice lack trunk somites and show a severely reduced PSM (Yoon and Wold, 2000). Both *Dll1* and *Msgn1* were correctly expressed in the PSM of *Cdx2*$^{-/-}$ embryos at E8.5, but the distribution of their transcripts was significantly reduced relative to that of control littermates (Fig. 4A-D). These results suggest that the PSM is correctly specified in *Cdx2*-null mutants but its production might not be sustained. Further support for this comes from the analysis of *Tbx6*, which is normally expressed in the primitive streak and nascent paraxial mesoderm and is downregulated upon somite formation (Chapman et al., 1996; Chapman and Papaioannou, 1998). *Tbx6* expression was markedly reduced in E8.5 *Cdx2*$^{-/-}$ mutants, particularly in the caudal-most embryo (Fig. 4E,F). Taken together with the cessation of somitogenesis seen in *Cdx2* mutants around E9.5 (Fig. 2), these results are consistent with a crucial function for Cdx2 in sustaining PSM production.

The axial truncation observed in *Cdx2*$^{-/-}$ embryos is reminiscent of the phenotype evoked by loss of a number of signaling molecules or transcription factors, including Fgfr1, Wnt3a and T, all of which are necessary for paraxial mesoderm ontogenesis (Wilkinson et al., 1990; Takada et al., 1994; Ciruna et al., 1997; Yoshikawa et al., 1997; Ciruna and Rossant, 2001). Wnt3a is expressed in the tail bud and PSM, and plays a role in cell proliferation in the PSM in which it functions to maintain a pool of paraxial mesoderm precursors in the tail bud (Takada et al., 1994; Greco et al., 1996; Yoshikawa et al., 1997). Wnt3a, together with the T-box transcription factors T and Tbx6, is also essential for specification of paraxial mesoderm (Herrmann et al., 1990; Chapman et al., 1996; Galceran et al., 1999; Streit and Stern, 1999; Yamaguchi et al., 1999b; Aulehla et al., 2007; Dunty et al., 2008). The migration of cells from the primitive streak is also dependent on both a caudorostral gradient of Fgf signaling (Yamaguchi et al., 1994; Sun et al., 1999; Ciruna and Rossant, 2001; Yang et al., 2002) and T function (Wilson and Beddington, 1996; Wilson and Beddington, 1997). This caudal Fgf gradient is opposed by a rostral high retinoic acid (RA) gradient, resulting from RA synthesis (by Raldh2) in the somites, and opposed by Cyp26a1-mediated RA catabolism in the tail bud (Abu-Abed et al., 2001; Sakai et al., 2001).

At E8.5, the anterior limit of *Wnt3a* expression was modestly reduced in *Cdx2* mutants relative to controls (Fig. 5A,B). T expression was also reproducibly reduced in the tail bud, but not the notochord, of mutants (Fig. 5E,F). *Fgf8* expression was also reduced

