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RESEARCH ARTICLE

Role of Cdx factors in early mesodermal fate decisions

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ABSTRACT

Murine cardiac and hematopoietic progenitors are derived from *Mesp1*$^+$ mesoderm. Cdx function impacts both yolk sac hematopoiesis and cardiogenesis in zebrafish, suggesting that Cdx family members regulate early mesoderm cell fate decisions. We found that Cdx2 occupies a number of transcription factor loci during embryogenesis, including key regulators of both cardiac and blood development, and that Cdx function is required for normal expression of the cardiogenic transcription factors *Nkx2-5* and *Tbx5*. Furthermore, Cdx and Brg1, an ATPase subunit of the SWI/SNF chromatin remodeling complex, co-occupy a number of loci, suggesting that Cdx family members regulate target gene expression through alterations in chromatin architecture. Consistent with this, we demonstrate loss of Brg1 occupancy and altered chromatin structure at several cardiogenic genes in Cdx-null mutants. Finally, we provide evidence for an onset of Cdx2 expression at E6.5 coinciding with egression of cardiac progenitors from the primitive streak. Together, these findings suggest that Cdx functions in multi-potential mesoderm to direct early cell fate decisions through transcriptional regulation of several novel target genes, and provide further insight into a potential epigenetic mechanism by which Cdx influences target gene expression.

KEY WORDS: Cdx, Mouse, Mesoderm, Cell fate, Transcription, Chromatin

INTRODUCTION

The murine Cdx genes, *Cdx1*, *Cdx2* and *Cdx4*, encode homeodomain transcription factors related to *caudal* in *Drosophila*. With the exception of early expression of *Cdx2* in the trophectoderm of the pre-implantation embryo, Cdx family members are sequentially activated during gastrulation commencing at embryonic day (E) 7.5, forming a nested expression domain in the primitive streak and subsequently in all three germ layers of the tail bud until approximately E13.5 (Beck, et al., 1995; Gamer and Wright, 1993; Meyer and Gruss, 1993; Strumpf et al., 2005). Cdx gene expression is then extinguished in all tissues except the hindgut endoderm and derivative intestinal epithelium, where *Cdx1* and *Cdx2* are maintained throughout adulthood (Silberg et al., 2000).

Whereas *Cdx2*$^{-/-}$ mutants are peri-implantation lethal, owing to a requirement for this transcription factor in the trophectoderm, *Cdx1*$^{-/-}$ and *Cdx2*$^{+/-}$ mice are viable and exhibit homeotic transformations of the cervical and thoracic vertebrae with concomitant shifts in Hox gene expression (Subramanian et al., 1995; Chawengsaksophak et al., 1997). *Cdx4*-null mutants are phenotypically normal; however, the loss of Cdx4 exacerbates *Cdx1*$^{-/-}$ or *Cdx2*$^{+/-}$ phenotypes, indicative of functional overlap between Cdx family members (Faas and Isaacs, 2009; Savory et al., 2011; van den Akker et al., 2002; van Nes et al., 2006; Young et al., 2009). The derivation and analysis of *Cdx2* conditional mutants (Savory et al., 2009a; Stringer et al., 2012) and compound mutant derivatives (van den Akker et al., 2002; Savory et al., 2011; Verzi et al., 2011; van Rooijen et al., 2012; Stringer et al., 2012; Grainger et al., 2010; Hryniuk et al., 2012) further substantiated the functional overlap between family members. Analysis of these mutants also revealed a requirement for Cdx genes in diverse processes in development and in the adult intestinal track. These include, for example, roles for Cdx proteins in specification and development of the intestine and its homeostasis in the adult (Beck et al., 1999, 2003; Chawengsaksophak et al., 1997; Gao et al., 2009; Grainger et al., 2010; Stringer et al., 2012; Hryniuk et al., 2012), yolk sac hematopoiesis (Lengerke et al., 2007; Wang et al., 2008; Brooke-Bisschop et al., 2017; Davidson and Zon, 2006; Davidson et al., 2003), neural tube closure, and elaboration of the post-otic embryo (Savory et al., 2009a, 2011; van Rooijen et al., 2012).

Although Cdx function has been shown to be crucial for numerous developmental programs, our understanding of the target genes governing these programs is incomplete. Cdx family members regulate target gene transcription through binding to a cognate response element (CDRE), of the consensus TTTATG, either in the proximity of their target genes (Suh et al., 1994; Subramanian et al., 1995) or at distal enhancer elements (Maier et al., 2005; Taylor et al., 1997; Gaunt, 2017). Although Cdx family members typically positively regulate transcription, there is a growing body of evidence that suggests they can also act as transcriptional repressors. The means by which Cdx factors regulate transcription is also poorly understood, although Cdx function has been shown to impact chromatin architecture (Verzi et al., 2013, 2010; Saxena et al., 2017; Neijts et al., 2017), consistent with their association with Brg1 (Smarca4) and other members of the SWI/SNF chromatin remodeling complex (Yamamichi et al., 2009; Nguyen et al., 2017).

In agreement with their functional overlap (van den Akker et al., 2002; Davidson and Zon, 2006; van Nes et al., 2006; Faas and Isaacs, 2009), Cdx family members appear comparable in their ability to occupy, and regulate, target genes (Charité et al., 1998; Savory et al., 2009a). Targets identified to date include a number of Hox genes, implicated in Cdx-dependent vertebral patterning (Shashikant et al., 1995; Charité et al., 1998; Subramanian et al., 1995; Pownall et al., 1996; Isaacs et al., 1998; van den Akker et al., 2002; Gaunt et al., 2003, 2008), *Cyp26A1*, which is essential for catabolism of retinoic acid (RA) and crucial for the maintenance of a caudal progenitor population driving axial elongation (Wingert et al., 2007; Savory et al., 2009a; Young et al., 2009; Martin and Kimelman, 2010), genes encoding products involved in intestinal differentiation and homeostasis (Beck et al., 1999, 2003; Chawengsaksophak et al., 1997; Gao et al., 2009; Grainger et al., 2010; Hryniuk et al., 2012), and targets necessary for yolk sac hematopoiesis (Davidson et al., 2003; Davidson and Zon, 2006; Lengerke et al., 2007; Wang et al., 2008; Brooke-Bisschop et al., 2017).

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To establish a more comprehensive understanding of Cdx function during mouse development, we conducted chromatin immunoprecipitation coupled with deep sequencing (ChIP-seq) using material from E8.5 embryos. This analysis revealed Cdx2 to be highly enriched at the loci of genes encoding numerous transcription factors, among which were a number of transcription factors involved in hematopoiesis and cardiogenesis. Consistent with this, we found that loss of Cdx function leads to heart anomalies and mis-expression of several cardiogenic genes, including gain of expression of cardiac markers in Cdx mutant yolk sacs concomitant with loss of expression of genes involved in yolk sac hematopoiesis. Finally, Brg1, a catalytic subunit of the SWI/SNF chromatin remodeling complex required for mesodermal differentiation (Alexander et al., 2015) and which interacts functionally with Cdx2 (Yamamichi et al., 2009; Nguyen et al., 2017), colocalized with Cdx2 at a number of cardiogenic loci. Moreover, Brg1 binding and chromatin structure was altered at these genes in Cdx mutants. As Cdx is essential for yolk sac hematopoiesis (Davidson and Zon, 2006; Davidson et al., 2003; Lengerke et al., 2007; Wang et al., 2008; Brooke-Bisschop et al., 2017), these findings suggest that Cdx function regulates chromatin remodeling events necessary for the coordinated expression of transcription factors that direct hematopoietic versus cardiac fates from a common progenitor population.

RESULTS

Cdx2 occupancy is enriched at transcription factor loci

To understand the means by which Cdx family members regulate developmental programs, Cdx2 occupancy in the E8.5 embryo was assessed by chromatin immunoprecipitation followed by deep sequencing (ChIP-seq). Of the 13,991 total peaks returned from this analysis, Cdx2 binding was found to be distributed primarily in introns (32% of peaks) and proximal to transcriptional start sites (20% of peaks; Fig. 1A,B). Genomic regions associated with Cdx2 occupancy were also highly enriched for canonical CDREs (Fig. 1C; Dearolf et al., 1989; Suh et al., 1994; Subramanian et al., 1995).

Putative Cdx target genes were selected for further analyses using a minimum peak score of 800 as a cut-off, based on our recovery of previously characterized Cdx2 targets with peak scores ranging from 800 (e.g. *Dll1*; Grainger et al., 2012) to 3100 (e.g. *Scl*; Savory et al., 2011; Brooke-Bisschop et al., 2017). Gene ontology (GO) term enrichment analysis was conducted using the 441 peaks annotated to a reference gene that met this threshold. This analysis revealed that Cdx2-occupied loci were significantly enriched for genes involved in transcription, positive and negative regulation of gene expression, and cell fate commitment (Fig. 1D), suggesting that Cdx2 coordinates the expression of gene regulatory networks essential to embryonic development. Consistent with this, 226 of the 441 peaks were associated with genes encoding factors related to regulation of transcription, and six corresponded to genes involved in signaling pathways (*Wnt3*, *Wnt7b*, *Wnt11*, *Cyp26A1*, *Bmp2* and *Dll1*). This analysis likely excluded potential candidate targets, owing to the relatively high stringency used for cutoff, as well as additional targets that may not be accessible at the developmental stage employed for this analysis. It is, however, interesting to note that a number of annotated peaks that fall below the cutoff are also enriched for genes encoding transcription factors and signaling molecules (Table S1).

