Development 134, 2315-2323 (2007) doi:10.1242/dev.001206

Wnt signaling is a key mediator of *Cdx1* expression in vivo

Nicolas Pilon[1,*], Karen Oh[1], Jean-René Sylvestre[1], Joanne G. A. Savory[2] and David Lohnes[2,†]

In the mouse, Cdx1 is essential for normal anteroposterior vertebral patterning through regulation of a subset of Hox genes. Retinoic acid (RA) and certain Wnts have also been implicated in vertebral patterning, although the relationship between these signaling pathways and the regulation of mesodermal Hox gene expression is not fully understood. Prior work has shown that *Cdx1* is a direct target of both Wnt and retinoid signaling pathways, and might therefore act to relay these signals to the Hox genes. Wnt and RA are believed to impact on *Cdx1* through an atypical RA-response element (RARE) and Lef/Tcf-response elements (LRE), respectively, in the proximal promoter. To address the roles of these regulatory motifs and pathways, we derived mice mutated for the LRE or the LRE plus the RARE. In contrast to RARE-null mutants, which exhibit limited vertebral defects, LRE-null and LRE+RARE-null mutants exhibited vertebral malformations affecting the entire cervical region that closely phenocopied the malformations seen in *Cdx1*-null mutants. Mutation of the LRE also greatly reduced induction of *Cdx1* by RA, demonstrating a requirement for Wnt signaling in the regulation of this gene by retinoids. LRE and LRE+RARE mutants also exhibited vertebral fusions, suggesting a defect in somitogenesis. As Wnt signaling is implicated in somitogenesis upstream of the Notch pathway, it is conceivable that Cdx1 might play a role in this process. However, none of the Notch pathway genes assessed was overtly affected.

KEY WORDS: Cdx1, Wnt, Lef/Tcf, Transcription, Anteroposterior patterning, Somitogenesis

INTRODUCTION

As the vertebrate embryo elongates, somites are produced in an anterior to posterior sequence by periodic segmentation of the paraxial mesoderm on either side of the presumptive neural tube. Somites subsequently differentiate into dermamyotome and sclerotome, the latter being the anlage of the vertebrae. Many vertebrae exhibit specific morphological characteristics according to their anteroposterior (AP) position and must therefore be subject to patterning along the major body axis.

It is well established that the Hox gene products are crucial mediators of AP vertebral patterning. The 39 murine Hox genes are distributed in four clusters, *Hoxa* to *Hoxd*, which are likely to have arisen from duplication of an ancestral complex related to the *Drosophila melanogaster* homeotic gene complex (HOM-C) (Duboule, 1998; Duboule and Dollé, 1989; Ferrier and Holland, 2001; Lemons and McGinnis, 2006). In the mouse, Hox transcripts are first detected at embryonic day 7.5 (E7.5) in the primitive streak, with expression subsequently expanding to a fixed rostral limit in the neurectoderm and paraxial mesoderm (Kmita and Duboule, 2003; Deschamps and van Nes, 2005). Both the onset and rostral limit of expression are generally related to the chromosomal location of a given Hox gene within a cluster, with 3′ members expressed earlier and reaching a more anterior limit than their 5′ counterparts. This results in staggered domains of Hox gene expression along the AP axis, which is believed to comprise a 'Hox code' for vertebral patterning (Burke et al., 1995; Gaunt, 1994; Kessel and Gruss, 1991). Grafting experiments in the chick have demonstrated that the cues controlling both vertebral AP patterning and somitic Hox gene expression are acquired early and are fixed in the anterior presomitic mesoderm prior to overt somite segmentation (Dubrulle and Pourquié, 2004; Christ et al., 1974; Christ and Ordahl, 1995; Kieny et al., 1972; Nowicki and Burke, 2000).

Understanding the molecular mechanisms involved in establishing Hox gene expression has been the focus of considerable research. A number of transcription factors that impact directly on Hox gene expression have been documented, including the vertebrate Cdx (caudal) gene family of homeodomain transcription factors, *Cdx1*, *Cdx2* and *Cdx4* (Beck et al., 1995; Gamer and Wright, 1993; Meyer and Gruss, 1993). In the murine embryo proper, *Cdx1* and *Cdx2* expression initiates in the primitive streak region at E7.5, followed by *Cdx4* at E8.5 (Epstein et al., 1997; Lohnes, 2003). This results in a nested, caudal-high pattern of *Cdx* expression in both ectodermal and mesodermal compartments, with *Cdx1* exhibiting a rostral-most limit of expression, followed by *Cdx2* and *Cdx4*. This pattern of transcript distribution is maintained until extinction of expression in the tail bud, with loss first of *Cdx1*, followed by *Cdx4* and *Cdx2*. These dynamic patterns of expression, together with the results of gain-of-function and transgenic reporter experiments, have been proposed to be indicative of a functional Cdx gradient that regulates the spatial expression of target genes along the major body axis (Marom et al., 1997; Charité et al., 1998; Gaunt et al., 2004).

Numerous studies have clearly demonstrated key roles for Cdx gene products in vertebral patterning, and this is believed to occur, at least in part, through direct regulation of Hox gene expression. Consistent with this, *Cdx1*-null and *Cdx2* heterozygotes, as well as *Cdx1;Cdx2*$^{+/-}$, *Cdx1;Cdx4* and *Cdx2;Cdx4* compound mutants, display vertebral homeosis of the cervical and anterior thoracic regions, which correlates with posterior shifts in the rostral mesodermal limit of several Hox genes (Chawengsaksophak et al., 1997; Subramanian et al., 1995; van Den et al., 2002, van Nes et al., 2006). Cdx-binding sites have also been identified in the promoter regions of numerous Hox genes (Subramanian et al., 1995), some of which have been shown to direct spatial expression in vivo (Charité et al., 1998; Gaunt et al., 2004). Gain- and loss-of-function studies in chick and *Xenopus* embryos are also consistent with roles for Cdx members in AP patterning via regulation of Hox gene expression (Bel-Vialar et al., 2002; Isaacs et al., 1998).

[1]Clinical Research Institute of Montreal, Montreal, Quebec, Canada. [2]Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, K1H 8M5, Canada.

*Present address: Centre de Recherche en Reproduction Animale, Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, Québec, Canada
†Author for correspondence (e-mail: dlohnes@uottawa.ca)

Accepted 29 March 2007

A number of signaling pathways impact on axial patterning, including retinoic acid (RA), Wnt and fibroblast growth factor (Fgf). However, the precise mechanisms by which these signaling molecules influence Hox gene expression as relates to vertebral patterning are not fully understood. Prior work has shown that *Cdx1* is a target of both RA and Wnt signaling, suggesting that Cdx1 might serve to relay information from Wnt and retinoid pathways to mesodermal Hox gene expression (Lohnes, 2003).

