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

Shoudong Ye, Ping Li, Chang Tong and Qi-Long Ying

Corresponding author: Qi-Long Ying, University of Southern California

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Thomas Schwarz-Romond

1st Editorial Decision 22 May 2013

Thank you very much for submitting your study placing Tfcp1l into the pluripotency network for consideration to The EMBO Journal editorial office.

The attached comments indicate general interest and novelty in your findings. However, they also emphasize that a better crystallization of Tfcp1l's contribution within the self-renewal circuitry and its position relative to Nanog, respective stronger and direct evidence for Nanog's regulation by Tfcp1l would be needed (please focus on the major comments of ref#2 to address this).

Conditioned on further experiments that address these two critical points, I am delighted to invite submission of a revised version.

(Please do consider textual changes/clarifications regarding minor comments of the referees as you find constructive and appropriate to improve presentation of your results.)

Please be reminded that The EMBO Journal considers only one round of revisions and the ultimate decision on publication will dependent on the outline and strength of your revised manuscript.

I am very much looking forward to your amended study and remain with best regards.
REFEREE REPORTS:

Referee #1:

Ye et al have performed an outstanding piece of work on the importance of Tfcp2l1 in the self-renewal of embryonic stem cells. They have dissected the LIF/Stat3 pathway and found that it converges with both the MEK and Wnt pathways, which reveals how the so-called 2i cocktail helps maintain ES cell pluripotency. This is an excellent piece of work that not only enlightens about stem cell self-renewal, but also provides insight into convergent signaling pathways.

Comments (minor)

1. Figure 1A: not clear what "gene signal" means. Please clarify or rename.
2. Results section ("Forced expression..."): "overnight" culturing jargon should be changed to more precise indication of timing.
3. Figure 2F: at least one additional pluripotency marker should be included.
4. Figure legend 2G: Nestin is not really a "neuronal" marker, change to "neural" or "neurectodermal"
5. Figure 3H: difficult to see that JAK inhibitor line on the graph. Change scale or color to make more apparent.
6. The results in Figure 8 are a bit preliminary and really not needed for this paper. I suggest these results be removed. Alternatively, the authors should perform a chromatin IP experiment. Since they make the claim that Tfcp2l1 regulates self-renewal by activating Nanog, they should perform a ChIP-PCR with wild-type and mutant Tfcp2l1 to show absent/decreased binding at the Nanog promoter in the mutant.
7. The authors may be overstating that Tfcp2l12l1 is downstream of Stat3 based upon their current evidence. Though JAK is known to mediate its effect through Stat3, it cannot be ruled out that Tfcp2l1 activation happens independent of Stat3. Inhibition of Tfcp2l1 expression in a Stat3 knockdown background would be required to make such a claim. Perhaps this alternative point could be presented in the discussion.
8. Given their interesting data on convergent pathways, might authors want to comment on the conflicting views of wnt signaling in ES cells?

Referee #2:

In this manuscript, Shoudong Ye and colleagues investigate the role of Tfcp2l1 in promoting ESC self-renewal and facilitating EpiSC reprogramming toward a naïve pluripotent state. While Tfcp2l1 is highly expressed in ESCs as well as in pre- and early post-implantation embryos (Pelton et al. 2002), its role in pluripotency was not fully elucidated yet. Here, Tfcp2l1 is identified as a potent self-renewal factor in both 129 and C57BL/6 ESCs, which can be independently and/or additively induced by LIF, Gsk3 (CHIR) and/or Mek (PD03) inhibitors - also known as 2i components. They show that Tfcp2l1 forced expression can substitute for LIF/Stat3 in both wild type and Stat3 KO ESCs in serum alone, recapitulates the effect of 2i components when combined with either CHIR or PD03 to sustain ESC self-renewal, and furthermore induces stable conversion of EpiSCs to ESCs in serum+LIF+2i conditions. Conversely, they find that Tfcp2l1 down regulation impairs the self-renewal ability of ESCs when grown in serum + LIF but not in 2i. Mechanistically, Tfcp2l1 is demonstrated to require an intact DNA-binding domain to mediate its function, possibly through the activation of Nanog in ESCs.

