The Hippo Signaling Pathway Components Lats and Yap Pattern Tead4 Activity to Distinguish Mouse Trophectoderm from Inner Cell Mass

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SUMMARY

Outside cells of the preimplantation mouse embryo form the trophectoderm (TE), a process requiring the transcription factor Tead4. Here, we show that transcriptionally active Tead4 can induce *Cdx2* and other trophoblast genes in parallel in embryonic stem cells. In embryos, the Tead4 coactivator protein Yap localizes to nuclei of outside cells, and modulation of Tead4 or Yap activity leads to changes in Cdx2 expression. In inside cells, Yap is phosphorylated and cytoplasmic, and this involves the Hippo signaling pathway component Lats. We propose that active Tead4 promotes TE development in outside cells, whereas Tead4 activity is suppressed in inside cells by cell contact- and Lats-mediated inhibition of nuclear Yap localization. Thus, differential signaling between inside and outside cell populations leads to changes in cell fate specification during TE formation.

INTRODUCTION

During mouse development, the first lineage specified is the trophoblast/placenta lineage, set aside during blastocyst formation. In the blastocyst, the trophoblast, or trophectoderm (TE), surrounds the inner cell mass (ICM), which will give rise to the fetus and other extraembryonic tissues. The homeodomain transcription factor Cdx2 is expressed in the TE at the blastocyst stage. Cdx2 is required for TE development and is sufficient to promote trophoblast fate in ICM-derived embryonic stem (ES) cells, including suppression of ICM and induction of TE genes (Niwa et al., 2005; Strumpf et al., 2005). Conversely, ICM fates are regulated by a distinct set of transcription factors, including

the POU family transcription factor Oct3/4 (encoded by *Pou5f1*). Prior to blastocyst formation, Cdx2 and Oct3/4 are initially coexpressed throughout the embryo (Dietrich and Hiiragi, 2007; Palmieri et al., 1994; Ralston and Rossant, 2008). Mutual antagonism between these two factors may contribute to the eventual segregation of their expression domains (Niwa et al., 2005), with Cdx2 in outside cells of the TE and Oct3/4 in inside cells of the ICM. However, molecular mechanisms that initially interpret inside/outside positional information within the embryo to establish this pattern are not known.

We and others recently showed that the TEAD/TEF family transcription factor Tead4 is essential for TE development and Cdx2 expression prior to the blastocyst stage (Nishioka et al., 2008; Yagi et al., 2007). This provided the first clue about molecular mechanisms acting upstream of the TE/ICM lineage distinction. However, whether Tead4 acted permissively or instructively in this process was unclear, since Tead4 itself was not restricted to outside cells (Nishioka et al., 2008).

Here, we sought to identify cofactors and signaling components that could impart positional information to spatially regulate Tead4 activity in the embryo. Many lines of evidence have suggested that TEAD-mediated transcription is regulated by the Ser/Thr kinase Hippo in *Drosophila*, or Stk3 (Mst) in mice. In *Drosophila*, Hippo inhibits the Yorkie (Yki) coactivator and suppresses cell proliferation (Huang et al., 2005). These activities are mediated by a Tead protein, Scalloped (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008b). In mammals, Hippo signaling comprises a growth-regulating pathway, which controls cell contact-mediated inhibition of proliferation (see reviews Pan, 2007; Reddy and Irvine, 2008; Saucedo and Edgar, 2007). In this context, cell-cell contact regulates nuclear accumulation of a Yki homolog, Yes-associated protein 1 (Yap1, Yap hereafter), through Hippo signaling and controls cell proliferation by regulating transcriptional activity of Tead proteins (Ota and Sasaki, 2008; Zhao et al., 2007, 2008). In the mouse embryo,
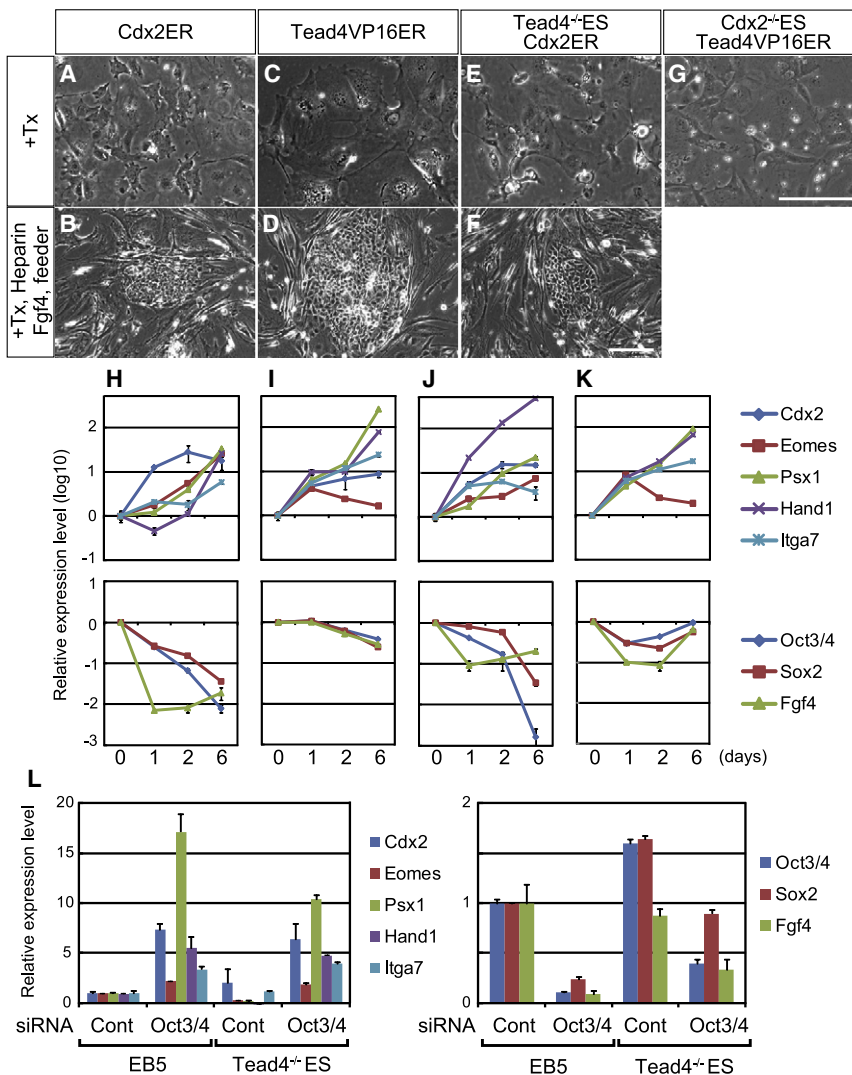
Figure 1. Tead4 Regulates Multiple Trophoblast Genes in ES Cells

(A–G) Morphologies of ES cells treated for 6 days with Tx in (A, C, E, and G) ES cell medium and in (B, D, and F) TS cell culture medium in the presence of feeder cells.

(H–K) Expression of trophoblast (upper panels) and ES/ICM (lower panels) genes in representative clones of (H) 5ECER4 (EB5 + Cdx2ER), (I) 5TVER7 (EB5 + Tead4VP16ER), (J) T4CER10 (Tead4$^{-/-}$ + Cdx2ER), and (K) CTVER5 (Cdx2$^{-/-}$ + Tead4VP16ER) cells after Tx treatment for the indicated time periods.

(L) Expression of trophoblast (left panel) and ES/ICM (right panel) genes in EB5 (control) and Tead4$^{-/-}$ ES cells after transfection of control or Oct3/4 siRNAs.

sufficiency of Tead4 to promote TE fate has not been reported. We examined the ability of Tead4 to promote trophoblast differentiation in ES cells. For comparison, we used the ES cell line 5ECER4 (Niwa et al., 2005), which stably expresses a tamoxifen (Tx)-inducible fusion between Cdx2 and a modified ligand-binding domain of the estrogen receptor (ER) (Cdx2ER). Consistent with previous analyses (Niwa et al., 2005), treatment of 5ECER4 cells with Tx led to flattened morphologies reminiscent of trophoblast cells (Figure 1A), induction of trophoblast genes, and downregulation of ES cell genes (Figure 1H). Using trophoblast stem (TS) cell culture conditions (Tanaka et al., 1998), TS-like cells could be derived from this line (Figure 1B). These observations are consistent with previous analyses (Niwa et al., 2005) and provide a standard against which to evaluate Tead4 activity in ES cells.

We next examined the ability of Tead4 to induce trophoblast fate in ES cells. We established ES cell lines stably expressing a Tx-inducible form of active Tead4 (Tead4VP16ER), which consisted of the Tead4 DNA-binding domain fused to the transcriptional activation domain of herpes simplex virus VP16, followed by the ER domain. After treatment with Tx, multiple independent clones exhibited morphological changes similar to Cdx2 overexpression (Figure 1C). Treated Tead4VP16-expressing clones also expressed trophoblast genes (Figure 1I), and TS-like cells could be derived under TS cell culture conditions (Figure 1D). Thus, constitutively active Tead4 is sufficient to promote trophoblast fate in ES cells.