RA signals through binding to the RA receptors (RARα, β and γ) which, together with a retinoid X receptor (RXRα, β or γ) heterodimeric partner, induces transcription of target genes through cis-acting RA-response elements (RAREs) (Altucci and Gronemeyer, 2001; Bastien and Rochette-Egly, 2001; Blomhoff and Blomhoff, 2006; Mark et al., 2006; Mic et al., 2003). *Cdx1* expression is attenuated in certain RAR-null mutant backgrounds, and is induced by RA treatment in vivo. Consistent with a direct relationship, the *Cdx1* promoter harbors an atypical RARE that is essential for a subset of *Cdx1* expression and function in vivo (Houle et al., 2000; Houle et al., 2003).

In the canonical Wnt pathway, Wnt occupation of frizzled receptor leads to stabilization of cytoplasmic β-catenin, which translocates to the nucleus and associates with members of the Lef/Tcf family of transcription factors (Lef1, Tcf1, Tcf3 and Tcf4) to activate transcription of target genes (Moon et al., 2002; Logan and Nusse, 2004). *Wnt3a* hypomorph *vestigial tail* (*vt*) mutants (Greco et al., 1996) exhibit vertebral defects associated with reduced *Cdx1* expression and posteriorized Hox gene expression (Ikeya and Takada, 2001; Prinos et al., 2001). Two Lef/Tcf-response elements (LREs) have been identified in the proximal *Cdx1* promoter that are candidates for response to Wnt (Lickert et al., 2000; Lickert and Kemler, 2002; Prinos et al., 2001).

Analysis of mice mutated for the *Cdx1* RARE has clearly demonstrated the in vivo relevance of retinoid signaling as regards *Cdx1* expression and function. To more definitely determine the importance of Wnt signaling alone or with RA in the regulation of *Cdx1*, we have derived mice in which the LRE, or the LRE plus the RARE, has been functionally inactivated by gene targeting in embryonic stem cells. Both the LRE and the LRE+RARE-null mutants exhibit near complete extinction of *Cdx1* expression as well as skeletal defects and posterior shifts in the expression of several Hox genes, which closely resembles the defects seen in *Cdx1*-null mutants. These data support a crucial role for Wnt signaling, through these specific LRE motifs, as a key regulator of *Cdx1* expression in vivo. Moreover, in agreement with prior work indicating a strong synergy between RA and Wnt signaling on the *Cdx1* promoter (Prinos et al., 2001), LRE-null mutant embryos were markedly refractory to induction of *Cdx1* by RA. Finally, recent data support a role for Wnt signaling in the regulation of Notch-dependent somitogenesis. A high incidence of vertebral fusions was observed in both LRE and LRE-RARE-null mutant offspring, suggesting that *Cdx1* might play a role in this process. However, we did not observe any overt changes in the expression of several members of the Notch signaling pathway in these backgrounds.

MATERIALS AND METHODS

Gene targeting and generation of mutant offspring

The isolation of relevant *Cdx1* genomic sequences has been described previously (Houle et al., 2003). The targeting vectors were generated from a 5.8 kb *Kpn*I-*Hin*dIII genomic fragment, subcloned into pBluescript KS+ (Stratagene), that encompassed the proximal *Cdx1* promoter, the first exon and part of the first intron (Fig. 1). The LRE sequences were mutated and *Sfu*I sites incorporated, as described previously (Prinos et al., 2001). For the LRE+RARE targeting construct, part of the RARE in the LRE mutant targeting vector was converted to a *Sac*I site (GAAGGGGAGCTCCCCCT; mutation underlined) using the Quick Change Site-Directed Mutagenesis Kit according to the manufacturer's (Stratagene) instruction. A bifunctional floxed thymidine kinase/neomycin resistance cassette (*loxPGK-TK-Neolox*) (Iulianella and Lohnes, 2002) was then subcloned into the unique *Xho*I site present in the intron of either construct.

R1 embryonic stem cells were cultured on murine embryonic fibroblasts under standard conditions. Cells were electroporated with 40 μg of linearized targeting vector and selected with G418 (200 μg/ml) for 10 days. Surviving clones were isolated, expanded and assessed for homologous recombination by genomic Southern blot analysis using *Sac*I digestion and hybridization with a probe 5′ to the sequences used to generate the targeting construct (probe A in Fig. 1). Positive clones were confirmed for the fidelity of recombination by Southern blot analysis using additional restriction enzymes and hybridization with an internal probe (probe B in Fig. 1). Incorporation of the mutated RARE in targeted clones was determined by genomic Southern blot analysis by virtue of the novel *Sac*I site introduced in this motif (Fig. 1), whereas integration of the mutated LRE was assessed by *Sfu*I digestion of a PCR product generated by primers flanking these sequences (Fig. 1).

Germline chimeras were derived by injection of targeted ES clones into C57BL/6 blastocysts according to standard procedures (Hogan et al., 1994). F1 animals from chimera-C57BL/6 outcrosses bearing the targeted allele were bred with homozygous *CMV-Cre* mice (Dupé et al., 1997) and offspring assessed for excision of the floxed *Tk-Neo* selection cassette by genomic Southern blot analysis as depicted in Fig. 1. Subsequent genotyping of established mutant lines was performed by PCR using primers flanking the *Xho*I site of the first intron (forward, 5′-ATCCTGGCGCAGTCCCTC-3′; reverse, 5′-AGGACAAGAGTGGTCGTGG-3′); the PCR product of the mutant allele exhibited an increased size owing to the presence of residual *loxP* sequences (Fig. 1).

Analysis of mutant offspring

Mice were mated overnight and noon of the day of vaginal plug detection was considered as E0.5. In situ hybridization and skeletal preparations were performed as previously described (Allan et al., 2001; Houle et al., 2003), using either wild-type littermates or CD1 offspring as controls. Embryos to be compared were stage-matched according to established criteria and processed in parallel. Probes for in situ hybridization were generated from previously described plasmids: *Cdx1* (Houle et al., 2000), *Hoxa3* (Manley and Capecchi, 1995), *Hoxd3* (Condie and Capecchi, 1993), *Hoxb4* (Folberg et al., 1999), *Hoxd4* (Featherstone et al., 1988) and *Lfng* (Mustonen et al., 2002). Plasmids for probes for *Notch1* and *Hes5* were a kind gift from C. C. Hui (The Hospital for Sick Children, Toronto, Canada) and the *Delta1* (*Dll1*) probe was kindly provided by O. Pourquié (Stowers Institute for Medical Research, Kansas City, MO). Embryo culture, Wnt3a treatment and oral RA gavage were carried out as described previously (Prinos et al., 2001; Houle et al., 2000).