Overall, the study is well crafted and executed, with two major concerns in the central conclusions
drawn from the findings: (1) the extent to which Tfcp2l1 is a limiting factor in ESCs, and (2) the specificity of Nanog over other core ESC factors that might be regulated by Tfcp2l1. These and other more minor points are expanded below.

1- The study suggests that Tfcp2l1 is a critical transcription factor at the crossroad of LIF- and 2i-mediated self-renewal pathways in ESCs but does not provide any clear explanation of what this would mean. Does Tfcp2l1 function in a dose-dependent manner, with critical expression levels to be secured through convergent signals? This could be perhaps explored by complementing Stat3/-ESC with different levels of ectopic Tfcp2l1 expression (Figure 3G). Additionally, the authors should further explore how the loss of Tfcp2l1 by RNAi and its impact on ESC self-renewal can be rescued in 2i (Figure 5H), checking at the first place whether 'residual' Tfcp2l1 expression might be enhanced in these conditions as compared to serum+ LIF.

2- The proposed model (Figure 8I) indicates Tfcp2l1 is acting via Nanog, yet Tcf2l1 is expected to activate many other transcription factors. This should be more clearly highlighted in Figure 2F by screening the expression levels of other pluripotency factors including Klf4 upon Dox-dependent induction of Tfcp2l1 in ESCs. Additionally, the authors need to provide direct evidence for Tfcp2l1 action on Nanog expression by identifying its critical binding site and validating in luciferase assays that Tfcp2l1 activates Nanog via this site. Assessing the level of Nanog reporter activity in wild type versus Tfcp2l1 Q234L/K236E ESC lines (Figure 8) will also be informative. The authors should furthermore validate that Nanog is required for Tfcp2l1-mediated action on EpiSC reprogramming to naïve ESCs. Although this would not formally exclude a role for any other factor, it would reinforce Nanog as a key downstream target of Tfcp2l1.

Other minor points:

- Figure 1A-B - could the authors please clarify in legend figure whether the cells were cultured in the presence of serum as in Figure 1D?

- Figure 1C - could the authors confirm that the PB-transgenes also remained expressed at similar levels when transferring cells into serum+LIF conditions?

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- Figure 2B - could the authors compare the level of Tfcp2l1 induction with its endogenous levels in serum +LIF and 2i +LIF conditions?

- Figure 2E as well as Figures 3, 5, 7 & 8: please add error bars and provide information about the number of biological replicates for these experiments.

- Figure 3F: this blot needs to be replaced (overexposed). Could the authors also please clarify the culture conditions used in this control experiment?

- Figure S1 is mislabeled.

Referee #3:

The authors found a new transcription factor, Tfcp2l1, to be a common target of the LIF/Stat3 and 2i-mediated signaling pathways involved in the maintenance of ESC self-renewal. The authors found that forced expression of Tfcp2l1 can sustain ESC self-renewal in the absence of LIF or in the absence of one of the two inhibitors in the 2i-containing culturing system. Moreover, the function of Tfcp2l1 in ESC self-renewal is dependent on NANOG. Additionally, the authors found Tfcp2l1 capable of reprogramming EpiSCs to ground pluripotency state ESCs. Overall, the data correlates well with the conclusions from the authors. However, there are minor comments to be addressed.
1) Nanog came up in the microarray data and should be incorporated in the scatter plot along with the other transcription factors in Figure 1A. Although the overall Nanog gene signal at each condition, LIF or LIF/2i, was much lower than Tfcp2l1, the ratio between LIF/2i and LIF was almost 2 folds, similar to Tfcp2l1 gene signal. This is important, since the data in this manuscript indicate Tfcp2l1 function to be upstream of Nanog. Thus, ultimately, the ground pluripotency state is dependent on Nanog, as previously shown (Niwa et al., 2009. Nature 460: 118-122).

2) A graphical representation showing the quantification of the alkaline phosphatase staining in Figure 1D is missing.

3) The resolution of the pictures in Figures 3B and 3E could be improved.

4) The Western blot in Figure 3F seem overexposed. The saturated signal mask the over expression of Tfcp2l1.

5) In Figure 5C, could the impairment of colony formation by knocking down Tfcp2l1 be rescued upon expression of any other pluripotent factor? This functional assay could be used to identify downstream targets of Tfcp2l1.

6) In Figure 6, could expression of Nanog be enough to reprogram Tfcp2l1-deficient EpiSCs.