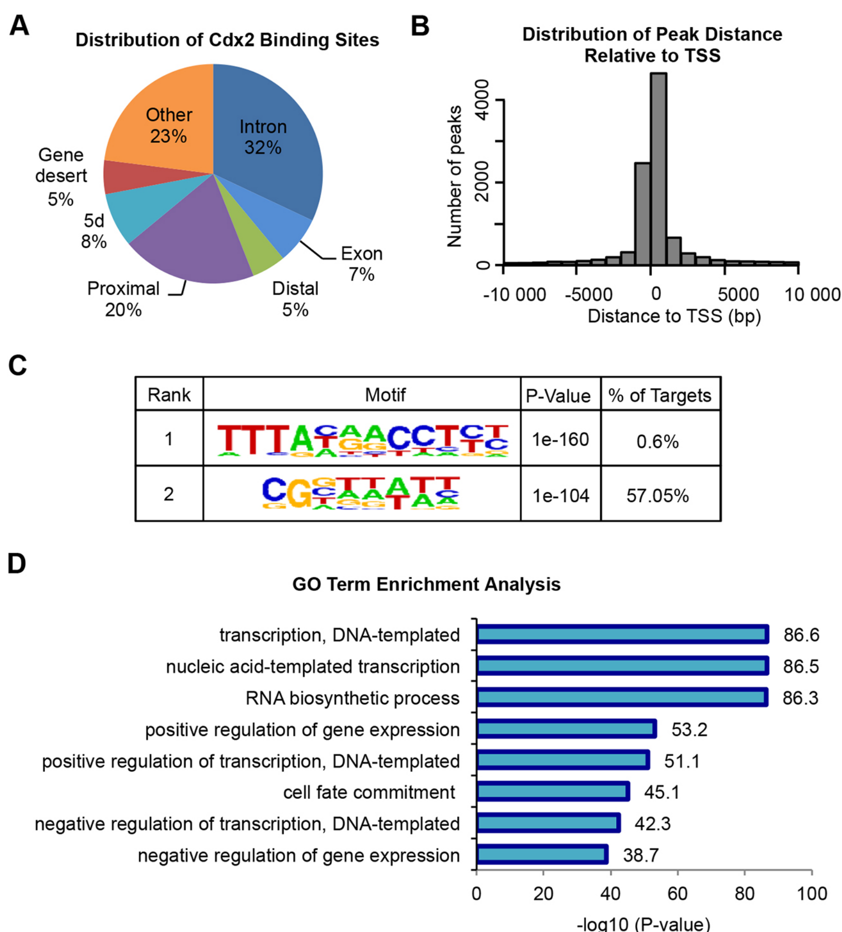

Fig. 1. Cdx2 binding distribution. (A) Relative distribution of Cdx2 genomic occupancy designated as proximal (within 2 kb of a TSS), distal (2-10 kb upstream of a TSS), 5d (10-100 kb upstream of a TSS), within gene deserts (≥100 kb upstream/downstream of a TSS), or other (all remaining peaks). (B) Distribution of peak distances relative to the nearest TSS. (C) Binding motifs with the greatest enrichment under Cdx2 peaks and their associated *P*-values. (D) GO term enrichment analysis of Cdx2-bound loci with peak scores of 800 or greater. A subset of GO terms with a fold enrichment of 5 or greater were considered and sorted by $\log_{10}$(*P*-value).

Among transcription-related targets, Cdx2 was enriched at a number of Hox genes (e.g. *Hoxa1*, *Hoxa2*, *Hoxb1*, *Hoxb2*, *Hoxb13*, *Hoxc4* and *Hoxd1*), consistent with the known roles for Cdx family members in vertebral patterning through regulation of Hox gene expression (Subramanian et al., 1995; Charité et al., 1998; van den Akker et al., 2002; Shashikant et al., 1995; Pownall et al., 1996; Isaacs et al., 1998; Gaunt et al., 2003, 2008). Certain transcriptional regulators of tissue patterning and specification were also enriched for Cdx2 occupancy (e.g. *Twist1*, *Irx2*, *Gsc*, *Pitx1*, *Pax1*, *Pax3*, *Pax9*, *Alx3*, *Sox1*, *Sox2*, *Foxa2* and *Foxd3*).

As previously described (Wang et al., 2008; Brooke-Bisschop et al., 2017), ChIP-seq identified *Scl* (*Tal1*), which encodes a master regulator of hematopoiesis, as a Cdx2 target (Fig. 2A; Shivdasani et al., 1995; van Handel et al., 2012; Org et al., 2015). In addition, other regulators of yolk-sac hematopoiesis, including *Lyl1*, *Lmo2* and *Meis1*, showed significant Cdx2 binding (Fig. 2A). Of particular note was the finding of a number of genes encoding cardiogenic transcription factors that were highly enriched for Cdx2 occupancy. Such genes encompassed some of the earliest known markers of cardiac progenitors, including *Gata4*, *Nkx2-5*, *Tbx5* and *Isl1*, as well as genes required for cardiac looping, such as *Hand1* and *Hand2* (Fig. 2B; reviewed by Srivastava, 2006).

We validated Cdx2 binding by ChIP-PCR within the genomic loci of select cardiac transcription factors, using primers flanking canonical CDREs within Cdx2 ChIP-seq peaks and chromatin isolated from E8.5 embryos (Fig. 2C). This analysis confirmed Cdx2 occupancy at a number of cardiac gene loci, including *Tbx5*, *Gata4*, *Hand1* and *Hand2* (Fig. 2D). Cdx2 binding to the promoter region of *Cyp26A1*, a previously characterized Cdx2 target (Savory et al., 2009a), was included as a positive control. Exon 4 of *Wnt3a* is not occupied by Cdx2 (Nguyen et al., 2017) and no increase in Cdx2 binding relative to IgG was seen at this region. Additionally, *Mef2c*, a cardiac marker specific to the second heart field (Verzi et al., 2005), was found to have a peak score of only 56.26 in the ChIP-seq dataset, and was not detected by ChIP-PCR. Thus, this locus served as a cardiac-specific negative control for Cdx2 binding in these experiments.

Cdx function is required for normal cardiac gene expression and heart tube morphogenesis

Cdx2$^{F/F}$*Actin::Cre-ER*^{T} mutants (Savory et al., 2009a) were crossed with germline Cdx1-nulls (Subramanian et al., 1995), generating *Cdx1*$^{-/-}$*Cdx2*$^{F/F}$*Actin::Cre-ER*^{T} compound mutants as previously described (Savory et al., 2011). Post-implantation *Cdx1-Cdx2* compound conditional null mutants (hereafter referred to as DKO) lose expression of *Cdx4*, becoming functionally Cdx-null and thus circumventing both the peri-implantation lethality of *Cdx2*-null mutation and functional overlap between family members (Savory et al., 2011, 2009a; van den Akker et al., 2002; van Nes et al., 2006;

