The transcription factor TFCP2L1 induces expression of distinct target genes and promotes self-renewal of mouse and human embryonic stem cells

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Xiaohu Wang[‡1], **Xiaoxiao Wang**[§1], **Shuyuan Zhang**[‡1], **Hongwei Sun**[‡1], **Sijia Li**[‡], **Huiwen Ding**[‡], **Yu You**[‡¶], **Xuewu Zhang**[‖], **and Shou-Dong Ye**[‡¶2]

From the [‡]*Center for Stem Cell and Translational Medicine, School of Life Sciences and the* [¶]*Institute of Physical Science and Information Technology, Anhui University, Hefei 230601, China, the* [§]*Department of Anesthesiology, Anhui Provincial Hospital, First Affiliated Hospital of University of Science and Technology of China, Hefei 230001, China, and the* [‖]*Department of Hematology, Institute of Hematology, First Affiliated Hospital of Zhejiang University, Hangzhou 310003, China*

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TFCP2L1 (transcription factor CP2-like 1) is a transcriptional regulator critical for maintaining mouse and human embryonic stem cell (ESC) pluripotency. However, the direct TFCP2L1 target genes are uncharacterized. Here, using gene overexpression, immunoblotting, quantitative real-time PCR, ChIP, and reporter gene assays, we show that TFCP2L1 primarily induces estrogen-related receptor *β* (Esrrb) expression that supports mouse ESC identity and also selectively enhances Kruppel-like factor 4 (Klf4) expression and thereby promotes human ESC self-renewal. Specifically, we found that in mouse ESCs, TFCP2L1 binds directly to the Esrrb gene promoter and regulates its transcription. Esrrb knockdown impaired Tfcp2l1's ability to induce interleukin 6 family cytokine (leukemia inhibitory factor)–independent ESC self-renewal and to reprogram epiblast stem cells to naïve pluripotency. Conversely, Esrrb overexpression blocked differentiation induced by Tfcp2l1 down-regulation. Moreover, we identified Klf4 as a direct TFCP2L1 target in human ESCs, bypassing the requirement for activin A and basic fibroblast growth factor in short-term human ESC self-renewal. Enforced Klf4 expression recapitulated the self-renewal–promoting effect of Tfcp2l1, whereas Klf4 knockdown eliminated these effects and caused loss of colony-forming capability. These findings indicate that TFCP2L1 functions differently in naïve and primed pluripotency, insights that may help elucidate the different states of pluripotency.

Embryonic stem cells (ESCs)[3] are derived from the inner cell mass of the preimplantation blastocyst (1–3). Under appropriate *in vitro* culture conditions, ESCs proliferate indefinitely without differentiation while retaining the capacity to generate cell lineages derived from all three primary germ layers (4). To date, although ESC-like cells from many species have been established, only ESCs derived from mice and rats possess the ability to generate germline-competent chimeric offspring and thus represent a "naïve" pluripotent state (1, 2, 5, 6). Interestingly, the available human ESCs (hESCs) are more similar to mouse postimplantation epiblast-derived stem cells (EpiSCs) than to mouse ESCs (mESCs) in their self-renewal requirements and morphology and thus represent a "primed" pluripotency state (3, 7, 8). mESC self-renewal can be maintained in two distinct culture systems: serum-containing medium supplemented with leukemia inhibitor factor (LIF) (9, 10) and serum-free N2B27 medium supplemented with two small molecule inhibitors (2i), CHIR99021 and PD0325901 (11). LIF supports self-renewal by inducing activation of signal transducer and activator of transcription 3 (STAT3) (12). CHIR99021 and PD0325901 maintain self-renewal through inhibition of glycogen synthase kinase 3 (GSK3) and mitogen-activated protein kinase kinase (MEK) (11), respectively. However, hESCs requires the activin A and basic fibroblast growth factor (bFGF) cytokines to maintain their identity (3). The addition of Wnt/β-catenin signaling inhibitors can further enable robust hESC propagation (13, 14). Understanding how these growth factors mediate intracellular signaling pathways controlling the unique pluripotent state and the similarities and differences between naïve and primed pluripotency are hot spots in current stem cell research.

Despite the difference in growth factor requirements between mESCs and hESCs, the core transcription factors gov-

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This article contains Tables S1–S6 and Figs. S1–S4.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE115075.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed: No. 111, Jiu Long Rd., School of Life Sciences, Anhui University, Hefei 230601, China. Tel.: 8655163861819; Fax: 8655163861819; E-mail: shdye@126.com.

[3] The abbreviations used are: ESC, embryonic stem cell; LIF, leukemia inhibitory factor; STAT3, signal transducer and activator of transcription 3; Esrrb, estrogen-related receptor β; Tfcp2l1, transcription factor CP2-like 1; Klf, Kruppel-like factor; PB, PiggyBac system; AP, alkaline phosphatase; qRT-PCR, quantitative real-time PCR; shRNA, short hairpin RNA; mESC, mouse ESC; hESC, human ESC; EpiSC, epibiast stem cell; GSK, glycogen synthase kinase; MEK, mitogen-activated protein kinase kinase; bFGF, basic fibroblast growth factor; Dox, doxycycline.

erning pluripotency are similar, such as the master pluripotency genes Oct4, Nanog, and Sox2 (15). Recently, transcription factor CP2-like 1 (Tfcp2l1) has been identified as an important pluripotent factor and has become one of the core markers to identify ESCs generated from many species (16–20). We and other groups reported that *Tfcp2l1* expression is high in the inner cell mass and mESCs, down-regulated in primed stem cells, and further reduced in differentiated cells (16, 17, 21, 22). Tfcp2l1 plays an essential role in maintaining ESC identity. In mESCs, it is a critical target in LIF- and 2i-mediated self-renewal (16, 17, 23). To date, only knockdown of *Tfcp2l1*, but not other STAT3 targets, is able to weaken the LIF/STAT3-mediated mESC self-renewal and EpiSC reprograming (16, 17). In addition, overexpression of *Tfcp2l1* can compensate for the function of 2i when combined with Klf2, another pluripotency gene (23). In contrast to naïve-type stem cells, Tfcp2l1 is expressed highly in early human embryos, while it declines *in vitro* in established primed hESCs (21, 24). However, overexpression of *Tfcp2l1* cannot reprogram hESCs into the naïve pluripotency state (18). Remarkably, enforced expression of *Tfcp2l1* promotes self-renewal, whereas its suppression leads to hESC differentiation toward endoderm and mesoderm specification (22, 24). Taken together, these findings suggest that the self-renewal–promoting function of Tfcp2l1 is conserved in mESCs and hESCs.

Tfcp2l1 has been proposed to act partially through repression of multiple lineage commitments (25). However, it is unclear whether Tfcp2l1 functions through direct activation of a selective pluripotent factor. To resolve this issue, we sought to identify genes directly regulated by Tfcp2l1 in mouse and human ESCs mainly based on gain- and loss-of-function analyses. These analyses identified Esrrb and Klf4 as two direct targets of Tfcp2l1 that are capable of mediating the self-renewal–promoting effects of Tfcp2l1 in mESCs and hESCs, respectively.

