A PiggyBac-Based Recessive Screening Method to Identify Pluripotency Regulators

Ge Guo1, Yue Huang2*, Peter Humphreys1, Xiaozhong Wang3, Austin Smith1*

1 Wellcome Trust Centre for Stem Cell Research, Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom, 2 Wellcome Trust Sanger Institute, Hinxton, United Kingdom, 3 Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois, United States of America

Abstract

Phenotype driven genetic screens allow unbiased exploration of the genome to discover new biological regulators. Bloom syndrome gene (Blm) deficient embryonic stem (ES) cells provide an opportunity for recessive screening due to frequent loss of heterozygosity. We describe a strategy for isolating regulators of mammalian pluripotency based on conversion to homozygosity of PiggyBac gene trap insertions combined with stringent selection for differentiation resistance. From a screen of 2000 mutants we obtained a disruptive integration in the Tcf3 gene. Homozygous Tcf3 mutants showed impaired differentiation and enhanced self-renewal. This phenotype was reverted in a dosage sensitive manner by excision of one or both copies of the gene trap. These results provide new evidence confirming that Tcf3 is a potent negative regulator of pluripotency and validate a forward screening methodology to identify modulators of pluripotent stem cell biology.

Introduction

Genome-wide loss of function screening in the diploid mammalian genome is hindered by the requirement for homozygosity. Although RNA interference approaches have been applied, this only reduces rather than eliminates gene expression, currently lacks genome coverage in the mouse, and is subject to off-target effects. An alternative possibility is to exploit embryonic stem (ES) cells deficient for the Bloom syndrome tumour suppressor gene (Blm) [1,2]. Blm encodes a RecQ helicase and mutant ES cells exhibit an elevated frequency of non-sister chromatid exchanges. Loss of heterozygosity (LOH) occurs at a rate of 4.2 ± 10⁻⁴ per cell per locus per generation. This incidence predicts that on average a homozygous mutant should arise from a single heterozygous cell within 14 duplication cycles. A previous functional screen using Blm-deficient ES cells identified homozygous retroviral gene trap mutations in the DNA mismatch repair (MMR) pathway [2]. From 10,000 gene traps, multiple hits were identified in one gene, mismatch homolog 6 (Msh6). This demonstrated the potential for homozygous screening for a selectable phenotype in ES cells, but also highlighted the insertion bias of retroviral mutagenesis.

PiggyBac (PB) transposition is highly efficient in human and mouse cells [3,4]. Recently PB transposition based gene trap mutagenesis was applied in a new MMR screen in Blm-deficient ES cells [5]. Homozygous mutations in all four known MMR factors were recovered from 14,000 PB insertions, consistent with evidence that PB transposition has a broader spectrum of genome coverage than retroviral insertion.

Self-renewal of mouse ES cells is traditionally maintained by culture in serum using the cytokine leukemia inhibitory factor (LIF) [6,7]. Upon withdrawal of LIF, ES cells commit to differentiation under the influence of serum-factors or, in serum-free conditions, of autocrine fibroblast growth factor 4 (Fgf4) [8]. Disruptions in genes that mediate commitment or repress pluripotency circuitry are anticipated to reduce dependency on LIF. Here we used a PB transposon gene trap system in Blm-deficient ES cells to conduct a pilot screen for recessive mutations that could confer differentiation resistance.

Results

Implementing a recessive screen requires a strategy to identify and isolate rare phenotypes of interest. In the context of ES cell self-renewal, rapid and stringent selection is required because a fraction of cells invariably escape initial commitment. Such cells will subsequently expand under paracrine stimulation if differentiated cells are not eliminated [9,10]. Rex1 (Zfp42) is a specific marker of naive undifferentiated ES cells [11]. It is down-regulated at the onset of differentiation more rapidly than the commonly used Oct4 marker (Fig. 1A). We therefore constructed a selectable Blm-deficient ES cell line by inserting eGFPproPuro into the Rex1 genomic locus via homologous recombination (Fig. 1B). The resulting NN97-5 cells expressed GFP in 60–80% of the population (Fig. 1C), consistent with the known mosaic expression of Rex1 in serum [11,12]. Upon plating for differentiation, the proportion of GFP positive cells declined rapidly (Fig. 1D). By day 5, only 2–3% of cells remained GFP positive.
We used a binary PB transposon delivery method for gene trap mutagenesis. This comprises a PB gene trap vector, pGG85, and a helper plasmid, pCAGPBase [4], that provides the transposase for vector/chromosome transposition (Fig. 2A). pGG85 carries a promoter-less gene trap cassette, SAIRESgeo [13]. The PB 5’ terminal repeat region (5TR) contains an RNA polymerase II promoter [14]. Therefore we positioned the SAIRESgeo cassette in opposite orientation towards the 3’ terminal (3TR) (Fig. 2A). We included loxP sites to enable reversion by Cre-mediated excision of the SAIRESgeo cassette.

