EZH2 is required for parathyroid and thymic development through differentiation of the third pharyngeal pouch endoderm

Cinzia Caprio¹,*, Gabriella Lania¹, Marchesa Bilio¹, Rosa Ferrentino¹, Li Chen² and Antonio Baldini¹,³,‡

ABSTRACT

The Ezh2 gene encodes a histone methyltransferase of the polycomb repressive complex 2 that methylates histone H3 lysine 27. In this study, we investigated whether EZH2 has a role in the development of the pharyngeal apparatus and whether it regulates the expression of the Tbx1 gene, which encodes a key transcription factor required in pharyngeal development. To these ends, we performed genetic in vivo experiments with mouse embryos and used mouse embryonic stem cell (ESC)-based protocols to probe endoderm and cardiogenic differentiation. Results showed that EZH2 regulates the Tbx1 gene locus in mouse embryos, and that suppression of EZH2 was associated with reduced expression of Tbx1 in differentiated mouse ESCs. Conditional deletion of Ezh2 in the Tbx1 expression domain, which includes the pharyngeal endoderm, did not cause cardiac defects but revealed that the gene has an important role in the morphogenesis of the third pharyngeal pouch (PP). We found that in conditionally deleted embryos the third PP was hypoplastic, had reduced expression of Tbx1, lacked the expression of Gcm2, a gene that marks the parathyroid domain, but expressed FoxN1, a gene marking the thymic domain. Consistently, the parathyroids did not develop, and the thymus was hypoplastic. Thus, Ezh2 is required for parathyroid and thymic development, probably through a function in the pouch endoderm. This discovery also provides a novel interpretational key for the finding of Ezh2 activating mutations in hyperparathyroidism and parathyroid cancer.

KEY WORDS: Ezh2, Parathyroids, Pharyngeal endoderm, Tbx1

INTRODUCTION

The polycomb chromatin remodelling complex (PcG) is a key transcriptional regulator that represses gene expression. EZH2 is part of the polycomb repressive complex 2 (PRC2) and has methyltransferase activity that methylates lysine 27 of histone H3 to generate H3K27me3. Although there is a similar gene in the mouse genome, named Eshl (Shen et al., 2008), Ezh2 is indispensable and its loss causes embryonic lethality at or before gastrulation (O’Carroll et al., 2001). However, in some cases functional redundancy has been noted (Ezhkova et al., 2011; Shen et al., 2008). Ezh2 is required for a number of developmental processes (Aloia et al., 2013). Specifically, several reports have addressed the role of Ezh2 during heart development using tissue-specific deletion of the gene. Ablation in the Nkx2-5/Cre recombination domain, which encompasses the first and second heart fields (SHF) as well as the pharyngeal endoderm, resulted in cardiac defects (Chen et al., 2012; He et al., 2012). However, ablation using the Mef2c-AHF-Cre driver, which induces recombination in the SHF and right ventricle, resulted in postnatal cardiomyocyte hypertrophy, but no morphogenetic defects of the outflow tract (Delgado-Olguín et al., 2012), suggesting that Ezh2 is dispensable during SHF development. Nevertheless, Ezh2 was shown to de-repress Tbx1 expression in human embryonic stem cells (ESCs) (Collinson et al., 2016). In addition, it has been shown that inactivation of the Eed gene, which encodes another component of the PRC2 complex, in the FoxN1 domain of the endodermic thymic primordia is associated with upregulation of Tbx1 gene expression in the developed thymus (Singarapu et al., 2018). These findings suggest that Tbx1 may be a target of PRC2. Tbx1 is an important player in the development of the SHF, cardiopharyngeal mesoderm and the pharyngeal endoderm. The gene is strongly implicated in DiGeorge/22q11.2 deletion syndrome, a developmental disorder that affects the pharyngeal apparatus (Baldini et al., 2017).

Here, we have addressed the question of whether Tbx1 is a target of EZH2. To this end, we have used genetically modified mouse lines and differentiating mouse ESCs. Results showed that the EZH2 protein binds to the Tbx1 gene and affects its expression in a tissue-specific manner. However, in contrast with the canonical function of EZH2, its loss is associated with reduced expression of the Tbx1 gene. Conditional deletion in the Tbx1 expression domain showed that Ezh2 is a modifier of the Tbx1 mutant phenotype and is required for parathyroid and thymic development.