Results

Esrrb is a direct target of Tfcp2l1 in mESCs

Previously, Smith and co-workers (26) developed a data-constrained, computational method and defined the simplest essential cassette for maintaining naïve pluripotency. This minimal set comprises 3 inputs (2i/LIF), 12 transcription factors (Oct4, Sox2, Nanog, Klf2, Esrrb, Tfcp2l1, Klf4, Sall4, Gbx2, STAT3, TCF3, and MEK), and 16 interactions, in which Esrrb and Sall4 are two potential direct targets of Tfcp2l1 (26). To validate this predicted relationship of Tfcp2l1, we designed five different approaches. First, we generated one mESC line that overexpressed FLAG-tagged mouse *Tfcp2l1* using a PiggyBac vector (PB-m*Tfcp2l1*) in which *Tfcp2l1* expression was efficiently enhanced (Fig. 1*A*). Quantitative real-time PCR (qRT-PCR) analysis revealed that overexpression of *Tfcp2l1* resulted in up-regulation of the *Esrrb* transcript but not *Sall4* (Fig. 1*B*). Second, to further validate that *Esrrb* is regulated by *Tfcp2l1*, we examined *Esrrb* transcription under different *Tfcp2l1* expression levels by using one mESC line that contains a doxycycline (Dox)-inducible mouse *Tfcp2l1* transgene (17), in which *Tfcp2l1* transcription was efficiently induced by Dox treatment (Fig. 1*C*). *Esrrb* has a similar expression pattern to *Tfcp2l1* following Dox addition (Fig. 1*C*). Third, to investigate whether *Tfcp2l1* down-regulation attenuates *Esrrb* expression, we constructed lentiviral vectors expressing mouse *Tfcp2l1*-specific shRNA sequences (*mTfcp2l1* sh#1, *mTfcp2l1* sh#2, and *mTfcp2l1* sh#3). Stable knockdown (70–80%) of *Tfcp2l1* transcript was observed following drug selection (Fig. 1*D*). As expected, the *Esrrb* transcript decreased in *Tfcp2l1* shRNA cells when compared with *scramble* control cells (Fig. 1*D*). Fourth, to explore how Tfcp2l1 regulates the expression of *Esrrb*, we mapped the binding sites of Tfcp2l1 by analyzing the deep sequencing (ChIP-seq) date of Tfcp2l1 (27). As shown in Fig. 1*E*, the Tfcp2l1 protein has many binding sites in the Esrrb locus (Fig. 1*E*). Finally, to further determine whether Esrrb is a direct target of Tfcp2l1, we analyzed the Tfcp2l1-binding consensus motifs (Fig. 1*F*) and predicted two potential binding sites within the Esrrb promoter region (from $-3000 \sim +1$) from the JASPAR CORE database (motif 1, $^{-1312}$CCAGCCTTGACTAG^{-1298}; and motif 2, $^{-574}$GCAGACTGGCCCAG^{-561}). We then performed ChIP–qRT-PCR in PB-m*Tfcp2l1* 46C mESCs and found a direct interaction between Tfcp2l1 and the *Esrrb* promoter region containing the motif 1 sequence (Fig. 1*G*). This suggested an interaction between Tfcp2l1 and the *Esrrb* promoter. To further determine whether Tfcp2l1 is a functional activator of the Esrrb promoter, *Tfcp2l1* was co-transfected with reporter vectors that drive the expression of luciferase under the control of *Esrrb* promoter fragments (*pGL3-Esrrb*), containing motif 1 or mutated motif 1 sequences (Mut) (Fig. 1*H*). Under these conditions, we observed a 2.7-fold increase in the WT promoter activity relative to the mutant sequence in PB-m*Tfcp2l1* cells (Fig. 1*I*). Collectively, these results suggest that Esrrb is positively regulated and activated by Tfcp2l1 in mESCs.

Esrrb mediates the self-renewal–promoting effects of Tfcp2l1 in mESCs

Tfcp2l1 overexpression can substitute for LIF or CHIR99021 to support mESC self-renewal (17, 23, 28). To determine whether Tfcp2l1 functions in maintaining mESC pluripotency depend on *Esrrb* in serum or serum-free conditions, we first decreased *Esrrb* expression in PB-*mTfcp2l1* mESCs with two shRNAs specific to mouse *Esrrb* mRNA (*mEsrrb* sh#1 and *mEsrrb* sh#2). qRT-PCR analysis confirmed that the *Esrrb* transcript levels were decreased (40–60%) in these cells (Fig. 2*A*). Next, we cultured these cells in serum-containing medium without LIF for 8 days and found that scramble control mESCs retained normal mESC morphology, positive AP activity, and expression of the pluripotency marker OCT4 (Fig. 2, *B–D*), whereas knockdown of *Esrrb* induced differentiation in PB-*mTfcp2l1* mESCs (Fig. 2, *B–D*). To further verify this observation, we seeded these cell lines in serum-free N2B27 medium supplemented with PD0325901. After three passages, we found that down-regulation of *Esrrb* extinguished the pluripotency supported by PB-*mTfcp2l1*, as indicated by the flat cell morphology and decreased AP activity (Fig. S1, *A* and *B*). Overall, these data suggest that Tfcp2l1 requires Esrrb to maintain the naïve pluripotency of mESCs.

Because knockdown of *Tfcp2l1* impairs the undifferentiated state of mESCs under LIF conditions, we consequently wanted