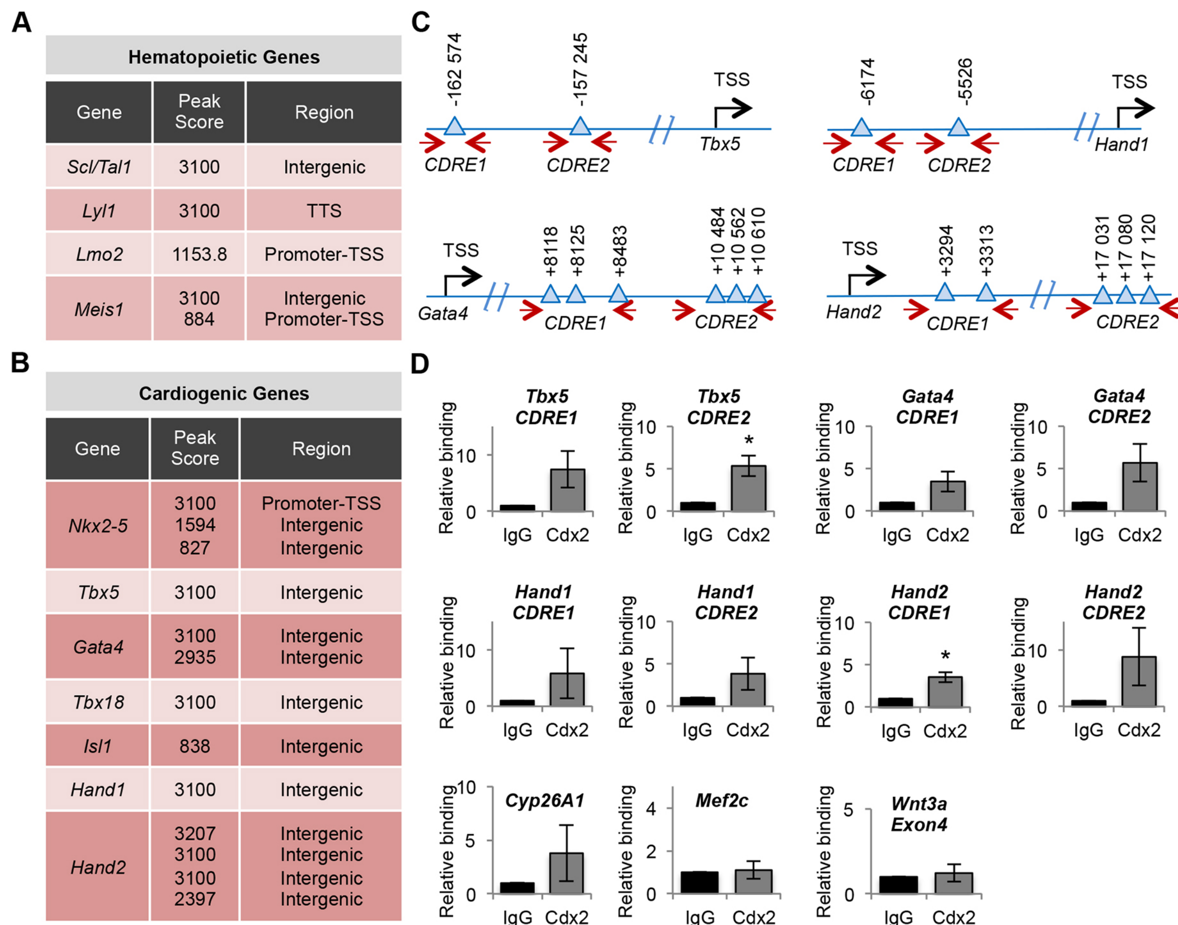

Fig. 2. Enriched Cdx2 binding at hematopoietic and cardiac gene loci. (A,B) Genes enriched for Cdx2 binding with known roles in hematopoiesis (A) or cardiogenesis (B) identified by ChIP-seq. Peak scores, indicative of the level of Cdx2 binding above background, are noted. (C) Schematics of CDREs, identified using TRANSFAC analysis of sequences under ChIP-seq peaks. Regions of complementarity for ChIP-PCR primers flanking CDREs (blue triangles) are indicated by red arrows. (D) Validation of Cdx2 binding at cardiac loci by ChIP-PCR using primers described in C. Error bars represent s.e.m. from three biological replicates. *Cyp26A1* was used as a positive control and *Wnt3a* Exon4 and *Mef2c* as negative controls. *$P \leq 0.05$ (determined by two-tailed *t*-test).

DEVELOPMENT

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Young et al., 2009). DKO mutants in which *Cdx2* deletion was induced at E5.5 (i.e. prior to the normal onset of embryonic Cdx expression) developed beating heart tubes at E9.5 but exhibited a distended pericardial sac and aberrant heart tube morphology, and typically died at E10.5 (Savory et al., 2011; Fig. 3A). Additionally, *in situ* hybridization (ISH) revealed that the ventricular marker *Mlc2v* (*Myl2*) (Franco et al., 1999) was reduced in intensity and spatially restricted in the mutant heart tubes relative to controls (Fig. 3B).

The heart defects observed in DKO mutants were variable, potentially as a result of differences in the embryonic stage at which *Cdx2* deletion was induced. To investigate this, tamoxifen was administered at E4.5 to assess whether loss of Cdx function at an earlier stage might exacerbate the cardiac phenotype. This earlier deletion, however, resulted in fully penetrant embryonic lethality, likely because of the requirement for Cdx2 during implantation (Chawengsaksophak et al., 1997). Although tamoxifen treatment at E6.5 also resulted in cardiac defects (Fig. S1), these were less severe than those elicited by Cdx2 deletion at E5.5, suggesting a narrow developmental window during which loss of Cdx function impacts heart development.

To characterize further the effect of Cdx loss of function on cardiogenesis, the expression of *Nkx2-5* and *Tbx5*, early markers of cardiac progenitors (Bruneau et al., 1999; Komuro and Izumo, 1993; Lints et al., 1993) and highly enriched for Cdx2 occupancy (Fig. 2B), were assessed by ISH. At E8.5, *Nkx2-5* transcripts were distributed in a triangle-like expression domain in DKO embryos rather than the cardiac crescent labeling typically observed in controls (Fig. 3C). At this stage, *Tbx5* transcripts were also localized to the cardiac crescent in control embryos, whereas DKO mutants exhibited broader lateral distribution of *Tbx5*-positive cells that tapered toward the midline (Fig. 3D). At E9.5, *Tbx5* expression in DKO embryos was even less spatially restricted relative to controls (Fig. 3E), consistent with prior observations in *cdx1a/4*-deficient zebrafish embryos (Lengerke et al., 2011).

Cdx represses cardiac gene expression in the yolk sac

Prior work has shown that Cdx function is required for proper yolk sac vascularization and hematopoiesis, and this may occur, in part, through Cdx-dependent regulation of *Scl* expression (Brooke-Bisschop et al., 2017; Wang et al., 2008). In this regard, both cardiac and yolk sac hematopoietic progenitors are derived from a common pool of multi-potential *Mesp1*$^+$ mesodermal precursors (Chan et al., 2013). *Mesp1*$^+$ mesoderm fated to give rise to early blood lineages must therefore initiate the expression of a hematopoietic gene expression program while simultaneously repressing the expression of genes involved in cardiogenesis (Chan et al., 2013). To determine whether the loss of hematopoietic potential in DKO mutant yolk sacs is accompanied by ectopic cardiac differentiation, indicative of a fate switch in the absence of Cdx, cardiac gene expression was assessed by qRT-PCR in E8.5 and E9.5 DKO yolk sacs and compared with littermate controls. Ectopic expression of *Tbx5* was observed in mutant yolk sacs at both stages (Fig. 4A), consistent with a role for Cdx in repressing cardiac differentiation in this lineage. In contrast, variable changes in expression were observed for yolk sac *Nkx2-5* and *cTnT* (*Tnnt2*) (Fig. 4B,C), suggesting that such Cdx-dependent repression may be specific to *Tbx5*.

Mesodermal Cdx function is essential for heart tube morphogenesis

In addition to mesoderm-intrinsic processes, signals from both the endoderm and the ectoderm impact heart development (Zaffran and Frasch, 2002). As the conditional mouse model employed here uses a *CMV-Actin::Cre-ER*^{T} transgene to induce global *Cdx2* gene deletion, the loss of Cdx function on cardiogenesis may occur

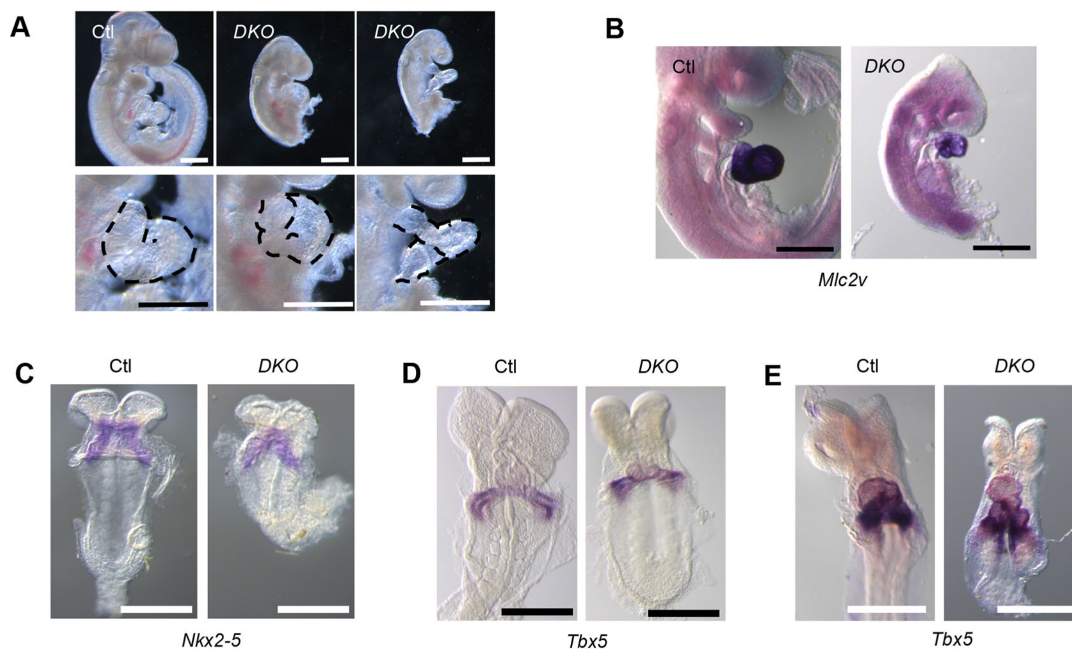

Fig. 3. Cardiac defects and altered expression of heart field markers in Cdx mutants. (A) Morphological changes to the heart tube in E9.5 Cdx mutants (DKO) relative to a stage-matched control (Ctl). Bottom panels show enlarged images with the heart tubes indicated by dashes. (B) *In situ* hybridization for *Mlc2v* in E9.5 DKO embryos and a stage-matched control (Ctl). Images are representative of 3/3 controls and 2/2 mutants. (C) *In situ* hybridization analysis of *Nkx2-5* expression in E8.5 wild-type (Ctl) and DKO embryos. *Nkx2-5* expression was affected in 4/4 *DKO* embryos, and only 1/6 controls. (D) *In situ* hybridization analysis of *Tbx5* in E8.5 Ctl and DKO embryos. *Tbx5* expression was affected in 3/3 *DKO* embryos, compared with 5 controls with normal expression. (E) *In situ* hybridization analysis of *Tbx5* in E9.5 Ctl and DKO embryos. *Tbx5* expression was affected in 1/2 *DKO* embryos compared with 7 normal controls. For each experiment, embryos were stage-matched and processed in parallel. Scale bars: 0.5 mm.

