Transcription coactivator Cited1 acts as an inducer of trophoblast-like state from mouse embryonic stem cells through the activation of BMP signaling

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Abstract
Trophoblast lineages, precursors of the placenta, are essential for post-implantation embryo survival. However, the regulatory network of trophoblast development remains incompletely understood. Here, we report that Cited1, a transcription coactivator, is a robust inducer for trophoblast-like state from mouse embryonic stem cells (ESCs). Depletion of Cited1 in ESCs compromises the trophoblast lineage specification induced by BMP signaling. In contrast, overexpression of Cited1 in ESCs induces a trophoblast-like state with elevated expression of trophoblast marker genes in vitro and generation of trophoblastic tumors in vivo. Furthermore, global transcriptome profile analysis indicates that ectopic Cited1 activates a trophoblast-like transcriptional program in ESCs. Mechanistically, Cited1 interacts with Bmpr2 and Smad4 to activate the Cited1–Bmpr2–Smad1/5/8 axis in the cytoplasm and Cited1–Smad4–p300 complexes in the nucleus, respectively. Collectively, our results show that Cited1 plays an important role in regulating trophoblast lineage specification through activating the BMP signaling pathway.

Introduction
The specification of extraembryonic trophectoderm (TE) and inner cell mass (ICM) at E3.5 is the first cell fate decision of mammalian development1,2. TE cells give rise to trophoblast lineages, thereafter mediating implantation and generating the functional placenta3. Given the indispensable role of the trophoblast for embryo development, a great deal of effort has been made to unravel the regulatory networks of trophoblast development.

Embryonic stem cells (ESCs) and trophoblast stem cells (TSCs), which are derivatives of ICM and TE respectively, retain the capacity to self-renew indefinitely and model their counterparts in vivo functionally4–6. ESCs are generally considered to have a weak ability to generate trophoblast lineages spontaneously due to their ICM origin7. Nonetheless, it was found that mouse ESCs can become trophoblast-like cells by forced expression of key trophoblast-associated factors such as Cdx28,9, Gata310, Arid3a11,12, or Brog513. Moreover, depletion of Oct4, an ESC core transcription factor14, leads to the commitment of ESCs into trophoblast-like cells15,16. The similar phenotype was observed in Tet1-depleted ESCs17. In addition, signaling pathways contribute to the ESC fate determination. BMP4 signaling was reported to enable ESCs to...
become trophoblast-like cells robustly. BMP and Activin/Nodal/TGF-β signaling pathways are two branches of the TGF-β superfamily, which use different sets of receptors and Smad transducers (Smad1/5/8 for BMP signaling and Smad2/3 for TGF-β signaling). These two branches often antagonize each other due to the competition for the common Smad4. BMP signaling also plays crucial roles for trophoblast and placenta development. However, the intracellular factors that can regulate its activity, thereby controlling trophoblast differentiation, remain to be discovered.

Analysis of published transcriptional datasets led us to identify Cited1 as a robust inducer of trophoblast-like state from mouse ESCs. Cited1 (Cbp/p300 Interacting Transactivator with Glu/Asp Rich Carboxy-Terminal Domain 1), formerly known as Msg132, was reported to interact with Smad4, estrogen receptor alpha and beta, and Cbp/p300. In addition, Cited1 could positively regulate TGF-β and BMP signaling through its association with the Smad/p300/Cbp-mediated transcriptional complex in melanoma and metanephros cells. During development, Cited1 plays a role in cell growth, pigmentation of melanocytes, early nephronic patterning and tumorigenesis. It also contributes to the terminal maturation of trophoblast subtypes and the organization of placenta structure in the mouse. Nonetheless, the precise role of Cited1 in the early stage of trophoblast development remains elusive.

Here, we investigate the role of Cited1 in ESC fate determination. Depletion of Cited1 dramatically compromises the capacity of ESCs to become trophoblast-like cells induced by BMP4. In contrast, ectopic Cited1 expression induces ESC trans-differentiation into trophoblast-like cells under the self-renewal culture condition and trophoblastic tumors with internal hemorrhage in vivo. Global transcriptional analysis shows that ectopic Cited1 expression initiates a trophoblast-like transcriptional program in ESCs. Mechanistically, Cited1 can associate with Bmp2 in the cytoplasm to enhance the phosphorylation of Smad1/5/8 and with Smad4 in the nucleus to enhance its transcriptional activity, respectively. Therefore, Cited1 could trigger a transition of ESCs from a self-renewal state to a trophoblast-like fate through activating the BMP signaling pathway.

