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# Interactions between *Cdx* genes and retinoic acid modulate early cardiogenesis

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ABSTRACT

*Cdx* transcription factors regulate embryonic positional identities and have crucial roles in anteroposterior patterning (AP) processes of all three germ layers. Previously we have shown that the zebrafish homologues *cdx1a* and *cdx4* redundantly regulate posterior mesodermal derivatives inducing embryonic blood cell fate specification and patterning of the embryonic kidney. Here we hypothesize that *cdx* factors restrict formation of anterior mesodermal derivatives such as cardiac cells by imposing posterior identity to developing mesodermal cells. We show that ectopic expression of *Cdx1* or *Cdx4* applied during the brief window of mesoderm patterning in differentiating murine embryonic stem cell (ESC) strongly suppresses cardiac development as assayed by expression of cardiac genes and formation of embryoid bodies (EB) containing "beating" cell clusters. Conversely, in loss-of-function studies performed in *cdx*-deficient zebrafish embryos, we observed a dose-dependent expansion of *tbx5a*$^{+}$ anterior-lateral plate mesoderm giving rise to cardiac progenitors. However, further cardiac development of these mesodermal cells required additional suppression of the retinoic acid (RA) pathway, possibly due to differential activity of inhibitory RA signals in *cdx* mutants. Together, our data suggest that *cdx* proteins affect cardiogenesis by regulating the formation of cardiogenic mesoderm and together with the RA pathway control the early development of cardiac precursor cells.

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## Introduction

The anteroposterior (AP) patterning of several embryonic tissues, including the developing mesoderm, is tightly regulated by *Hox* gene expression patterns, especially by precise positioning of their anterior expression boundaries (Kmita and Duboule, 2003). Although the mechanisms of *Hox* activation along the developing AP axis are not completely understood, one plausible model is the instructional (morphogen) gradient hypothesis that proposes that retinoic acid (RA), FGF and Wnt establish *Hox* expression boundaries at threshold concentrations (Deschamps and van Nes, 2005; Gaunt, 2000). Members of the caudal-related family of homeobox (Cdx) proteins have been proposed to mediate positional information between morphogen pathways and downstream *Hox* genes (Allan et al., 2001).

The *Cdx* gene family derives from the ancestral ParaHox cluster and comprises *Cdx1*, *Cdx2*, and *Cdx4* in mammals and *cdx1a*, *cdx1b* and *cdx4* in zebrafish. In the developing embryo, *Cdx* expression is induced within the primitive streak/tailbud (Gaunt et al., 2003; Gaunt et al., 2005) and later, protein levels are distributed along a posterior-to-anterior concentration gradient, probably due to decay in protein concentration in cells moving out of this region (Beck et al., 1995; Gamer and Wright, 1993; Meyer and Gruss, 1993). Consistent with this expression pattern, *Cdx* genes play major roles during patterning of the AP axis and regulation of axial elongation during development (Chawengsaksophak et al., 2004; van den Akker et al., 2002). For example, loss- and gain-of-function studies performed in mice have identified roles for *Cdx* genes during the patterning of paraxial mesoderm and the development of the somites and vertebrae (reviewed by (Young and Deschamps, 2009)). More recently, *Cdx* genes have been associated with the expansion and patterning of posterior tissues (Davidson et al., 2003; Davidson and Zon, 2006; Shimizu et al., 2005; Wingert et al., 2007), the embryonic kidney (Wingert et al., 2007), and the specification of hematopoietic cell fate, a function that can be rescued by specific *hox* genes (Davidson et al., 2003; Davidson and Zon, 2006; Lengerke et al., 2007; McKinney-Freeman et al., 2008). Molecularly, *Cdx* genes are well known as master regulators

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of *Hox* gene expression (Lohnes, 2003). Presumably due to similar effects of downstream *Hox* genes, redundancies between *Cdx* family members have been reported in different systems (Davidson and Zon, 2006; Lengerke et al., 2007; McKinney-Freeman et al., 2008). These redundant effects complicate *in vivo* loss of function studies in mice, where a *Cdx*-knockout mouse model has been challenging to create due to essential early roles of *Cdx2* during placenta formation (Strumpf et al., 2005). During development, expression of *Cdx* genes is induced and maintained by morphogens such as Wnt, FGF and RA (Lengerke et al., 2008; Lohnes, 2003; Pilon et al., 2006). However, recent data suggest a more complex model, and show that *Cdx* genes themselves can modulate morphogen expression levels (e.g. maintenance of posterior Wnt signaling and clearance of retinoic acid in the "posterior growth zone") (Lengerke et al., 2008; Young et al., 2009).

To date, there have been no reports implicating *Cdx* genes as regulators of heart development. At early gastrula stage, cardiac precursor cells are found at the anterior region of the primitive streak. During gastrulation, they leave the primitive streak and migrate anterolaterally to form the precardiac mesoderm within the left and right anterior lateral plate mesoderm. Here, commitment to the heart lineage occurs in response to endoderm-derived signals such as BMP, FGF and Wnt-antagonists (reviewed by (Nakajima et al., 2009)) and in accordance to retinoic acid exposure (Keegan et al., 2005). Given the prominent role of *Cdx* genes during early patterning processes, we hypothesized that they play roles in the development of anterior mesoderm derivatives such as cardiac cells. In this report we analyze the impact of *Cdx* genes on cardiac development from mouse ESC and during *in vivo* zebrafish embryo development by performing functional studies and analyzing expression of markers indicating commitment to the cardiac lineage such as *Nkx2.5* and *Mesp1* (Bondue et al., 2008; David et al., 2008).

## Materials and methods

### Cell culture and differentiation

i*Cdx1*, i*Cdx4* and parental Ainv15 murine ESC (Kyba et al., 2002; Lengerke et al., 2008; McKinney-Freeman et al., 2008; Wang et al., 2008) were cultured as reported on irradiated mouse embryonic fibroblasts in Dulbecco modified Eagle medium with 15% fetal calf serum (HyClone Laboratories, Logan, UT), 1000 U/ml leukemia inhibitory factor (Chemicon International, Temecula, CA), 2 mM penicillin/streptomycin/glutamine (Invitrogen, Carlsbad, CA), 0.1 mM nonessential amino acids (Invitrogen), and 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St Louis, MO) at 37 °C/5% $CO_2$ (Kyba et al., 2002). Media was refreshed daily, and cultures were passaged with trypsine (Invitrogen) every 2 to 3 days.

