Combining competition assays with genetic complementation strategies to dissect mouse embryonic stem cell self-renewal and pluripotency

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Substantial scientific interest has been dedicated recently to the crucial factors that control the pluripotent state of stem cells. To gain a comprehensive understanding of the molecular mechanisms regulating mouse embryonic stem cell (mESC) self-renewal and lineage differentiation, we have developed a robust method for studying the role of a particular gene in these processes. This protocol describes detailed procedures for the design and generation of the complementation rescue system and its application in dissecting the network of pluripotency-associated factors, using mESCs as a model. Specifically, three main procedures are described: (i) screening pluripotency-associated factors by competition assay; (ii) setting up an inducible complementation rescue system; and (iii) dynamically studying the pluripotency network response to target depletion. Completion of the competition assay and complementation rescue system takes 35 and 30 d, respectively, and an additional 16 d to study the dynamic molecular effects of a gene of interest in the pluripotency network.

INTRODUCTION

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) hold great promise for biomedicine as a major source for generating differentiated cells for transplantation-based therapies, as well as for developing new avenues to study the etiology of diseases. They are distinguished from somatic cells by their capacity for unlimited self-renewal and differentiation potential into all cell lineages of an organism. It is important to have a comprehensive understanding of the molecular mechanisms controlling ESC self-renewal and the decisions governing the differentiation of ESCs into a wide range of mature cells in order for ESCs/iPSCs to fulfill their expectations in regenerative therapy. Over the past several years, much effort has been made to identify and understand the molecular mechanism regulating ESC self-renewal and pluripotency. A number of transcription factors (e.g., Oct4 (also known as Pou5f1), Sox2, Nanog, Esrrb, Tbx3, Tcl1, Tcf3, Foxo1 and Foxo3a)[1–2], histone modifiers (Wdr5, Ezh1 and Dpy30)[3–5] and chromatin remodeling molecules (the SWI/SNF complex and Tip60-p400)[6–8] have been characterized. In addition, signaling pathways have been proposed to integrate internal and/or external information via output in the nuclear transcriptional and epigenetic networks to control the ESC pluripotent state[9–12]. Recently, analyses of pluripotency protein-protein interaction networks and direct targets of these pluripotency factors have provided additional insights into how these factors collaborate with each other to regulate gene expression[13–20]. Uncovering new factors in self-renewal signaling and integrating their functions into the known pluripotency network will allow us to understand the molecular mechanisms controlling self-renewal and differentiation and more efficiently manipulate the pluripotent state for future application in regenerative medicine.

Development of the protocol

RNA interference (RNAi) system. mESCs are particularly attractive research tools for screening or for the identification of critical genes in ESC self-renewal and pluripotency because of their simplified culture conditions, fast growth rate and reduced expense compared with human ESCs. In recent years, many groups, including ours, have developed and applied multiple approaches to analyze and catalog the essential factors required for maintaining the ESC pluripotent state[2,4,6–7,21–26]. Gene deletion and RNAi-mediated gene silencing are two of the most common approaches chosen to study specific gene functions in ESCs. Although gene deletion is the ultimate tool for developing insights into the role of a specific gene, its time-consuming nature precludes high-throughput analysis. In addition, if a particular gene is required for maintaining normal cellular function, it will not be feasible to conduct future studies in the complete-null phenotype because of lethality. In contrast, RNAi, a natural process for suppressing gene function either by site-specific cleavage followed by degradation of the target mRNA or by inhibiting mRNA translation, still leaves open the possibility of context-dependent cell survival[27–28]. It is currently the most potent gene knockdown method used in animal systems[29–30]. The variability in knockdown efficiency across particular sequences and clones provides an additional benefit when studying the function of a gene associated with cell survival. Taking advantage of its rapidity, specificity and ease of handling, the RNAi-mediated gene knockdown system has provided a feasible approach for high-throughput functional genomics screening in organisms that were previously inaccessible to such systemic genetic perturbation. Small interfering RNA (siRNA) and short hairpin RNA (shRNA) are the most common RNAi approaches to deplete gene function in mESCs[6,21,26,31]. siRNAs are synthesized as 21- to 23-nt double-stranded RNA molecules. They are unwound by the RNA-induced silencing complex (RISC), and the antisense strand of siRNA serves as a template for recognizing complementary mRNA. Once its complementary strand is matched, the RISC complex with a bound siRNA-mRNA induces mRNA degradation and turns off future gene expression[32]. shRNAs are designed to produce a hairpin-shaped structure that contains

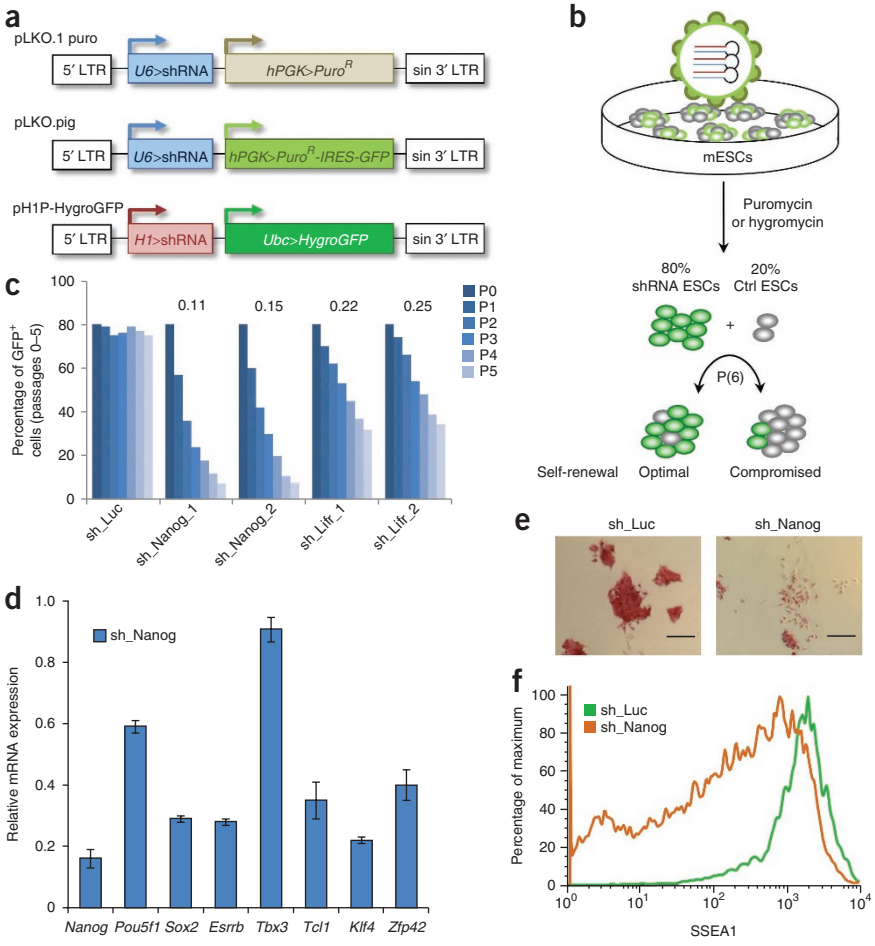

Figure 1 | Fluorescence-based competition assay and mESC pluripotency characterization. (**a**) Schematic maps of lentivirus-based shRNA constructs (pLKO.1 puro, pLKO.pig and pH1P-HygroGFP) used in gene knockdown in this protocol. (**b**) Competition assay is applied to identify essential pluripotency factors required for mESC self-renewal. (**c**) Knockdown of Nanog and Lifr results in impaired CCE self-renewal. shRNA targeting the luciferase gene (sh_Luc) does not significantly alter the GFP$^+$ population over six passages. The knockdown efficiency of Nanog and Lifr by two distinct shRNAs (sh_Nanog_1 and sh_Nanog_2 for Nanog, and sh_Lifr_1 and sh_Lifr_2 for Lifr) is shown above each time-course experiment. (**d**) qRT-PCR analysis of the expression of pluripotency genes (*Nanog*, *Pou5f1*, *Sox2*, *Esrrb*, *Tbx3*, *Tcl1*, *Klf4* and *Zfp42*) upon depletion of Nanog by pLKO.pig Nanog shRNA. The relative mRNA expression is compared with CCE cells infected with control Luc shRNA. Error bars indicate means ± s.e.m. for $n = 3$. (**e**) Knockdown of Nanog by pLKO.pig Nanog shRNA in CCE cells showed both differentiated morphologies and low AP activity. Scale bars, 50 µm. (**f**) SSEA1 staining shows reduced SSEA1 expression in CCE cells in response to knockdown of Nanog.

a sense strand, an antisense strand and a short loop between the sense and antisense sequences. The targeting sequence of shRNA varies in length from 19–29 nt. Similarly to siRNAs, shRNAs are also processed by Dicer and incorporated into the RISC complex.

The siRNA-mediated gene knockdown approach can be achieved by using a standard transfection method, but it only permits a transient 3- to 5-d knockdown of target genes. In contrast, shRNAs can be designed and inserted into DNA plasmids, transduced into cells using different delivery systems and, most importantly, stably expressed by lentivirus-meditated genome integration, thus executing stable and lasting silencing of target genes, which provides a perfect system for generating stable clones and studying the long-term effect of cellular function on target knockdown[29,33–40]. The two main advantages of shRNA over siRNA are long-term gene repression and easier transduction of cells that are relatively difficult to transfect (e.g., mESCs and human ESCs). Taking advantage of shRNA, we use the lentivirus-mediated shRNA knockdown system for studying specific gene function in mESCs. Two shRNA knockdown systems have been modified and successfully used in our laboratory[2,4,40]. One is the pH1P-HygroEGFP system, in which the shRNA is expressed from an *H1* promoter, whereas a *ubiquitin-C (Ubc)* promoter drives the expression of a hygromycin-green fluorescent protein (GFP) fusion gene[2,41] (**Fig. 1a**). Another is the pLKO.pig system, modified from the pLKO.1 puro vector[42], in which the shRNA is expressed from a *U6* promoter, whereas a human *PGK* promoter drives the expression of a gene encoding puromycin resistance (Puro^R), followed by an internal ribosome entry site (IRES) and GFP **Fig. 1a**)[4,40]. A number of shRNA design websites provide a free service for predicting a proper region in your gene of interest for RNAi-mediated gene knockdown on the basis of similar principles. In our laboratory, we typically opt for shRNA design provided by the RNAi consortium (TRC)[42].

The design and cloning of shRNA targeting your gene of interest into pLKO.1 puro, pLKO.pig or pH1P-HygroGFP vectors (**Fig. 1a**) are described in PROCEDURE Steps 1–12.

Competition assay system. To investigate the function of a specific gene in regulating ESC self-renewal and pluripotency, we use the above-mentioned lentivirus-based, shRNA-mediated loss-of-function approach combined with a fluorescence-based competition assay for our primary screening for putative candidates. The idea of a competition assay is based on the fact that mESCs have a much shorter G1 phase (usually 1–2 h) than differentiated cells do (usually 6–12 h)[43–44]. We anticipate that if a given shRNA affects mESC self-renewal and further induces differentiation, the growing population of shRNA-transduced mESCs will markedly decrease in comparison with control shRNA-transduced mESCs. Briefly, we coculture test shRNA-transduced GFP-positive (GFP$^+$) ESCs (CCE line) with control shRNA-transduced GFP-negative (GFP$^-$) cells in a 4:1 ratio in the presence of leukemia inhibitory factor (Lif). The ratio of GFP$^+$/GFP$^-$ is measured at every passage ending with passage six (**Fig. 1b**,**c**). As a decrease in the cell population might also be due to simple changes in cell cycle kinetics or compromised cell survival, the measurement of pluripotency transcription factors such as Oct4, Sox2, Nanog, Esrrb, Tbx3 and Tcl1; mESC surface marker stage-specific embryonic antigen 1 (SSEA1); and alkaline phosphatase (AP) activity should be applied to support the finding of the competition assay (**Fig. 1d–f**).

