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Embryonic stem cell self-renewal pathways converge on the transcription factor Tfcp2l1

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Mouse embryonic stem cell (mESC) self-renewal can be maintained by activation of the leukaemia inhibitory factor (LIF)/signal transducer and activator of transcription 3 (Stat3) signalling pathway or dual inhibition (2i) of glycogen synthase kinase 3 (Gsk3) and mitogen-activated protein kinase kinase (MEK). Several downstream targets of the pathways involved have been identified that when individually overexpressed can partially support self-renewal. However, none of these targets is shared among the involved pathways. Here, we show that the CP2 family transcription factor Tfcp2l1 is a common target in LIF/Stat3- and 2i-mediated self-renewal, and forced expression of Tfcp2l1 can recapitulate the self-renewal-promoting effect of LIF or either of the 2i components. In addition, Tfcp2l1 can reprogram post-implantation epiblast stem cells to naïve pluripotent ESCs. Tfcp2l1 upregulates Nanog expression and promotes self-renewal in a Nanog-dependent manner. We conclude that Tfcp2l1 is at the intersection of LIF- and 2i-mediated self-renewal pathways and plays a critical role in maintaining ESC identity. Our study provides an expanded understanding of the current model of ground-state pluripotency.

Introduction

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass of the pre-implantation blastocyst (Smith, 2001). ESCs were first established from mice (Evans and Kaufman, 1981; Martin, 1981), and more recently, from rats (Buehr et al, 2008; Li et al, 2008). Attempts to derive authentic ESCs from other mammals have so far failed, highlighting the need to understand more about how the ESC state is established and maintained.

Mouse ESC (mESC) self-renewal can be maintained in two distinct culture systems: serum-containing medium supplemented with leukaemia inhibitor factor (LIF) (Smith et al, 1988; Williams et al, 1988), and serum-free N2B27 medium supplemented with two small molecule inhibitors (2i), CHIR99021 (CHIR) and PD0325901 (PD03) (Ying et al, 2008). LIF supports self-renewal by inducing activation of signal transducer and activator of transcription 3 (Stat3) (Niwa et al, 1998). CHIR and PD03 maintain self-renewal through inhibition of glycogen synthase kinase 3 (Gsk3) and mitogen-activated protein kinase kinase (MEK), respectively. When overexpressed, several LIF/Stat3 downstream targets, including Klf4, Gbx2, Pim1, Pim3, Pramel7, Rhox5, and c-Myc, can partially recapitulate the self-renewal-promoting effect of LIF/Stat3 signalling (Cartwright et al, 2005; Aksoy et al, 2007; Cinelli et al, 2008; Bourillot et al, 2009; Hall et al, 2009; Casanova et al, 2011; Tai and Ying, 2013). Knockdown of any of those Stat3 downstream targets, however, does not abolish LIF/Stat3-mediated mESC self-renewal, suggesting that LIF/Stat3 might activate multiple downstream targets with cross-compensatory functionality, ensuring the integrity of the self-renewal program. It is also possible that the master downstream targets of LIF/Stat3 have yet to be identified.

CHIR inhibits Gsk3, leading to the stabilization and nuclear translocation of β-catenin and subsequent activation of canonical Wnt/β-catenin signalling. CHIR-induced activation of Wnt/β-catenin signalling abrogates T-cell factor 3 (Tcf3)-mediated repression of the pluripotency network, and this has been proposed to be a key mechanism underlying CHIR's role in promoting mESC self-renewal (Wray et al, 2011). One of the key targets of Tcf3 repression is Esrrb, whose expression is essential for mESC self-renewal mediated by inhibition of Gsk3 by CHIR (Martello et al, 2012). CHIR alone is not sufficient for maintaining mESC self-renewal, and addition of PD03 is required. PD03 enhances Nanog expression, which may account for the self-renewal-promoting effect of this small molecule (Miyanari and Torres-Padilla, 2012).

Although LIF and 2i promote mESC self-renewal through seemingly independent pathways, they might converge on the same downstream targets to exert their self-renewal-promoting effect. However, no direct downstream connection has yet been established. LIF can replace PD03 or CHIR in the presence of either to recapitulate the combined self-renewal-sustaining effect of 2i in serum-free culture (Wray et al, 2010), indicating possible overlap in the self-renewal machinery activated by LIF and 2i. This notion is further supported by the facts that Stat3-null mESC self-renewal can be maintained by 2i (Ying et al, 2008), and LIF/PD03 is sufficient to maintain β-catenin-null ESCs that are not responsive to CHIR for self-renewal (Lyashenko et al, 2011). Uncovering how these molecular pathways overlap will provide further insight into not only how ESCs maintain their intrinsic properties, but also how stem cells may be exploited for medical and technological purposes. Such an advance might also facilitate the development of conditions for the derivation of authentic ESCs from species other than mice and rats.

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Here, we identified transcription factor CP2-like 1 (Tfcp2l1, also called Tcfcp2l1 or CRTR-1) as a common downstream target of LIF/Stat3, CHIR, and PD03, that when overexpressed, can recapitulate the self-renewal-promoting effect of each.

Results

Identification of genes differentially induced/ suppressed in mESCs by LIF and LIF/2i

Under feeder-free conditions, LIF alone is not sufficient to maintain self-renewal of ESCs derived from non-129 strains of mice such as C57BL/6 and BALB/c, and addition of 2i is required (Gertsenstein *et al*, 2010; Wray *et al*, 2010; Ye *et al*, 2012). We hypothesized that 2i promotes ESC self-renewal by inducing the expression of pluripotency genes insufficiently induced by LIF or through suppressing differentiation-inducing genes. We performed DNA microarray analysis to identify genes differentially expressed in C57BL/6 mESCs treated with LIF or LIF/2i. We identified 446 genes upregulated and 841 genes downregulated by 1.5-fold or greater in LIF/2i compared with LIF alone (GEO ID Number: GSE46369). We focussed on genes that are specifically expressed in pluripotent stem cells and are upregulated in the LIF/2i condition, and identified five candidates: *Tbx3*, *Klf2*, *Klf4*, *Nanog*, and *Tfcp2l1* (Figure 1A). The differential expression levels of these candidate genes in LIF/2i and LIF alone were confirmed by quantitative real-time

PCR (qRT–PCR) (Figure 1B). We then overexpressed these genes in C57BL/6 mESCs (Figure 1C), and all were able to support feeder-free C57BL/6 mESC self-renewal in the presence of LIF alone (Figure 1D and E). The results for *Tbx3*, *Klf2*, *Klf4*, and *Nanog* were consistent with previous reports that overexpression of these genes promoted self-renewal in 46C and E14 mESCs without exogenous LIF (Hall *et al*, 2009; Niwa *et al*, 2009). *Tfcp2l1* is preferentially expressed in ESCs (Ivanova *et al*, 2006), but its function in ESC self-renewal has not been characterized. This prompted us to undertake further analysis of the role of Tfcp2l1 in mESC self-renewal.

Tfcp2l1 overexpression can substitute for PD03 or CHIR in 2i to maintain mESC self-renewal