Cdx2 Is a Major Target of Tead4

Tead4 is genetically upstream of Cdx2 during TE formation in the embryo (Nishioka et al., 2008; Yagi et al., 2007), suggesting that Tead4 is not required for Cdx2-mediated trophoblast gene expression in ES cells. To test this hypothesis, we established feeder-free Tead4$^{-/-}$ ES cell lines (Nishioka et al., 2008) that stably express Cdx2ER, and we examined their ability to adopt

Tead1$^{-/-}$; Tead2$^{-/-}$ mutants die soon after implantation due to reduced cell proliferation and increased apoptosis (Sawada et al., 2008). Tead1/2 interact genetically with Yap (Sawada et al., 2008), suggesting that the roles of these genes in Hippo signaling are conserved in mice.

We examined the role of Tead4 in TE development using both ES cells and preimplantation embryos. We show that active Tead4 can promote multiple trophoblast genes in parallel, including Cdx2. We next show that, in the embryo, the Tead coactivator Yap localizes to nuclei only in outside cells and is excluded from inside cell nuclei by the Hippo signaling pathway component Lats. These observations suggest that Tead4/Yap interpret positional information along the inside/outside axis of the embryo to restrict expression of Cdx2 and TE fates to outside cells.

RESULTS

Tead4 Instructively Regulates Multiple Trophoblast Genes in ES Cells

Although Tead4 is required for establishment of the TE lineage in the mouse embryo (Nishioka et al., 2008; Yagi et al., 2007), the

trophoblast fate. After treatment with Tx, multiple independent clones exhibited similar trophoblast-like morphologies (Figure 1E). In addition, trophoblast genes were upregulated, whereas ES genes were downregulated (Figure 1J), suggesting that *Tead4* is not required for Cdx2-mediated induction of trophoblast differentiation in ES cells. To examine the requirement for *Tead4* in long-term TS-like potential, we cultured these cells under TS conditions. Colonies with TS-like morphology could be derived, but could not be maintained as TS cells (Figure 1F and data not shown), indicating that *Cdx2* cannot fully substitute for *Tead4* in the trophoblast lineage.

As another means by which to examine the epistatic relationship between *Tead4* and *Cdx2*, we established *Cdx2*$^{-/-}$ ES cells (Niwa et al., 2005) stably expressing Tead4VP16ER, and we examined their ability to adopt trophoblast fate. After treatment with Tx, multiple independent clones also exhibited trophoblast-like morphology (Figure 1G) and trophoblast gene expression (Figure 1K). Tead4 can therefore regulate trophoblast gene expression independently of *Cdx2*. However, TS-like colonies could not be derived from these cells, suggesting that *Cdx2* is essential for proper trophoblast lineage development. Taken together, these observations suggest that Tead4 promotes trophoblast fate through both *Cdx2*-dependent and -independent pathways, and that Cdx2 is a major mediator of *Tead4*-dependent changes in trophoblast gene expression.

Tead4 Is Dispensable for *Cdx2* Expression when *Oct3/4* Levels Are Reduced

In ES cells, Oct3/4 suppresses *Cdx2*, and reduction of *Oct3/4* leads to upregulation of *Cdx2* expression and formation of TS-like cells (Niwa et al., 2000, 2005). Because *Tead4* is required for *Cdx2* expression and TE development in vivo (Nishioka et al., 2008; Yagi et al., 2007), we next asked whether *Tead4* is required for *Cdx2* expression even if *Oct3/4* expression levels are reduced. We examined the requirement for *Tead4* in inducing *Cdx2* expression after siRNA-mediated knockdown of *Oct3/4*. In control wild-type (EB5) ES cells, *siOct3/4* transfection led to reduced expression of *Oct3/4* and other ES genes, including *Sox2* and *Fgf4* (Figure 1L). Knockdown of *Oct3/4* also led to increased expression of *Cdx2* and other trophoblast genes (Figure 1L). Interestingly, *Oct3/4* knockdown in *Tead4*$^{-/-}$ ES cells (Nishioka et al., 2008) led to essentially the same changes in gene expression as in wild-type ES cells (Figure 1L), indicating that *Tead4* is not required for expression of *Cdx2* and other trophoblast genes as long as *Oct3/4* levels are reduced. However, immediate induction of *Cdx2* by Tead4VP16 in ES cells was not accompanied by a clear reduction of *Oct3/4* at day 1 (Figure 1I), suggesting that Tead4 can induce *Cdx2* expression by overcoming Oct3/4-mediated suppression. Therefore, in the presence of Oct3/4, Tead4 is required to induce *Cdx2* expression.

Tead4 Regulates *Cdx2* as a Transcriptional Activator In Vivo

These observations suggested that Tead4 can instructively induce *Cdx2* expression and trophoblast fate. However, Tead4 is expressed ubiquitously in preimplantation embryos (Nishioka et al., 2008), raising the question as to how its activity is restricted to outer cells of the nascent TE. We hypothesized that Tead4

activity must be regulated along the inside/outside axis. To test this hypothesis, we first examined the ability of variant forms of Tead4 to induce Cdx2 expression in inside cells when overexpressed. During normal development, Cdx2 is initially ubiquitously expressed and becomes progressively downregulated in inside cells and upregulated in outside cells during blastocyst formation (Ralston and Rossant, 2008). Because levels of Cdx2 are highly variable among embryos and among inside cells of individual embryos during this process (Dietrich and Hiiragi, 2007; Ralston and Rossant, 2008), we examined populations of embryos in which these constructs were ubiquitously overexpressed by RNA injection from the 2-cell stage (Figure 2A).

At the 20- to 30-cell stages, embryos were classified into three phenotypic categories, depending on the level of Cdx2 expression detected in inside cells. That is, type I embryos exhibited undetectable levels of Cdx2 in inside cells, type II embryos exhibited low levels of Cdx2 relative to outside cells, and type III embryos exhibited high levels of Cdx2 (comparable to outside cell levels). At this stage, the majority of water or *β-globin* mRNA-injected embryos exhibited either no Cdx2 (type I) or weak Cdx2 expression (type II) in inside cells, whereas only 8% of embryos on average exhibited strong Cdx2 expression (type III) in a few inside cells (Figures 2C and 2E; see Figure S1 available online). Expression of either full-length Tead4 or a repressor-modified form of Tead4, Tead4EnR (a fusion between the Tead4 containing the DNA-binding domain and the repression domain of *Drosophila* Engrailed), did not lead to changes in Cdx2 expression (Figures 2B and 2E). In contrast, overexpression of Tead4VP16 led to a significant increase in the number of type III embryos exhibiting elevated Cdx2 expression in inside cells (Figures 2B, 2D, and 2E), consistent with our observations in ES cells. The number of inside cells expressing high levels of Cdx2 also increased after Tead4VP16 overexpression (Figure 2D; Figure S1). Injection of higher doses of Tead4VP16 RNA led to increased lethality (not shown). By contrast, overexpression of an unrelated transcription factor fused to the VP16 activation domain (Foxa2VP16) did not affect Cdx2 expression (Figure 2E).

Taken together, these results suggest that the Cdx2-inducing activity of Tead4 is dependent on the presence of an exogenous activation domain. Preimplantation embryos also express *Tead1* and *Tead2* (Nishioka et al., 2008), and these factors are known to bind similar DNA motifs as Tead4 (Sawada et al., 2008). Interestingly, overexpression of activator-modified Tead1 (Tead1VP16) also increased the frequency of type III embryos (Figure 2E), raising the possibility that other Tead proteins may participate in regulation of Cdx2 expression during embryogenesis.