PBase mediated vector-chromosome transposition is very efficient. To restrict the number of integrations it is important to determined an appropriate ratio of transposase and transposon vector [5]. Electroporation of 2×10⁶ ES cells with 1 µg pGG85 and 3 µg pPBase yielded 500–1,000 G418 resistant colonies. Splinkerette PCR amplification [15] from 24 randomly picked clones indicated one or two PB insertions in most clones (Fig. 2B and 2C). We therefore employed this 1:3 ratio.

The screening strategy is depicted schematically in Figure 2D. A pilot scale gene trap library was prepared by transfecting a total of

---

**Figure 1. Generation of Rex1 reporter cells.** A. qRT-PCR analysis of Rex1 and Oct4 mRNA during monolayer differentiation in N2B27. B. Strategy to create the Rex1GIP knock in allele. C. Flow cytometry of a representative Rex1-Egfp profile in undifferentiated NN97-5 cells. E. Flow cytometry of Rex1-Egfp population in NN97-5 cells during monolayer differentiation in N2B27.

doi:10.1371/journal.pone.0018189.g001
10^7 NN97-5 cells in 5 electroporations as above. After twelve days under selection in G418, plates were harvested in two separate pools, each containing about one thousand clones and expanded for a further 48 hours. This period of 14 days since transfection is sufficient to allow for at least one homozygous conversion event at the majority of loci. Cells from each pool were then separately plated in N2B27 medium without serum and LIF. These conditions lead to neural differentiation of ES cells [16]. Untransfected NN97-5 cells were plated as a control. Five days later, puromycin was applied for two days to remove differentiating Rex1 negative cells. LIF was added at the same time to maximize self-renewal of persisting undifferentiated cells. Recovered cells were replated for a second round of differentiation. Ten days later, over 100 undifferentiated colonies were evident in pool 1, while pool 2 and the NN97-5 control plates showed only around 10 colonies. Twenty colonies were picked from pool 1 for further analysis.

Expanded clones were assessed for resistance to differentiation. Six clones produced mostly undifferentiated ES cells in monolayer neural differentiation conditions. The remainder showed high levels of differentiation (Fig. 3A and Table 1). We used splinkerette PCR amplification and sequence analysis to identify the insertion sites. All 6 carry the same PB integration in the third intron of the T-cell factor 3 (Tcf3) gene (Fig. 3B and 4A). This insertion was also identified in 4 of the differentiating clones (Table 1). We examined Tcf3 expression by RT-PCR in Tcf3 mutants (Fig. 4B). Tcf3 mRNA was undetectable in non-differentiating clones but present in the differentiating clones. This indicates that differentiating cultures with the Tcf3 insertion might be heterozygous.

To establish a causative link between the Tcf3 mutation and differentiation deficiency, a homozygous Tcf3 gene trap clone, P1-2, was transfected with a Cre expression plasmid. Cre recombination should remove the gene trap cassette and revert the