RESULTS

Inactivation of the *Cdx1* LRE and RARE

Prior work suggests that *Cdx1* is regulated by both retinoid and Wnt signaling pathways through an atypical RARE and two LREs, respectively, in the proximal *Cdx1* promoter. These elements are capable of responding to exogenous RA and Wnt3a in tissue culture (Lickert et al., 2000; Prinos et al., 2001), and data from transgenic models is consistent with a role for these motifs in regulating spatial-temporal expression of *Cdx1* in vivo (Lickert and Kemler, 2002). A crucial role for the *Cdx1* RARE has been clearly established by gene targeting, although ablation of this element in vivo does not fully recapitulate the findings from transgenic analysis (Houle et al., 2003). RA and Wnt have also been shown to synergistically activate the *Cdx1* promoter in tissue culture models (Prinos et al., 2001). However, the precise in vivo role of the LRE, alone or in concert with the RARE, has not been thoroughly investigated.

To evaluate the importance of the LRE and the interplay between retinoid and Wnt signaling in the control of *Cdx1* expression in vivo, we derived mice mutated for the LRE or the LRE+RARE. Targeting

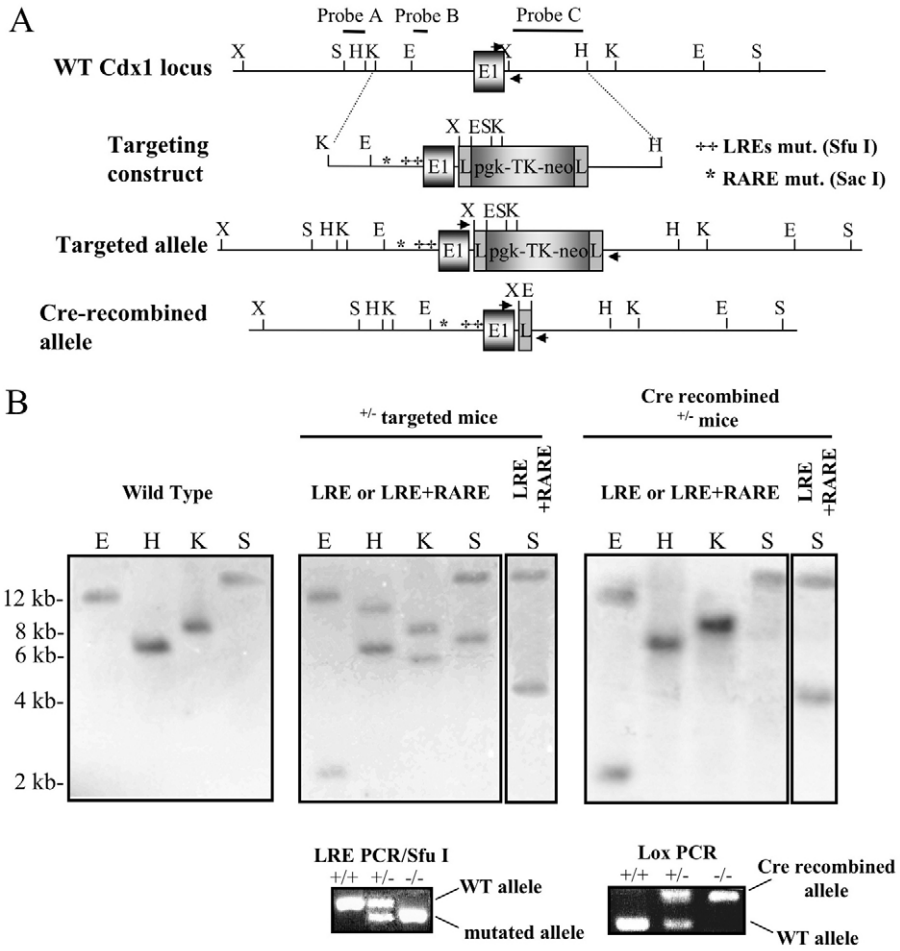

Fig. 1. Targeting of the LRE and RARE in the mouse *Cdx1* promoter. (**A**) Schematic of the wild-type (WT) *Cdx1* locus, targeting vector, targeted allele and *Cre*-recombined allele. Probe A (5′ external probe) was used to screen for targeted ES cells, probe B (5′ internal probe) was used to confirm the predicted targeting event and probe C (3′ internal probe) was used to confirm excision of the floxed neomycin selection cassette. E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sac*I; E1, Exon 1; *, mutated RARE; ++, mutated LRE. (**B**) Southern blot analysis of DNA from wild-type (left), heterozygous targeted (middle) and heterozygous *Cre*-recombined (right) offspring using probe B and the indicated restriction endonucleases (see A). Concomitant integration of mutated LRE and LRE+RARE within the targeted allele was determined by the introduction of novel restriction sites: for the RARE mutation, a *Sac*I restriction site was observed by Southern blot analysis (last lane of middle and right panels); for the LRE mutation, novel *Sfu*I restriction sites were assessed by restriction of a PCR product spanning the LRE (lower left panel; see also Materials and methods).

constructs containing a mutated LRE or mutated LRE+RARE were used to generate recombinant ES clones (Fig. 1). Chimeras generated from targeted cells for either mutation gave germline transmission. Subsequent excision of the selection cassette was effected by crossing F1 offspring with *CMV-Cre* mice (Fig. 1B). All of the resulting heterozygotes passed the mutant allele to their offspring at the predicted mendelian ratio.

We used previously described mutations to inactivate the LRE (Prinos et al., 2001). However, novel point mutations were introduced to inactivate the RARE. To determine if this element was devoid of function, the mutated RARE was amplified from LRE-RARE homozygote mutant offspring and subcloned into a previously described *Cdx1* reporter vector (Houle et al., 2000), replacing the cognate wild-type element. This mutant RARE reporter failed to respond to exogenous RA in transfection assays in F9 embryocarcinoma cells (data not shown), demonstrating that this element was functionally inactive.