Murine ESC was differentiated in embryoid bodies (EB) as described previously (Kyba et al., 2002; Lengerke et al., 2008). Briefly, confluent cultures were harvested and resuspended at a concentration of 100 cells/20µl in EB differentiation media composed of Iscove modified Dulbecco medium (IMDM) plus 15% fetal calf serum (StemCell Technologies, Vancouver, BC), 2 mM penicillin/streptomycin/glutamine (Invitrogen), 4.5 mM monothioglycerol (Sigma-Aldrich), 200 µg/ml holo-transferrin (Sigma-Aldrich), and 50 µg/ml ascorbic acid (Sigma-Aldrich). EB were cultured in 20 µl hanging drops for 48 hours and then transferred and cultured in 10 $cm^2$ petri dishes for an additional 4 days at 37 °C/5% $CO_2$ while shaking at 50 rpm. For ectopic gene expression, doxycycline (1.0 µg/ml; Sigma-Aldrich) was added as indicated.

### Flow cytometry and cell sorting

EB cells were stained with PE-conjugated anti-Flk1 (BD Pharmingen). Flow cytometry was performed on a FACSCalibur from Becton Dickinson. Flk1 positive and Flk1 negative cells were isolated with magnetic anti-PE microbeads (Miltenyi Biotech). Purity after sorting was >90%.

### Functional cardiomyocyte assessment

At day 6 of EB differentiation 150–200 EB were transferred from the shaking petri dishes onto gelatinized six-well dishes (Gelatine 1%, Biochrom AG) and further cultured in EB differentiation medium. At days 7, 9 and respectively 11, EB containing beating areas were scored by counting a total number of at least 50 to 100 EB for each condition.

### Real-time RT-PCR

Cells were harvested in RLT Buffer (supplied with RNeasy Mini Kit, Qiagen), and total RNA was isolated according to manufacturer instructions, including on-column DNAse treatment. cDNAs were prepared according to the manufacturer's protocol using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative PCR was performed using SYBR Green reagent (Eurogentec) on a Light Cycler480 Real-time PCR instrument (Roche Applied Science). Primer sequences were used as previously reported (Lengerke et al., 2007) or as listed in Table 1. The annealing temperatures that were used are listed in Table 1. All primers were used at 150 nM.

### Zebrafish husbandry, genetic strains, chemical treatments and morpholinos

Zebrafish were maintained and staged as described (Kimmel et al., 1995). The *cdx4* ($kgg^{tv205}$) mutant allele was maintained on the Tübingen strain, and incrosses of heterozygous adults were used to obtain *cdx4*−/− embryos. Doubly deficient *cdx1a/4* embryos were generated by injecting *cdx4*−/− embryos at the 1-cell stage with *cdx1a* morpholino (CAGCAGATAGCTCACGGACATTTTC) as described (Davidson et al., 2003). Both retinoic acid signaling antagonists, diethylaminobenzaldehyde (DEAB) (Sigma) and the RARα antagonist R0-41-5253 (Biomol International), were dissolved in 100% dimethyl sulfoxide (DMSO) to make 0.1 M stock solutions, and aliquots were stored at −80 °C. All-trans retinoic acid (RA) Sigma was dissolved in 100% DMSO to make a 1 M stock, and aliquots were stored at −80 °C. For chemical treatments, embryos were incubated in $1.6\times10^{-5}$ M DMSO (vehicle control), $1.6\times10^{-5}$ M DEAB/DMSO in E3 embryo media, $1\times10^{-7}$ M Ro-41-5253/DMSO in E3 embryo media, or $1.6\times10^{-5}$ M

**Table 1**
Primer sequences.

| Gene | Forward primer | Reverse primer | Annealing temperature |
|---|---|---|---|
| *Cdx1* | GTA AGA CCCGAACCA AGGAC | GGA ACCAGATCTTTA CCTGC | 58 °C |
| *Cdx4* | GAGGAAGTCAGAGCTGGCAGTTA | GGCTCTGCGATTCTGAAACC | 60 °C |
| *Gata4* | AGGGTGAGCCTGTATGTA ATGCCT | AGGACCTGCTGGCGTCTTAGATTT | 63 °C |
| *Isl-1* | GCA AGGACA AGA AACGCAGCATCA | ACTGGGTTAGCCTGTAAACCACCA | 63 °C |
| *Mesp1* | TACGCAGAA ACAGCATCCCAGGAA | CCAGGTTTCTAGAAGAGCCAGCAT | 63 °C |
| *Nkx2.5* | CAA GTGCTCTCCTGCTTTCC | CCAGCTCCACTGCCTTCTG | 60 °C |
| *MyoD1* | ATCCCTAAGCGACACAGA ACAGGGAA | TGCAGTCGATCTCTCAAAGCACCT | 63 °C |
| Myogenin | ACAATCTGCACTCCCTTACGTCCA | TCTCAGTTGGGCATGGTTTCGTCT | 63 °C |
| GAPDH | AATGCATCCTGCACCACCAACTGCTT | AGTGATGGCATGGACTGTGGTCAT | 64 °C |
| Actin | TCTTGGGTATGGAATCCTGTGGCA | ACTCCTGCTTGCTGATCCACATCT | 59 °C |

DEAB with $1 \times 10^{-8}$ M RA in E3 embryo media (DEAB rescue study), between 60% epiboly and the 15 somite stage. The *cdx4* mutation, *cdx1a* morpholino, and RA antagonists produced fully penetrant effects as described (Wingert et al., 2007). Following chemical incubation, embryos were rinsed in E3 media then fixed in 4% paraformaldehyde (PFA) in 1X Pbst for gene expression analysis. Whole-mount in situ hybridization of zebrafish embryos was performed as described (Davidson, et al., 2003), using established antisense probe construction as reported for *krox20, mhc, myoD, pax2a* (Wingert, et al., 2007), *nkx2.5* (Serbedzija, et al., 1998), *tbx5a* (Begemann and Ingham, 2000) and *cmlc2* (Yelon, Horne, Stainier, 1999). For each reported gene expression pattern, at least 15 embryos were examined. Embryos were deyolked and flat-mounted on glass slides, then photographed using a Nikon Eclipse 80i microscope and Nikon CoolPix 4500 digital camera. Nikon Elements Basic Research software was used with a Nikon DS-Fi1 color camera system to measure the areas of *nkx2.5* and *cmlc2*-expressing cells.

### *Statistics*

For all experiments, error bars represent the standard error, and *P* values are derived via the application of a 2-tailed, unpaired Student *t* test. $^{*}p<0.01$, $^{**}p<0.005$.