Nuclear Localization of Yap Anticipates Cdx2 Expression in the Outer Cells

Our analyses of constitutively active Tead4 both in ES cells and in the early embryo suggested that Tead4 activity is regulated along the inside/outside axis of the embryo. Tead proteins are known to act in conjunction with the coactivator protein Yap (Vassilev et al., 2001), whose nuclear localization is regulated by phosphorylation (Zhao et al., 2007). *Yap* mRNA was detected throughout preimplantation development by RT-PCR (Figure 3A), prompting us to examine the localization of Yap protein throughout preimplantation development. During
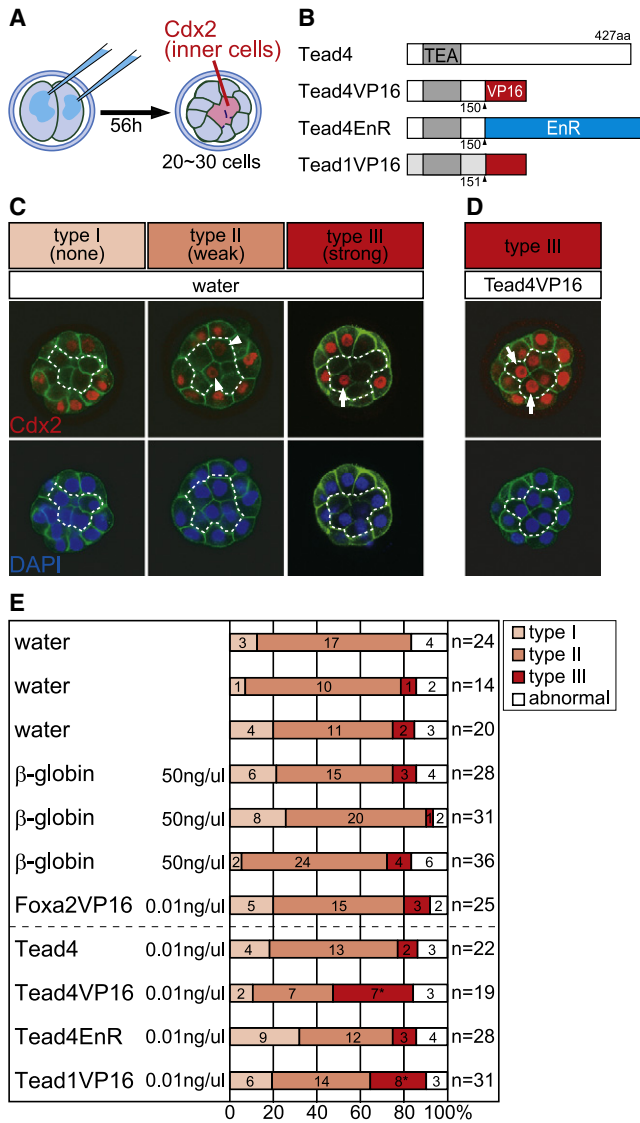
Figure 2. Tead4 Instructively Regulates Cdx2 Expression in Embryos

(A) Scheme for RNA injection for the experiments shown in this figure and Figures 4 and 6. Expression of Cdx2 was examined in inside cells after 56 hr of culture.

(B) Tead variants used in this study.

(C) Examples of three classes of water-injected embryos exhibiting different levels of Cdx2 (red) in inside cells (indicated by a dotted line). Inside cells were identified based on Z-series confocal images of embryos stained with nuclei (DAPI, blue) and cell membranes (β-catenin, green). Inside cells exhibited weak (arrowheads) or strong (arrow) Cdx2 expression.

(D) A representative embryo injected with *Tead4VP16* RNA.

(E) Graph summarizing the effects of *Tead* RNA injection on Cdx2 expression in inside cells. Numbers in each column represent the number of embryos in each category. Asterisks indicate that the differences were statistically significant compared to the *β-globin* RNA-injected group (p < 0.05).

Figure 3. Nuclear Yap Anticipates Outer Cell-Restricted Expression of Cdx2

(A) RT-PCR analysis of gene expression in pools of 50 embryos for each stage indicated.

(B) Immunofluorescence localization of Cdx2 and Yap proteins during preimplantation development. Cdx2 is still detected in some inside cells, although Yap is not (arrowheads).

(C) Yap and Cdx2 localization in nuclei of the TE in the mid/late blastocyst.

(D) Levels of nuclear Yap proteins during preimplantation development. To account for changes in fluorescence due to changing focal planes, Yap fluorescence levels were normalized to corresponding DAPI fluorescence levels for each nucleus.

(E) Immunofluorescence localization of Wwtr1. Embryo cell number is indicated in (B), (C), and (E).

development, nuclear Yap was first detected in embryos at the 4-cell stage, although the signal was weak and variable among embryos and among individual blastomeres (Figure 3B and data not shown). By th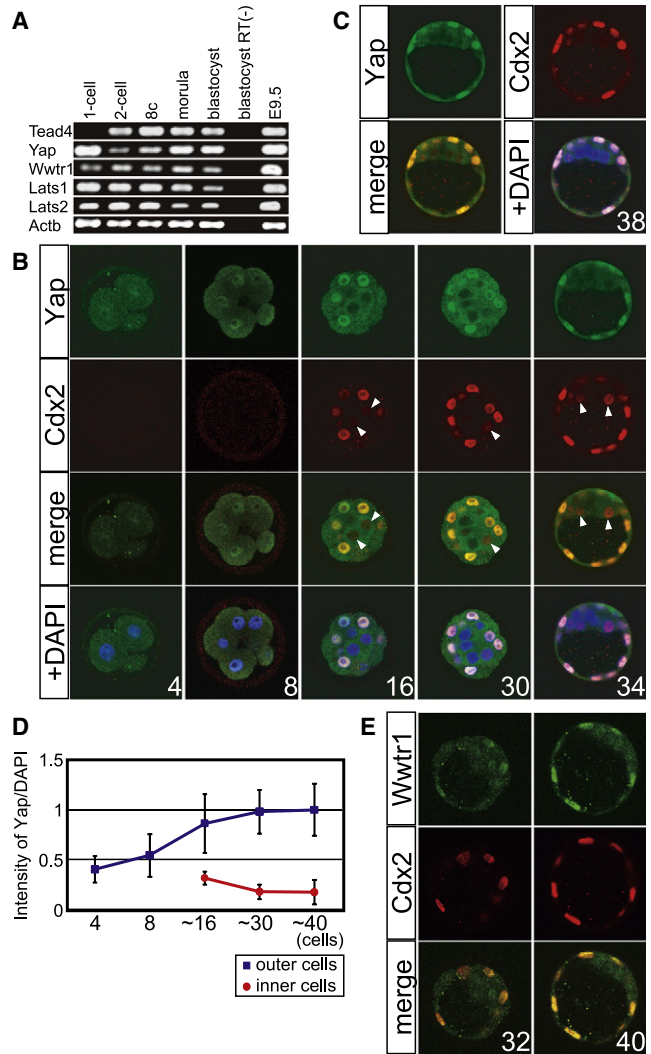e early 8-cell stage, nuclear Yap was detected in all blastomeres, even in embryos that had not yet undergone compaction (n = 22) (Figure 3B). After the 8-cell stage, levels of nuclear Yap increased in outside cells up to the 30-cell stage and remained constant thereafter, whereas nuclear Yap decreased in inside cells and Yap appeared to be excluded from the nuclei (Figures 3B and 3D). At the mid/late blastocyst stage, nuclear Yap was restricted to outside cells of the TE and was not detected within cells of the ICM (Figure 3C).
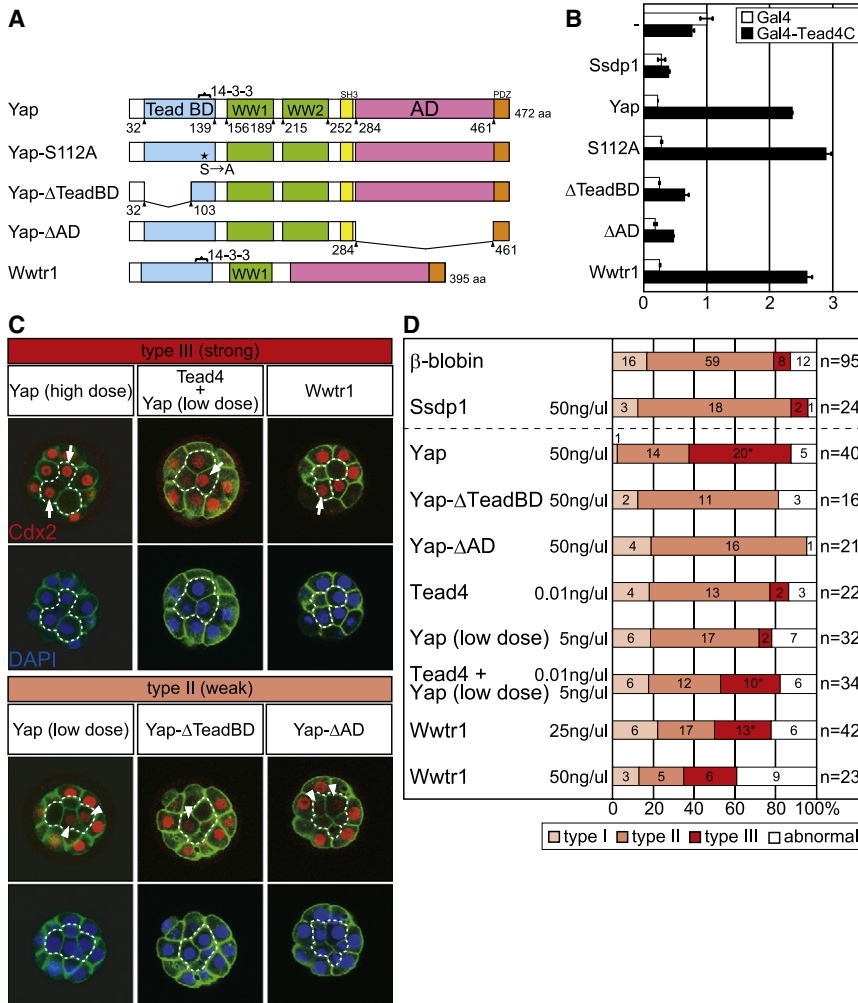
Figure 4. Nuclear Yap Regulates Cdx2 Expression

(A) Yap variants used in this study (TeadBD, Tead-binding domain; WW1 and WW2, WW domains; AD, activation domain; 14-3-3, 14-3-3-binding site; SH3, SH3-binding site; PDZ, PDZ domain-binding site). Residue numbers within the Yap protein were indicated. The domain structure of Wwtr1 was defined based on sequence homology to Yap.