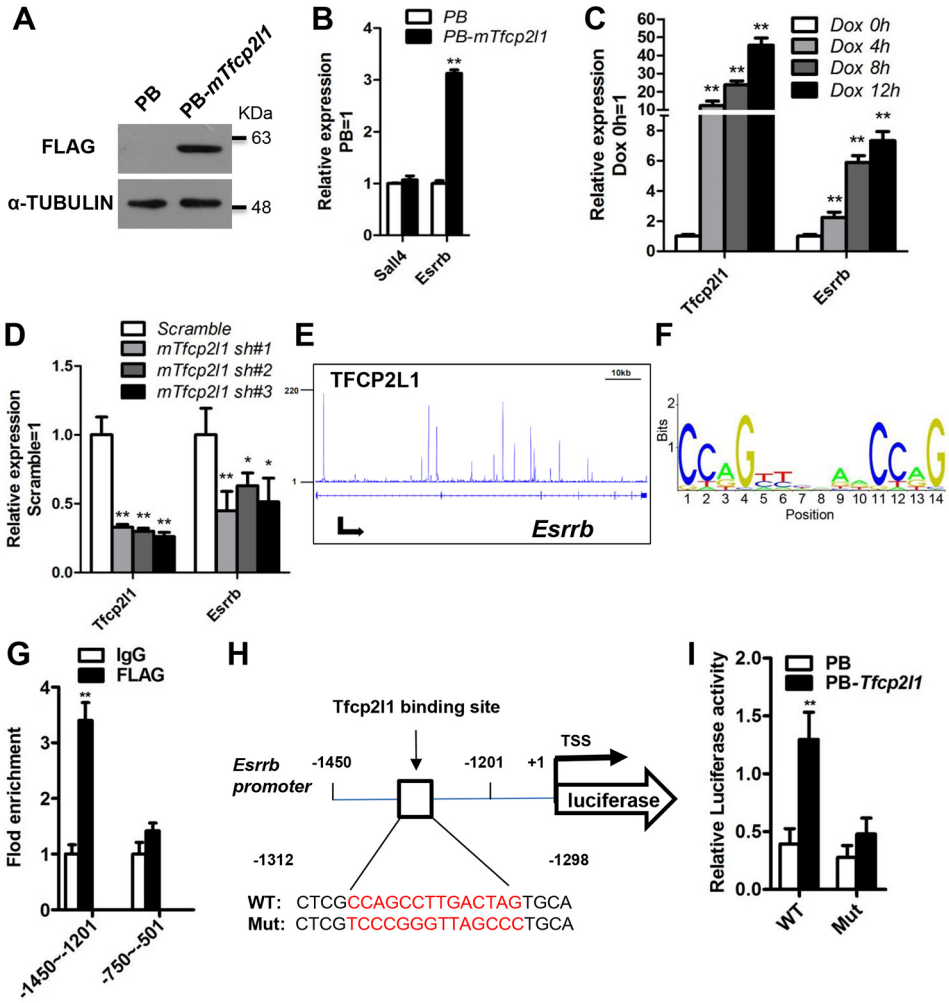


Figure 1. Mouse Tfcp2l1 directly stimulates *Esrrb* expression in mESCs. *A*, Western blotting analysis of the FLAG protein in FLAG-tagged mouse *Tfcp2l1* (PB-m*Tfcp2l1*) 46C mESCs cultured in LIF/serum. α-Tubulin was used as a loading control. *B*, qRT-PCR analysis of *Sall4* and *Esrrb* expression levels in PB and PB-m*Tfcp2l1* 46C mESCs cultured in the presence of LIF. The data are presented as the means ± S.D. of three biological replicates. **, $p < 0.01$ *versus* PB. *C*, qRT-PCR analysis of *Esrrb* expression in mouse *Tfcp2l1*-inducible mESCs treated with Dox for different times. The data are presented as the means ± S.D. of three biological replicates. **, $p < 0.01$ *versus* 0 h. *D*, qRT-PCR analysis of *Esrrb* expression in 46C mESCs infected with scramble or mouse *Tfcp2l1* shRNA lentivirus (mTfcp2l1 sh#1, mTfcp2l1 sh#2, and mTfcp2l1 sh#3). The data are presented as the means ± S.D. of three biological replicates. *, $p < 0.05$; **, $p < 0.01$ *versus* scramble. *E*, gene tracks represent Tfcp2l1 binding at the indicated gene loci. The *x* axis represents the linear sequence of genomic DNA, and the *y* axis represents the total number of mapped reads. *F*, predicted consensus binding motif of *Tfcp2l1* target loci from the JASPAR CORE database. *G*, Tfcp2l1 binds to the Esrrb promoter. ChIP assays were performed using anti-Flag and control IgG antibodies, and fold enrichment in the indicated regions of the Esrrb promoter was examined using qRT-PCR. Two fragments of the Esrrb promoter are indicated by −1450 to −1201 and −750 to −501. The data are presented as the means ± S.D. of three biological replicates. **, $p < 0.01$ *versus* IgG. *H*, the positions of one putative Tfcp2l1 binding site in *Esrrb* promoter. *TSS*, transcription start site. *I*, luciferase activity analysis of PB cells overexpressing the WT Esrrb promoter plasmid or FLAG-m*Tfcp2l1* cells transfected with the WT or mutant *Esrrb* promoter reporter plasmid. The data are presented as the means ± S.D. of three biological replicates. **, $p < 0.01$ *versus* PB.

to test whether elevated expression of *Esrrb* diminishes this phenotype. First, we overexpressed *HA*-tagged mouse *Esrrb* using a PB vector (PB-*mEsrrb*), which caused significant increases in ESRRB (Fig. 2*E*). Second, we decreased *Tfcp2l1* expression in PB and PB-*mEsrrb* mESCs with two shRNAs specific to mouse Tfcp2l1 mRNA (*mTfcp2l1* sh#1 and *mTfcp2l1* sh#2). qRT-PCR analysis confirmed that the Tfcp2l1 transcript levels were decreased (40–60%) in these cells (Fig. 2*F*). After being cultured in serum-containing medium without LIF for 8 days, only the PB-*mEsrrb* mESCs infected with the scramble or *Tfcp2l1* shRNA lentivirus displayed ESC morphological characteristics and expressed the pluripotency marker OCT4, whereas PB mESCs infected with the scramble or Tfcp2l1 shRNA lentivirus differentiated (Fig. 2, *G* and *H*). Notably, Tfcp2l1 protein and Esrrb protein also interact in mESCs (29). Therefore, Tfcp2l1 promotes mESC self-renewal via Esrrb at the protein and transcriptional levels.

Esrrb is required for Tfcp2l1 to induce naïve pluripotency in EpiSCs

Both Esrrb and Tfcp2l1 can reprogram postimplantion embryo derived EpiSCs to naïve pluripotency (16, 17, 23, 28). To assess whether Esrrb modulates the ability of Tfcp2l1 to reprogram EpiSCs, CD1 mouse EpiSCs, isolated from embryonic day 5.5 embryos (14), were transfected with a PB construct containing a *FLAG*-tagged mouse *Tfcp2l1* (PB-*mTfcp2l1*) (Fig. 3*A*) and then infected with the *scramble* control or mouse *Esrrb* shRNA lentivirus (*mEsrrb* sh#1 and *mEsrrb* sh#2) (Fig. 3*B*). After exposure to reprograming conditions including 2i/LIF for 12 days, the EpiSCs transfected with empty vector PB differen-

