Notch and Hippo Converge on Cdx2 to Specify the Trophectoderm Lineage in the Mouse Blastocyst

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SUMMARY

The first lineage choice in mammalian embryogenesis is that between the trophectoderm, which gives rise to the trophoblast of the placenta, and the inner cell mass, from which is derived the embryo proper and the yolk sac. The establishment of these lineages is preceded by the inside-versus-outside positioning of cells in the early embryo and stochastic expression of key transcription factors, which is then resolved into lineage-restricted expression. The regulatory inputs that drive this restriction and how they relate to cell position are largely unknown. Here, we show an unsuspected role of Notch signaling in regulating trophectoderm-specific expression of Cdx2 in cooperation with TEAD4. Notch activity is restricted to outer cells and is able to influence positional allocation of blastomeres, mediating preferential localization to the trophectoderm. Our results show that multiple signaling inputs at preimplantation stages specify the first embryonic lineages.

INTRODUCTION

Upon fertilization, the unicellular mammalian zygote undergoes a series of equal cell divisions that in 4 days produces a 60- to 100-cell blastocyst, in which the first embryonic lineages—the trophectoderm (TE) and the inner cell mass (ICM)—have been established (Stephenson et al., 2012). How this initial lineage choice occurs and what genetic components constitute the system that controls this process have been actively studied for the past 20 years. Key transcriptional regulators of these early lineages identified in the mammalian preimplantation embryo include OCT4, NANOG, and SOX2 for the ICM and CDX2 for the TE (Cockburn and Rossant, 2010). These factors determine blastocyst lineages, but the onset of their expression is stochastic (Dietrich and Hiragi, 2007), and later restriction to a particular cell type occurs as a downstream effect of earlier events related to the position of blastomeres in the embryo (Rossant and Tam, 2009).

This process is believed to involve differences in polarity and adhesion between inner and outer cells that are related to differential activation of the Hippo signaling pathway (Hirate et al., 2013; Nishioka et al., 2009), resulting in sustained and restricted expression of genes, such as Cdx2 in the outer cells of the future trophectoderm (Cockburn and Rossant, 2010). Hippo signaling is switched off in outer cells, leading to nuclear localization of the transcriptional coactivator YAP, which can then activate downstream target genes through interaction with the transcription factor TEAD4. Embryos lacking Tead4 fail to develop the TE, and Cdx2 expression is not maintained (Nishioka et al., 2008; Yagi et al., 2007). Correspondingly, overexpression of LATs2 kinase (an activator of Hippo) reduces expression of CDX2 in outer cells, whereas embryos lacking Lats1 and Lats2 express CDX2 in inner cells (Lorthongpanich et al., 2013; Nishioka et al., 2009). Similar results are obtained when other components of the pathway, such as Nfat2 and Amot, are disrupted (Cockburn et al., 2013; Hirate et al., 2013). How these components are integrated to fully define lineage restriction in the blastocyst is largely unknown, and additional inputs remain to be identified (Wennekamp et al., 2013). Reconstruction of this process will require detailed understanding of the transcriptional control of the key lineage regulators acting in the preimplantation embryo.

With this aim in mind, we have searched for cis-regulatory elements responsible for trophectoderm-restricted expression of Cdx2, and through the analysis of one such enhancer, we have uncovered a role of the Notch signaling pathway. We find...
that Notch is active specifically in outer cells of the blastocyst. Analysis of double mutants for Tead4 and the Notch effector Rbpj shows that the Notch and Hippo pathways converge on Cdx2 to activate its expression in the trophectoderm, uncovering an unexpected role for Notch signaling during preimplantation development (Souilhol et al., 2006). Furthermore, we show that forced activation of Notch directs blastomeres to the trophectoderm.

RESULTS

Characterization of a Trophodermic-Specific Enhancer from Cdx2

By means of transient transgenic analysis in mouse preimplantation embryos, we identified a cis-regulatory element located 5’ of Cdx2 that drives reporter expression in the trophoderm (TE) of the blastocyst (Figures S1A and S1B available online). Using fragment #3 of this TE enhancer (TEE) (Figures S1A and S1B) linked to a H2B-mRFP reporter gene, we then generated several transgenic lines for further study. The reporter is only occasionally active in a few cells at the eight-cell stage (Figure 1A), and upon compaction, it starts to be present in the outer cells of the morula (Figure 1B), attaining strong activity at the 16-cell stage (Figure 1C). At the blastocyst stage, TEE-driven reporter activity is localized throughout the TE and excluded from the ICM (Figure 1D). We also generated TEE transgenic lines using a lacZ reporter, which behaved identically to the H2B-mRFP reporter lines (Figure S1O). Tracking blastomeres in the mRFP reporter lines up to the 3.5 dpc blastocyst revealed a steady temporal increase in the number expressing mRFP (Figure 1E). Reporter activity in either the mRFP or lacZ TEE lines was found throughout the TE, although occasionally we detected cells with lower levels of expression. Immunohistochemical analysis of the association between reporter activity and endogenous Cdx2 showed concordant expression in >75% of blastomeres (CDX2+/TEE+, CDX2−/TEE−; Figure 1F), confirming that TEE activity closely matches the expression of CDX2 at preimplantation stages (Dietrich and Hiragi, 2007; Strumpf et al., 2009).