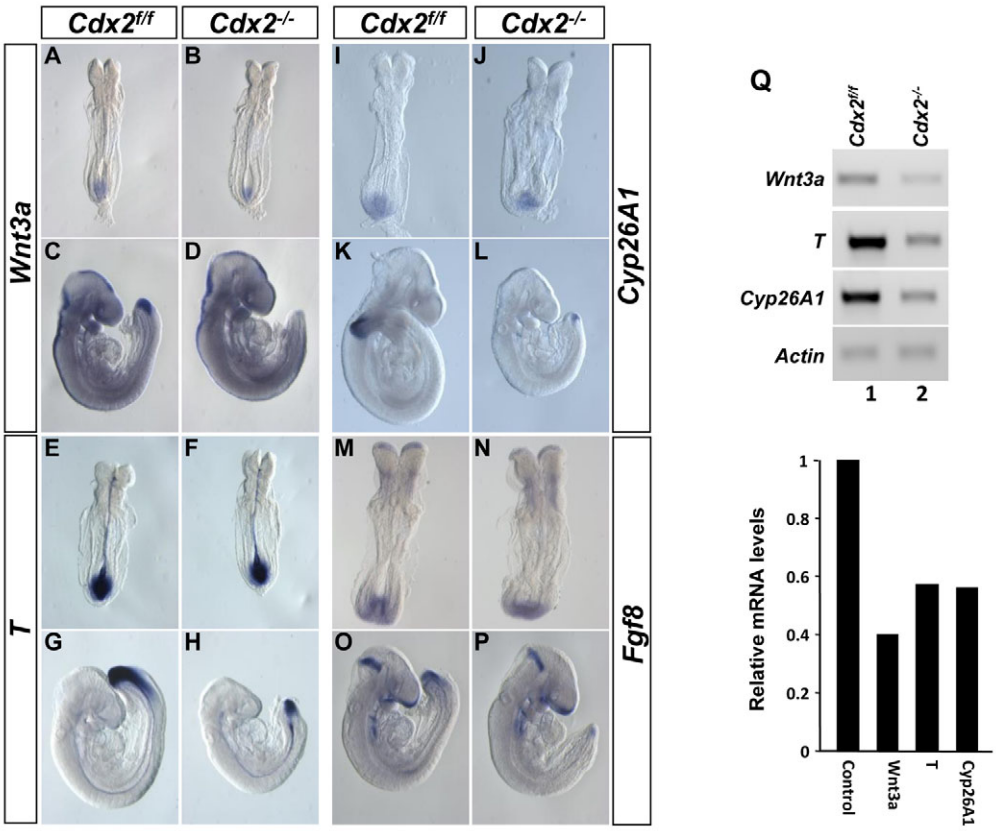

Fig. 5. The expression of genes involved in paraxial mesoderm specification in *Cdx2* mutants. (**A-P**) In situ hybridization of E8.5 (A,B,E,F,I,J,M,N) and E9.5 (C,D,G,H,K,L,O,P) control (A,C,E,G,I,K,M,O) and *Cdx2*$^{-/-}$ mutant (B,D,F,H,J,L,N,P) embryos. (**Q**) Relative transcript abundance as assessed by semi-quantitative RT-PCR. Agarose gel of representative RT-PCR analysis (top). Relative expression was calculated by scanning densitometry using β-actin as an internal control (bottom).

in E8.5 *Cdx2*$^{-/-}$ embryos relative to control littermates (Fig. 5M,N), although *Cyp26a1* expression was more strongly affected (Fig. 5I,J). At E9.5, the expression of *Wnt3a*, *T* and *Fgf8* were all markedly reduced compared with controls (Fig. 5C,D,G,H,O,P), whereas *Cyp26a1* expression was almost absent (Fig. 5K,L). These observations were confirmed by semi-quantitative RT-PCR from E8.5 caudal embryo explants (Fig. 5Q).

The above results suggest that nascent paraxial mesoderm ontogenesis is dependent on Cdx2. Consistent with this, *Wnt3a*, *T* and *Tbx6* mutants all impact on paraxial mesoderm ontogenesis and all exhibit mis-specification of this tissue into ectopic neural tubes (Wilkinson et al., 1990; Takada et al., 1994; Chapman et al., 1996; Yoshikawa et al., 1997; Yamaguchi et al., 1999b). To investigate whether a similar outcome manifests in *Cdx2*$^{-/-}$ mutants, we analyzed the expression of the neural marker *Sox2* (Kamachi et al., 1998; Wood and Episkopou, 1999) and found it to be unchanged (see Fig. S3 in the supplementary material). Moreover, there was no irregularity in the folding of the neural tube along the AP axis, another commonality between *Wnt3a*, *T*, *Tbx6* and *Cyp26a1* mutants (Takada et al., 1994; Abu-Abed et al., 2001; Sakai et al., 2001). Thus, Cdx2 loss does not appear to result in mis-specification of paraxial mesoderm.

Wnt5a is co-expressed with Cdx2 in the primitive streak and PSM, and *Wnt5a*-null mutants recapitulate some aspects of *Cdx2* loss-of-function. Moreover, the *Wnt5a* message was reduced in *Cdx2*$^{-/-}$ mutants suggesting that it is downstream of *Cdx2* (see Fig. S4 in the supplementary material). However, T, Fgf8 and Tbx6, which were all affected in *Cdx2*$^{-/-}$ embryos, are not perturbed in *Wnt5a* mutants (Yamaguchi et al., 1999a), suggesting that the loss of Wnt5a is not the primary basis for the *Cdx2*-null phenotype.

Loss of Cdx2 alters retinoid signaling in the PSM

The distribution of RA during development is regulated by a balance between its synthesis and degradation. Retinaldehyde dehydrogenases, of which Raldh2 is most crucial at the stages of interest, generate RA initially in the primitive streak region around E7.5, with subsequent production shifting to the trunk region around E8.5-E9.5 (Duester, 2008). RA biosynthesis is counterbalanced by the catabolic activity of members of the Cyp26 family of P450 cytochrome oxidases (White et al., 1996; Fujii et al., 1997; Ray et al., 1997; Hollemann et al., 1998). In this regard, Cyp26a1 is expressed in the tail bud at E8.5 and its loss leads to caudal regression and vertebral defects consistent with RA teratogenesis (Sakai et al., 2001; Abu-Abed et al., 2003). *Cyp26a1* is also a direct RA target gene, suggesting that it might operate in a feedback loop to limit retinoid signaling in the caudal embryo from E8.5 onwards (Iulianella et al., 1999; Abu-Abed et al., 2003; Sirbu et al., 2005).