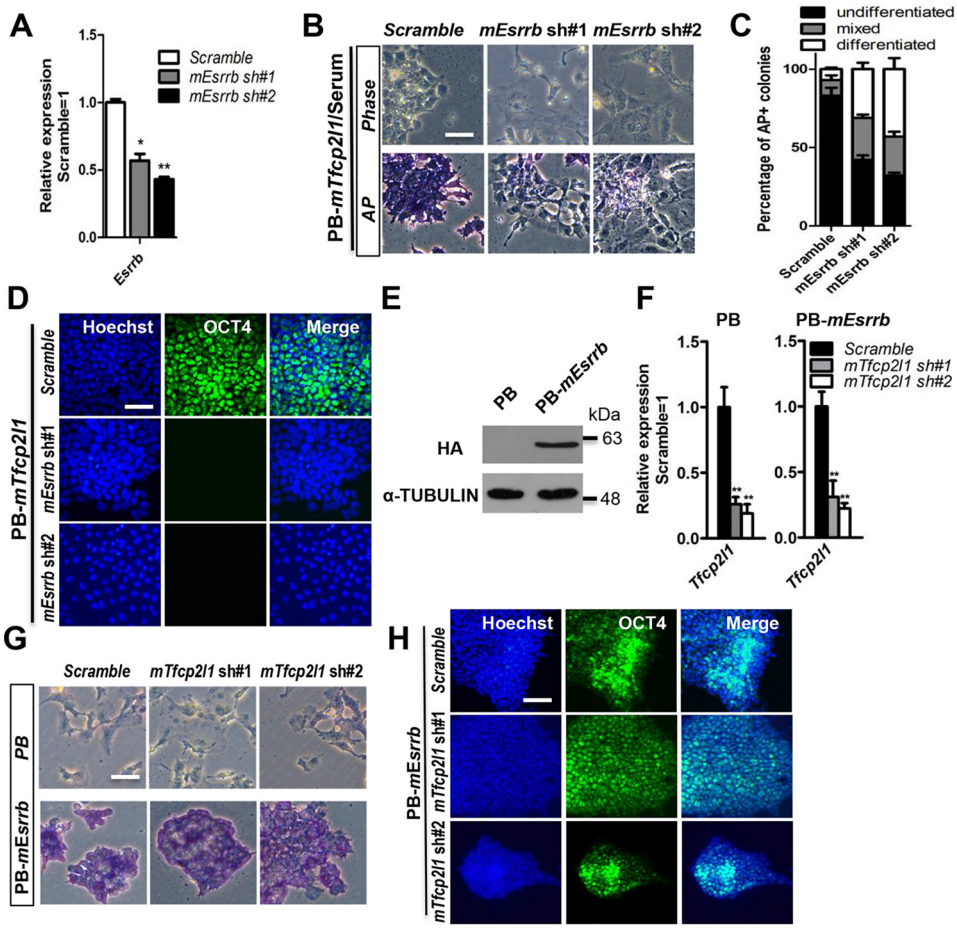


Figure 2. Esrrb is critical for mouse Tfcp2l1 to support the ground state of mESCs. *A*, qRT-PCR analysis of *Esrrb* expression in 46C mESCs infected with scramble or mouse *Esrrb* shRNA lentivirus (mEsrrb sh#1 and mEsrrb sh#2). The data are presented as the means ± S.D. of three biological replicates. *, $p < 0.05$; **, $p < 0.01$ *versus* scramble. *B*, phase-contrast images and AP staining of scramble control and m*Esrrb* shRNA mESCs that are overexpressing PB-m*Tfcp2l1* and cultured in serum without LIF for 8 days. *Bar*, 100 μm. *C*, quantification of AP positive colonies in Fig. 2*B*. The data are presented as the means ± S.D. of three biological replicates. *D*, immunofluorescence staining of *OCT4* in PB-m*Tfcp2l1*/m*Esrrb* shRNA 46C mESCs. The nuclei were counterstained with Hoechst 33342 (Hoechst). *Bar*, 100 μm. *E*, Western blotting analysis of the HA protein in HA-tagged mouse *Esrrb* (PB-m*Esrrb*)-expressing 46C mESCs cultured in LIF/serum. α-Tubulin was used as a loading control. *F*, qRT-PCR analysis of *Tfcp2l1* expression in PB and PB-m*Esrrb* mESCs infected with scramble or m*Tfcp2l1* shRNA lentivirus. The data are presented as the means ± S.D. of three biological replicates. **, $p < 0.01$ *versus* scramble. *G*, AP staining of the scramble control and m*Tfcp2l1* shRNA mESCs overexpressing PB or PB-m*Esrrb* and cultured in serum without LIF for 8 days. *Bar*, 100 μm. *H*, immunofluorescence staining of *OCT4* in PB-m*Esrrb*/m*Tfcp2l1* shRNA 46C mESCs. *Bar*, 100 μm.

tiated or died, whereas an average of 28 AP-positive colonies were generated from 1×10^5 PB-*mTfcp2l1*/scramble transfectants in three independent replicates. In parallel experiments, an average of 13 and 9 colonies were obtained from PB-*mTfcp2l1*/*mEsrrb* sh#1 and PB-*mTfcp2l1*/*mEsrrb* sh#2 transfectants, respectively (Fig. 3, *C* and *D*). These ESC-like colonies tolerated single-cell dissociation and could be continuously expanded in LIF/serum. At the same time, they acquired the mESC-specific transcriptional program and expressed high levels of *Nanog*, *Stella*, *Rex1*, and *Nr0b1*, but a low level of the EpiSC-specific marker *Fgf5* (Fig. 3*E*); this suggests that they had been reprogrammed into a naïve state. Collectively, these results indicate that augmented *Esrrb* expression is likely important for *Tfcp2l1* to convert mouse EpiSCs to the ESC state.

KLF4 is critical for TFCP2L1 to maintain hESC identity

Ectopic expression of TFCP2L1 can bypass the requirement for activin A and bFGF in short-term self-renewal of hESCs, whereas suppression of TFCP2L1 abolishes hESC pluripotency (22, 24). To screen the functional targets of human TFCP2L1, we performed an RNA-Seq analysis. We identified 623 genes that were up-regulated and 449 genes that were down-regulated at least 2-fold by human *TFCP2L1 overexpression* (PB-*hTfcp2l1*) compared with empty vector PB (GEO accession no GSE115075) (Fig. 4*A*). Among these increased genes, we focused on pluripotency genes and identified KLF4 and KLF5. qRT-PCR was then used to confirm the mRNA levels of these two candidates in PB and PB-*hTfcp2l1* cells (Fig. 4*B*). Subsequently, to examine whether the expression of the two candidates decreased during hESC differentiation, such as TFCP2L1, we performed monolayer differentiation. As shown in Fig. 4*C*, *KLF5* is expressed in both pluripotent hESCs and differentiated cells, whereas the *Klf4* transcript is down-regulated as hESCs differentiate (Fig. 4*C*). In particular, the change in *KLF4* expression is similar to that seen for *TFCP2L1*, as well as other pluripotency markers, such as *OCT4* and *NANOG* (Fig. 4*C*), which implies that KLF4 contributes to hESC maintenance and that there is association between KLF4 and TFCP2L1.