Results
Cited1 is highly expressed in trophoblast lineages in vitro and in the trophectoderm of early mouse embryos
To identify transcription-related factors involved in the early TE formation during mouse embryonic development, we analyzed published microarray data of ESCs, TSCs, and TSC-like cells derived by Oct4 knockdown (KD) in ESCs. We compared 3 sets of genes, including top 100 genes highly expressed in TSCs versus ESCs, top 1% of upregulated genes upon Oct4 KD in ESCs and 1502 transcription-associated factors from a commercial library (Table S1) and found that 8 genes were shared by all 3 gene sets. They were Cited1, Irf3, Mxd2 and known TE lineage markers Elf5, Cdx2, Gata3, Gata2, and Id2 (Fig. 1a). Cited1 was chosen for further investigation, since its knockout (KO) mice showed placenta defects and its function in ESC fate determination remained unclear.

We began with examining the expression pattern of Cited1 and found that its transcript and protein levels were significantly higher in TSCs than in ESCs, having a similar pattern to known trophoblast markers (Cdx2, Gomes, or Elf5) (Fig. 1b, c). In contrast, Oct4 was only found in ESCs. We further determined Cited1 expression profile in two trophoblast induction models. First, Oct4 was depleted in the ZHBTc4 mouse ESC line, in which the endogenous Oct4 loci were replaced with tetracycline-regulated Oct4 transgene. Cited1 expression was induced by Oct4 KD, together with upregulation of known trophoblast markers, such as Cdx2, Gata3, and Hand1 (Fig. 1d, e). Second, Cited1 expression was examined during BMP4-induced trophoblast differentiation from ESCs. With the addition of BMP4, ESC colonies were gradually converted to flattened epithelia with a cobblestone appearance at day 6 (Fig. 1f). The effective trophoblast induction was further indicated by increased expression of trophoblast markers (Fig. 1g). Krt7, a pan- trophoblast marker, was expressed in the majority of BMP4-induced cells (Figures S1A-S1C). Moreover, teratomas generated from BMP4-treated cells contained typical structures of trophoblastic hemorrhagic lesions with large amounts of trophoblastic giant cells surrounding blood-filled lacunas (Figure S1D). Importantly, Cited1 was also significantly upregulated by BMP4 treatment (Fig. 1g, h). Therefore, results from both models suggest the close association of Cited1 with trophoblast lineage induction.

Additionally, we found that the expression level of Cited1 increased gradually during TSC differentiation induced by FGF4 withdrawal, in a manner similar to pan-trophoblast markers (Dlx3, Krt7, and Ptx1) but different from TSC markers (Elf5, Cdx2 and Gomes) (Figure S2A). The finding indicates that Cited1 might also function in differentiated trophoblast cells.

At last, we determined the expression of Cited1 in early mouse embryos from the 2-cell to blastocyst stage by immunofluorescence staining (Fig. 1i). Cited1 was detectable in all these stages and primarily localized in the cytoplasm. Interestingly, Cited1 was mostly found in the outer layer cells, if any in the inner cells, of embryos at 8-cell and morula stages. At the blastocyst stage,
Fig. 1 (See legend on next page.)
Cited1 was expressed with a higher level in TE cells than in ICm cells. As controls, Cdx2 was found in the nucleus of TE cells in blastocysts, while Oct4 and Cnot3 (a known cytoplasmic protein) were mainly detected in the nucleus and cytoplasm of ICm cells, respectively (Fig. 1i and S2B). Taken together, Cited1 is highly expressed in the cells of trophoblast lineages both in vitro and in vivo, suggesting its potential role for trophoblast development.

Cited1 depletion compromises BMP4-induced trophoblast differentiation

To test the function of Cited1 in trophoblast induction from ESCs, we generated Cited1 KO ESCs using the CRISPR/Cas9 system. A construct containing sequences of Cas9, a puromycin-resistant cassette and a gRNA targeting the third exon of Cited1 was transfected into ESCs (Fig. 2a). Six resistant colonies were picked and validated by genomic DNA PCR and Sanger sequencing. Biallelic deletion of Cited1 was verified in all six clones, four of which contained frame-shift mutations (KO #1, KO #2, KO #4) or large fragment deletion (KO #3) (Fig. 2b and S3). Western blotting analysis further validated deletion of Cited1 in these clones (Fig. 2c), which were then utilized to determine whether Cited1 would play a role in BMP4-induced trophoblast differentiation.