To determine whether *Tfcp2l1* expression is induced by CHIR or PD03 or both, we analysed its expression in mESCs treated with one or both of these small molecules in the presence of LIF. *Tfcp2l1* expression was induced in all three conditions (Figure 2A). To determine the function of Tfcp2l1 in mESC self-renewal, we used an inducible cassette exchange (ICE) system (Iacovino *et al*, 2011) to generate an mESC line harbouring a doxycycline (Dox)-inducible *Tfcp2l1* transgene (i-Tfcp2l1 mESCs). Western blot analysis confirmed that Tfcp2l1 expression in these cells was efficiently induced by Dox treatment (Figure 2B). i-Tfcp2l1 mESCs as well as control mESCs harbouring a Dox-inducible *EGFP* transgene (i-EGFP mESCs) could be efficiently maintained in the serum-free N2B27/2i condition with or without Dox. Withdrawal of 2i
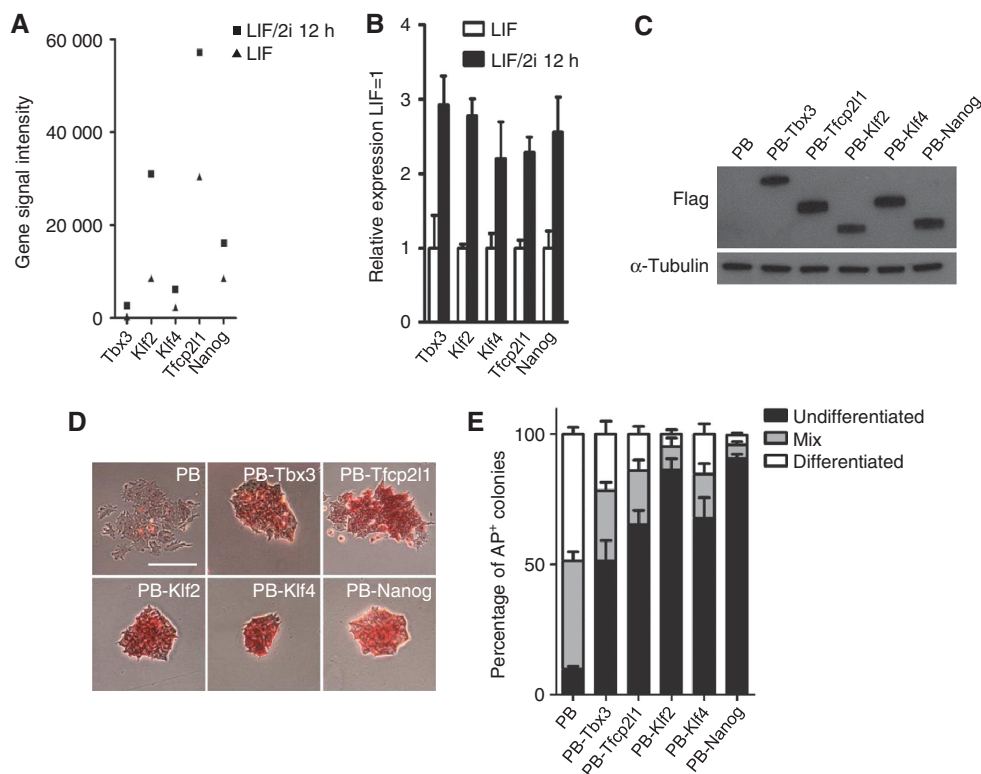
Figure 1 2i induces multiple transcription factors to promote mESC self-renewal. (**A**) Scatter plots of DNA microarray data showing *Tfcp2l1*, *Klf2*, *Klf4*, *Nanog*, and *Tbx3* gene signal intensities for C57BL/6 mESCs cultured in serum/LIF/2i or serum/LIF for 12 h. (**B**) qRT–PCR analysis of *Tbx3*, *Klf2*, *Klf4*, *Tfcp2l1*, and *Nanog* expression in C57BL/6 mESCs cultured in the presence of serum/LIF/2i or serum/LIF for 12 h. Error bars are the s.d. of three biological replicates. (**C**) Western blot analysis of indicated Flag-tagged proteins in C57BL/6 mESCs cultured in LIF/2i. α-Tubulin is a loading control. (**D**) Alkaline phosphatase staining of colonies arising from transfectants cultured in serum/LIF for two passages. Scale bar, 100 μm. (**E**) Quantification of alkaline phosphatase-positive colonies. Data represent mean ± s.d. from triplicate experiments. Source data for this figure is available on the online supplementary information page.
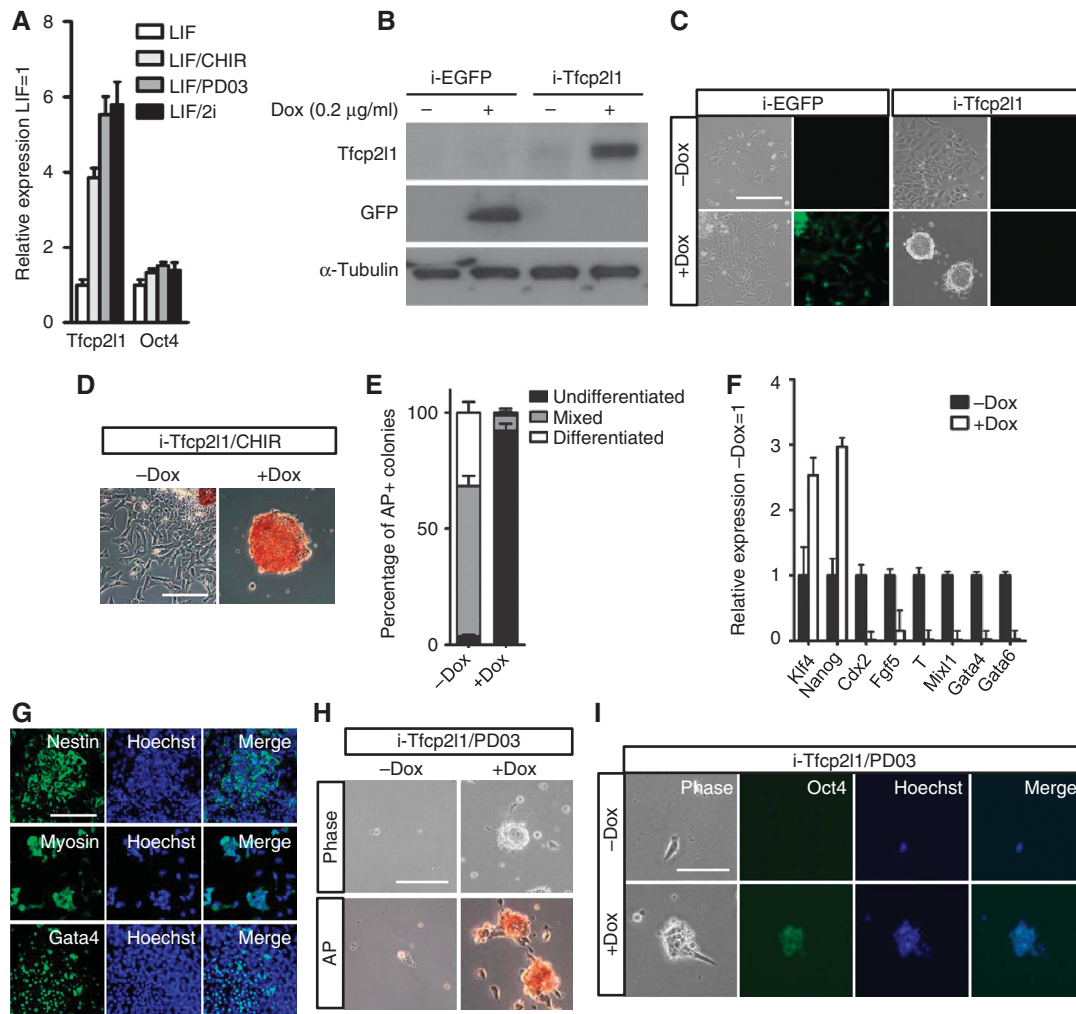
Figure 2 Enforced Tfcp2l1 phenocopies PD03 or CHIR to promote ground-state pluripotency. (**A**) qRT–PCR analysis of *Tfcp2l1* expression in 46C mESCs treated for 12 h with LIF, CHIR, and/or PD03 as indicated. Data represent mean ± s.d. of three biological replicates. (**B**) Western blot analysis of GFP and Tfcp2l1 expression in i-EGFP and i-Tfcp2l1 mESCs cultured in the presence or absence of 0.2 µg/ml Dox for 12 h. (**C**) i-EGFP and i-Tfcp2l1 mESCs were cultured in N2B27/CHIR for five passages in the presence or absence of Dox. Scale bar, 100 µm. (**D, E**) Alkaline phosphatase staining of i-Tfcp2l1 mESCs cultured in the presence or absence of Dox and quantification of alkaline phosphatase-positive colonies. Scale bar, 100 µm. Data represent mean ± s.d. from triplicate experiments. (**F**) qRT–PCR analysis of *Klf4*, *Nanog*, *Cdx2*, *Fgf5*, *T*, *Mixl1*, *Gata4*, and *Gata6* expression in i-Tfcp2l1 mESCs cultured in N2B27/CHIR with or without Dox. Data represent mean ± s.d. of three biological replicates. (**G**) Immunostaining for neural marker Nestin, cardiomyocyte marker Myosin, and primitive endoderm marker Gata4 in i-Tfcp2l1 mESC-derived differentiated cells. Hoechst was used for nuclear staining. Scale bar, 100 µm. (**H, I**) Morphology, alkaline phosphatase staining, and immunostaining of i-Tfcp2l1 mESCs cultured in N2B27/PD03 with or without Dox for five passages. Scale bars, 100 µm. Source data for this figure is available on the online supplementary information page.

resulted in cell death or differentiation within five passages even in the presence of Dox, suggesting that artificial Tfcp2l1 expression cannot replace 2i for the maintenance of mESC self-renewal. This prompted us to examine the relationship between Tfcp2l1 and each 2i component in maintaining mESC self-renewal. i-Tfcp2l1 mESCs could be continually passaged in N2B27/ CHIR plus Dox while retaining typical ESC morphology and an alkaline phosphatase-positive staining profile (Figure 2C–E). qRT–PCR analysis revealed high-level expression of the pluripotency genes *Klf4* and *Nanog* and low-level expression of genes associated with differentiation (Figure 2F), and immunofluorescence staining showed positive expression of the pluripotency markers Oct4 and SSEA1 (Supplementary Figure S1). Following the removal of Dox, i-Tfcp2l1 mESCs retained the capacity to differentiate into Nestin-positive neurons, Myosin-positive myocardial cells, and Gata4-positive primitive endoderm cells *in vitro* (Figure 2G). Notably, Dox-induced Tfcp2l1 expression did not

alter the phosphorylation level of extracellular signal-regulated kinase (ERK), suggesting that the ability of Tfcp2l1 to replace PD03 is not attributable to inhibition of ERK phosphorylation (Supplementary Figure S2). In N2B27/PD03 plus Dox, undifferentiated i-Tfcp2l1 mESCs could also be continually passaged, although they proliferated more slowly than did i-Tfcp2l1 mESCs in CHIR or 2i (Figure 2H and I). Taken together, these findings demonstrate that Tfcp2l1 transcription can be upregulated by both CHIR and PD03, and that forced expression of Tfcp2l1 can recapitulate the effect of PD03, and partially that of CHIR, in promoting mESC self-renewal in the 2i condition.