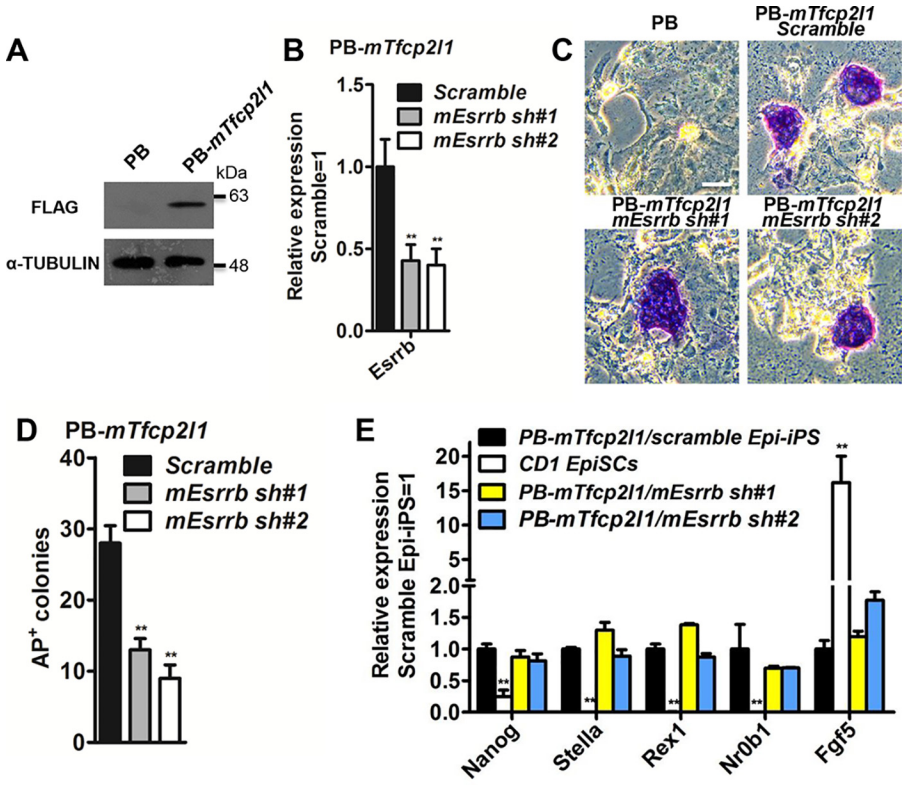
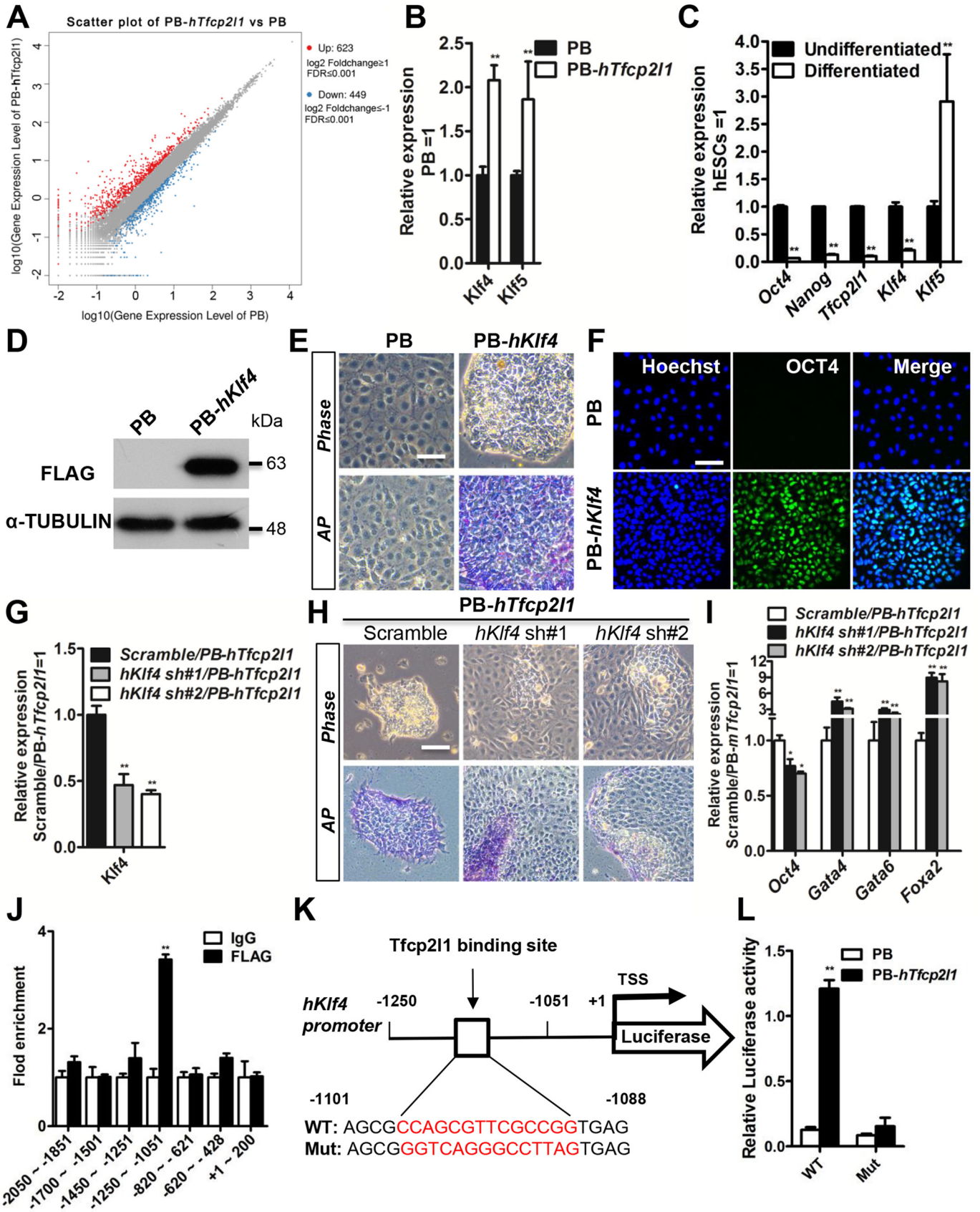


Figure 3. Esrrb plays an important role in the induction of naïve pluripotency downstream of mouse Tfcp2l1. *A*, FLAG-tagged mouse *Tfcp2l1* (PB-m*Tfcp2l1*) was introduced into CD1 EpiSCs, and the protein level of FLAG was determined by Western blotting. The cells were cultured in activin A, basic FGF, and IWR1 conditions. *B*, qRT-PCR analysis of *Esrrb* expression levels in m*Esrrb* knockdown EpiSCs overexpressed PB-m*Tfcp2l1*. The data are presented as the means ± S.D. of three biological replicates. **, $p < 0.01$ *versus* scramble. *C*, AP staining of colonies generated from scramble control and m*Esrrb* knockdown EpiSCs transfected with PB-mTfcp2l1. The cells were cultured in serum medium in the presence of LIF/2i for 12 days. *Bar*, 100 μm. *D*, quantification of the AP-positive colonies generated from scramble and m*Esrrb* knockdown EpiSCs carrying the m*Tfcp2l1* transgene. The cells were cultured in serum medium supplemented with LIF/2i for 12 days. The data are presented as the means ± S.D. of three biological replicates. *E*, comparison of marker gene expression in the indicated Epi-iPS cells and EpiSCs. *Nanog*, *Stella*, *Rex1*, and *Nr0b1* are mESC markers, whereas *Fgf5* is an EpiSC marker. The data are presented as the means ± S.D. of three biological replicates. **, $p < 0.01$ *versus* CD1 EpiSCs. *Epi-iPS*, induced pluripotent stem cells generated from EpiSCs.