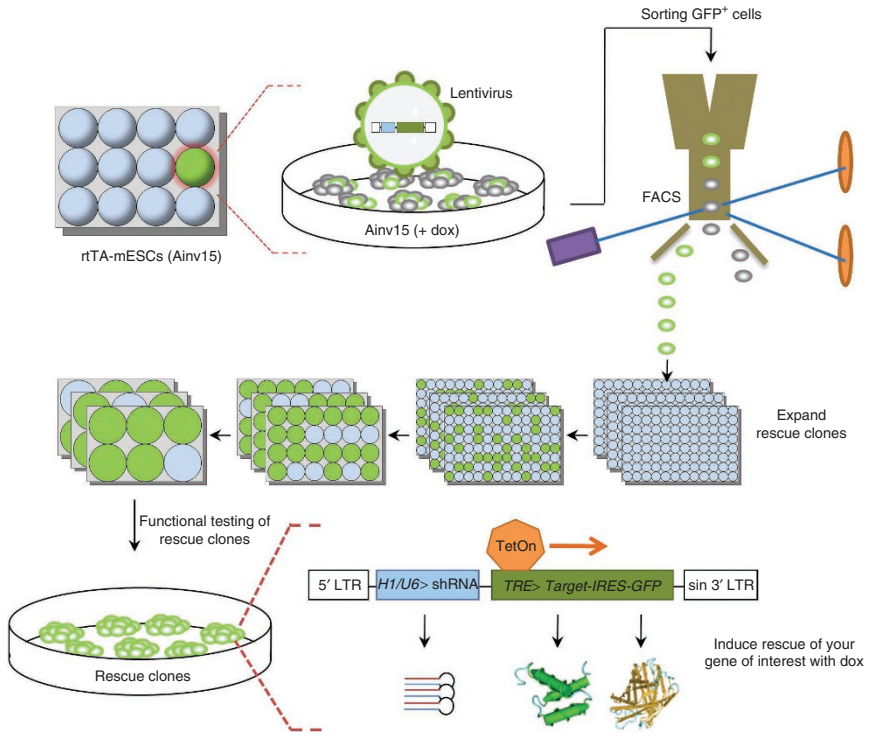

Figure 2 | Genetic complementation system setup and overview of steps involved in screening and expanding rescue clones. Setup starts with transduction of the complementation rescue construct, which carries a constitutively expressed shRNA and a TRE-controlled immune-deficient gene and GFP, into transactivator (rtTA)-expressing mouse Ainv15 ESCs. In the fluorescence-activated cell sorting (FACS) diagram shown here, the system isolates single cells and seeds them in 96-well dishes. After ~16 d of culture and expansion, the rescue clones should be isolated and then characterized.

Genetic complementation rescue system. Although RNAi knockdown is broadly applied in studying specific gene function in ESCs[21,26], eliminating the possibility of off-target effects of sequences in either siRNAs or shRNAs is essential before more comprehensive investigation of any candidate gene[2]. To solve these problems, we have developed a genetic complementation rescue system to evaluate the results of the competition assay[2,4,41]. Briefly, this system contains two expression cassettes, a shRNA designed to deplete a target gene product and an inducible shRNA-'immune' expression system that rescues this depletion in order to evaluate and confirm the target-specific effect of the shRNA. This complementation vector is specially designed to modify the previously described pLKO.pig or pH1P-HygroGFP, which we used for gene knockdown and the competition assay, by replacing *hPGK-Puro^R-IRES-GFP* or *Ubc-HygroGFP* with tetracycline response element *(TRE)-'RNAi-immune target gene'-IRES-GFP*. This engineered lentivirus-based rescue vector contains a human *U6* or *H1* promoter–driven shRNA targeting an endogenous mRNA and a *TRE* promoter driving an exogenous shRNA-immune rescue mRNA and GFP, which is translated via an *IRES* element. This system requires an ESC line, such as the Ainv15 line, which expresses the TetOn rtTA transactivator[45]. In the absence of doxycycline hydrochloride (dox), both the rescue mRNA and GFP are not expressed. In the presence of dox, the exogenous shRNA-immune mRNA is expressed and rescues the knockdown effect caused by the constitutively expressed shRNA, also producing a GFP reporter (**Fig. 2**). A schematic experimental procedure is outlined in **Figure 2**. Importantly, this method can be applied not only to identifying ESC pluripotency genes, but also to fine time- and dose-dependent regulation of target gene expression, which aids in dissecting the dynamic biological decision underlying cell fate and lineage commitment upon knockdown of a specific target gene[41].

Application of the method

Recently, we have applied this methodology to analyzing and visualizing the temporal alternations of pluripotency-associated factor gene expression upon knockdown of a given target gene by integrating the knockdown effects into a dynamic picture of transcriptional activity and pluripotency-associated factor kinetics[2,41]. This approach can also be applied to the study of the flow of widespread cellular changes through different molecular regulatory layers, ranging from the epigenetic landscape to the transcriptome and finally the proteome, which we have described previously[41]. Notably, this system can serve as a platform for studying the set of perturbations that trigger changes in histone methylation/acetylation, DNA methylation, gene transcription and protein phosphorylation by high-throughput analyses. This will permit studies that can provide novel insights into the multilayered regulation of mESC self-renewal and pluripotency. By measuring the dynamic alteration of gene expression, particularly among pluripotency transcription factors, this inducible complementation rescue system allows us to monitor the subsequent cascade of events that leads to the collapse of the core mESC pluripotency network[13,16–17,19–20,46]. We describe how to identify features in dynamic transcriptional systems and exploit this information to study the nature of pluripotency network regulation using the *Nanog* rescue clone as an example (see PROCEDURE Steps 65–89).

Comparison of the competition assay and genetic complementation rescue system

The competition assay offers a number of advantages over traditional approaches to identifying factors involved in mESC self-renewal and pluripotency. First, the competition assay exploits the rapid proliferation rate of pluripotent mESCs (8–12 h per cell cycle), which is faster than that of differentiated cells. Usually, mESCs have a very short G1 phase (1–2 h). In contrast, differentiated cells (e.g., mouse embryonic fibroblast (MEFs)) transit the G1 phase in 6–12 h and many adult cells take even longer[43]. Without the disadvantage of the traditional proliferation assays, such as the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, in which the cell growth rate is significantly affected by cell density, the competition assay provides a more stable culture condition for maintaining an equal number of cells across different passages and eliminating the variation stemming from direct cytotoxicity immediately after gene knockdown. Second, in contrast

with the direct measurement of the expression of pluripotency factors by quantitative real-time PCR (qRT-PCR) and immunostaining, the flow cytometry–based competition assay provides a convenient and inexpensive platform for the primary screening. Third, after the identification of putative pluripotency-associated factors by the competition assay, the genetic complementation rescue system offers a tightly controlled system with which to confirm that the effect on mESC self-renewal and pluripotency is directly attributable to target knockdown and not to shRNA off-target effects. Fourth, the genetic complementation rescue system permits the study of the time-course effects of target knockdown on the pluripotency network. Notably, measurements from such a system can be applied to the integration of experimental and computational techniques for dissecting the dynamic biological decisions underlying cell fate and lineage commitment upon knockdown of a specific target gene[41].

However, potential weaknesses and limitations do exist in this methodology. First, additional time and effort are required to establish a lentivirus-based shRNA expression system to knock down the specific genes in mESCs compared with direct knockdown by siRNAs. Second, cell cycle and apoptosis-related genes usually are selected by the competition assay because their knockdown results in defects of cell proliferation and survival. Hence, some of these might later prove to be false positives. Further confirmation, including examining pluripotency transcription factor expression, AP and SSEA1 staining, tetraploid complementation assay and even chimera formation/germ-line transmission, is required to conclude whether the candidates identified by the competition assay are worth pursuing with complementation rescue clones.

Third, some pluripotency factors might have multiple cellular functions (e.g., they might prevent cell apoptosis and maintain cell survival) that intersect with their control of pluripotency. In our hands, we have encountered difficulties in obtaining enough cells to initiate the competition assay. To obtain enough cells, a long culture period is required; this results in 'clone selection,' in which mESCs maintain a certain level of gene knockdown but undergo only minor differentiation. They might serve as false negatives. Fourth, PCR cloning for the coding region of candidate genes is essential for the generation of complementation rescue clones. The huge size of some candidates (>8 kb) might make cloning more challenging, although several efficient high-fidelity PCR enzymes (e.g., PfuUltra DNA polymerase and Phusion DNA polymerase) can be used to perform this long-fragment amplification. Fifth, stability of rescue clones varies. We realize that some rescue clones will lose the rescue gene and GFP expression during culture, perhaps because of inactivation of the lentivirus depending on the genomic insertion site. Repeat sorting to pick up single clones would be required if this occurs.

Experimental design

Positive and negative controls. shRNAs targeting Nanog, Sox2, Pou5f1, Esrrb, Foxo1 and Foxo3a are ideal as positive controls because of their essential functions in maintaining mESC identity[1–2]; Luciferase (Luc) shRNA (5′-CTTACGCTGAGTACTTCGA-3′)[47] or a scrambled shRNA, in which the nucleotides of the original nonscrambled target shRNA sequence are randomly reordered, thereby preventing effective targeting, can be used as a negative control.

Effect of knockdown of pluripotency factors in the competition assay. If a transduced shRNA interferes with ESC self-renewal, cell propagation will slow down and GFP^+/GFP^- ratios will rapidly decline. In contrast, if a transduced shRNA does not affect ESC pluripotency, GFP^+/GFP^- ratios will have no or little change (**Fig. 1c**). We use Luc shRNA, which does not affect ESC pluripotency, as a negative control. Ideally, the GFP^+ population of Luc shRNA–transduced cells remains at around 80% after six passages. In parallel, Nanog and Lifr shRNAs (5′-GCCAACCTGTACTATGTTTAA-3′, 5′-GACAGTGAGGTGCATATAC-3′, 5′-GCAGAGATACAGCTTAGTAAA-3′ and 5′-GCCTCATTTCTCCGGTTACAA-3′ for sh_Nanog_1, sh_Nanog_2, sh_Lifr_1 and sh_Lifr_2, respectively) are used as strong and mild positive controls. Usually, the GFP^+ populations of Nanog and Lifr shRNA–transduced cells are around 8 and 35% after six passages (12 d) in mouse CCE cells (**Fig. 1c**). It is worth cautioning that knockdown efficiency of the target gene is a major factor affecting the result of the competition assay. Although both Nanog and Lifr are well-defined pluripotency factors controlling ESCs, Nanog functions as a direct core pluripotency transcription factor, whereas Lifr-Stat3 functions as a peripheral factor. Therefore, knockdown of Nanog causes a more severe decrease in the GFP^+ population than knockdown of Lifr.

Variability in the competition assay. To eliminate variability in the competition assay, we usually execute the competition assay in biological triplicate. To rule out the possibility of nonspecific effects stemming from a specific shRNA, more than two different shRNAs with similar knockdown efficiencies are crucial to confirm the result of the competition assay. As shown in **Figure 1c**, two distinct shRNAs targeting either Nanog or Lifr produce comparable GFP^+ fractions after six passages (7.5% GFP^+ in sh_Nanog_1 versus 7.7% GFP^+ in sh_Nanog_2; 32% GFP^+ in sh_Lifr_1 versus 35% GFP^+ in sh_Lifr_2). Furthermore, the same batch of reagents of mESC medium components (especially ESGRO(LIF) and FBS), and the same multiplicity of infection (MOI) of virus for infecting CCE cells, should be used throughout the competition assay.

