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Cdx regulates Dll1 in multiple lineages

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ABSTRACT

Vertebrate *Cdx* genes encode homeodomain transcription factors related to *caudal* in *Drosophila*. The murine *Cdx* homologues *Cdx1*, *Cdx2* and *Cdx4* play important roles in anterior–posterior patterning of the embryonic axis and the intestine, as well as axial elongation. While our understanding of the ontogenic programs requiring Cdx function has advanced considerably, the molecular bases underlying these functions are less well understood. In this regard, *Cdx1-Cdx2* conditional mutants exhibit abnormal somite formation, while loss of Cdx1-Cdx2 in the intestinal epithelium results in a shift in differentiation toward the Goblet cell lineage. The aim of the present study was to identify the Cdx-dependent mechanisms impacting on these events. Consistent with prior work implicating Notch signaling in these pathways, we found that expression of the Notch ligand *Dll1* was reduced in Cdx mutants in both the intestinal epithelium and paraxial mesoderm. Cdx members occupied the *Dll1* promoter both *in vivo* and *in vitro*, while genetic analysis indicated interaction between Cdx and Dll1 pathways in both somitogenesis and Goblet cell differentiation. These findings suggest that Cdx members operate upstream of *Dll1* to convey different functions in two distinct lineages.

Introduction

The endoderm, ectoderm and mesoderm germ layers are formed during gastrulation, with each contributing to distinct cellular lineages. For example, the vertebrae of the axial skeleton and their associated muscles and tendons, the skeletal muscles of the body wall and limbs, as well as the dermis of the back are derived from transient blocks of paraxial mesoderm, known as somites. Somites are produced in a periodic fashion via condensation of paraxial presomitic mesoderm, with a new somite pair being formed approximately every 120 min in the mouse. Once generated, somites undergo subsequent differentiation into dermatome, myotome, and sclerotome, which are the anlagen of the dermis, skeletal muscle of the trunk and limbs, and vertebrae, respectively (Dequeant and Pourquie, 2008).

The periodicity of somite condensation and their placement along the axis are under tight spatio-temporal regulation. The positioning of somite condensation is believed to rely on the interaction of caudal-high gradients of fibroblast growth factors (FGFs) and Wnt proteins, which are opposed by a gradient of retinoic acid (RA) produced in more anterior regions. These opposing cues are believed to establish a determination front which dictates the location of somite condensation along the A-P axis (Olivera-Martinez and Storey, 2007; Wahl et al., 2007). The periodicity of somite segmentation is governed by a molecular "clock", the activity of which is reflected by the oscillating expression of a number of genes. For example, members of the Notch pathway including *Lfng, Hes1*, and *Hes7*; Wnt pathway components such as *Axin2;* and Fgf pathway members *Spry2* and *Dusp6* (Feller et al., 2008; Gomez et al., 2008; Gridley, 2006) oscillate during somitogenesis. Expression of many of these genes is typically initiated in the caudal region of the tailbud and propagate anteriorly through the presomitic mesoderm to the determination front, where segmentation is initiated (Olivera-Martinez and Storey, 2007).

Like the mesoderm, the endoderm also undergoes extensive patterning along the A-P axis, leading to the mature gastrointestinal tract. This patterning is reflected by the regionalization of the intestinal epithelium into esophagus, stomach, small and large intestines, as well as the development of accessory organs such as the pancreas, liver and salivary glands (Barrow, 2006; Kwon et al., 2008; Rajewsky, 2006; Wells and Melton, 1999). Endoderm patterning is incompletely understood, but relies on a number of transcription factors including Cdx2, as well as signaling molecules such as Wnt, Shh and RA, which emanate from both the endoderm and the underlying mesoderm (Bayha et al., 2009; Bergsten et al., 2001; Gao et al., 2009; Grainger et al., 2010; Wells and Melton, 1999; Zacchetti et al., 2007).

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The mature small intestinal epithelium is composed of villi and invaginating crypts which define the crypt-villus axis, and is maintained by intestinal stem cells in two distinct populations at or near the base of each crypt (Barker et al., 2007; Sangiorgi and Capecchi, 2008). These stem cells give rise to rapidly proliferating transit-amplifying cells which subsequently exit mitosis and differentiate into the mature cells of the intestinal epithelium. These are grouped into either absorptive cells (enterocytes) or the secretory cells (Goblet, enteroendocrine and Paneth cells) (Wells and Melton, 1999).

The intestinal mucosa is the most rapidly renewing epithelium in the body. Turnover occurs every 5–7 days in the mouse, with the exception of the Paneth cells which reside in the base of the crypt for approximately 21 days (Wells and Melton, 1999). This rapid turnover necessitates tight coordination of proliferation and differentiation of precursor populations, which is regulated by a transcription network that includes Wnt and Notch pathways, among others (Crosnier et al., 2006; de Lau et al., 2007; Fre et al., 2005).