**Figure 2. piggyBac mutagenesis and monolayer differentiation screen.** A. Binary piggyBac gene trap system composed of gene trap vector, pGG85, and transposase expressing helper plasmid, pCAGG-PBase. B. G418 resistant colonies produced by co-electroporation of 1 μg of pGG85 and 3 μg of helper plasmid. C. Splinkerette PCR amplified genome junction flanking PB insertions indicating the number of PB inserts in each clone. D. Schematic representation of monolayer differentiation screen.
induced mutation (Fig. 4C). Transfected cells were plated at low density for clonal expansion. By RT-PCR we identified clones that express wild type Tcf3 mRNA (Fig. 4D). These included one clone, CreA12, which expressed both the gene trap transcript and the wild type Tcf3 mRNA (Fig. 4D). Sub-cloning confirmed that CreA12 was not a mixed population but a clone in which only one Tcf3 allele had been repaired. Consistent with heterozygosity, Tcf3 transcript level in CreA12 cells was around 50% of that in parental NN97-5 cells (Fig. 4E). Whereas P1-2 cells formed abundant undifferentiated ES cell colonies in serum-free culture without LIF, homozygous repaired CreD10 cells rapidly differentiated (Fig. 4F). Heterozygous CreA12 cells initially formed a mixture of undifferentiated and differentiated cells, but by day 9 had mostly differentiated with few remaining ES cells. Phenotype reversion confirms that the Tcf3 mutation is causal for enhanced self-renewal. Partial resistance to differentiation explains why heterozygous clones could be recovered in the screen and indicates dosage sensitive activity of Tcf3.

In the absence of LIF, serum induces heterogeneous non-neural differentiation of ES cells [10]. We tested P1-2 cells in these conditions and observed that a large fraction of cells retained undifferentiated ES cell morphology and Oct4 expression (Fig. 5A). They also maintained a high proportion of Rex1-GFP positive cells (Fig. 5B). In contrast, CreD10 cells showed rapid loss of GFP while Tcf3 heterozygous CreA12 cells showed a more gradual reduction. We examined clonal propagation in the absence of LIF, a rigorous test of self-renewal efficiency. CreD10 cells produced only fully differentiated and mixed colonies (Fig. 5C). In contrast P1-2 cells formed entirely ES cell containing colonies. These colonies showed more differentiation than in the presence of LIF, however, and were smaller (Fig. 5D). Thus Tcf3 deletion confers heightened resistance to differentiation in serum but does not substitute fully for LIF.

To rule out any effect specific to the Blm-deficient genetic background, we used siRNA to knock down Tcf3 in wild type Rex1 reporter ES cells. qRT-PCR showed that Tcf3 mRNA was reduced to less than 20% two days after Tcf3 siRNA transfection. This effect was transient and after six days Tcf3 mRNA was restored (Fig. 6A). In Tcf3 siRNA treated cells Rex1 expression levels remained high in serum or serum-free differentiation.
conditions for 2–3 days (Fig. 6B and 6C). Tcfβ3 knockdown also allowed transient clonal expansion in serum without LIF. Compact alkaline phosphatase positive undifferentiated ES cell colonies were present in siRNA treated cultures 5 days after transfection and plating, while control siRNA treated cells formed only differentiated colonies (Fig. 6D).

Tcfβ3 is the predominant Tcf in ES cells [17]. Other Tcfs are mediators of canonical Wnt/β-catenin induced transcriptional activation, but the role of Tcfβ3 in this pathway is less well-defined [17]. Despite the lack of Tcfβ3, P1-2 cells retained TOPFlash reporter activation in response to Wnt3a (Fig. 7B). Furthermore they showed induction of chromosomal Wnt target genes, Axin2, Cdx1 and T-brachyury (Fig. 7C). Absence of Tcfβ3 therefore does not impede canonical Wnt signalling in ES cells.

Genome location analyses suggest that Tcfβ3 binds to promoters of several pluripotency genes including Oct4, Nanog, and Klf4 [18,19]. Through interaction with Groucho family members Tcfβ3 is proposed to repress pluripotent gene expression [17]. We detected near two folds increase in the expression of the core pluripotency genes, Oct4, Klf4 and Nanog in P1-2 cells when compared to the reverted CreD10 cells. However, when compared with NN97-5 cells only Klf4 showed significantly increased expression (Fig. 7A). This biological variation between parental line and subclone indicates that the repressive effect of Tcfβ3 on individual genes may be modest and environmental factors. Nonetheless, the increased expressions of Klf4 or Nanog are notable because either of these is sufficient to increase resistance to differentiation [20,21,22,23].

