

# Maternal *Cdx2* is dispensable for mouse development

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## SUMMARY

In many invertebrate and vertebrate species, cell fates are assigned through the cellular inheritance of differentially localized maternal determinants. Whether mammalian embryogenesis is also regulated by deterministic mechanisms is highly controversial. The caudal domain transcription factor CDX2 has been reported to act as a maternal determinant regulating cell fate decisions in mouse development. However, this finding is contentious because of reports that maternal *Cdx2* is not essential for development. Notably, all of the previously published studies of maternal *Cdx2* relied on injected RNA interference constructs, which could introduce experimental variation. Only deletion of the maternal gene can unambiguously resolve its requirement in mouse development. Here, we genetically ablated maternal *Cdx2* using a Cre/lox strategy, and we definitively establish that maternal *Cdx2* is not essential for mouse development.

**KEY WORDS:** Blastocyst, Maternal-effect gene, Trophectoderm, Caudal

## INTRODUCTION

In animals, early embryonic development is regulated by maternal genes, which are transcribed in the female germline during oogenesis. The extent to which maternal genes participate in embryo patterning varies among species. For example, in fruit fly embryos, differentially localized maternal factors define body axes, whereas in frog and nematode, maternal factors, in addition to information from the site of sperm entry, regulate early cell fate decisions. In mice, maternal factors have been identified that are essential for embryo viability (Li et al., 2010), but mouse early embryos are thought to undergo regulative, rather than mosaic, development (Johnson, 2009; Rossant and Tam, 2009; Yamanaka et al., 2006). Therefore, the contribution of maternal factors to embryo patterning remains speculative.

Mouse *Cdx2*, the ortholog of the *Drosophila* maternal-effect gene *caudal* (Frohnhofer and Nüsslein-Volhard, 1986; Mlodzik et al., 1985), is essential for early development, and loss of zygotic *Cdx2* disrupts trophectoderm development, leading to preimplantation lethality (Strumpf et al., 2005). Whether maternal *Cdx2* is also required for development is unresolved. Studies of the requirement for maternal *Cdx2* in development have yielded conflicting results (Jedrusik et al., 2010; Wang et al., 2010a; Wu et al., 2010). Notably, these reports relied on RNA interference (RNAi) to reduce *Cdx2* levels, leaving open the possibility that the differing phenotypes resulted from differing degrees of gene inactivation. To resolve unambiguously the requirement for maternal *Cdx2* in development, *Cdx2* must be deleted from the oocyte prior to fertilization using a conditional null allele. We use this approach to show that *Cdx2* is not a maternal-effect gene in mouse.

## MATERIALS AND METHODS

### Mouse strains

The following alleles or transgenes were maintained in an outbred (CD1) background: *Cdx2<sup>m1Fbe</sup>* (a null allele) (Chawengsaksophak et al., 1997), *Tg(Zp3-cre)93Kmw* (de Vries et al., 2000) and *Cdx2<sup>fl</sup>* (a new conditional allele). All animal work conformed to the guidelines and regulatory standards of the University of California Santa Cruz Institutional Animal Care and Use Committee.

### Generation of the *Cdx2* conditional allele

To generate the *Cdx2* conditional allele, the targeting construct was created by PCR amplification of 129X1/SvJ genomic DNA using PfuTurbo Hotstart DNA polymerase (Stratagene) to amplify regions of the *Cdx2* locus. Amplified regions were assembled in ploxPF1neo plasmid [gift of Dr James Shayman (Hiraoka et al., 2006)]. *PvuI*-linearized plasmid was then electroporated into R1 ES cells. *NheI*-digested genomic DNA from a total of 480 neo-resistant clones was then screened by Southern blot using a probe complementary to the 3' region. Ten positive clones were then digested with *ScaI* and screened by Southern blot using the 5' probe and by PCR using primers P1 and P2 (5'-GAATACGTCGTGTAATTAGCA-3' and 5'-CAAAGCCAACAACCTGGAC-3'). A single correctly targeted clone was selected for injection into C57BL/6J blastocysts to produce chimeric mice. Germline transmission was observed in 4/11 male chimeras, which were then bred to establish founder *Cdx2<sup>fl/+</sup>* mice. Subsequently, the neo cassette was removed by crossing founder lines to the B6;SJL-Tg(ACTFLPe)9205Dym/J mouse (Rodriguez et al., 2000).