To determine whether the reduction in Cyp26a1 in *Cdx2*-null mutants impacted retinoid signaling, we compared the activity of the RA-responsive *RARE-hpslacZ* transgene (Rossant et al., 1991) between mutant and wild-type embryos. In agreement with previous results (Sakai et al., 2001), transgene expression was robust in the trunk, but absent from the tail bud, of control E8.5 embryos (Fig. 6A,C). In *Cdx2*$^{-/-}$ embryos there was a posterior expansion of

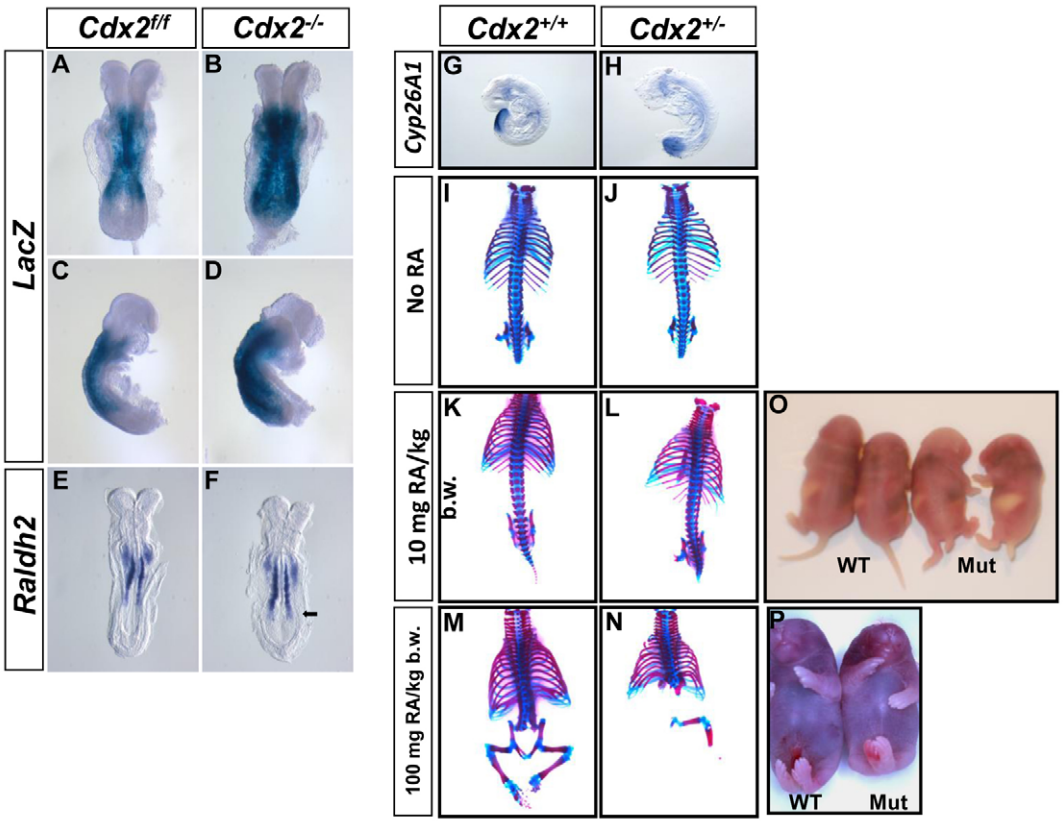

Fig. 6. The domain of retinoid bioactivity is expanded in *Cdx2*-null mutants. (**A-F**) The expression of a *RARE-lacZ* reporter in control (A,C) and *Cdx2*$^{-/-}$ (B,D) embryos. Caudal expansion of X-Gal staining was observed in the mutants (B,D). The expression of *Raldh2* was modestly posteriorized in the *Cdx2*$^{-/-}$ mutant embryos (E,F, arrow). (**G**,**H**) *Cyp26a1* expression in control and *Cdx2*$^{+/-}$ embryos. (**I-N**) Dose-dependent effects of RA on *Cdx2*$^{+/-}$ embryos. A comparison of wild-type and *Cdx2* heterozygous E18.5 skeletons collected after RA administration at E8.5 (dose is indicated to the left). (**O**,**P**) External abnormalities in *Cdx2*$^{+/-}$ mutants exposed to RA in utero. Loss of the tail (O) and fused hindlimbs (P) were observed in the mutants.

transgene activity into the tail bud (Fig. 6B,D), consistent with an increase in RA in the caudal region of the mutants. The expression domain of Raldh2 was also extended posteriorly, although not to the same extent as RA reporter expression (Fig. 6E,F). Thus, altered RA biodistribution might contribute to the axial truncation phenotype in *Cdx2*-null mutants.

Exposure of late gastrulation stage embryos to exogenous RA evokes concentration-dependent axial patterning defects ranging from vertebral homeosis to caudal agenesis (Kessel and Gruss, 1991; Kessel, 1992). The reduction in Cyp26a1 expression in the tail bud of *Cdx2* mutants is predicted to render the embryo more susceptible to the effects of exogenous RA. As *Cdx2*$^{-/-}$ mutants die at mid-gestation, pregnant mice from a *Cdx2*$^{+/-}$ outcross were given a single dose of RA at E8.5 and fetuses were examined at term; *Cdx2*$^{+/-}$ embryos exhibited an attenuation of Cyp26a1 expression intermediate to wild-type and null mutants (Fig. 6G,H). At 10 mg RA/kg body weight, no gross external abnormalities were apparent in control embryos, whereas *Cdx2*$^{+/-}$ embryos consistently presented with a shortened tail (Fig. 6K,L,O). Increasing the dose of RA to 100 mg/kg body weight led to loss of the tail in wild-type littermates, whereas *Cdx2*$^{+/-}$ offspring exhibited fused hindlimbs (sirenomelia) in addition to agenesis of caudal vertebrae (Fig. 6M,N,P). This sirenomelic phenotype is reminiscent of *Cyp26a1*$^{-/-}$ offspring (Abu-Abed et al., 1998; Sakai et al., 2001) and is consistent with a functionally significant decrease in Cyp26a1 activity in *Cdx2* mutants.