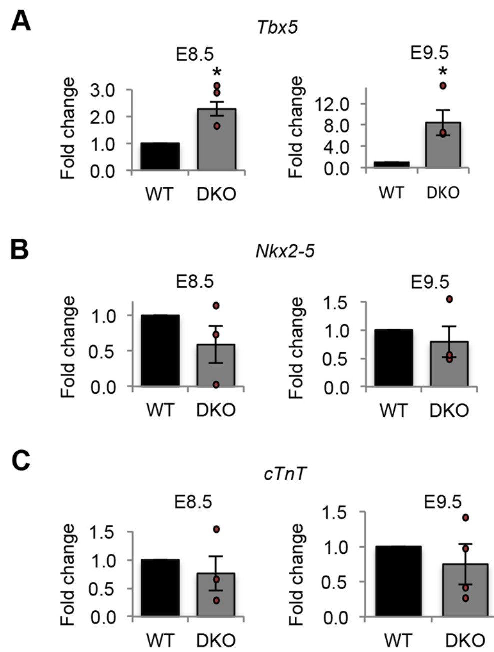

Fig. 4. Cardiac genes are ectopically expressed in Cdx-deficient yolk sacs. (A-C) *Tbx5* (A), *Nkx2-5* (B) and *cTnT* (C) expression levels were assessed in E8.5 and E9.5 yolk sacs collected from DKO or littermate control (WT) embryos by qRT-PCR or RT-PCR. Error bars represent s.e.m. from a minimum of three independent experiments. $^{*}P\leq 0.05$ (determined by two-tailed *t*-test).

through non-mesodermal pathways. To investigate this, we utilized a *Mesp1-Cre* knock-in allele (*Mesp1*$^{+/Cre}$; Saga et al., 1999) to effect *Cdx2* deletion. *Mesp1* expression initiates at E6.5, and descendant cells contribute to cardiac progenitors of both the first and second heart fields, hematopoietic and endothelial progenitors in the yolk sac, and myogenic mesoderm (Devine et al., 2014; Chan et al., 2013; Saga et al., 1999).

At E11.5, *Cdx1*$^{-/-}$*Cdx2*$^{F/F}$*Mesp1*$^{+/Cre}$ mutants appeared smaller than littermate controls, lacked red blood cells, exhibited a malformed heart with pericardial edema (Fig. 5A), and were never recovered beyond E12.5. E8.0 *Cdx1*$^{-/-}$*Cdx2*$^{F/F}$*Mesp1*$^{+/Cre}$ mutants and littermate controls were assessed for *Nkx2-5* expression, which marks cardiac progenitors of both the first and second heart fields (Stanley et al., 2002). In contrast to Cdx DKO mutants, *Nkx2-5* expression in these embryos was indistinguishable from controls (Fig. 5B), suggesting that the cardiac defects observed in *Cdx1*$^{-/-}$*Cdx2*$^{F/F}$*Mesp1*$^{+/Cre}$ mutants either manifest after E8.0, or occur through mechanisms that do not impact *Nkx2-5*.

In E9.5 embryos, expression of *Mlc2v* was comparable in the prospective ventricles in *Cdx1*$^{-/-}$*Cdx2*$^{F/F}$*Mesp1*$^{+/Cre}$ mutants and littermate controls (Fig. 5C,D). However, the positioning of the heart was affected in the mutants, which also exhibited a narrowed outflow track (Fig. 5D, arrowhead) and underdevelopment of the right ventricle. These observations suggest that *Mesp1*$^{+/Cre}$-mediated deletion of *Cdx2* results in a milder cardiac phenotype than that elicited by *CMV-Actin::CreER*^{T}. Consistent with this, although the tail buds of E9.5 *Cdx1*$^{-/-}$*Cdx2*$^{F/F}$*Mesp1*$^{+/Cre}$ embryos were foreshortened relative to controls, they did not exhibit the severe axial truncation consistently observed in DKO mutants (Fig. 5C; Savory et al., 2011). These differences may be due to Cdx deletion in axial progenitors in the tail bud of *Cdx1*$^{-/-}$*Cdx2*$^{F/F}$ *CMV-Actin::CreER*^{T} mutants, whereas *Mesp1*$^{+/Cre}$ is not anticipated to impact this niche (Saga et al., 1999; Chan et al., 2013).

Cre-negative *Cdx1*$^{-/-}$*Cdx2*$^{F/F}$ embryos did not display obvious cardiac defects, nor did *Cre*$^{+}$ littermates heterozygous for *Cdx2* (*Cdx1*$^{-/-}$*Cdx2*$^{+/F}$Mesp1$^{+/Cre}$; data not shown), consistent with prior findings (Subramanian et al., 1995; van den Akker et al., 2002). These defects were also not induced by expression of *Cre* alone in either model (*Mesp1-Cre* or *CMV-Actin::CreER*^{T}), with or without tamoxifen treatment (data not shown).

Cardiogenesis requires Cdx function in the yolk sac

In addition to aberrant cardiac morphogenesis, *Cdx1*$^{-/-}$*Cdx2*$^{F/F}$ Mesp1$^{+/Cre}$ mutants exhibited defective yolk sac vascularization similar to that observed in DKO mutants (Fig. S2A; Brooke-Bisschop et al., 2017). Deficiency in yolk sac hematopoiesis can lead to pericardial edema (Shivdasani et al., 1995; Moser et al., 2004). It is therefore possible that the cardiac defects in Cdx mutants are secondary to the failure of yolk sac hematopoiesis. To assess this, a *Tie2*$^{+/Cre}$ transgenic line, in which expression of the Cre recombinase is largely restricted to endothelial and hematopoietic lineages (Tang et al., 2010), was used to delete *Cdx2* in a *Cdx1*-null background as previously described (Brooke-Bisschop et al., 2017). In addition to being smaller than littermate controls and deficient in yolk sac hematopoiesis (Fig. 5E,F; Brooke-Bisschop et al., 2017), *Cdx1*$^{-/-}$*Cdx2*$^{F/F}$*Tie2*$^{+/Cre}$ embryos displayed aberrant cardiac morphology at E10.5, with distended pericardial sacs and a narrow, misshapen heart tube (Fig. 5F). It may be that the loss of Cdx function impacts cardiac development secondary to yolk sac hematopoiesis; however, mis-expression of *Tbx5* and *Nkx2-5* in DKO mutant embryos was observed at E8.5, before circulatory defects are anticipated to influence cardiac morphogenesis. Furthermore, Cdx2 binding occurs at the genomic loci of a number of cardiac targets, also at E8.5 (Fig. 1B-D). Although it is impossible to distinguish between the two, these data suggest that the aberrant cardiac morphogenesis observed in Cdx mutants likely results from a combination of defects that are both primary and secondary to Cdx deletion.

Cdx2 expression is coincident with cardiac progenitor specification

Cdx2 expression has been reported in the chorion, ectoplacental cone, primitive streak and allantois as early as E7.5 (Beck et al., 1995); however, the contribution made by *Cdx2*$^{+}$ cells and their descendants to the cardiac lineage is unknown. Moreover, this pattern of *Cdx2* expression is inconsistent with a role in cardiac mesoderm specification from *Mesp1*$^{+}$ progenitors, which commences at E6.5 (Saga et al., 1996; 2000; Bondue et al., 2008). To address these discrepancies, we analyzed single cell RNA-seq data generated by Scialdone and colleagues (Scialdone et al., 2016), which revealed that *Cdx2* transcripts are indeed present in the murine embryo as early as E6.5 (Fig. 6A-D). *Cdx1* transcripts were also identified at this stage, whereas *Cdx4* transcripts were rare (Figs S3 and S4). *Cdx2*$^{+}$ cells were found within the epiblast at E6.5 in nascent mesoderm, posterior mesoderm, allantois and blood progenitors, among others (Fig. 6C). Whole-mount immunohistochemistry also revealed Cdx2 in the E6.5 embryo, including the anterior region of the primitive streak (Fig. 6E), corresponding with the region associated with cardiac mesoderm specification and egression. In addition, *Cdx2* transcripts were detected in E6.5 embryos by RT-PCR in both embryonic and extra-embryonic material (Fig. 6F), and *Cdx1*, and to a lesser extent *Cdx4*, were also expressed (Fig. S5). Consistent with previous reports (Beck et al., 1995; Mcdole and Zheng, 2012), Cdx2 levels were more robust at E7.5 (Fig. 6E). Taken together, these data demonstrate that Cdx2 is present in the appropriate region and at the appropriate developmental stage to be able to impact cardiac progenitors directly.

