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Cdx4 is a Cdx2 target gene

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ABSTRACT

The products of the *Cdx* genes, Cdx1, Cdx2 and Cdx4, play multiple roles in early vertebrate development, and have been proposed to serve to relay signaling information from Wnt, RA and FGF pathways to orchestrate events related to anterior-posterior vertebral patterning and axial elongation. In addition, Cdx1 and Cdx2 have been reported to both autoregulate and to be subject to cross regulation by other family members. We have now found that *Cdx4* expression is significantly down regulated in *Cdx2*$^{-/-}$ mutants suggesting previously unrecognized cross-regulatory interactions. Moreover, we have previously shown that Cdx4 is a direct target of the canonical Wnt signaling pathway, and that Cdx1 physically interacts with LEF/TCF members in an autoregulatory loop. We therefore investigated the means by which Cdx2 impacted on *Cdx4* expression and assessed potential interaction between Cdx2 and canonical Wnt signaling on the *Cdx4* promoter. We found that the *Cdx4* promoter was regulated by Cdx2 in transient transfection assays. Electrophoretic mobility shift assays showed that Cdx2 bound to predicted Cdx response elements in the Cdx4 promoter which, when mutated, significantly reduced activity. Consistent with these data, chromatin immunoprecipitation assays from embryos demonstrated occupancy of the *Cdx4* promoter by Cdx2 *in vivo*. However, we failed to observe an interaction between Cdx2 and components of the canonical Wnt signaling pathway. These findings suggest that, while both canonical Wnt and Cdx2 can regulate the activity of the Cdx4 promoter, they appear to operate through distinct mechanisms.

1. Introduction

The vertebrate Cdx genes, *Cdx1*, *Cdx2* and *Cdx4*, encode homeodomain transcription factors related to the *Drosophila* gene *caudal*. In the mouse, Cdx genes are sequentially activated beginning at the late primitive streak stage leading to a nested expression pattern 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 in the nascent mesoderm with an anterior limit in the posterior hindbrain (Meyer and Gruss, 1993). *Cdx2* exhibits an early onset of expression in the trophectoderm at E3.5; expression 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 (Beck et al., 1995). At tail bud stages, *Cdx2* expression continues in the posterior neural plate and the endoderm, and is eventually confined to the hindgut endoderm posterior to the foregut/mid gut junction from E12.5 onwards (Duprey et al., 1988; Silberg et al., 2000). *Cdx4* is initially detected at E7.5 in the base of the allantois and posterior primitive streak and subsequently in the unsegmented paraxial mesoderm with a rostral limit posterior to the most recently formed somite (Gamer and Wright, 1993). *Cdx4* is also found in the neural ectoderm, with expression slightly more rostral than its domain in the paraxial mesoderm, and is extinguished by E10.5 (Lohnes, 2003).

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The complex developmental expression pattern of the Cdx genes suggest they play important roles in the early embryo. Functional studies reveal that Cdx members are critical regulators of antero-posterior (AP) patterning in a variety of vertebrate and invertebrate embryos (Chawengsaksophak et al., 1997; Duprey et al., 1988; Frumkin et al., 1991; Hunter and Kenyon, 1996; Mlodzik et al., 1985; Northrop and Kimelman, 1994; Savory et al., 2009a; Subramanian et al., 1995). Later in development, Cdx2 proteins regulate gut development and intestinal-specific gene expression, again impacting on patterning events along the AP gut axis (Chen et al., 2009; Faas and Isaacs, 2009; Flores et al., 2008; Grainger et al., 2010). Cdx2 and Cdx4 have also been implicated in certain pathological situations. For example, reduced levels of *Cdx2* in colon cancers correlates with enhanced tumor progression, suggesting that Cdx2 may play a tumor suppression role (Bonhomme et al., 2003; Brabletz et al., 2004; Mallo et al., 1997). Conversely, *Cdx4* has been shown to be aberrantly expressed in a subset of acute myeloid leukemia (AML) patients, indicative of a role in the etiology of this cancer (Bansal et al., 2006; Scholl et al., 2007). Given the important roles of *Cdx* genes in development, understanding the mechanisms governing their expression is of considerable importance.

Promoter analysis performed in diverse vertebrate model systems has shown that Cdx family members are downstream of a number of signaling pathways, including fibroblast growth factor (FGF), Wnt and retinoic acid (RA) (Lohnes, 2003). In particular, *Cdx1* has been shown to be directly regulated by both RA and canonical Wnt (Faas and Isaacs, 2009; Houle et al., 2000, 2003; Ikeya and Takada, 2001; Lickert et al., 2000; Pilon et al., 2007; Prinos et al., 2001) while *Cdx4* is also a direct target gene of Wnt signaling pathway (Pilon et al., 2006). More recent evidence suggests that Cdx and canonical Wnt comprise a positive feedback loop involved in elaboration of the posterior embryo (Faas and Isaacs, 2009; Savory et al., 2009a).