RESULTS

EZH2 localizes to the Tbx1 gene in mouse embryos

Published data sets show that the mouse Tbx1 gene region is enriched for H3K27me3 in various stages of mouse ESC differentiation (Wamstad et al., 2012) (Fig. S1A). To establish whether H3K27me3 enrichment also occurs in vivo, we performed quantitative chromatin immunoprecipitation (qChIP) of embryonic day (E)9.5 mouse embryos and tested H3K27me3 enrichment at three loci. We found high enrichment at all the loci tested (Fig. 1A). Next, we performed qChIP using an anti-EZH2 antibody

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and found localization of the EZH2 protein at Tbx1 gene loci. We detected an effect of the genotype only at the exon 3 locus in Tbx1cre+/+ embryos, in which we found a slight increase in EZH2 enrichment compared to wild-type embryos (Fig. 1B).

We next tested the expression of Tbx1 and Wnt5a, a Tbx1 target, by qRT-PCR on RNA from somite-matched whole E8.5 embryos with the genotypes Tbx1+/−; Ezh2+/− versus Tbx1+/−; Ezh2+/+. Results showed that Ezh2 heterozygosity had no significant effect on Tbx1 or Wnt5a expression (Fig. 1C).

The lack of significant expression changes of Tbx1 in Ezh2 heterozygous mutant embryos may be because of the heterogeneity of the tissue tested (whole embryo) or because the heterozygous deletion is insufficient to significantly reduce the availability of the EZH2 protein to the chromatin. Therefore, we switched to a mouse ESC-based system to study differentiated cell types.

Loss of EZH2 or inhibition of its enzymatic activity during differentiation of ESCs downregulates Tbx1 gene expression

To test whether EZH2 may affect Tbx1 gene expression in two of the critical tissues in which it has a developmental function, we used in vitro differentiation protocols to obtain cardiogenic mesoderm and definitive endoderm from mouse ESCs. We targeted Ezh2 exon 16, encoding part of the methyltransferase domain, using CRISPR/Cas9 technology in E14Tg2A.4 mouse ESCs (Fig. 2A). We selected two clones (1C and 1D) that exhibited homozygous mutation of Ezh2 and no EZH2 protein expression (Fig. 2A). We subjected these cells (here referred to as Ezh2−/− cells) to cardiac mesoderm/heart field progenitor differentiation (Andersen et al., 2018), and to endoderm differentiation (Loh et al., 2014).

During cardiac differentiation, we tested Gata4, Tbx1 and cTnt (also known as Tnnt2) expression, the first two being markers of cardiac progenitors, and the latter of cardiomyocytes (Fig. 2B). We found that the Ezh2 homozygous deletion led to earlier differentiation and expression of these markers compared to wild-type cells. qRT-PCR confirmed and quantified these results (Fig. 2C).

Endodermal differentiation provided a substantially different picture. Because the protocol was designed for human ESCs, we first induced epiblast differentiation and then proceeded with endodermal differentiation. As shown in the marker expression panel (Fig. 3A), at D5, all cell lines robustly expressed the epiblast markers Fgf5, Sox17 and Brachyury (T); and at D7, wild-type cells expressed the endodermal marker FoxA2 and also Tbx1, which is strongly expressed in the

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During cardiac differentiation, we tested Gata4, Tbx1 and cTnt (also known as Tnnt2) expression, the first two being markers of cardiac progenitors, and the latter of cardiomyocytes (Fig. 2B). We found that the Ezh2−/− cell lines, although positive for these markers, differentiated earlier than the wild-type parental line in two independent experiments. For example, expression of the cardiac troponin gene (cTnt), a marker of cardiomyocyte differentiation, appeared at day 4 in mutant cells, but at day 6 in wild-type cells (Fig. 2B). Conversely, Tbx1 expression appeared later in differentiation and at a lower intensity. qRT-PCR confirmed and quantified these results (Fig. 2C).