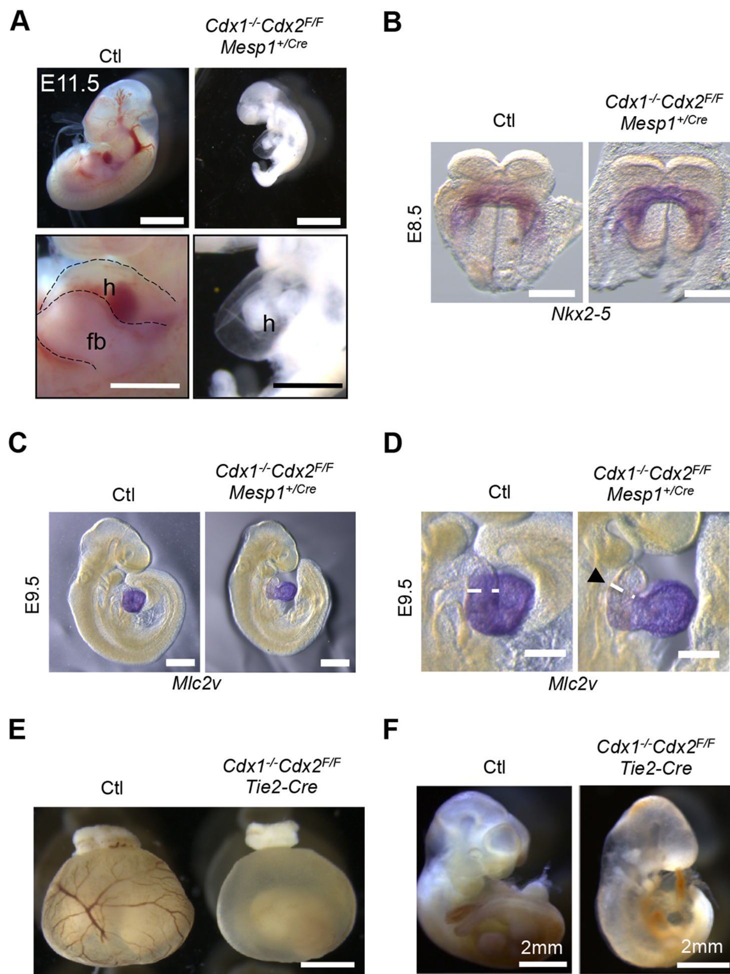

Fig. 5. Aberrant cardiac morphogenesis in *Cdx1$^{-/-}$Cdx2$^{F/F}$Mesp1$^{+/Cre}$* mutants. (A) Representative images of a *Cdx1$^{-/-}$Cdx2$^{F/F}$Mesp1$^{+/Cre}$* mutant and littermate control (Ctl) at E11.5. An enlarged view of the mutant heart shown in the panel below illustrates a distended pericardial sac, malpositioning of the heart, and the absence of blood typical of these mutants. Images are representative of 7/9 mutants and 14 littermate controls. fb, forelimb bud; h, heart. (B-D) *In situ* hybridization analysis of *Nkx2-5* and *Mlc2v* in *Cdx1$^{-/-}$Cdx2$^{F/F}$Mesp1$^{+/Cre}$* mutants and littermate controls. Images are representative of 1 mutant and 8 littermate controls for *Nkx2-5* staining, and 2/3 mutants and 5 littermate controls for *Mlc2v* staining. Axial truncation in E9.5 *Cdx1$^{-/-}$Cdx2$^{F/F}$Mesp1$^{+/Cre}$* mutants is shown in D. Arrowhead indicates the outflow tract, which is narrowed in the *Cdx1$^{-/-}$Cdx2$^{F/F}$Mesp1$^{+/Cre}$* mutant, as demonstrated by the dashed line. (E) Representative images of *Cdx1$^{-/-}$Cdx2$^{F/F}$Tie2-Cre* (n=3) and littermate control (n=3) yolk sacs at E10.5. (F) Representative images of 4/4 *Cdx1$^{-/-}$Cdx2$^{F/F}$Tie2-Cre* mutant embryos and 1/1 littermate control at E10.5. Scale bars: 0.25 mm (B,C); 0.5 mm (D); 1 mm (A, bottom); 2 mm (A, top; E,F).

Cdx2-positive cells frequently co-expressed *Mesp1* (Fig. 6G), but rarely expressed *Tbx5* or *Mef2c*, markers of the first and second heart fields, respectively (Fig. 6I,J; Bruneau et al., 1999; Verzi et al., 2005). Although there was a weak correlation between *Cdx2* expression and that of additional heart markers (Fig. S6), these genes, as well as Cdx, are also expressed in other mesodermal lineages. Correlations between *Cdx1* or *Cdx4* expression and the expression of *Mesp1*, *Tbx5* and *Mef2c* were similar to those observed between *Cdx2* and these transcripts (Scialdone et al., 2016; data not shown), in agreement with functional overlap among Cdx family members. Conversely, a subset of *Cdx2*$^{+}$ cells co-expressed *Scl* (Fig. 6H) and other hematopoietic markers (Fig. S6). Taken together, these data are consistent with a role for Cdx2 in repressing cardiogenic, and promoting hematopoietic, differentiation in *Mesp1*$^{+}$ progenitors.

Cdx2 co-occupies cardiogenic loci with Brg1

Brg1, the catalytic subunit of the SWI/SNF chromatin remodeling complex, is required for mesoderm induction and cardiac progenitor specification in differentiating embryonic stem cells (ESCs; Alexander et al., 2015), as well as heart development (Stankunas et al., 2008; Hang et al., 2010). Cdx2 physically associates with Brg1, and other members of the SWI/SNF complex, and Cdx2-dependent expression of at least some targets is associated with chromatin remodeling events (Nguyen et al., 2017; Verzi et al., 2010, 2013).

To determine whether a similar association between Cdx2 and Brg1 was observed at cardiogenic loci, the Cdx2 ChIP-seq data were intersected for Brg1 chromatin occupation derived from ChIP-seq analysis of ESCs at the mesoderm stage of directed differentiation to cardiomyocytes (Alexander et al., 2015). This exercise revealed a number of cardiac loci occupied by both Cdx2 and Brg1, including *Tbx5*, *Nkx2-5*, *Gata4*, *Hand1* and *Hand2*, whereas the *Isl1* locus was occupied by Cdx2 only (Fig. 7A).

To determine whether Brg1 occupies Cdx2-bound cardiac genes in a Cdx-dependent manner, ChIP-PCR was performed for Brg1, comparing wild-type with DKO embryos at E9.5 (Fig. 7B). Brg1 binding was impaired within Cdx2-occupied regions of *Tbx5*, *Gata4* and *Nkx2-5* genes as well as the previously characterized Cdx targets *Cyp26A1* and *Dll1* (Savory et al., 2009a; Grainger et al., 2012). These findings are consistent with a model whereby Cdx2 recruits Brg1 to select cardiac targets to regulate gene expression via SWI/SNF chromatin remodeling.

To determine whether impaired Brg1 binding to cardiac targets results in altered chromatin organization, formaldehyde-assisted isolation of regulatory elements (FAIRE) coupled with PCR was used to compare chromatin accessibility between wild type and DKO mutants. Given the ectopic cardiac gene expression observed in yolk sacs, and that hematopoietic yolk sac mesoderm shares a common *Mesp1*$^{+}$ progenitor with the cardiac lineage, yolk sacs from wild-type and DKO embryos at E9.5 were used for these experiments. Changes in chromatin accessibility at Cdx2-occupied CDREs were observed at *Tbx5*, *Gata4* and *Nkx2-5* loci, whereas accessibility at the β-actin locus remained unaffected in DKO yolk sacs, and thus served as a negative control (Fig. 7C). Although these changes were variable and did not reach statistical significance,

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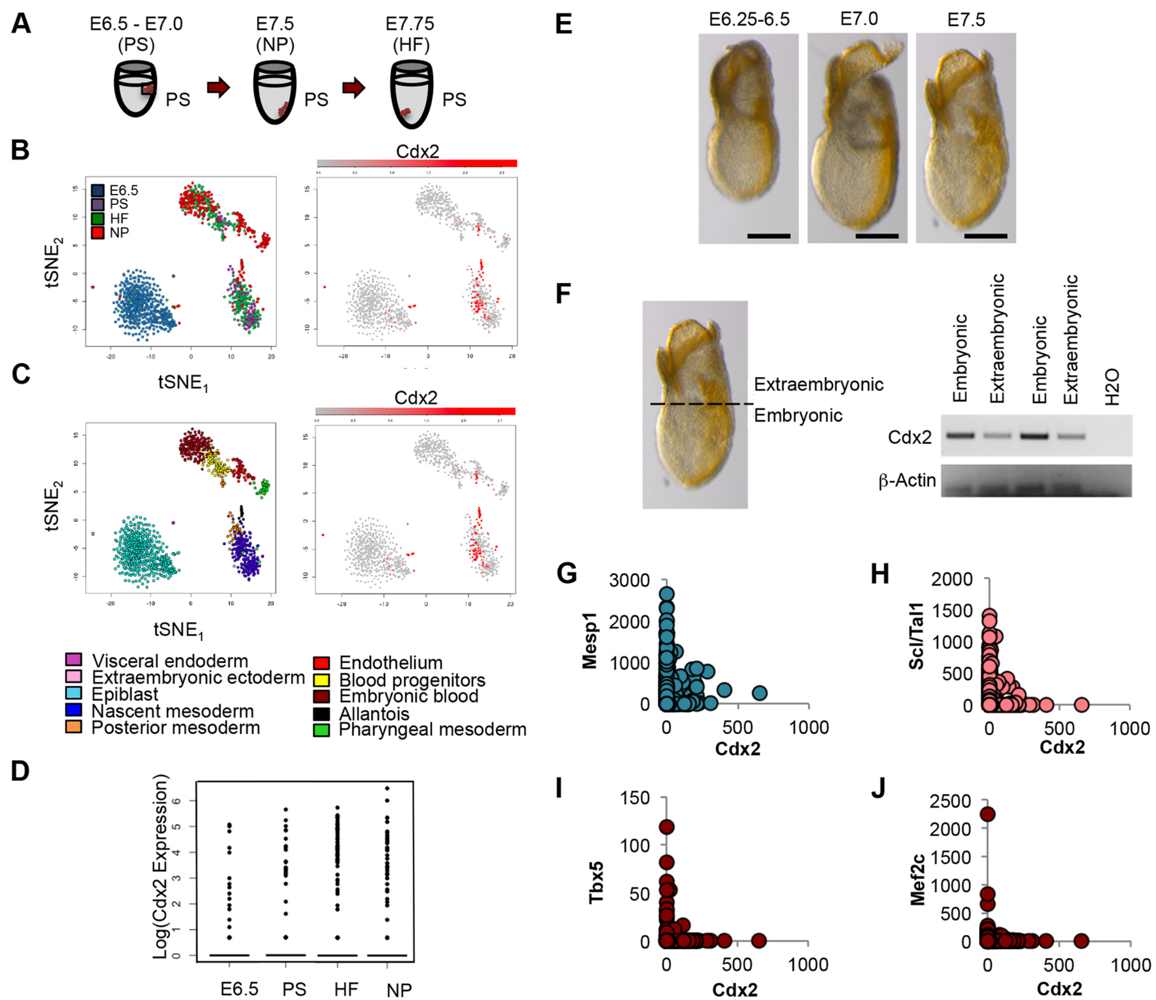