7) As shown in Figure 7, the use of a Nanog null ESC line determines the role of Tfcp2l1 in ESC self-renewal to be irrelevant in the absence of Nanog. This suggests potential targets of Nanog to hold the capacity of maintaining the naïve pluripotent state in ESCs.

8) In Figure 8B, the inability of the Tfcp2l1 Q234L/K236E mutant construct to uphold self-renewal should be rescued upon ectopic expression of Nanog, or any other pluripotent factor with the ability to bypass Tfcp2l1 DNA-binding activity.

9) Regarding the relevance of this work, it is known that over expression of several genes, including Nanog, Klf4, Klf2 and c-Myc can reprogram EpiSCs into naïve pluripotent ESCs (De Los Angeles et al., 2012. Current Opinion in Gen & Dev. 22: 272-282). Thus, Tfcp2l1 is an addition to this list. What would be the placement of these known genes in an epistatic pathway(s) associated to the maintenance of the naïve pluripotent state? Klf4 is upstream of Nanog (Zhang et al., 2010. J Biol Chem. 285: 9180-9189), and so is Tfcp2l1. Accordingly, over expression of Nanog, on its own, can sustain naïve pluripotency in ESCs. Therefore, how redundant is the role of Klf4 versus Tfcp2l1? Are additional targets of Tfcp2l1 required to sustain the ground state of pluripotency in ESCs, besides Nanog.

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Ye et al have performed an outstanding piece of work on the importance of Tfcp2l1 in the self-renewal of embryonic stem cells. They have dissected the LIF/Stat3 pathway and found that it converges with both the MEK and Wnt pathways, which reveals how the so-called 2i cocktail helps maintain ES cell pluripotency. This is an excellent piece of work that not only enlightens about stem cell self-renewal, but also provides insight into convergent signalling pathways.

**Comments (minor)**

1. **Figure 1A:** not clear what "gene signal" means. Please clarify or rename.

--- “Gene signal” means gene signal intensity on microarrays. We have renamed “gene signal” to “gene signal intensity”.

2. **Results section ("Forced expression... ")**: "overnight" culturing jargon should be changed to more precise indication of timing.

---We have changed “overnight” to the actual time (12 hours) in the text.
3. **Figure 2F**: at least one additional pluripotency marker should be included.

---We have included another pluripotency marker, Klf4.

4. **Figure legend 2G**: Nestin is not really a "neuronal" marker, change to "neural" or "neurectodermal"

---We have changed “neuronal” to “neural” as suggested.

5. **Figure 3H**: difficult to see that JAK inhibitor line on the graph. Change scale or colour to make more apparent.

---We have changed the scale (new Figure 3I).

6. The results in Figure 8 are a bit preliminary and really not needed for this paper. I suggest these results be removed. Alternatively, the authors should perform a chromatin IP experiment. Since they make the claim that Tfcp2l1 regulates self-renewal by activating Nanog, they should perform a ChIP-PCR with wild-type and mutant Tfcp2l1 to show absent/decreased binding at the Nanog promoter in the mutant.

--- In a previous ChiP-seq study by Chen X et. al., (Cell 133:1106-17, 2008), several putative Tfcp2l1 binding sites on the Nanog promoter were identified. We performed luciferase reporter assays to measure Nanog promoter activity in mESCs transfected with wild-type Tfcp2l1 or Tfcp2l1\textsubscript{Q214L,K216E} expression constructs. Our results indicate that Nanog promoter activity is increased by wild-type Tfcp2l1, but not by Tfcp2l1\textsubscript{Q214L,K216E}. These results have been included in new Figure 8G.

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--- We agree that additional experiments are required in order to draw a conclusion that Tfcp2l1 is a direct downstream target of Stat3. We did not perform these experiments, because in the related manuscript from Austin Smith’s group, data have been presented to demonstrate that Tfcp2l1 is indeed a direct downstream target of Stat3.