Forced expression of Tfcp2l1 can maintain self-renewal in the absence of LIF/Stat3 signalling

As LIF can be used to replace CHIR or PD03 in the presence of either in maintaining mESC self-renewal (Wray *et al*, 2010), we sought to determine whether there is any connection

between LIF signalling and *Tfcp2l1* expression. We evaluated the transcriptional responsiveness of *Tfcp2l1* to LIF stimulation in 46C mESCs. 46C mESCs were cultured in the absence of LIF for 12 h, and the expression of *Tfcp2l1* as well as the direct Stat3 targets *Socs3* and *Klf4* (Hall *et al*, 2009) was then analysed by qRT–PCR, which showed that expression of all three genes was significantly downregulated (Figure 3A). We next cultured the i-Tfcp2l1 mESCs in serum medium in the presence of different concentrations of Dox (Figure 3B). Following LIF withdrawal, i-Tfcp2l1 mESCs remained undifferentiated when Dox was present at a concentration of 0.2 μg/ml or higher (Figure 3B–F), whereas they rapidly differentiated when Dox was absent or present only at a low concentration (0.02 μg/ml) (Figure 3B–E). We then compared *Tfcp2l1* expression induced by various concentrations of Dox with endogenous *Tfcp2l1* expression induced by LIF

and 2i. Because 0.02 μg/ml of Dox induced lower *Tfcp2l1* expression than did LIF or 2i (Figure 3D), we chose 0.2 μg/ml of Dox to achieve a more-appropriate level of *Tfcp2l1* expression for all the following experiments.

To determine whether Stat3 is required for Tfcp2l1-promoted mESC self-renewal, we overexpressed Tfcp2l1 in *Stat3*-null mESCs (Figure 3G) and cultured them in serum without LIF or 2i (Ying *et al*, 2008). These cells remained undifferentiated even after multiple passages, whereas *Stat3*-null mESCs transfected with an empty vector died or differentiated after one passage (Figure 3H). These results indicate that Tfcp2l1 promotes mESC self-renewal independent of Stat3.

Binding of LIF to the LIF receptor/gp130 complex can trigger three signalling pathways: janus kinase (JAK)/Stat3, phosphoinositide 3-kinase (PI3K)/AKT, and mitogen-activated
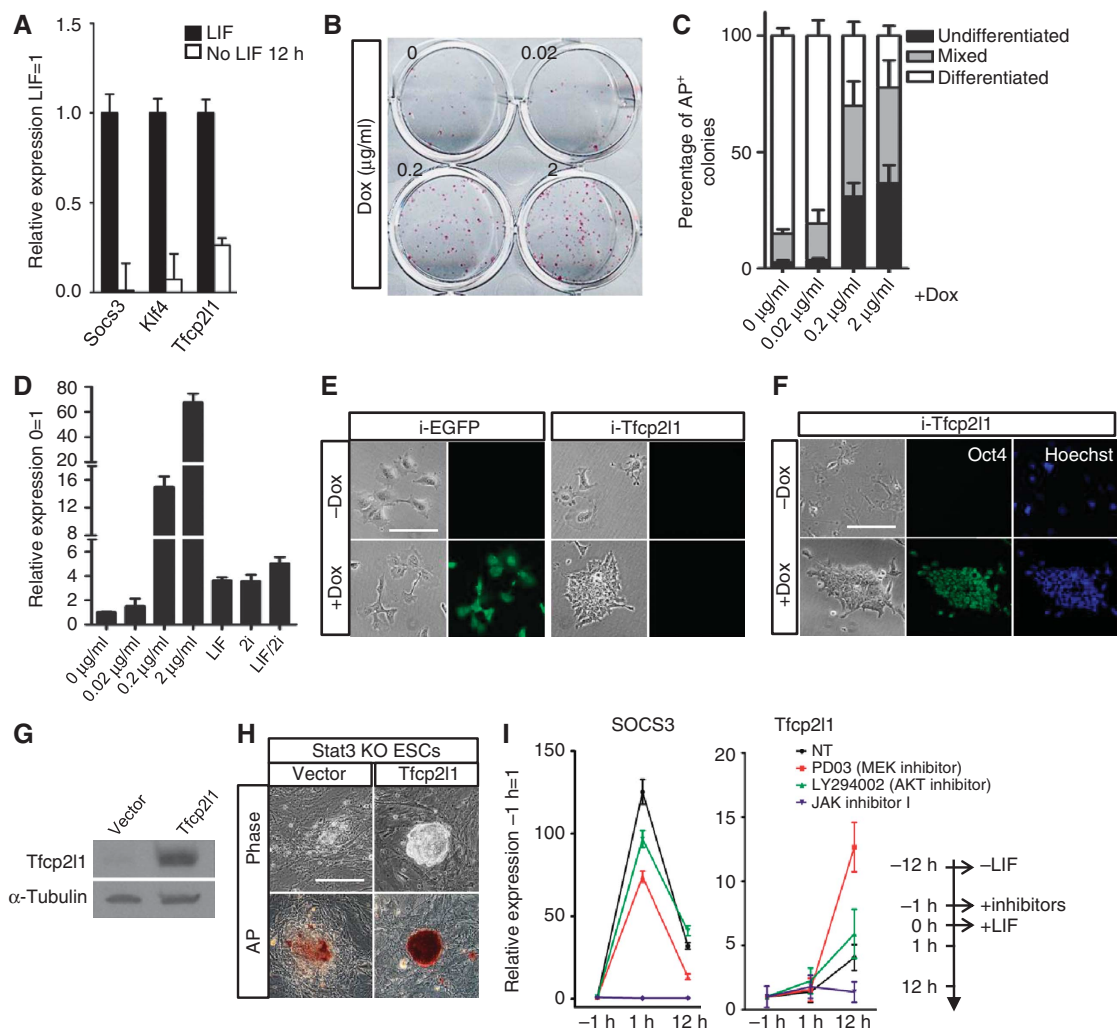
Figure 3 Enforced Tfcp2l1 promotes mESC self-renewal independent of LIF/Stat3. (**A**) qRT–PCR analysis of *Socs3*, *Klf4*, and *Tfcp2l1* expression in 46C mESCs deprived of LIF for 12 h. Data represent mean ± s.d. of three biological replicates. (**B**) Alkaline phosphatase staining of i-Tfcp2l1 mESCs cultured in the absence or presence of different concentrations of Dox for 8 days. (**C**) Quantification of alkaline phosphatase-positive colonies. Data represent mean ± s.d. from triplicate experiments. (**D**) qRT–PCR analysis of *Tfcp2l1* in i-Tfcp2l1 mESCs cultured in the indicated conditions for 12 h. (**E**) Morphology of i-EGFP and i-Tfcp2l1 mESCs cultured in serum medium without LIF for 8 days. Scale bar, 100 μm. (**F**) Immunostaining of i-Tfcp2l1 mESCs after 8 days in the presence or absence of Dox. Scale bar, 100 μm. (**G**) Western blot analysis of Tfcp2l1 expression in empty or Tfcp2l1 vector-transfected Stat3 knockout ESCs cultured in N2B27/2i. (**H**) Morphology and alkaline phosphatase staining of Stat3 knockout ESCs transfected with empty or Tfcp2l1 vector and cultured in serum without LIF for one passage. Scale bar, 100 μm. (**I**) mESCs were deprived of LIF for 12 h, and then treated with indicated kinase inhibitors for 1 h before LIF stimulation for 12 h, as indicated on timeline (lower right). *Socs3* and *Tfcp2l1* transcripts were evaluated at − 1, 1, and 12 h by qRT–PCR. Data represent mean ± s.d. of three biological replicates. Source data for this figure is available on the online supplementary information page.

protein kinase (MAPK) (Hirai *et al*, 2011). To determine which of these signalling pathways are involved in the induction of *Tfcp2l1* by LIF, we treated 46C mESCs with inhibitors specific for JAK, PI3K, and MAPK, and then assessed the induction of *Tfcp2l1* by LIF. Upregulation of *Socs3* by LIF stimulation was completely prevented by JAK inhibitor I but not by the PI3K and MAPK inhibitors (Figure 3I). The presence of JAK inhibitor I also prevented the induction of *Tfcp2l1* by LIF, whereas PI3K inhibitor had no effect, and treatment with MAPK inhibitor PD03 was associated with strong *Tfcp2l1* induction as expected (Figure 3I). Taken together, our results suggest that LIF-induced expression of *Tfcp2l1* is mediated by Stat3, but not by PI3K or MAPK. These observations indicate that a high level of Tfcp2l1 is an important factor in liberating mESCs from LIF dependence and in promoting self-renewal downstream of Stat3.

Given that CHIR, PD03, and LIF exhibit a similar ability to induce Tfcp2l1 expression, we wanted to examine whether these three mediators depend on one another for their upregulative effect. We pre-treated mESCs with JAK inhibitor I (Stat3 signalling inhibitor), 53AH (Wnt signalling inhibitor) (Willems *et al*, 2011), or bFGF (activator of FGF/MEK signalling pathway) for 1 h before adding LIF, CHIR, or PD03. Twelve hours after the addition of LIF, CHIR, or PD03, *Egr1*, a target of the FGF/MEK signalling pathway, was induced by bFGF but repressed by MEK inhibitor PD03 (Figure 4A). *Axin2*, a target of the Wnt signalling pathway, was induced by CHIR but repressed by 53AH (Figure 4B); and *Socs3*, a target of the LIF/Stat3 signalling pathway, was induced by LIF but repressed by JAK inhibitor I (Figure 4C). *Tfcp2l1* expression was considerably elevated following treatment with LIF, CHIR, or PD03, even in the presence of irrelevant signalling pathway inhibitors/activator (Figure 4A–C), demonstrating that CHIR, PD03, and LIF can induce *Tfcp2l1* transcription independent of one another.