LRE and LRE+RARE mutations phenocopy loss of Cdx1 function

The murine axial skeleton is normally composed of occipital bones, derived from condensation of the four rostral-most somites, and a vertebral column composed of seven cervical (C1-C7), 13 thoracic (T1-T13), six lumbar (L1-L6), three or four sacral (S1-S3/S4) and 31 caudal vertebrae. Many vertebrae exhibit specific morphological characteristics related to their position along the AP axis. The first cervical vertebra (C1, or atlas) exhibits thick neural arches and possesses a ventrally located tubercle, the anterior arch of the atlas (AAA; see Fig. 2A, for example). The neural arches of C2 are intermediate to those of C1 and more-caudal cervical vertebrae. C2 also possesses a second vertebral body, the dens axis, which articulates with C1. C3, C4 and C5 are morphologically similar, whereas C6 is distinguished by ventrally protruding anterior tuberculi. The thoracic vertebrae are characterized by the presence of ribs, the first seven of which (T1-T7) are attached to the sternum.

RARE$^{+/-}$ offspring do not exhibit any overt vertebral anomalies (Table 1, Fig. 2A) (Houle et al., 2003). By contrast, approximately 40% of LRE$^{+/-}$ and LRE+RARE$^{+/-}$ offspring exhibited a C2 to C1 transformation, indicated by a broader neural arch on C2. This transformation was, however, less expressive in LRE$^{+/-}$ offspring as an ectopic AAA was never observed in this background (Table 1, Fig. 2B,C). By contrast, the penetrance and expressivity of C2 to C1 anterior homeotic transformation (including a C2 AAA) in the LRE+RARE$^{+/-}$ mutants was comparable to that observed in *Cdx1* heterozygote offspring (Table 1).

The vertebral defects inherent to RARE mutants are essentially restricted to C2 and represent only a subset of the malformations exhibited by *Cdx1*-null mutants, which are affected throughout the cervical and anterior thoracic region (Table 1, Fig. 2D,E) (Subramanian et al., 1995). In contrast to RARE loss-of-function, ablation of the LRE or the LRE+RARE resulted in anterior homeotic transformations and vertebral fusions that largely recapitulated the defects seen in *Cdx1*-null offspring (Table 1, Fig. 2F-H). C1 displayed a reduced neural arch and loss of the AAA and was fused to, or in close apposition with, the basioccipital bone. C2 to C1, C3 to C2, C6 to C5 and C7 to C6 transformations were also observed, as evidenced

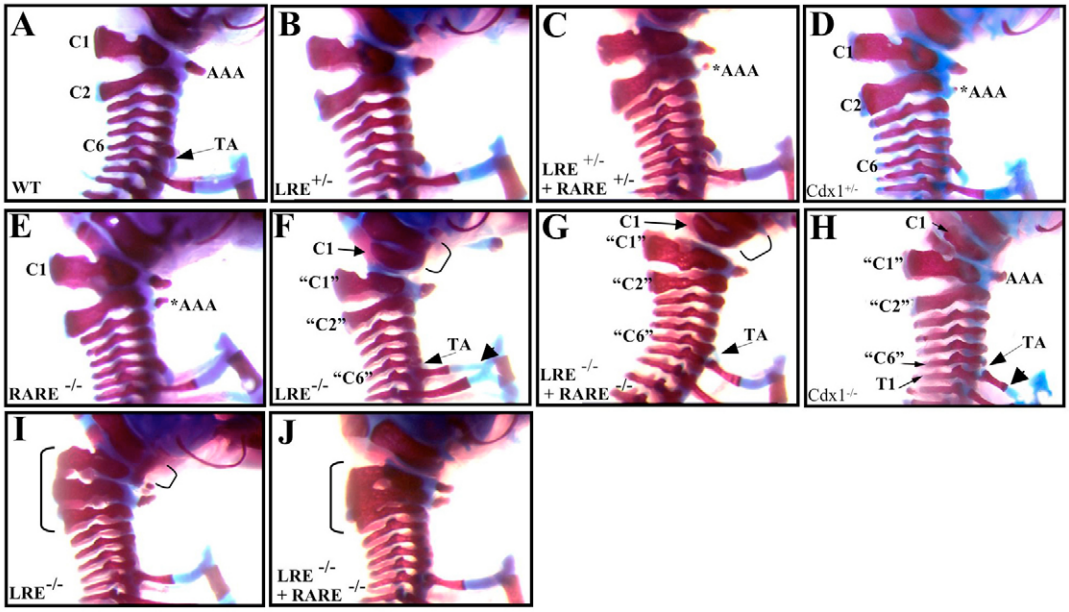

Fig. 2. Skeletal analysis of vertebral patterning defects. Cervical region of whole-mount mouse skeletal preparations from (**A**) wild-type, (**B**) LRE$^{+/-}$, (**C**) LRE+RARE$^{+/-}$, (**D**) *Cdx1*$^{+/-}$, (**E**) RARE$^{-/-}$, (**F**,**I**) LRE$^{-/-}$, (**G**,**J**) LRE+RARE$^{-/-}$ and (**H**) *Cdx1*$^{-/-}$ offspring. Note the C2 to C1 transformations evidenced by a broader C2 neural arch and an ectopic anterior arch of the atlas (*AAA in C-E). Anterior transformation of C1 was assessed by loss of the anterior arch of the atlas and anterior malposition (C1 arrow in F,G,H) and fusion with the basioccipital bone (small bracket in F,G,I). Anterior transformation of C2, C3 and C7 are denoted "C1", "C2" and "C6", respectively, in F-H as determined by morphological criteria as described in Material and methods. A partial rib associated with presumptive T1, which did not reach the sternum, is indicated by the short arrow (F,H). Fusions between adjacent vertebrae in LRE and LRE+RARE-null mutants are indicated by the long bracket (I,J). AAA, anterior arch of the atlas; *AAA, ectopic anterior arch of the atlas; TA, tuberculum anterior; C, cervical vertebrae; T, thoracic vertebrae. Quotation marks indicate presumptive anterior transformations.

by the morphological criteria described above. However, both the LRE and the LRE+RARE-null mutants were distinguished from *Cdx1*-null offspring by a high incidence of fusions between neural arches of the first two or three cervical vertebrae (Table 1, Fig. 2I,J). Moreover, relative to *Cdx1* mutants, fusion between C1 and the basioccipital bone was less expressive in the LRE and LRE-RARE-null backgrounds. Together, with expression analysis (below), these data suggest that some residual Cdx1 function exists in these backgrounds.