(B) Effects of Yap variants on the transcription activity of Gal4-Tead4C in NIH 3T3 cells.

(C) Representative embryos showing the effects of overexpression of Yap variants on Cdx2 (red) levels. Membrane (green) and nuclei (blue) are also shown.

(D) Graph summarizing the effects of Yap variants on Cdx2 levels in inside cells. The asterisks indicate that the differences were significant compared to the β-globin RNA-injected group.

Since *Cdx2* expression begins after compaction around the 8-cell stage (Ralston and Rossant, 2008), nuclear localization of Yap appears to precede expression of Cdx2. In addition, restriction of Yap to outside cells appears to precede that of Cdx2, since nuclear Yap was restricted to outside cells from the 16-cell stage onward, whereas Cdx2 is not clearly restricted to outside cells until later stages (Figure 3B) (Dietrich and Hiiragi, 2007; Niwa et al., 2005; Ralston and Rossant, 2008). Thus, restriction of Yap to outside cell nuclei precedes restriction of Cdx2 expression to outside cells, suggesting that Yap could play a role in Tead4-mediated patterning of Cdx2 expression along the inside/outside axis during blastocyst formation.

Yap and Wwtr1 Regulate Cdx2 Expression in Preimplantation Embryos

We next asked whether Yap could induce Cdx2 expression in inside cells of the embryo. Importantly, Yap cooperatively increased transcription induced by Gal4-Tead4C (a fusion protein of the DNA-binding domain of yeast Gal4 and the C-terminal cofactor-binding domain of Tead4) in NIH 3T3 cells (Figure 4B), confirming the ability of Yap to enhance Tead4-mediated transcriptional activity. Next, *Yap* mRNA was injected into both blastomeres of the 2-cell embryo, and phenotypes

were categorized as described above. Overexpression of *Yap* led to a significant increase in the frequency of type III embryos exhibiting high levels of Cdx2 in inside cells (Figures 4C and 4D). In contrast, neither Yap-ΔTeadBD, lacking the Tead binding domain, nor Yap-ΔAD, lacking the transactivation domain, were able to enhance Tead4-mediated transactivation in NIH 3T3 cells (Figures 4A and 4B), and both constructs failed to increase Cdx2 expression in embryos (Figures 4C and 4D). Thus, Yap activity may depend on interaction with Tead. Overexpression of an unrelated coactivator protein, Ssdp1 (Nishioka et al., 2005), had no effect on Tead4-mediated transcription in NIH 3T3 cells, or on Cdx2 expression in embryos (Figures 4B and 4D), confirming the specificity of our observations. Finally, to examine whether Yap can confer Cdx2-inducing ability on unmodified Tead4 (lacking VP16), we examined the ability of unmodified Tead4 to induce Cdx2 expression in the presence of Yap. Injection of either full-length *Tead4* mRNA or low-dose (5 ng/μl) *Yap* mRNA alone had no effect on Cdx2 expression, whereas their coinjection significantly increased Cdx2 expression (Figures 4C and 4D). Taken together, our observations suggest that Yap can induce *Cdx2* expression cooperatively with Tead4.

Although Yap is sufficient to upregulate Cdx2 in inside cells, $Yap^{-/-}$ mutant embryos exhibit normal TE development (Morin-Kensicki et al., 2006). This observation suggested that a Yap-related protein could compensate for the absence of *Yap* during early development. We therefore examined the Yap-related protein Wwtr1 (TAZ) (Mahoney et al., 2005). *Wwtr1* was detected in preimplantation embryos; high levels of Wwtr1 protein were detected in outside cell nuclei (Figures 3A and 3E), whereas low, but detectable, levels of Wwtr1 were detected in inside cell nuclei. Importantly, overexpression of *Wwtr1* was sufficient to increase Cdx2 expression in inside cells (Figures
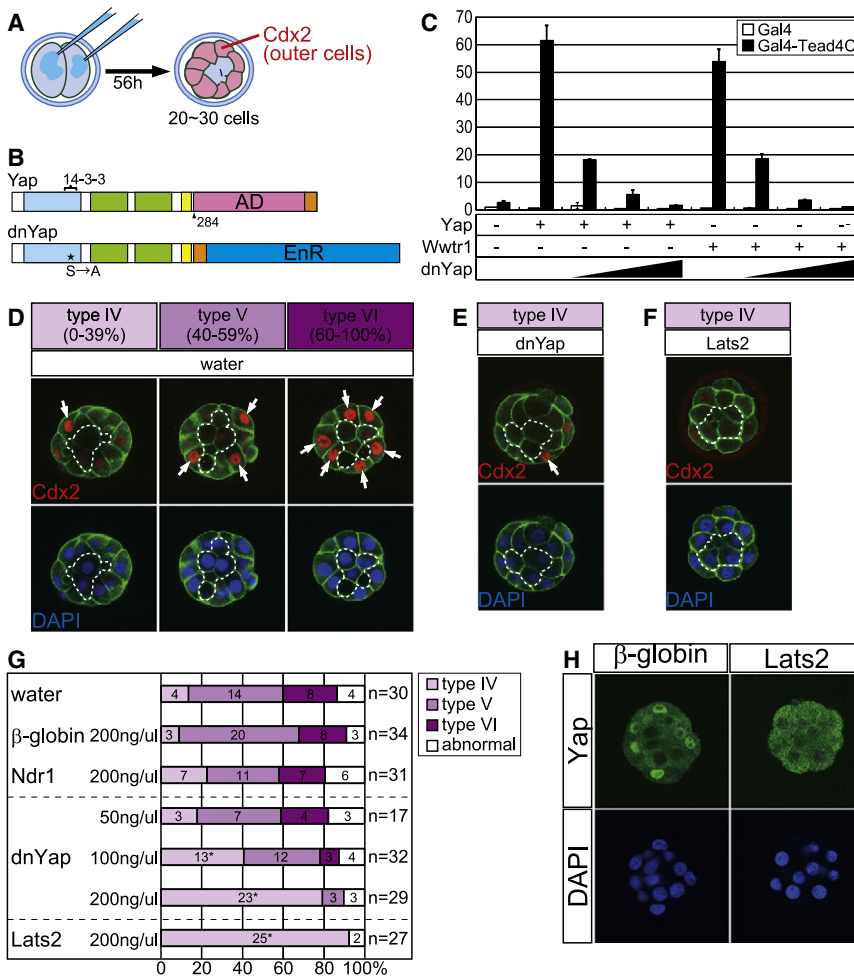
Figure 5. Requirements of Nuclear Yap/Wwtr1 in Cdx2 Expression

(A) Scheme for RNA injection and analysis of Cdx2 in outside cells after 56 hr of culture.

(B) Schematic representation of the structure of dnYap.

(C) Luciferase assay after overexpression of dnYap in HeLa cells.

(D) Examples of embryos classified according to the number of outside cells with strong Cdx2 (red, arrows). Membrane (green) and nuclei (blue) are also shown.

(E and F) Representative embryos injected with (E) *dnYap* or (F) *Lats2* RNA.

(G) Graph summarizing phenotypes resulting from injection of RNAs for *dnYap* or *Lats2* on expression of Cdx2 in outside cells. The asterisks indicate that the differences were significant compared to the control β-globin-injected group.

(H) Effects of *Lats2* RNA injection on subcellular localization of Yap proteins.

4C and 4D), consistent with the hypothesis that Wwtr1 plays a Yap-like role in the early embryo.