### Downregulation of Tfcp2l1 impairs mESC self-renewal in LIF condition

To test whether *Tfcp2l1* knockdown has an effect on LIF/Stat3- or 2i-mediated mESC self-renewal, we infected 46C mESCs with lentiviruses encoding two short-hairpin RNAs (shRNAs) specific for *Tfcp2l1* mRNA (shTfcp2l1). Stable knockdown (70–80%) of *Tfcp2l1* transcript levels was observed following drug selection (Figure 5A). sh*Tfcp2l1*

mESCs formed fewer colonies than did mESCs expressing scramble control shRNAs, and these colonies exhibited a comparatively flatter morphology, even in the presence of LIF (Figure 5B and C). Accordingly, these sh*Tfcp2l1* cells expressed a low level of the pluripotency markers *Oct4*, *Sox2*, *Nanog*, and *Klf4*, but high levels of the differentiation-related genes *Cdx2*, *Nestin*, *T*, *Gata6*, and *FoxA2* (Figure 5D). To exclude off-target effects of the two shRNAs used in *Tfcp2l1* knockdown, we modified the *Tfcp2l1* expression construct to encode *Tfcp2l1* mRNA harbouring synonymous mutations at the two sites targeted by our shRNAs (see Materials and methods), and tested whether transfection with this mutant construct could rescue the knockdown phenotypes. Stable mutant-Tfcp2l1 transfectants formed significantly more alkaline phosphatase-positive colonies in serum/LIF than did empty-vector controls following infection with sh*Tfcp2l1* lentiviruses (Figure 5E and F). These results reveal that Tfcp2l1 can mediate LIF/Stat3-dependent self-renewal in ESCs. sh*Tfcp2l1* mESCs cultured in 2i maintained a higher level of *Tfcp2l1* expression than did sh*Tfcp2l1* mESCs in serum + LIF (Figure 5G). Consistent with this, sh*Tfcp2l1* mESCs cultured in 2i maintained an undifferentiated phenotype, although they formed smaller colonies than did mESCs expressing scramble control shRNA (Figure 5H).

Tfcp2l1 facilitates epiblast stem cells reprogramming

Tfcp2l1 expression, like that of *Oct4* and *Nanog*, is robust in undifferentiated mESCs, but declines abruptly upon induction of embryoid body (EB) formation (Figure 6A), indicating that *Tfcp2l1* is a pluripotency marker. On the basis of this finding and previous reports that direct transfection with other Stat3 targets such as *Gbx2*, *Klf4*, *Klf2*, or *Nanog* can reprogram epiblast stem cells (EpiSCs) to a state of naïve pluripotency (Guo *et al*, 2009; Guo and Smith, 2010; Yang *et al*, 2010; Tai and Ying, 2013), we tested whether transfection of EpiSCs with Tfcp2l1 could induce naïve pluripotency.

We induced the differentiation of 46C mESCs into EpiSCs, and examined the expression of typical ESC markers. 46C EpiSCs stained negative for alkaline phosphatase and expressed a higher level of *Fgf5* and a lower level of *Tfcp2l1*, *Rex1*, and *Nanog* compared with 46C mESCs (Figure 6B and C). 46C EpiSCs were then transfected with a PiggyBac vector encoding *Tfcp2l1* and analysed by western blotting for
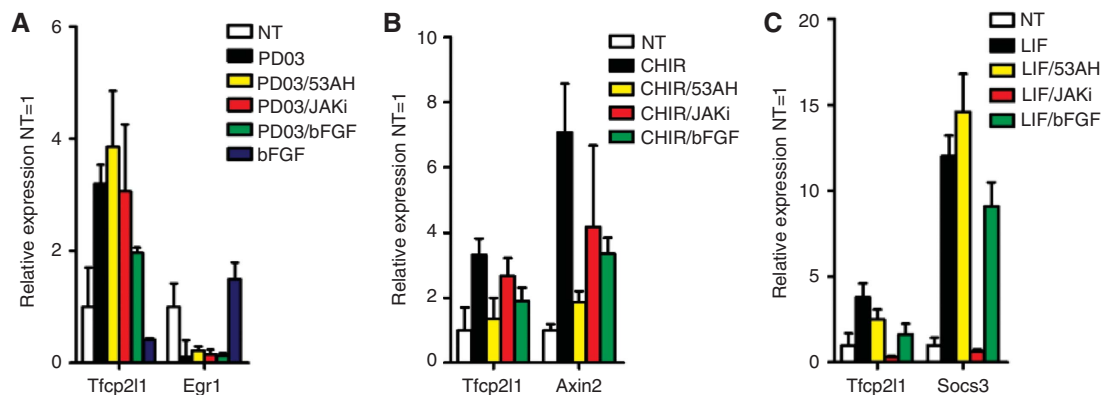

Figure 4 PD03, CHIR, and LIF induce Tfcp2l1 independently. (**A–C**) mESCs were deprived of LIF for 12 h, and then treated with 1 μm 53AH, 10 μM JAK inhibitor I, or 20 ng/ml bFGF for 1 h before LIF, CHIR, or PD03 stimulation. *Egr1*, *Axin2*, *Socs3*, and *Tfcp2l1* transcript levels were evaluated 12 h later by qRT–PCR. Data represent mean ± s.d. of three biological replicates.
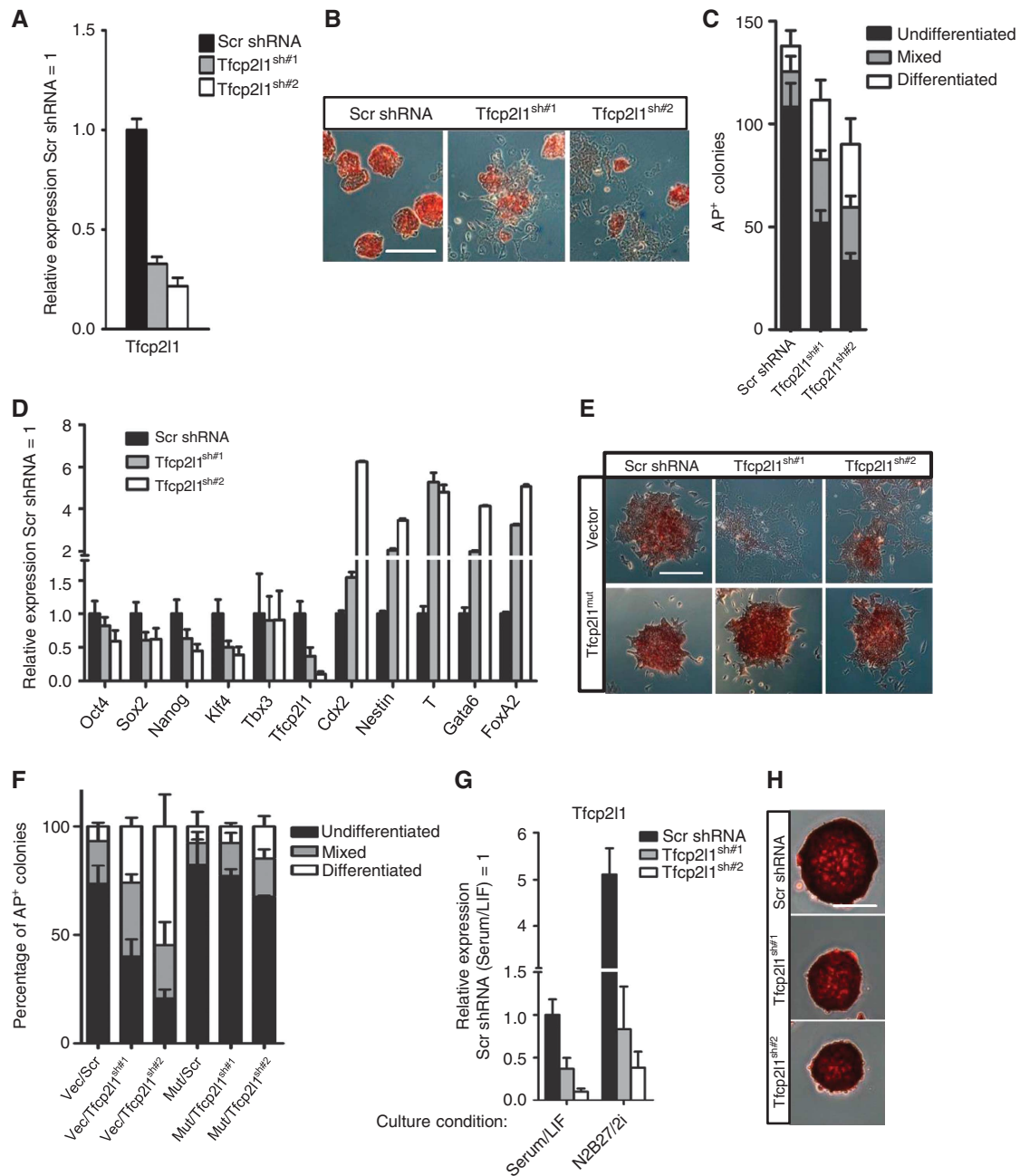
Figure 5 Knockdown of Tfcp2l1 impairs Stat3- but not 2i-mediated mESC self-renewal. (**A**) qRT–PCR analysis of *Tfcp2l1* expression in shRNA knockdown cells. The transcript level was normalized against scramble shRNA control. Data represent mean ± s.d. of three biological replicates. (**B**) Alkaline phosphatase staining of shTfcp2l1 mESC colonies cultured in serum/LIF condition for 8 days. Scale bar, 100 μm. (**C**) Quantification of alkaline phosphatase-positive shTfcp2l1 mESC colonies. Data represent mean ± s.d. from triplicate experiments. (**D**) qRT–PCR analysis of ESC pluripotency marker (*Oct4, Sox2, Nanog, Klf4,* and *Tbx3*) and differentiation-associated gene (*Cdx2, Nestin, T, Gata6,* and *FoxA2*) expression in shTfcp2l1 ESCs cultured in the presence of LIF. Transcript levels were normalized against scramble shRNA control. Data represent mean ± s.d. of three biological replicates. (**E, F**) Alkaline phosphatase staining and quantification of alkaline phosphatase-positive empty vector and mutant-Tfcp2l1 (Tfcp2l1^mut) colonies transfected with Tfcp2l1 shRNA lentivirus and cultured in serum/LIF medium. Scale bar, 100 μm. (**G**) qRT–PCR analysis of *Tfcp2l1* expression in *Tfcp2l1* shRNA knockdown and scramble shRNA control cells cultured in serum/LIF or N2B27/2i. Data represent mean ± s.d. of three biological replicates. (**H**) Alkaline phosphatase staining of shTfcp2l1 mESC colonies cultured in N2B27/2i for five passages. Scale bar, 100 μm.