Endodermal differentiation provided a substantially different picture. Because the protocol was designed for human ESCs, we first induced epiblast differentiation and then proceeded with endodermal differentiation. In the schematic shown in Fig. 3A, time D0 to D5 refer to days of epiblast differentiation, whereas D5 to D7 refer to days of endodermal differentiation (definitive endoderm). As shown in the marker expression panel (Fig. 3A), at D5, all cell lines robustly expressed the epiblast markers Fgf5, Sox17 and Brachyury (T); and at D7, wild-type cells expressed the endodermal marker FoxA2 and also Tbx1, which is strongly expressed in the
pharyngeal endoderm. However, we could not recover mutant cells at D7 in three independent differentiation experiments. In fact, virtually all cultured mutant cells died between D5 and D6. Thus, with this protocol, the complete loss of EZH2 in these cells prevented endodermal differentiation, expansion or survival. To gain additional information, we inhibited the enzymatic activity of EZH2 using GSK126. We added GSK126 during the endoderm differentiation segment of the protocol (days D5 to D7) at four different concentrations (1 to 4 µM) (Fig. 3B). Under these conditions, we did not observe the culture failures observed with different combinations (1 to 4 µM) (Fig. 3B). Under these conditions, we did not observe the culture failures observed with different combinations.

**Deletion of Ezh2 in the Tbx1 expression domain affects pharyngeal endoderm but not cardiovascular development**

Next, we tested whether Ezh2 may modify the Tbx1 mutant phenotype. We tested the Tbx1 haploinsufficiency phenotype and used three different strategies: (1) crosses with germ line mutants (Tbx1<sup>+/−</sup> and Ezh2<sup>−/−</sup>); (2) conditional mutation of Ezh2 (Tbx1<sup>Cre</sup> and Ezh2<sup>−/−</sup>); and (3) EZH2 enzymatic inhibition by GSK126 treatment during endoderm differentiation. Overall, our data indicate that loss of EZH2 is associated with reduced expression of the Tbx1 gene during cardiac mesoderm and endoderm differentiation.

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In the mouse, the thymus and parathyroid primordia are derived from adjacent but distinct domains of the third pharyngeal pouch (PP) endoderm, and they express FoxN1 and Gcm2, respectively. Tbx1 is expressed in the early endoderm that gives rise to both primordia but it is later turned off in the thymic domain (Bain et al., 2016; Huynh et al., 2007). Thus, Tbx1Cre-induced recombination, as visualized by the reporter allele RosamT−mG, spans the entire pouch at E11.5, when the domains are clearly distinct (Fig. 6A-A′).

In Ezh2cko embryos, the third PP appeared to be smaller and rounder than in controls (Fig. 6B-D), FoxN1 was readily detectable but Gcm2 expression was not, with one exception where we detected 1-2 positive cells (Fig. 6C,D). Tbx1 expression, revealed by RNAscope, was barely detectable (Fig. 6F), consistent with reduced expression after GSK126 treatment observed in endoderm in vitro. Immunofluorescence with an antibody against TBX1 revealed a very low signal in the third PP of Tbx1Cre+/Ezh2fl/fl embryos, but was undetectable in the third PP of Ezh2−/− embryos (arrows in Fig. S3A-C). We found no obvious difference in the intensity of the signal in adjacent mesodermal tissue (arrowheads in Fig. S3). Despite the absence of Gcm2 expression, we could see the presence of a FoxN1− dorsal region of the mutant pouch (Fig. 6D, arrowhead), a region normally associated with Gcm2 expression. This suggests that the pouch, although smaller, might be correctly patterned, but failed to activate Gcm2 expression.

**Table 1. Ink injection experiments to detect fourth pharyngeal arch artery defects**

| Genotype (E10.5) | Normal | Fourth PAA defects |
|------------------|--------|-------------------|
| **A** Tbx1+/-    | 16     | 0                 |
| Tbx1Cre/+        | 0      | 8 (100%)          |
| Tbx1Cre/+;Ezh2+/− | 0      | 8 (100%)          |
| **B** Genotype (E10.5) GSK126 | Normal | Fourth PAA defects |
| Tbx1+/-          | Vehicle 10 | 0                 |
|                  | Treated 7    | 0                 |
| Tbx1+/-          | Vehicle 1    | 20 (95%)          |
|                  | Treated 1    | 19 (95%)          |
| **C** Genotype (E10.5) | Normal | Fourth PAA defects |
| Tbx1+/-; Ezh2+/− | 6      | 0                 |
| Ezh2+/−          | 7      | 0                 |
| Tbx1−/−          | 3      | 7 (70%)           |
| Tbx1+/-; Ezh2−/− | 1      | 7                 |

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Ezh2 is required for parathyroid formation