Wild-type (WT) ESCs displayed typical trophoblast-like morphology at day 6 of BMP4 treatment, with significant upregulation of trophoblast genes (Hand1, Gata3, Cdx2, Krt7, Krt18, Pdx1, Dlx3, Esx1, and Mash2) and down-regulation of pluripotency genes (Oct4, Sox2, Nanog, and Klf4). However, Cited1 KO compromised the trophoblast conversion. BMP4-induced cobblestone-like morphology in WT ESCs was not observed in Cited1 KO cells (Fig. 2d). At molecular levels, dramatically elevated expression of trophoblast marker genes in BMP-treated cells was significantly reduced by Cited1 deletion (Fig. 2e). Of note, Cited1 depletion attenuated the BMP4-induced downregulation of Oct4 and Nanog, whereas Sox2 and Klf4 remained down (Fig. 2f). The phenomenon suggests that Cited1 could be only involved in the regulation of a subset of pluripotency markers during this differentiation process. These results indicate an indispensable role of Cited1 in trophoblast differentiation.

**Forced expression of Cited1 activates trophoblast gene expression**

We then asked whether forced expression of Cited1 would be able to induce ESCs to trans-differentiate into a trophoblast state. To test the possibility, Cited1 was overexpressed in ESCs under a self-renewal condition with serum and leukemia inhibitory factor (LIF) (Figure S4A). Two days after transfection, the majority of Cited1-transfected cells lost the doomed morphology with negative or significantly reduced intensities of alkaline phosphatase (AKP) staining, an indicator of the undifferentiated state (Fig. 3a). These cells had significantly higher levels of trophoblast genes such as Gata3, Hand1, Elf5, Cdx2, Krt7, Krt18, Pdx1, Esx1, Dlx3, Eomes, Mash2, and Rhox9 (Fig. 3b, upper panel). As positive controls, Cdx2 and Gata3, which are known to efficiently induce trophoblast differentiation, were also overexpressed (Fig. 3b, middle and bottom panels). The expression of trophoblast markers was comparable between Cited1-overexpressing cells and positive control cells. In contrast, the expression of three germ layer (ectoderm, mesoderm and endoderm)-associated genes was only slightly or not altered (Fig. 3c), suggesting that Cited1 specifically activated the trophoblast lineage markers. Moreover, pluripotency-associated markers decreased mildly in Cited1-transfected cells (Fig. 3d), implicating that Cited1 might disrupt ESC self-renewal through the direct activation of trophoblast genes rather than repression of pluripotency genes. Additionally, Cited1-transfected cells had significantly enhanced cell growth rates (Figure S4B), in line with previous reports in Wilms’ tumor and intestinal tumor cells. To exclude the possibility that the phenotype caused by Cited1 overexpression was ESC
Fig. 2 (See legend on next page.)
line dependent, same experiments were performed in another mouse ESC line (CGR8 ESC line) and consistent results were obtained (Figures S4C-S4E). Furthermore, we characterized the Cited1-overexpressing cells with a prolonged culture up to day 6 and determined the expression of pluripotency and lineage markers. Among the trophoblast markers tested, the majority solely mark trophoblast lineages, including Elf5, Pdx1, Ess1, Dlx3, Mash2, and Rhox9, while some markers are known to also express in mesendodermal cells, such as Gata3, Hand1, and Cdx2. To test the possibility of mesendoderm induction by Cited1 overexpression, we examined the expression of additional mesendoderm markers (Lhx1, Cxcr4, Nodal, Runx2, Gsc, Pitx2, Fgf8, and Wnt3) besides T and Mixl1. Our result revealed that Cited1 overexpression gradually and robustly activated trophoblast marker expression with only mild or moderate alterations in three germ layer-associated genes (Figures S4F and Fig. 3e). Immunofluorescence staining results also indicated decreased and enhanced levels of Oct4 and pan-trophoblast marker Krt7, respectively, at day 6 after Cited1 transfection (Fig. 3f, g), while the key mesodermal marker (T) was not induced at all (Figure S4G). In particular, FACS analysis revealed high percentages of Krt7+ cells (about 56%) in Cited1-overexpressing cells (Fig. 3h, i). Together, forced Cited1 expression activated trophoblast genes and induced ESCs to exit from a self-renewal state. We next tested whether Cited1 overexpression could cause similar phenotypes in the absence of LIF, a differentiation condition. Similar results were obtained (Figures S5A-S5E). To evaluate the ability of Cited1 to control ESC fate determination at a genome-wide scale, we analyzed the global transcriptional changes caused by Cited1 overexpression in ESCs. Cell morphology and marker gene expression revealed that deletion of the CR2 region from the full length or presence of CR2 region alone abolished the capacity of Cited1 to activate trophoblast genes (Figures S5G and S5H), suggesting that the CR2 region was indispensable for Cited1 to execute its function in ESCs, although the region alone was not sufficient to be functional.