expression of Tfcp2l1 (Figure 6D). Empty-vector control 46C EpiSCs died or differentiated within 8 days of being switched to serum/LIF/2i, whereas *Tfcp2l1*-46C EpiSCs gave rise to many ESC-like colonies with dome-shaped morphology (Figure 6E). We picked up three of these colonies and transferred them to normal mESC culture medium (serum/LIF without 2i). After two passages, these cell populations retained robust alkaline

phosphatase activity (Figure 6F). To address the possibility that these results reflect mixed populations of reprogrammed 46C cells and original 46C mESCs, we derived EpiSCs from embryonic day 5.5 CD1 mouse embryos, and then introduced *Tfcp2l1*. One million transfected EpiSCs were cultured for 10 days in serum/LIF/2i and gave rise to ~80 alkaline phosphatase-positive ESC-like colonies (Figure 6G and H).
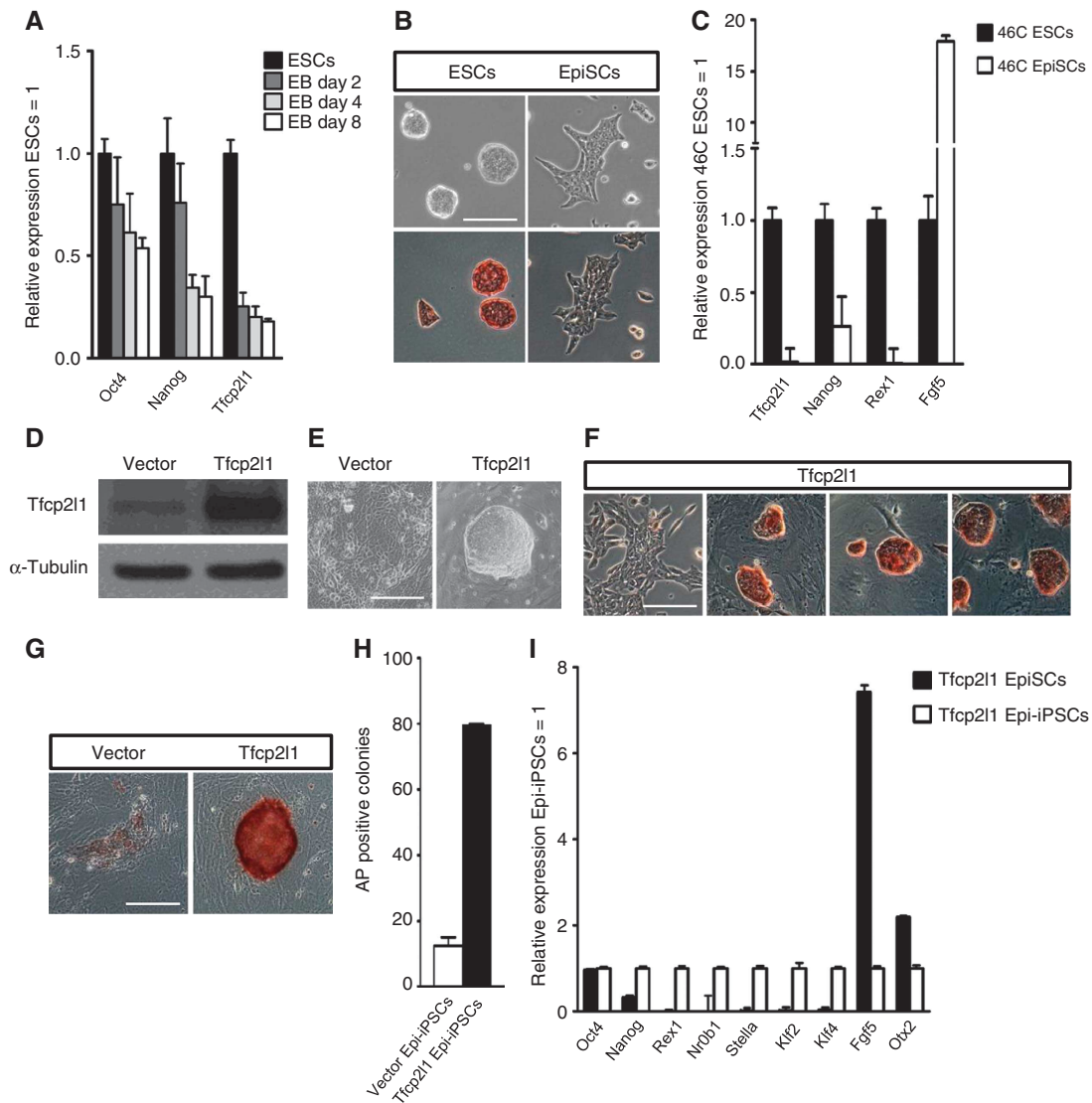
Figure 6 Tfcp2l1 reprograms EpiSCs to naïve pluripotency. (**A**) 46C mESCs and 46C-derived embryoid bodies were harvested. *Oct4, Nanog*, and *Tfcp2l1* transcript levels were analysed by qRT–PCR. Data represent mean ± s.d. of three biological replicates. (**B**) Morphology and alkaline phosphatase staining of 46C mESCs and 46C mESC-derived EpiSCs cultured in Activin A, bFGF, and 53AH for eight passages. Scale bar represents 100 μm. (**C**) Expression patterns of *Tfcp2l1, Nanog, Rex1*, and *Fgf5* in 46C mESCs and EpiSCs. Data represent mean ± s.d. of three biological replicates. (**D**) Western blot analysis of Tfcp2l1 in 46C EpiSCs with stable Tfcp2l1 transgene expression. (**E**) Morphology of empty and Tfcp2l1 vector EpiSC transfectants cultured in serum medium supplemented with LIF/2i for 8 days. Scale bar represents 100 μm. (**F**) Alkaline phosphatase staining of Tfcp2l1 EpiSCs and three Tfcp2l1 Epi-iPSC (induced pluripotent stem cell) clones. Scale bar represents 100 μm. (**G, H**) 10^5 empty and Tfcp2l1 vector-transfected CD1 EpiSCs cultured in serum medium supplemented with LIF/2i. After 10 days, alkaline phosphatase-positive colonies were photographed and counted under a microscope. Scale bar represents 100 μm. (**I**) Comparison of gene marker expression in Tfcp2l1 Epi-iPS cells and CD1 EpiSCs. *Nanog, Klf2, Klf4, Nr0b1, Rex1*, and *Stella* are ESC markers, whereas *Fgf5* and *Otx2* are EpiSC markers. Data represent mean ± s.d. of three biological replicates. Source data for this figure is available on the online supplementary information page.

These converted ESC-like cells could be continuously expanded in serum/LIF without feeders, and expressed high levels of the naïve pluripotency markers *Nanog, Rex1, Nrob1, Stella, Klf2*, and *Klf4*, but low levels of the EpiSC-specific markers *Fgf5* and *Otx2* (Figure 6I). In contrast, far fewer colonies arose from empty-vector transfectants and these colonies died or differentiated after passaging. These results show that Tfcp2l1 is capable of converting EpiSCs into a state of naïve pluripotency.

Tfcp2l1 contributes to ground-state pluripotency via Nanog

The expression of pluripotency genes *Oct4, Sox2, Klf4*, and *Nanog* was downregulated in sh*Tfcp2l1* mESCs (Figure 5D).