Effect of LRE and LRE-RARE mutation on *Cdx1* expression

Cdx1 expression was compared between wild-type, LRE-null and LRE+RARE-null embryos by whole-mount in situ hybridization. In agreement with previous analysis (Meyer and Gruss, 1993), *Cdx1* transcripts were first detected in the late primitive streak region in wild-type embryos at E7.5 (Fig. 3A). Expression peaked around E8.0 in the tail bud and then declined until E9.5, by which time transcripts in the caudal embryo were barely detectable (Fig. 3B,C). Relative to controls, *Cdx1* expression in the primitive streak was barely detectable at earlier stages, with a weak signal in some LRE-null and LRE+RARE-null mutants at the early head-fold stage (E7.75; Fig. 3D,G). As observed in controls, expression peaked around E8.0 in both LRE-null and LRE+RARE-null mutants, although transcript levels remained greatly reduced and were no longer detected after the 4- to 5-somite stage (Fig. 3E,F,H,I; note that LRE and LRE+RARE mutants were stained four times longer than controls, to reveal residual expression).

Altered Hox gene expression in LRE and LRE+RARE mutants

Cdx1-null mice exhibit vertebral defects that reflect those of certain Hox group 3- and group 4-null mutants (Subramanian et al., 1995). Consistent with this relationship, the rostral mesodermal limit of expression of a number of these Hox genes is posteriorized in *Cdx1*-null mutants (Allan et al., 2001; Houle et al., 2003). By contrast, only Hox group 4 genes are affected in RARE mutant offspring, in agreement with the restricted effects on C2 morphogenesis seen in this background.

To determine the relationship between Hox gene expression and the vertebral defects in LRE and LRE+RARE mutants, we assessed the expression patterns of *Hoxa3*, *Hoxd3*, *Hoxb4* and *Hoxd4*. In wild-type embryos at E9.5, the mesodermal limit of expression of *Hoxa3* and *Hoxd3* is at the fifth somite (Fig. 4A,D) (Condie and Capecchi, 1993; Gaunt, 1988; Sham et al., 1992). As for *Cdx1* (but not RARE) mutants, expression of these genes was posteriorized by one somite in both LRE and LRE+RARE-null mutants (Fig. 4B,C,E,F). The normal rostral limit of *Hoxb4* and *Hoxd4* in the mesoderm is at somite 6 (Fig. 4G,J) (Featherstone et al., 1988; Gaunt et al., 1989). Again, expression was posteriorized by one somite in both LRE and LRE+RARE-null mutants (Fig. 4H,I,K,L); an identical posteriorization is also seen in both *Cdx1* and RARE-null mutants (Houle et al., 2003; van Den et al., 2002). These results are in agreement with skeletal analyses (Fig. 2), and underscore a crucial role for Wnt signaling in vertebral patterning through regulation of *Cdx1*.

Table 1. Vertebral phenotypes of *Cdx1* mutants

Phenotype	WT *n=14* (%)	RARE$^{+/-}$* *n=15* (%)	LRE$^{+/-}$ *n=17* (%)	LRE+RARE$^{+/-}$ *n=18* (%)	*Cdx1*$^{+/-}$† *n=16* (%)	RARE$^{-/-}$* *n=13* (%)	LRE$^{-/-}$ *n=12* (%)	LRE+RARE$^{-/-}$ *n=29* (%)	*Cdx1*$^{-/-}$‡ (%)
Vertebra 1									
Malformed NA	—	—	—	—	3 (17)	4 (25)	13 (100)	12 (100)	29 (100)
Fusion to occipitals	—	—	—	—	—	—	4 (31)	8 (67)	29 (100)
AAA fused to V2	—	—	2 (13)	8 (47)	—	—	1 (8)	4 (33)	—
Vertebra 2									
C1 identity§	—	—	6 (40)	8 (47)	10 (56)	14 (88)	13(100)	12 (100)	29 (100)
Malformed NA	—	—	2 (13)	2 (12)	2 (11)	—	11 (85)	10 (83)	—
Vertebra 3									
C2 identity	—	—	—	—	—	—	13 (100)	11 (92)	29 (100)
Malformed NA	—	—	—	—	1 (6)	—	8 (61)	7 (58)	4 (14)
NA fusions									
V1-V2	—	—	—	1 (6)	1 (6)	—	5 (38)	2 (17)	—
V2-V3	—	—	—	—	1 (6)	—	—	—	—
V1-V2-V3	—	—	—	—	—	—	6 (46)	4 (33)	—
Vertebra 7									
C6 identity	—	—	—	—	—	—	12 (92)	10 (83)	28 (97)
Vertebra 8									
C7 identity	—	—	—	—	—	—	—	—	11 (38)
Incomplete or fused rib	—	—	—	—	—	1 (6)	2 (15)	—	18 (62)

The number of embryos displaying each phenotype is shown, with the percentage of the total for that genotype in parentheses.
AAA, Anterior arch of the atlas.
NA, Neural arch.
*From Houle et al. (Houle et al., 2003).
†From Béland et al. (Béland et al., 2004).
‡From Allan et al. (Allan et al., 2001).
§AAA and/or thick NA.

Response of LRE-null and LRE+RARE-null mutants to exogenous Wnt and RA

To verify if the LRE is required for regulation by Wnt signaling ex vivo, embryos were cultured in the absence or presence of Wnt3a-conditioned medium, and *Cdx1* expression assessed. As described previously (Prinos et al., 2001), *Cdx1* expression was modestly but reproducibly induced by Wnt3a-conditioned medium in wild-type embryos at all stages assessed (Fig. 5A,B and data not shown). By contrast, induction was markedly attenuated in LRE and LRE+RARE mutants at E7.5-8.5 (Fig. 5C-F and data not shown; note that mutants were stained four times longer than controls to reveal residual expression).

Targeted mutation of the RARE blocks the response of a *Cdx1* reporter to RA in tissue culture, but does not completely prevent induction in vivo, perhaps owing to the presence of alternate RAREs (Houle et al., 2000; Houle et al., 2003; Gaunt et al., 2003). Prior work has also shown a strong synergistic interaction between RA and Wnt3a on the *Cdx1* promoter in embryocarcinoma cells (Prinos et al., 2001). To determine the potential relevance of this interaction in vivo, we assessed *Cdx1* expression in LRE-null embryos 4 hours after RA treatment in utero. Whereas treatment clearly induced *Cdx1* expression at E7.5 and E8.5 in wild-type controls (Fig. 6A-D), this effect was markedly reduced in LRE-null embryos at E7.5 (Fig. 6E,F) and E8.5 (Fig. 6G,H). Given that Wnt signaling is active in the caudal embryo at these stages, these results are consistent with previous findings in tissue culture, and underscore a role for RA and Wnt signaling through these specific motifs in coordinately regulating *Cdx1* transcription.