Examination of shRNA knockdown efficiency. It is an essential step to measure shRNA knockdown efficiency in order to determine whether the biological change upon shRNA introduction really comes from knockdown of your gene of interest rather than from nonspecific effects. qRT-PCR is a routine method applied to determine knockdown efficiency. To obtain an accurate knockdown efficiency measurement, the primer design for qRT-PCR is extremely crucial. We design our qRT-PCR primer sets by using PrimerBank, a public database for PCR primers (http://pga.mgh.harvard.edu/primerbank/) for gene expression detection. Predesigned primer sets of genes of either human or mouse species can be searched by either GenBank accession number, National Center for Biotechnology Information (NCBI) protein accession number, NCBI gene ID, NCBI gene symbol or keyword. We usually seek a knockdown efficiency of >70% before proceeding with any given shRNA for future study. Furthermore, the effect of knockdown on protein expression should be determined by immunoblotting if the appropriate antibody is available.

shRNA-immune gene. Generation of an inducible complementation rescue construct to rule out potential shRNA off-target effects relies on a so-called shRNA-immune gene, thus allowing

the evaluation of the specific effect of target downregulation on mESC self-renewal and pluripotency. If the shRNA targets the 3′ untranslated region (UTR) of a target gene, the unmodified coding sequence of that target gene product is suitable for rescuing the shRNA-mediated effects. However, if the shRNA targets the coding region of a target gene, the shRNA-immune gene product can be generated by mutating 3 nt in the middle of the shRNA target sequence (shown in boldface below) while preserving the amino acid sequence. For instance, the *Wdr5* shRNA2 targeting sequence is GCC-GT**T**-CA**T**-TT**C**-AAC-CGT-GAT (176-Ala-Val-His-Phe-Asn-Arg-Asp-182). The *Wdr5* rescue gene should be cloned and its targeted sequence mutated to GCC-GT**c**-CA**c**-TT**t**-AAC-CGT-GAT in order to prevent Wdr5 shRNA2 knockdown, thereby taking advantage of the wobble base and introducing no amino acid–level changes[4]. The effect of the shRNA on the 'immune' gene should be determined by qRT-PCR and immunoblotting to ensure that the construct is not being degraded by the shRNA.

mESC pluripotency characterization. Several methods are routinely used in our laboratory to characterize mESC pluripotency. AP staining is a simple, rapid and sensitive method for phenotypic visualization of undifferentiated mESCs that exploits the high level of AP activity in pluripotent mESCs and extreme decrease of AP expression during mESC differentiation. Flow cytometry analysis of SSEA1 provides another characterization of the mESC pluripotent state. Furthermore, measuring mRNA expression of pluripotency factors, such as Oct4, Sox2 and Nanog, by qRT-PCR provides a more direct and precise way to evaluate the pluripotent status of mESCs. This method provides a quantitative assay for detecting the switching of the global cellular pluripotency state between undifferentiated and differentiated cells. For more details, please see **Box 1**. Moreover, to evaluate *in vivo* pluripotency higher standards can be applied, such as the tetraploid complementation assay and chimera formation/germ-line transmission assay[48]. The tetraploid complementation assay examines the ability of a tetraploid wild-type blastocyst that contributes exclusively to extraembryonic tissue injected with cells from a modified ESC line (e.g., by transduction with an shRNA cassette targeting a particular gene) to form a new embryo proper; this new embryo is composed entirely of the injected ESCs and is a useful tool to study *in vivo* pluripotency. The chimera formation/germ-line transmission assay determines whether modified mESCs injected into blastocysts are able to contribute efficiently to all tissues, including germ-line cells, and form chimeric mice[2]. Owing to the fact that they are very time-consuming, these two methods are usually only used to evaluate the potency of final cherry-picked hits.

Sample preparation for qRT-PCR analysis and primer design. To estimate the effect of a specific perturbation (e.g., knockdown of a gene of interest) on the mESC pluripotency network, qRT-PCR analysis is used to monitor changes in the gene expression levels after the perturbation. RNA of collected cell samples is extracted using TRIzol and followed by the purification with the RNeasy mini kit. Thereafter, RNA is converted to double-stranded cDNA. Quantitative PCR is performed by using SYBR Green on the LightCycler480 real-time PCR system. For more details, please see **Box 1**. Gene-specific primers are obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/), a public resource for PCR primers.

Box 1 | Methods for characterizing mESCs pluripotency ● TIMING variable; 40 min–8 h

AP staining ● TIMING 40–60 min

The following steps describe AP staining on mESCs growing in one six-well plate.

1. Aspirate away the cell culture medium and wash each well twice with 2 ml of 1× DPBST (see REAGENT SETUP).
2. Add 2 ml of fix solution for each well and incubate at room temperature for 1–2 min.

! CAUTION Do not overincubate the cells with fix solution. Fixation longer than 2 min causes loss of AP activity.

3. Aspirate away the fix solution and wash each well with 2 ml of 1× DPBST.

! CAUTION Do not let the wells completely dry out.

4. Prepare AP staining solution. Mix AP staining solutions A and B in a ratio of 1:1. For one well of a six-well cell culture plate, mix 0.5 ml of AP staining solution A and 0.5 ml of AP staining solution B in a 15-ml conical tube.

▲ CRITICAL STEP Freshly prepare AP staining solution and use it as soon as possible after preparation, ideally within 1 min after mixing AP staining solutions A and B. Discard the remaining solution.

5. Aspirate away the 1× DPBST and add 2 ml of fresh AP staining solution to each well.
6. Incubate the cell plate in the dark at room temperature for 10–20 min.

▲ CRITICAL STEP To avoid nonspecific staining, check the color change and stop the staining when the color turns bright.

7. Stop the staining reaction by aspirating the AP staining solution, and then wash the wells with 2 ml of 1× DPBS each.

▲ CRITICAL STEP Use 1× DPBS instead of 1× DPBST to wash.

8. Cover the wells with 1× DPBS to prevent drying.
9. Check the staining result under a phase contrast microscope.
10. Store the plate at 4 °C for future purposes.

SSEA1 staining ● TIMING 1–2 h

1. Trypsinize cells and centrifuge at 300*g*, 4 °C, for 5 min (see **Box 3**).
2. Wash cells three times with 1× DPBS (supplemented with 0.5% (wt/vol) BSA).

(continued)

Box 1 | (continued)

3. Fc-block the cell sample with 1 µg of mouse IgG per 10^5 cells (4×10^6 cells per ml) for 15 min at room temperature prior to staining. Do not wash away the excess blocking IgG from this step.
4. Transfer 25 µl cells (1×10^5 cells) in a 5-ml polypropylene round-bottom tube. Add 10 µl of phycoerythrin (PE)-conjugated anti-SSEA1 antibodies.
5. Incubate at 4 °C for 30–45 min.
6. For control analysis, treat cells in a separate tube with PE-labeled mouse IgM antibody.
7. After the incubation, wash the cells twice with 5 ml 1× DPBS buffer (supplemented with 0.5% (wt/vol) BSA) to remove the excess anti-SSEA1 antibodies. Centrifuge at 300*g*, 4 °C, for 5 min.
8. Resuspend the cells in 200–400 µl of 1× DPBS for flow cytometric analysis using BD LSR II.

qRT-PCR analysis ● TIMING 6–8 h

1. Collect the cell samples from the six-well culture plate. Dissolve the cell pellet in 800 µl of TRIzol reagent. Extract mRNA from cell samples using the RNeasy mini kit according to the manufacturer's protocol. Measure RNA concentration by NanoDrop ND-1000 spectrophotometer.
▲ **CRITICAL STEP** Perform DNase treatment to prevent DNA contamination.
■ **PAUSE POINT** Store RNA at −80 °C for later use.
2. Perform reverse-transcription reaction with 1 µg of RNA by using the high-capacity cDNA reverse transcription kit to synthesize cDNA.
■ **PAUSE POINT** Store cDNA at −20 °C for later use.
3. Dilute cDNA in DNase- and RNase-free water tenfold.
4. Use primer pairs for measuring pluripotency gene expression as described in **Table 1**. Measure endogenous mRNA expression with primer pairs in the 3′ UTR of your gene of interest and the total (exogenous+endogenous) mRNA expression using primer pairs in the coding region of your gene of interest (see PROCEDURE Steps 63–87 for samples of *Nanog* rescue clone).
5. Set up qPCR in a LightCycler 480 multiwell plate 384. Each well should contain 5 µl of FastSYBR Green master mix, 0.5 µl of forward primer (10 µM), 0.5 µl of reverse primer (10 µM), 2 µl of diluted cDNA and 2 µl of water. β-Actin is used as a qPCR internal control.
6. Perform qPCR in a LightCycler 480 real-time PCR machine or other appropriate instrument. PCR parameters: 1 cycle at 95 °C for 20 s and 40 cycles of 95 °C for 3 s, followed by 60 °C for 30 s, according to the manufacturer's protocol.
7. Collect results and analyze the gene expression data. Determine the amount of each amplification gene product, as described previously[51].

MATERIALS

REAGENTS

- CCE ESCs (ATCC, cat. no. SCRC-1023)
- Ainv15 ESCs (rtTA-expressing ES cells, a gift from G. Daley; ATCC, cat. no. SCRC-1029)
- Human embryonic kidney (HEK) 293T cells (ATCC, cat. no. CRL-11268)
- mESC medium (see REAGENT SETUP)
- MEF cells (Global Stem, cat. no. GSC-6001G)
- Dulbecco's modified Eagle medium (DMEM) with high glucose (Invitrogen, cat. no. 11965)
- Hyclone FBS (defined; Thermo Scientific, cat. no. SH30070.03)
- Penicillin-streptomycin (Invitrogen, cat. no. 15140)
- L-Glutamine (Invitrogen, cat. no. 25030)
- Sodium pyruvate (Invitrogen, cat. no. 11360-070)
- MEM nonessential amino acids (MEM NEAA, Invitrogen, cat. no. 11140)
- β-Mercaptoethanol (B-ME; Sigma, cat. no. M7522) **! CAUTION** It is acutely toxic; avoid inhalation and contact with skin and eyes.
- ESGRO (LIF) (Millipore, cat. no. ESG1107)
- Dulbecco's phosphate-buffered saline (DPBS; Invitrogen, cat. no. 14287072)
- Gelatin solution (0.1% (wt/vol); Millipore, cat. no. ES-006-B)
- Gelatin (Sigma, cat. no. G-1890)
- TrypLE Express (1×), phenol red (Invitrogen, cat. no. 12605-010)
- DMSO (Fisher Scientific, cat. no. BP231-1)
- pLKO.1 puro plasmid (Addgene, Plasmid no. 8453)
- pCMV-dR8.2 dvpr plasmid (Addgene, Plasmid no. 8455)
- pCMV-VSV-G plasmid (Addgene, Plasmid no. 8454)
- AgeI (5,000 U ml^{-1}; New England Biolabs, cat. no. R0552L)
- EcoRI (20,000 U ml^{-1}; New England Biolabs, cat. no. R0101L)
- SmaI (20,000 U ml^{-1}; New England Biolabs, cat. no. R0141L)
- XbaI (20,000 U ml^{-1}; New England Biolabs, cat. no. R0145L)
- Hexadimethrine bromide (commonly referred to as polybrene; Sigma, cat. no. H9268)
- Puromycin (Sigma, cat. no. P8833)
- Opti-MEM I reduced-serum medium (Invitrogen, cat. no. 11058-021)
- Freezing medium (see REAGENT SETUP)
- SuperFect transfection reagent (Qiagen, cat. no. 301305)
- FastSYBR Green master mix (Applied Biosystems, cat. no. 4385612)
- BD Matrigel (BD, cat. no. 356230)
- Iscove's DMEM (IMDM; Cellgro, cat. no. 15-016-CV)
- AP staining kit (includes fix solution, AP staining solution A, AP staining solution B; Stemgent, cat. no. 00-0009)
- Tween 20 (Acros Organics, cat. no. 23336-2500)
- Anti-SSEA1-phycoerythrin (R & D systems, cat. no. FAB2155P)
- BSA (Fisher Scientific, cat. no. BP1605-100)
- RNeasy mini kit (Qiagen, cat. no. 74104)
- QIAprep spin miniprep kit (250; Qiagen, cat. no. 27106)
- MinElute gel extraction kit (250; Qiagen, cat. no. 28606)
- TRIzol reagent (Invitrogen, cat. no. 15596-018)
- High-Capacity cDNA reverse transcription kit (Applied Biosystems, cat. no. 4368814)
- Agarose (Fisher Scientific, BP160-500)
- T4 DNA ligase (New England Biolabs, cat. no. M0202L)
- LB broth (RPI, cat. no. L24060-500.0)
- LB agar (RPI, cat. no. L24030-500.0)
- Ampicillin (Fisher Scientific, BP1760-25)
- Doxycycline (Fisher Scientific, cat. no. BP2653-1)
- Hygromycin B in PBS (50 mg ml^{-1}; Invitrogen, cat. No. 10687-010)
- pH1P-HygroGFP, pLKO.pig and pTRE-IRES2-EGFP (+ XcmI) plasmids (available from I.R.L.'s laboratory upon request)