Western-blotting analysis indicated that neither Oct4 nor Nanog protein are appreciably increased in Tcfβ3 deficient cells (Fig. 7D). We therefore examined cellular expression by immunofluorescent staining because Nanog is heterogeneous in ES cells in serum [24]. This dynamic heterogeneity is postulated to underlie ES cell susceptibility to differentiation [24,25,26]. Compared with NN97-5 cells, P1-2 cells cultured in serum with LIF showed more uniform immunofluorescent staining for Nanog (Fig. 7E). We quantified staining intensity relative to Oct4 over 25 fields using CellProfiler software [27]. Scatter plots of mean fluorescence intensities confirm that the fraction of low or non-expressing cells within the Oct4 positive population is reduced in Tcfβ3 deficient cells (Fig. 7F). Thus absence of Tcfβ3 stabilises expression of Nanog within individual ES cells, even though overall expression level may not be significantly altered. Interestingly there was also a modest shift in the Oct4 profile towards higher expression, consistent with evidence that Tcfβ3 may repress Oct4 [18].

### Discussion

In this study, we piloted a recessive screening strategy to identify genes modulating ES cell differentiation and self-renewal. There are three key components in this approach. First, use of PB transposon mutagens offers significant advantages for genome wide screens. They have much higher chromosomal integration efficiencies than plasmids and do not appear to have the bias for hot spots seen with retroviral vectors [4,5]. Second, rapid and stringent selection is critical in an ES cell self-renewal screen to minimise paracrine interactions between residual undifferentiated ES cells and differentiating progeny [20]. Oct4 is widely used as a reporter and selection driver, but it is not optimal because expression reduces only gradually. Moreover, in early derivatives of ES cells, including stable EpiSC cell lines, Oct4 is fully maintained [28]. Indeed we found that selection for Oct4 was of limited utility over the time course of monolayer differentiation, with high background necessitating multiple rounds of replating. In contrast Rex1 selection allowed mutants to be isolated after only a single round of secondary plating. Third, it is essential to demonstrate reversion of phenotype in order to confirm causality. Using the PB vector reversion can readily be achieved by excision of the gene trap cassette with Cre recombinase.

From 2,000 gene traps, we isolated ES cells with enhanced self-renewal. All 5 non-differentiating clones had a gene trap insertion disrupting the Tcfβ3 gene and no Tcfβ3 mRNA was detectable in these cells. The integration site was identical in these clones indicating that they arose from the same original PB insertion. Some colonies exhibited partially differentiation-resistant phenotype and also contained this Tcfβ3 insertion. The presence of Tcfβ3 mRNA in these cells indicates either that they have not converted to homozgyosity or that they are mixed clones. Complete Tcfβ3 deficiency greatly reduced differentiation and allowed ES cell expansion without exogenous LIF, even at clonal density. These findings are consistent with recent studies linking Tcfβ3 to the core

#### Table 1. Monolayer neural differentiation of individual gene trap clones.

| Gene trap clones | Monolayer Differentiation | Gene trap clones | Monolayer Differentiation |
|------------------|---------------------------|------------------|---------------------------|
| P1-1*            | D                         | P1-12*           | Non D                     |
| P1-2*            | Non D                     | P1-13*           | Non D                     |
| P1-3             | D                         | P1-14*           | Non D                     |
| P1-4*            | D                         | P1-15*           | Non D                     |
| P1-5             | D                         | P1-16            | D                         |
| P1-6             | D                         | P1-17            | D                         |
| P1-7             | D                         | P1-18            | D                         |
| P1-8             | D to flat cells           | P1-19*           | Non D                     |
| P1-9             | D                         | P1-20*           | D                         |
| P1-10            | D                         | P2-1             | D                         |
| P1-11*           | D                         | P2-2             | D                         |

* Monolayer neural differentiation of twenty clones from gene trap mutation pool 1 is presented. Clones with Tcfβ3 mutation are labelled with ‘*’. Two clones from mutant pool 2 were also included as a control for monolayer differentiation assay. “D” represents clones showing extensive neural differentiation. “Non-D” represents cells showing predominantly undifferentiated ES cell morphology. P1-8 cells differentiated to flat non-neural cells.