Notch signaling and somitogenesis in LRE+RARE mutants

Fusions between one or more of the first three cervical vertebrae were frequently observed in LRE and LRE+RARE mutants, suggesting a defect in somitogenesis. Both Wnt and RA signaling have been implicated in this process, the former operating upstream of the Notch pathway and impacting on the somite 'clock', whereas RA has been proposed to antagonize Fgf8-dependent events related to the wavefront (Aulehla and Herrmann, 2004; Iulianella et al., 2003; Dubrulle et al., 2001; Dubrulle and Pourquié, 2004). Moreover, RA, Wnt, Fgf and Notch pathways have all been linked to regulation of Hox gene expression and vertebral patterning (Cordes et al., 2004; Dubrulle et al., 2001; Yamaguchi et al., 1994; Zakany et al., 2001).

As *Cdx1* is a target of both Wnt and retinoid signaling pathways, it could conceivably serve as an intermediary in somitogenic events upstream of Notch. To this end, we compared expression of the Notch pathway components *Dll1*, *Lfng*, *Notch1*, *Mesp2* and *Hes5* between wild-type and LRE+RARE mutants. The vertebral fusions seen in LRE and LRE+RARE mutants involved the first three cervical vertebrae, which are derived from somites 5-7. As the somite determination front is located in a region corresponding to four prospective somites in the unsegmented paraxial mesoderm (Dubrulle et al., 2001), expression patterns were compared using embryos of zero to five somites. No overt alterations in transcript levels or distribution were observed for these particular markers in these mutant backgrounds (see Fig. S1 in the supplementary material and data not shown).

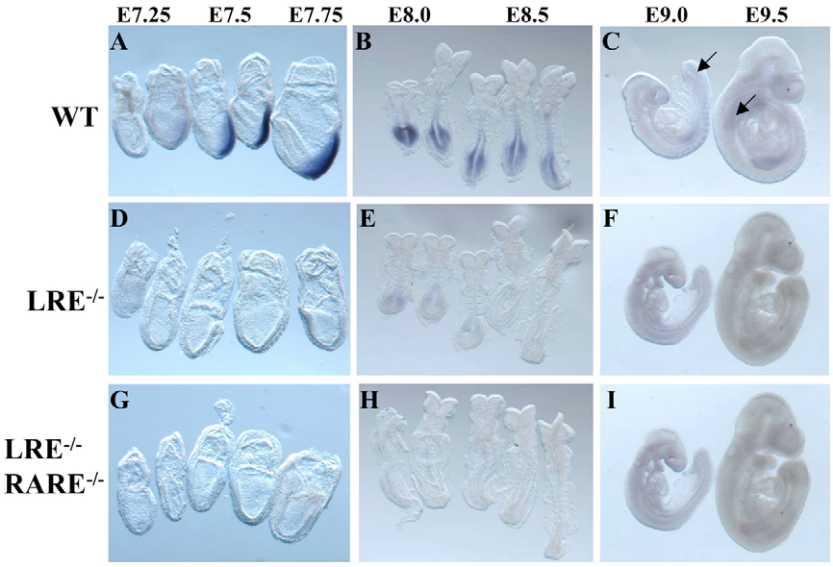

Fig. 3. *Cdx1* expression in LRE and LRE+RARE mutants. Whole-mount in situ hybridization analysis of *Cdx1* expression in wild-type mouse embryos (**A-C**), and in LRE (**D-F**) and LRE+RARE (**G-I**) homozygous mutant embryos. All embryos were processed in parallel, although mutant specimens were stained four times longer than the wild type to reveal residual expression. Approximate chronological stage is shown above. (A,D,G). The onset of *Cdx1* expression at late primitive streak in wild-type controls (A) was delayed to early head-fold stage in the mutants (D,G), and subsequently transcript levels were reduced (compare wild-type controls in B with mutants in E,H). *Cdx1* transcripts became undetectable after the 4- to 5-somite stage in both mutant lines (E,F,H,I), whereas expression was readily detected in wild-type controls (B,C). Arrows in C indicate *Cdx1* expression in mesoderm in the tailbud region at E9.5.

DISCUSSION

We and others have previously documented that *Cdx1* is a Wnt target gene, and that the Wnt signal is likely to manifest, at least in part, through two LREs in the proximal promoter (Lickert et al., 2000; Lickert and Kemler, 2002; Prinos et al., 2001). *Cdx1* is also regulated by RA through an atypical RARE, and mutagenesis studies clearly demonstrate a role for retinoid signaling in directing a subset of *Cdx1* expression. Moreover, strong synergistic interaction between RA and Wnt signaling on transcription from the *Cdx1* promoter has been described in embryocarcinoma cells, and this interaction is dependent on the LRE and RARE (Prinos et al., 2001). To more precisely address the role of Wnt and RA signaling in the regulation of *Cdx1* expression, we derived and analyzed murine lines lacking the LRE or LRE+RARE.

We used previously described point mutations to functionally inactivate the *Cdx1* LRE. To minimize potential secondary effects on the proximal promoter, the selection cassette was cloned distally in intron one. Some enhancer activity has been attributed to intron one sequences of the chick *Cdx1* homolog *CdxA* (Gaunt et al., 2003). These sequences are conserved in the mouse, but are approximately 1 kb 3′ of the residual *loxP* sequences. It would therefore appear unlikely that these exogenous *lox* sequences would impact on *Cdx1* expression through interference with these enhancer sequences, although this has not been formally tested.