The TE Enhancer Is Active in Cdx2 and Tead4 Mutant Blastocysts

We next aimed to determine the upstream regulatory factors acting on the TEE to restrict Cdx2 expression. To date, the only transcription factors proposed to regulate Cdx2 in the trophoderm are Cdx2 itself, through an autoregulatory loop (Niwa et al., 2005), and TEAD4, acting together with Yap downstream of the Hippo pathway (Nishioka et al., 2009). To test whether these factors are required for TEE activity in the blastocyst, we first examined homozygous Cdx2 knockout (KO) mice (Strumpf et al., 2005) containing the mRFP reporter, finding that TEE reporter activity was still restricted to the TE (Figures S2A and S2B) and that the number of positive cells was the same as in wild-type (WT) littermates (Figures S2C and S2D). We next analyzed the regulation of the TEE by the Hippo pathway during development (Souilhol et al., 2006). Furthermore, we show that forced activation of Notch directs blastomeres to the trophectoderm.

The Notch Signaling Pathway Is Active in the Trophoderm of the Blastocyst

To identify other transcriptional inputs acting on the TEE, we searched the minimal 1.3 kb sequence active in the TE (fragment #6; Figures S1A and S1B) for putative transcription factor binding sites; this search detected four high-confidence sites for RBPJ (Tun et al., 1994), the transcriptional effector of the Notch pathway. RBPJ acts together with the intracellular domain of Notch, the product of the proteolytic cleavage of the receptor upon its activation that is then translocated to the nucleus (Kopan and Ilagan, 2009). Additionally, we detected two putative TEAD binding sites (Anbanandam et al., 2006) (Figure S3A).

This observation suggested that Notch could have a role in development of the blastocyst, but to date there is scant evidence available for the expression of Notch pathway components at this stage (Cormier et al., 2004; Wang et al., 2004), and no studies have addressed their differential localization. To examine the activation status of the Notch pathway during preimplantation stages, we used a mouse line containing a CBF/H2B-Venus reporter, which contains multiple RBPJ binding sites driving the expression of a nuclear-targeted Venus fluorescent protein and that faithfully reports the activation state of the...
Figure 1. A cis-Regulatory Element Upstream of Cdx2 Drives Restricted Expression in the Trophectoderm

Figure 1 (A–D) H2BmRFP reporter expression driven by the Cdx2 trophoderm enhancer (TEE) at (A) the noncompacted eight-cell stage, (B) compacted eight-cell morula stage, (C) 16-cell stage, and (D) blastocyst stage. H2BmRFP was detected by immunohistochemistry, and nuclei were stained with DAPI. Scale bars, 10 μm.
The reporter was active in a few cells at the eight-cell stage, and activity became progressively restricted to outer cells by the 3.5 dpc blastocyst stage (Figures 3A–3C). Treatment of embryos from this line with the γ-secretase inhibitor RO4929097 (RO), a well-characterized and widely used tool that interferes with Notch signaling by blocking the processing of the receptor (Münch et al., 2013), resulted in a strong downregulation of the reporter (Figure 3D). We next examined the distribution by immunofluorescence of the endogenous NOTCH1 intracellular domain (N1ICD), finding that it is localized to the nucleus only in the outer cells of the blastocyst (Figures 3E and S3B). Together, these data show that the Notch signaling pathway is active specifically in the developing TE.

The TE Enhancer Contains Functional RBPJ and TEAD Binding Sites

To investigate the function of the sites identified in the TEE, we transfected HEK293 cells with the wild-type minimal TEE and versions mutated in the RBPJ sites alone (TEE<sub>RBPJmut</sub>), the TEAD sites alone (TEE<sub>TEADmut</sub>), or in the RBPJ and the TEAD sites (TEE<sub>RBPJ/TEADmut</sub>) (Figure 4A). We then tested their response to constitutively active forms of N1ICD (Kopan et al., 1994) and TEAD4 (Tead4VP16) (Nishioka et al., 2009), expressed either alone or in combination. Whereas
the wild-type TEE responded to N1ICD, the TEE^RBPJmut version did not (Figure 4B), demonstrating that this regulatory element is a transcriptional target of the Notch pathway. Tead4VP16 alone did not affect the wild-type TEE, consistent with the results from Figure 3. The Notch Signaling Pathway Is Active in the Trophectoderm

(A–C) CBF:H2B-Venus reporter expression (green) at (A) the noncompacted eight-cell stage, (B) morula stage, and (C) blastocyst stage.

(D) Treatment of embryos form the CBF:H2B-Venus line with the γ-secretase inhibitor RO4929097 (RO) downregulates reporter activity as compared to controls (DMSO).

(E) Immunodetection of Notch1 intracellular domain (N1ICD, red). Nuclei were stained with DAPI. Scale bars, 10 μm.

See also Figure S3.

5TVER7 ES cells (Figure 2E); however, it was sufficient to transactivate the TEE^RBPJmut version (Figure 4B). This suggests that TEAD4 acts together with N1ICD/RBPJ to activate the TEE but cannot activate it when RBPJ is bound in the absence of N1ICD (and thus acts as a repressor [Lai, 2002]). Mutation of the RBPJ sites lifts this restriction, allowing transactivation of the TEE by TEAD4. The TEE^TEADmut or TEE^RBPJ/TEADmut versions did not respond to either factor (Figure 4B). Surprisingly, transgenic embryos carrying the TEE^RBPJmut or the TEE^TEADmut version retained TE lineage-restricted activity (Figures 4C–4E), whereas TEE activity was abolished when the RBPJ and TEAD sites were both mutated (Figure 4F). The lack of response of the TEE^TEADmut to N1ICD in vitro, while retaining activity in vivo suggests that TEAD sites are critical for enhancer activity in HEK cells, but not in the blastocyst.