Fig. 6. Cdx2 is expressed in mesodermal progenitors. (A) Schematic of embryonic development between E6.5 and E7.75 corresponding to the time points at which single cell RNA-seq (Scialdone et al., 2016) was performed as shown in B,C. Red circles represent cardiac progenitors as they are specified and migrate anterolaterally to form the heart fields. HF, head fold; NP, neural plate; PS, primitive streak. (B) *Cdx2* transcripts in cells isolated from embryos at E6.5 (epiblast, blue), primitive streak (E7.0, purple), head fold (E7.5, green) and neural plate (E7.75, red) stages. (C) Cells in B were color-coded according to cell lineage as indicated in the key. tSNE, t-distributed stochastic neighbor embedding. (D) *Cdx2* transcript abundance was plotted as log expression according to embryonic stage using the single cell RNA-seq dataset generated by Scialdone and colleagues (Scialdone et al., 2016). (E) Immunohistochemistry illustrating the presence of Cdx2 protein in the primitive streak region of wild-type embryos at E6.5-E7.5. Scale bars: 0.25 mm. (F) Left: Image of an E7.5 embryo with the boundary between the embryo proper and the extra-embryonic region indicated by a dashed line. Right: *Cdx2* transcripts were detected in both embryonic and extra-embryonic material from independent E6.5 embryos by RT-PCR. β-Actin was used as a loading control. (G-J) Single cell RNA-seq data (Scialdone et al., 2016) was used to assess correlations between cells expressing *Cdx2* and *Mesp1* (mesoderm; G), *Tbx5* (first heart field; I), *Mef2c* (second heart field; J), or *Scl* (hematopoietic progenitors; H) between E6.5 and E7.75.

likely owing to the cellular heterogeneity of the yolk sac, they are consistent with Cdx-dependent chromatin organization and in agreement with prior observations in other systems (Verzi et al., 2010, 2013; Nguyen et al., 2017; Saxena et al., 2017).

DISCUSSION

ChIP-seq analysis of Cdx2 occupancy in E8.5 embryos revealed a marked enrichment at loci encoding transcription factors, suggesting that Cdx coordinates gene regulatory networks essential to numerous developmental processes, a finding consistent with the complex phenotype of Cdx-null mutants (Beck, et al., 1995; Gamer and Wright, 1993; Meyer and Gruss, 1993; van den Akker et al., 2002; Strumpf et al., 2005; Savory et al., 2009a, 2011; van Rooijen et al., 2012). A subset of these transcription factors are involved in cardiogenesis and hematopoiesis, both of which are known roles for Cdx (Lengerke et al., 2007; Wang et al., 2008; Brooke-Bisschop et al., 2017). Cardiac defects observed in Cdx DKO embryos, together with altered gene expression patterns, suggest that Cdx function is required for normal heart development, potentially governing fate decisions between hematopoietic and cardiac lineages from a common progenitor pool. Finally, Brg1 was recruited to a subset of cardiogenic loci in a Cdx-dependent manner. Together with the finding of altered chromatin architecture at some of these genes, these observations lead us to propose a model in which Cdx factors, transiently expressed in mesodermal progenitors within the primitive streak, occupy the loci of fate-determining transcription factors with Brg1 and the SWI/SNF complex, leading to establishment of the chromatin architecture required for the fidelity of subsequent gene expression programs at later developmental stages (Fig. 8).

Cdx2 as a master developmental regulator

ChIP-seq analysis revealed that many of the robust Cdx2 peaks localized to transcription factor loci. Consistent with this, GO

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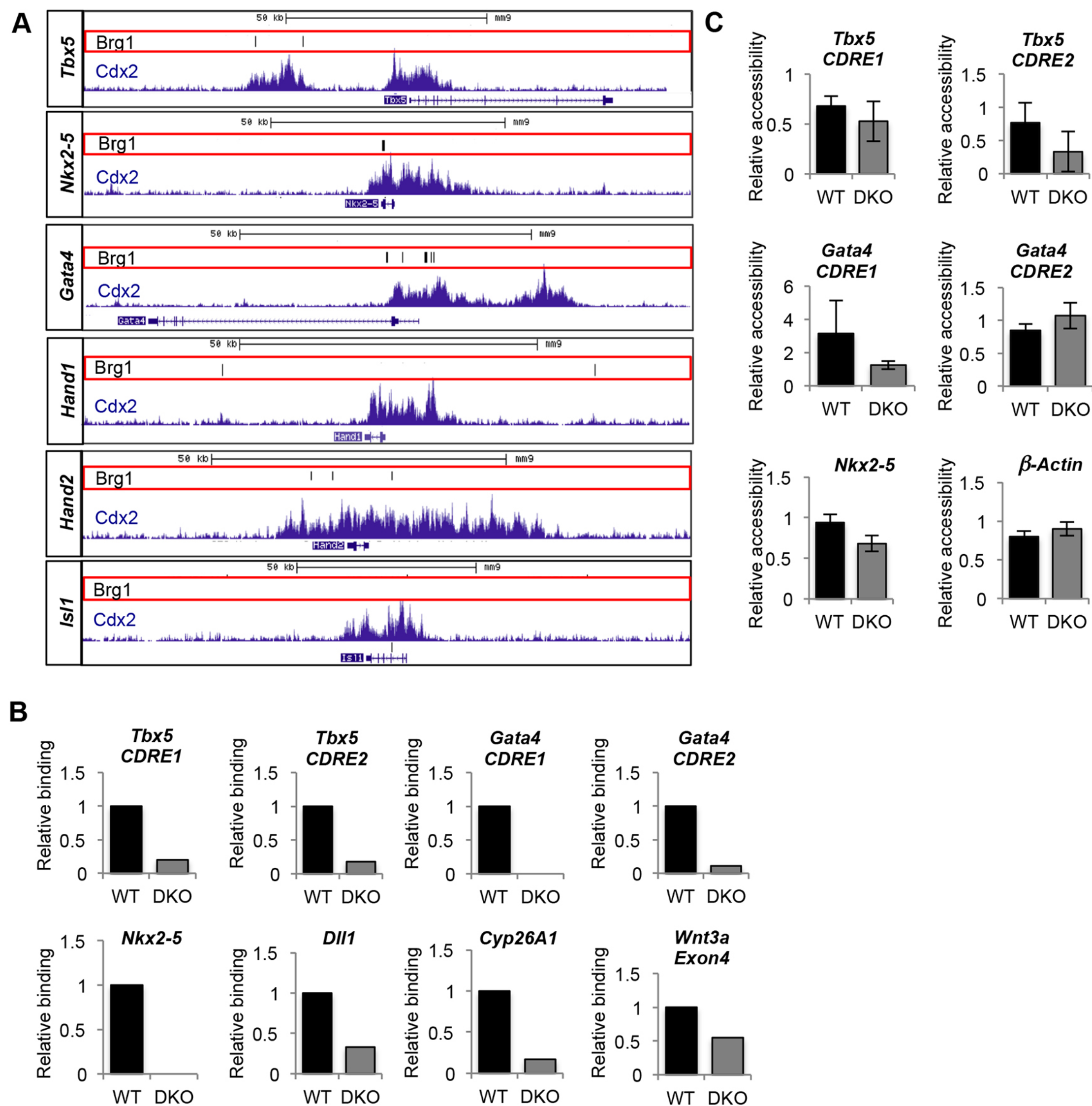

Fig. 7. Cdx2 and Brg1 co-occupy cardiac transcription factor loci. (A) Brg1 ChIP-seq tracks from ESCs at the mesoderm stage of directed differentiation to cardiomyocytes (Alexander et al., 2015) were intersected with Cdx2 ChIP-seq data from E8.5 embryos at *Tbx5*, *Nkx2-5*, *Gata4*, *Hand1*, *Hand2* and *Isl1* genes. Brg1 tracks are outlined in red with regions of enriched binding indicated by black vertical bars; Cdx2 tracks are indicated below in blue. (B) ChIP-PCR experiments assessing Brg1 binding at Cdx2-occupied cardiac loci in wild-type (WT) and DKO embryos at E9.5. Graphs are representative of two independent experiments in which similar results were obtained. (C) FAIRE-PCR-mediated assessment of chromatin accessibility in wild-type and DKO yolk sacs at E9.5. Values were normalized to input. Error bars represent s.e.m. from a minimum of three independent experiments. β-Actin was used as a negative control.

terms highly over-represented in this dataset included 'transcription', 'positive and negative regulation of gene expression' and 'cell fate determination', suggesting that Cdx2 impacts the expression of a number of transcriptional networks implicated in development. Indeed, transcription factors represented 226 of the 441 annotated peaks exhibiting binding above the threshold of 800. This association is further reflected in some of the phenotypes exhibited by Cdx mutants. For example, $Cdx1^{-/-}$ and $Cdx2^{+/-}$ mice and compound mutant derivatives thereof typically display vertebral homeotic transformations associated with altered expression of Hox target genes (Subramanian et al., 1995; Chawengsaksophak et al., 1997). Cdx DKO and triple-knockout mutants exhibit precocious axial truncation, which may be ascribed, at least in part, to illicit RA signaling in the tailbud caused by loss of *Cyp26A1* expression (Savory et al., 2009a; Young et al., 2009; Martin and Kimelman, 2010).