Because Gcm2 is required for parathyroid development (Günther et al., 2000; Liu et al., 2007), we tested whether Ezh2 deletion in the Tbx1 domain affected parathyroid development. We sectioned E16.5 embryos and examined the neck/mediastinic region by haematoxylin-eosin staining and by Gcm2 in situ hybridization. Wild-type embryos exhibited clearly detectable bilateral Gcm2-stained parathyroid glands adjacent to the thyroid lobes (Fig. 7A-A′). Tbx1Cre/+ embryos had very small and ectopic parathyroid glands (Fig. 7B), similar to Tbx1Cre/+;Ezh2fl/+ embryos (Fig. 7C), whereas in Ezh2cko embryos, we could not detect any parathyroid tissue in the entire mediastinic region in coronal and transverse sections. An example is shown in Fig. 7D.

DISCUSSION

We initiated this work in search of upstream chromatin regulators of the Tbx1 gene. Published literature suggested that EZH2/PRC2 could be a suppressor of Tbx1 expression (Collinson et al., 2016; Singaravelu et al., 2018), thus a potential target for a compensatory treatment for gene haploinsufficiency. Indeed, ChIP experiments performed on mouse embryos showed that EZH2 localizes to the Tbx1 gene. However, we did not find any convincing evidence that EZH2 suppresses Tbx1 gene expression. On the contrary, we found that loss of EZH2, or inhibition of its methyltransferase activity, was associated with reduced expression of the gene in certain contexts.

Tbx1 is required in a population of mesodermal cells, the SHF, for the development of the cardiac outflow tract and the pharyngeal apparatus (Rana et al., 2014; Xu et al., 2004; Zhang et al., 2006). It is also required in the pharyngeal epithelia, including the pharyngeal endoderm, for the fourth PAA formation and for the development of thymus and parathyroids (Calmont et al., 2009; Hasten and Morrow, 2019; Jackson et al., 2014; Zhang et al., 2005). Thus, we used mouse ESC-based cardiac mesoderm and endoderm differentiation protocols to test the effect of loss of Ezh2 on Tbx1

Fig. 4. Loss of Ezh2 in the Tbx1 expression domain causes severe thymic hypoplasia in E18.5 embryos. (A-D) Heart and thymus preparations from E18.5 embryos. Note the small thymus in Ezh2cko embryos (arrows). (E) Section of an E18.5 control (Tbx1Cre/+;Ezh2fl/fl) thymus stained with DAPI (blue for nuclei) and immunostained with an anti-E-Cadherin (CDH1) antibody (red). (F) Section of an Ezh2cko thymus at the same magnification as in E. An adjacent section is shown magnified in F′. Note the large number of CDH1-lined cysts, which are also present, but in a very small number, in control embryos (arrows).
expression. In both systems, loss of EZH2 had a significant impact, although the effect on endoderm differentiation was the most dramatic, as we could not recover any endodermally differentiated cells. However, inhibition of EZH2 enzymatic activity, rather than genetic knockout, allowed us to obtain endodermally differentiated cells for analysis. We observed that suppression of EZH2 reduced the expression of Tbx1 in cardiac mesoderm and definitive endoderm in cultured cells.

![Fig. 5. Normal FoxN1 but altered Tbx1 gene expression in E16.5 Ezh2 mutant thymi. Quantitative real time PCR of FoxN1 and Tbx1 gene expression in thymi of the genotype indicated. FoxN1 gene expression (left) did not change in any of the genotypes tested. Tbx1 gene expression (right) was significantly reduced in the double heterozygotes, but increased, and very variable from embryo to embryo, in the Ezh2^cko. Data are mean±s.e.m. Figures above the blue segments indicate P values, calculated by an unpaired two-tailed Student’s t-test using GraphPad software.]