- Distilled H_2O
- ddH_2O

EQUIPMENT

- Class-II cabinet with aspirator for tissue culture (Nuair, Class II, Type A1B3)
- Forma Scientific CO_2 water-jacketed incubator (37 °C, 5% CO_2; Forma Scientific)
- Water bath, Precision 180 Series (37 °C)
- Hemocytometer (Bright-line, Improved Neubauer, 0.1 mm deep)
- Cell culture tabletop centrifuge (Eppendorf, cat. no. Centrifuge 5810F)
- Refrigerator (4 °C) and freezer (−20 °C)
- Liquid nitrogen storage tank
- Pipettes (10, 20, 200 and 1,000 µl for tissue culture use only; Gilson)
- Cryotubes (1.8-ml tubes; Nalgene, cat. no. 5000-0012)
- Microcentrifuge tubes (1.5 ml; Crystalgen, cat. no. 033429775)
- Phase-contrast microscope (Nikon, TMS)
- Fluorescence microscope (Nikon, Eclipse, TE2000-U)
- Plastic disposable pipettes (1, 5, 10 and 25 ml)
- Tissue culture plate (10 cm; BD Falcon, cat. no. 353003)
- Flat-bottomed six-well tissue culture plate (BD Falcon, cat. no. 353046)
- Flat-bottomed 12-well tissue culture plate (BD Falcon, cat. no. 353043)
- Flat-bottomed 24-well tissue culture plate (BD Falcon, cat. no. 353047)
- Flat-bottomed 96-well tissue culture plate (BD Falcon, cat. no. 353072)
- Conical tubes (15 ml; BD Falcon, cat. no. 352099)
- Conical tubes (50 ml; BD Falcon, cat. no. 352098)
- CryoTube vials (Nunc, cat. no. 377267)
- Cryo 1 °C freezing container 'Mr. Frosty' (Nalgene, cat. no. 5100-0001)
- Polypropylene round-bottom tube (5 ml; BD Falcon, cat. no. 352063)
- Polystyrene round-bottom tube (5 ml; BD Falcon, cat. no. 352058)
- Cell-strainer caps for tubes
- NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies)
- Fisherbrand 9-inch Pasteur pipette (Fisher Scientific, cat. no. 13-678-6B)
- Filter unit (500 ml, 0.22 µm; Nalgene, cat. no. 5660020)
- Filter unit (150 ml, 0.22 µm; Nalgene, cat. no. 5650020)
- Low-protein-binding filter unit (0.45 µm; Corning Life Sciences, cat. no. 0976155)
- Amicon Ultra-15 centrifuge filter units (Millipore, cat. no. UFC903024)
- Petri dishes (BD Falcon, cat. no. 351029)
- LightCycler 480 real-time PCR system (Roche, cat. no. 04545885001)
- LightCycler 480 multiwell plate 384, white (Roche, cat. no. 04729749001)
- Glass beaker (2 liters; Kimax, cat. no. 14005)
- Heat block (VWR, cat. no. 12621-104)
- Wide Mini-Sub cell GT system, horizontal electrophoresis system (Bio-Rad, cat. no. 170-4405)
- Power supply (PS 500×T DC)
- DAKO-Cytomation MoFlo high-speed sorter (Beckman Coulter, formerly Dako)
- BD LSR II flow cytometer (BD Biosciences)

REAGENT SETUP

Gelatin solution (0.1%, wt/vol) To prepare 1 liter of 0.1% (wt/vol) gelatin solution, dissolve 1 g of gelatin powder in 100 ml of 10× DPBS and add 900 ml of distilled H_2O. Autoclave the solution. The solution can be stored at 4 °C for up to 1 month.

Gelatin-coated culture dishes Coat culture plates with a sufficient volume of 0.1% (wt/vol) gelatin solution for at least 15 min in a 37 °C incubator. Use 10 ml, 2 ml, 1 ml, 0.5 ml or 100 µl of 0.1% (wt/vol) gelatin solution for each well in a 10-cm 6-well, 12-well, 24-well or 96-well plate, respectively. Before use, aspirate off the gelatin and seed cells immediately. Gelatin-coated plates can remain in a 37 °C incubator for up to 7 d before use.

B-ME (100×, 10 mM) To prepare 100 ml of 100× B-ME, add 37 µl of B-ME in 100 ml of DPBS and filter-sterilize through a 100-ml 0.22-µm filter unit. Store the solution at 4 °C for up to 1 month.

mESC culture medium To prepare 500 ml of mESC culture medium, mix 400 ml of DMEM, 75 ml of FBS, 5 ml of penicillin-streptomycin, 5 ml of L-glutamine, 5 ml of sodium pyruvate, 5 ml of MEM NEAA, 5 ml of 100× B-ME and 50 µl of ESGRO(LIF) , and then filter-sterilize through a 500-ml 0.22-µm filter unit. Store the medium at 4 °C for up to 1 month.

293T/MEF culture medium To prepare 500 ml of 293T/MEF culture medium, mix 435 ml of DMEM, 50 ml of FBS, 5 ml of penicillin-streptomycin, 5 ml of L-glutamine and 5 ml of sodium pyruvate; filter-sterilize through a 500-ml 0.22-µm filter unit. Store the medium at 4 °C for up to 1 month.

Freezing medium To prepare 10 ml of freezing medium, mix 5 ml of FBS, 4 ml of DMEM and 1 ml of DMSO. Store the medium at 4 °C for up to 1 month.

MEF feeder cells MEF feeder cells are used for culturing Ainv15 ES cells. Dissect out E13.5–14.5 mouse embryos and culture them for embryonic fibroblast growth. Expand the established fibroblast cell culture and mitotically inactivate by γ-irradiation treatment. Freeze these irradiated MEFs (2×10^6 cells per vial)[49]. Commercial MEFs are also available (see REAGENTS).

MEF feeder cells on coated culture dishes The maintenance of Ainv15 mESCs requires survival signals from MEF feeder cells. To prepare MEF feeder cell–coated culture dishes, thaw a cryovial of Global Stem commercial MEFs (see REAGENTS) and plate them onto four gelatin-coated 10-cm or six-well tissue culture dishes. ▲ **CRITICAL** The dilution of MEFs seeded on culture dishes should be determined by the frozen feeder cell concentration or performed according to the provided protocol.

Matrigel aliquots Take Matrigel out of the −20 °C freezer and thaw it on ice overnight. For a bottle of 10 ml of Matrigel, add an equal volume (10 ml) of IMDM and 100 µl of penicillin-streptomycin. Mix the Matrigel well and prepare 2-ml aliquots in 5-ml polystyrene round-bottom tubes. ▲ **CRITICAL** Matrigel solidifies at room temperature (25 °C).

Matrigel-coated culture dishes Thaw Matrigel aliquots for at least 3–4 h on ice. Meanwhile, cool a six-well culture plate on ice or in a freezer for at least 15 min and keep the plate on ice before use. Add ~1 ml of Matrigel into the first well and gently shake the plate until the well is covered with Matrigel. Remove extra Matrigel and add it into the next well. Repeat this for all six wells. Store the remaining Matrigel at 4 °C for up to 2 months before use. Leave the Matrigel-coated plate on ice for 30 min and then remove any remaining Matrigel from wells by aspiration. Place the Matrigel-coated plate into the 37 °C, 5% CO_2 incubator overnight. The plate is ready to use the next day.

DPBST (1×) To prepare 100 ml of DPBST, add 50 µl of Tween 20 into 100 ml of 1× DPBS to obtain a final concentration of 0.05% (vol/vol). Mix well and store at room temperature for up to 1 year before use.

PROCEDURE

Design of shRNA to knock down your gene of interest ● TIMING 1 h

1| *Day 1*. Design your shRNA forward and reverse oligos using Public TRC Portal (http://www.broadinstitute.org/rnai/public/). Well-designed shRNAs targeting genes of either human or mouse species can be searched for by official gene symbol or transcript ID (XM_ or NM_ sequences). For instance, the official gene symbol of *Mus musculus* WD repeat domain 5 is *Wdr5* and its reference transcript ID is NM_080848.2. Searching for *Wdr5* in this shRNA design tool will return ten distinct clone IDs and their associated target sequence information (e.g., match position and region (coding sequence or 3′ UTR) and adjusted score) and details on oligo design for arrayed cloning. For pLKO.1 puro and pLKO.pig vectors, the designed shRNA duplex consists of two oligos, a forward oligo (5′-CCGG-21bp_sense_target_sequence-CTCGAG-21bp_antisense_target_sequence-TTTTTG-3′) and a reverse oligo (5′-AATTCAAAAA-21bp_sense_target_sequence-CTCGAC-21bp_antisense_target_sequence-3′). This set of primers is used for directly cloning the annealed shRNA duplex

into AgeI- and EcoRI-digested pLKO.1 or pLKO.pig. For shRNAs with the pH1P-HygroGFP vector, the designed shRNA duplex consists of two oligos, a forward oligo (5′-CCGG-21bp_sense_target_sequence-CTCGAG-21bp_antisense_target_sequence-TTTTTTGT-3′) and a reverse oligo (5′-CTAGACAAAAAA-21bp_sense_target_sequence-CTCGAC-21bp_antisense_target_sequence-3′). This set of primers is used for direct cloning of the annealed shRNA duplex into pH1P-HygroGFP digested by SmaI and XbaI. The CTCGAG sequence is designed to form a loop structure between the sense and antisense strands of the shRNA.

Generate shRNA-expressing construct ● TIMING 4 d

2| *Day 2*. Resuspend the oligos, previously ordered from commercial vendors, at a concentration of 20 μM in sterile DNase/RNase-free ddH_2O.