## Results

### *Endogenous and ectopic Cdx1 and Cdx4 expression in differentiating murine ESC*

ESC differentiation into EB recapitulates the commitment events of early embryonic development and can be documented as temporal waves of tightly controlled transcription factors such as *Cdx* genes (Fig. 1A) and lineage-specific gene expression (Keller, 2005). Brachyury-positive, primitive-streak like mesodermal cells emerge sequentially between days 2 and 4 of EB development (Fig. 2A). Gene expression indicative of mesodermal commitment to specific fates occurs slightly later, around days 3 to 4 of EB development for hematopoietic (not shown) and days 4 to 5 of EB development for cardiac cell fates with specified hematopoietic and cardiac cells peaking around day 6 of EB development (Keller, 2005) (Fig. 2A). Focusing on days 0 to 11 of EB development, we assessed the expression pattern of murine *Cdx1* and *Cdx4* by performing gene expression analysis by real-time PCR (Fig. 1A). As previously reported, undifferentiated mouse ESC express only very low levels of *Cdx4* but higher levels of *Cdx1* (McKinney-Freeman et al., 2008). When ESC was differentiated as EB, *Cdx1* and *Cdx4* expression increases showing enhanced expression around days 1 and 3 of differentiation for *Cdx1* and slightly later, at day 3, for *Cdx4*. After day 3 and respectively day 4, *Cdx1* and *Cdx4* expression rapidly wanes (Fig. 1A). *Cdx1* sometimes shows a second peak of slightly enhanced expression at later time-points (e.g. day 6 to day 8, Fig. 1A and data not shown), probably reflecting expression in developing endodermal tissues. Thus, as previously reported (McKinney-Freeman et al., 2008), *Cdx* genes are expressed in an overlapping temporal manner with *Cdx1* slightly preceding *Cdx4* and overall both genes active in the time-window when mesoderm is formed and patterned to specific fates. This *in vitro* expression pattern is in accordance with the *in vivo* expression data reporting most intense *Cdx* induction at the time of gastrulation in the primitive streak. The earliest mesodermal cells to adopt a cardiac fate are the cardioangioblasts which can be isolated as Flk1 positive cells at day 4 of EB development (Kattman et al., 2006). We detected *Cdx1* and *Cdx4* transcripts in this population, although at lower levels than in the corresponding Flk1 negative fraction containing progenitors of other tissues such as hematopoietic, renal and endodermal lineages (Fig. 1B).

To test the effect of *Cdx1* and *Cdx4* expression on cardiogenesis, we used two previously generated mouse ESC lines with *Cdx1* and *Cdx4* under the control of a tetracycline inducible promoter, respectively (Davidson et al., 2003; Kyba et al., 2002; McKinney-Freeman et al., 2008). Doxycycline exposure of day 2.25 EB generated from inducible *Cdx1* (i*Cdx1*) and *Cdx4* (i*Cdx4*) ESC enhances *Cdx* gene transcripts (Fig. 1C) and protein levels (McKinney-Freeman et al., 2008) within their endogenous expression window in a tightly regulated manner

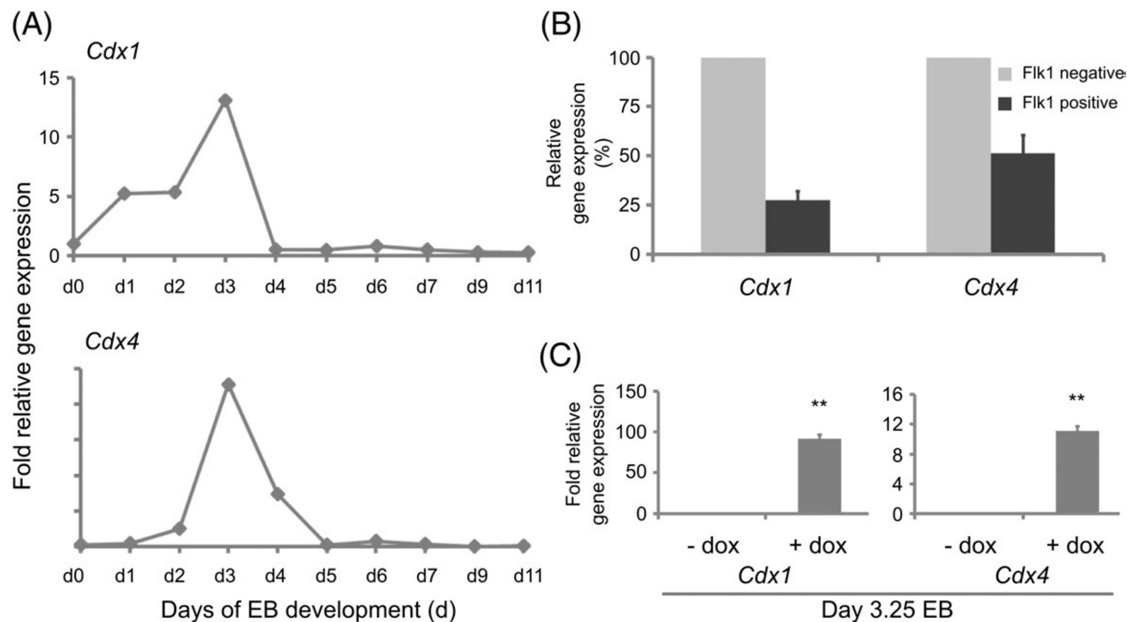


**Fig. 1.** (A) Time course of *Cdx1* and *Cdx4* expression in differentiating EB shows an expression peak around day 3 of EB development. Mouse ESC was differentiated in EB and samples collected between day 1 and day 11 of EB development were subjected to real-time PCR analysis. Results are shown as fold expression relative to expression in undifferentiated ES cells. Shown are data from one representative experiment. (B) Expression of *Cdx1* and *Cdx4* in Flk1 positive cardioangioblasts isolated at day 4 of EB development. Results are shown as % expression relative to Flk1 negative cells isolated at the same point in time. Shown are data from two biological experiments which showed a purity >90% in the sorted Flk1 positive population. (C) Ectopic *Cdx1* and *Cdx4* gene induction within the endogenous expression window. i*Cdx1* and respectively i*Cdx4* ES cells were differentiated until day 2.25 in basic differentiation medium to allow mesoderm induction and afterwards further cultured in basic differentiation medium +/− doxycycline. Induction of *Cdx1* and respectively *Cdx4* induction were analyzed by real-time PCR performed 24 hours later on day 3.25 EB. Results are shown as relative fold expression in comparison to non-induced controls collected at the same time-point. Gene expression levels summarize endogenous and if applicable ectopic levels. Shown are summarized results from three experiments.