To confirm the function of the identified binding sites, we combined microinjection of the mutated TEE versions for binding sites of one pathway with pharmacological disruption of the other (Figure 4G). To disrupt the Notch pathway, we treated microinjected embryos with RO, that as shown above blocks Notch activity in the TE (Figure 3D). As for the Hippo pathway, we used Verteporfin (VP), a small molecule that inhibits TEAD-YAP association (Liu-Chittenden et al., 2012). To confirm that VP disrupts Hippo signaling as expected, we treated embryos from the mRFP reporter line and examined reporter activity together with endogenous CDX2 expression (Figure S4A). A high percentage of VP-treated embryos phenocopied Tead4^−/− embryos (Figure 2B), with strong reduction of CDX2 but TEE activity still present (Figure S4A).

As expected, treatment of TEE^RBPJmut microinjected embryos with RO or TEE^TEADmut microinjected embryos with VP had no
significant effect on TE specificity of reporter activity (Figures 4H, S4B, and S4C). RO treatment of TEE\textsuperscript{TEADmut} microinjected embryos strongly reduced the number of transgenic embryos showing TE-specific expression compared to controls microinjected with TEE\textsuperscript{TEADmut} and incubated with DMSO or with wild-type TEE treated with RO (Figures 4H and S4C). This result confirms that attenuation of Notch signaling diminishes activity of a TEE that lacks functional TEAD sites.

In the converse experiment, treatment with VP of embryos microinjected with the TEE\textsuperscript{TEADmut} significantly reduced TE specificity, as compared to controls incubated with DMSO or with wild-type TEE treated with VP (Figures 4H and S4C). This shows that interfering with the transcriptional activity of YAP and TEAD4 when the TEE RBPJ sites are missing decreases enhancer activity.

Taken together, these results show that transcriptional inputs from both pathways are responsible for full enhancer activity through the specific binding sites identified, acting in a redundant fashion in vivo.

**Notch and Hippo Pathways Converge on Cdx2 Regulation**

To further explore the regulation of the TEE by Notch, we tested its activity in the absence of Notch signaling by breeding the mRFP-TEE line in the Rbpj KO background (Oka et al., 1995). Consistent with the activity of TEE\textsuperscript{RBPJmut} in the TE (Figure 4D), Rbpj\textsuperscript{+/−} and Rbpj\textsuperscript{−/−} embryos showed normal TEE-driven expression, as is the case for Tead4\textsuperscript{+/−} embryos (Figures 5A–5C). These embryos also had normal numbers of cells and of TEE+ blastomeres, although Rbpj\textsuperscript{−/−} embryos showed a possible but statistically nonsignificant tendency to have fewer TEE+ cells than did WT embryos (Figure S5A). To test for interaction between the Notch and Hippo on the TEE, we generated embryos in the mRFP-TEE line containing different combinations of Tead4 and Rbpj mutant alleles (Figures 5D and 5E). Double heterozygote embryos (Rbpj\textsuperscript{+/−};Tead4\textsuperscript{+/−}) contained significantly fewer than did normal TEE+ cells (Figures 5D and S5A), and this effect was more marked in Rbpj\textsuperscript{−/−};Tead4\textsuperscript{−/−} embryos, where only a few mRFP cells were detected in the TE (Figures 5E and S5A). This was not due to disruption of overall cell number or the inside–outside distribution of blastomeres in these embryos, as these parameters showed no significant difference compared with wild-type, Tead4\textsuperscript{+/−}, Rbpj\textsuperscript{+/−}, or Rbpj\textsuperscript{−/−} blastocysts (Figure S5B). Furthermore, we observed no difference in the total number of CDX2+ cells per embryo in the different genotypes (Figure S5B), detecting outer blastomeres that express CDX2, but not the reporter (Figures 5C–5E, arrowheads). Unexpectedly, we did not recover any double homozygote knockout embryos at 3.5 dpc (Figure S5C), suggesting that the combined lack of these two factors causes the death of embryos at an earlier stage.

We next investigated whether Notch was required for endogenous levels of Cdx2 expression. Quantification of CDX2 protein per blastomere revealed significantly below-normal expression in Rbpj\textsuperscript{−/−}, Rbpj\textsuperscript{−/−};Tead4\textsuperscript{−/−}, and Rbpj\textsuperscript{−/−};Tead4\textsuperscript{−/−} embryos, but not in Rbpj or Tead4 single heterozygotes (Figure 5F). The effect of Notch on Cdx2 expression was confirmed by treating embryos from the two-cell to blastocyst stages with the γ-secretase inhibitor RO, which significantly decreased mRNA expression of Cdx2, but not of Oct4 or Nanog. We also examined changes in expression of other TE-expressed genes, finding that neither Gata3 nor Eomes changed significantly (Figure S5G), suggesting a specific requirement of Notch for Cdx2 expression. Notch on its own does not impact events downstream or parallel to Cdx2 function, which could be explained by the earlier requirement of Cdx2 as compared to Gata3 and Eomes (Ralston et al., 2010; Strumpf et al., 2009) and is consistent with Notch signaling not being strictly required for trophectoderm development (Sohlhol et al., 2006). In line with the genetic analysis, combined treatment of embryos from the mRFP-TEE transgenic line with the pharmacological disruptors of both the Notch and Hippo pathways previously used (RO+VP) strongly reduced TEE activity and endogenous CDX2 expression (Figure S5D).

Notch signaling and RBPJ are thus necessary for proper expression of endogenous Cdx2 in the embryo, in cooperation with TEAD4. Our results thus show that the Notch and Hippo pathways act together in the preimplantation embryo, affecting TEE activity, CDX2 expression, and embryo viability.