3| Prepare 50 μl of the oligo duplex mixture by mixing 5 μl of 10× NEB buffer 2 (NEB buffers come with restriction enzymes), 5 μl of forward oligo, 5 μl of reverse oligo and 35 μl of sterile DNase/RNase-free ddH_2O.

4| Boil 1 liter of water in a 2-liter glass beaker on the heating block.

5| Incubate the tube of the oligo duplex mixture in the boiling water for 5–8 min.

6| Turn off the heater and let the temperature of the shRNA duplex mixture gradually decrease to room temperature.
▲ **CRITICAL STEP** Cooling down the shRNA duplex mixture slowly is essential for annealing shRNA oligos perfectly. It usually takes 4–6 h to cool samples from 100 °C to room temperature. The annealed shRNA duplex can be kept at room temperature for up to 1 month.

7| *Day 3*. To clone shRNA into pLKO.1 puro or pLKO.pig vector with AgeI and EcoRI, use 6 μg of vector, 7 μl of 10 × NEB buffer 1, 6 μl of AgeI (5,000 U ml^{-1}), 4 μl of EcoRI (20,000 U ml^{-1}) and add ddH_2O to 70 μl. Incubate the digestion reaction at 37 °C for 1 h 40 min. To clone shRNA into pH1P-HygroGFP vector, use 6 μg of vector, 7 μl of 10× NEB buffer 4, 6 μl of SmaI (20,000 U ml^{-1}), 4 μl of XbaI (20,000 U ml^{-1}) and add ddH_2O to 70 μl. Incubate the digestion reaction at 37 °C for 1 h 40 min.
▲ **CRITICAL STEP** A longer digestion time results in lower ligation efficiency because of vector overdigestion. Although the enzyme activity of SmaI at 37 °C is only 50%, it still works well under these digestion conditions.

8| Run the digested vector on a 0.8% (wt/vol) agarose gel at 100 V for 30 min.

9| Cut and extract the digested vector from the agarose gel with a gel extraction kit (Qiagen) and resuspend it in 30 μl of the elution buffer provided in the kit (10 mM Tris·Cl, pH 8.5) the kit or in water.

10| Ligate the digested vector with annealed shRNA oligos by using 1 μl of the annealed oligo mixture, 0.5 μl of digested vector, 1 μl of 10× NEB T4 DNA ligase buffer, 1 μl of NEB T4 DNA ligase and 6.5 μl of ddH_2O. Incubate the ligation mixture at room temperate for 1 h (pLKO.1 puro or pLKO.pig system) or 16 °C overnight (pH1P-HygroGFP system).

11| Transform 5 μl of the shRNA-vector ligation product into 50 μl of DH5α-competent cells and heat-shock them at 42 °C for 50 s; plate the bacteria on LB agar containing 50–100 μg ml^{-1} ampicillin and incubate at 37 °C overnight.

12| *Days 4 and 5*. Pick up antibiotic-resistant clones, inoculate them into 5 ml of LB broth supplemented with 50–100 μg ml^{-1} ampicillin and grow them overnight at 37 °C; isolate plasmids by using the miniprep kit and check shRNA insertion by sequencing.
▲ **CRITICAL STEP** As the shRNA hairpin structure may be difficult to sequence, a specific protocol may be required to sequence the shRNA.
? TROUBLESHOOTING

Fluorescence-based competition assay setup ● TIMING 35 d

13| *Days 6–14*. Generate lentiviruses, prepared as described in **Box 2**.

14| *Day 14*. Collect CCE cells by trypsinization (**Box 3**). Count cell numbers using a hemocytometer and seed the cells in a gelatin-precoated 12-well plate at a density of 2 × 10^5 cells per well. Grow cells at 37 °C and 5% CO_2 overnight.

Box 2 | Generation, concentration and titer determination of lentiviral particles ● TIMING 8 d

The lentivirus-based shRNA knockdown system not only allows for silencing of a specific gene's expression in difficult-to-transfect cells but also permits long-term gene repression. The following simple steps will easily provide a high titer of lentiviral particles for knockdown of a specific gene in mESCs or other types of cells.

1. *Day 1*. For each transfection, plate 2 × 10^6 293T cells in a 10-cm tissue culture plate. Incubate cells in a humidified atmosphere at 37 °C, 5% CO_2 overnight. It may be convenient to prepare three 10-cm plates for each lentiviral shRNA vector and aliquot concentrated virus for future use.

▲ **CRITICAL STEP** Although 293T cells are regularly maintained in penicillin-streptomycin–containing medium, in this step cells are cultured without antibiotics (DMEM high glucose supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine and 1 mM sodium pyruvate) to permit increased cell growth and viral production.

2. *Day 2*: Prepare all transfection reagents in 5-ml polypropylene round-bottom tubes. Mix 8 μg of pLKO.pig shRNA (or pH1P-HygroGFP) plasmid, 6 μg of packaging plasmid pCMV-dR8.2 dvpr and 2 μg of encoding plasmid pCMV-VSV-G in 300 μl of Opti-MEM I reduced-serum medium. Vortex briefly and incubate the DNA mixture at room temperature for 15 min. In the meantime, dilute 22 μl of SuperFect transfection reagent in 300 μl of Opti-MEM I reduced-serum medium and incubate both tubes at room temperature for 15 min.

▲ **CRITICAL STEP** Perform the transfection late in the afternoon, because the transfection mix should only be incubated with the cells for 12–15 h.

3. Mix the DNA cocktail with the diluted SuperFect transfection reagent and incubate it at room temperature for 30 min.

4. While the mixture is incubating, take the 293T cells from the incubator. Carefully aspirate the culturing medium and add 3 ml of fresh Opti-MEM I reduced-serum medium.

▲ **CRITICAL STEP** Do not wash 293T cells with 1× DPBS, which may further detach or wash away cells.

5. After 30 min of incubation, gently add the DNA-SuperFect mixture dropwise to the 293T cell culture plate. Incubate the plates at 37 °C, 5% CO_2 for 3 h.

6. Add 10 ml of regular culture medium with penicillin-streptomycin into each plate after 3 h incubation.

7. *Day 3*. In the morning of day 3, replace the medium with 10 ml of fresh mESC culture medium without ESGRO(LIF) and grow cells at 37 °C, 5% CO_2 for an additional 30–36 h. During incubation, 293T cells will generate lentiviral particles and secrete them in the supernatants.

! **CAUTION** Wear appropriate protective gear/clothing (lab coat and gloves) when performing lentivirus-related experiments. The experiments should be conducted in a biohazard hood and all governmental and institutional biohazard committee guidelines should be followed. All material and equipment (such as culture plates and disposable pipettes) that have been in contact with viral particles should be soaked in 10% (vol/vol) bleach for at least 20 min before discarding them in biohazard bags or containers, and autoclaved.

8. *Day 4*. In the afternoon of day 4, collect the viral supernatant and pass it through a 0.45-μm low-protein-binding filter to remove cell debris.

9. To concentrate the viral supernatant, use Amicon Ultra-15 centrifuge units. Add 10–15 ml of the supernatant to the filter unit tube and centrifuge at 1,600*g* at 4 °C for 20 min. The final volume should be ~0.5 ml. If the virus is not being used immediately, store at −80 °C.

10. To determine the titer of lentiviruses, infect 293T cells that have been preseeded in a six-well dish (2 × 10^5 cells per well) with concentrated virus at serially prepared 1:100, 1:1,000 and 1:10,000 dilutions containing 8 μg ml^{-1} polybrene. Mix thoroughly and grow 293T cells at 37 °C and 5% CO_2 overnight.

11. *Day 5*. Aspirate the medium and replace it with 2 ml of fresh 293T/MEF medium. Grow 293T cells at 37 °C and 5% CO_2.

12. *Days 6 and 7*. Grow the lentivirus-infected 293T cells for an additional 2 d.

13. *Day 8*. Determine the percentage of GFP-expressing cells by flow cytometry and calculate the biological titer of the concentrated lentiviruses as described by Kutner *et al.*[52].

▲ **CRITICAL STEP** After 24 h of culture, CCE cells should have reached 50% confluence. Higher confluence will result in lower lentivirus infection efficiency.

15| *Day 15*. Aspirate medium and replace with 0.5 ml of fresh mESC culture medium supplemented with 16 μg ml^{-1} polybrene.

16| Infect the cells by adding concentrated lentiviruses (MOI = 10). Slightly shake the plate with your hands and gently swirl the dish to ensure uniform distribution of the viruses within the medium. The final concentration of polybrene is 8 μg ml^{-1}. Grow cells at 37 °C and 5% CO_2 overnight.

▲ **CRITICAL STEP** In our experience, mESCs (e.g., CCE and Ainv15 cells) are more difficult to infect with lentiviruses than somatic cells (e.g., MEFs). If necessary, increase the MOI (e.g., 20).

Box 3 | Cell maintenance and culture procedure ● TIMING ~1.5 h (plus incubation time)

Thawing CCE, Ainv15 mESCs and 293T cells ● TIMING 15 min

1. Take a cryovial of frozen cells from storage in liquid nitrogen.
2. Place the cryovial immediately in a water bath (37 °C).

▲ CRITICAL STEP Thaw the cells as quickly as possible (<1 min) by lightly swirling the vial.

3. Prepare 10 ml of 293T/MEF culture medium in a 15-ml conical tube. Rinse the cryovial with 70% (vol/vol) ethanol before opening. Transfer the cells into fresh medium. Centrifuge at 300*g*, 4 °C, for 5 min.
4. Aspirate away supernatant containing cytotoxic DMSO, add 10 ml of fresh culture medium (mESC culture medium for CCE and Ainv15 cells, and 293T/MEF culture medium for 293T cells), resuspend the cell pellet and plate the cells into a new dish. Use 10-cm gelatin-coated culture dishes, MEF feeder cell–coated culture dishes and regular tissue culture–treated dishes for CCE, Ainv15 and 293T cells, respectively.
5. Put the plated cells into the incubator at 37 °C, 5% CO_2.

▲ CRITICAL STEP Freshly thawed cells need time to recover. Culture the cells for at least two or three passages before setting up your experiment.

Subculture of CCE, Ainv15 mESCs and 293T cells ● TIMING 30 min

mESCs and 293T cells are routinely maintained in 10-cm tissue culture plates in a humidified atmosphere at 37 °C, 5% CO_2. Maintain CCE mESCs on gelatin-coated plates and grow Ainv15 mESCs on culture plates coated with MEF feeder cells. Examine the morphology of mESCs every day under a phase-contrast microscope.

▲ CRITICAL STEP It is important to prewarm cell culture medium in a 37 °C water bath before use.

▲ CRITICAL STEP As the passage number of 293T cell lines can affect transfection efficiency and protein expression, it is important to avoid culturing up to high passage numbers (>25 passages).

Trypsin treatment of CCE, Ainv15 mESCs and 293T cells ● TIMING 30 min

Passage CCE, Ainv15 and 293T cells regularly approximately every 2 d upon reaching 90% confluence. The trypsin treatment for these cells is the same. In general, follow these steps:

1. Gently aspirate away cell culture medium and wash cells with 5–6 ml of fresh 1× DPBS to remove serum that could inhibit trypsin activity, adding 1× DPBS to the side of the well.
2. Aspirate away the 1× DPBS.
3. Add 1 ml of prewarmed TrypLE Express solution directly to the cell culture plate, and incubate the plate at 37 °C, 5% CO_2 for 3–5 min.
4. Take the cell culture plate out, gently tap or shake it, and then add 10 ml of fresh cell culture medium with 10% (vol/vol) FBS to stop trypsin activity. Mix cells with a pipette and transfer them into a 15-ml conical tube. Centrifuge at 300*g*, 4 °C, for 5 min.
5. Prepare new plates for cells. Use 10-cm gelatin-coated culture dishes, MEF feeder cell–coated culture dishes and regular tissue culture plates for CCE, Ainv15 and 293T cells, respectively.
6. After briefly spinning down, aspirate the cell supernatant. Add fresh cell culture medium and resuspend and passage CCE, Ainv15 and 293T cells (with a 1:10, 1:10 or 1:6 dilution, respectively), to the new culture dish for regular maintenance. The splitting ratio varies according to different experimental purposes.