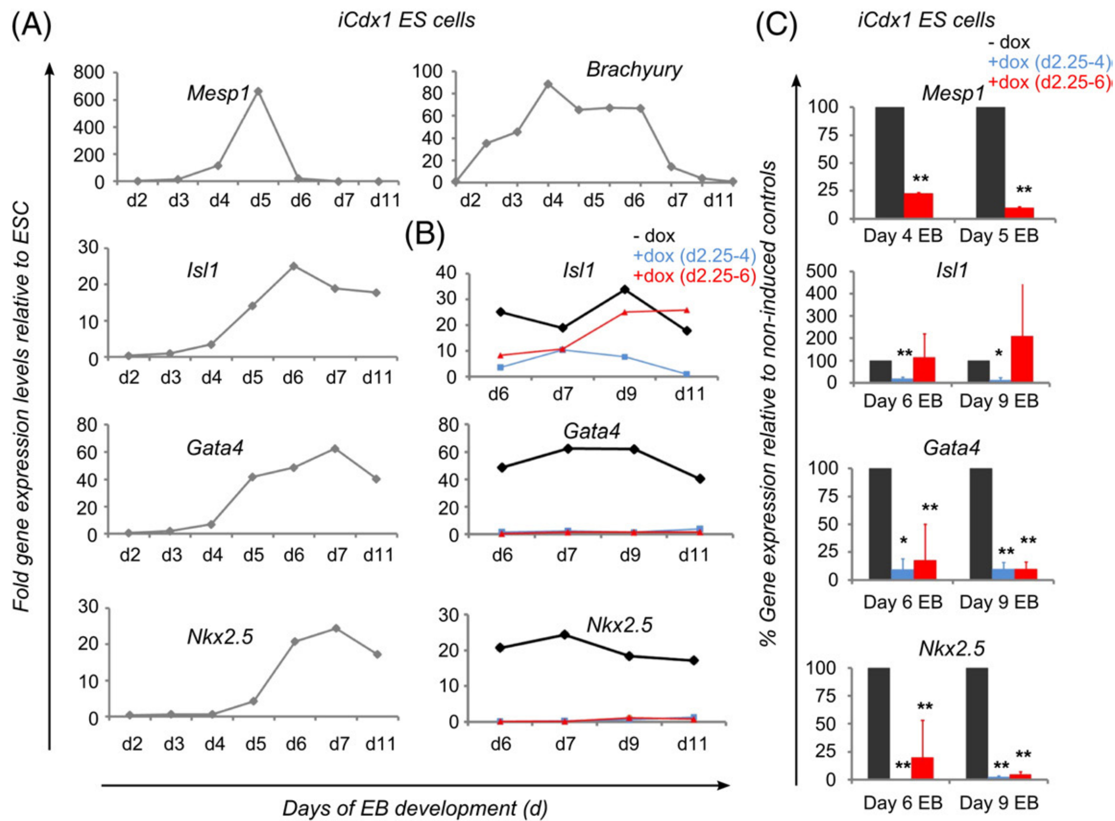


**Fig. 2.** Expression pattern of genes involved in mesoderm and cardiac development during *in vitro* EB differentiation. Suppressive effects of *Cdx* induction on cardiac genes. (A) Markers of early cardiac development such as *Mesp1*, *Isl-1*, *Gata4* and *Nkx2.5* are upregulated during EB differentiation shortly after establishment of the mesodermal marker *Brachyury*. *Mesp1* shows a sharp peak of expression and extinguishes rapidly upon activation of other cardiac genes such as *Nkx2.5*. i*Cdx1* ES cells were differentiated in basic differentiation medium in the absence of doxycycline and samples collected at several time-points. Gene expression analysis was performed by real-time PCR analysis and results are shown as fold gene expression relative to expression levels in undifferentiated ES cells. Shown are data from one representative experiment for each marker. (B, C) Continuous suppression of *Isl-1*, *Gata4* and *Nkx2.5* following early induction of *Cdx1* by addition of doxycycline between days 2.25 to 6 (B, C) or as a brief pulse between days 2.25 to 4 only (C). A, B: Shown are data from one representative experiment; C: shown are summarized data from at least two independent experiments for each analyzed condition.

(Supplemental Fig. 1A). Moreover, previously documented auto- and cross-regulation of *Cdx* genes (Beland et al., 2004; Lengerke et al., 2008; Young et al., 2009; Savory et al., 2011) may contribute to the enhanced gene expression levels documented by PCR (Fig. 1C). As expected, no induction of *Cdx* was reported by addition of doxycycline in parental cells (McKinney-Freeman et al., 2008; Supplemental Fig. 1C).

### *Mesodermal and cardiac differentiation from iCdx1 and iCdx4 ES cells*

Since individual ESC lines have been reported to behave differently with respect to cardiac development, we first examined cardiac development in our ESC lines by performing gene expression time-courses and analyzing the formation of functional cardiac cells at different time-points. The i*Cdx1* and i*Cdx4* ESC lines, which have been generated in the same Ainv15 ES cell background (Kyba et al., 2002), behaved similarly in these assays (data not shown) and experiments performed in these ESC are shown in summary (Figs. 2A and 3A).

As expected, cardiac gene expression was induced around day 4 of development, after mesoderm had been initiated as indicated by the expression of *Brachyury*. These developmental kinetics are congruent with previously reported results showing that cardiac progenitors first arise at day 4 of EB development in the form of a Flk1 positive population (Kattman et al., 2006; Kouskoff et al., 2005), slightly after the development of the blood and vascular lineages at day 3 (Fehling et al., 2003). Of note, the earliest marker reported to induce cardiac development, *Mesp1,* showed an induction peak around days 4 and 5, before extinguishing abruptly. *Nkx2.5* transcripts, which initiate following cardiac progenitor specification, followed *Mesp1* expression and showed strong up-regulation at day 6 of EB development. *Isl-1* and *Gata4* showed a rather early induction, consistent with their role in early cardiac specification, but expression did not wane at later time-points, indicating an on-going involvement in cardiac development and/or differentiation of other cell lineages.

Of note, doxycycline treatment alone did not impact differentiation of parental Ainv15 cells lacking transgenes (Supplemental Fig. 1B and C).

### *Cdx1 or Cdx4 activation during mesoderm formation suppresses the formation of beating cardiomyocytes in differentiating murine ESC*

To assess the role of *Cdx* during cardiac development, i*Cdx1* and i*Cdx4* ESC were cultured in the presence or absence of doxycycline. In the cultures supplemented with doxycycline, addition was performed between days 2.25–6 or days 2.25–4 of EB differentiation, in order to enhance *Cdx* expression within their endogenous expression window (Fig. 1A). At day 6, EB were transferred onto gelatinized plates and cultured in basic differentiation medium without doxycycline for another 1 to 5 days. Development of cardiac cells was assessed by scoring the development of EB presenting "beating" areas (Wu et al., 2006). In some experiments, a few beating areas were observed as early as day 7 (Fig. 3A). At day 9 consistent beating was observed in approximately 75% of EB throughout the experiments, with no progression occurring at later time-points such as day 11 (Fig. 3A).