8. **Given their interesting data on convergent pathways, might authors want to comment on the conflicting views of Wnt signalling in ES cells?**

---We believe that activation of the Wnt/β-catenin signal in ES cells can activate programs responsible for both self-renewal and lineage commitment, and that it exerts a self-renewal effect only when the lineage commitment effect is blocked. The genes induced by Wnt/β-catenin with a positive effect in promoting ES cell self-renewal include Tfcp2l1, Esrrb, Nanog, and Klf2. The genes induced by Wnt/β-catenin that negatively regulate ES cell self-renewal have not been well-studied, but potential candidates include: T, Cdx1, Cdx2, and Axin2. Further studies are required to understand how activated β-catenin exhibits diverse, sometimes opposite roles in ES cells.
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1- The study suggests that Tcfp2l1 is a critical transcription factor at the crossroad of LIF- and 2i-mediated self-renewal pathways in ESCs but does not provide any clear explanation of what this would mean. Does Tcfp2l1 function in a dose-dependent manner, with critical expression levels to be secured through convergent signals? This could be perhaps explored by complementing Stat3/- ESCs with different levels of ectopic Tcfp2l1 expression (Figure 3G). Additionally, the authors should further explore how the loss of Tcfp2l1 by RNAi and its impact on ESC self-renewal can be rescued in 2i (Figure 5H), checking at the first place whether 'residual' Tcfp2l1 expression might be enhanced in these conditions as compared to serum+ LIF.

--- Our results demonstrate that Tcfp2l1 transcription is upregulated by LIF, CHIR, and PD03 and increased expression of Tcfp2l1 can recapitulate the effect of LIF, PD03, and partially that of CHIR, in promoting mESC self-renewal. We performed additional experiments to determine whether Tcfp2l1 functions in a dose-dependent manner. As shown in new Figure 3B-D, Tcfp2l1 does function in a dose-dependent manner in promoting mESC self-renewal. We cultured shTcfp2l1 mESCs in serum+LIF and 2i conditions and compared their ‘residual’ Tcfp2l1 expression levels. We found that basal and residual Tcfp2l1 expression levels are both significantly higher in 2i compared to that in serum+LIF (new Figure 5G). This might partially explain why Tcfp2l1 knockdown impairs mESC self-renewal in serum+LIF but not in 2i. Besides Tcfp2l1, 2i can upregulate several other pluripotency genes such as Esrrb, Nanog, and Klf2. Upregulation of these genes by 2i might also compensate for the loss of Tcfp2l1 in promoting mESC self-renewal.

2- The proposed model (Figure 8I) indicates Tcfp2l1 is acting via Nanog, yet Tcfp2l1 is expected to activate many other transcription factors. This should be more clearly highlighted in Figure 2F by screening the expression levels of other pluripotency factors including Klf4 upon Dox-dependent induction of Tcfp2l1 in ESCs. Additionally, the authors need to provide direct evidence for Tcfp2l1 action on Nanog expression by identifying its critical binding site and validating in luciferase assays that Tcfp2l1 activates Nanog via this site. Assessing the level of Nanog reporter activity in wild type versus Tcfp2l1 Q234L/K236E ESC lines (Figure 8) will also be informative. The authors should furthermore validate that Nanog is required for Tcfp2l1-mediated action on EpiSC reprogramming to naïve ESCs. Although this would not formally exclude a role for any other factor, it would reinforce Nanog as a key downstream target of Tcfp2l1.
---We performed additional experiments to identify pluripotency genes activated by Tfcp2l1. Among the 5 pluripotency genes we tested, only Nanog and Klf4 were upregulated upon induction of Tfcp2l1 in i-Tfcp2l1 mESCs (new Figure 7A). We performed luciferase reporter assays to measure Nanog promoter activity in mESCs expressing wild-type Tfcp2l1 or Tfcp2l1Q214L/K216E. Our results indicate that Nanog promoter activity is increased by wild-type Tfcp2l1, but not by Tfcp2l1Q214L/K216E. These results have been included in new Figure 8G. Based on a published study by Chen X et. al (Cell 133:1106-17, 2008), we identified a putative Tfcp2l1 binding site on the Nanog promoter, CCGACTTAAGCTGG. We generated a Nanog promoter luciferase reporter in which this site was mutated to: AACAGAAGTCAACT. However, TOPFlash activity was still induced by Tfcp2l1. We are currently trying to identify other potential Tfcp2l1 binding sites on the Nanog promoter and validate them by luciferase assays. To validate the role of Nanog in Tfcp2l1-mediated EpiSC reprogramming, we knocked down Nanog in Tfcp2l1-overexpressing mEpiSCs. As shown in new Figure 7J, Nanog knockdown reduces Tfcp2l1-mediated EpiSC reprogramming efficiency by 40%. We attempted several times to convert Nanog null mESCs into EpiSCs in the bFGF/activin A condition, but all attempts failed.