**Notch Activation Increases CDX2 Levels and Drives Blastomeres to the Trophectoderm**

To further explore the role of Notch in trophectoderm development, we overexpressed the active form of NOTCH1 in the blastocyst by crossing a R26-stop-N1ICD-ires-EGFP line (Murtaugh et al., 2003) with a line carrying a maternal Sox2-Cre allele (Hayashi et al., 2003). Most blastocysts obtained were viable (80%; n = 58), as shown by the presence of a blastocoele and proper CDX2 expression in outer cells (Figures 6A and S6A–S6C). Although this strategy predicts uniform recombination in all cells of the blastocyst (Figure S6G), we found mosaic expression of the reporter at 3.5–4.0 dpc (23% EGFP−; blastomeres; Figures 6A and 6E). The degree of mosaicism in R26-stop-N1ICD-ires-EGFP embryos ranged from zero recombined cells to fully recombined blastocysts (Figures S6A–S6C). This was confirmed by PCR detection of the nonrecombined allele in blastocysts obtained from this cross (Figure S6E), and we were also able to detect mosaic recombination using a R26-stop-YFP reporter and the maternal Sox2-Cre allele (Sriniivas et al., 2001) (Figures S6F and S6H).

We took advantage of this mosaicism to investigate whether activation of the Notch pathway could upregulate endogenous Cdx2. Quantification of CDX2 staining at the blastocyst stage showed that blastomeres overexpressing N1ICD have higher levels of CDX2 protein (Figure 6B). The same pattern was seen when we analyzed only outer cells (those that would normally express Cdx2) (Figure 6C). These results complement the earlier observation of reduced CDX2 in Rbpj\textsuperscript{−/−} mutant embryos (Figure 5F), confirming that the Notch pathway directly regulates Cdx2 expression.

In this set of experiments, we also noticed that EGFP+ blastomeres were more often localized to the TE (Figure 6A), so we analyzed the spatial distribution of blastomeres overexpressing N1ICD in more detail. Whereas EGFP− blastomeres from mosaic embryos showed an inside–outside distribution similar to that of blastomeres from wild-type blastocysts, EGFP+ blastomeres were significantly more abundant in outside positions (Figure 6D). Analysis of individual blastocysts revealed that those
Role of Notch in Trophoderm Specification

A

B

C

D

E

F

G

H

(legend on next page)
overexpressing N1ICD had significantly more outside cells (75% versus 65% in wild-type embryos) and that EGFP+ cells were overrepresented in this population compared with inside cells (81.3% versus 64%; Figure 6E). These results indicate that blastomeres in which the Notch pathway is active localize preferentially to the outer TE population and that cells can be directed to this population by forced overexpression of the active form of Notch1.

**DISCUSSION**

In this study, we have dissected the transcriptional regulation of Cdx2 in the preimplantation mouse embryo, and by characterizing an early TE-specific enhancer, we have identified Notch signaling as a component of the process. The role of Notch in cell-fate decisions during development is well established (Koch et al., 2013), but previously no function had been described in the initial lineage choices in the early embryo, because of the fact that no loss-of-function mutant of the Notch pathway has a phenotypic effect on preimplantation development (Souilhol et al., 2006). We find that Notch directly regulates Cdx2 expression in cooperation with TEAD4 and can also influence the fate of blastomeres by driving them to outer positions in the embryo. This cooperation is also supported by the genetic interaction of the two pathways, as no double homozygous mutants for Tead4 and Rbpj are obtained at preimplantation stages. Although other instances of interaction between Notch and Hippo have been described (Barry and Camargo, 2013; Camargo et al., 2007; Chen et al., 2011; Graves et al., 2012; Tschaharganeh et al., 2013), the results of this study show that the Hippo and Notch pathways act in parallel and converge during the first lineage specification in the blastocyst. In this scenario, outside cells more prone to a TE fate would have the Notch pathway active and the Hippo pathway inactive. It is interesting to note that in *Drosophila* wing epithelial cells, inactivation of Hippo signaling results in apical accumulation of the Notch receptor (Genevet et al., 2009).

Compound mutants for Rbpj and Tead4 show a reduction in the total number of TEE-positive cells, but not of CDX2-positive cells. However, they have below-normal expression levels per blastomeres, suggesting that additional regulatory inputs act on Cdx2 in the blastocyst. A possible mechanism is autoregulation of Cdx2 (Barros et al., 2011; Cockburn and Rossant, 2010; Niwa et al., 2005), which would maintain and stabilize Cdx2 expression levels after initial inputs from Notch and Tead4.

Sustained activity of the Notch pathway in outer cells would ensure that they maintain a TE fate, allowing for correct specification of blastomeres that reposition during cell division. Therefore, the forced activation of the pathway achieved by overexpressing N1ICD drives cells to relocate to the outside and adopt a TE fate. It could be argued that Notch simply elevates Cdx2 levels and that this is the trigger for cell relocation (Jedrusik et al., 2008). However, the fact that blastomeres mutant for Cdx2 can adopt an outer position argues against this possibility (Raistan and Rossant, 2008) and suggests that Notch must target other mechanisms operating in outer cells that result in early lineage segregation.

The Notch signaling pathway’s role in generating heterogeneity from an otherwise homogenous group of cells has been extensively studied (Artavanis-Tsakonas et al., 1999). On the other hand, it has also been shown how Notch can produce local homogeneity in a tissue by signaling between adjacent cells (de la Pompa and Epstein, 2012; Lewis, 1998; Neves et al., 2011). It would be too early to speculate which of these mechanisms, lateral inhibition or lateral induction, is involved in lineage determination in the blastocyst. Future work on how pathway components are differentially localized during preimplantation development and on the precise timing of expression of ligands and receptors will be key to resolve the role of Notch in TE specification.