Induction of either *Cdx1* or *Cdx4* under these conditions strongly suppressed beating at all points in time, suggesting that the inhibitory effects were not due to differences in ESC differentiation dynamics but rather a specific developmental effect on cardiogenesis (Fig. 3B–D). A

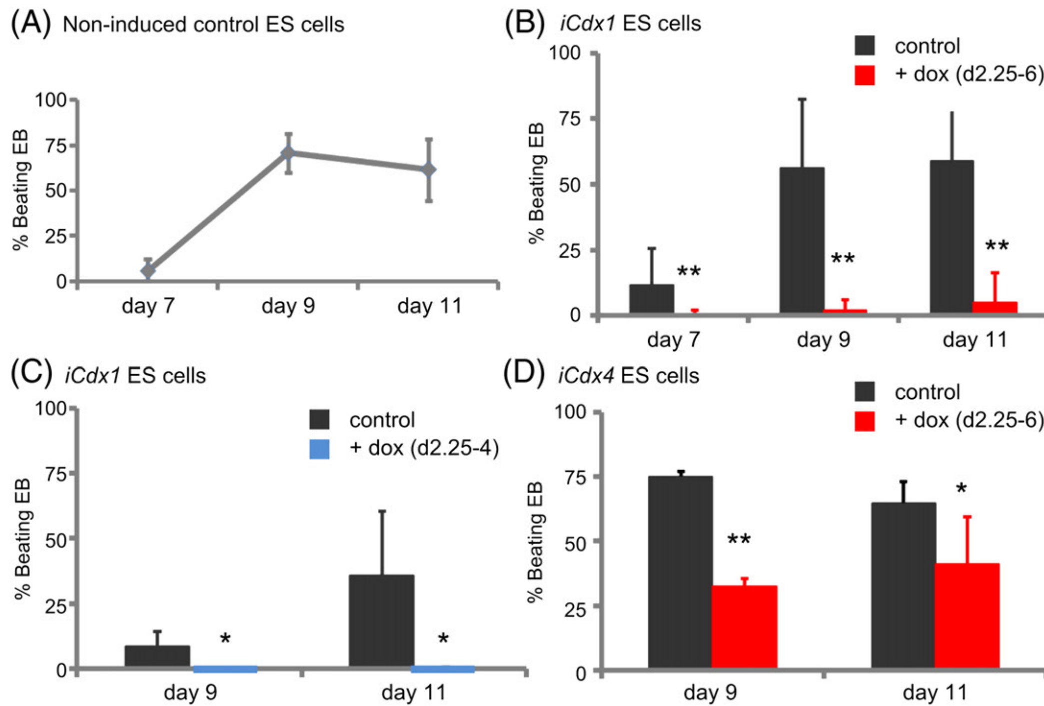


**Fig. 3.** *Cdx1* and *Cdx4* suppress the formation of "beating" cardiomyocytes from differentiating EB. (A) Time-course of development of "beating" clusters in i*Cdx* ES cells differentiated in basic differentiation medium without addition of doxycycline first detects functional cardiomyocytes around day 7 of EB development with reproducible development in high numbers at days 9 and 11 across experiments. Shown are summarized data on % beating EBs in non-induced i*Cdx1* and i*Cdx4* EB collected in nine independent experiments. (B, C, D) Exposure to doxycycline during EB development (from days 2.25 to 6 and respectively from days 2.25 to 4) strongly suppresses cardiomyocyte development from both i*Cdx1* and i*Cdx4* ES cells at all analyzed time-points. Results are shown as % beating EB in doxycycline induced cells in comparison to non-induced controls. Shown are data from at least three independent experiments for each analyzed condition.

brief pulse of doxycycline between days 2.25 and 4 was sufficient to inhibit cardiogenesis (Fig. 3C), consistent with an early effect of *Cdx* on cardiac development. The suppressive effect was stronger for *Cdx1* than *Cdx4*, supporting data collected in other murine organs where *Cdx4* showed less potent effects than *Cdx1* (Deschamps and van Nes, 2005; Lengerke et al., 2008; McKinney-Freeman et al., 2008). However, the more pronounced effect of *Cdx1* in our system could also be due to the fact that ectopic activation resulted in a greater increase in gene expression relative to endogenous levels documented at this developmental stage.

*Cdx induction inhibits the expression of cardiac genes, including the early marker Mesp1*

We next examined cardiac marker expression in i*Cdx1* and i*Cdx4* ESC and EB at several time-points. Up-regulation of *Cdx* expression at day 2.25 inhibited the induction of *Gata4*, *Nkx2.5* and *Mesp1* (Figs. 2B and C, 4A and B). Interestingly, modulation of *Isl-1* expression was different when *Cdx* activation was performed in the time-window between days 2.5–4 or continued until day 6, suggesting that in addition to an early role at the level of mesoderm, *Cdx* may impact the differentiation of *Isl-1* regulated tissues at later time-points (Figs. 2B and C, 4A and B). Overall, ectopic *Cdx1* showed a stronger suppressive effect than ectopic *Cdx4*, confirming the results in functional assays (Fig. 3B and D).

*Cdx morphants display a dose-dependent enhancement in tbx5a$^+$ cardiac mesoderm*

Next, we examined whether *Cdx* genes function to restrict cardiac fates during heart development *in vivo* using the zebrafish embryo as a model. As *cdx4* and *cdx1a* have established partially redundant roles in mesoderm patterning in the zebrafish (Davidson and Zon, 2006), we analyzed the expression of cardiac lineage markers in *cdx4* deficient and *cdx4/1a* doubly-deficient embryos using whole mount *in situ* hybridization. The expression of *tbx5a*, a marker of the anterior lateral plate mesoderm that includes the precursor cardiomyocyte population, was expanded posteriorly in both *cdx4* deficient embryos and *cdx1a/4* doubly-deficient embryos (Fig. 5). Conversely, the expression domain of *pax2a*, an intermediate mesoderm marker, was reduced and shifted posteriorly (Fig. 5). These data suggest that broad mesodermal patterning was altered in the absence of *cdx* activity. However, the expression domains of *nkx2.5* and *cmlc2*, which are specific to cardiac precursors, were not altered in *cdx* deficient embryos (Figs. 6 and 7; Supplemental Fig. 2). This finding was surprising given the changes in mesoderm gene expression and the expression changes subsequent to *Cdx* overexpression in ESC and EB cultures.