Identification of novel Cdx target genes

Expression analysis placed several genes known to be involved in PSM ontogenesis downstream of *Cdx2*, but did not distinguish between indirect and direct events. Cdx response elements (CDREs) of the consensus TTTATG have been identified in a number of target genes (Knittel et al., 1995; Subramanian et al., 1995; Charité et al., 1998). As a first step to identifying Cdx2 targets implicated in posterior development, we used binding site algorithms to examine the 5′ proximal promoter region of selected genes acutely attenuated in *Cdx2* mutants and identified multiple potential CDREs within the 5′ proximal *Wnt3a* (Fig. 7A), *Cyp26a1* (Fig. 8A) and *T* (Fig. 9A) promoters. By contrast, no CDREs were identified in the 5′ proximal *Wnt5a* promoter.

Chromatin immunoprecipitation (ChIP) analysis using chromatin from E8.5 embryos revealed Cdx2 occupancy of the *Wnt3a*, *Cyp26a1* and *T* promoters in intervals harboring the putative CDREs (Fig. 7B, Fig. 8B, Fig. 9B). Electrophoretic mobility shift assay (EMSA) was then used to determine whether one or more of these potential CDREs could be occupied directly. Indeed, Cdx2 bound strongly to one or more of the potential CDRE motifs in the *Wnt3a* (Fig. 7C), *Cyp26a1* (Fig. 8C) and *T* (Fig. 9C) promoters, and a weaker association with two other elements of the *Cyp26a1* (Fig. 8C) and *T* promoters (data not shown) was also observed. Note that the binding in each of these three potential targets was comparable to that seen for the previously characterized *Hoxb8* CDRE (Charité et al., 1998).

The *Wnt3a* and *Cyp26a1* CDREs were further investigated to determine whether they could mediate Cdx function in tissue culture. Luciferase reporter constructs harboring either wild-type promoter sequences, or identical reporters mutated for the CDREs, were transfected into P19 cells with or without a *Cdx2* expression vector. *Wnt3a* (Fig. 7D) and *Cyp26a1* (Fig. 8D) reporters showed a 10- to 15-fold increase in promoter activity in the presence of Cdx2 that was highly dependent on the single CDRE identified in the *Wnt3a* promoter (Fig. 7D). In the case of *Cyp26a1*, analysis of a series of reporters harboring various combinations of mutated CDREs revealed that CDREs 1 and 3 are necessary for maximal reporter induction (Fig. 9D), whereas CDRE 2, which is a perfect