### Embryo genotyping

Embryo genotyping was performed blind, without prior knowledge of phenotypes. Genomic DNA was extracted from individual embryos using the Red Extract-N-Amp Kit (Sigma) in a final volume of 10 µl extraction/neutralization buffers. Subsequently, 0.5–1 µl lysate was used for PCR detection of the various alleles using the following primers (5'-3'): wild type and *Cdx2<sup>m1Fbe</sup>*, AGGGACTATTCAAACCTACAGGAG, TAAAAGTCAACTGTGTTCCGATCC and ATATTGCTGAAGAG-CTTGCGGGC; *Zp3Cre*, GCGGTCTGGCAGTAAAACTATC and GTGAAACAGCATTGCTGCTACTT; and wild type, *Cdx2<sup>fl</sup>* and *Cdx2<sup>del</sup>*, P1 and P2 (see above).

### Immunofluorescence, embryo culture and microscopy

Embryos were collected from timed natural matings by flushing dissected oviducts or uteri with M2 medium (Millipore). Embryos were either fixed and stained as previously described (Ralston and Rossant, 2008) or cultured in KSOM (Millipore) at 37°C and 6% CO<sub>2</sub> to monitor morphological development. Primary antibodies included mouse anti-CDX2 (Biogenex CDX-88), rabbit anti-NANOG (Reprocell), rat anti-CDH1 (Sigma) and rat

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anti-KRT8 (Troma-1; R. Kemler, Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA 52242, USA). Secondary antibodies, nuclear stain (Draq5) and confocal microscopy methods were as described (Ralston and Rossant, 2008), using a Leica SP5 confocal microscope and 20 $\times$ , 0.7 NA objective.

#### Real-time PCR

To obtain oocytes, female mice were superovulated by subcutaneous injections of 5 IU each pregnant mare serum (PMS) and human chorionic gonadotropin (HCG) (Sigma), 46 hours apart. MII oocytes were collected 23 hours after HCG injection from dissected ampullae. Oocytes were then denuded of cumulus cells by incubation and gentle pipetting in 300  $\mu$ g/ml bovine type IV-S hyaluronidase (Sigma) in M2 medium. RNA was extracted from  $\sim$ 20 pooled oocytes or individual blastocysts using the PicoPure RNA Isolation Kit (Arcturus) according to the manufacturer's instructions. As previously described (Ralston et al., 2010), cDNA was prepared and amplified by SYBR Green-based relative quantification PCR using a Roche LightCycler 480. For each primer pair, the PCR efficiency was empirically determined from a standard curve, and this was used to calculate measurements using the  $\Delta C_T$  method using Roche software. Primers were (5'-3'): *Actb*, CTGAACCCTAAGGCCAACC and CCAGAGGCATACAGGGACAG; *Cdx2*, AACCTGTGCGAGTGGATG and TCTGTGTACACCACCCGGTA.

## RESULTS AND DISCUSSION

### Oocytes express lower levels of *Cdx2* than do embryonic stem cells

As a first step toward determining whether maternal *Cdx2* is important for development, several groups have characterized the level of protein (CDX2) and mRNA (*Cdx2*) present in mouse oocytes and ovaries. By immunohistochemistry, CDX2 was undetectable in mouse ovaries (Beck et al., 1995). Similarly, CDX2 was not detected in mouse oocytes by mass spectrometry (Wang et al., 2010b). Post-fertilization, CDX2 is not detectable at the 2- or 4-cell stages by immunofluorescence (Ralston and Rossant, 2008), suggesting that maternal *Cdx2* is not a major source of CDX2 in the embryo. *Cdx2* mRNA levels were reportedly low, but detectable, in oocytes (Jedrusik et al., 2010; Wang et al., 2010a). Both these studies reported lower levels of *Cdx2* in oocytes than in blastocysts, but neither group examined *Cdx2* levels in a cell type in which *Cdx2* is functionally irrelevant. For example, embryonic stem (ES) cells do not require *Cdx2* (Chawengsaksophak et al., 2004) and could be used as a negative control. We therefore compared levels of *Cdx2* mRNA in blastocysts, oocytes and ES cells by reverse transcription and relative quantification real-time