Retinoid signaling has been implicated in early stages of *Cdx1* expression (Houle et al., 2000; Houle et al., 2003), whereas Wnt signaling and autoregulation have been suggested to be involved at both early and later stages (Lickert and Kemler, 2002; Beland et al.,

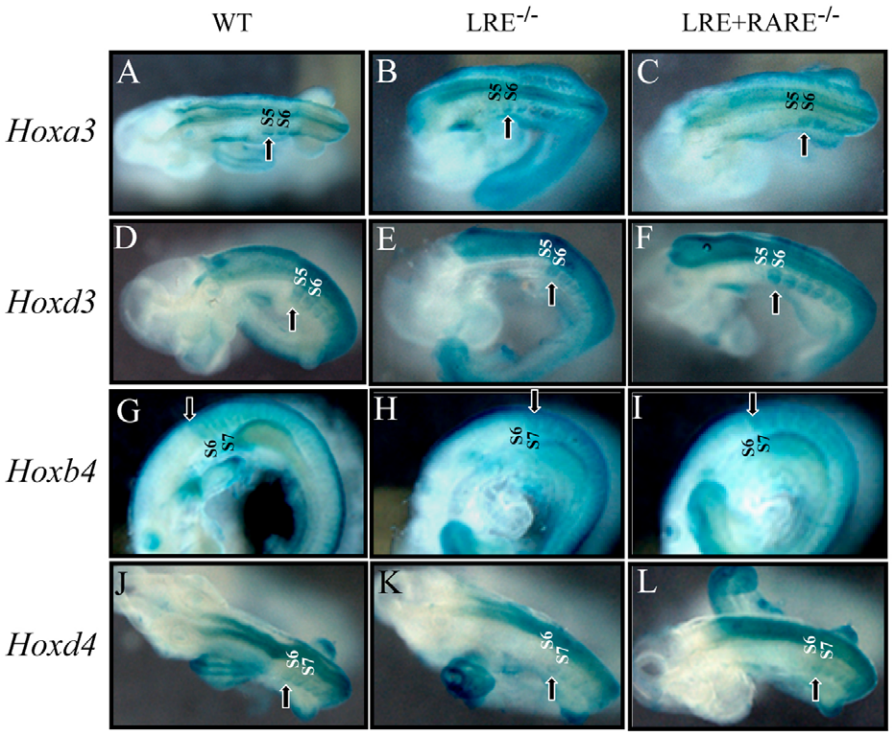

Fig. 4. Hox gene expression in LRE and LRE+RARE mutants. Expression of (**A-C**) *Hoxa3*, (**D-F**) *Hoxd3*, (**G-I**) *Hoxb4* and (**J-L**) *Hoxd4* in E9.5 wild-type (A,D,G,L), LRE mutant (B,E,H,K) and LRE+RARE mutant (C,F,I,L) mouse embryos. The rostral somitic expression boundary (arrows) was posteriorized by one somite for each Hox gene examined in both mutant backgrounds, as compared with wild-type controls. S, somite.

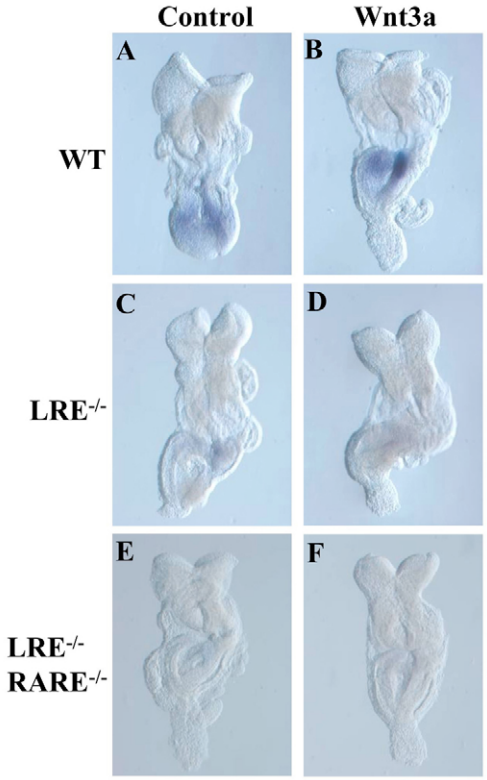

Fig. 5. Regulation of *Cdx1* by Wnt3a. Expression of *Cdx1* in E8.5 wild-type (**A**,**B**), LRE mutant (**C**,**D**) and LRE+RARE mutant (**E**,**F**) mouse embryos following ex vivo culture with control (A,C,E) or Wnt3a-conditioned (B,D,F) media. Embryos of a given genotype were cultured and stained in parallel. Mutants were stained four times longer than controls to reveal residual expression of *Cdx1*.

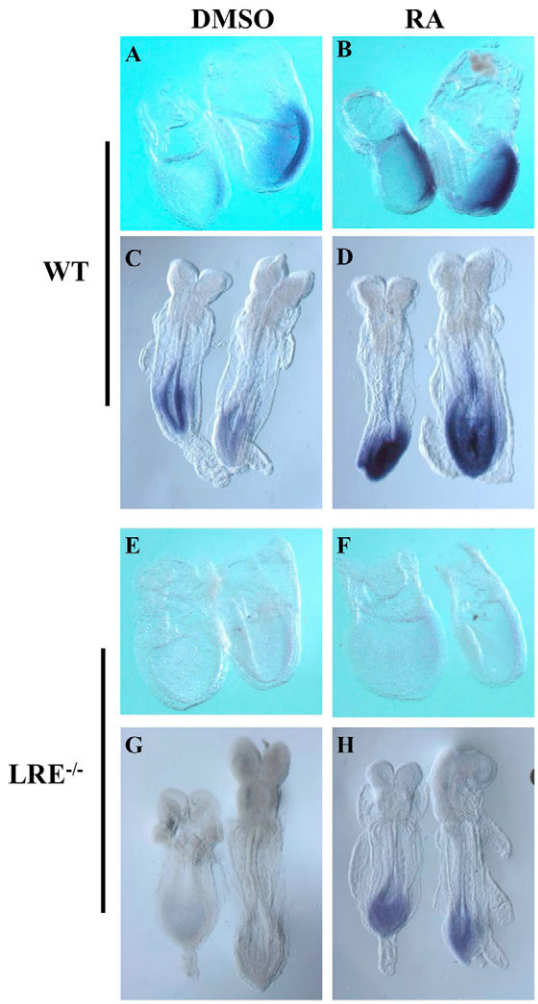

Fig. 6. Regulation of *Cdx1* by RA. Expression of *Cdx1* in E7.5 (**A**,**B**,**E**,**F**) or E8.5 (**C**,**D**,**G**,**H**) wild-type (A-D) and LRE mutant (E-H) mouse embryos following in utero treatment with DMSO vehicle (A,C,E,G) or RA (100 mg/kg; B,D,F,H). Embryos were processed and stained in parallel, with mutants stained four times longer than controls to reveal residual expression.