doi:10.1371/journal.pone.0018189.t001
Figure 4. Tcf3 gene trap mutants. A. Tcf3 gene trap (Tcf3\textsuperscript{tp}) and Cre-reverted (Tcf3\textsuperscript{rev}) alleles. Cre recombination deletes the gene trap cassette to leave a reverted allele retaining the PB terminal repeats. B. RT-PCR analysis of Tcf3 expression in gene trap mutants. Tcf3 mRNA was not detected in clones P1-2, P1-12 and P1-19 but evident in clones P1-1, P1-11 and P1-20. C. Diagram showing generation of het or homozygous reverted cells. D. RT-PCR analysis of Tcf3 gene trap (Ex3-SA) and Tcf3 wild type (Ex3-Ex7) transcripts. CreA12-1 and CreD10-4 are subclones of CreA12 and CreD10. E. qRT-PCR analysis of Tcf3 expression. F. After 9 days monolayer differentiation multiple ES cell colonies formed from Tcf3 homozygote P1-2, but not from parental NN97-5 or revertant CreA12 or CreD10 cells.

doi:10.1371/journal.pone.0018189.g004
pluripotent transcription factor network [17,18]. Isolation from a stringent genetic screen independently establishes the importance of Tcf3 for ES cell differentiation. The more homogenous expression of Nanog in Tcf3 mutants indicates that repression by Tcf3 contributes significantly to the heterogeneous and fluctuating pattern observed in serum [24,26]. This effect is rather subtle in terms of quantitative gene expression at the population level, but is likely to be biologically significant at the single cell level. With Tcf3 deleted, Nanog is maintained more evenly in all cells and the population is therefore more resistant to inductive cues for commitment. In a separate study we present evidence that the potent impact of glycogen synthase kinase-3 inhibition on ES cell self-renewal is in large part mediated by Tcf3 derepression [29]. Genome location studies suggest that Tcf3 may directly repress multiple components of the pluripotent circuitry [18,19]. We hypothesise that the strong phenotype of Tcf3 deletion reflects cumulative impact of general derepression of the pluripotency network rather than dramatic up-regulation of specific targets.

In summary, this study demonstrates the feasibility of recessive genetic screening for pluripotency regulators using a PB-based gene trap in Blm-deficient ES cells configured for Rex1 selection. The more homogenous expression of Nanog in Tcf3 mutants indicates that repression by Tcf3 contributes significantly to the heterogeneous and fluctuating pattern observed in serum [24,26]. This effect is rather subtle in terms of quantitative gene expression at the population level, but is likely to be biologically significant at the single cell level. With Tcf3 deleted, Nanog is maintained more evenly in all cells and the population is therefore more resistant to inductive cues for commitment. In a separate study we present evidence that the potent impact of glycogen synthase kinase-3 inhibition on ES cell self-renewal is in large part mediated by Tcf3 derepression [29]. Genome location studies suggest that Tcf3 may directly repress multiple components of the pluripotent circuitry [18,19]. We hypothesise that the strong phenotype of Tcf3 deletion reflects cumulative impact of general derepression of the pluripotency network rather than dramatic up-regulation of specific targets.

In summary, this study demonstrates the feasibility of recessive genetic screening for pluripotency regulators using a PB-based gene trap in Blm-deficient ES cells configured for Rex1 selection. This screen could readily be scaled up and applied in different culture conditions. Ideally, ES cells with inducible deletion of Blm would be used to minimise the incidence of background mutations [30]. Importantly, reversible insertional mutagenesis is a more robust screening methodology than RNAi based approaches, which although flexible inevitably suffer from variable penetrance and off-target effects.

**Methods**

**ES cell culture and differentiation**

Mouse ES cells were routinely maintained on gelatin coated tissue culture plates in medium containing serum and LIF as described [31]. The monolayer neural differentiation protocol is detailed in full elsewhere [8]. In brief, cells were dissociated with trypsin and washed once in PBS to remove residual FCS, and then plated in N2B27 medium at a density of 2\times10^4 cells/cm^2. Medium was changed every second day. For non-neural differentiation, cells were plated at similar density with either recombinant BMP-4 (10 ng/ml, R&D systems) or 10% FCS. For colony assays 600 fully dissociated ES cells were plated per 90 mm tissue culture plate. Colonies were stained for alkaline phosphatase (Sigma Aldrich, cat number 86R1KT). Colonies were scored based on alkaline phosphatase staining as pure ES cells, mixed or completely differentiated.