Other minor points:

Figure 1A-B - could the authors please clarify in legend figure whether the cells were cultured in the presence of serum as in Figure 1D?

---These cells were cultured in serum medium. We have added this information to the figure legend.

Figure 1C - could the authors confirm that the PB-transgenes also remained expressed at similar levels when transferring cells into serum+LIF conditions?

---The PB-transgenes are stably integrated into the genome, so their expression should remain stable under different culture conditions.

Figure 1D - could the authors quantify the number of undifferentiated, mixed and differentiated colonies in each condition?

---We quantified the number of AP positive colonies in each condition (new Figure 1E).

Figure 2B - could the authors compare the level of Tcfp2l1 induction with its endogenous levels in serum +LIF and 2i +LIF conditions?

---We compared the level of Tfcp2l1 induction with its endogenous levels in serum+LIF and 2i+LIF conditions (see new Figure 3D). We found that administration of Dox at 0.2 µg/ml was sufficient to maintain i-Tfcp2l1 mESC self-renewal, while 0.02 µg/ml Dox failed to do this.

Figure 2E as well as Figures 3, 5, 7 & 8: please add error bars and provide information about the number of biological replicates for these experiments.

---We have added the error bars and provided information about the number of biological replicates for each experiment in these Figures.
Figure 3F: this blot needs to be replaced (overexposed). Could the authors also please clarify the culture conditions used in this control experiment?

---We have replaced this blot (new Figure 3G). The empty vector- and Tfcp2l1-overexpressing Stat3 null ESCs were cultured in N2B27/2i. We have added this information to the figure legend.

Figure S1 is mislabelled

---The figure shows immunostaining results for mESCs expressing Tfcp2l1 in the presence of CHIR, and the Figure label describes the result.

Referee #3:
The authors found a new transcription factor, Tfcp2l1, to be a common target of the LIF/Stat3 and 2i-mediated signalling pathways involved in the maintenance of ESC self-renewal. The authors found that forced expression of Tfcp2l1 can sustain ESC self-renewal in the absence of LIF or in the absence of one of the two inhibitors in the 2i-containing culturing system. Moreover, the function of Tfcp2l1 in ESC self-renewal is dependent on NANOG. Additionally, the authors found Tfcp2l1 capable of reprogramming EpiSCs to ground pluripotency state ESCs.

Overall, the data correlates well with the conclusions from the authors. However, there are minor comments to be addressed.

1) Nanog came up in the microarray data and should be incorporated in the scatter plot along with the other transcription factors in Figure 1A. Although the overall Nanog gene signal at each condition, LIF or LIF/2i, was much lower than Tfcp2l1, the ratio between LIF/2i and LIF was almost 2 folds, similar to Tfcp2l1 gene signal. This is important, since the data in this manuscript indicate Tfcp2l1 function to be upstream of Nanog. Thus, ultimately, the ground pluripotency state is dependent on Nanog, as previously shown (Niwa et al., 2009. Nature 460: 118-122).

---We strongly agree and have incorporated Nanog data into the scatter plot shown in Figure 1A. We also provided Nanog qRT-PCR and overexpression results in Figure 1B-E.

2) A graphical representation showing the quantification of the alkaline phosphatase staining in Figure 1D is missing.

---We have quantified the number of AP positive colonies of mESCs transfected with different transgenes (see new Figure 1E).

3) The resolution of the pictures in Figures 3B and 3E could be improved.

---We have replaced these pictures (new Figures 3E and F).

4) The Western blot in Figure 3F seems overexposed. The saturated signal mask the over expression of Tfcp2l1.

---We have replaced this picture (new Figure 3G).
5) In Figure 5C, could the impairment of colony formation by knocking down Tfcp2l1 be rescued upon expression of any other pluripotent factor? This functional assay could be used to identify downstream targets of Tfcp2l1.