The early mouse embryo has been proposed to be a self-organizing system (Wennenkamp et al., 2013), such that individual cells choose their fate according to a combination of intrinsic and extrinsic cues. In this scenario, the robustness for TE specification would be conferred through combinatorial inputs that would initiate and refine the expression of TE factors, such as Cdx2. The partially redundant activities of these inputs would be integrated via regulatory elements, such as the Cdx2 TEE, and could underlie the regulatory capacities of the mammalian embryo and the initial stochastic lineage-specific transcriptional programs.

The structure of the Cdx2 TE-enhancer described here can be used to screen for cis-regulatory elements that integrate signals from both pathways, with a similar organization of RBPJ and TEAD sites and thereby identify other components of the TE specification gene regulatory network. Future work will provide information about how modulation of Notch, together with other signaling pathways, leads to the differentiation of the totipotent cell and how symmetry is first broken during mammalian development.
EXPERIMENTAL PROCEDURES

Construct Generation for Microinjection

Cdx2 genomic regions were amplified by PCR using bacterial artificial chromosome (BAC) RP245I065 as a template. This BAC covers the whole intergenic region containing mouse Cdx2 and was obtained from the BACPAC Resources Center (http://bacpac.chori.org/). The restriction enzyme strategy or primers used for PCR, together with the lengths of corresponding amplified fragments, were as follows: fragment #1, GGTGGAAGTGAAGAAGATCAG (5,428 bp product size), fragment #2, 1,856 bp ApaI digestion of fragment #1, fragment #3, 3,572 bp ApaI digestion of fragment #1, fragment #4, TGCTAACACAGCTCCCTGCA and AAAGCAGGGAAGAGCACT GCTCC (766 bp), fragment #5, GACTGGCTGCCTTACCAGAG and TCTTCCAAAGACGCTGGAGT (1,487 bp), fragment #6, CACACGGATGATGTCTGG and AACAGGGACAGGTGAGATGG (1,329 bp), fragment #7, GCCTAGGATGCTGACTGAGG and CCCAAGTTGGAAAGGTTTGA (809 bp), and fragment #8, ATCTCACCTGTCCCTGTTGG and CCCTGGGTGAAGTGAAGAAG (1,107 bp).

As a positive control, we used the Pou5f1 distal enhancer element (Oct4DE) (Pernaute et al., 2010; Yeom et al., 1996). Each fragment was subcloned in pGEM-T Easy Vector and then excised and cloned into a modified pBluescript vector (Yee and Rigby, 1993) containing either a lacZ reporter gene or H2BmRFP1 reporter gene under the control of the human beta-globin minimal promoter and including an SV40 polyadenylation signal. Constructs were linearized, and plasmid sequences were removed before microinjection.

Transient Transgenic Analysis

For the generation of transient transgenics, F1 (C57Bl/6xCBA) females were superovulated to obtain fertilized oocytes as previously described (Nagy et al., 2003). Each construct was microinjected into the pronucleus of fertilized oocytes at E0.5 at a concentration range from 3–6 ng/µl. Microinjected oocytes were cultured in microdrops of M16 medium (Sigma) covered with mineral oil (Sigma) at 37°C, 5% CO2 until the blastocyst stage. A minimum of 50 blastocysts were used to calculate the percentage of lacZ or H2BmRFP-positive embryos per construct. When using the empty vector containing only the minimal promoter and the lacZ reporter as a negative control, we routinely obtained low-level punctuated lacZ expression.

Figure 5. TEE Activity and Cdx2 Expression Require Transcriptional Inputs from Notch and TEAD4

(A–E) TEE activity (red) and CDX2 immunodetection (green) in (A) Tead4+/−, (B) Rbpj+/−, (C) Rbpj−/−, (D) Rbpj+/−;Tead4+/−, and (E) Rbpj−/−;Tead4−/− mutant embryos. Nuclei were stained with DAPI. Arrowheads in (C)–(E) indicate TEE−; Cdx2+ outer blastomeres. Maximal projections of merged images are shown in the right panels. Scale bars, 10 µm.

(F) Quantified Cdx2 expression in outer cells of wild-type blastocysts (n = 89; three embryos) and in Tead4+/− (n = 140; three embryos), Rbpj+/− (n = 128, 4 embryos), Rbpj−/− (n = 150; four embryos), Rbpj+/−;Tead4+/− (n = 115; four embryos), and Rbpj−/−;Tead4−/− allelic combinations (n = 159; three embryos). Boxes span the 25th to the 75th percentile; internal horizontal lines indicate median values; and whiskers show minima and maxima. ***p < 0.001 by Bonferroni posttest.

(G) Relative expression of Cdx2, Gata3, Eomes, Oct4, and Nanog in pools of 25 embryos (n = 6) treated from the two-cell stage until blastocyst stage with DMSO or RO. Data are means ± SEM. **p < 0.01 by Student’s t test. See also Figure S5.
expression or weak H2BmRFP expression in approximately 10% of blastocysts. For lacZ staining, blastocysts were fixed in buffer containing 1% formaldehyde, 0.2% glutaraldehyde, 2 mM MgCl₂, 5 mM ethyleneglycoltetracetic acid (EGTA), and 0.02% Igepal for 5 min at room temperature. After washes in PBS, blastocysts were transferred to X-Gal staining solution for 24 hr at room temperature in the dark. For H2BmRFP detection, embryos were fixed in 4% paraformaldehyde for 10 min at room temperature and either analyzed for endogenous fluorescence or immunostaining. To visualize nuclei, embryos were incubated in DAPI at 1 μg/ml (Vector Laboratories).