Freezing down CCE, Ainv15 mESCs and 293T cells ● TIMING 15 min

Freeze cells in exponential growing phase. One confluent plate of cells can be frozen down into three to five cryovials.

1. Trypsinize the cells and centrifuge them at 300*g*, 4 °C, for 5 min.
2. Prepare fresh freezing medium (see REAGENT SETUP).
3. Aspirate away the supernatant and add 3–5 ml of freezing medium. Resuspend the cell pellet and aliquot the cells into three to five cryovials, 1 ml each.
4. Place the cryovials into a 'Mr. Frosty' cryocontainer, providing 1 °C min^{-1} cooling, and then freeze the container with the cells at −80 °C overnight.
5. Transfer the cryovials into a liquid nitrogen tank the next day.

17| *Day 16*. At 24 h after infection, replace polybrene/virus-containing medium with 1 ml of fresh mESC culture medium.

18| *Day 17*. Collect the CCE cells by trypsinization (**Box 3**) and split them at a ratio of 1:5 into one well of a gelatin-coated six-well plate in 3 ml of mESC culture medium.

19| *Day 18*. Discard the medium and add 3 ml of fresh mESC culture medium.

20| *Day 19*. Collect the CCE cells by trypsinization (**Box 3**) and split them at a ratio of 1:10 into one well of a gelatin-coated six-well plate in 3 ml of mESC culture medium.

21| *Day 20*. Discard the medium and add 3 ml of fresh mESC culture medium.

22| *Day 21*. On day 6 after infection, collect the CCE cells by trypsinization (**Box 3**) and split them at a ratio of 1:8 into one well of a gelatin-coated six-well plate in 3 ml of mESC culture medium supplemented with 2 μg ml^{-1} puromycin (for pLKO.1 puro and pLKO.pig systems) or 250 μg ml^{-1} hygromycin (for pH1P-HygroGFP system).
▲ **CRITICAL STEP** Before using a drug to select lentivirus-transduced positive cells, the concentration of puromycin and hygromycin should be tested to determine the optimal concentration needed to kill parental mESCs. In our hands, 1–2 μg ml^{-1} puromycin and 200–250 μg ml^{-1} hygromycin cause complete mESC (e.g., CCE and E14T cells) death within 2–3 d and 7–10 d, respectively.
▲ **CRITICAL STEP** For some core pluripotency factors, such as Nanog, Oct4, Foxo1 and Foxo3a, knockdown compromises cell growth and survival so severely that few cells remain after selection. If this occurs, expand cells to five 10-cm tissue culture plates before selection in order to increase the total number of cells available after drug selection.

23| *Days 22–24*. Discard the medium and replace it with 3 ml of puromycin-containing medium every day. Ideally, after 3 d of selection, most surviving mESCs should be GFP^+ and should harbor an shRNA.
? TROUBLESHOOTING

24| *Day 25*. Collect shRNA-expressing CCE cells by trypsinization (**Box 3**). Count the cell numbers with a hemocytometer. Mix 8 × 10^5 shRNA-expressing CCE cells (GFP^+) and 2 × 10^5 control shRNA-expressing CCE cells (GFP^-) in one well of a gelatin-coated six-well plate in 3 ml of mESC culture medium supplemented with 2 μg ml^{-1} puromycin.
▲ **CRITICAL STEP** We use CCE cells infected with lentiviruses carrying the pLKO.1 puro–Luc shRNA construct, which does not contain GFP expression, as a GFP^- control for the competition assay. In our hands, CCE cells expressing Luc shRNA do not show any differences in ESC morphology, growth rate or pluripotency gene expression in comparison with parental cells.

25| Put 1 × 10^6 shRNA-expressed CCE cells (GFP^+) aside for RNA isolation in order to measure shRNA knockdown efficiency, the expression of pluripotency factors (**Box 1**) and to further confirm the result of the competition assay.
■ **PAUSE POINT** All the cell samples can be dissolved in TRIzol and stored at −80 °C to permit mRNA isolation at a future date.

26| *Day 26*. Discard the medium and add 3 ml of fresh mESC culture medium supplemented with 2 μg ml^{-1} puromycin.

27| *Days 27–38*. Split cells at a ratio of 1:8 into one well of the gelatin-coated six-well plate in 3 ml of mESC culture medium supplemented with 2 μg ml^{-1} puromycin every 2 d and replace with fresh medium every day. Repeat this procedure for six passages (~12 d).

28| *Day 39*. After six passages, collect the cells by trypsinization (**Box 3**) and resuspend them in 2 ml of 1× DPBS.

29| Pass the suspended cells through a 5-ml polystyrene round-bottom tube with a cell-strainer cap to collect single cells and remove large aggregated cells.

30| Analyze the GFP^+ population by flow cytometry using the GFP or FL1 channel. Use the percentage in the Lifr-shRNA sample as a positive cutoff because Lif-Lifr-Stat3 signaling is required for mESC self-renewal[10]. One would expect that knockdown of pluripotency-related genes would cause more severe defects in propagation than *Lifr* knockdown.

31| *Day 40*. Confirm the competition assay result with knockdown efficiency and the expression of pluripotency factors upon depletion of your gene of interest (**Box 1**). Identify an shRNA with sufficiently high knockdown efficiency for use in the rescue complementation system.
▲ **CRITICAL STEP** Use AP and SSEA1 staining to demonstrate that knockdown of your gene of interest causes changes in mESC morphology, AP activity and SSEA1 expression (**Box 1**).
? TROUBLESHOOTING

Genetic complementation rescue assay ● TIMING 30 d
32| *Days 41–48*. Generate lentivirus particles carrying individual rescue constructs, prepared as described in **Box 2**.

33| *Day 47*. Maintain the Ainv15 mESC line, which expresses the TetOn rtTA transactivator, on a layer of MEFs as feeders in mESC culture medium at 37 °C and 5% CO_2. Follow standard mESC culture procedures (see REAGENT SETUP and **Box 3**).

34| Collect a 10-cm dish of Ainv15 cells by trypsinization (**Box 3**). Resuspend the cells in 10 ml of mESC medium and seed them on an uncoated tissue culture dish. Maintain the cells at 37 °C in a humidified incubator containing 5% CO_2 for 30 min to allow the MEFs to attach to the dish.
▲ **CRITICAL STEP** This procedure is used for separating MEFs from Ainv15 cells. Usually, 30 min of incubation is enough to allow MEFs but not Ainv15 cells to attach to noncoated dishes. Longer incubation might cause Ainv15 cells to also attach to the dishes. Check cells under the microscope. MEFs should attach to the bottom of the tissue culture dish, but Ainv15 cells should remain in suspension.

35| Shake the 10-cm dish gently and collect supernatants in 15-ml tubes. Pipette up and down five times using a 10-ml sterile pipette to resuspend the cells.

36| Count cell numbers using a hemocytometer and seed the cells on a Matrigel-precoated 12-well plate (see REAGENT SETUP) at a density of 2×10^5 cells per well; grow cells at 37 °C and 5% CO_2 overnight.
▲ **CRITICAL STEP** After 24 h of culture, Ainv15 cells should have reached 50% confluence. Higher confluence will result in lower lentiviral infection efficiency.
▲ **CRITICAL STEP** If Matrigel-coated plates are not available, gelatin-coated plates can be used as an alternative. However, the infection efficiency of Ainv15 cells growing on gelatin-coated plates is lower than that of the cells growing on Matrigel-coated plates.

37| *Day 48*. Aspirate medium and replace with 0.5 ml of fresh mESC culture medium with 16 μg ml^{-1} polybrene.

38| Infect the cells by adding desired amounts of concentrated lentivirus particles carrying individual rescue constructs (MOI = 10). Slightly shake the plate with your hands to mix viruses homogenously in medium. Gently swirl the dish to ensure uniform distribution of the viruses within the medium. The final concentration of polybrene is 8 μg ml^{-1}.

39| *Day 49*. At 24 h after infection, replace the polybrene/virus-containing medium with 1 ml of fresh mESC culture medium supplemented with 1–1.5 μg ml^{-1} dox.
▲ **CRITICAL STEP** To prevent shRNA-induced mESC differentiation and detect GFP expression, maintain rescue clones in mESC culture medium supplemented with 1–1.5 μg ml^{-1} dox.

40| Prepare gelatin-coated six-well plates (see REAGENT SETUP) for the next few days of experiments.

41| *Day 50*. Collect the Ainv15 cells by trypsinization (**Box 3**) and split them at a ratio of 1:5 into one well of a six-well plate with MEFs in 3 ml of mESC culture medium supplemented with 1–1.5 μg ml^{-1} dox.

42| *Day 51*. Discard the medium and add 3 ml of fresh mESC culture medium supplemented with 1–1.5 μg ml^{-1} dox.

43| *Day 52*. Collect the Ainv15 cells by trypsinization (**Box 3**) and split them at a ratio of 1:8 into one 10-cm dish with MEFs in 3 ml of mESC culture medium supplemented with 1–1.5 μg ml^{-1} dox.

44| *Day 53*. Discard the medium and add 3 ml of fresh mESC culture medium supplemented with 1–1.5 μg ml^{-1} dox.

45| For each rescue clone, prepare three gelatin-coated 96-well plates with MEFs (**Box 3**) for the next day's experiment.

46| *Day 54*. Discard the medium from 96-well plates and add 150 μl of fresh mESC culture medium supplemented with 1–1.5 μg ml^{-1} dox into each well. Keep the plates at 37 °C in the incubator before using them for cell sorting.

47| Check GFP expression in lentivirus-infected Ainv15 cells under the fluorescence microscope. Usually, we are able to detect GFP expression in 10–30% of rescue clones.
? TROUBLESHOOTING

48| Collect the cells by trypsinization (**Box 3**) and resuspend them in 1 ml of 1× DPBS supplemented with 5% (vol/vol) FBS. Keep cells on ice before sorting GFP^+ cells.

49| Pass the suspended cells through a 5-ml polystyrene round-bottom tube with a cell-strainer cap to collect single cells and remove large aggregated cells.
▲ **CRITICAL STEP** Keep cells on ice before sorting GFP^+ cells.

50| Use a single-cell sorting machine (we routinely use a DAKO-MoFlo Cytomation sorter but other cell sorters are suitable as well) to sort single GFP$^+$ cells into three 96-well plates containing MEF feeder cells (see REAGENT SETUP) with 150 µl of fresh mESC culture medium supplemented with 1–1.5 µg ml^{-1} dox for each rescue clone.

51| *Days 55–60*. Discard the medium and add 150 µl of fresh mESC culture medium supplemented with 1–1.5 µg ml^{-1} dox into each well. Maintain cells at 37 °C and in 5% CO_2 and change the medium every day.