Next, we tested the requirement for *Yap* and *Wwtr1* in Cdx2 expression in early embryos. Whereas loss of either *Yap* or *Wwtr1* alone does not lead to abnormalities in preimplantation development (Hossain et al., 2007; Makita et al., 2008; Morin-Kensicki et al., 2006), $Yap^{-/-};Wwtr1^{-/-}$ embryos died before the morula stage (16–32 cells), prior to establishment of inside and outside cell populations (Table S1). Thus, *Yap* and *Wwtr1* are required prior to lineage specification, precluding analysis of their requirement during lineage specification. However, use of a dominant-negative Yap (dnYAP), allowed us to address this issue.

To create dnYap, the transcriptional activation domain of Yap was replaced with the *Drosophila* Engrailed repression domain, and Yap-S112 was converted to A (equivalent to human YAP-S127A) (Figure 5B). Phosphorylation of Yap-S112 by the protein kinase Lats promotes cytoplasmic localization of Yap through interaction with the cytoplasmic scaffold protein 14-3-3 (Basu et al., 2003; Dong et al., 2007; Zhao et al., 2007). In HeLa cells, dnYap strongly suppressed activation of Tead4 by either Yap or Wwtr1 (Figure 5C), confirming the dominant-negative activity of this construct. Next, we overexpressed *dnYap* in embryos by RNA injection as described above, and we examined Cdx2 expression (Figure 5A), with the prediction that dominant-nega-

tive Yap would decrease Cdx2 expression in outside cells. Overexpression of dnYap did not cause early lethality, but did alter Cdx2 expression in early blastocysts. Embryos were classified into three categories, based on the fraction of outside cells exhibiting high levels of nuclear Cdx2: embryos exhibiting Cdx2 in 0%–39% of outside cells (type IV), in 40%–59% of outside cells (type V), and in 60%–100% of outside cells (type VI) (Figure 5D). Overexpression of *dnYap* RNA significantly increased the frequency of type IV, while decreasing the frequency of type V and type VI embryos in a dose-dependent manner (Figures 5E and 5G). Thus, dnYap decreased expression of Cdx2 in outside cells, and regulation of Cdx2 expression appears to be dependent on Tead4 coactivator activity. These results are consistent with the hypothesis that Yap and Wwtr1 act together with Tead4 to regulate Cdx2 expression in outside cells during blastocyst formation. In addition, other domains of Yap/Wwtr1 proteins may be required for viability at very early stages.

Lats Regulates Yap Localization during Preimplantation Development

We next sought to examine how Yap becomes localized to nuclei of outside cells prior to TE formation. In cultured cells, Lats1/2-mediated phosphorylation of Yap/Wwtr1 leads to their cytoplasmic localization (Dong et al., 2007; Hao et al., 2008; Lei et al., 2008; Zhang et al., 2008a; Zhao et al., 2007). We therefore examined the localization of phosphorylated Yap (p-Yap) by using an antibody raised against p-Yap. Prior to the blastocyst stage, p-Yap was detected in the cytoplasm of inside cells, and at the blastocyst stage high levels of cytoplasmic p-Yap were detected within ICM cells (Figure 6A). Protein phosphatase treatment of embryos eliminated this pattern (Figure 6A, right), confirming the specificity of the antibody. These observations
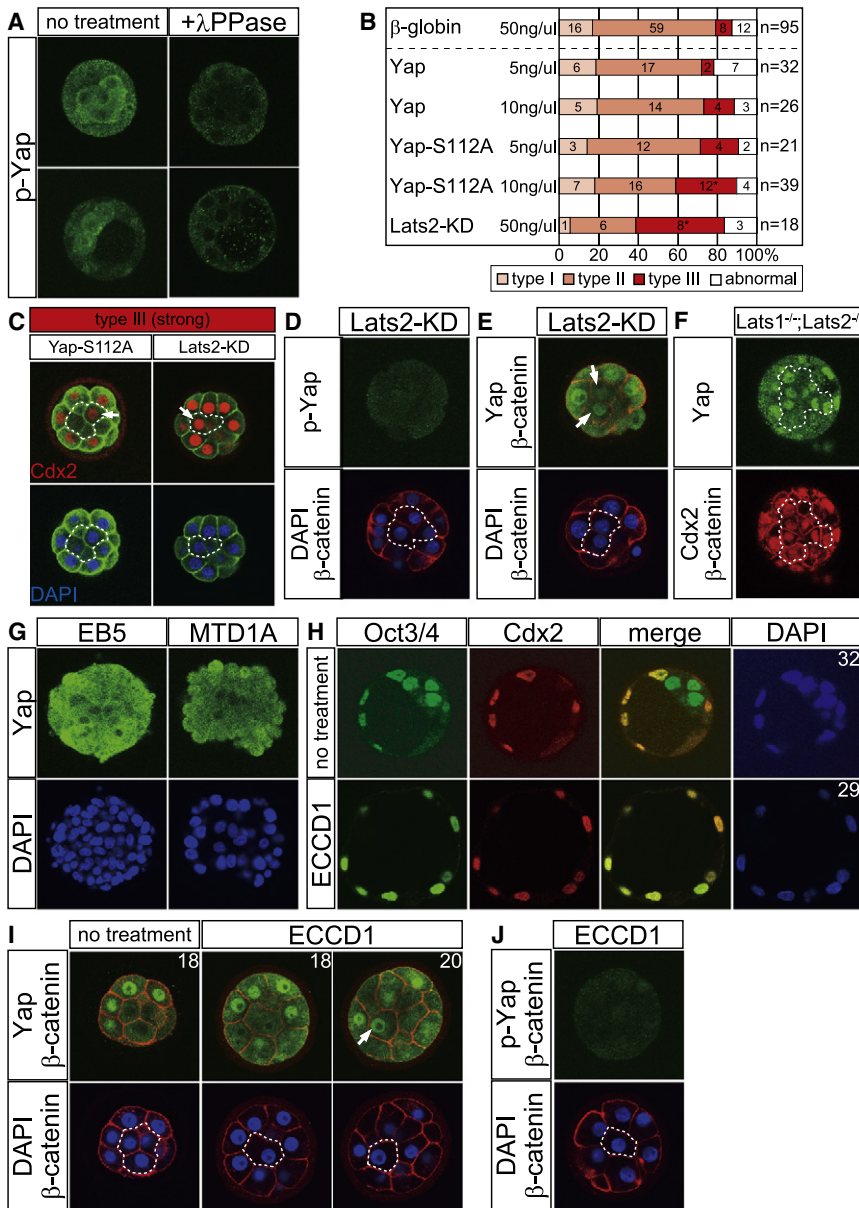
Figure 6. Cell Adhesion and Lats2 Suppress Nuclear Yap Localization in Inside Cells

(A) Immunofluorescence localization of phosphorylated Yap (p-Yap) in preimplantation embryos. A stronger p-Yap signal was detected in inside cells of morula-stage embryos and in the inner cell masses of blastocyst-stage embryos. Treatment with lambda protein phosphatase (λPPase) eliminated p-Yap from inside cells.

(B) Graph summarizing the effects of *Yap-S112A* or *Lats2-KD* on Cdx2 expression in inside cells. The asterisks indicate that the differences were significant compared to the control β-globin-injected group.

(C) Representative embryos showing the effects of injection of *Yap-S112A* or *Lats2-KD* on Cdx2 expression (red). Membrane and nuclei were counterstained with green and blue, respectively.

(D and E) Representative embryos showing reduction of the (D) p-Yap signal and (E) increased nuclear Yap in embryos injected with *Lats2-KD* RNA.

(F) A representative *Lats1⁻/⁻*; *Lats2⁻/⁻* embryo showing increased nuclear Yap and strong Cdx2 expression in the inner cells.

(G) Immunofluorescence localization of Yap in cell aggregates of two cell lines, EB5 (ES) and MTD1A.

(H) Representative embryos after treatment with an anti-E-Cadherin antibody, ECCD1 (bottom).

(I) Altered Yap localization in ECCD1-treated embryos. ECCD1-treated embryos, which failed to exclude Yap from nuclei (center) or exhibited obvious nuclear accumulation of Yap (right) (embryo cell number indicated).

(J) Levels of p-Yap in ECCD1-treated embryos.

suggest that Yap phosphorylation leads to its cytoplasmic localization in inside cells of the preimplantation embryo.

We next examined factors regulating Yap localization during TE formation. In cultured cells, Hippo signaling suppresses the nuclear accumulation of Yap through the activities of Lats1 and Lats2 (Dong et al., 2007; Hao et al., 2008; Ota and Sasaki, 2008; Zhang et al., 2008a; Zhao et al., 2007). We examined localization of Yap in embryos after *Lats2* overexpression. As expected, *Lats2* overexpression greatly reduced nuclear Yap accumulation (Figure 5H). Moreover, Cdx2 levels were also significantly downregulated in these embryos (Figures 5F and 5G), consistent with a requirement for nuclear Yap localization in Cdx2 expression. *Lats2*-injected embryos also failed to form blastocoels after extended culture (data not shown), also characteristic of *Tead4⁻/⁻* embryos (Nishioka et al., 2008), consistent with a loss of Yap/Tead activity in both situations. Importantly,

Lats2 overexpression did not dramatically alter cell number, compared with control embryos, suggesting that the effects of *Lats2* on Yap localization and Cdx2 levels were not an artifact of altered developmental timing (Figures 5F and 5H and data not shown). By contrast, overexpression of a Lats-related kinase, Ndr1 (Stk38), had no effect on Cdx2 expression (Figure 5G), indicating specificity of the *Lats2* overexpression phenotype. Taken together, these observations suggest a model in which *Lats* kinases restrict Yap-dependent Tead4 activity to outside cells of the embryo to restrict Cdx2 expression to the nascent TE.