**Generation of TEE Mouse Lines**

Three independent transgenic mouse lines were obtained for the fragment #3 TEE constructs linked to each lacZ or H2BmRFP. All lines reproduced the TE-restricted expression pattern in early preimplantation stages. Genotyping was performed by PCR with primers SV40 (TCACTGCATTCTAGTTGTGG) and 5′-rev comp (CTGATCTTCTTCATGGCCAG), which generate a 150 bp product for the TEE-lacZ lines, and 5′Cdx2 (GCAAGTGGCTGATCTTCTTC) and mRFP (GAAGCCGTACTGGAACTGAGG), which generate a 900 bp product for the TEE-H2BmRFP lines. PCR conditions were 95°C for 5 min, 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Embryos were collected from crosses of TEE-lacZ- and TEE-H2BmRFP males with outbred superovulated ICR females.

**Mouse Breeding**

The different mouse lines used are described in the main text. Adults were genotyped by PCR of tail-tip DNA using primers and conditions previously described for each line. For preimplantation embryos, genotyping was performed by PCR with primers SV40 (TCACTGCATTCTAGTTGTGG) and 5′-rev comp (CTGATCTTCTTCATGGCCAG) and scale bars, 10 μm. The following antibodies and dilutions were used: monoclonal mouse anti-Cdx2 (Mu392-UC, BioGenex) 1:200, rabbit polyclonal living colors DsRed (632496 Clontech) 1:500, rabbit polyclonal living colors GFP (632460 Clontech) 1:200, mouse anti-TEAD4 (ab58310 Abcam) 1:100, and rabbit anti-Cleaved NOTCH1 (Val1744) (2421, Cell Signaling Technology) 1:100. Cleaved NOTCH1 was also immunodetected with amplification of 3′Sox2-Cre cross (n = 313 N1OE+; 93 N1OE− blastomeres/ten embryos).

(C) Quantified CDX2 expression in outer N1OE+ (n = 247) and N1OE− (n = 59) blastomeres. In (B) and (C), boxes span the 25 th to 75 th percentile; internal horizontal lines indicate median values; and whiskers show minima and maxima. ***p < 0.001 by Student’s t test.

(D) Inside/outside distribution of all N1OE+ and N1OE− blastomeres compared with the distribution in wild-type (WT) blastomeres (n = 304; six embryos). ***p < 0.001 by chi-square test.

(E) Distribution per embryo of inside (gray) and outside cells (white) in wild-type (WT; n = 6) and N1ICD-overexpressing embryos (N1OE; n = 10). For N1OE embryos, the contribution of EGFP-positive cells (green) to each population is also shown. See also Figure S6.

**Immunohistochemistry of Preimplantation Embryos**

Immunohistochemistry was performed as previously described (Dietrich and Hillagi, 2007). The following antibodies and dilutions were used: monoclonal mouse anti-Cdx2 (MU392-UC, BioGenex) 1:200, rabbit polyclonal living colors DsRed (632496 Clontech) 1:500, rabbit polyclonal living colors GFP (632460 Clontech) 1:200, mouse anti-TEAD4 (ab58310 Abcam) 1:100, and rabbit anti-Cleaved NOTCH1 (Val1744) (2421, Cell Signaling Technology) 1:100. Cleaved NOTCH1 was also immunodetected with amplification of expression pattern in early preimplantation stages. Genotyping was performed by PCR with primers SV40 (TCACTGCATTCTAGTTGTGG) and 5′-rev comp (CTGATCTTCTTCATGGCCAG), which generate a 150 bp product for the TEE-lacZ lines, and 5′Cdx2 (GCAAGTGGCTGATCTTCTTC) and mRFP (GAAGCCGTACTGGAACTGAGG), which generate a 900 bp product for the TEE-H2BmRFP lines.

PCR conditions were 95°C for 5 min, 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Embryos were collected from crosses of TEE-lacZ or TEE-H2BmRFP males with outbred superovulated ICR females.

**Mouse Breeding**

The different mouse lines used are described in the main text. Adults were genotyped by PCR of tail-tip DNA using primers and conditions previously described for each line. For preimplantation embryos, genotyping was performed by PCR with primers SV40 (TCACTGCATTCTAGTTGTGG) and 5′-rev comp (CTGATCTTCTTCATGGCCAG) and scale bars, 10 μm. The following antibodies and dilutions were used: monoclonal mouse anti-Cdx2 (Mu392-UC, BioGenex) 1:200, rabbit polyclonal living colors DsRed (632496 Clontech) 1:500, rabbit polyclonal living colors GFP (632460 Clontech) 1:200, mouse anti-TEAD4 (ab58310 Abcam) 1:100, and rabbit anti-Cleaved NOTCH1 (Val1744) (2421, Cell Signaling Technology) 1:100. Cleaved NOTCH1 was also immunodetected with amplification of expression pattern in early preimplantation stages. Genotyping was performed by PCR with primers SV40 (TCACTGCATTCTAGTTGTGG) and 5′-rev comp (CTGATCTTCTTCATGGCCAG), which generate a 150 bp product for the TEE-lacZ lines, and 5′Cdx2 (GCAAGTGGCTGATCTTCTTC) and mRFP (GAAGCCGTACTGGAACTGAGG), which generate a 900 bp product for the TEE-H2BmRFP lines.