We examined the induction of these genes in i-Tfcp2l1 mESCs upon Dox treatment, and detected strong upregulation of *Nanog* and *Klf4* (Figure 7A). Knockdown of *Klf4* in i-Tfcp2l1 mESCs (Supplementary Figure S3A) failed to impair the self-renewal-promoting effect of Tfcp2l1 (Supplementary Figure S3B). Nanog is one of the most important members in the core pluripotency network of mESCs (Silva *et al*, 2009). It is a Stat3 downstream target and forced Nanog expression can bypass the need for LIF/Stat3 signalling in the clonal expansion of mESCs (Chambers *et al*, 2003; Niwa *et al*, 2009). Although mESCs in serum/LIF express heterogeneous levels of Nanog, the addition of MEK inhibitor can increase the fraction of Nanog-positive cells and the expression level of *Nanog* with no alteration in the levels of *Oct4* (Figure 7B)
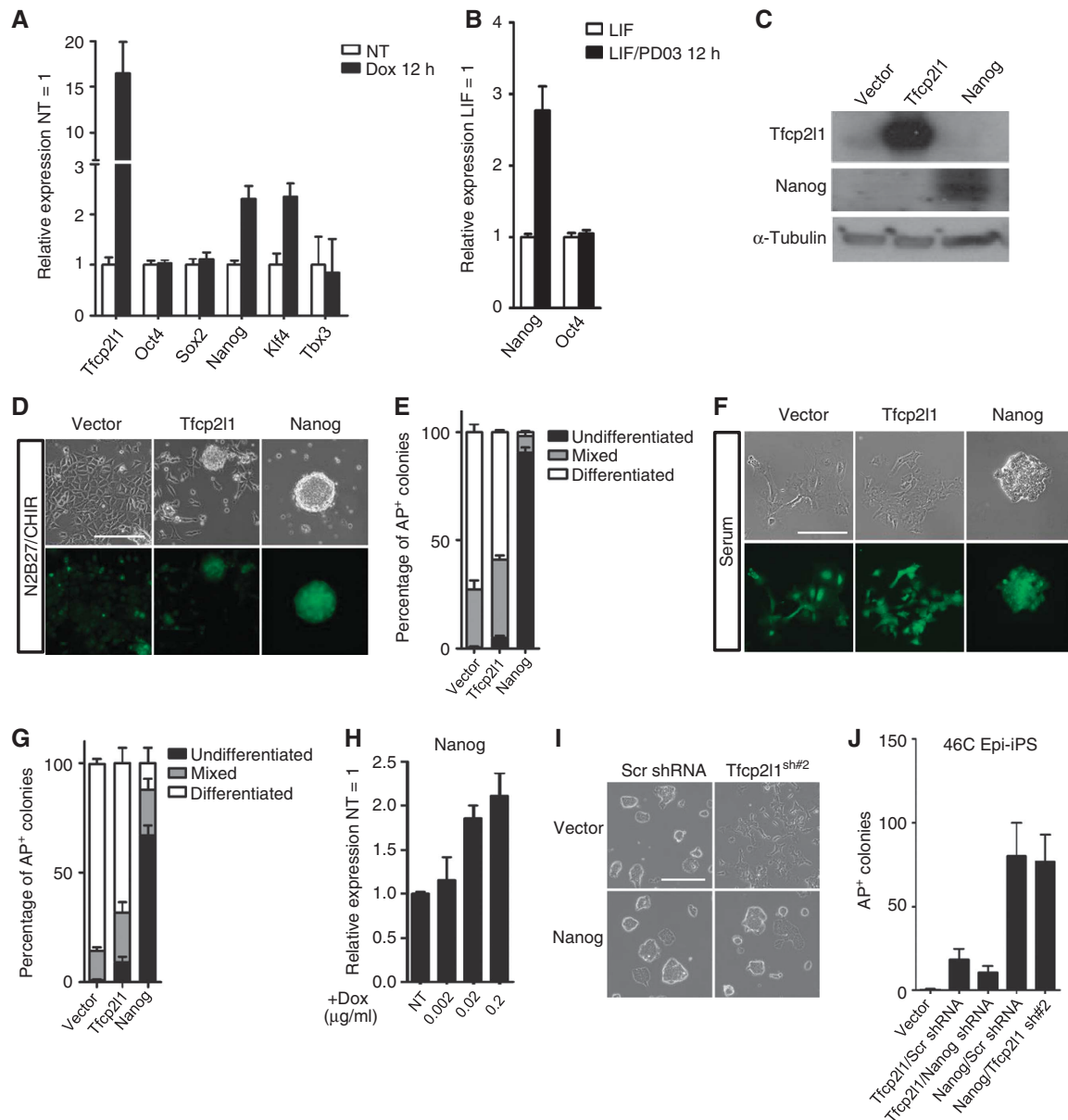
Figure 7 Nanog is critical for the function of Tfcp2l1 in mESCs. (**A**) qRT–PCR analysis of *Tfcp2l1*, *Oct4*, *Sox2*, *Nanog*, *Klf4*, and *Tbx3* expression in i-Tfcp2l1 mESCs cultured in the presence or absence of Dox for 12 h. Data represent mean ± s.d. of three biological replicates. (**B**) qRT–PCR analysis of *Nanog* and *Oct4* gene expression in 46C mESCs cultured in the presence of LIF with or without PD03 for 12 h. (**C**) Expression vectors for Tfcp2l1 or Nanog were introduced into Nanog-null mESCs, and the protein levels were assayed by western blotting. (**D**, **E**) Morphology and quantification of alkaline phosphatase-positive Nanog-null mESC colonies expressing Tfcp2l1 or Nanog transgene cultured in N2B27/CHIR. Nanog-null mESCs are GFP positive. Scale bar, 100 μm. Data represent mean ± s.d. from triplicate experiments. (**F**, **G**) Morphology and quantification of alkaline phosphatase-positive Nanog-null mESC colonies expressing Tfcp2l1 or Nanog transgene cultured in serum medium. Scale bar represents 100 μm. (**H**) *Nanog* expression in i-Tfcp2l1 mESCs treated with different concentrations of Dox. Data represent mean ± s.d. of three biological replicates. (**I**) Morphology of Nanog-overexpressing or empty vector control 46C mESCs infected with scramble or Tfcp2l1 shRNA lentivirus. Scale bar, 100 μm. (**J**) 46C EpiSCs transfected with indicated constructs were cultured in 2i/LIF for 10 days and stained for alkaline phosphatase activity. Alkaline phosphatase-positive colonies were quantified. Data represent mean ± s.d. from triplicate experiments. Source data for this figure is available on the online supplementary information page.

(Silva *et al*, 2009). To test whether Nanog is critical for Tfcp2l1 expression-based maintenance of self-renewal, we introduced constitutive *Tfcp2l1* and *Nanog* transgenes into Nanog-null mESCs (Figure 7C) in which GFP is a Nanog knockout signal and is expressed constitutively (Figure 7D and F). Stable transfectants were obtained and then transferred into N2B27 plus CHIR. Nanog-null mESCs overexpressing Nanog formed tightly compact colonies and stained mostly positive for alkaline phosphatase (Figure 7D and E) (Chambers

et al, 2007). However, *Tfcp2l1* transfectants differentiated (Figure 7D and E), as they did when cultured in serum only (Figure 7F and G). Previous Tfcp2l1 ChiP-seq data have shown sharp binding peaks associated with the *Nanog* promoter (Chen *et al*, 2008). Consistent with this, we found that *Nanog* expression in i-Tfcp2l1 mESCs increased in a Dox dose-dependent manner (Figure 7H), and that elevated Nanog expression could rescue the differentiation phenotype induced by *Tfcp2l1* knockdown (Figure 7I). We also found

that decreased *Nanog* expression impaired the ability of Tfcp2l1 to mediate the reprogramming of EpiSCs to naïve-state ESCs (Figure 7J). These results suggest that *Nanog* is a downstream Tfcp2l1 target, and that the function of the latter in mESC self-renewal is dependent on Nanog.

Tfcp2l1 maintains mESC self-renewal in a manner dependent upon DNA-binding activity

The transcription factors of the CP2 family have a conserved N-terminal domain involved in DNA binding and critical for their regulatory activity (Shirra *et al*, 1994). A Tfcp2 mutant
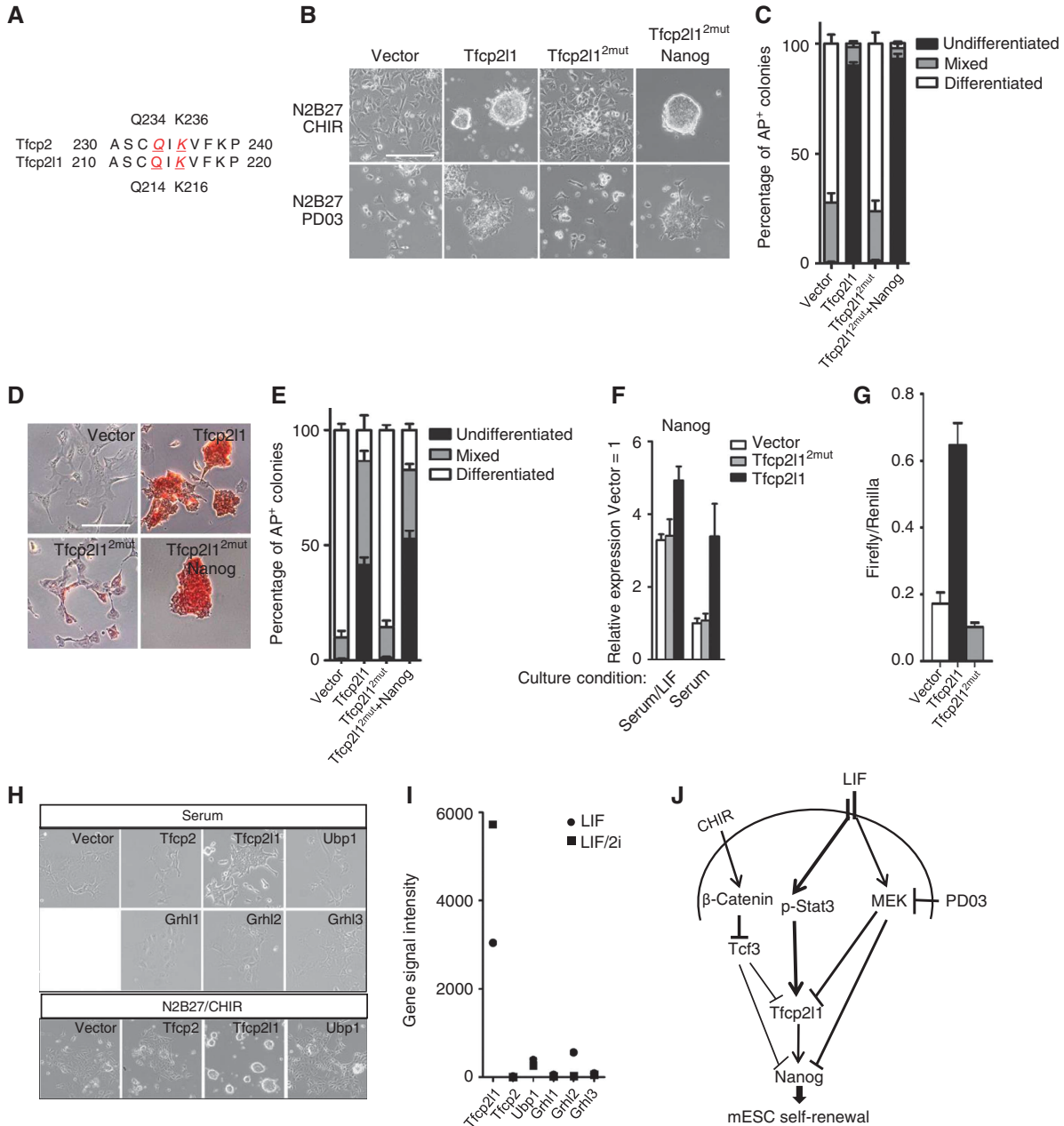
Figure 8 DNA-binding activity is required for the function of Tfcp2l1 in mESCs. (**A**) BLAST protein sequence of Tfcp2 (amino acids 230–240) and Tfcp2l1 (amino acids 210–220). (**B, C**) Morphology and quantification of alkaline phosphatase-positive colonies arising from empty vector, Tfcp2l1, and Tfcp2l1^{2mut} transfectants cultured in N2B27/CHIR or N2B27/PD03 for three passages. Scale, 100 µm. Data represent mean ± s.d. from triplicate experiments. (**D, E**) Alkaline phosphatase staining and quantification of positive colonies arising from empty vector, Tfcp2l1 and Tfcp2l1^{2mut} transfectants cultured in serum medium for 10 days. Scale bar, 100 µm. Data represent mean ± s.d. from triplicate experiments. (**F**) qRT–PCR analysis of *Nanog* expression in empty vector, Tfcp2l1, and Tfcp2l1^{2mut} transfectants after withdrawal of LIF for 12 h (Serum) or in the presence of LIF (Serum/LIF). Data represent mean ± s.d. of three biological replicates. (**G**) Luciferase reporter assays of *Nanog* promoter construct transiently transfected into empty vector, Tfcp2l1 and Tfcp2l1^{2mut} mESCs. Data represent mean ± s.d. from triplicate experiments. (**H**) Morphology of different CP2 family member transfectants cultured for three passages in normal mESC medium without LIF or in N2B27 with CHIR or PD03. Scale bars, 100 µm. (**I**) Gene signal intensities of different CP2 family members in C57BL/6 mESCs cultured in LIF or LIF/2i. (**J**) Tfcp2l1 at the crossroads of LIF/Stat3, Wnt/β-catenin, and FGF/MEK signalling pathways. Tfcp2l1 expression is independently induced by LIF, CHIR, and PD03 by activation of Stat3, activation of Wnt/β-catenin, and suppression of FGF/MEK pathways, respectively. Elevated Tfcp2l1 expression can recapitulate the separate effects of LIF and PD03, and partially that of CHIR, in promoting mESC self-renewal. Tfcp2l1 induces and requires Nanog expression for its self-renewal-promoting effect.