![Fig. 6. Third pharyngeal pouch anomalies in Ezh2^cko embryos at E11.5. (A-A’): Sagittal sections from the same pouch at two different lateral levels stained with a FoxN1 probe by in situ hybridization (left panels). The same sections were then stained with anti GFP (green) and anti CDH1 (red) antibodies. FoxN1 stains the ventral domain of the pouch, whereas GFP, indicating the recombination of the Rosa^M^T_m^G^ allele driven by Tbx1^cre^, stains the entire pouch (centre panels), as does CDH1 immunostaining (right panels). (B-D) Two-colour in situ hybridization of Gcm2 (blue) and FoxN1 (brown) in control (Tbx1^Cre/+; Ezh2^flox/+^) (B) and in two Ezh2^cko^ embryos (C-D). C shows the only Ezh2^cko^ pouch in which we could see cells stained with Gcm2. In the other embryos no Gcm2 signal was detected, as in D. The arrowhead indicates a FoxN1^-^ and Gcm2^-^ region. (E,F) RNAscope in situ hybridization using a Tbx1 probe (green). Cell nuclei are stained with DAPI (blue). (E) Control embryo (Tbx1^cre^/+, Ezh2^flox^/+^). (F) Ezh2^cko^ embryo, which shows a barely detectable signal. Dor, dorsal side of pouch. In all sections anterior is up, posterior is down. Scale bars: 100 µm.]
In vivo experiments revealed a role of Ezh2 in the pharyngeal endoderm when ablated in the Tbx1 expression domain. Indeed, we found that Ezh2 is required for the development of the third PP, which includes the primordia of the thymus and parathyroids. In E11.5 Ezh2−/− embryos, the third PP was overall hypoplastic, exhibiting a FoxN1+ region, indicative of thymic specification (Blackburn et al., 1996; Gordon et al., 2001), but lacked Gcm2 expression, which is required for parathyroid development (Günther et al., 2000; Liu et al., 2007; Peissig et al., 2018). Consistent with this, Ezh2+/− embryos exhibited thymic hypoplasia and lacked parathyroids, and in Tbx1+/− and Tbx1Cre+/−;Ezh2+/− embryos the glands were ectopic and hypoplastic. Parathyroid hypoplasia has been reported in Tbx1 heterozygous mutants (Hasten and Morrow, 2019). Thus, Ezh2 is a critical factor in endoderm development and, consequently, it affects the morphogenesis of organs derived from it.

In contrast with a clear effect in the third PP, we found that in a Tbx1 heterozygous mutant background Ezh2 loss did not cause cardiac outflow tract defects. This result is consistent with data generated with a different SHF driver, Me2c-AHF-Cre, and which also showed no outflow tract defects (Delgado-Olguin et al., 2012). Thus, all data considered, Ezh2 appears to be dispensable for SHF-dependent outflow tract development. These findings do not completely exclude a role of PRC2 in the SHF because loss of Ezh2 might be compensated by Ezh1.

It has been reported that the conditional deletion of Eed, encoding another PRC2 component, in the FoxN1 expression domain causes postnatal thymic hypoplasia and dysfunction (Singarapu et al., 2018). The phenotype was partly attributed to upregulation of the Tbx1 gene and suppression of the FoxN1 gene. Our data are in apparent contrast to those results because we found no evidence that Ezh2 may suppress Tbx1 expression, although we did see variable expression in mutant thymi, nor did we see FoxN1 suppression. However, the Tbx1Cre driver is activated much earlier than the Foxn1Cre driver, and the loss of the Ezh2 gene may be partially compensated by Ezh1.

In summary, we found that the Tbx1 gene is a likely target of Ezh2 and that its suppression is associated with reduced expression of Tbx1 in differentiated mouse ESCs and in embryos, at the third PP endoderm. We also observed loss of Gcm2 expression. Consistently, we observed severe developmental anomalies of the endodermally derived thymus and parathyroids. The mechanisms by which Ezh2 regulates Tbx1 and Gcm2 expression may be indirect because PRC2 is known to be a repressor, but there are exceptions (Jiao et al., 2020; Kim et al., 2018).

Interestingly, Ezh2 is activated in human parathyroid malignancies (Cetani et al., 2019) and in hyperparathyroidism (Duan et al., 2015), suggesting that the gene has a major role in the transcriptional program of this gland. Finally, 22q11.2DS patients, who are haploinsufficient for Tbx1, are often, but not always, affected by hypocalcaemia and parathyroid hypoplasia, and therefore it would be of interest to determine whether variants of the Ezh2 gene affect the penetrance and/or expressivity of this clinically relevant phenotype.