52| *Day 61*. Check GFP$^+$ rescue clone growth in the 96-well plates with a fluorescence microscope and mark the wells containing GFP$^+$ clones.
▲ **CRITICAL STEP** In some wells, you might observe that there is more than one clone growing in the same well, which means that they come from two individual cells. We would not suggest picking cells from these wells. Moreover, owing to occasional errors in the cell sorter, some clones are GFP$^-$ and not useful for future characterization. Usually, 75% of colonies that grow are derived from single cells when using DAKO-MoFlo Cytomation.
? TROUBLESHOOTING

53| Discard the medium, wash the cells with 100 µl of 1× DPBS and add 20 µl of 1× TrypLE Express for 3 min at 37 °C to allow cells to detach from the 96-well plates (also see **Box 3**).

54| Add 150 µl of mESC culture medium supplemented with 1–1.5 µg ml^{-1} dox in each well and pipette up and down slightly to resuspend the cells; add the suspended cells into one well of the 24-well plate with MEFs in 1 ml of mESC culture medium supplemented with 1–1.5 µg ml^{-1} dox.
▲ **CRITICAL STEP** Longer trypsinization and inappropriate pipetting of rescue clones during expansion will cause cell differentiation.

55| *Days 62–66*. Discard the medium and add 2 ml of fresh mESC culture medium supplemented with 1–1.5 µg ml^{-1} dox to each well. Maintain cells at 37 °C and 5% CO_2 and change the medium every day.

56| *Day 67*. Check GFP expression of expanded rescue clones and mark strong GFP-expressing clones without a differentiated morphology.
▲ **CRITICAL STEP** Some rescue clones might look differentiated or lose GFP expression during expansion. In our hands, only ~30% of expanded rescue clones show undifferentiated mESC morphology and GFP$^+$ expression.

57| Collect the identified GFP$^+$ rescue clones by trypsinization (**Box 3**). Split the cells at a ratio of 1:5 into one well of a gelatin-coated six-well plate in 3 ml of mESC culture medium supplemented with 1–1.5 µg ml^{-1} dox.

58| Discard the medium and add 3 ml of mESC culture medium supplemented with 1–1.5 µg ml^{-1} dox.

59| Collect the cells by trypsinization (**Box 3**). Split the cells at a ratio of 1:8 into one well of a six-well plate with MEFs in 3 ml of mESC culture medium (without dox) and one well of six-well plate with MEFs in 3 ml of mESC culture medium supplemented with 1–1.5 µg ml^{-1} dox.

60| *Day 68*. Discard the medium and add 3 ml of mESC culture medium supplemented with or without dox.

61| *Day 69*. Collect cells by trypsinization (**Box 3**) and remove MEFs according to the procedure in Steps 34 and 35.

62| Spin down the suspended cells briefly, remove the supernatant and lyse cells in 800 µl of TRIzol for future mRNA isolation and qRT-PCR analysis (**Box 1**).
■ **PAUSE POINT** Store all TRIzol-dissolved samples at −80 °C before mRNA extraction at a later date.

63| Compare mRNA expression between knockdown (−dox) and rescue clones (+dox) to make sure that addition of dox rescues the knockdown effect of the 'target' gene. Pick up the proper rescue clones with these properties.
▲ **CRITICAL STEP** Generally, we select more than five distinct clones for each rescue construct. The proper clones for a rescue experiment should be chosen based on how closely the gene expression level of the clones grown in the presence of dox resembles that of the parental Ainv15 cells.
▲ **CRITICAL STEP** Adjusting the concentration of dox to increase or decrease the induction of the rescue gene is an alternative way to optimize restoration of gene expression.
? TROUBLESHOOTING

64| *Day 70*. Freeze down the desired rescue clones as stocks (**Box 3**).

Testing dox-inducible rescue clones: thaw and maintain *Nanog* rescue clones ● TIMING 7 d

65| *Day 71*. Plate irradiated MEF cells (5×10^4 cells per well) on a gelatin-coated six-well tissue culture plate (see REAGENT SETUP and **Box 3**) in regular MEF medium. Maintain MEFs at 37 °C with 5% CO_2 overnight until use.

66| *Day 72*. Discard the medium and seed *Nanog* rescue clones at a density of 5×10^4 cells per well on the confluent MEF feeder layer and culture them at 37 °C with 5% CO_2.
▲ **CRITICAL STEP** After plating the *Nanog* rescue clones on MEFs, maintain cells in mESC culture medium supplemented with 1 μg ml^{-1} of dox instead of regular culture medium. Replace the medium every day with fresh medium. The rescue clone colonies should be observable under the microscope after 2 d.

67| *Days 73–77*. Maintain the *Nanog* rescue clones that were generated by using the *Nanog* rescue construct, which contains *H1* promoter–driven Nanog shRNA targeting the 3′ UTR of Nanog mRNA and exogenous *Nanog* cDNA driven by the *TRE* promoter (**Fig. 3**)[1,9], in mESC culture medium supplemented with 1 μg ml^{-1} of dox by following the regular mESC culture protocol (**Box 3**) until use. Usually, cells are ready for use after 1 week of recovery.

Time-course experiment setup ● TIMING 9 d

68| *Day 78*. Collect the cells by trypsinization (**Box 3**) and remove the MEFs according to the procedure in Steps 34 and 35.

69| Count the cell numbers with a hemocytometer.

70| Seed 3×10^5 cells into a 10-cm gelatin-coated dish in 10 ml of mESC culture medium supplemented with 1 μg ml^{-1} of dox (see REAGENT SETUP). Prepare four dishes with this density of cells for a future time-course experiment.
▲ **CRITICAL STEP** Aspirate excess gelatin before seeding cells. Work quickly and do not allow the wells to dry out.
▲ **CRITICAL STEP** Establish your time-course accurately, design your time points and select the days on which you want to collect your samples.

71| Maintain the cells at 37 °C with 5% CO_2 in a tissue culture incubator overnight.

72| *Day 79*. After 24 h of culture, check and take pictures of the cell morphology and GFP expression with a fluorescence microscope.

73| Discard the supernatant, collect the cells from one dish, dissolve them in 800 μl of TRIzol and store them at −80 °C. This is the day 0 (D0) sample.

74| Discard the medium from the remaining three dishes and wash the cells twice with 10 ml of 1× DPBS.

75| Add 10 ml of fresh mESC culture medium without dox in each dish. From this point until the end of the experiment, culture three dishes of the *Nanog* rescue clone with mESC culture medium without dox and collect cells at the corresponding time points previously established.
▲ **CRITICAL STEP** Wash cells properly to remove any traces of dox present in the previous medium. Complete removal of residual dox from rescue clones is important to ensure that there is no exogenous gene expression that could interfere with the shRNA knockdown effect.

76| *Day 80*. After 24 h of maintenance with dox-free mESC culture medium, check and take pictures of the cell morphology and GFP expression using a fluorescence microscope.

77| Discard the supernatant from one dish, collect the cells, dissolve them in 800 μl of TRIzol and store them at −80 °C. This is the day 1 (D1) sample.

78| Discard the medium from the remaining two dishes and add 10 ml of fresh mESC culture medium without dox per dish.

79| *Day 81*. Discard the medium and add 10 ml of fresh mESC culture medium without dox per dish.

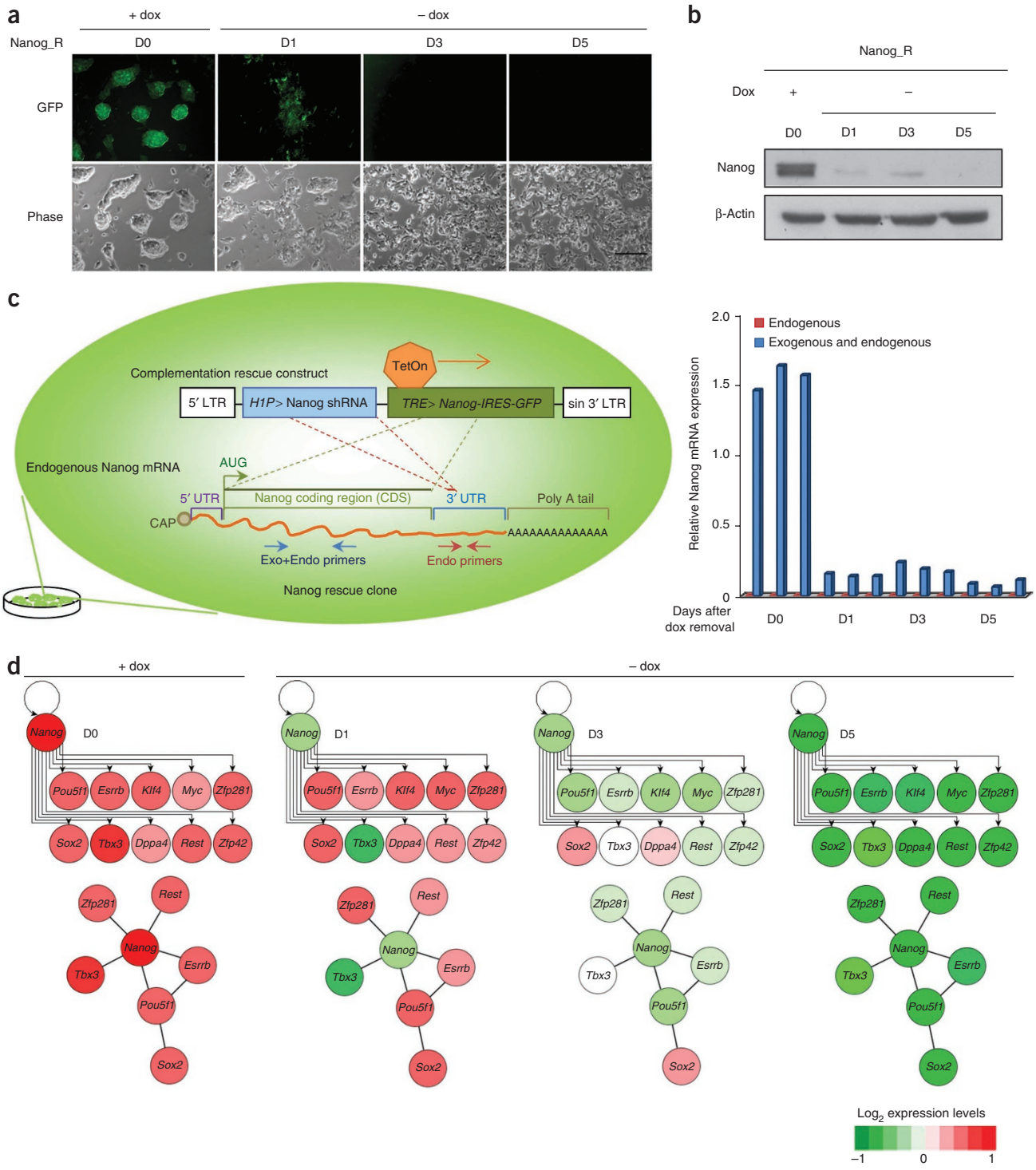

Figure 3 | Dynamic knockdown of Nanog impairs mESC pluripotency network. (**a**) Withdrawal of dox in the *Nanog* rescue clone leads to loss of both Nanog and GFP and shows a differentiated phenotype. Scale bar, 100 μm. (**b**) Immunoblotting reveals loss of Nanog expression upon dox withdrawal. (**c**) Left, schematic description of qPCR primer sets Endo and Exo+Endo (designed to target endogenous or total Nanog mRNA, respectively) in complementation rescue clones. In *Nanog* rescue clones, Nanog shRNA is designed to target the 3′ UTR of the Nanog transcript. The shRNA-immune *Nanog* gene–coding region is PCR-amplified and cloned under the *TRE* promoter. Therefore, Nanog shRNA only targets and knocks down endogenous Nanog mRNA. Exogenous Nanog mRNA is transcribed upon dox treatment. During maintenance, endogenous Nanog mRNA is continually knocked down by Nanog shRNA, and exogenous Nanog mRNA remains expressed in dox-containing media. Two distinct primer sets (Endo and Exo+Endo) are designed to detect either endogenous or both endogenous and exogenous Nanog mRNA by targeting either the 3′ UTR region or the coding sequence (CDS) of Nanog mRNA, respectively. Right, time-course experiment shows continuous downregulation of endogenous Nanog across multiple time points; in contrast, exogenous Nanog is gradually downregulated upon dox removal. (**d**) The collapse of the Nanog-targeting pluripotency genes and its pluripotency interactome upon knockdown of Nanog. qRT-PCR analysis of the expression of Nanog-regulated (upper panel) and -interacted (lower panel) pluripotency factors upon depletion of Nanog. The network is created in yEd Graph Editor. Red and green colors represent upregulation and downregulation of noted pluripotency gene expression, respectively.