containing two amino-acid substitutions, Q234L and K236E, exhibits no DNA-binding ability (Shirra *et al*, 1994). The sites modified in the Tfcp2 mutant correspond to two homologous sites within the DNA-binding domain of Tfcp2l1, Q214, and K216. To test the functional role of these sites, we generated a Tfcp2l1 mutant containing the amino-acid substitutions Q214L/K216E (Tfcp2l1^{2mut}) (Figure 8A). Tfcp2l1^{2mut}-overexpressing ESCs cultured in N2B27/CHIR differentiated (Figure 8B and C). The same result was obtained when Tfcp2l1^{2mut} mESCs were cultured in serum medium without LIF (Figure 8D and E). As expected, elevated *Nanog* could rescue the self-renewal defect in Tfcp2l1^{2mut} mESCs (Figure 8B and D). *Nanog* expression in Tfcp2l1^{2mut} mESCs cultured in serum/LIF declined following LIF withdrawal, but remained high in Tfcp2l1 mESCs in the absence of LIF (Figure 8F). Consistent with this result, a luciferase reporter assay showed that wild-type Tfcp2l1 but not Tfcp2l1^{2mut} could activate the *Nanog* promoter (Figure 8G). These results indicate that the intact DNA-binding domain of Tfcp2l1 is essential for Tfcp2l1-mediated induction of *Nanog* and maintenance of ESC self-renewal. Future exploration of the effectors regulating, binding, or being controlled by this region will extend our understanding of the transcriptional network that governs pluripotency.

Considering that members of the CP2 family are highly conserved, we wanted to assess whether they have functional redundancy in mediating mESC self-renewal. We cloned all factors of the CP2 family (*Tfcp2*, *Tfcp2l1*, *UBP1*, *Grhl1*, *Grhl2*, and *Grhl3*) and introduced the corresponding expression constructs into mESCs. Only *Tfcp2l1* was able to promote ESC self-renewal either in serum in the absence LIF or in N2B27/CHIR (Figure 8H). Microarray analysis showed the endogenous expression level of the other CP2 genes to be low compared with *Tfcp2l1*, and that their expression, unlike that of *Tfcp2l1*, cannot be induced by 2i (Figure 8I). These results show that other members of the CP2 family do not function or respond in mESCs in the same manner as *Tfcp2l1*.

Discussion

Our experiments show that Tfcp2l1 is a common downstream target of mESC self-renewal-promoting pathways induced by LIF, CHIR, and PD03, and that overexpression of Tfcp2l1 can substitute for each of these mediators for the maintenance of mESC self-renewal. Forced expression of Tfcp2l1 also suffices for the reprogramming of EpiSCs to a naïve pluripotent state. Our study therefore establishes Tfcp2l1 as a common and critical mediator of self-renewal-promoting pathways in ESCs (Figure 8J).

Previous gene expression profiling and ChiP-seq analysis of ESCs have suggested potential integration of Tfcp2l1 into the pluripotency gene regulatory network, but the functional significance of Tfcp2l1 in maintaining pluripotency has not been established (Chen *et al*, 2008). Tfcp2l1 contains DNA-binding and oligomerization domains similar to those of other CP2 family members Tfcp2, Ubp1, Grhl1, Grhl2, and Grhl3 (Rodda *et al*, 2001). All these CP2 family members, including Tfcp2l1, have been implicated in promoting epidermal development (Wilanowski *et al*, 2002; Kokoszynska *et al*, 2008). Interestingly, Tfcp2l1 is the only CP2 family member that is highly expressed in mESCs, is induced by LIF, CHIR, and PD03, and can promote mESC self-renewal

(Figure 8H and I). Tfcp2l1 has been proposed to be a downstream target of canonical Wnt/β-catenin (Patel *et al*, 2011; Martello *et al*, 2012) and FGF/MEK signalling pathways (Patel *et al*, 2011). CHIR induces the expression of Tfcp2l1 likely by activating the Wnt/β-catenin pathway, as inhibition of this pathway in mESCs results in failure of Tfcp2l1 to exhibit upregulated expression in the presence of CHIR (Figure 4B). How inhibition of FGF/MEK induces Tfcp2l1 expression, however, remains unknown. Induction of Tfcp2l1 by LIF is mediated through activation of Stat3 and is independent of Wnt/β-catenin and FGF/MEK signalling pathways (Figure 4C). It will be of interest to investigate how three seemingly independent signalling pathways induce the expression of the same downstream target, Tfcp2l1.

LIF, CHIR, and PD03 act synergistically to maintain mESC self-renewal. When used individually, none of them is sufficient to maintain mESC self-renewal under feeder- and serum-free conditions, and the combined use of any two of the three is required (Wray *et al*, 2010), suggesting that these agents drive complementary or overlapping programs for maintaining mESC self-renewal. mESCs are susceptible to differentiative cues, and thus maintenance of the mESC state requires self-renewal-promoting and/or differentiation-suppressing signals provided by LIF, CHIR, and PD03. It is likely that these signals need to reach a certain threshold in order to counteract differentiation-inducing signals. Tfcp2l1 appears to be an LIF-, CHIR-, and PD03-induced self-renewal-promoting factor whose overexpression acts additively with any of these three agents to reach the threshold. shRNA-mediated knockdown of *Tfcp2l1* impairs mESC self-renewal maintained by serum/LIF but not by 2i (Figure 5B and H). This could be due to the fact that the expression of residual *Tfcp2l1* in 2i is higher than in serum/LIF (Figure 5G). It is equally possible that 2i produces a stronger self-renewal signal than serum/LIF does, and that the 2i self-renewal signal is still above the threshold required for ESC maintenance even after knockdown of *Tfcp2l1*.

Apart from its role in promoting mESC self-renewal, Tfcp2l1 also acts as a transcription factor capable of reprogramming EpiSCs to the naïve ground state of ESCs. Tfcp2l1 is highly and uniformly expressed in the mouse inner cell mass, from which ESCs are derived, but barely detectable in post-implantation epiblasts (Rogers *et al*, 1991; Pelton *et al*, 2002). Tfcp2l1 interacts with Esrrb and Klf5, the two transcription factors that can replace Klf4 in 'Yamanaka factor'-mediated reprogramming (Takahashi and Yamanaka, 2006; van den Berg *et al*, 2010). Tfcp2l1 binds to the promoter regions of *Klf4* and *Nanog*, indicating a potential role in regulating their transcription (Chen *et al*, 2008). Klf4, Klf5, and Nanog have all been shown to be capable of reprogramming EpiSCs to an ESC state (Hall *et al*, 2009; Yang *et al*, 2010; Festuccia *et al*, 2012). Our finding that Tfcp2l1 can also convert EpiSCs into ESCs therefore seems to be consistent with its reported relationship to other mediators of reprogramming. Whether the reprogramming activity of Tfcp2l1 requires the presence of Klf4/5 and/or Nanog or can instate naïve pluripotency independent of these factors warrants further investigation. Tfcp2l1 is also highly expressed in the inner cell mass of human blastocysts, but becomes significantly downregulated during derivation of human ESCs, which share defining features with mouse EpiSCs (O'Leary *et al*, 2012), suggesting that Tfcp2l1 might

also play a role in establishing and maintaining naïve pluripotency in human ESCs, as it does in mESCs.