In the canonical pathway, Notch receptors bind Delta-Serrate-Lag-2 (DSL) ligands, which include the Delta-like (Dll) homologues in mammals. Ligand binding to the Notch receptor initiates two proteolytic cleavages leading to the release and nuclear translocation of the Notch intracellular domain (NICD). NICD then participates in the transcriptional regulation of target genes via association with CSL transcription factors (D'Souza et al., 2008; Fortini, 2009). In the intestine, both the maintenance of the intestinal stem cell niche and differentiation of the transit-amplifying (TA) cell population are dependent on Notch signaling (Pellegrinet et al., 2011; Wells and Melton, 1999). In particular, Notch activation of *Hes1* favors enterocyte differentiation, while high *Math1* activity results in differentiation into the secretory lineages. Consistent with this, loss of *Math1* results in a depletion of secretory lineages without affecting enterocytes (Yang et al., 2001), while loss of *Hes1* results in an increase in secretory lineages at the expense of enterocytes (Jensen et al., 2000). Cross-talk between these two transcription factors is also evidenced by the finding that Hes1 represses *Math1* expression (Jensen et al., 2000; Zheng et al., 2000). Involvement of Notch signaling in intestinal differentiation is further underscored by the outcome of *Pofut1* or *RBP-J* mutation, gamma secretase inhibition or through simultaneous loss of either *Notch1* and *Notch2* or *Dll1* and *Dll4,* all of which bias TA cell differentiation into Goblet cells (Guilmeau et al., 2008; Pellegrinet et al., 2011; Riccio et al., 2008c; van Es et al., 2005).

Cdx genes encode homeodomain transcription factors related to *Drosophila caudal*. The three *Cdx* murine homologues, *Cdx1*, *Cdx2* and *Cdx4,* exhibit overlapping patterns of expression in the posterior embryo and play overlapping roles in vertebral patterning, axial elongation, and neural tube closure (Beck, 2004; Beck et al., 1995; Lohnes, 2003; Savory et al., 2009b, 2011a; van den Akker et al., 2002; van Nes et al., 2006). Cdx2 also plays key roles in patterning of the definitive endoderm and, together with Cdx1, is essential for maintenance of the intestinal epithelium in the adult (Beck et al., 1999; Gao et al., 2009; Grainger et al., 2010; Verzi et al., 2010, 2011).

Loss of Cdx2, or both Cdx1 and Cdx2, results in an increase in Goblet cells in the small intestine as well as defects in somitogenesis (Crissey et al., 2011; Savory et al., 2009a, 2011a; Verzi et al., 2011). Consistent with a role for Notch signaling in these processes, we found that expression of *Dll1* was compromised in both the intestinal epithelium and in the tail bud of Cdx mutant embryos, with concomitant impact on downstream differentiation effectors in the intestine. Non-allelic non-complementation studies revealed a genetic interaction between *Cdx* and *Dll1* mutant alleles in both paraxial mesoderm and in the intestinal epithelium. Finally, we identified two potential Cdx response elements in the *Dll1* promoter, and found that Cdx proteins occupy this region *in vivo*. These findings are consistent with *Dll1* operating downstream of Cdx members, possibly through a direct regulatory interaction. This study illustrates that Cdx function can manifest, in part, through a common molecular pathway in distinct lineages.

Materials and methods

Mice

Cdx1$^{-/-}$, *Cdx2*$^{f/f}$, *Dll1*$^{f/f}$, *actin*-Cre ER^{T} and *villin*-Cre ER^{T} mice have been previously described (Brooker et al., 2006; el Marjou et al., 2004; Santagati et al., 2005; Savory et al., 2009a; Subramanian et al., 1995). *Cdx2* deletion was effected by Tamoxifen (Tam) administration at E13.5 in *villin*-Cre ER^{T} (Grainger et al., 2010) or at E5.5 in *actin*-Cre ER^{T} (Savory et al., 2009a) backgrounds. Embryos were subsequently harvested at E6.5-E9.5 for investigation of somitogenesis, while gastrointestinal tracts were harvested at E18.5. Non-transgenic littermates were used as controls in both instances.

Histological analysis

E18.5 intestinal tracts were sectioned and processed for histological staining as previously described (Grainger et al., 2010). Slides were mounted using Permount (Fisher) and images captured using a Zeiss Mirax Midi Scanner (Zeiss). Goblet cells were quantified as PAS-positive cells relative to the total number of nuclei captured from 5 random fields from each sample. Data was accrued from a minimum of 3 independent samples.

In situ hybridization

In situ hybridization (ISH) of gastrointestinal sections was carried out as previously described (Grainger et al., 2010) using probes for *Hes1* and *Dll1* (Schroder and Gossler, 2002). The probe for *Math1,* corresponding to the first 500 bp of the transcript, was derived by RT-PCR. Whole mount ISH was performed as previously described, with probes for *Mox1*, *Uncx4.1* and *Paraxis* (Houle et al., 2000; Savory et al., 2011a). Embryos were photographed using a Leica MZ16FA microscope.

Quantitative polymerase chain reaction (qPCR) and semi-quantitative reverse-transcriptase PCR (RT-PCR)

RNA was extracted from embryonic (E)18.5 small intestine using Trizol reagent (Invitrogen) and used to generate cDNA by standard procedures. cDNA was subsequently amplified by semi-quantitative RT-PCR or qPCR using oligonucleotides specific for *Dll1*, *Math1*, *TFF3, IFABP* or *β-actin* with SsoFast EvaGreen Supermix (qPCR, BioRad) or GoTaq (RT-PCR, Promega), according to the manufacturer's recommendations. qPCR was performed using the MX3005P (Agilent Technologies) and results were analyzed using the $2^{-\Delta\Delta Ct}$ method (Schefe et al., 2006), normalized to *β-actin.* For specificity, the dissociation curve was considered for each amplicon. RT-PCR was performed over a series of cycles and samples within the linear range used for analysis. Data in both cases is reflective of at least 3 different biological sample sets (specific PCR conditions and primer sequences available in supplementary information).

Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described (Pilon et al., 2006) using chromatin generated from wild-type E8.5 embryos or E18.5 intestinal tracts. PCR was directed over regions encompassing potential CDREs, or distal (control) intervals by standard methods. Oligonucleotide sequences used for amplification are available in supplementary information.

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out as previously described (Houle et al., 2000; Pilon et al., 2006). GST or GST-Cdx2 fusion proteins were used