**Teratomas derived from Cited1-overexpressing ESCs exhibit trophoblastic features**

To test the differentiation potential of Cited1-transfected cells in vivo, we injected the cells subcutaneously into NOD/SCID mice for teratoma formation. Cells transfected with an empty vector were used as a control. Teratomas generated from Cited1-overexpressing cells (referred to as to Cited1 OE teratomas) were aggressive, being about 4-fold bigger and heavier than those from control ESCs (Fig. 4a, b). Histological analysis of teratoma sections showed that cell types from all three germ layers could be identified in both groups of teratomas (Fig. 4c). However, only Cited1 OE teratomas contained evident and numerous internal hemorrhage foci (Fig. 4a, d), with 92 out of 100 randomly observed sections containing such bleeding regions. Moreover, large numbers of clustered or scattered giant cells with a characteristic of the large cytoplasm and nuclei could be easily observed in these regions (Fig. 4e, left image). Multinucleated trophoblast cells could also be found (Fig. 4e, right image). In addition, immunohistochemical analysis with antibodies against Placental lactogen 1, a specific marker of giant cells, further validated the presence of giant cells (Fig. 4f, left image). Therefore, Cited1-transfected ESCs were able to generate trophoblastic teratomas in vivo.

**Overexpression of Cited1 induces a trophoblast transcriptional program**

To evaluate the ability of Cited1 to control ESC fate determination at a genome-wide scale, we analyzed the global transcriptional changes caused by Cited1 overexpression in ESCs. Cells were collected at day 1 and day 2 after transfection and genome-wide transcript profiles
Fig. 3 (See legend on next page.)
were determined by Affymetrix microarray analysis (Fig. 5a). With a cutoff threshold of two-fold and p value < 0.05, 40 and 696 differentially expressed genes (DEGs) were identified at day 1 and day 2, respectively (Fig. 5b; Table S2). Twelve DEGs were randomly selected for qRT-PCR validation (Fig. 5c). Top 60 DEGs induced by Cited1 overexpression was further analyzed by GO (Gene Ontology). GO terms related to embryonic organ development and embryonic placenta development were enriched (Fig. 5d), in accordance with the phenotype caused by Cited1 overexpression in vitro and in vivo.

Several studies have reported that overexpression of key trophoblast genes (Cdx2 or Gata3) could induce mouse ESCs to become trophoblast-like cells. To know whether Cited1 overexpression would induce similar transcriptional programs, we compared the global gene expression profiles of Cited1-overexpressing cells with previously published microarray data from Cdx2- and Gata3-overexpressing cells. Interestingly, 60.1% (462 out of 696) of the DEGs induced by Cited1 overexpression were overlapped with the DEGs induced by either Cdx2 or Gata3 (Fig. 5e), suggesting that Cited1-overexpressing cells gained similar molecular features to trophoblast-like cells induced by overexpressing Cdx2 or Gata3.

We next performed gene set enrichment analysis (GSEA) to compare our microarray data of Cited1-overexpressing cells with the following gene sets: top 1% of upregulated Cdx2 OE-specific genes (368 genes), Gata3 OE-specific genes (384 genes) or Oct4 KD-specific genes (204 genes) or TSC-specific genes (TSCs vs. ESCs, 313 genes), and ESC-specific genes (ESCs vs. differentiated ESCs, 218 genes). The analysis revealed that Cited1 OE-specific DEGs were strongly positively correlated with the TSC-specific gene sets and the gene profiles of trophoblast-like cells generated by Cdx2 OE or Gata3 OE, or Oct4 KD (Fig. 5f). In contrast, these Cited1 OE-specific DEGs displayed a poor correlation with ESC-specific gene sets. Taken together, the analysis indicates that ectopic expression of Cited1 in ESCs can result in the loss of ESC identity and evoke an overall startup of trophoblast-like gene expression program.