2004; Prinos et al., 2001). In agreement with this, we found that the LRE mutants exhibited skeletal defects affecting the whole cervical region that essentially phenocopied the malformations observed in *Cdx1*-null mutants. Consistent with these observations, in situ hybridization analysis revealed that *Cdx1* expression was markedly reduced throughout its normal window of expression. These data further suggest that Wnt signaling might be required even before RA, as loss of the LRE impacts on C1 patterning, whereas C2 is the first vertebra affected in RARE mutants. However, as other RAREs might be involved in regulating *Cdx1* (Houle et al., 2003; Gaunt et al., 2003), the full import of retinoid signaling as regards *Cdx1* expression is likely to remain unresolved at present. Irrespective, these findings underscore a crucial role for Wnt signaling in regulating *Cdx1*.

Prior analysis of Wnt in affecting *Cdx1* in vivo utilized primarily the *Wnt3a*$^{vt\text{-}vt}$ hypomorph, which exhibits a low incidence of vertebral defects and a marginal reduction in *Cdx1* expression (Prinos et al., 2001; Ikeya and Takada, 2001). *Wnt3a*-null mutants also exhibit a reduction in *Cdx1* expression and a completely penetrant C2 to C1 transformation (Ikeya and Takada, 2001). However, other vertebral defects observed in *Cdx1*-null mutants are not manifested in either *Wnt3a*$^{vt\text{-}vt}$ or *Wnt3a*-null offspring. This is in contrast to the phenotype of LRE mutants, suggesting that *Cdx1* is regulated by other Wnts present in the primitive streak/tail bud, such as Wnt3 or Wnt8 (Bouillet et al., 1996; Liu et al., 1999; Takada et al., 1994). Conversely, the residual *Cdx1* expression in LRE-null offspring, and the occasional minimal response to exogenous Wnt3a seen in embryo culture (data not shown), suggests the presence of other functional motifs conveying the Wnt signal. Indeed, additional potential LREs have been described in the 5′ promoter region of the *Cdx1* locus (Lickert et al., 2000). These other putative elements are, however, clearly unable to effectively compensate in the absence of the proximal LRE sequences.

Combinatorial signaling and *Cdx1* expression

The importance of the *Cdx1* RARE and LRE in directing expression in vivo has been suggested by transgenic studies (Lickert and Kemler, 2002), and a definitive role for the RARE has been further demonstrated by targeted mutagenesis (Houle et al., 2003). In this regard, loss of the RARE has a limited effect on *Cdx1* expression in vivo and affects morphogenesis of only the second cervical vertebra. By contrast, mutation of the LRE impacted on *Cdx1* expression at all stages and resulted in a nearly complete recapitulation of the *Cdx1*-null phenotype. Subsequent loss of the RARE in an LRE mutant background resulted in only a slight additional effect on expression and vertebral patterning, as expected from the dominant

impact of the LRE mutation. The combinatorial contribution of Wnt and retinoid signaling to *Cdx1* expression and function was, however, clearly evidenced by comparison of LRE$^{+/-}$ and LRE+RARE$^{+/-}$ skeletons, where a more complete C2 to C1 transformation was observed in the latter background.

A strong synergy between retinoid and Wnt signaling in inducing transcription from the *Cdx1* promoter has been observed in tissue culture models, and this interaction requires both the RARE and the LRE (Prinos et al., 2001). Although RA still induces *Cdx1* in vivo in RARE mutants, perhaps owing to other RAREs (Houle et al., 2003; Gaunt et al., 2003), the response to RA was greatly attenuated in the LRE mutant background in the present study. This suggests that retinoid signaling is reliant on the LRE to mediate full induction of *Cdx1* transcription. Although the mechanism underlying this cooperativity is speculative, it might relate to the architectural role of Lef/Tcf transcription factors (Dragan et al., 2004), or might be indicative of chromatin modification events affected by Wnt signaling, which is essential for productive interaction of the retinoid receptors with the promoter. In any event, this finding clearly underscores a cooperative role for Wnt and RA in the regulation of *Cdx1* in vivo.

Cdx1 and Hox gene expression

In situ hybridization analysis of LRE-null and LRE+RARE-null embryos revealed identical posterior shifts in expression of Hox paralog group 3 and 4 genes, as has been observed in *Cdx1*-null mutants (Houle et al., 2003; van Den et al., 2002). By contrast, only Hox group 4 genes are affected in RARE mutants. In this regard, *Cdx1* expression in RARE-null mutants is properly initiated but transcript levels are reduced (Houle et al., 2003). This suggests Hox paralog group 3 genes might require a lower threshold of *Cdx1* activity than group 4 genes, leading to normal C1 patterning in RARE mutants, as previously discussed (Houle et al., 2003) (see also Charité et al., 1998; Gaunt et al., 2003; Gaunt et al., 2004; Marom et al., 1997; Deschamps and van Nes, 2005). However, timing of expression is also an essential component of Hox-mediated skeletal patterning (Juan and Ruddle, 2003; Dollé et al., 1989). The delay in onset of *Cdx1* transcription seen in the LRE and LRE+RARE mutants might therefore underlie the defects in morphogenesis of C1, although it is difficult to differentiate such a mechanism from dosage effects.

Is Cdx1 function linked to somite segmentation?

LRE and LRE+RARE mutants exhibit a high incidence of fusions between the first three cervical vertebrae, suggesting a link between Cdx1 and somitogenesis. The lack of similar defects in *Cdx1*-null mutants might be related to residual function in the LRE and LRE+RARE mutant backgrounds, eventually impacting on events related to sclerotome patterning. In this regard, it has been shown that Hox gene expression is subject to Notch-mediated signaling in the anterior presomitic mesoderm (Zakany et al., 2001), and also that Notch functions downstream of Wnt signaling in this domain. Moreover, haploinsufficiency of Dll1, as well as gain or loss of Lfng function, results in homeotic transformations affecting the cervical and thoracic vertebrae (Cordes et al., 2004). As *Cdx1* is a direct Wnt target, it is conceivable that its product could contribute to Wnt-dependent regulation of Notch signaling processes involved in somitogenesis. However, in situ hybridization analysis did not reveal any overt alterations in *Delta1*, *Notch1*, *Hes5* or *Lfng* in LRE+RARE mutant embryos, although we cannot exclude the possibility that their oscillatory patterns of expression might be subtly affected.

We thank P. Gruss, M. Capecchi, M. Featherstone, I. Thesleff, C. C. Hui, O. Pourquié and G. Martin for kind gifts of plasmids; Qinzhang Zhu and Michel Robillard for microinjection; and A. Nagy for R1 cells. This work was supported by funding from the CIHR. N.P. is supported by a postdoctoral fellowship from the CIHR and D.L. by the FRSQ.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/12/2315/DC1

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