---We performed additional experiments to identify pluripotency genes activated by Tfcp2l1. Among the 5 pluripotency genes we tested, only Nanog and Klf4 were upregulated upon induction of Tfcp2l1 in i-Tfcp2l1 mESCs (new Figure 7A). Tfcp2l1 failed to promote Nanog null mESC self-renewal, but Tfcp2l1’s self-renewal-promoting effect is not affected by Klf4 knockdown (new Figure S3A, B). These results suggest that Nanog is a key target of Tfcp2l1 in promoting mESC self-renewal. It would be of interest to determine whether there are other downstream targets of Tfcp2l1 that are important in mediating Tfcp2l1’s self-renewal-promoting effect.

6) In Figure 6, could expression of Nanog be enough to reprogram Tfcp2l1-deficient EpiSCs.

--- Yes, Nanog overexpression can reprogram Tfcp2l1-knowckdown EpiSCs to naive state ESCs(new Figure 7J).

7) As shown in Figure 7, the use of a Nanog null ESC line determines the role of Tfcp2l1 in ESC self-renewal to be irrelevant in the absence of Nanog. This suggests potential targets of Nanog to hold the capacity of maintaining the naive pluripotent state in ESCs.

--- We agree. Esrrb is one of the potential targets of Nanog that hold the capacity of maintaining the naïve pluripotent state in ESCs (Festuccia N et al, Esrrb is a direct Nanog target gene that can substitute for Nanog function in pluripotent cells. Cell Stem Cell. 2012,11:477-90). We found that overexpression of Esrrb can rescue self-renewal defective Tfcp2l1 knockdown mESCs (data not shown).

8) In Figure 8B, the inability of the Tfcp2l1 Q234L/K236E mutant construct to uphold self-renewal should be rescued upon ectopic expression of Nanog, or any other pluripotent factor with the ability to bypass Tfcp2l1 DNA-binding activity.

---Our results suggest that ectopic expression of Nanog can rescue the inability of the Tfcp2l1Q214L/K216E mutant to promote mESC self-renewal (new Figures 8B-E).

9) Regarding the relevance of this work, it is known that over expression of several genes, including Nanog, Klf4, Klf2 and c-Myc can reprogram EpiSCs into naïve pluripotent ESCs (De Los Angeles et al., 2012. Current Opinion in Gen & Dev. 22: 272-282). Thus, Tfcp2l1 is an addition to this list. What would be the placement of these known genes in an epistatic pathway(s) associated to the maintenance of the naïve pluripotent state? Klf4 is upstream of Nanog (Zhang et al., 2010. J Biol Chem. 285: 9180-9189), and so is Tfcp2l1. Accordingly, over expression of Nanog, on its own, can sustain naïve pluripotency in ESCs. Therefore, how redundant is the role of Klf4 versus Tfcp2l1? Are additional targets of Tfcp2l1 required to sustain the ground state of pluripotency in ESCs, besides Nanog?

--- Tfcp2l1, Nanog, and Klf4, when overexpressed individually, can promote ESC self-renewal and reprogramming of EpiSCs to naïve-state ESCs. As the reviewer indicated, Klf4 is upstream of Nanog. Tfcp2l1 can upregulate both Nanog and Klf4 (new Figure 7A), suggesting that Tfcp2l1 is upstream of Klf4 and Nanog. Knockdown of Nanog, but not Klf4, impairs Tfcp2l1’s effect in promoting ESC self-renewal and reprogramming of EpiSCs to ESCs (new Figure S3, and new
Figures 7D-G). Overexpression of Tfcp2l1, but not Klf4, can maintain Stat3 null mESC self-renewal (new Figure 3H. Also see the related manuscript from Austin Smith’s group and Hall J. et al. Cell Stem Cell 5:597-609, 2009). Knockdown of Tfcp2l1, but not Klf4, impairs LIF/Stat3-mediated ESC self-renewal (see the related manuscript from Austin Smith’s group and Jiang J. et al. Nat Cell Biol. 10:353-60, 2008). These results suggest that Klf4 is not a key mediator of Tfcp2l1 functionality and their roles in promoting ESC self-renewal are not redundant. Apart from Nanog, we have not identified any other targets of Tfcp2l1 that are required to sustain the ground state of pluripotency. We feel that further studies are needed to determine the placement of Tfcp2l1, Nanog and Klf4 in an epistatic pathway associated with the maintenance of the naïve pluripotent state.