Cdx and mesodermal specification

Cdx2 was found to be resident at a number of genes encoding regulators of yolk sac hematopoiesis, consistent with previous work underscoring a requirement for Cdx in hematopoiesis in diverse vertebrate species (Brooke-Bisschop et al., 2017; Lengerke et al., 2007; Davidson and Zon, 2006; Davidson et al., 2003; Wang et al., 2008). In the present study, Cdx2 binding was also enriched at the loci of numerous cardiogenic transcription factors (e.g. *Nkx2-5*, *Tbx5*, *Gata4*, *Isl1*, *Hand1* and *Hand2*).

Prior work has suggested a role for Cdx in early mesoderm fate decisions (Mendjan et al., 2014), including suppression of cardiogenesis (Lengerke et al., 2011; Rao et al., 2016). Although the basis underlying these observations is poorly understood, occupancy of cardiogenic and hematopoietic transcription factors by Cdx2, coupled with ectopic cardiogenic gene expression in DKO mutants, is consistent with a role for Cdx2 in suppressing cardiogenesis and promoting hematopoietic gene expression

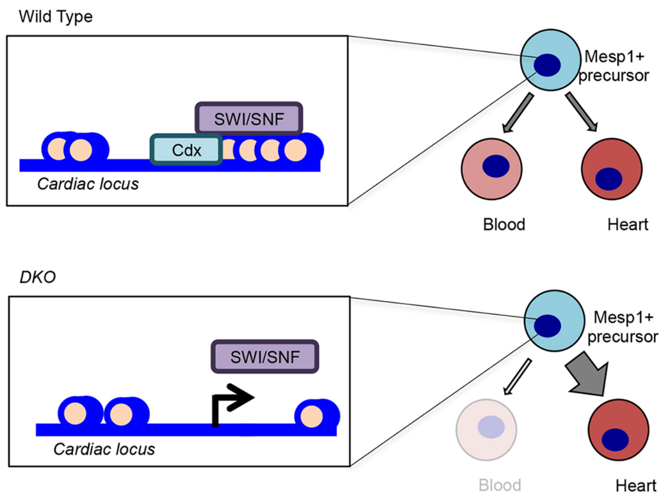

Fig. 8. Proposed model for Cdx function in early mesoderm. Cdx recruits Brg1 and the SWI/SNF complex to cardiac loci in wild-type *Mesp1*$^+$ mesoderm, resulting in chromatin remodeling and subsequent gene silencing. This, in turn, maintains the balance between differentiation to cardiac and hematopoietic lineages from *Mesp1*$^+$ precursors. In Cdx DKO mutants, Brg1 is no longer recruited to cardiac targets, which results in increased chromatin accessibility and ectopic cardiac gene expression, perturbing the balance between commitment to hematopoietic and cardiac cell fates.

programs within a common *Mesp1*$^+$ mesodermal precursor, an event believed to occur during early gastrulation at E6.5 (Saga et al., 1999; Chan et al., 2013). However, the onset of Cdx gene expression in the embryo proper is reported to occur at around E7.5 (Beck et al., 1995; Gamer and Wright, 1993; Meyer and Gruss, 1993; Strumpf et al., 2005), and is therefore inconsistent with a role in this specification event. Recent single cell RNA-seq performed and published by Scialdone et al. (2016) suggests that these early *Mesp1*$^+$ progenitors do, in fact, express Cdx family members as early as E6.5. This was further supported by whole-mount immunohistochemistry, which revealed Cdx2 protein in the primitive streak at E6.5, thus placing Cdx in the appropriate developmental context to influence early *Mesp1*$^+$ mesoderm specification events. Furthermore, an onset for Cdx function at the early primitive streak stage supports previously described roles for Cdx family members in early post-otic embryonic development (van Rooijen et al., 2012; Chawengsaksophak et al., 2004, 1997; Savory et al., 2009a; Amin et al., 2016; Subramanian et al., 1995) and craniorachichisis (Savory et al., 2011).

In contrast to its role at hematopoietic loci, Cdx2 occupancy at cardiac loci is likely inhibitory, as previous studies using directed differentiation of mouse and human ESCs *in vitro* demonstrated increased differentiation to cardiac fates in the absence of Cdx function (Lengerke et al., 2011; Rao et al., 2016) and decreased cardiac gene expression in models of Cdx gain of function (Lengerke et al., 2011). Consistent with this, single cell RNA-seq revealed that all three Cdx family members are expressed in *Mesp1*$^+$ progenitor cells in the mouse, but are not co-expressed with *Tbx5* or *Mef2c*, suggesting that Cdx expression is incompatible with the acquisition of a cardiac fate. A role for Cdx in suppressing cardiogenesis is further evidenced by ectopic *Nkx2-5* and *Tbx5* expression in the cardiac crescent of DKO mutants, and ectopic *Tbx5* expression in DKO yolk sacs.

Our observation that *Tbx5* expression is misregulated in DKO mutants is consistent with previous work in zebrafish that revealed expanded *tbx5a* expression in *cdx1a/4* compound mutants (Lengerke et al., 2011). Moreover, ectopic *Tbx5* expression in DKO mutant yolk sacs indicates that Cdx-deficient yolk sacs maintain sufficient plasticity to be redirected to a cardiac fate. Notably, a similar role has been described for *Scl* (Van Handel et al., 2012; Org et al., 2015), which functions downstream of Cdx (Brooke-Bisschop et al., 2017; Wang et al., 2008; Davidson and Zon, 2006; Davidson et al., 2003). Although Cdx may impact mesodermal cell fate decisions in part via *Scl*, our finding of direct binding of Cdx2 to numerous cardiogenic loci, together with the mis-expression of some of these genes in DKO mutants, suggests that Cdx also plays a direct role in repressing cardiogenic gene expression.

Although *Tbx5* expression was consistently upregulated in DKO mutant yolk sacs, random alterations in *Nkx2-5* expression suggests that Cdx factors may not repress all cardiac targets similarly within a given context. In this regard, in the zebrafish, embryonic *nkx2-5* expression remains unaffected in Cdx mutants until they are treated with RA antagonists, suggesting that retinoid-mediated compensatory mechanisms are necessary to effect *nkx2-5* expression in the absence of Cdx (Lengerke et al., 2011). RA signaling not only regulates *Cdx1* expression (Houle et al., 2000, 2003; Prinos et al., 2001) but is also involved in heart development, and altered retinoid signaling can lead to aberrant cardiac mesoderm specification and subsequent defects in cardiac morphogenesis (Zile, 1998; Dickman and Smith, 1996). This suggests that murine Cdx factors are necessary, but not sufficient, for the regulation of certain Cdx targets, such as *Nkx2-5*, and that RA, or other signaling pathways, impact expression of such genes in Cdx mutants.

Defects in yolk sac hematopoiesis, such as those observed in Cdx mutants (Savory et al., 2011; Brooke-Bisschop et al., 2017; Lengerke et al., 2008; Wang et al., 2008; Davidson and Zon, 2006; Davidson et al., 2003), can also affect cardiac development (Shivdasani et al., 1995; Moser et al., 2004). Consistent with this, *Cdx1*$^{-/-}$*Cdx2*$^{F/F}$*Tie2-Cre* mutants exhibited aberrant cardiogenesis, suggesting that the absence of blood contributes to cardiac defects in these mutants. Nevertheless, cardiac defects in DKO mutants are preceded by altered expression of *Nkx2-5* and *Tbx5* as early as E8.5, likely before yolk sac hematopoiesis is sufficiently compromised to impact heart tube morphogenesis. Furthermore, Cdx2 binding to a number of cardiac loci supports a direct role for Cdx during heart field specification and argues against aberrant cardiogenesis in DKO mutants being entirely secondary to circulatory deficiencies.

Cdx factors as integrators of extracellular signaling

Although similar morphological defects were observed in Cdx mutant heart tubes produced by *CMV-Actin::CreER*^{T} and *Mesp1-Cre* mediated recombination, perturbations in the expression of cardiac transcription factors were only observed in *CMV-Actin::CreER*^{T} mutants, arguing against Cdx function in *Mesp1*$^+$ progenitors as causal to these heart defects. It may be that cell Cdx-dependent non-autonomous cues instructive to cardiac morphogenesis originating from the endoderm and/or ectoderm are disrupted only in the *CMV-Actin::CreER*^{T} mutant model. For instance, BMP2 expressed in the endoderm (Schultheiss et al., 1997; Somi et al., 2004) is required for the formation of a heart in zebrafish (Frasch, 1995) and *Xenopus* (Shi et al., 2000), as is its homolog *dpp* in *Drosophila* (Kishimoto et al., 1997). Although cardiac mesoderm is induced in BMP2-deficient mice, subsequent heart morphogenesis is perturbed (Zhang and Bradley, 1996). In this regard, we identified *Bmp2* as a potential Cdx2 target gene by ChIP-seq in E8.5 embryos, the stage at which the heart tube emerges and commences looping. Thus, Cdx regulation of BMP signaling from non-mesodermal germ layers may be preserved in *Mesp1-Cre*

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driven Cdx mutants, yet lost in *CMV-Actin::CreER*^{T} mutants, possibly contributing to the stronger phenotype in the latter. Further work will be required to examine this potential relationship.