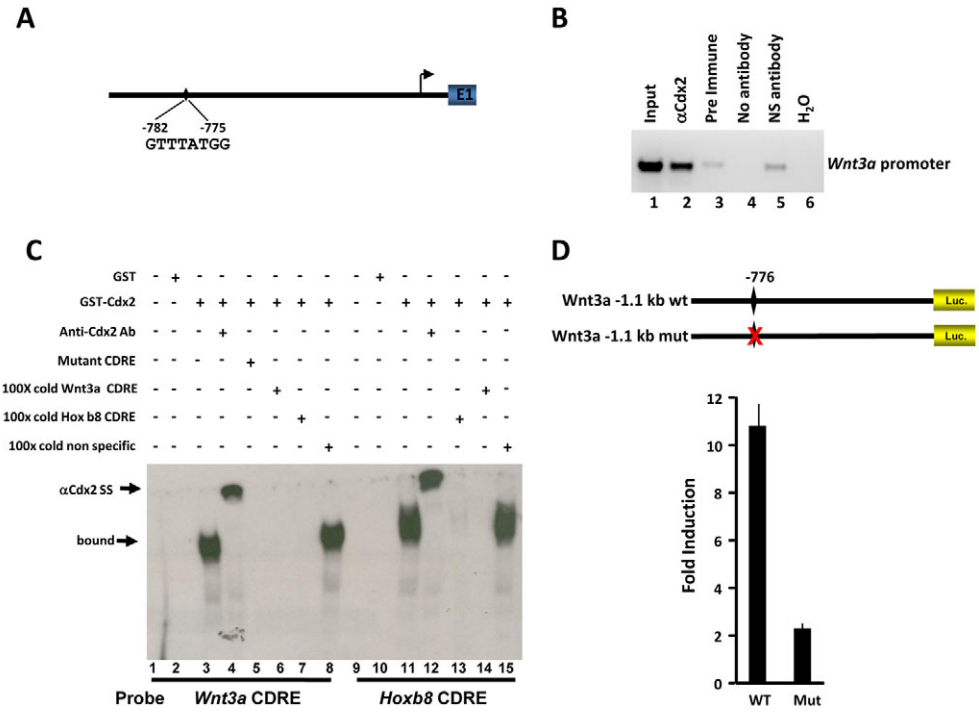

Fig. 7. Cdx2 directly regulates *Wnt3a* expression. (**A**) Schematic representation of the 5′ proximal region of the *Wnt3a* promoter showing the relative position of putative Cdx response element (CDRE; diamond), as identified by TESS. Numbering is relative to the transcription start site. (**B**) ChIP of *Wnt3a* promoter sequences from E8.5 embryos illustrating specific occupancy of the proximal promoter by Cdx members. (**C**) EMSA illustrating specific binding of Cdx2 to sequences corresponding to the putative CDREs highlighted in A in vitro (lane 3). Binding to the native sequence is specifically competed by a 100-fold excess of either unlabelled self oligonucleotide (lane 6) or the CDRE from the *Hoxb8* locus (lane 7) (Charité et al., 1998). Specificity was further demonstrated by a supershift by anti-Cdx2 (lane 4). (**D**) The *Wnt3a* proximal promoter responds to Cdx2 in vitro. Luciferase reporter constructs derived from the *Wnt3a* proximal promoter, together with a reporter lacking the putative CDREs shown in A, are depicted in the upper portion. The fold induction of reporter activity mediated by Cdx2 co-transfection is shown in the lower portion. All transfections were normalized relative to β-galactosidase and performed in triplicate; error bars indicate s.d. from the mean.

consensus motif, made no contribution, suggesting that not all canonical CDREs are functional in vivo. Finally, both Cdx1 and Cdx4 elicited similar CDRE-dependent responses from the *Wnt3a* and *Cyp26a1* promoters (data not shown), consistent with the functional redundancy common among these family members (Savory et al., 2009).

To assess Cdx2-dependent regulation of *T*, we employed a transgenic reporter strategy using promoter sequences previously shown to be sufficient to recapitulate *T* expression in nascent mesoderm in vivo (Clements et al., 1996; Yamaguchi et al., 1999b). Consistent with this prior work, wild-type promoter sequences directed the expression of a *lacZ* reporter in nascent mesoderm, but not the notochord, in 5 of 8 transgenic embryos at E8.5-E9.5 (Fig. 9E). By contrast, *lacZ* expression was absent in all of the transgenic embryos generated from a promoter mutated for the CDREs (0/8; Fig. 9E). It is notable that these mutant transgenics were devoid of *lacZ* expression, whereas *T* expression was readily detected by in situ hybridization in *Cdx2*-null mutants. This is probably due to functional redundancy among Cdx family members, as expression of *T*, *Wnt3a* and *Cyp26a1* is essentially abolished in E8.5 *Cdx1;Cdx2* double-null mutants (J.G.A.S. and D.L., unpublished). It is also notable that at least one of the CDREs identified in the murine *T* promoter is conserved in both rat and human (Fig. 9D), which is suggestive of evolutionary retention of this functional hierarchy. Taken together, these findings are consistent with *Wnt3a*, *Cyp26a1* and *T* as direct Cdx target genes.

DISCUSSION

To circumvent the early lethality inherent to *Cdx2* loss-of-function, we generated a floxed allele and used a tamoxifen-regulated Cre to effect inactivation at post-implantation stages. Mutants generated using this approach revealed a crucial role for Cdx2 in axial elongation, consistent with previous work (Chawengsaksophak et al., 2004). Our data also reveal a pivotal role for Cdx2 in directly regulating the expression of a number of non-Hox genes that are crucial for paraxial mesoderm ontogenesis, including *Wnt3a*, *T* and *Cyp26a1*. These findings suggest that Cdx2 is pivotal both to Hox-independent programs that regulate axial elongation and to Hox-dependent AP vertebral patterning.

Cdx2 operates in transcriptional networks essential for paraxial mesoderm ontogenesis

In vertebrates, three events take place in the primitive streak and tail bud that are crucial to the generation of PSM and the coordinated extension of the primary body axis (Dubrulle and Pourquié, 2004). First, control of cell proliferation is crucial in order to maintain a pool of progenitor cells necessary for the continuation of axis elongation at the proper rate. Second, the cells must migrate from the primitive streak or tail bud. Finally, cells must be appropriately specified to the paraxial mesoderm lineage. This series of events is regulated by an interactive cascade of transcription factors and signaling molecules, a number of which are downstream of *Cdx2*.