In addition to being subject to direct regulation by a number of signaling pathways, Cdx members also exhibit both auto- and cross-regulation (Beland et al., 2004; Bonhomme et al., 2008; Chawengsaksophak et al., 2004; Crissey et al., 2008; Lorentz et al., 1997; Prinos et al., 2001; Xu et al., 1999). In particular, *Cdx1* is required to maintain its own expression *in vivo* (Beland et al., 2004) while loss of *Cdx2* leads to a marked reduction in *Cdx4* (Chawengsaksophak et al., 2004; Savory et al., 2009a). *Cdx* genes are also subject to feedback inhibition. For example, overexpression of *Cdx2* can downregulate expression of *Cdx1 in vitro,* while Cdx2 has been shown to inhibit β-catenin-stimulated expression of *Cdx1* in human colon cancer cell lines (Domon-Dell and Freund, 2002). In this regard, recent work has shown that Cdx2 can bind to β-catenin and prevent its interaction with the Tcf transcription factors providing a possible explanation for this latter inhibitory function (Guo et al., 2010) Taken together, these data suggest that expression of Cdx genes is governed by complex and interactive sets of positive and negative regulatory mechanisms.

The loss of expression of *Cdx4* in *Cdx2* conditional null mutant embryos (Savory et al., 2009a), together with the overlapping expression of *Cdx* family members and demonstrated interaction between these genes, suggested a direct regulatory hierarchy between Cdx2 and *Cdx4*. In the present study, we present data in support of this concept. In addition, although Cdx4 is also a direct Wnt target (Pilon et al., 2006), and canonical Wnt and Cdx1 synergize to regulate *Cdx1* (Beland et al., 2004), no such interaction was seen on the Cdx4 promoter, suggesting that these two pathways operate independently in the context of this promoter.

2. Results

Cdx family members display an overlapping and dynamic pattern of expression along the developing axis from late gastrulation to tail bud stages. Analysis of the *Cdx2* conditional mutant revealed a significant reduction in the expression of *Cdx4* (Fig. 1 and Chawengsaksophak et al., 2004; Savory et al., 2009a). This observation, together with the auto- and cross-regulation previously observed between other Cdx members (Beland et al., 2004; Chawengsaksophak et al., 2004; Lorentz et al., 1997; Prinos et al., 2001; Xu et al., 1999), suggested that *Cdx4* lies downstream of Cdx2 during embryonic development. We now present evidence that *Cdx4* is a direct Cdx target gene.

2.1. *Cdx4 expression is reduced in Cdx2*$^{-/-}$ *conditional mutant embryos*

Cdx4 expression was examined by whole mount immunohistochemistry in wild type (WT), *Cdx1*$^{-/-}$, *Cdx2*$^{-/-}$ and *Cdx1/2* Double Knock out (DKO) mutants at embryonic day (E) 8.5. In agreement with previous findings (Chawengsaksophak et al., 2004; Savory et al., 2009a), Cdx4 expression was significantly reduced in the *Cdx2* mutant background (Fig. 1 compare C with D); note that the rostral limit of Cdx4 was also reduced in the Cdx2 null embryo. This reduction in protein levels was likely a consequence of reduced *Cdx4* transcript abundance, as suggested by semi-quantitative RT-PCR analysis using RNA from caudal explants of E8.5 embryos (Fig. 1G). In contrast, Cdx4 expression was only marginally affected in *Cdx1*$^{-/-}$ embryos relative to controls (Fig. 1A and B) suggesting a minor role for Cdx1 in regulating Cdx4 expression. Expression was not detectably perturbed in *Cdx2* heterozygous embryos (Fig. 1G) suggesting that a single copy of Cdx2, together with Cdx1, is sufficient to maintain expression. Conversely, we observed an almost complete loss of Cdx4 expression in *Cdx1/2* double mutants (Fig. 1F and G) consistent with contributions from both Cdx1 and Cdx2 on the Cdx4 promoter.

2.2. *Cdx1 and Cdx2 localize to the Cdx4 promoter in vivo and regulate the promoter in vitro*

To further explore the basis for regulation of *Cdx4* we first sought to determine whether Cdx members occupied the Cdx4 promoter *in vivo*. To this end, ChIP analysis, using chromatin from E8.5 embryos, revealed that both Cdx1 and Cdx2 occupied the proximal region of the *Cdx4* promoter *in vivo* (Fig. 2C). Binding was specific to this interval, as similar association was not seen in immunoprecipitations with non-specific and control antibodies (Fig. 2C).