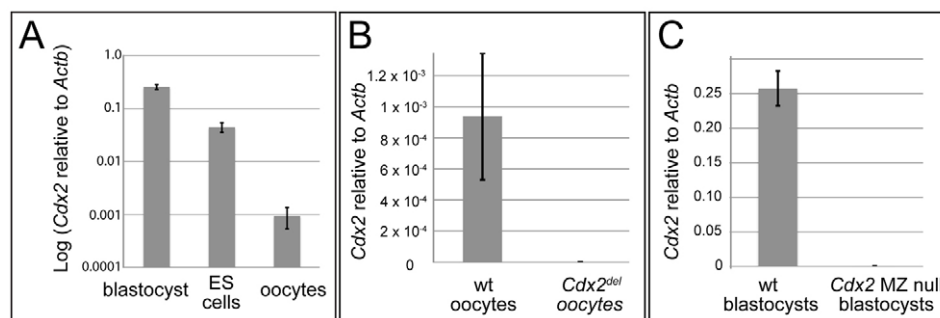
PCR (qPCR). Consistent with previous reports, we detected higher levels of *Cdx2* mRNA in blastocysts than in oocytes. However, *Cdx2* mRNA levels were more than an order of magnitude lower in oocytes than in ES cells (Fig. 1A). This suggests that the level of oocyte *Cdx2* mRNA is functionally irrelevant.

### Generation of viable mice lacking maternal *Cdx2*

Our qPCR analysis indicated that *Cdx2* is unlikely to be required for development, and we next sought a means to test this hypothesis. Several groups have examined the requirement for maternal *Cdx2* by injecting siRNA constructs into oocytes and zygotes (Jedrusik et al., 2010; Wang et al., 2010a; Wu et al., 2010). Curiously, these groups obtained contradictory findings, even after injection of identical constructs (Wu and Schöler, 2011). Groups led by Wu and Wang reported that maternal *Cdx2* is not required for development (Wang et al., 2010a; Wu et al., 2010), whereas Jedrusik et al. (Jedrusik et al., 2010) reported that maternal *Cdx2* is required for development. Since different methods of injection might lead to different experimental outcomes (Morris, 2011), we used a non-invasive, genetic strategy to remove *Cdx2* from the maternal germline.

First, we created a conditional allele of *Cdx2* (supplementary material Fig. S1A-D). In this allele, Cre-mediated recombination between loxP sites leads to deletion of the *Cdx2* transcription start site and introduction of a nonsense frame shift. We then used this allele to create adult females carrying *Cdx2* null oocytes using the female germline-specific *Zp3-Cre* (de Vries et al., 2000) (supplementary material Fig. S1E). *Zp3-Cre* is expressed specifically in oocytes, where it causes recombination of floxed alleles (de Vries et al., 2000) very early in oogenesis (Lan et al., 2004). We first confirmed that *Cdx2* mRNA was ablated in oocytes from *Cdx2* germline null (*Cdx2<sup>fl</sup>/Cdx2<sup>fl</sup> or del*; *Zp3-Cre/+*) females by qPCR (Fig. 1B).

We then bred *Cdx2* germline null females to wild-type males, with the expectation that if maternal *Cdx2* were required for development then no viable offspring would result. However, we obtained comparable numbers of viable offspring from *Cdx2* germline null and control females (7.3 $\pm$ 1.5 and 7.0 $\pm$ 0.8 pups/litter, respectively). We examined whether Cre-mediated excision had occurred in these crosses by PCR genotyping pups and embryos collected from *Cdx2* germline null females. This confirmed that Cre-mediated excision was highly penetrant, as the excised allele was detected in almost all progeny examined (6/6 pups and 70/72 blastocysts). We also determined that, although only around half of



**Fig. 1. Relative quantification of *Cdx2* levels by qPCR.** (A) Average *Cdx2* levels, normalized to  $\beta$ -actin (*Actb*), in E3.5 blastocysts, oocytes and ES cells. Averages were calculated from three biological replicate measurements: three wild-type (wt) blastocysts, three ES cell lines (R1, E14 and G4), and oocytes from three mice. (B) Levels of *Cdx2*, relative to *Actb* mRNA, in wild-type and *Cdx2* MZ null oocytes (average of three biological replicates for each genotype). (C) Average levels of *Cdx2*, relative to *Actb* mRNA, in single wild-type blastocysts ( $n=4$ ) and *Cdx2* MZ null blastocysts ( $n=5$ ) at E3.5. Error bars indicate s.d. of biological replicates.

the progeny inherit the *Zp3-Cre* transgene (11/25 blastocysts), excision occurs in all progeny (25/25 blastocysts), consistent with excision prior to the first meiotic division (de Vries et al., 2000; Lan et al., 2004). We infer that germline *Cdx2* is dispensable for female fertility and embryo development.