To evaluate the ability of KLF4 to promote hESC self-renewal, we constructed a human *KLF4*-overexpressing hESC line with the PB vector (PB-*hKlf4*) (Fig. 4*D*). After cultured in hESC basal medium without activin A and bFGF for 6 days, PB cells differentiated, whereas PB-*hKlf4* cells maintained an undifferentiated morphology and sustained AP-positive activity (Fig. 4*E*). Accordingly, immunofluorescence showed high expression of the pluripotency marker OCT4 (Fig. 4*F*). However, similar to *TFCP2L1* overexpressing hESCs (22), PB-*hKlf4* ESCs exhibited some evidence of differentiation at the edge of each colony and could not been maintained for long-term cultures. Therefore, overexpression of *KLF4* enables short-term self-renewal of hESCs.

To demonstrate that Klf4 is a key downstream mediator of Tfcp2l1, we knocked down the human *KLF4* transcript (*hKlf4* sh#1 and *hKlf4* sh#2) in PB-*hTfcp2l1* transfectants (Fig. 4*G*). Knockdown of *KLF4* resulted in PB-*hTfcp2l1* hESC differentiation in the absence of activin A and bFGF, as indicated by the flat cell morphology, decreased AP activity and low expression of OCT4 (Fig. 4, *H* and *I*). Conversely, they highly expressed differentiation genes, such as *GATA4*, *GATA6*, and *FOXA2* (Fig. 4*I*), suggesting that knockdown of KLF4 eliminates the ability of TFCP2L1 to promote self-renewal. To determine whether TFCP2L1 directly binds to the KLF4 locus, we analyzed the TFCP2L1-binding consensus motifs (Fig. 1*F*) and investigated the role of TFCP2L1 in the regulation of *KLF4* expression. To detect whether TFCP2L1 binds to the KLF4 promoter, we predicted several potential binding sites within the KLF4 gene (from −2500 to +200) from the JASPAR CORE database. Then we used ChIP–qRT-PCR in PB-*hTfcp2l1* hESCs and found the direct interaction of TFCP2L1 with the KLF4 promoter (Fig. 4*J*). To further characterize the role of TFCP2L1 in *KLF4* expression, we first constructed WT and mutant pGL3-*Klf4* cells (Fig. 4*K*) and transfected the modified mESCs with the *TFCP2L1* gene. Under these conditions, we observed that *TFCP2L1* overexpression enhanced the luciferase activity in pGL3-*Klf4* cells. *TFCP2L1* overexpression did not increase the KLF4 promoter activity in mutant pGL3-*Klf4* cells (Fig. 4*L*), indicating that KLF4 is a direct target of TFCP2L1. To further investigate whether TFCP2L1 protein and KLF4 protein interact in hESCs, we performed co-immunoprecipitation in *flag*-tagged *TFCP2L1* and *HA*-tagged *KLF4* overexpressing hESCs. We found that the TFCP2L1 and KLF4 proteins also interact (Fig. S2). Taken together, these results suggest that KLF4 is a direct target of TFCP2L1 and that the function of the latter in hESC self-renewal is largely dependent on KLF4. It will be of great interest to investigate whether KLF4 regulates its own expression via TFCP2L1.

To verify that ESRRB is not essential for human TFCP2L1 to promote hESC self-renewal and that Klf4 is dispensable for


A
Scatter plot of PB-hTfcp2l1 vs PB
Up: 623
log2 Foldchange≥1
FDR≤0.001
Down: 449
log2 Foldchange≤-1
FDR≤0.001
log10(Gene Expression Level of PB-hTfcp2l1)
log10(Gene Expression Level of PB)
B
PB
PB-hTfcp2l1
Relative expression PB =1
Klf4
Klf5
C
Undifferentiated
Differentiated
Relative expression hESCs =1
Oct4
Nanog
Tfcp2l1
Klf4
Klf5
D
PB
PB-hKlf4
kDa
FLAG
63
α-TUBULIN
48
E
PB
PB-hKlf4
Phase
AP
F
Hoechst
OCT4
Merge
PB
PB-hKlf4
G
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hKlf4 sh#1/PB-hTfcp2l1
hKlf4 sh#2/PB-hTfcp2l1
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-620 ~ -428
+1 ~ 200
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PB
PB-hTfcp2l1
Relative Luciferase activity
WT
Mut

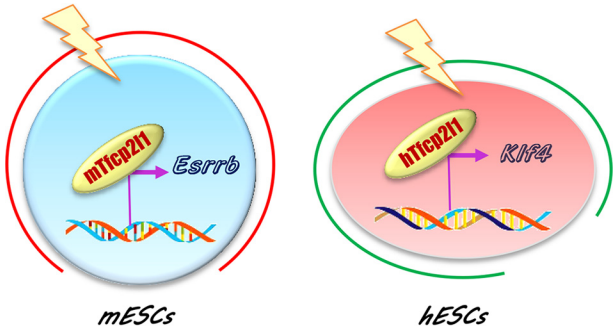

Figure 5. Diagram of the Tfcp2l1 function in mESC and hESC maintenance. Tfp2l1 functions differently in naïve and primed state ESCs. Specifically, Tfcp2l1 supports mESC stemness mainly though induction of *Esrrb*, whereas it promotes hESC self-renewal largely via up-regulation of *Klf4*.

mouse Tfcp2l1 to support mESC stemness, we repressed human *ESRRB* in PB-*hTfcp2l1* hESCs (Fig. S3*A*) and decreased mouse *Klf4* in PB-*mTfcp2l1* mESCs (Fig. S4, *A* and *B*), respectively. As expected, all of these ESCs remained undifferentiated (Figs. S3, *B* and *C*, and S4, *C* and *D*). These observations further demonstrate that the molecular mechanism by which Tfcp2l1 promotes self-renewal is different in naïve and primed pluripotency states.

Discussion

Tfcp2l1 plays an important role in maintaining mouse and human ESC identity. Although many modulators that regulate *Tfcp2l1* expression have been explored, such as the LIF/STAT3 signaling pathway (16, 17), Wnt/β-catenin signaling pathway (17, 23, 28), JMJD1A (30) and Oct4 (31), it remains unclear how Tfcp2l1 safeguards ESC pluripotency. It may act as either an activator or as a repressor of transcription in ESCs. To resolve these issues, we sought to determine the direct targets of Tfcp2l1 and identified the Esrrb and Klf4 transcription factors. Surprisingly, our findings suggest that Tfcp2l1 preferentially activates *Esrrb* to reinforce the pluripotent status of mESCs, whereas Tfcp2l1 largely depends on *Klf4* to promote hESC self-renewal (Fig. 5). To the best of our knowledge, this may be the first report demonstrating that one transcription factor (Tfcp2l1) maintains the undifferentiated state of naïve and primed pluripotency through induction of different downstream targets.