Cited1 interacts with Bmpr2 to activate BMP signaling and induce trophoblast trans-differentiation

We were interested in how Cited1 activated trophoblast-like transcriptional program. Cited1 was reported to bind nuclear Smads to enhance their transcriptional activity and hyperactivation of BMP4-Smad1/5/8 signaling was known to induce trophoblast differentiation in ESCs. Therefore, we examined whether the phenotype induced by Cited1 overexpression would be associated with the activation of BMP-Smad1/5/8 signaling. We first evaluated levels of phosphorylated Smad1/5 (pSmad1/5) and Smad2 (pSmad2) in Cited1-overexpressing cells. Levels of pSmad1/5 were dramatically elevated 1 day after Cited1 overexpression (Fig. 6a), indicating that Cited1 activated BMP-Smad1/5 signaling. Meanwhile, the levels of pSmad2 slightly decreased, hinting at a potential involvement of Cited1 in the regulation of Nodal/Activin signaling. To verify whether the activation of BMP-Smad1/5 signaling was responsible for Cited1-induced trophoblast-like trans-differentiation, BMP signaling inhibitors, including Noggin (a ligand antagonist), LDN193189 and K02288 (inhibitors targeting type I receptors), were respectively added to the culture media 6 h after Cited1 transfection. SB431542, a TGF-β signaling inhibitor, was used as a negative control. The effectiveness of inhibitors was verified by western blotting (Fig. 6b). Results from both cell morphology and marker gene expression analysis at day 3 indicated that Noggin or K02288, or LDN193189 treatment significantly compromised trophoblast trans-differentiation induced by Cited1 overexpression (Fig. 6c, d, left panel). In contrast, SB431542 enhanced the expression of trophoblast markers induced by Cited1 overexpression (Fig. 6d, right panel), which was in line with the reports that SB431542 promotes human ESC differentiation into trophoblast and that Nodal/activin signaling regulates BMP signaling.
Fig. 4 (See legend on next page.)
in mouse ESCs\textsuperscript{45,46}. The results were reproducible in another mouse ESC line (Figure S6). Therefore, activation of BMP signaling might play a major role for Cited1 to induce trophoblast-like trans-differentiation.

To explore how Cited1 activated BMP signaling, we determined the transcript levels of BMP signaling ligands in Cited1-overexpressing cells at day 3 and found that most ligands tested had comparable levels to those in control cells (Figure 6e). Since ectopic Cited1 mainly located in the cytoplasm (Figure S7A), we examined if Cited1 could bind BMP receptors. Co-immunoprecipitation (Co-IP) assays showed that ectopic Cited1 interacted with endogenous Bmpr2 in ESCs (Figure 6f), suggesting Cited1 might activate BMP-Smad1/5 signaling via its interaction with Bmpr2. To determine the role of Bmpr2 for Cited1's function, two Bmpr2 KO ESC lines were generated by the CRISPR/Cas9 system (Figure S7B). Cited1 overexpression could not activate trophoblast marker expression in both lines of Bmpr2 KO ESCs (Figure 6g), indicating that Bmpr2 might be a major mediator of Cited1 to activate trophoblast-associated gene expression, although it is not clear how Cited1 modulates the function of Bmpr2.

Cited1 links to Smad4 and p300 to enhance the transcriptional activity of BMP4 signaling effectors

The observations that the level of pSmad1/5 was only moderately reduced in Cited1 KO cells after BMP4 treatment (Figure 7a) and that a small proportion of Cited1 located in the nucleus (Figure S7A) prompted us to consider whether Cited1 would have a nuclear function in addition to its interaction with Bmpr2. As Cited1 was previously reported to bind Smad4 and could form complexes with Smad1/5/8 and p300/Cbp to enhance the activity of BMP signaling\textsuperscript{34,47}, we speculated that Cited1 might also positively regulate BMP4 signaling through associating with Smads. To test the possibility, Co-IP assays were performed in ESCs. Indeed, Flag-Cited1 and endogenous Smad4 formed complexes (Figure 7b). As Cited1 is a coactivator of the p300/Cbp-mediated transcription, we further tested whether the induction of trophoblast genes by Cited1 overexpression was dependent on p300. Expression of p300 was silenced by three sets of shRNAs specifically targeting different coding sequences (shRNA#5, #6, #8), respectively. KD of p300 abolished the activation of trophoblast genes induced by Cited1 overexpression (Figure 7c), revealing the dependence of Cited1 on p300 for induction of trophoblast gene expression. Taken together, we propose that Cited1 induces a trophoblast transcriptional signature in ESCs through interacting with Bmpr2, Smad4, and p300 to activate BMP signaling (Figure 7d).