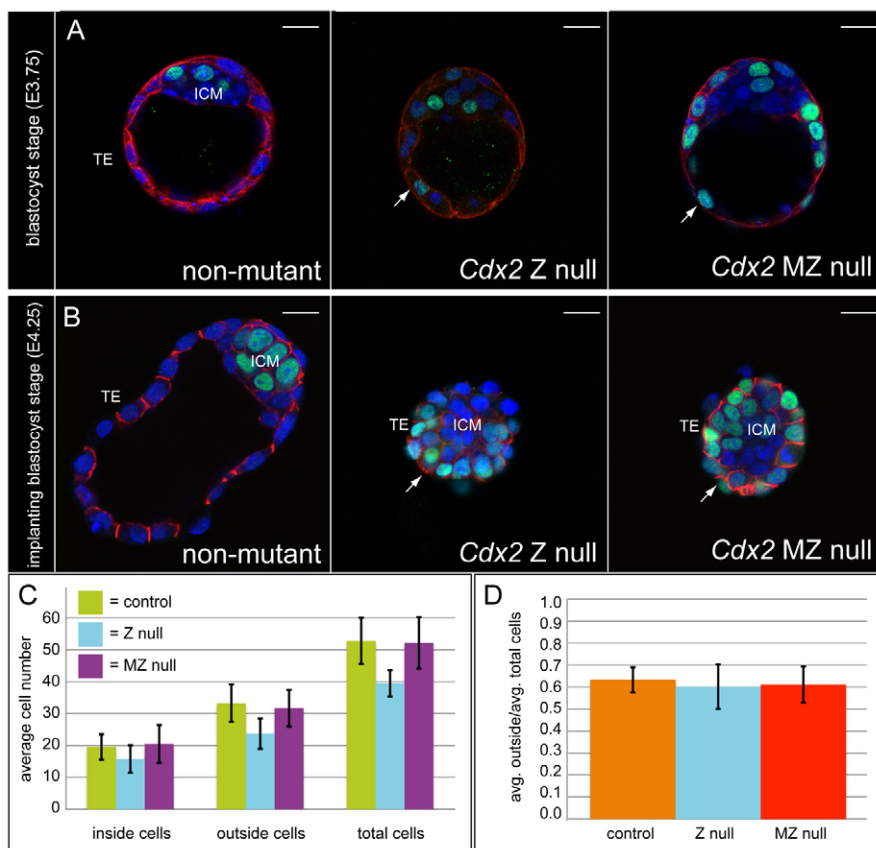
### Maternal *Cdx2* is not required for trophectoderm specification

Jedrusik et al. (Jedrusik et al., 2010) suggested that maternal *Cdx2* is required for trophectoderm cell polarization and cell fate specification prior to the establishment of inner and outer cell populations because zygotic *Cdx2* knockdown, which presumably interfered with both maternal and zygotic *Cdx2*, led to phenotypes that were more severe than that of the *Cdx2* zygotic (Z) null (Jedrusik et al., 2010). *Cdx2* maternal-zygotic (MZ) knockdown phenotypes included prolonged cell cycle during cleavage stages, lethality prior to the blastocyst stage in over half of the embryos and formation of a blastocyst with a reduced number of trophectoderm cells in the remaining embryos. In addition, molecular defects were noted in *Cdx2* MZ knockdown embryos, including failure to express cell polarity genes, including E-cadherin (*Cdh1*) and keratin 8 (*Krt8*), and lineage markers such as *Nanog* (Jedrusik et al., 2010). By contrast, another group reported that knockdown of *Cdx2* in the zygote did not disrupt formation of the blastocyst, but phenocopied *Cdx2* Z null embryos (Wu et al., 2010). Accordingly, *Cdx2* MZ knockdown blastocysts expressed CDH1 and KRT8, whereas NANOG was ectopically expressed in the trophectoderm, consistent with the *Cdx2* Z null phenotype (Ralston and Rossant, 2008; Strumpf et al., 2005). *Cdx2* MZ knockdown blastocysts then collapsed around implantation stage (Wu et al., 2010), as do *Cdx2* Z null blastocysts (Strumpf et al.,

2005). Subsequently, differences among phenotypes resulting from *Cdx2* MZ knockdown have been discussed in the literature (Bruce, 2011; Johnson, 2011; Wu and Schöler, 2011), but no consensus has been reached to explain the differing phenotypes.