Although we previously reported that pluripotency gene Nanog is important for Tfcp2l1 to promote mESC self-renewal, it is unclear whether Nanog is directly controlled by Tfcp2l1 (17). In this study, we determined that Esrrb is a direct target of Tfcp2l1 and could mediate the influence of Tfcp2l1 on maintaining and inducing naïve pluripotency (Figs. 1–3). This is unsurprising, given that both Tfcp2l1 and Esrrb share many similar and associated features in mESCs. First, both genes are specific markers of pluripotency that are negatively regulated by the GSK3/TCF3 axis, and overexpression of either phenocopies the GSK3 inhibitor effect if supporting mESC pluripotency (17, 23, 28). Second, their expression levels both are dramatically reduced upon mESC differentiation into EpiSCs (16, 17, 32). Thus, overexpression of either factor not only renders mESCs independent of LIF but also reprograms EpiSCs to enter a naïve pluripotency state (16, 17, 32, 33). Third, LIF/STAT3 signaling is necessary for the up-regulation of *Esrrb* expression induced by Nanog and the GSK3 inhibitor in partially reprogrammed cells (pre-iPSCs) (34), although Esrrb was shown to be a direct downstream target of Nanog and Wnt/β-catenin signaling in mESCs (28, 32). Importantly, overexpression of *Esrrb* can resume the reprogramming halted by inhibition of LIF/STAT3 signaling (34). *Esrrb* thus serves as an LIF activity–dependent downstream effector for the establishment of complete pluripotency establishment (34). Nevertheless, Esrrb is not a direct target of STAT3 (16, 17, 33). Tfcp2l1, as one of the most important direct targets of STAT3 (16, 17), may bridge the indirect interactions between the LIF/STAT3 pathway and Esrrb. Of note, LIF/serum conditions allow self-renewal of *Esrrb*-null mESCs (28, 32), whereas other groups found that knockdown of *Esrrb* impairs LIF-mediated mESC self-renewal (35, 36). These inconsistencies may be due to the cross-compensatory functions between Esrrb and other genes and may be triggered by complete loss but not down-regulation of Esrrb. For example, *Esrrg* can replace *Esrrb* to induce the generation of iPSCs (37). It will be of great interest to identify the gene redundant with Esrrb in *Esrrb*-null mESCs.

In disagreement with the role in mESCs, elevated expression of TFCP2L1 does not induce ESRRB expression in human ESCs (Fig. 4*A*). We further explored this and demonstrated that KLF4 is a direct target of TFCP2L1, and it can mediate TFCP2L1-induced self-renewal in hESCs (Fig. 4, *E–I*). In fact, great progress has been made around the roles of Klf4 and Tfcp2l1 in mESCs, but little was known about their roles in hESCs until now. In mESCs, both genes are direct targets of the LIF/STAT3 signaling pathway and up-regulate *Nanog* expression (16, 17, 38). Meanwhile, they maintain and induce naïve

Figure 4. KLF4 mediates the function of human TFCP2L1 in HES2 hESCs. *A*, scatter plot of the genes modified by human *Tfcp2l1* overexpression (PB-h*Tfcp2l1*). A Benjamini Hochberg false discovery rate of 0.01 was applied. *B*, qRT-PCR analysis of *Klf4* and *Klf5* expression in PB and PB-h*Tfcp2l1* hESCs. The data are presented as the means ± S.D. of three biological replicates. **, $p < 0.01$ *versus* PB. *C*, comparison of *Oct4*, *Nanog*, *Tfcp2l1*, *Klf4*, and *Klf5* expression between undifferentiated hESCs and differentiated cells cultured in basal medium for 8 days. The data are presented as the means ± S.D. of three biological replicates. **, $p < 0.01$ *versus* undifferentiated. *D*, Western blotting analysis of the FLAG protein in FLAG-tagged human *Klf4* (PB-h*Klf4*) hESCs. α-Tubulin was used as a loading control. *E*, phase-contrast images and AP staining of PB and PB-h*Klf4* hESCs cultured in basal medium for 6 days. *Bar*, 100 μm. *F*, immunofluorescence analysis of *OCT4* expression in PB and PB-h*Klf4* cells. OCT4 is a marker of undifferentiated hESCs. The nuclei were counterstained with Hoechst. *Bar*, 100 μM. *G*, qRT-PCR analysis of *Klf4* expression in PB-h*Tfcp2l1* hESCs infected with scramble or human *Klf4* shRNA lentivirus (hKlf4 sh#1 and hKlf4 sh#2). The data are presented as the means ± S.D. of three biological replicates. **, $p < 0.01$ *versus* scramble/PB-h*Tfcp2l1*. *H*, phase-contrast images and AP staining of PB-h*Tfcp2l1* hESCs infected with scramble or h*Klf4* shRNA lentivirus and cultured in basal medium for 6 days. *Bar*, 100 μm. *I*, qRT-PCR analysis of *Oct4*, *Gata4*, *Gata6*, and *FoxA2* expression in PB-hTfcp2l1 hESCs infected with scramble or h*Klf4* shRNA lentivirus. The data are presented as the means ± S.D. of three biological replicates. *, $p < 0.05$; **, $p < 0.01$ *versus* scramble/PB-h*Tfcp2l1*. *J*, *Tfcp2l1* binds to the *Klf4* promoter. ChIP assays were performed using anti-Flag and control IgG antibodies and fold enrichment within the indicated regions of the *Klf4* promoter was examined using qRT-PCR. The data are presented as the means ± S.D. of three biological replicates. *, $p < 0.05$; **, $p < 0.01$ *versus* IgG. *K*, the position of one putative Tfcp2l1 binding site in *Klf4* promoter. *L*, PB or FLAG-h*Tfcp2l1* cells were transfected with the indicated *Klf4* promoter reporter plasmids for 48 h, and then the luciferase activity was measured. The data are presented as the means ± S.D. of three biological replicates. **, $p < 0.01$ *versus* PB.

pluripotency (16, 17, 39). In hESCs, the *KLF4* transcript level was markedly reduced upon hESC differentiation, which is similar to *TFCP2L1* (22, 40). Elevated expression of *KLF4* transcription inhibits neural differentiation of hESCs and enhances pluripotency marker expression (41). On the other hand, KLF4 is a Yamanaka factor that can induce iPSCs from human fibroblasts (42). All of these data highlight an important characteristic of KLF4 in hESCs and imply that the interaction between TFCP2L1 and KLF4 is likely a key contribution of Tfcp2l1 to promote hESC self-renewal. Interestingly, in mESCs, Klf4 has overlapping functions with Klf2 and Klf5 in LIF/serum medium (43, 44). However, in hESCs, we found that knockdown of *KLF4* is enough to trigger differentiation in *TFCP2L1*-overexpressing hESCs (Fig. 4, *G–I*). These may be caused by the different culture conditions. In addition, KLF proteins exhibit unequal potency (44). For example, depletion of *Klf2* is sufficient to abolish mESC self-renewal in 2i conditions (45). Furthermore, no single Klf is sufficient to restore self-renewal of triple-knockout mESCs (44). Further studies are necessary to address the detailed mechanism of TFCP2L1 and KLF4 in maintaining hESC pluripotency.

In summary, we found two distinct transcription factors downstream of Tfcp2l1 that are functionally important for the self-renewal of mouse and human ESCs. These results provide new insights into the fully understanding of the regulatory circuitry that maintains naïve and primed pluripotency and will help to derive ESCs from different species and ultimately to safely use ESCs for future translational applications.