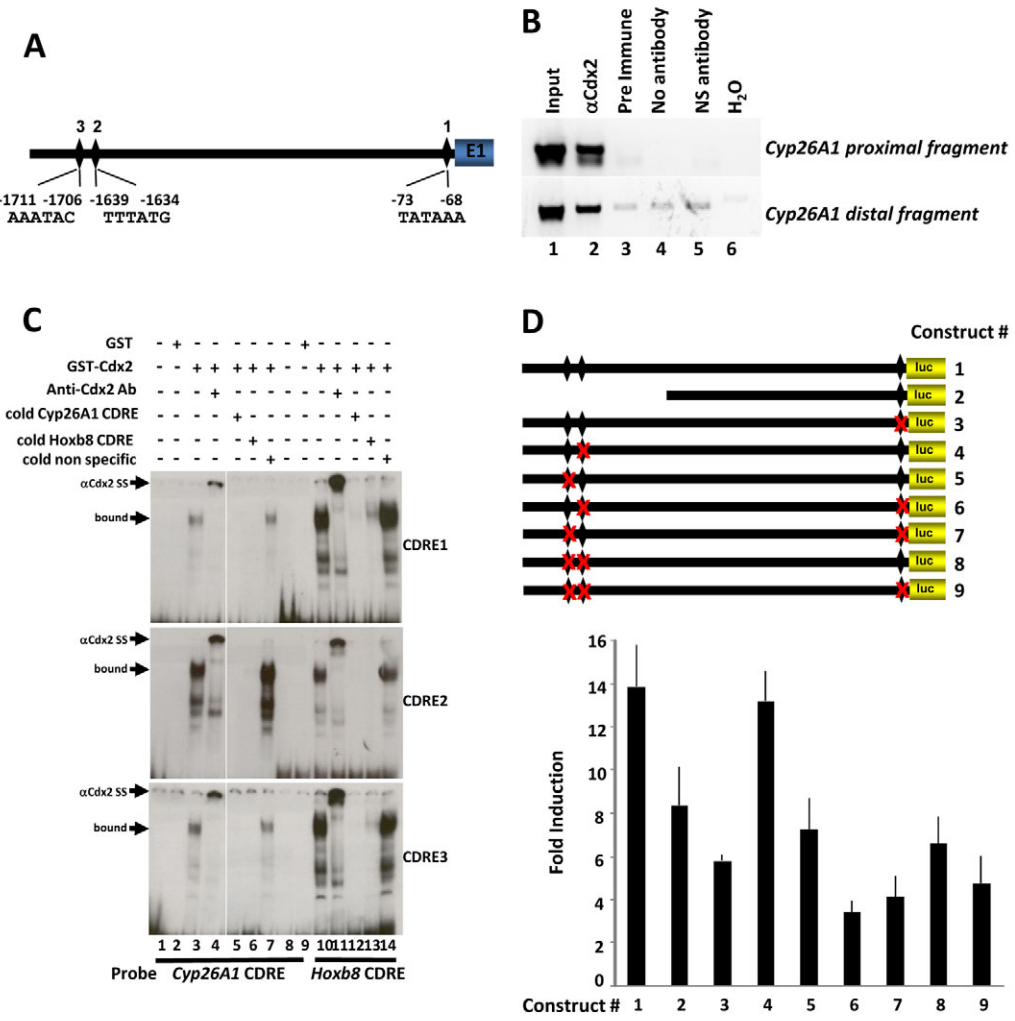

Fig. 8. Regulation of the *Cyp26a1* promoter by Cdx2. (**A**) Schematic representation of the 5′ proximal region of the *Cyp26a1* promoter showing the relative position of putative CDREs (diamonds) identified by TESS. Numbering is relative to the transcription start site. (**B**) ChIP of *Cyp26a1* promoter sequences from E8.5 embryos illustrating specific occupancy by Cdx2. (**C**) EMSA illustrating specific binding of Cdx2 to sequences corresponding to the putative CDREs (diamonds in A) in vitro. Binding was assessed as in Fig. 7. (**D**) Luciferase reporter assays performed using wild-type and mutant constructs derived from the *Cyp26a1* promoter, assessed as in Fig. 7.

Wnt3a is required for the proliferation of cells within the caudal embryo (Takada et al., 1994; Greco et al., 1996; Yoshikawa et al., 1997), as well as for specification of paraxial mesoderm (Takada et al., 1994). Some of these functions are mediated by *T*, which is a direct Wnt3a target gene required for specification of paraxial mesoderm (Yamaguchi et al., 1999b). *Tbx6* is likewise essential for normal specification of paraxial mesoderm and, as a direct T target, might mediate some of the effects of Wnt3a and T in this process (Chapman et al., 1996; Chapman and Papaioannou, 1998). Fgf signaling is also required for mesoderm specification (Ciruna and Rossant, 2001) and to maintain a pluripotent precursor population for the continued generation of paraxial mesoderm (Yamaguchi et al., 1994; Sun et al., 1999). Finally, *Cyp26a1*, together with *Raldh2*, establishes fields of retinoid signaling in the developing embryo (Duester, 2008) that restrict RA to the trunk region at E8.5, where it provides a countervailing signal to Fgf to establish the determination front for somite condensation (Duester, 2007).