*Expansion of nkx2.5 and cmlc2 expressing cardiac cells in cdx mutants requires simultaneous inhibition of the RA pathway*

During cardiac specification in the zebrafish embryo, retinoic acid (RA) signaling plays an essential role in restricting the number of cardiac progenitors (Keegan et al., 2005). RA is synthesized during development through the sequential action of enzymes that include *retinaldehyde dehydrogenases* (*raldh*s or *aldh*s), and RA signals are conferred by binding to complexes of retinoic acid receptors (RARs, RXRs) (Duester, 2008). Chemical modulation of RA signaling by exposure to RA antagonists causes expansion of the cardiomyocyte pool (Keegan, et al., 2005). We hypothesized that the presence of endogenous RA signaling was sufficient to limit cardiac expansion in the setting of *cdx* deficiency. To test this idea, we treated *cdx4*-deficient and *cdx1a/4*-deficient embryos with diethylaminobenzaldehyde (DEAB), a pan-raldh inhibitor, or RO-41-5253, a RARα-antagonist. Interestingly, exposure to DEAB or RO-41-5253 induced a significant expansion in the *nkx2.5*-expressing in *cdx4*-deficient embryos, and *cdx1a/4*-deficient embryos showed an even more dramatic expansion

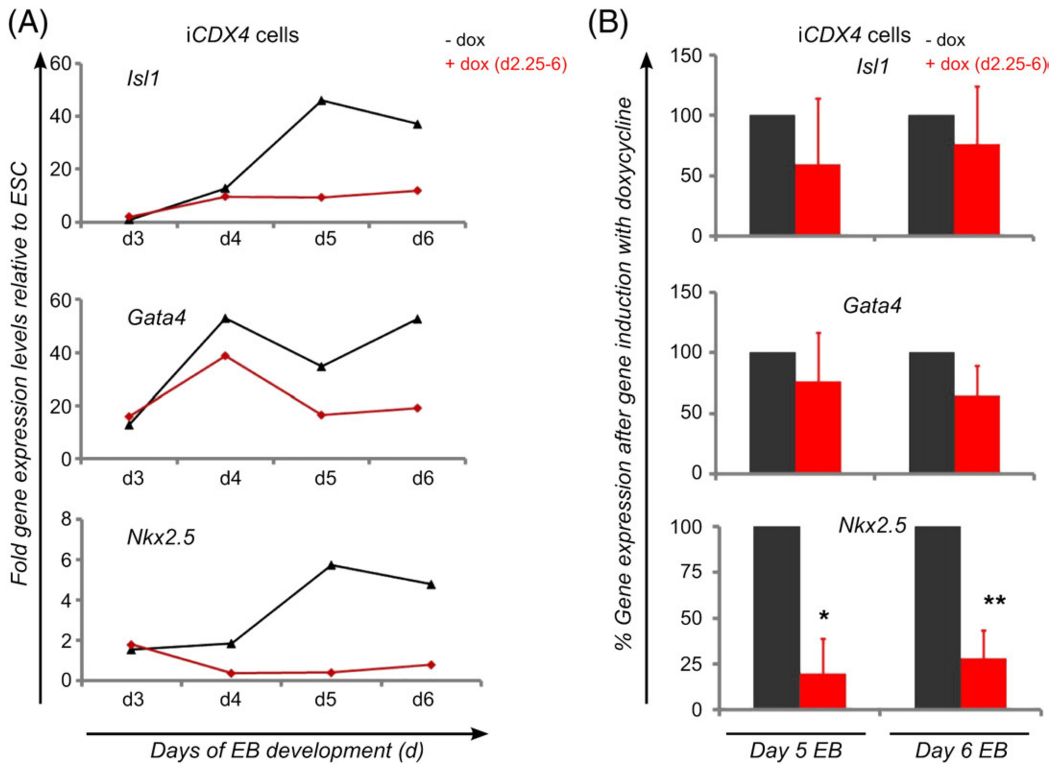


**Fig. 4.** Effect of ectopic Cdx4 on the expression of cardiac genes in differentiating murine ES cells. (A) Time-course analysis in samples collected from i*Cdx4* ES cells differentiating as EB with or without exposure to doxycycline during days 2.25 to 6 of development shows continuous suppression of *Gata4*, *Nkx2.5* and *Isl-1*. Results are shown as fold expression levels relative to gene expression in undifferentiated ES cells. Shown are data from one representative experiment showing more pronounced effects. (B) Gene expression analysis in day 5 and day 6 i*Cdx4* EB previously differentiated in basic medium with or without exposure to doxycycline as indicated shows strong suppression of *Nkx2.5* while only mild, non-significant effects were seen on expression of *Isl-1* and *Gata4*. Shown are data on % gene expression relative to non-induced controls collected at the same time-points in at least two independent experiments.

(Fig. 6; Supplemental Fig. 2A and C). Next, we examined the expression of the cardiac myosin gene *cmlc2* in *cdx*-deficient animals when RA levels were reduced. Both *cdx4*-deficient and *cdx1a/4*-deficient embryos exposed to DEAB displayed significant expansions of *cmlc2*-expressing cells, as compared to wild-type embryos similarly treated with DEAB (Fig. 7; Supplemental Fig. 2B and D). In addition, the expansion of the *cmlc2* field in *cdx*-deficient embryos was rescued when they were treated with a combination of DEAB and RA (Fig. 7; Supplemental Fig. 2B and D). This finding provides evidence that the restoration of RA levels is sufficient to reduce the expansion of cardiac progenitors. Taken together, these data confirm our hypothesis that RA is the critical signaling molecule that restricts the expansion of cardiac fate in a *cdx1a/4*-deficient background.

## Discussion

*Cdx* transcription factors regulate positional identities and have crucial roles in anteroposterior patterning processes during embryonic development, where they have been especially studied as regulators of *Hox* gene expression in the paraxial mesoderm (reviewed by (Deschamps et al., 1999)). More recent data collected in zebrafish indicate an additional involvement of *Cdx* genes in the patterning and formation of posterior mesodermal tissues such as embryonic blood and kidney (Davidson et al., 2003; Wingert et al., 2007). Studies in mouse and human pluripotent stem cells suggest conservation of pathways and redundant roles for *Cdx1* and *Cdx4* during mesoderm specification to blood in mammalian cells (Lengerke et al., 2009;

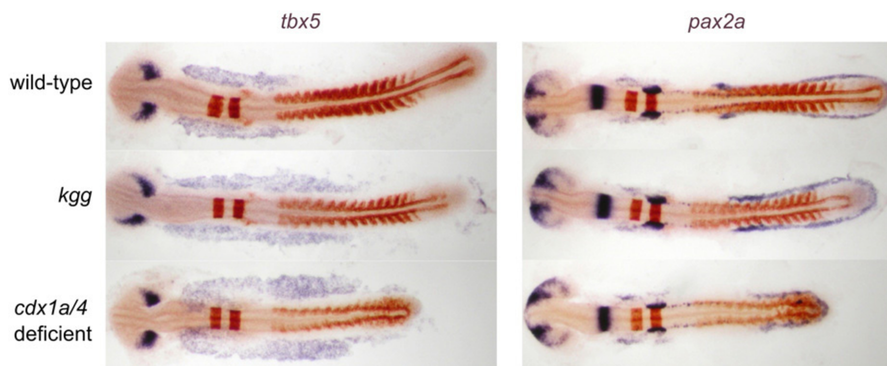


**Fig. 5.** Whole mount *in situ* hybridization analysis of *tbx5a* expression in wildtype, *cdx4 and cdx1a/4*-deficient embryos shows expansion of cardiogenic anterior-lateral plate mesoderm. The intermediate mesoderm field, marked by *pax2a* expression, shows a posterior shift and reduction in cell number, as reported previously. The effect was more pronounced in *cdx1/4* double deficient embryos than in *cdx4*−/− embryos. Shown are zebrafish embryos at 15-somite stage. Purple staining was used for *tbx5* and *pax2a* and red staining for *krox20* and *myoD* as landmarks of other tissues.