Experimental procedures

Cell culture

For maintenance of mESCs, 46C mESCs (46), provided by Qi-Long Ying (University of Southern California), were cultured in 0.1% gelatin-coated dishes at 37 °C in 5% carbon dioxide. The medium used for routine maintenance was Dulbecco's modified Eagle's medium (TransGen Biotech) supplemented with 10% fetal bovine serum (FND500, ExCell Bio), 1× minimal essential medium nonessential amino acids (11140-050, Invitrogen), 2 mM GlutaMAX (21051024, Invitrogen), 0.1 mM β-mercaptoethanol (M3148, Sigma), and 1000 units/ml LIF (LIF1010, Millipore). For serum-free culture, mESCs were maintained in N2B27 medium (11) supplemented with PD0325901 (1 μM, Sigma) and CHIR99021 (3 μM, Sigma).

For maintenance of CD1 mouse EpiSCs and HES2 hESCs, EpiSCs were seed in 0.1% gelatin-coated dishes, whereas hESCs were cultured on plates precoated with Matrigel (BD Biosciences). hESCs were cultured in N2B27 medium supplemented with 10% KSR (Invitrogen), activin A (10 ng/ml; 120–14E, Peprotech), bFGF (10 ng/ml; AF-100–18B, Peprotech), and 4 μM IWR1 (I0161, Sigma). EpiSCs were cultured in mESC medium supplemented with Activin A, bFGF and IWR1. Y27632 (1 μM; Tocris) was added when they were passaged. For passaging, the cells were dissociated with a calcium trypsin KSR solution every 3–5 days (47, 48).

Plasmid construction

The coding regions of the *Tfcp2l1*, *Esrrb*, and *Klf4* genes were inserted into the BglII and XhoI sites of the PiggyBac transposon vectors carrying a Flag or HA tag. The shRNA-expressing plasmids were constructed according to the pLKO.1-TRC protocol (Addgene plasmid no. 10878). The target-specific shRNA sequences for *Tfcp2l1*, *Esrrb*, and *Klf4* were inserted into the AgeI and EcoRI sites. The shRNA sequences are as follows: *mEsrrb* sh#1, CGATTCATGAAATGCCTCAAA; *mEsrrb* sh#2, GCCGAGGACTATATCATGGAT; *hKlf4* sh#1: GCCAGAATTGGACCCGGTGTA; and *hKlf4* sh#2, GCCTTACACATGAAGAGGCAT. Details of the mouse and human Tfcp2l1 shRNA vectors have been reported in our previous studies (17, 22). The target specific *shRNA* sequences for *Tfcp2l1*, *Esrrb*, and *Klf4* used in this study are listed in Table S1.

Western blotting analysis

Western blotting was performed according to a standard protocol. Briefly, the cells were lysed in radioimmune precipitation assay buffer (P0013B, Beyotime Biotechnology, China). The protein concentrations of the samples were estimated with a BCA kit (PA115, Tiangen Biotech), and then the samples were separated on 10% PAGE gels and electrotransferred onto polyvinylidene difluoride membranes. Probing was performed with specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies. We used the following primary antibodies: Flag (F1804, Sigma, 1:2000), HA (3724S, Cell Signaling Technology, 1:2000), and α-tubulin (32-2500, Invitrogen, 1:5000). Signals were detected using a Pro-Light horseradish peroxidase kit (PA112, Tiangen Biotech).

qRT-PCR

Total RNA was extracted using the TRIzol Up Plus RNA kit (ER501-01, TransGen Biotech). cDNA was synthesized from 1 μg of total RNA using the TransScript all-in-one first-strand cDNA synthesis SuperMix for qPCR (One-Step gDNA removal, AT341-02, TransGen Biotech) according to the manufacturer's instructions. qRT-PCR was carried out with Top Green qPCR SuperMix (AQ131-04, TransGen Biotech) in a PikoReal real-time PCR machine (Thermo Scientific). The relative expression level was determined by the 2-ΔCq method and normalized to β-actin expression. The primers used are listed in Table S2.

Immunostaining

Immunostaining was performed via standard protocols. Briefly, the cells were fixed in 4% paraformaldehyde for 20 min and incubated at 37 °C in blocking buffer (PBS containing 5% BSA and 0.2% Triton X-100). The cells were then incubated with the Oct4 primary antibody at 4 °C overnight. After three times washes with PBS, the cells were then incubated with secondary antibody for 1 h at 37 °C. The nuclei were stained with Hoechst 33342 (H3570, Invitrogen, 1:10,000) for 30 s.

AP activity assay

The cells were fixed in 4% paraformaldehyde for 2 min at room temperature, washed in PBS, and incubated in AP staining reagent (85L2-1KT, Sigma) for 30 min at room temperature in the darkroom. After two washes with PBS, the cells were visualized under a Leica DMI8 microscope.

EpiSC reprogramming

A total of 1×10^5 transfectants were plated in 0.1% gelatin-coated 6-well plates and cultured in mESC medium supplemented with LIF and 2i for 12 days. Then the number of AP-positive clones generated from the CD1 EpiSCs was counted under a Leica DMI8 microscope.

ChIP assay

ChIP was performed using the ChIP assay kit (P2078; Beyotime Biotechnology) according to the manufacturer's protocol. Anti-FLAG antibody (F1804, Sigma) was used for immunoprecipitation, and IgG was used as a control antibody. ChIP enrichment was determined by qRT-PCR. The primer sequences and locations within the *Esrrb* and *Klf4* genes are listed in Tables S3 and S4, respectively.

Luciferase assay

The promoter regions of Esrrb (from −1450 to −1201) and Klf4 (from −1250 to −1051) were cloned into pGL3-base plasmid; the new constructs were named pGL3-*Esrrb* and pGL3-*Klf4*, respectively. The primers sequences are listed in Tables S5 and S6. They were co-transfected into 46C mESCs or hESCs, along with the Tfcp2l1-overexpressed construct, or empty vector, with a *Renilla* luciferase plasmid. After 48 h, luciferase activities were measured with the dual luciferase assay kit. (FR201, TransGen Biotech).

Co-immunoprecipitation

Cell extracts were prepared using Nonidet P-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Nonidet P-40, and protease inhibitors). The supernatant was collected and incubated with 10 μl of anti-FLAG affinity gel (SG4110-16, GNI, Tokyo, Japan) for 2 h at 4 °C. The beads were then washed six times with lysis buffer and resuspended in 100 μl of 1× SDS sample buffer for Western blotting analysis.

Accession number

RNA-seq data have been deposited to the NCBI Gene Expression Omnibus under the accession number GSE115075.

Statistical analysis

All data are reported as the means ± S.D. Student's *t* test was used to determine the significance of differences using GraphPad Prism 6. $p < 0.05$ was considered statistically significant. Sample size and *p* values have been included in the figure legends.

Author contributions—Xiaohu Wang, S. Z., and H. S. investigation; Xiaohu Wang, Xiaoxiao Wang, S. Z., and H. D. methodology; Xiaoxiao Wang and Y. Y. formal analysis; H. S. and S.L. validation; X. Z. resources; S.-D. Y. project administration.

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