Discussion

In this study, we report that transcription coactivator Cited1 can induce mouse ESCs into a trophoblast-like state through activation of the BMP signaling pathway. Interestingly, after Cited1 overexpression, trophoblast-related genes were massively upregulated whereas pluripotency genes were only slightly downregulated, indicating that ectopic Cited1 could directly activate a trophoblast-like transcriptional signature. The down-regulation of pluripotency genes could be a secondary event.

Although some markers (such as Gata3, Hand1, and Cdx2) induced by Cited1 have been implicated in both mesendoderm and trophoblast differentiation, our results showed that Cited1 activated trophoblast genes more robustly than mesendoderm sole genes (Figures 3b, e and S4f). Moreover, Cited1 overexpression did not induce expression of mesendoderm master gene T (Figure S4G). Combined with the phenotype of typical trophoblastic tumors generated from Cited1-overexpressing cells (Figure 4) and a genome-wide trophoblast-like gene expression program evoked by ectopic Cited1 (Figure 5), we conclude that Cited1 induces trophoblast-like differentiation from ESCs under a self-renewal condition. However, we do not exclude the possibility that Cited1 might also function in the mesendoderm development under certain contexts.

BMP4 is an import signal to trigger trophoblast differentiation. However, the intracellular factors that can directly activate the BMP pathway remain to be discovered. Our study uncovers that Cited1 associates with and functionally depends on Bmpr2 to activate Smad1/5, the effectors of BMP signaling. Furthermore, our results reveal that Cited1 forms protein complexes with Smad4.
Fig. 5 (See legend on next page.)
and requires p300 to activate trophoblast gene expression, in a similar way to its activation of the TGF-β or BMP pathway in other cell contexts reported previously. Taken together, this study shows that Cited1 directly activates the BMP pathway to induce the conversion of ESCs into trophoblast-like cells via two complementary mechanisms in the cytoplasm and nucleus, respectively.

**Experimental procedures**

**Cell culture**

Mouse E14T, CGR8, and ZHBTc4 ESCs (kind gifts of Dr. Austin Smith from the University of Cambridge) were cultured on gelatin-coated plates in the ESC medium consisting of GMEM (Glasgow’s minimum essential medium; Invitrogen, cat 11710–035) supplemented with 10% fetal bovine serum (FBS; Gibco), 1 mM sodium pyruvate (Gibco), 0.1 mM non-essential amino acids (NEAA; Gibco), 2 mM l-glutamine (Gibco), 100 μM β-mercaptopoethanol (Sigma), 100 U/mL penicillin and 100 μg/mL streptomycin (Hyclone), and 1000 U/mL recombiant leukemia inhibitory factor (LIF; Millipore). Mouse TSCs (a kind gift of Dr. Shaorong Gao from Tongji University) were cultured as described previously.

**Trophoblast Trans-differentiation Induced by BMP4**

For trophoblast induction by BMP4, mouse ESCs were plated in Matrigel-coated six-well plates at a density of 1.0 × 10⁵ cells per well in a trophoblast induction medium for 6 days. The trophoblast induction medium contains KnockOut DMEM/F-12 (Invitrogen, cat 12660–012), 64 μg/mL l-ascorbic acid-2-phosphate magnesium (ACC), insulin-transferrin-selenium (Life Technologies), 543 μg/mL NaHCO₃ (Sigma), 1 μg/mL heparin (Sigma), 2 mM l-glutamine (Life Technologies), 100 U/mL penicillin and 100 μg/mL streptomycin (Hyclone), and 10 ng/mL BMP4 (HUMANZYME).

**Inhibitors and antibodies used in western blot analysis**

Western blotting was carried out with following primary antibodies: Cited1 (made in our lab or Genetex, GTX114559), Oct4 (made in our lab), Sox2 (made in our lab), phospho-Smad1/5 (Cell Signaling Technology, #9516), phospho-Smad2 (Cell Signaling Technology, #3101), total Smad5 (Cell Signaling Technology, #9517), total Smad2/3 (Cell Signaling Technology, #3102), Smad4 (Proteintech, 10231–1-AP), Noggin (Abcam, ab96826), Elf5 (Santa Cruz, sc-9645), T (R&D, AF2085), Placental lactogen 1 (Santa Cruz, sc-376436), Flag (Abmart, M20018F), β-Actin (HuaBio, M1210–2) and α-Tubulin (Sigma, T5168). The inhibitors were purchased from STEMCELL (SB431542: #72232), Selleckchem (LDN193189: S2618, K02288: S7359; protease inhibitor cocktail: B14002; phosphatases inhibitor: B15002) and PeproTech (Noggin: #120–10 C), respectively.