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the signal with a tyramide amplification kit (TSA) coupled to Cy3 (1:100; PerkinElmer). Nuclei were visualized by incubating embryos in DAPI at 1 μg/ml.

**Pharmacological Inhibitor Treatments**

Two or four-cell embryos were cultured in drops of M16 medium (Sigma) covered with mineral oil (Sigma) at 37°C, 5% CO2, containing the corresponding pharmacological inhibitor or control (DMSO) until the blastocyst stage. The following inhibitors and concentrations were used: 10 μM of the γ-secretase inhibitor RO4929097 (Stiffler et al., 2013) and 2.5 μM of the TEAD/YAP inhibitor Verteporfin (Sigma) (Ju-Chittenden et al., 2012).

**Cell Culture**

5TVER7-ES cells were cultured on gelatin-coated dishes with 1,000 U/ml LIF (ESGRO-LIF; Millipore) or as previously described (Nishioka et al., 2009). Tead4VP16ER was induced by incubation of cells for 48 hr with 4-hydroxy-2-methoxyflavone (Sigma) at 0.1 μg/ml in EMFI-CM medium (Tanaka et al., 1998) in the presence of FGF4 (1:1,000 dilution; R&D Systems) and R840 (Sigma). Cell transfections were performed with Lipofectamine 2000 (Invitrogen) and 400 ng DNA (eGFP, empty vector, Oct4ME, or TEE), followed by growth for 48 hr.

**Quantitative PCR**

RNA was isolated from 5TVER7 cells with the RNeasy Mini Kit (Qiagen) and reverse transcribed using the Quantitect Kit (Applied Biosystems). For γ-secretase inhibitor experiments, RNA from pools of 25 embryos was isolated using the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems) and reverse transcribed using the QuantiTect Kit (QIAGEN). For knockdown approaches based on Dicer (Joung et al., 2008), 250 ng of the short hairpin RNA (shRNA) of the target was transfected into the ESGRO-LIF (Millipore) as previously described (Nishioka et al., 2009). The following primers were used: Actin, CAGAAGAGAGGATACCTGTCTG (forward) and TACTCCTGCTTGGTATCCACAT (reverse), Cdx2, TCAACATCGCCACACACC TTCCT (forward) and TGTCCTACGGTGCTG (reverse), Eomes, TTACATCTCAGAAGACAGAGCTCAT (forward) and GAGGTAACCTCTGCTATCC TTGCAAGGCC (reverse), Gata3, GGTTGCGTGATAGTCTGAG (forward) and CCAAGTGGAGGATAGTCTGAG (reverse), Nanog, CTACCAAGGCTCTGCTATCGAAGATC (forward) and AAGGCGTCCTGCTGCTGCTTCTCATGAC (reverse), and Ywhaz, CCGTGTAGGAGGGCTGATTG CAT (forward) and TCTGGTGGAGGAGCATG (reverse). Nuclei were segmented in 3D reconstructions based on DAPI staining with an 8 μm isosurface. After computer segmentation, segments were visually inspected and corrected when necessary. The number of nuclei staining positive for TEE, CDX2, or GFP was evaluated visually (eight-cell stage embryos) or by segmentation (IMARIS software; E2.5 to E4.5). CDX2 protein level was estimated from unmodified mean fluorescence intensities within segmented nuclei. Mean DAPI fluorescence intensity was used to minimize error caused by staining and confocal imaging variability. CDX2 intensity values for each blastomere were normalized to the mean DAPI fluorescence intensity for each nucleus, and these ratios were normalized to the average mean DAPI intensity per whole embryo.

**Imaging and Quantification**

Confocal images of microinjected or antibody-stained embryos were acquired with a Leica SP5 confocal microscope. Images were acquired with a 63× objective and 2× zoom every 2.5 μm. Images of lacZ-stained embryos were obtained with a Zeiss DiMIRE2 inverted microscope. Images were prepared for figures using Adobe Photoshop CS5.

For quantification, unmodified images were analyzed as previously described using IMARIS imaging software (v. 7.6.3) (Bitplane) (Dietrich and Hiragi, 2007), with some modifications. Nuclei were segmented in 3D reconstructions based on DAPI staining with an 8 μm isosurface. After computer segmentation, segments were visually inspected and corrected when necessary. The number of nuclei staining positive for TEE, CDX2, or GFP was evaluated visually (eight-cell stage embryos) or by segmentation (IMARIS software; E2.5 to E4.5). CDX2 protein level was estimated from unmodified mean fluorescence intensities within segmented nuclei. Mean DAPI fluorescence intensity was used to minimize error caused by staining and confocal imaging variability. CDX2 intensity values for each blastomere were normalized to the mean DAPI fluorescence intensity for each nucleus, and these ratios were normalized to the average mean DAPI intensity per whole embryo.

**Statistics**

Statistical analyses were performed with GraphPad Prism 5. Data are presented as means ± SEM or ± SD as indicated in the figures. Differences were considered statistically significant at p < 0.05. p values were calculated by t test for comparisons of two groups and ANOVA with Bonferroni posttest for multiple pairwise comparisons. For the data presented in Figures 1H, 4D, and 6E, a chi-square test was performed. All data used for quantitative analysis are supplied in Table S1.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.06.019.