Cdx proteins act as intermediaries that relay caudalizing signals to regulate expression of transcription factors, such as the Hox genes (Shashikant et al., 1995; Charité et al., 1998; Subramanian et al., 1995; Pownall et al., 1996; Isaacs et al., 1998; van den Akker et al., 2002; Gaunt et al., 2003, 2008). Consistent with this, previous work has demonstrated Wnt- and RA-dependent regulation of Cdx family members (Houle et al., 2000, 2003; Prinos et al., 2001; Ikeya and Takada, 2001; Pilon et al., 2006). Directed differentiation of ESCs to cardiomyocytes *in vitro* mimics signaling gradients in the primitive streak, and requires an initial phase of Wnt signaling for mesoderm induction (Lindsley et al., 2006; Gadue et al., 2006; Haegel et al., 1995), followed by Wnt inhibition to permit differentiation to cardiac cell fates (Lickert et al., 2002; Ueno et al., 2007; Burridge et al., 2012; Yang et al., 2008; Murry and Keller, 2008; Marvin et al., 2001; Schneider and Mercola, 2001; Tzahor and Lassar, 2001); continued Wnt signaling promotes mesodermal progenitor commitment to alternative lineages, including blood (Woll et al., 2008; Wang et al., 2008; Rao et al., 2016). Such models have placed Cdx function downstream of Wnt to repress cardiogenesis, thereby promoting differentiation to such alternative mesodermal lineages (Lengerke et al., 2011; Mendjan et al., 2014; Rao et al., 2016). Thus, cardiac inhibition by Cdx factors as shown here, coupled with activation of hematopoietic regulators, is consistent with their role downstream of Wnt during mesodermal cell fate decisions.

Cdx and chromatin remodeling

Cdx2 has been shown to influence chromatin accessibility in the intestinal epithelium (Verzi et al., 2010, 2013), and interacts with Brg1 at some target loci in other models (Nguyen et al., 2017; Yamamichi et al., 2009). Furthermore, Cdx2 appears to be required for Brg1 recruitment to cardiac genes. Chromatin organization within these regions was also disrupted in DKO yolk sacs, suggesting that the interaction between Cdx2 and Brg1 at cardiac genes impacts chromatin accessibility. It is possible that Cdx2 recruits Brg1 and SWI/SNF chromatin remodeling activity to cardiogenic loci to establish non-permissive chromatin, thereby epigenetically silencing gene expression in non-cardiac lineages at later developmental stages. However, not all Cdx2-bound loci were co-occupied by Brg1. For example, at the *Tbx5* gene locus, Brg1 binding was robust at the Cdx2 ChIP-seq peak most distal to the transcriptional start site (TSS), whereas the more proximal peak was occupied by Cdx2 alone (Fig. 7A). Cdx2 and Brg1 were also present at the *Hand1* locus, although Brg1-bound regions flanked Cdx2 peaks, suggesting that these factors may both impact *Hand1* expression, but do not co-occupy genomic intervals at this particular gene. Conversely, *Isl1* was occupied by Cdx2 alone, suggesting that regulation by Cdx at some cardiac targets might occur independent of SWI/SNF complex recruitment. Although we cannot exclude Brg1 occupation of such genes in lineages not surveyed here, and/or at other times, these observations suggest that Cdx2-dependent transcription occurs through Brg1-dependent and -independent mechanisms.

In summary, the findings presented here suggest a model in which Cdx factors regulate gene expression through epigenetic mechanisms following recruitment of the SWI/SNF complex. This putative mechanism allows for Cdx$^+$ cells and their progeny to initiate the appropriate gene regulatory networks at developmental stages when cell fate decisions are made, after Cdx expression is extinguished.

MATERIALS AND METHODS

Mice

Cdx1$^{-/-}$*Cdx2*$^{F/F}$, *CMV-Actin::CreER*^{T}, *Tie2*$^{+/Cre}$, and *Mesp1*$^{+/Cre}$ mouse lines have been previously described (Savory et al., 2009a; Santagati et al., 2005; Saga et al., 1999, 2000; Kisanuki et al., 2001; Tang et al., 2010). *Cdx1*$^{-/-}$*Cdx2*$^{F/F}$ females were paired with *Cdx1*$^{-/-}$*Cdx2*$^{F/F}$ *CMV-Actin::CreER*^{T} males for timed matings, with noon of the day of vaginal plug discovery considered E0.5, and Cdx2 deletion induced with 2 mg tamoxifen in corn oil by oral gavage at E5.5. *Mesp1*$^{+/Cre}$ or *Tie2*$^{+/Cre}$ animals were crossed into the *Cdx1*$^{-/-}$*Cdx2*$^{F/F}$ line, and *Cdx1*$^{-/-}$*Cdx2*$^{+/F}$*Mesp1*$^{+/Cre}$ or *Cdx1*$^{-/-}$*Cdx2*$^{+/F}$*Tie2*$^{+/Cre}$ males were paired with *Cdx1*$^{-/-}$*Cdx2*$^{F/F}$ females for timed matings, as above. Animals were maintained according to the guidelines established by the Canadian Council on Animal Care and Animal Care and Veterinary Services at the University of Ottawa.

Antibodies

Rabbit polyclonal anti-Cdx2 antibodies have been previously described (Savory et al., 2009b). Brg1 antibodies were purchased from Abcam (ab110641). Control rabbit IgG antibodies were purchased from Sigma (i8140).

Chromatin immunoprecipitation (ChIP) and ChIP-seq

Embryos from timed matings were dissected, crosslinked with 1% formaldehyde, and ChIP performed as previously described (Pilon et al., 2006) with the following modifications: chromatin was sonicated for 1-min intervals six times using a Branson Sonifier 450 at output 3 with 30% duty cycle, and 10 µg of polyclonal rabbit-anti-Cdx2 (Savory et al., 2009b) and pre-immune rabbit-anti-IgG (Sigma) was coupled to 200 µl of magnetic protein G Dynabeads (Invitrogen). Samples were purified using the QIAquick PCR Purification Kit (Qiagen) and library preparation and sequencing performed by the Genome Quebec and McGill Innovation Centre using the mm9 reference genome. ChIP experiments were performed as above using 5 µg of antibody against Cdx2 (Savory et al., 2009b), or IgG control (Sigma), coupled to 50 µl of protein A/G Sepharose beads (Santa Cruz Biotechnology). DNA purification was followed by PCR using primers directed against sequences flanking putative Cdx response elements (CDREs), identified by TRANSFAC, within genomic intervals associated with enriched Cdx2 occupancy as determined by ChIP-seq. Primer sequences are listed in Table S2.

ChIP-seq tracks for Brg1 binding in ESCs at the mesoderm stage of differentiation to cardiomycotes in culture (Alexander et al., 2015) were intersected with Cdx2-ChIP seq data using the UCSC genome browser (https://genome.ucsc.edu/).

GO term analysis

Cdx2 ChIP-seq peaks with peak scores greater than 800 was assembled and used for GO term enrichment analysis. Gene IDs were uploaded to the Gene Ontology Consortium enrichment analysis tool (www.geneontology.org/page/go-enrichment-analysis), and only those GO terms with a fold enrichment of 5 or greater were considered. GO terms were sorted by *P*-value and plotted as $-\log_{10}(P\text{-value})$.

In situ hybridization

Embryos were dissected at E8.5 or E9.5 in chilled PBS, pooled according to genotype, stage-matched based on somite number (or head morphology in cases of axial truncation) and processed in parallel as described (Houle et al., 2000; Wilkinson et al., 1990). Probes were generated from previously described plasmids encoding *Nkx2-5* (Komuro and Izumo, 1993), *Tbx5* (Bruneau et al., 1999) or *Mlc2v* (Molkentin et al., 1997).

Whole-mount immunohistochemistry

Whole-mount immunohistochemistry was performed using antibodies against Cdx family members on E6.0-E7.5 embryos as previously described (Qiu et al., 1997; Savory et al., 2009b).

Reverse-transcription quantitative PCR (RT-qPCR)

Embryos or yolk sacs were collected at E8.5-9.5 and RNA extracted using Trizol (Gibco BRL) according to the manufacturer's instructions. cDNA

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was synthesized by standard methods and amplified by semi-quantitative RT-PCR with GoTaq (Promega) using a DNA Engine Tetrad 2 (Bio-Rad), or by RT-qPCR using SYBR green (Promega) and the CFX 96 (Bio-Rad) thermocycler. Either β-actin or *Gapdh* was used as an input control, and RT-qPCR results were analyzed using the $2^{-\Delta\Delta Ct}$ method. Oligonucleotides (listed in Tables S3 and S4) were validated before use and dissociation curves were considered for each amplicon to ensure specificity.

RNA-seq

Single cell RNA-seq was originally performed using E6.5-E7.75 whole embryos by Scialdone et al. (2016). Single cell read counts were downloaded from http://gastrulation.stemcells.cam.ac.uk and plotted against expression levels of Cdx family members.

FAIRE-PCR

Experiments were performed using E9.5 yolk sacs isolated from wild-type and DKO mutant embryos. Chromatin was sheared for seven 1-min intervals on ice using a Branson Sonifier 450 with a 30% duty cycle on power output 3, and DNA accessibility was assessed according to previously published protocols (Simon et al., 2012). Primers are listed in Table S5.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.E.F., J.G.A.S., D.L.; Methodology: T.E.F., B.H., J.G.A.S., R.R.; Validation: T.E.F.; Formal analysis: T.E.F., J.G.A.S., D.L.; Investigation: T.E.F., D.L.; Resources: D.L.; Writing - original draft: T.E.F.; Writing - review & editing: T.E.F., D.L.; Visualization: R.R., T.E.F.; Supervision: D.L.; Project administration: D.L.; Funding acquisition: D.L.

Data availability

ChIP-seq data have been deposited in GEO under accession number GSE128858.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.170498.supplemental

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