**Mouse embryo collection and immunofluorescence analysis**

Mouse embryo collection and immunofluorescence were carried out as described previously. Briefly, pre-implantation embryos were flushed out from the oviducts or uterus of pregnant female mice and were subsequently fixed in 4% paraformaldehyde. Immunofluorescence staining was performed using primary antibodies against Cdx2 (Biogenex, MU392A-UC), Oct4 (Santa Cruz, sc-101534) or Cited1 (Genetex, GTX114559), following standard protocols for whole embryo staining. Image data were acquired with a Zeiss LSM 780 microscope. The cell immunofluorescence staining was performed with following steps: ESCs on coverslips were firstly fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2 % Triton X-100 for 10 min, and blocked with 3% bovine serum albumin (BSA) for 30 min. Next, cells were incubated with the primary anti-Cdx2 antibody (1:200 diluted), or anti-Elf5 antibody (1:200 diluted), or anti-Krt7 antibody (1:200 diluted), overnight at 4 °C. The next day, cells were incubated with the fluorescent Cy3-goat anti-mouse antibody (1:200 diluted), or FITC-goat anti-mouse antibody (1:200 diluted), or Cy3-rabbit anti-goat antibody (1:200 diluted), for 1 h at room temperature in the dark, respectively. The nuclei were further stained with 4′,6-diamindino-2-phenylindole (DAPI; Invitrogen) for 3 min. Finally, coverslips were dried and affixed to slides using a fluorescent mounting medium.
Fig. 6 (See legend on next page.)
Immunofluorescence

Immunofluorescence was performed as described by the laboratory of Dr. Janet Rossant (http://lab.research.sickkids.ca/rossant/labresources/). An antibody was used against Placental lactogen 1 (sc-376436; 1:50 dilution).

Mesoderm induction

For mesoderm induction by BMP4 and CHIR99021, mouse ESCs were plated in gelatin-coated six-well plates at a density of 1.5 × 10^4 cells per well in a mesoderm induction medium for 3 days. The mesoderm induction medium contains N2B27 medium supplemented with 1% knock-out serum replacement (KOSR, Gibco), 0.1% bovine serum albumin (Gibco) and BMP4 at 10 ng/mL (HUMANZYMEn) for 2 days. Cells were then changed to a DMEM-based medium, supplemented with 15% KOSR, 0.5% DMSO (Sigma) and CHIR99021 (Stem cell) at 1 μM.  

Flow cytometry analysis

Cell cultures were dispersed as single-cell suspensions by 0.25% trypsin containing EDTA (Invitrogen). After fixation in a 4% paraformaldehyde/PBS solution for 15 min and permeabilization in 1.0% Triton X-100/PBS for 15 min, nonspecific binding was reduced by exposure to 5% donkey serum in 0.2% Triton X-100/PBS for 30 min at room temperature. The cells were incubated with primary anti-Krt7 (Santa Cruz, sc-53263) antibody (1:25 diluted) for 1 h at room temperature, followed by incubation with FITC-donkey anti-mouse antibody (1:200 diluted) for 30 min at room temperature. Between all steps, cells were washed three times with a PBS buffer. All procedures were carried out in the dark at room temperature. Cells were analyzed on a Beckman Coulter Gallios flow cytometer system. Final data were prepared in FlowJo (version 10) software. All flow cytometry analyses were carried out three times and representative results are shown.

Plasmids and transfection

The coding sequences of full-length Cited1 [NCBI Reference Sequence: NM_001276474.1] and its truncations were amplified using Hi-Fi KOD polymerase (Takara) from complementary DNA (cDNA) of E14T mouse ESCs. Amplified fragments were subcloned into the pPyCAGIP vector (a kind gift of Dr. Ian Chambers from University of Edinburgh). Three short-hairpin RNAs (shRNAs) targeting p300 mRNA were synthesized (by Shanghai Shengong) and subcloned into pLKO.1-puro (Addgene) vector. Primers used for gene cloning and shRNA targeting sequences are listed in Table S3. For transfection, ESCs were plated in six-well plates at a density of 0.2 million cells per well 1 day before transfection. The transfection was performed using Lipofectamine™2000 (Invitrogen) mixed with appropriate plasmids according to the manufacturer’s instruction.