**ACKNOWLEDGMENTS**

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Role of Notch in Trophectoderm Specification


Notch and Hippo Converge on Cdx2 to Specify the Trophectoderm Lineage in the Mouse Blastocyst

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 1: Identification of the Cdx2 TEE. (A) Diagram of the Cdx2 locus showing the fragments tested in transient transgenic embryos, the specific activity of the fragments in the TE, and the percentage of embryos for each construct showing any sign of reporter activity. (B) Representative transient transgenic embryos showing lacZ reporter activity driven by those constructs showing specific activity in the TE. (C) Temporal dynamics of a stable lacZ-TEE line for construct #3 at the 8-cell, 16-cell and blastocyst stages.

Figure S2, related to Figure 2: The Cdx2 TEE is not an auto-regulatory element. (A-B) Activity of the TEE, detected by immunohistochemistry with anti-mRFP antibody (red), and immunodetection of CDX2 (green) in (A) wild type (wt) and (B) Cdx2 mutant blastocysts. Nuclei were stained with DAPI (blue). (C) Average cell number in wild type and Cdx2 mutant blastocysts. (D) Percentage of TEE-positive cells per embryo in wild type (n=149, 4 embryos) and Cdx2 mutant blastocysts (n=145, 3 embryos). (E) Average cell number in wild type blastocyst, Tead4 heterozygotes and Tead4 mutant blastocysts. Differences in cell number among the genotypes are not significant. (F) Percentage of TEE-positive cells per embryo in wild type blastocysts (n=218, 3 embryos), Tead4 heterozygotes (n=464, 8 embryos) and Tead4 mutant homozygotes (n=78, 2 embryos). Data are means ± s.d.

Figure S3, related to Figure 3: Evidence for a role of the Notch signaling pathway in the TE. (A) Sequence of the 1.3 kb TEE, highlighting putative binding sites for RBPJ (blue) and TEAD (green). (B) Immunodetection of N1ICD with the signal enhanced by using a tyramide amplification kit (red). Nuclei were stained with DAPI (blue). Merged image is shown in the right panel. Scale bars, 10 μm.
**Figure S4, related to Figure 4:** The RBPJ and TEAD binding sites of the TEE are functional. (A) Effect of the TEAD–YAP inhibitor Verteporfin (VP) on TEE activity (red) and endogenous CDX2 (green). (B) TEE\textsuperscript{TEAD} mut activity in transient transgenic embryos treated with DMSO, VP or RO. (C) TEE\textsuperscript{RBPJ} mut activity in transient transgenic embryos treated with DMSO, RO or VP. Nuclei were stained with DAPI. Scale bars, 10 μm.

**Figure S5, related to Figure 5:** Phenotype of embryos with different Rbpj;Tead4 allelic combinations. (A) Percentage of TEE-positive cells per embryo in wild type blastocysts (n=162, 3 embryos) and in Tead4\textsuperscript{+/+} (n=200, 3 embryos), Rbpj\textsuperscript{+/+} (n=187, 5 embryos), Rbpj\textsuperscript{-/-} (n=115, 3 embryos), Rbpj\textsuperscript{+/+};Tead4\textsuperscript{+/+} (n=238, 5 embryos) and Rbpj\textsuperscript{-/-};Tead4\textsuperscript{+/+} allelic combinations (n=176, 2 embryos). *p<0.05, ***p<0.001 by Bonferroni post test. (B) Average number of inside and outside cells positive or negative for CDX2 in wild type (n=164, 3 embryos), Tead4\textsuperscript{+/+} (n=200, 3 embryos), Rbpj\textsuperscript{+/+} (n=179, 4 embryos), Rbpj\textsuperscript{-/-} (n=214, 4 embryos), Rbpj\textsuperscript{+/+}; Tead4\textsuperscript{+/+} (n=179, 4 embryos) and Rbpj\textsuperscript{-/-};Tead4\textsuperscript{+/+} allelic combinations (n=217, 3 embryos) quantified in Figure 4F. Data are means ± s.d. (C) Distribution (%) of embryos for the different allelic combinations of Rbpj and Tead4, compared with the expected distribution. Dead embryos inside the zona, which could include double homozygotes, were observed but could not be genotyped due to DNA degradation (Table S1). (D) TEE activity (red) and CDX2 immunodetection (green) in embryos from the mRFP line treated with DMSO or RO+VP from 4 cells until blastocyst stage (left panel). Maximal projections of merged images are shown in the right panels. Nuclei were stained with DAPI. Scale bars, 10 μm.
Figure S6, related to Figure 6: Mosaicism of maternal Sox2-Cre activity in the blastocyst. (A) Mosaicism of reporter expression (green) from the R26-stop-N1ICD-ires-EGFP line when recombined by maternal Sox2-Cre. (B) Example of a blastocyst with a very high proportion of non-recombined cells. (C) Example of a blastocyst with recombination occurring in all cells. (D) Average cell number in wild type (wt) and N1ICD-overexpressing (N1OE) blastocysts. Data are means ± s.d. (E) Detection of mosaic recombination in blastocysts by PCR of Neo. The varying degree of detection of the non-recombined allele in different embryos is shown, ranging from non-recombined (embryo 2, shown in panel B), mosaic recombination (white arrowhead), to full recombination of the Neo allele (embryo 5, shown in panel C). Negative control (-) and H2O samples are indicated. (F) Reporter mosaicism (cytoplasmic, green) from the R26-stop-YFP line when recombined by maternal Sox2-Cre. (G-H) Breeding strategy for the ♂R26-stop-N1ICD-ires-EGFP X ♀Sox2-Cre cross (G) and for the ♂R26-stop-YFP X ♀Sox2-Cre cross. (H). White arrowheads in (A-C, F) mark non-recombined cells. Nuclei in (A-C and F) were stained with DAPI. Scale bars, 10 μm.