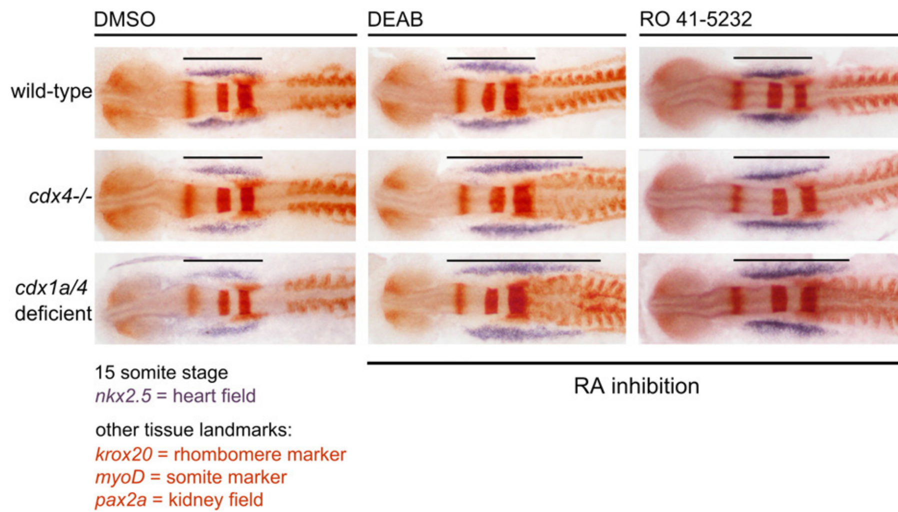


**Fig. 6.** Excessive *tbx5* positive anterior-lateral plate mesoderm formed in *cdx*-deficient embryos requires additional inhibition of the retinoic acid pathway in order to adopt cardiac fate and form *nkx2.5* expressing cardiac cells. Shown are wildtype, cdx4−/− and cdx1/4 deficient embryos analyzed at 15-somite stage after treatment with the retinoic acid inhibitors DEAB, Ro-41-5253, or DMSO (vehicle) as a control. *cdx*-dose-dependent effects are observed with both inhibitors.

2007; 2008; McKinney-Freeman et al., 2008). Generation of blood formation has been intensively studied on the molecular level in different models and *Cdx* genes have shown to regulate blood formation by the induction of posterior *Hox* genes (Davidson et al., 2003; Davidson and Zon, 2006; Lengerke et al., 2008; Wang et al., 2008), in a partially Wnt-dependent fashion (Lengerke et al., 2008), as well as via regulation of RA production (de Jong et al., 2010).

The current report analyzes whether the regulatory effects of *Cdx* genes on early mesoderm development extend to cardiac cells, which are derived from more anterior mesoderm. We show in mouse EB *in vitro* and zebrafish embryos *in vivo* that *Cdx* genes in cooperation with RA may restrict the development of mesoderm prone to undergo cardiac differentiation and the early development of cardiac cells. In EB, a pulse of *Cdx* expression, during the window of mesoderm patterning, is sufficient to suppress cardiac development and marker expression, whereas loss-of-function studies in zebrafish show that *cdx* genes restrict the size of the *tbx5a*$^{+}$ "pre-cardiac" anterior lateral plate mesoderm domain. Both results argue for an early effect of *Cdx* genes on heart development, at the level of mesoderm.

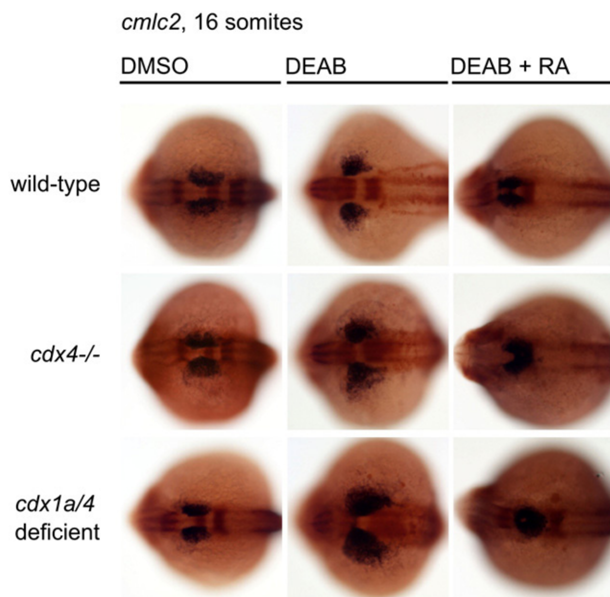


**Fig. 7.** Suppression of RA levels expands cardiac myosin expressing-cells (*cmlc2*) in *cdx*-deficient embryos, and can be rescued by exogenous RA treatment. Expression of *cmlc2* expression in wildtype, *cdx4* and *cdx1a*/*4*-deficient embryos incubated with DMSO (control), DEAB, or DEAB and RA, was assayed by whole mount *in situ* hybridization at the 16 somite stage. *cdx* mutants evinced dramatic expansion of cardiac mesoderm when RA production was abrogated, and this phenotype was rescued by concomitant DEAB and RA treatment.

The effects of *Cdx* overexpression on heart development are unlikely to be due to non-specific toxicity as we have previously shown that EB morphology and cell number are unaffected by *Cdx* overexpression and treatment with doxycycline alone had no impact on cardiac differentiation in our system (Supplemental Fig. 1B and C). In addition, we found that induction of *Cdx1* and *Cdx4* during a similar time-window to that reported here promotes hematopoietic development (Lengerke et al., 2008). We explored the effects of *Cdx* induction on other mesodermal derivatives, such as muscle but could find no consistent alteration (Supplemental Fig. 3). However, possible shifts in formation of posterior rather than anterior muscle would not be detectable by our method of whole EB assessment by PCR. Together, these findings argue that the inhibitory effect on the cardiac lineage is specific and not the result of general toxic effects of ectopic *Cdx* expression.