RNA isolation, reverse transcription and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using the TRIzol Reagent (Invitrogen) and was reverse-transcribed to cDNA by the ReverTra Ace (Toyobo, TRT-101). The real-time qPCR was performed using the SYBR® Premix Ex Taq™ II (Takara) mixed with appropriate primers on a ViiA 7 real-time PCR system (Life Technologies). Gapdh was used as an internal control to normalize the relative expression level of each gene.

Teratomas

For Cited1 experiment, ESCs were transplanted with the Cited1 expression plasmid or an empty pPyCAGIP vector. One day later, the cells were selected with 1 μg/mL puromycin (Sigma) for additional 2 days and then harvested. Subsequently, 1.5 million transplanted cells were subcutaneously injected into NOD/SCID mice. Teratomas were collected 4 weeks later for histological analysis. For trophoblast induction experiment, ESCs were cultured in a trophoblast induction medium for 6 days to transdifferentiate into trophoblast-like cells. Then 1.5 million cells were subcutaneously injected into NOD/SCID mice. Teratomas were collected 6 weeks later for histological analysis.
Fig. 7 (See legend on next page.)
Western blot analysis and immunoprecipitation (IP)

Collected cells were washed twice with PBS and lysed in the Co-IP buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 2 mM EDTA, 0.5% NP-40, and protease inhibitors). Protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher) according to the manufacturer’s instructions. Total proteins were separated by SDS-PAGE gels and transferred to nitrocellulose membranes (GE Healthcare). Membranes were incubated with specific primary antibodies, and the antibody-protein complexes were detected by HRP-conjugated secondary antibodies (Jackson) and Pierce ECL Western Blotting Substrate (Thermo Fisher). All western blot analyses were carried out at least three times and representative results are shown. The IP assays were conducted as previously described20. Anti-Flag M2 magnetic beads (Sigma, M8823) were added to the cell lysate for incubation overnight.

Alkaline phosphatase (AKP) staining assays

AKP staining assays were performed according to the manufacturer’s instructions (VECTOR Blue Alkaline Phosphatase Substrate Kit, SK-5300, Vector Lab).

Genome editing with CRISPR-Cas9 approach

The sgRNAs targeting Cited1 and Bmpr2 locus were designed using the CRISPR Design Tool (http://crispr.mit.edu/) and synthesized by the Shanghai Shenggong Company. Annealed sgRNA oligos were inserted into the pSpCas9(BB)-2A-Puro (PX459) (Addgene Plasmid #48139). E14T ESCs were plated onto gelatin-coated six-well plates to grow for 1 day, and then were transfected with sgRNA-PX459 plasmids targeting Cited1 and Bmpr2 using Lipofectamine™ 2000, respectively. One day after transfection, cells were selected by 1.5 μg/mL puromycin for 2 days. Then puromycin-resistant ESCs were replated at the density of 10 thousand cells per 10-cm dish and cultured for 6 days without selection. Colonies were picked up and verified by PCR and Sanger sequencing individually. The sgRNA sequences targeting Cited1 or Bmpr2 are included in Table S3.

Microarray analysis

E14T ESCs were transiently transfected with a Cited1 expression construct or an empty pPyCAGIP vector. One day later, transfected cells were selected in 1 μg/mL puromycin. Independent biological triplicates of either Cited1 construct-transfected cells or control construct-transfected cells at day 1 and day 2 were collected for RNA isolation with Trizol. Microarray analysis was carried out with the Affymetrix GeneChip® Mouse Genome 430 2.0 Arrays. The whole procedures, including RNA extraction, cDNA synthesis, labeling, hybridization, washing and scanning, were conducted according to the standard Affymetrix protocol by the Shanghai Biotechnology Corporation. Differentially expressed genes (DEGs) with a fold change > 2 were selected for further analysis. The selected genes were grouped in functional categories based on Gene Ontology database (GO: http://www.geneontology.org/), and gene set enrichment analysis (GSEA) were also made.12 The microarray data from this publication have been submitted to the GEO database with an access number of GSE103414.

Statistical analysis

All qRT-PCR data are presented as the mean ± SD of three independent experiments and analyzed by the unpaired Student’s t-test. p < 0.05 was considered statistically significant. *p < 0.05, **p < 0.01, ***p < 0.001.
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Y.X., XL, and HL, conception and design, collection and/or assembly of data, data analysis, and interpretation, manuscript writing; Z.F., X.Z., Y.Z., C.Z., J.G., F.T., Y.H., collection and/or assembly of data; Y.J., GH, conception and design; Y.J., HL, editing, approval, and submission of the manuscript. Y.X. and XL contributed equally.

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