**Rex1 knock-in**

The Rex1 targeting region in AB2.2 BAC clone (bMQ-381F12, provided by Wellcome Trust Sanger Institute), was first replaced with eGFPIrespuro using bacterial recombineering [32]. To generate the Rex1 targeting vector the 5’ homology arm and the 3’ homology arms including the eGFPIrespuro cassette were amplified by PCR and cloned into pBluescript by three-way ligation. The targeting vector was transfected into Blm mutant or E14Tg2aIVC ES cells by electroporation. Following 7 days puromycin (1 μM) selection ES cell colonies were picked and expanded. Genomic
Figure 6. siRNA knockdown of Tcf3 in Blm wild type cells. **A.** qRT-PCR analysis of Tcf3 knockdown in Tcf3 siRNA treated ES cells and control siRNA treated cells. **B.** Graph shows population of Rex1-EGFP positive cells in Tcf3 siRNA and control siRNA treated cells after 2 days in monolayer differentiation with or without serum. **C.** qRT-PCR analysis of Rex1 expression in Tcf3 siRNA or control siRNA treated cells in monolayer differentiation with or without serum. **D.** Images showing a typical AP positive ES cell colony formed in Tcf3 siRNA treated cells after 5 days in serum while only differentiated colonies formed from control siRNA treated cells. Error bar represents standard deviation from three individual plating.

doi:10.1371/journal.pone.0018189.g006
PCR was used to identify targeted clones. RT-PCR confirmed that only targeted clones expressed the fused transcript including first exon of *Rex 1* and the gGFP-IresPuro knock-in cassette.

**PB gene trap system**

PB 5′TR and PB 3′TR with LoxP sites were amplified by PCR from plasmid PB-SB-PGK-Neo-bP1 [34] and ligated to pBluescript to generate pGG81. An oligo linker was inserted to pGG81 to generate pGG83 containing multiple cloning sites. The *SAIRES*geo cassette was generated by four-way ligation of *IRES* fragment from pCA1 [33], the PCR amplified splice acceptor (S) fragment and the *LacZ/Neo* fragment from RGTV1 [2] into pBlueScript. *SAIRES*geo was then inserted to the pGG83 to generate the PB based gene trap vector, pGG83. Splinkerette PCR was performed as described [34]. In brief, genomic DNA was digested with *Bst*II and then ligated with Splinkerette oligo adapter. The genome and PB insertion junction was amplified with HMSP-1/PB-SP1 primers and then nested PCR using HMSP-2/SP-SP2 primers. PCR reaction was treated with Exonuclease I (New England Biolabs) to degrade single strand oligonucleotides, followed by ethanol precipitation for sequencing with SP3 primers.

**Luciferase assay**

Cells were co-transfected with TOPFlash and Renilla plasmids using LipofectamineTM 2000 (Invitrogen). Luciferase assay was performed using Dual Luciferase Reporter Assay System (Promega). Recombinant mouse Wnt-3A was purchased from R&D Systems.

**siRNA knock down**

Tcf3 siRNA (ON-TARGETplus SMARTpool L-04861-01-0005) and the control siRNA (ON-TARGETplus Non-targeting pool D-001810-10-05) were purchased from Dharmacon. 10 nM siRNA or control was used for each transfection with LipofectamineTM 2000 (Invitrogen). Luciferase assay was performed using a CyAn flow cytometer (DakoCytomation).

**Quantitative RT-PCR**

Total RNA was prepared using RNeasy mini Kit (Qiagen). First strand cDNA was synthesised using SuperscriptTM III reverse transcriptase (Invitrogen) and Oligo-dT priming. Real time PCR was performed using Taqman probes (Applied Biosystems) or the universal probe library (Roche). Relative expression was determined using the delta Ct method. Standard deviation was calculated on three PCR triplicates.

**Flow cytometry analysis**

For live cell analysis, ES cells were collected in PBS with 3% FCS. ToPro-3 (Invitrogen) was added to cells at a final concentration of 0.05 nM for staining of dead cells. Analyses were performed using a CyAn flow cytometer (DakoCytomation).

**Acknowledgments**

We are grateful to Allan Bradley for discussion. We thank Rachael Walker for flow cytometry and Isabel Eyres for technical support.

**Author Contributions**

Conceived and designed the experiments: GG AS. Performed the experiments: GG. Analyzed the data: GG AS. Contributed reagents/materials/analysis tools: YH PH XW. Wrote the paper: GG AS.
11. Toyooka Y, Shimosato D, Murakami K, Takahashi K, Nica H (2008) Identification and characterization of subpopulations in undifferentiated ES cell culture. Development 135: 909–918.