Given that Yap phosphorylation regulates its localization and activity, we reasoned that a phosphorylation-defective Yap would have enhanced Cdx2-inducing activity. To test this, we overexpressed *Yap-S112A* (Figure 4A). Injection of low doses (5 ng/μl and 10 ng/μl) of *Yap-S112A* RNA led to dose-dependent and significant increases in type III embryos with elevated Cdx2 expression and was more effective than wild-type *Yap* at these doses (Figures 6B and 6C). These observations support the proposal that Lats-mediated changes in Yap phosphorylation can alter the Cdx2-inducing activity of Yap during preimplantation development.

Next, we examined the requirement for Lats in the regulation of Yap localization during preimplantation development. The mouse has two *Lats* genes, both of which are expressed throughout preimplantation development (Figure 3A). Since null mutations in either gene do not disrupt preimplantation development (McPherson et al., 2004; St. John et al., 1999; Yabuta et al., 2007), we overexpressed a catalytically inactive (kinase dead; KD) variant of Lats2, designed to dominantly inhibit both Lats1 and Lats2. As predicted, embryos overexpressing *Lats2-KD* exhibited clearly reduced p-Yap levels (Figure 6D), as well as Yap accumulation in nuclei of inside cells (Figure 6E) and a significant increase in Cdx2 in inside cells (type III embryos) (Figures 6B and 6C). Finally, to genetically explore the role of Lats1/2, we generated a null allele of *Lats1*, and we examined embryos obtained from intercrossing $Lats1^{+/-}$; $Lats2^{+/-}$ mice. Consistent with the analysis of *Lats2-KD* overexpression, $Lats1^{-/-}$; $Lats2^{-/-}$ double mutant embryos exhibited nuclear accumulation of Yap and strong Cdx2 expression in inside cells (n = 6/7) (Figure 6F; Figure S2). Taken together, these observations strongly suggest that Lats1/2 regulate Yap localization during preimplantation development.

Cell-Cell Contact Inhibits Nuclear Accumulation of Yap in Inside Cells

In cultured cells, the subcellular localization of Yap and therefore Tead activity are controlled by cell-cell contacts via the Hippo signaling pathway (Ota and Sasaki, 2008; Zhao et al., 2007). We therefore asked whether cell contact is also involved in the regulation of Yap localization in preimplantation embryos. To examine whether the degree of cell-cell contact can regulate the subcellular localization of Yap in three-dimensional cell aggregates, we first examined its localization in cultured aggregates of a mouse ES cell line, EB5. Yap was detected only in the cytoplasm of cells internal to the aggregates, whereas Yap was detected in the nucleus and cytoplasm of outer cells of aggregates (Figure 6G, left). Similar results were obtained for aggregates of an epithelial cell line, MTD1A (Figure 6G, right). These correlative results suggest that circumferential cell contacts may inhibit the nuclear localization of Yap.

We next disrupted E-cadherin-mediated cell adhesion by using the E-cadherin blocking antibody ECCD1. As reported (Shirayoshi et al., 1983), treatment of compacted 8-cell embryos with ECCD1 led to decompaction. Culture of these embryos led to the reestablishment of cell adhesion, recompaction, and blastocoel formation, although the timing of this latter process was premature, resulting in ICM sizes that were either small or undetectable (Figure 6H; Figure S3; data not shown) (Shirayoshi et al., 1983). As expected based on the severe reduction of ICM in older ECCD1-treated embryos, Yap was not strictly excluded from nuclei of inside cells among ECCD1-treated embryos examined shortly after recompacting (18- to 22-cell stages, n = 6/8) (Figure 6I, middle). In fact, inside cells exhibited levels of nuclear Yap comparable to those of outside cells in some embryos (n = 2/8) (Figure 6I, right). Conversely, p-Yap levels were clearly reduced in inside cells of ECCD1-treated embryos (Figure 6J). Thus, continuous maintenance of circumferential cell-cell contact or adhesion is a prerequisite for the proper regulation of Yap phosphorylation and repression of Yap accumulation in the nuclei of inside cells.

Cell Position Can Regulate Yap Localization and Cell Fate

The observation that the degree of cell contact correlates with Yap localization and activity provided a potential link between embryo topology and cell fate specification. To test this hypothesis, we examined Yap localization and Cdx2 expression after the manipulation of cell position, by using two different approaches. First, we forced cells to occupy an inside position in reaggregated embryos. Embryos were dissociated at the 8-cell stage, a stage at which Yap is nuclear in all cells, and prior to the creation of inside cells. In nondissociated 8-cell embryos, apicobasal cell polarization can be visualized by examining Ezrin, which localizes to the apical cell pole (Louvet-Vallee et al., 2001). However, in individual 1/8 blastomeres, this pattern was lost, as was nuclear Yap (Figure 7A). Next, we aggregated individual blastomeres from three different embryos into one large chimera, with some cells now occupying a position internal to the others. In these reaggregated embryos, outside cells reestablished polarity, and nuclear Yap and Cdx2 were detected. In contrast, neither nuclear Yap nor Cdx2 were detected in inside cells (Figure 7B).

As a second approach to manipulating cell position, we examined the dynamics of Yap localization and Cdx2 expression in the regenerating TE after immunosurgery (Rossant and Lis, 1979; Spindle, 1978). After immunosurgery, most embryos had reestablished a morphologically distinct TE layer and blastocoel by 24 hr (n = 4/7), and nuclear Yap and Cdx2 were detected in outside cells of all regenerates (Figure 7C, n = 7/7). At 12 hr after immunosurgery, some cells appeared to be flattening on the surface of the ICM (n = 5/5), and nuclear Yap and Cdx2 were detected in these cells, although levels were apparently weaker compared with levels seen in later stages of regeneration (Figure 7D, n = 4/5). Together, these observations support the hypothesis that cell position influences the cell fate in preimplantation embryos by regulating subcellular localization of Yap.

DISCUSSION

Tead4 Instructively Regulates Multiple Transcription Factors to Promote Trophoblast Development