The potential role of Tfcp2l1 in regulating Nanog expression suggested by previous ChIP-seq analysis (Chen *et al*, 2008) was functionally demonstrated in our study: increased expression of *Tfcp2l1* upregulates Nanog expression, whereas knockdown of *Tfcp2l1* downregulates Nanog expression (Figures 5D, 7A, and H). It is noteworthy that Nanog expression has been shown to be indirectly regulated by LIF, CHIR, and PD03 (Pereira *et al*, 2006; Niwa *et al*, 2009; Martello *et al*, 2012; Miyanari and Torres-Padilla, 2012). LIF/Stat3 regulates Nanog expression through induction of Klf4 (Niwa *et al*, 2009); CHIR treatment releases the suppression of *Nanog* by Tcf3 (Pereira *et al*, 2006; Martello *et al*, 2012); PD03 elevates Nanog expression by inducing a transient switch from monoallelic to biallelic expression (Silva *et al*, 2009; Miyanari and Torres-Padilla, 2012). Tfcp2l1's self-renewal-promoting effect in mESCs depends on the presence of Nanog, which together with the foregoing findings, suggests that Nanog is a critical mediator of Tfcp2l1-promoted ESC self-renewal.

In summary, our study reveals a novel function of Tfcpl21 in establishing and maintaining pluripotency of mESCs. To our knowledge, Tfcp2l1 and Nanog are the only two transcription factors identified as common downstream targets of LIF, CHIR, and PD03 that possess the capacity for both promoting mESC self-renewal and reprogramming to a naïve ESC state. Understanding how Tfcp2l1 and Nanog cooperate with other downstream targets of LIF, CHIR, and PD03 to maintain the pluripotent ESC state might facilitate the development of new culture conditions for the derivation of authentic ESCs from species other than mice and rats, as the fundamental molecular basis of pluripotency is likely shared among different species.

Materials and methods

Cell culture
mESCs were cultured on 0.1% gelatin-coated dishes, at 37°C in 5% CO_2. Medium for routine maintenance was GMEM (Sigma, G5414) supplemented with 10% FCS (HyClone), 1% MEM non-essential amino acids (Invitrogen), 2 mM GlutaMax (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen), and 100 units/ml LIF (prepared in-house). For serum-free culture, mESCs were maintained in N2B27 (Ying *et al*, 2008) supplemented with 3 μM CHIR99021 and 1 μM PD0325901 (Synthesized in the Division of Signal Transduction Therapy, University of Dundee, UK).

Generation of inducible EGFP and Tfcp2l1
Tfcp2l1 was cloned from mESC cDNA with KOD Hot Start DNA Polymerase (EMD) and inserted into the *Sal*I and *Not*I sites of the p2Lox vector. A2Lox.cre mESC was seeded onto DR4 MEFs in a 3.5-cm dish and transfected with 2 μg p2Lox-Tfcp2l1 or p2Lox-EGFP plasmid using the Lipofectamine™ LTX Reagent (Invitorgen) according to the manufacturer's instructions. Selection was initiated the next day by adding 300 μg/ml of G418 (Gibco) and maintained for 3 weeks. Surviving colonies were isolated and replated onto DR4 MEFs for expansion.

In vitro differentiation of i-Tfcp2l1 ESCs
i-Tfcp2l1 ESCs were plated onto uncoated Petri dishes, in serum medium. Cells were allowed to grow in suspension for 8 days, and the resulting EBs were digested and then plated onto 0.1% gelatin-coated dishes, in serum medium. The expression of markers for the three germ layers was examined by immunostaining at day 11. For neural and cardiac cell differentiation, we applied the $4^-/4^+$ method (Bain *et al*, 1995).

Overexpression and knockdown plasmid construction
The coding regions of CP2 family genes and *Nanog* were cloned from mESC cDNA with KOD and inserted into the PiggyBac vector. Overlapping PCR was used to generate the Tfcp2l1$^{Q214L/K216E}$ mutant. Base substitutions in the relevant encoding region, as indicated by italics, were AGCTGC*CTG*ATC*GAG*GTGTTCAA. For generating the Tfcp2l1 mutant immune to Tfcp2l1sh#1- and Tfcp2l1sh#2-mediated knockdown, the following mutations corresponding to Tfcp2l1 shRNA-targeted regions were introduced: GCTCTT*TAACGCAATAA AGGGGAGAAACGTAAGACCGAAGA*. For RNA interference in mESCs, shRNA constructs were designed to target 21 base-pair gene-specific regions of *Tfcp2l1* and were then cloned into plko.1-TRC (*Age*I and *Eco*RI sites). The targeted sequences are as follows: *Tfcp2l1* sh#1: GCAGGAATGTGAGGCCAAAGA; *Tfcp2l1* sh#2: GCTCT TCAATGCCATCAAAGG; *Klf4*: GTCAGCTTGTGAATGGATAAT.

Cell transfection and virus production
For gene overexpression, ESCs or EpiSCs were transfected with 2 μg of PiggyBac inserted with genes plus 2 μg transposase vector using LTX according to the manufacturer's instructions. Selection was begun the next day by adding puromycin to a final concentration of 2 μg/ml and continued for 1 week. For the knockdown experiment, Plko.1-TRC-based lentiviral vectors and packaging plasmids pMD2.G and psPAX2 were transfected into 293FT cells using LTX. Supernatant was collected after 48 h and passed through 0.45 μm filters (Millipore). ESCs were cultured in the viral supernatant in the presence of 8 μg/ml polybrene (Sigma) for 24 h. Colonies were then selected for 1 week in 2 μg/ml of puromycin.

Luciferase reporter assay
Nanog promoter (-1611 to $+50$) was inserted into pGL3-basic plasmid (Promega), and co-transfected with a Renilla luciferase plasmid (Promega) into mESCs overexpressing Tfcp2l1 or Tfcp2l1^{2mut} or empty vector control mESCs. Cells were harvested after 48 h, and the luciferase activity of the lysate was measured with the Dual-Luciferase reporter assay system (Promega).

qRT–PCR
Total RNA was extracted using the RNEasy Mini Kit (QIAGEN). cDNA was synthesized from 1 μg total RNA using the QuantiTect Reverse Transcription Kit (QIAGEN) according to the manufacturer's instructions. qRT–PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) in an ABI7900HT Real-Time PCR machine (Applied Biosystems). Target gene expression was normalized to GAPDH expression. The primers used are listed in Supplementary Table S1.

Alkaline phosphatase activity assay
The alkaline phosphatase activity of mESCs cultured on gelatin-coated plates was detected using the Alkaline Phosphatase Kit (Sigma).

Western blotting
Cells were lysed in ice-cold RIPA cell buffer (TEKNOVA) supplemented with protease inhibitors (Thermo Scientific). The proteins were separated with a 4–15% PAGE gel (Bio-Rad) and electrotransferred onto a PVDF membrane. Probing was performed with specific primary antibodies and HRP-conjugated secondary antibodies. The primary antibodies used were Tfcp2l1 (N-20; Santa Cruz, 1:200), Nanog (AF2729; R&D, 1:500), Flag (M2; Sigma, 1:2000), GFP (4B10; CST, 1:200), α-tubulin (32-2500; Invitrogen, 1:2000), p-ERK (E-4; Santa Cruz, 1:1000), and ERK1 (K-23; Santa Cruz, 1:1000).

Immunofluorescence staining
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). Cells were incubated in the presence of primary antibodies at 4°C overnight and then washed three times in 1 × PBS. Cells were then incubated with Alexa Fluor 488 (Invitrogen, 1:1000) secondary antibody for 1 h at 37°C. Nuclei were stained with Hoechst (Invitrogen, 1:10 000). The primary antibodies and dilutions used are Oct4 (sc-5279; Santa Cruz, 1:200), SSEA1 (sc-21702; Santa Cruz, 1:100), Nestin (2Q178; Santa Cruz, 1:100), Gata4 (G4; Santa Cruz, 1:100), and Myosin (MF-20; DSHB, 1:50).

EpiSC derivation and reprogramming

For ESC-to-EpiSC differentiation, 5000 46C mESCs were plated into one 0.1% gelatin-coated well of a 12-well plate and cultured in serum medium supplemented with Activin A (10 ng/ml; Peprotech), bFGF (10 ng/ml; Peprotech), and 53AH (1 μm; Cellagen Technology). Cells were passaged every 3–4 days. For EpiSC derivation, E5.5 embryos from CD1 mice were cultured in the Activin A/bFGF/53AH medium. Cells were used between passages 8 and 15. For reprogramming, 1×10^5 transfectants were seeded onto one T75 flask. After 24 h, the medium was changed to mESC medium supplemented with LIF/2i and replaced every second day. The number of alkaline phosphatase-positive clones was counted microscopically. EpiSC reprogrammed to ESC-like clones were picked after 20 days and subsequently expanded in mESC medium with LIF. Animal experiments were performed according to the investigator's protocols approved by the USC Institutional Animal Care and Use Committee.

Accession numbers

Our Microarray data set has been deposited in the GEO database with ID number GSE46369.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Conflict of interest

The authors declare that they have no conflict of interest.

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