12. Wray J, Kalkan T, Smith AG (2010) The ground state of pluripotency. Biochem Soc Trans 38: 1027–1032.

13. Mountford PS, Smith AG (1995) Internal ribosome entry sites and dicistronic RNAs in mammalian transgenesis. Trends Genet 11: 179–184.

14. Cary LC, Goebel M, Corasao BG, Wang HG, Rosen E, et al. (1989) Transposon mutagenesis of baculoviruses: analysis of Trichoplusia ni transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. Virology 172: 156–169.

15. Devon RS, Porteous DJ, Brookes AJ (1995) Splinkerettes—improved vectorrettes for greater efficiency in PCR walking. Nucleic Acids Res 23: 1644–1645.

16. Ying QL, Stavridis M, Griffiths D, Li M, Smith A (2003) Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Mol Cell Biol 26: 7479–7491.

17. Pereira L, Yi F, Merrill BJ (2006) Repression of Nanog gene transcription by Tcf3 limits embryonic stem cell self-renewal. Mol Cell Biol 26: 7479–7491.

18. Cole MF, Johnstone SE, Newman JJ, Kagey MH, Young RA (2008) Tcf3 is an integral component of the core regulatory circuitry of embryonic stem cells. Genes Dev 22: 746–753.

19. Tam WL, Lim CY, Han J, Zhang J, Ang YS, et al. (2008) Tcf3 regulates embryonic stem cell pluripotency and self-renewal by the transcriptional control of multiple lineage pathways. Stem Cells.

20. Chambers I, Colby D, Robertson M, Nichols J, Lee S, et al. (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113: 643–655.

21. Li Y, McClintick J, Zhong L, Edenberg HJ, Yoder MC, et al. (2005) Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Krü. Blood 105: 635–637.

22. Hall J, Guo G, Wray J, Eyres I, Nichole J, et al. (2009) Oct4 and LIF/Stat3 additively induce Krüppel factors to sustain embryonic stem cell self-renewal. Cell Stem Cell 5: 597–609.

23. Nica H, Ogasawara, Shimosato D, Adachi K (2009) A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. Nature 460: 118–122.

24. Chambers I, Silva J, Colby D, Nichols J, Nijmeijer B, et al. (2007) Nanog safeguards pluripotency and mediates germline development. Nature 450: 1230–1234.

25. Silva J, Smith A (2008) Capturing pluripotency. Cell 132: 532–536.

26. Kalmar T, Lin G, Hayward P, Munoz-Descalzo S, Nichols J, et al. (2009) Regulated fluctuations in nanog expression mediate cell fate decisions in embryonic stem cells. PLoS Biol 7: e1000149.

27. Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang HI, et al. (2006) CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biol 7: R100.

28. Rathjen J, Lake JA, Bettes MD, Washington JM, Chapman G, et al. (1999) Formation of a primitive ectoderm like cell population, EPL cells, from ES cells in response to biologically derived factors. J Cell Sci 112(Pt 5): 601–612.

29. Wray J, Kalkan T, Gomez-Lopez S, Eckardt D, Kemler R, et al. (2011) Inhibition of glycogen synthase kinase-3 alleviates Tcf3 repression of the pluripotency network and increases embryonic stem cell resistance to differentiation. Nat Cell Biol. In press.

30. Yuan H, Horie K, Kondoh G, Kono M, Maeda Y, et al. (2004) Genome-wide phenotype analysis in ES cells by regulated disruption of Bloom’s syndrome gene. Nature 429: 896–899.

31. Smith AG (1991) Culture and differentiation of embryonic stem cells. J Tiss Cult Meth 13: 89–94.

32. Liu P, Jenkins NA, Copeland NG (2003) A highly efficient recombineering-based method for generating conditional knockout mutations. Genome Res 13: 476–484.

33. Abram CL, Page MJ, Edwards PA (1997) A new retroviral vector, CA1, to identify and select for cells expressing an inserted gene in vitro and in vivo. Gene 196: 187–189.

34. Mikkels H, Allen J, Knipscheer P, Romeijn L, Hart A, et al. (2002) High-throughput retroviral tagging to identify components of specific signaling pathways in cancer. Nat Genet 32: 153–159.