Although *Tead4* is required for *Cdx2* expression in vivo, the unpatterned expression of *Tead4* (Nishioka et al., 2008) made it difficult to predict that Tead4 restricts Cdx2 expression to outside cells during TE formation. We have shown that the activity of Tead4 is regulated, and that its activation is sufficient to regulate multiple factors in parallel to promote trophoblast fate specification in the ES cell model (Figure 7E). These observations are consistent with our analysis of *Tead4* mutant embryos (Nishioka et al., 2008), and with the fact that the *Tead4* mutant phenotype is more severe than loss of *Cdx2* alone (Strumpf et al., 2005). Thus, Tead4 appears to act at the top of a hierarchy of trophoblast-specific transcription factors, among which *Cdx2* plays a central role. However, we do not yet know whether Tead4 regulates *Cdx2* directly, since the *Cdx2* trophoblast enhancer has not been identified. Whereas Tead4-Yap activates Cdx2 in the outer cells, the uniform expression of Oct3/4 up to the late blastocyst stage would suggest that *Cdx2* receives persistent suppressive input from Oct3/4 (Dietrich and Hiiragi, 2007; Niwa et al., 2005). Tead4 may, therefore, promote Cdx2
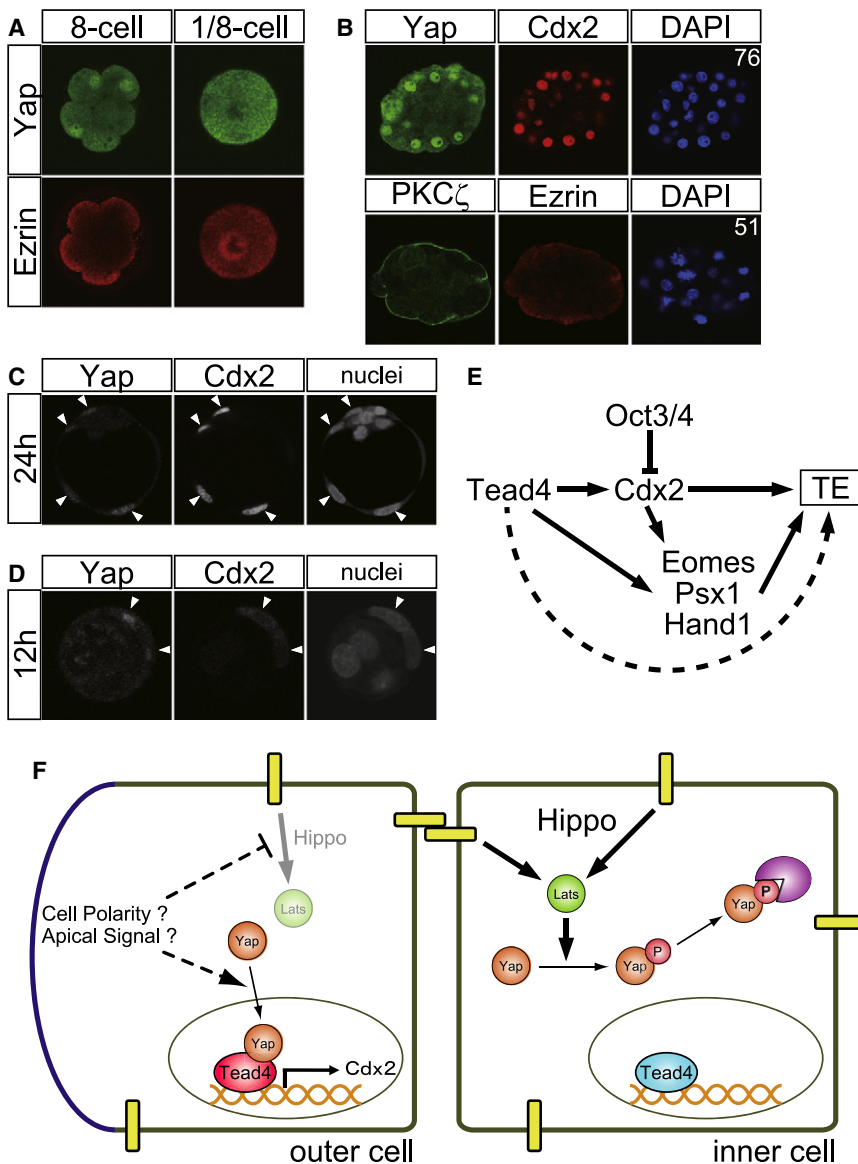
Figure 7. Cell Position Controls Nuclear Localization of Yap

(A) Altered Yap localization and cell polarity (Ezrin) in dissociated blastomeres of the 8-cell embryo.

(B) Localization of Yap, PKCζ, and Ezrin in reaggregated embryos.

(C and D) Yap localization and Cdx2 expression in isolated inner cell masses (C) 24 hr and (D) 12 hr after immunosurgery. Arrowheads indicate Yap-positive nuclei.

(E) A model of the transcriptional network regulating TE development.

(F) A model of cell position-dependent fate specification in preimplantation embryos. See Discussion for details.

Yap is phosphorylated by Lats and is excluded from the nuclei. As a consequence, Tead4 remains inactive, and these cells adopt an ICM fate. In the outside cells, lower levels of Yap phosphorylation allow for its nuclear accumulation, which leads to activation of Tead4. In turn, active Tead4 induces trophoblast genes, including *Cdx2*, and promote TE fate.

Although cell position appears to influence Yap localization, the exact mechanisms underlying this phenomenon remain elusive. One likely mechanism is the difference in the degree of cell-cell contacts. Inside cells are surrounded entirely by outside cells, whereas outside cells have an outside-exposed surface. Thus, the degree of cell contact could influence Lats- and/or Hippo-mediated cell signaling. Cell contact-based changes in Hippo signaling have been proposed to explain cell contact-mediated inhibition of proliferation in cultured cells (Ota and Sasaki, 2008; Zhao et al., 2007), and cell contacts are actually required for the suppression of nuclear Yap in inside cells of preimplantation embryos.

Although our observations generally support the Inside-Outside model of early lineage specification, they do not rule out involvement of the Polarity Model. In addition to cell-cell contact, other information, such as cell polarization or the presence of an exposed apical surface, may also contribute to differential Yap localization in the early embryo, for example by restricting the localization or activity of Hippo signaling components. In support of this, dissociated blastomeres, which do not receive cell contact information and also lose polarity, did not exhibit nuclear Yap. As two transmembrane receptors, Fat and CD44, are known upstream regulators of Hippo signaling (Bennett and Harvey, 2006; Hamaratoglu et al., 2006; Morrison et al., 2001; Silva et al., 2006; Willecke et al., 2006), it is tempting to speculate that signaling through these proteins may transmit cell contact information to Lats/Yap in preimplantation embryos.

expression by overcoming this negative input. Thus, Tead4 instructively regulates multiple transcription factors to promote trophoblast development.

Lats and Yap Convert Positional Information into Cell Fate Information

Two classical models of cell fate specification during preimplantation development are the Inside-Outside Model, in which, topological differences dictate cell fates (Tarkowski and Wroblewska, 1967), and the Polarity Model, wherein differential inheritance of information present along the apicobasal axis dictates both cell position and fate (Johnson and Ziomek, 1981). These models are not mutually exclusive and provide a framework for interpreting our results (Figure 7F). As we have shown, two components of the Hippo signaling pathway, Lats and Yap, are involved in the establishment of position-dependent Tead4 activity and cell fate specification. In inside cells,

Although recently it has been shown that Ras-MAPK signaling promotes TE development (Lu et al., 2008), its relationship to Tead4-Yap remains unknown. Interestingly, MAPK signaling negatively regulates Tead activity in cultured cells (Thompson et al., 2003).

Our model places importance on the suppression of Tead4 activity in inside cells to establish differential Tead4 activity along the inside-outside axis. Active Tead4 induces and/or reinforces *Cdx2* expression, overcoming Oct3/4-mediated repression in outside cells, whereas inactive Tead4 together with Oct3/4 may suppress *Cdx2* expression in inside cells. Inactive Tead4 likely acts as a repressor and suppresses *Cdx2* expression, as switching roles between activator and repressor is a typical feature of transcription factors at the end of signaling pathways (Barolo and Posakony, 2002). Continuous operation of this mechanism throughout preimplantation development likely ensures position-dependent cell fate specification, whereas inside and outside daughters are produced from mothers that are initially outside (Fleming, 1987). This system would confer a degree of developmental flexibility on preimplantation mouse embryos. Recently identified asymmetric distribution of *Cdx2* mRNA (Jedrusik et al., 2008) may also be involved in this process.

A Role for Hippo Signaling in Preimplantation Embryos

The Hippo signaling pathway mediates cell contact-mediated growth inhibition in cultured cells (Lei et al., 2008; Ota and Sasaki, 2008; Zhao et al., 2007, 2008), but our evidence suggests a slightly different role during preimplantation development. Although cell contact is still involved, growth inhibition is not, since changes in Yap localization suppressed Cdx2 expression without affecting cell number. A similar role in cell fate specification has been observed in *Drosophila* photoreceptor differentiation (Mikeladze-Dvali et al., 2005), suggesting that Hippo signaling may regulate distinct cellular outcomes, depending on the context.

EXPERIMENTAL PROCEDURES

Cell Culture

EB5 ES cells were cultured on gelatin-coated dishes in the absence of feeder cells in ES medium (Glasgow modification of Eagle's medium (GMEM) supplemented with 10% (v/v) FCS, 1000 U/ml LIF, 1× sodium pyruvate, 1× nonessential amino acids, 10^{-4} M β-mercaptoethanol) (Niwa et al., 1998) containing 10 μg/ml blasticidin S. 5ECER4 ES cells (Niwa et al., 2005) were cultured in ES medium containing 10 μg/ml blasticidin S and 1 μg/ml puromycin. To establish ES cells stably expressing Tead4VP16ER (5TVER7 and 5TVER16), EB5 ES cells were electroporated with linearized pCAG-Tead4VP16ER-IP and were selected with 1 μg/ml puromycin in ES cell medium containing 10 μg/ml blasticidin S. *Tead4*$^{-/-}$ ES cells and derivatives were maintained in serum-free CultiCell medium (Stem Cell Sciences, Japan) (Ogawa et al., 2004). To establish *Tead4*$^{-/-}$ ES cells stably expressing Cdx2ER (T4CER9 and T4CER10), *Tead4*$^{-/-}$ ES cells (#1–5) (Nishioka et al., 2008) were electroporated with linearized pCAG-Cdx2ER-IP (Niwa et al., 2005) and were selected with 1 μg/ml puromycin in CultiCell serum-free ES medium. To establish *Cdx2*$^{-/-}$ ES cells stably expressing Tead4VP16ER (CTVER5 and CTVER20), dko23-5 cells (Niwa et al., 2005) were electroporated with linearized pCAG-Tead4VP16ER-IP and selected with 1 μg/ml puromycin in ES cell medium containing 10 μg/ml blasticidin S and 200 μg/ml G418. In dko23-5 ES cells, expression of *Oct3/4* does not change during differentiation, because *Oct3/4* is expressed from a transgene (Niwa et al., 2005). To induce transgenes, 5ECER4, T4CER9, and T4CER10 were induced with 1 μg/ml tamoxifen in ES medium. 5TVER7 and 5TVER16 were induced with 0.1 μg/ml tamoxifen, and CTVER5

and CTVER20 were induced with 0.2 μg/ml tamoxifen; higher doses of tamoxifen resulted in significant cell death of 5TVER7, 5TVER16, CTVER5, and CTVER20. Induction of ES cell differentiation into TS cells was performed as previously described (Niwa et al., 2005).