To resolve the debate, we bred *Cdx2* germline null (*Cdx2<sup>fl/fl</sup> or fl<sup>del</sup>; Zp3-Cre/+*) females with *Cdx2<sup>null/+</sup>* (Chawengsaksohak et al., 1997) males to generate embryos lacking both maternal and zygotic *Cdx2* (*Cdx2* MZ null). In contrast to the study by Jedrusik et al. (Jedrusik et al., 2010) and consistent with studies by Wu et al. and Wang et al. (Wu et al., 2010; Wang et al., 2010a), we found that *Cdx2* MZ null embryos reached the blastocyst stage and then collapsed around the time of implantation (11/11 embryos; data not shown). We confirmed that *Cdx2* mRNA was ablated in *Cdx2* MZ null blastocysts (Fig. 1C). Thus, *Cdx2* MZ null embryos morphologically phenocopied *Cdx2* Z null embryos. In fact, MZ null and Z null embryos cultured side by side from 1-cell to implantation stages underwent cleavages, compaction, cavitation, expansion and collapse within the same time frame ( $n=11$  MZ null and  $n=10$  Z null cultured embryos; data not shown), indicating no difference in cell cycle length or morphology between *Cdx2* MZ null and Z null embryos. Importantly, we observed no phenotypic differences among *Cdx2<sup>del/del</sup>*, *Cdx2<sup>del/null</sup>* and *Cdx2<sup>null/null</sup>* embryos, and none of these maintained an expanded blastocoel or hatched from the zona, indicating that deleted and null alleles are functionally equivalent.

To examine the consequences of simultaneous loss of maternal and zygotic *Cdx2* on a molecular level, we examined the expression of CDH1, KRT8 and NANOG in *Cdx2* MZ null blastocysts. We found that these proteins were all still detectable in *Cdx2* MZ null blastocysts (Fig. 2A,B). Notably, NANOG was ectopically expressed in trophectoderm cells of *Cdx2* MZ null



**Fig. 2. Loss of maternal *Cdx2* does not worsen the *Cdx2* zygotic null phenotype.** (A) Expression of KRT8 (red) and NANOG (green) in confocal transverse sections of preimplantation mouse blastocysts at E3.75 (nuclei, blue). Images are representative of  $n=20$  control (*Cdx2<sup>+/+</sup>* or wild type),  $n=6$  Z null,  $n=5$  MZ null blastocysts. In control blastocysts, KRT8 is restricted to the trophectoderm (TE) and NANOG is restricted to the inner cell mass (ICM). In *Cdx2* Z null and MZ null blastocysts, KRT8 is still expressed in the TE and NANOG is ectopically expressed in the TE (arrows). (B) Expression of CDH1 (red) and NANOG (green) in implantation stage blastocysts at E4.25 (nuclei, blue). Control blastocysts are expanded and *Cdx2* Z null and MZ null blastocysts are collapsed. CDH1 and NANOG are detectable in *Cdx2* Z null and MZ null blastocysts and NANOG is ectopically expressed in the TE of both mutants (arrows). Representative of  $n=7$  control,  $n=7$  Z null,  $n=9$  MZ null blastocysts. (C) Average numbers of inside, outside and total cells in control (*Zp3-Cre/+*;  $n=28$ ), *Cdx2* Z null ( $n=4$ ) and *Cdx2* MZ null ( $n=22$ ) blastocysts at E3.5. Inside and outside cells were counted on the basis of morphological position in the blastocyst. (D) Data from C showing the average proportion of outside cells per embryo, indicating no difference in the proportion of TE cells for any genotype ( $P>0.05$ ,  $t$ -tests). Error bars indicate s.d. Scale bars: 20  $\mu$ m.



blastocysts and Z null blastocysts (Fig. 2A,B), as previously reported for *Cdx2* Z null (Strumpf et al., 2005) and knockdown (Wu et al., 2010) blastocysts. In addition, KRT8 was expressed at a slightly lower level in both *Cdx2* MZ null and Z null blastocysts (Fig. 2A), consistent with previous studies of *Cdx2* Z null (Ralston and Rossant, 2008) and knockdown (Wu et al., 2010) blastocysts. At later time points, when the embryos had collapsed, CDH1 localization appeared identical in *Cdx2* Z null and MZ null embryos (Fig. 2B), consistent with disrupted trophectoderm cell polarity in the absence of *Cdx2* (Strumpf et al., 2005; Wu et al., 2010). Finally, we did not detect a significant difference in the proportion of trophectoderm cells in *Cdx2* MZ null embryos compared with *Cdx2* Z null or control blastocysts (Fig. 2C,D). Thus, neither trophectoderm cell polarity nor initial cell fate depends on maternal *Cdx2*. The findings presented here therefore show that maternal *Cdx2* is not required for mouse development.

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#### Competing interests statement

The authors declare no competing financial interests.

#### Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.086025/-/DC1>

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