**Materials and Methods**

**Mouse lines**

We used the following mouse lines: Tbx1Cre/+ (Huynh et al., 2007); Tbx1+/− (Lindsay et al., 2001), Ezh2tm1TaraGrea! (Su et al., 2003); Me2c-AHFCre (Verzi et al., 2005); and RosamTm1TaraGrea!, a Cre reporter line (Muzumdar et al., 2007). All lines were maintained in a congenic C57/B6N background. To generate an Ezh2 null allele, Me2c-AHFCre females, which express Cre in the germline (Ehlers et al., 2014) were crossed with Ezh2+/− mice. Ezh2+/− progeny were crossed with wild-type mice and we used the second generation of Ezh2+/− mice to exclude mosaicism. All animals were genotyped according to the original reports. To evaluate the fourth PAA phenotype, we injected India ink into the heart of E10.5 embryos. Subsequently, embryos were fixed in 4% paraformaldehyde (PFA), dehydrated and clarified in the pharyngeal outflow tract. These findings do not completely exclude a role of PRC2 in the SHF because loss of Ezh2 might be compensated by Ezh1.

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Cell cultures
E14Tg2A.4 feeder-free mouse ESCs (strain 129/Ola, BayGenomics) were cultured in feeders-free conditions in Glasgow’s minimum essential medium (Sigma-Aldrich, G5154) supplemented with 15% ESC-screened fetal bovine serum (FBS) (US Euroclone, CHA30070L), 0.1 mM non-essential amino acids, 0.1 mM β-mercaptoethanol, 0.1 mM L-glutamine, 0.1 mM sodium pyruvate and 10^3 U/ml ESGRO leukaemia inhibitory factor (Millipore, ESG1107). Cardiac differentiation was performed according to Andersen et al. (2018). The organoids were collected every 2 days to monitor cardiac differentiation. We performed epiblast differentiation according to a published protocol (Fiorenzano et al., 2016). Endoderm differentiation was performed using a commercial kit (PSC Definitive Endoderm Induction Kit, Gibco, A3062601) based on published data (Loi et al., 2014). For experiments with the inhibitor GSK126, the inhibitor was dissolved in DMSO (Sigma-Aldrich, 1 to 4 μM final concentration in the medium) and added to the culture at day 5 (beginning of endoderm differentiation). The same volume of DMSO was added to control cultures.

CRISPR/Cas9-mediated targeting of the Ezh2 gene was performed using a guide (g)RNA sequence (Fig. 2) that was selected, using the tool crispr.mit.edu, to have the lowest off target probability. The sequence was cloned into a plasmid (Thermo Fisher Scientific, A21174) encoding the single gRNA and the Cas9 protein, and an orange fluorescent protein. The plasmid was transfected into mouse ESCs using X-tremeGENE, along with an oligonucleotide as a homologous recombination template. Transfected cells were FACs-purified and plated at clonal density. Forty-three clones were picked, expanded and screened by PCR to identify clones that had undergone homologous recombination. Targeted clones were sequenced confirmed and then tested by western blot to verify the loss of the EZH2 protein. All cell cultures used in this work were regularly tested to exclude mycoplasma contamination.

Chromatin immunoprecipitation
E9.5 embryos were collected and fixed in 1% formaldehyde for 12 min, stored at -30°C and, after chromatin extraction, we immunoprecipitated 6 μg of chromatin. ChIP experiments were carried out as described previously (Lania et al., 2016)

Histone extraction
Histone extraction was performed as previously described (Gordon et al., 2001; Shechter et al., 2007). Briefly, embryos were homogenized with hypotonic lysis buffer [10 mM Tris-Cl (pH 8.0), 1 mM KCl, 1.5 mM MgCl2, 1 mM DTT and protease inhibitors] and incubated for 30 min. To extract histones, the nuclei were collected by centrifugation at 10,000 g and resuspended in acid extraction buffer (0.4 N H2SO). The histone preparation was concentrated by precipitation with trichloroacetic acid and resuspended in deionized water for western blot analysis. Antibodies are listed in Table 2.

Immunofluorescence
For immunofluorescence analysis, tissues were embedded in paraffin and cut into 10 μm sections. Sections were rehydrated and briefly microwaved to boiling point in 10 mM sodium citrate (pH 6.0). Slides were then incubated for 2 h at 4°C in 50:50 v/v 30% sucrose/PBS 1× optimal cutting temperature (OCT) compound, then embedded in OCT compound and cut into 10 μm sections. Sections were rehydrated and briefly microwaved to boiling point in 10 mM sodium citrate (pH 6.0). Slides were then incubated for 2 h at 4°C in 50:50 v/v 30% sucrose/PBS 1× optimal cutting temperature (OCT) compound, then embedded in OCT compound and cut into 10 μm sections. To perform the immunofluorescence analysis following in situ hybridisation on E11.5 embryos, at the end of the in situ hybridisation protocol, slides were washed in PBS-0.5% Triton X-100 and incubated with primary antibody. Signal was revealed as described above.