ES cell transfection with siRNA was performed by using Lipofectamine 2000 as previously described (Hough et al., 2006), by using feeder-free conditions. For Oct3/4 knockdown, three predesigned Stealth siRNAs (Stealth Select RNAi) targeting *Oct3/4* (Pou5f1-MSS237605, Pou5f1-MSS237606, and Pou5f1-MSS237607) were obtained from Invitrogen. Pou5f1-MSS237605 and Pou5f1-MSS237606 clearly reduced *Oct3/4*, whereas Pou5f1-MSS237607 exhibited a weaker effect. The two former siRNAs were therefore used and produced similar results. The representative result with Pou5f1-MSS237605 siRNA is shown in a figure. The Stealth RNAi Negative Control Medium GC Duplex (Invitrogen) was used for control experiments.

MTD1A (Hirano et al., 1987), NIH 3T3, and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% Fetal Calf Serum (DMEM + 10% FCS).

For detailed information about plasmids, see Supplemental Data.

Luciferase Assay

NIH 3T3 or HeLa cells were seeded at a density of 1×10^5 cells/well on 12-well plates 24 hr before transfection. A DNA mixture consisting of effector (50 ng), pCMV-Gal4 (BD) or pCMV-Gal4-Tead4C (50 ng), pG4-TK-Luc (200 ng), and pCS2-β-gal (50 ng) were transfected for 24 hr with 2 μl FuGENE HD (Roche). Lysate preparation, luciferase, and β-galactosidase assays were performed as described (Sasaki et al., 1999). Luciferase activities were normalized to β-galactosidase activities. For each experiment, values from two samples were averaged and are presented with standard errors.

RT-PCR

Total RNA was isolated from ES cells or embryos by using Trizol reagent (Invitrogen) by following the manufacturer's instruction. cDNA was prepared from 1 μg total RNA by using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare) or Superscript III reverse transcriptase (Invitrogen) and Oligo-dT primers (Invitrogen) per manufacturers' instructions. cDNA was diluted 1:200 for quantitative PCR reactions. Primers and conditions for Quantitative-PCR (Q-PCR) reactions for *Cdx2*, *Eomes*, *Psx1*, *Hand1*, *Itga7*, *Oct3/4*, *Sox2*, *Fgf4*, and *Gapdh* are described by Niwa et al. (2005). Q-PCR was performed by using SYBR Premix Ex Taq (Takara Bio, Kyoto Japan) and an ABI PRISM 7900HT (Applied Biosystems). Expression of each gene was normalized to the expression of *Gapdh*. Average results and standard errors from three independent measurements are presented.

Mouse Lines

Wild-type litters were obtained by crossing C57BL/6 and [C57BL/6xDBA]F1 mice. *Yap*^{tm1Smil} mice (Morin-Kensicki et al., 2006) were crossed with *Actb:Cre* transgenic mice to remove the neomycin cassette flanked by *loxP* sites. Resulting mice (*Yap*^{Δtm1}) are referred to as *Yap* mutant mice in this paper. *Wwtr1* mutant mice (*Taz*^{lacZ}) were previously described (Makita et al., 2008). *Lats2* mutant mice were previously described (Yabuta et al., 2007). The *Lats1* mutant allele was generated by homologous recombination in ES cells in H.N.'s laboratory. Exon 1 (E1), containing a translation initiation codon, was replaced with a cassette containing the *Pgk* promoter, the *neomycin resistance* gene, and the *Pgk* polyA signal (Figure S4A), resulting in generation of a null allele. Details for the generation and characterization of *Lats1* mutants will be described elsewhere by N.Y. and H.N. Mice were housed in environmentally controlled rooms in the Laboratory Animal Housing Facility of the RIKEN Center for Developmental Biology, under the institutional guidelines for animal and recombinant DNA experiments.

Embryo Culture and Embryo Manipulation

Embryo culture was performed as previously described (Nishioka et al., 2008). Treatment of embryos with ECCD1 (Takara Bio, Kyoto Japan) was performed as previously described (Shirayoshi et al., 1983). Dissociation of 8-cell-stage embryos was performed as previously described (Dietrich and Hiiragi, 2007), and blastomeres were reaggregated by gentle rocking in U-bottom, MPC-coated 96-well plates (Nunc).

RNA Injection

Poly(A)-tailed RNA was synthesized from cDNAs cloned into the pcDNA3.1-poly(A)83 plasmid (Yamagata et al., 2005), and purified RNAs were injected into both blastomeres of 2-cell-stage embryos according to standard protocols (Hogan et al., 1994). Details of plasmids used are provided in Supplemental Data.

Immunofluorescent Staining

Immunofluorescent staining of embryos was performed by following standard protocols. Inside cells were identified by acquiring Z-series confocal images of the stained embryos with LSM510 META (Zeiss). Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature, and then washed in PBS + 0.2% goat serum (PBSS) for 5 min. Embryos were subsequently permeabilized with 0.2% Triton X-100 in PBS for 20 min at room temperature, washed in PBSS for 5 min, blocked with 2% goat serum in PBS (blocking solution), and incubated overnight with primary antibodies diluted in blocking solution at 4°C. After washing in PBSS for 5 min, embryos were incubated with the following secondary antibodies diluted in PBSS for 1 hr at room temperature: Alexa Fluor 488 goat anti-rabbit (Molecular Probes, A11034; 1:4000) and/or Alexa Fluor 594 goat anti-mouse (Molecular Probes, A11005; 1:4000). Nuclei were visualized by staining with 4,6-diamidino-2-phenylindole dilactate (DAPI; Molecular Probes, D3571). For detection of p-Yap, all solutions up to primary antibody were supplemented with Phosphatase Inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan) at 1:100. For λPPase treatment, ∼10 embryos were incubated with 2000 U Lambda Protein Phosphatase (Sigma P9614) in 100 ml λPPase buffer supplied by the manufacturer at 30°C for 1 hr prior to blocking.

Statistics

Statistical analyses were performed with Prism4 statistical software (Graph-Pad) by using Fisher's exact probability test. For all comparisons, experimental results were compared with control results (β-globin-injected). For experiments shown in Figures 2, 4, and 6, the frequencies of type III embryos were compared. For experiments shown in Figure 5, frequencies of type IV embryos were compared. Statistically significant differences (p < 0.05) are indicated by asterisks.

Quantification of Immunofluorescent Signals

Confocal images of the stained embryos were acquired with LSM510 META (Zeiss). Average pixel intensities of the Yap and DAPI in nuclear cross-section were measured by using MetaMorph software (Molecular Devices). Yap nuclear signal values were normalized to the DAPI signal. Average values from multiple embryos are presented with standard deviation.

Immunosurgery

Embryos were harvested around E3.0, zonae pellucida were removed, and embryos were subsequently incubated with nonpreadsorbed rabbit anti-mouse lymphocyte antibody (Cedarlane) diluted 1:8 in KSOM for 25 min in a 37°C incubator. Embryos were washed through five droplets of KSOM and then incubated as described above in anti-rabbit Alexa Fluor 488 (1:400, Molecular Probes) for 15 min. Embryos were again washed in KSOM, and then incubated as described above for 8 min in guinea pig complement (Cedarlane) diluted 1:4 in KSOM, and lysed cells were removed by extensive flushing through a pulled glass needle. Resulting inner cell masses were screened for efficient removal of TE by brief examination of fluorescence signal by fluorescence microscopy. Efficiently lysed ICMs were then incubated in KSOM as described above until the indicated time points, then harvested for immunostaining and confocal analysis as described (Ralston and Rossant, 2008). Antibodies used included rabbit anti-YAP (Cell Signaling; 1:100); mouse anti-Cdx2 (Biogenex; 1:200); anti-mouse Alexa 546, anti-mouse Alexa 488, and Draq5 (Molecular Probes; all at 1:400), and all images were collected during a single confocal session with identical confocal settings.

SUPPLEMENTAL DATA

Supplemental Data include four figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00077-X.

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