The *in vitro* development of ESC is highly dynamic and gene expression as well as the functional capacity of the cells depends on their developmental stage. The early cardiac-suppressing effect of *Cdx* genes was not due to impaired developmental dynamics, as our time-course of marker gene expression and assessment of beating areas showed that suppression could be detected at all analyzed time-points. In contrast, activation of *Cdx* genes at later developmental time-points (e.g. after gastrulation and the formation of *Nkx2.5* expressing cardiac progenitor cells) cannot reverse the developmental program and moreover does not show any inhibitory effect on cardiac cell development (Ehrman and Yutzey, 2001).

A number of studies have shown that the *Cdx* genes display both gene-specific as well as redundant activities. Knock-out experiments in the mouse have demonstrated that individual *Cdx* genes have slightly different impacts on vertebral patterning. *Cdx1*-null mice mainly show abnormalities in the cervical and thoracic region, partially following the rules defined for anterior transformations (van den Akker et al., 2002, and references therein), while *Cdx2* and *Cdx4* give rise to transformations of posterior cervical vertebrae and elements of the thoracic region, with much higher severity and penetrance for *Cdx2* compared to *Cdx4* (van Nes et al., 2006). Defects in sternal ribs suggest additional disruption of contributions to the paraxial mesoderm in *Cdx* mutants (van den Akker et al., 2002; Kato and Aoyama, 1998; Pinot, 1969). In zebrafish, *cdx4*-deficient fish display diminished blood development,

and further suppression of *cdx1a* results in complete ablation of posterior embryonic blood, consistent with redundancy (Davidson et al., 2003; Davidson and Zon, 2006). In the mouse, the most severe *Cdx* loss-of-function allelic combination possible, the *Cdx1/2/4* null embryo, has not been examined as *Cdx2* is necessary for early placenta development (Strumpf et al., 2005). More recently, conditional *Cdx2* knockout mice have been generated to study functions in specific lineages such as the enterocytes and the intestinal epithelium (Gao and Kaestner, 2010; Grainger et al., 2010). Redundant roles of *Cdx1* and *Cdx4* are found in hematopoietic development of murine ESC (Lengerke et al., 2008; McKinney-Freeman et al., 2008). Our data are consistent with *Cdx1* and *Cdx4* having similar, and redundant, suppressive effects on cardiac development.

Despite *cdx*-deficiency having a widespread effect on mesoderm patterning in zebrafish embryos, including a posterior expansion in *tbx5a*$^+$ anterior lateral mesoderm, there was no significant change in the number of *nkx2.5* and respectively *cmlc2* expressing cardiac cells. During gastrulation, Keegan and colleagues (2005) have demonstrated that blocking RA signaling leads to an excess of cardiac precursors being specified, most likely at the expense of other tissues such as the pectoral fin mesenchyme. Since RA was shown to restrict cardiac development and interactions between *cdx* and RA have been previously reported in the development of other organs, we tested whether the development of cardiac cells in *cdx*-deficient embryos was inhibited by RA signaling. A significant increase in *nkx2.5* and respectively *cmlc2* expressing cells was observed in *cdx*-deficient fish concomitantly treated with RA inhibitors, in comparison to untreated fish or treated wildtype controls. This dual requirement for *cdx*-deficiency and RA inhibition in order to reveal a cardiac phenotype is most likely due to the *raldh2* expression domain being posteriorly expanded in *cdx*-deficient embryos (de Jong et al., 2010; Shimizu et al., 2006; Wingert et al., 2007). Thus, the increase in *tbx5a*$^+$ "pre-cardiac" anterior lateral plate mesoderm tissue in *cdx*-deficient embryos is offset by a concomitant increase in the cardiac-suppressive effects of RA signaling.

The work presented here suggests that the *Cdx* genes act to localize specific tissues within the developing mesoderm (e.g. posterior derivatives such as the blood and kidney at the expense of cardiac cells in the anterior lateral plate). This hypothesis is also supported by studies of other germ layers. In the zebrafish ectoderm, *cdx4* is required to establish the boundary between the hindbrain and spinal cord territories. Loss of *cdx4* results in posterior expansion of the segmented hindbrain at the expense of the spinal cord and, conversely, overexpression of *cdx4* has a posteriorizing effect (Shimizu et al., 2006; Skromne et al., 2007). As seen in our studies, *cdx1a/4*-deficient embryos show enhanced phenotypes consistent with functional redundancy between the genes (Shimizu et al., 2006; Skromne et al., 2007). Within the endoderm, *cdx4* confers posterior identity and regulates the localization of the developing pancreas (Kinkel et al., 2008).

Interestingly, important interactions between the *cdx* genes and retinoic acid (RA) were also found. While several reports demonstrated that RA can specify the pancreas in vertebrates (Chen et al., 2004; Martín et al., 2005; Molotkov et al., 2005; Stafford et al., 2004; Stafford and Prince, 2002), treatment of zebrafish embryos with RA only induces insulin-producing cells in the anterior endoderm, whereas simultaneous inhibition of *Cdx* function is required to induce this effect posteriorly. These data in the endoderm support our results in the developing mesoderm, and suggest that the normal (posterior) expression of *Cdx* genes may in general regulate cell fate by modulating inhibitory RA signals activity.

Taken together, our study has identified a strong *Cdx*-mediated suppression of cardiac development, reinforcing a general regulatory role of *Cdx* genes within the developing mesoderm. Moreover, our data reveal novel interactions between *Cdx* genes and the RA pathway, and suggest that *Cdx* genes regulate activity of inhibitory RA signals, a function which may be conserved across germ layers.

## Conclusions

This study performed in zebrafish embryo and mouse embryonic stem cells suggests that *Cdx* genes modulate early cardiogenesis presumably by acting on the level of mesoderm and regulating together with RA the development of cardiac precursors. In zebrafish embryos, loss of *cdx4* and/or *cdx1a* induced a profound, dose-dependent expansion of the anterior lateral plate mesoderm. Interestingly, further development of *nkx2.5*$^+$ cardiac progenitors and *cmlc2*$^+$ cells in cdx deficient fish requires suppression of the RA pathway, indicating close interactions between *cdx* genes and the retinoic acid pathway during early cardiogenesis.

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## Authorship

Contribution: C.L., R.W., M.B., M.G., M.K. and A.G.S. designed and performed experiments and analyzed results. C.L. and R.W. wrote the manuscript. C.L., R.W., G.Q.D. and A.J.D. designed the research and reviewed the manuscript. All authors contributed to editing the manuscript.

## Conflict-of-interest disclosure

The authors declare no competing financial interests.

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