RNAseq in situ hybridization
RNAseq experiments were performed according to the manufacturer’s instructions. Embryos were fixed in 4% PFA at 4°C, then dehydrated using a standard ethanol series and embedded in paraffin. Selected sections of 6 μm were hybridized with RNAscope Probe Mm-Tbx1 (ACD, 481911, probe C1).

Table 2. Oligonucleotides and antibodies used in this work

| Oligonucleotides (5′–3′)                      | Antibodies                                                      |
|---------------------------------------------|-----------------------------------------------------------------|
| Tbx1 F (gRT-PCR)                            | Anti H3K27me3 (ChIP)                                            |
| Tbx1 R (gRT-PCR)                            | Anti EZH2 (ChIP)                                                |
| Tbx1 R (RT-PCR)                             | Anti EZH2 (western blot)                                       |
| Tbx1 F (RT-PCR)                             | Anti H3K27me3 (western blot)                                   |
| E2pcrKO-F                                   | Anti H3 (western blot)                                          |
| E2pcrKO-R                                   | Anti β-actin (western blot)                                    |
| Ezh2 F                                      | Anti E-cadherin (CDH1, immunofluorescence)                      |
| Ezh2 R                                      | Anti GFP (immunofluorescence)                                  |
| Sox17 F                                     | Anti TBX1 (immunofluorescence)                                 |
| Sox17 R                                     | Pgdm80 A                                                       |
| Wnt5a F                                     | PGDM80 R                                                       |
| Wnt5a r                                     | Anti β-actin (western blot)                                    |
| Fgf5 F                                     | Anti β-actin (western blot)                                    |
| Brachyury F                                 | Anti β-actin (western blot)                                    |
| Brachyury R                                 | Anti β-actin (western blot)                                    |
| Foxa2 F                                    | Anti β-actin (western blot)                                    |
| Foxa2 R                                    | Anti β-actin (western blot)                                    |
| Foxn1 F                                    | Anti β-actin (western blot)                                    |
| Gapdh F                                     | Anti β-actin (western blot)                                    |
| Gapdh R                                     | Anti β-actin (western blot)                                    |
| Gata4 F                                     | Anti β-actin (western blot)                                    |
| Gata4 R                                     | Anti β-actin (western blot)                                    |
| cTnT F                                     | Anti β-actin (western blot)                                    |
| cTnT R                                     | Anti β-actin (western blot)                                    |
| 490-Tbx1 F, ChIP                            | Anti β-actin (western blot)                                    |
| 490-Tbx1 R, ChIP                            | Anti β-actin (western blot)                                    |
| Intron1-Tbx1 F, ChIP                        | Anti β-actin (western blot)                                    |
| Intron1-Tbx1 R, ChIP                        | Anti β-actin (western blot)                                    |
| Ex-3-Tbx1 F, ChIP                           | Anti β-actin (western blot)                                    |
| Ex-3-Tbx1 R, ChIP                           | Anti β-actin (western blot)                                    |

In situ hybridization
For in situ hybridization, antisense RNA probes were labelled using a digoxigenin RNA and fluorescein RNA labelling kit (Roche). The probes used were for Gcm2 (Addgene, 41031) and FoxN1 (Addgene 41032) (Gordon et al., 2001). E11.5 mouse embryos were cryoprotected by serial dilution of sucrose/1× PBS (10%, 20% and 30% sucrose) at 4°C and then incubated for 2 h at 4°C in 50:50 v/v 30% sucrose/PBS 1× optimal cutting temperature (OCT) compound, then embedded in OCT compound and cut into 10 μm sections. To perform the in situ hybridization at E16.5, embryos were embedded in OCT compound as described above.

RNA extraction and qRT-PCR
Total RNA was isolated from P19C16, mouse ESCs and embryos using TRIzol. RNA (0.2-1 μg) was retrotranscribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) and diluted tenfold with RNase-free H2O. Gene expression was quantified by qRT-PCR using 2 μl of the reverse transcription reaction and the Power SYBR Green PCR Master Mix (Applied Biosystems), and normalized relative to Gapdh expression. Statistical evaluation was performed using a two-tailed Student’s t-test. Primers used are listed in Table 2.
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