The cessation of posterior elongation in *Cdx2* mutants coincided with reduced expression of Wnt3a, T, Tbx6, Cyp26a1 and Fgf8 in the caudal embryo, and the reduction of paraxial mesoderm markers including *Dll1* and *Msgn1*. We identified functional CDREs in the promoter regions of *Wnt3a*, *T* and *Cyp26a1*, which, together with Cdx2 occupancy of these promoters in vivo, suggests that all three genes are direct Cdx2 targets. Although we cannot exclude the possibility that *Dll1*, *Fgf8* and *Msgn1* are not also directly regulated by Cdx2, *Dll1* is downstream of *Wnt3a* (Aulehla et al., 2003; Galceran et al., 2004; Aulehla et al., 2007), whereas *Msgn1* is regulated by T (Goering et al., 2003). Moreover, exogenous RA or a *Cyp26a1* mutation can result in attenuation of Fgf8 expression in the caudal embryo (Iulianella et al., 1999; Wasiak and Lohnes, 1999; Sakai et al., 2001). These observations are consistent with *Dll1*, *Msgn1* and *Fgf8* being affected in *Cdx2* mutants in a secondary manner.

Embryonic exposure to exogenous RA induces malformations of the axial skeleton, the nature of which depends on the dosage and the stage of exposure (Kessel and Gruss, 1991; Kessel, 1992; Lohnes et al., 1993). *Cyp26a1* functions to protect the posterior region of the embryo from the teratogenic effect of RA and to exclude endogenous RA from the tail bud (Abu-Abed et al., 2001; Sakai et al., 2001; Ribes et al., 2007). Among other defects, *Cyp26a1*-null mutants exhibit hindlimb fusion (sirenomelia) a phenotype not seen in wild-type

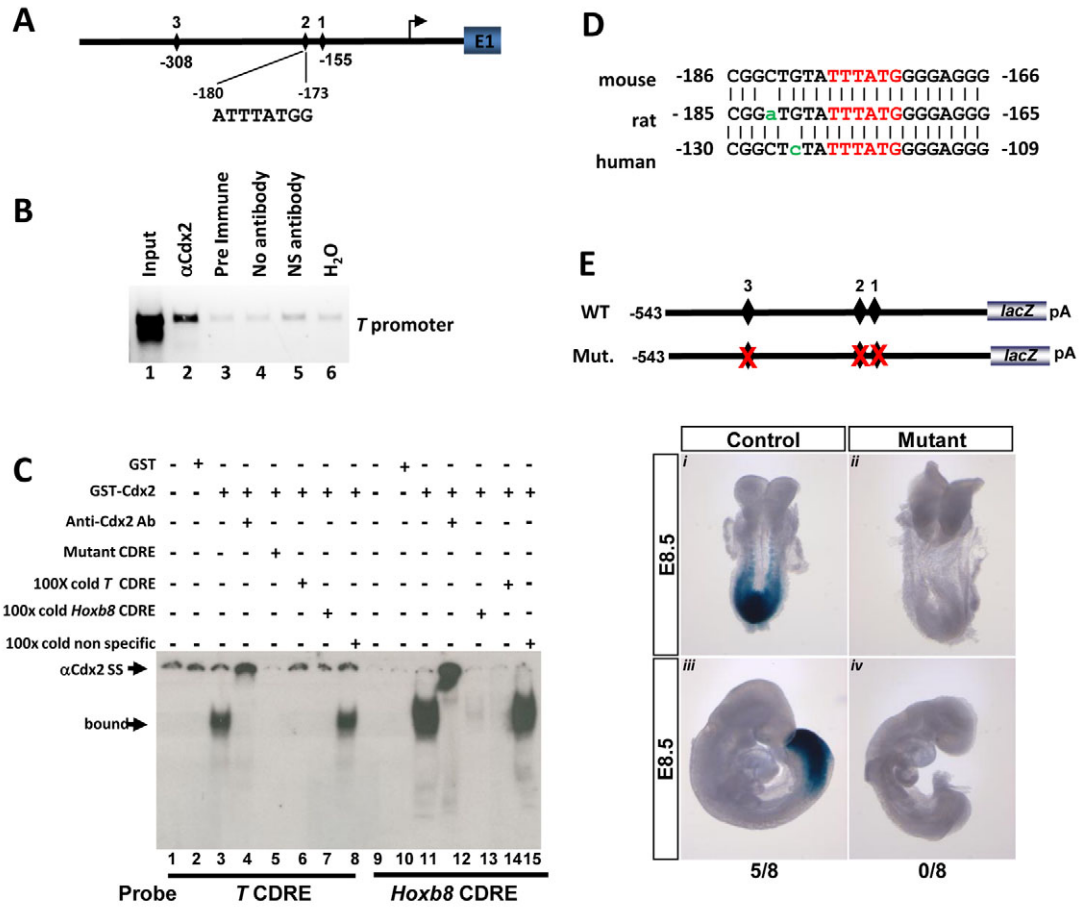

Fig. 9. *T* is a direct Cdx2 target gene. (**A**) Schematic of the proximal *T* promoter showing three putative CDREs. (**B**) ChIP analysis specifically localizes Cdx2 to the proximal *T* promoter in E8.5 embryos. (**C**) Cdx2 associates specifically to the second CDRE of the *T* promoter, as determined by EMSA; binding assays were conducted as described for Fig. 5. (**D**) CDRE in the mouse *T* promoter is conserved in the rat and human *T* promoters; numbering is relative to the transcription start site. (**E**) A schematic representation of wild-type and mutant *T* transgenic reporter constructs (top). The expression of the *lacZ* reporter driven by the wild-type (**i**,**iii**) or mutant (**ii**,**iv**) *T* promoters in transgenic embryos recovered at E8.5 (i,iii) or E9.5 (ii,iv) (bottom). Shown is the number of *lacZ*-positive embryos relative to the number of embryos positive for the transgene.

embryos exposed to exogenous RA. Our finding that *Cdx2*$^{+/-}$ embryos were both more sensitive to RA treatment and exhibited sirenomelia are consistent with a crucial role for Cdx2 in RA signaling in the caudal embryo through direct regulation of *Cyp26a1* expression.

Functional overlap among Cdx members

Cdx2-null mutants exhibited reduced expression of Cdx4. As Cdx members exhibit extensive functional overlap, at least as regards vertebral patterning (van den Akker et al., 2002; Savory et al., 2009), the *Cdx2*-null phenotype observed in the present study might be due to loss-of-function of both *Cdx2* and *Cdx4*. Consistent with this, the CDRE in the *Wnt3a* promoter is responsive to all Cdx members, and all Cdx members bound the *T* CDREs in vitro and occupied the *T*, *Cyp26a1* and *Wnt3a* promoters in vivo (data not shown). Such functional overlap might also underlie the observation that mutation of CDREs in the *T* promoter completely abrogated expression of a transgenic reporter, whereas residual T expression was still detected in the *Cdx2* mutant background at comparable stages. In this regard, the expression of Wnt3a and T is essentially absent in nascent mesoderm in *Cdx1;Cdx2* double-mutants at E8.5 (J.G.A.S. and D.L., unpublished), consistent with functional redundancy among Cdx members.

A new model for Cdx function

The effect of Cdx2 loss on the development of the caudal embryo begins to manifest around E8.5, shortly after the onset of expression of *Cdx2* in the embryo proper (Beck et al., 1995), whereas expression of *T* and *Wnt3a* is initiated much earlier (Takada et al., 1994; Chapman et al., 1996). These findings suggest that Cdx function is required to maintain expression of *T* and *Wnt3a* at later stages, whereas other pathways contribute to their initiation at earlier stages of expression. Conversely, Cdx signaling might have a more immediate role in initiating *Cyp26a1* expression in the tail bud around E8.5.

It is notable that caudalizers, including RA, canonical Wnt and Fgf, are involved in both the development of the posterior embryo and in vertebral patterning. The latter function could be mediated, at least in part, through direct regulation of expression of Cdx family members (Lohnes, 2003). Based on these observations, our present findings suggest a model whereby Cdx2 functions directly upstream of factors involved both in axis elongation and in AP patterning, and therefore integrates aspects of retinoid, Fgf and Wnt signaling involved in these processes (Fig. 10). In this regard, this model is consistent with the previously described *Wnt3a-Cdx* feedback loop in *Xenopus* (Faas and Isaacs, 2009). In addition, Cdx2 has also been shown to govern endoderm patterning and specification of the colon through Hox-independent means (Gao et al., 2009). Finally, it is notable that, in

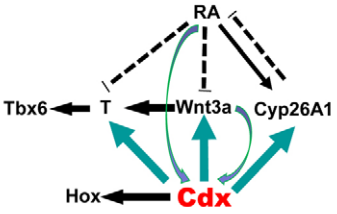

Fig. 10. Cdx-dependent transcriptional networks integrate axis elongation and somite AP patterning. Schematic of selected target genes directly activated by Cdx in the presomitic mesoderm. See text for details.

Drosophila, *cad* is required for specification of the posterior embryo through regulation of expression of gap and pair-rule genes (Levine et al., 1985; Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987) and is subsequently needed for gastrulation and hindgut patterning. Three other genes, *fkh*, *byn* and *wg*, which are related to murine *HNF-3* (*Foxm1* – Mouse Genome Informatics), *T* and *Wnt*, are also required for *Drosophila* hindgut gastrulation. The overlapping expression patterns and cross-regulation of *cad*, *fkh*, *byn* and *wg*, certain aspects of which are conserved in the vertebrate homologues *Cdx*, *T*, *HNF-3* and *Wnt*, have led to the hypothesis that these genes constitute an evolutionarily conserved 'cassette' that functions during gastrulation (Wu and Lengyel, 1998; Lengyel and Iwaki, 2002). Our finding of a central role for Cdx2 within this cassette, aspects of which appear to be reflected across diverse vertebrate species, emphasizes a conserved role for Cdx/cad in AP pattering and elaboration of the posterior embryo.

Acknowledgements

We thank M. Robillard and Q. Zhu for ES cell injections; T. Yamaguchi, M. Petkovich, G. Duester, A. Iulianella, O. Pourquié and A. McMahon for providing cDNAs for in situ hybridization probes; J. Rossant for the RARE reporter line; and A. Nagy for R1 ES cells. We acknowledge E. Tibbo and S. Dawson for excellent animal husbandry. This work was supported by funding from the Canadian Institutes of Health Research to D.L.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/24/4099/DC1

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