80| *Day 82*. After 72 h of maintenance with dox-free mESC culture medium, check and take pictures of the cell morphology and GFP expression with a fluorescence microscope.

81| Discard the supernatant from one dish, collect the cells, dissolve them in 800 µl of TRIzol and store them at −80 °C. This is the day 3 (D3) sample.

82| Discard the medium from the remaining dishes and add 10 ml of fresh mESC culture medium without dox.

83| *Day 83*. Discard the medium and add 10 ml of fresh mESC culture medium without dox.

84| *Day 84*. After 120 h of maintenance with dox-free mESC culture medium, check and take pictures of the cell morphology and GFP expression with a fluorescence microscope.

85| Discard the supernatant from the last dish, collect the cells, dissolve them in 800 µl of TRIzol and store them at −80 °C. This is the day 5 (D5) sample.

86| *Day 85*. Extract RNA from D0, D1, D3 and D5 *Nanog* rescue clone samples and perform qRT-PCR to measure pluripotency gene expression (**Box 1**). The sequence of primers used for qRT-PCR in this study is available in **Table 1**.
▲ **CRITICAL STEP** Instead of measuring pluripotency gene expression, AP staining and SSEA1 staining should be applied to evaluate whether the knockdown of your gene of interest alters mESC morphology, AP activity or ESC surface marker SSEA expression (**Box 1**).

87| *Day 86*. Identify Nanog direct targets and protein-protein interaction partners from previous publications and alternative databases of DNA interactions[13–18,50].

88| Establish the new nodes of the Nanog-associated mESC pluripotency network and represent this network using yEd graph editor (http://www.yworks.com/en/products_yed_about.html).

89| Color the nodes of the network according to the gene expression levels from your qPCR results.

TABLE 1 | Primers used for qRT-PCR.

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
Nanog (Endo)	CCTGGCCTTGAACTCAGAGA	GGTGCCCAGCATGTTCTAAG
Nanog (Exo)	CTGCTCCGCTCCATAACTTC	GCTTCCAAATTCACCTCCAA
Pou5f1	GGATGGCATACTGTGGACCT	GTTGGTTCCACCTTCTCCAA
Esrrb	CTAGTTGCGGCTCCTTCATC	TGGCGTTAAGCATGTACTCG
Klf4	CTGAACAGCAGGGACTGTCA	GAGGGGACTTGTGACTGCAT
Myc	GAGTGCATTGACCCCTCAGT	GAATCGGACGAGGTACAGGA
Zfp281	CGCAGTGCGTGTTATCCTC	TCCTTCTTGAAAGTCATGTCCG
Sox2	CACAACTCGGAGATCAGCAA	CTCCGGGAAGCGTGTACTTA
Tbx3	CGTCTCAGGCCTAGAATCCA	GTGTTGTTGGAGGTGGAAGG
Dppa4	TTGAGGAACGTCCCTGACTC	GCAGGTATCTGCTCCTCTGG
Rest	ACCGCTGTGGCTACAATACC	CTTTCCTGGGGAAATGGTTT
Zfp42	CCGGGATGAAAGTGAGATTAGC	TCACCTCGTATGATGCACTCT
Tcl1	AAATTCCAGGTGATCTTGCG	TGTCCTTGGGGTACAGTTGC
Actb	ACCAACTGGGACGACATGGAGAAG	TACGACCAGAGGCATACAGGGACA

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
12	No colonies	Failure to form shRNA duplex	Allow the shRNA duplex mixture cool down slowly. Place a foil cover on top of the beaker to prevent the temperate from dropping too fast
		Overdigested plasmid	Use a shorter digestion if the restriction enzyme concentration is already high
		Low transformation	Use competent cells with a high transformation efficiency
	No shRNA insertion	Incomplete digestion	Check the activity of the restriction enzyme
	Unreadable DNA sequencing result	Hairpin structure of shRNA interferes with DNA sequencing	Choose a specific protocol for sequencing shRNA, as provided by commercial sequencing services or sequence core facilities
23	Few or no cells survive after selection	Low viral titer	Check the quality of plasmids used for transfection by NanoDrop. Usually, high quality of plasmids is required for good transfection efficiency
			Make sure 293T cells are healthy and grow in log phase
			Make sure the filter used for removing cell debris has a low protein-binding affinity
			Longer incubation times at a lower temperature can also prevent lentivirus degradation and increase lentivirus production. Usually, we allow 293T cells to produce lentivirus at 32 °C for 48 h
		Your gene of interest is involved in cell cycle and apoptosis	Use a lower titer of lentivirus to infect cells or use an alternative shRNA targeting another region of your gene of interest. Try several different concentrations of lentivirus to obtain a proper knockdown efficiency that minimizes cell toxicity
		Inappropriate drug concentration	Use a lower drug concentration to select cells expressing lower copies of shRNAs and drug-selection genes
31	Lower knockdown efficiency	Low viral titer	Please see TROUBLESHOOTING for Step 23
		Inappropriate shRNA design	Design more shRNAs or use other available databases to design shRNAs
47	No GFP expression in rescue clones	Wrong construct design	Make sure your rescue construct is correct
52	Few GFP cells after sorting	Inappropriate sort gating	Use stringent sorting conditions, including sorting high GFP-expressing clones
		Inappropriate sorting instrument	Consult with flow cytometry core facility to make sure the sorting instrument is appropriate for your purpose
63	Low rescue efficiency	Inappropriate immune construct design	Make sure that the immune construct is correct and not knocked down by shRNA
			Make sure that the design of the immune gene contains a Kozak consensus sequence to increase translation efficiency
		Large size of rescue gene	Increase the concentration of dox to increase induction ability

● TIMING

Step 1, shRNA design: day 1, 1 h

Steps 2–12, shRNA construct: day 2, 1 h; day 3, 8 h; day 4, 1 h; day 5, 5 h

Step 13, lentiviral generation: day 6, 0.5 h; day 7, 6 h; day 8, 0.5 h; day 9, 4 h; day 10, 0.5 h; day 11, 0 h; day 12, 0 h; day 13, 5 h

Steps 14–31, competition assay: day 14, 0.5 h; day 15, 0.5 h; day 16, 0.5 h; day 17, 1 h; day 18, 0.5 h; day 19, 1 h; day 20, 0.5 h; day 21, 1 h; day 22, 0.5 h; day 23, 0.5 h; day 24, 0.5 h; day 25, 3 h; day 26, 0.5 h; day 27, 1 h; day 28, 1 h; day 29, 1 h; day 30, 1 h; day 31, 1 h; day 32, 1 h; day 33, 1 h; day 34, 1 h; day 35, 1 h; day 36, 1 h; day 37, 1 h; day 38, 1 h; day 39, 6 h; day 40, 8 h

Step 32, lentiviral generation: day 41, 0.5 h; day 42, 6 h; day 43, 0.5 h; day 44, 4 h; day 45, 0.5 h; day 46, 0 h; day 47, 0 h; day 48, 5 h

Steps 33–64, genetic complementation rescue assay: day 47, 1 h; day 48, 1 h; day 49, 1 h; day 50, 1 h; day 51, 0.5 h; day 52, 1 h; day 53, 0.5 h; day 54, 4 h; day 55, 0.5 h; day 56, 0.5 h; day 57, 0.5 h; day 58, 0.5 h; day 59, 0.5 h; day 60, 0.5 h; day 61, 6 h; day 62, 0.5 h; day 63, 0.5 h; day 64, 0.5 h; day 65, 0.5 h; day 66, 0.5 h; day 67, 6 h; day 68, 0.5 h; day 69, 6 h; day 70, 3 h

Steps 65–67, thaw and maintain rescue clones: day 71, 0.5 h; day 72, 0.5 h; day 73, 0.5 h; day 74, 0.5 h; day 75, 0.5 h; day 76, 0.5 h; day 77, 0.5 h

Steps 68–89, examine rescue clones: day 78, 2 h; day 79, 2 h; day 80, 1 h; day 81, 0.5 h; day 82, 1 h; day 83, 0.5 h; day 84, 1 h; day 85, 8 h; day 86, 6 h

Box 1, methods for characterizing mESCs pluripotency: variable; 40 min–8 h

Box 2, generation, concentration and titer determination of lentiviral particles: 8 d

Box 3, cell maintenance and culture: ~1.5 h (plus incubation time)

ANTICIPATED RESULTS

Four distinct shRNAs should be designed to target each gene of interest, and the sequence chosen to target the coding region and/or 3′ UTR must not target any other genes. By using the shRNA design website from TRC, we usually identify two out of four sequences with more than 70–80% knockdown efficiency[1]. By applying these lentivirus-based shRNAs for the fluorescence-based competition assay, we expect that if your gene of interest is required for mESC pluripotency maintenance, efficient knockdown of this gene by the designed shRNA sequences should show more severely reduced propagation compared with knockdown of Lifr. The GFP^+ populations of shRNA-transduced cells should be <35% after six passages in mouse CCE cells (the percentage GFP^+/GFP^- of knockdown of Lifr is 35%). The later qRT-PCR experiment should show reduced pluripotency factor expression upon knockdown of the tested gene. If this gene is involved in maintaining mESC pluripotency, knockdown of this gene usually shows more than a 50–70% decrease of Nanog, Oct4, Sox2, Esrrb, Tbx3 and Tcl1 expression (**Fig. 1d**). In addition, lower SSEA1 expression and AP activity should be detected upon knockdown of your gene of interest (**Fig. 1e**,**f**).

For rescue clone setup (**Fig. 2**), we start with 288 single cells (three 96-well plates) in the initial screening for selecting rescue clones. In the end, we expect to have more than 20 individual rescue clones that show a slightly diverse rescue effect. The concentration of 1–1.5 μg ml^{-1} dox is usually able to fully rescue the expression of your gene of interest. Under dox-containing culture conditions, rescue clones show normal mESC morphology, and the expression of pluripotency transcription factors (e.g., Nanog, Oct4, Sox2, Esrrb, Tbx3 and Tcl1) is comparable to that of the control rescue clone or parental cells. Under dox-free culture conditions (Steps 65–89), rescue clones develop a differentiated morphology, reduced AP activity and lower expression of SSEA1 and pluripotency transcription factors.

By using this system, you will be able to study your gene of interest involved in mESC identity toward an understanding of the triggers and dynamic changes that occur upon cell fate determination. Here we use *Nanog* as an example and demonstrate dynamics of Nanog knockdown effect on the transcriptional regulatory circuit. We build up the ESC pluripotency network and incorporate the expression of pluripotency transcription factors upon knockdown of Nanog at different time points upon dox withdrawal. In the presence of dox, *Nanog* rescue clones show mESC morphology and express GFP, which indicates that exogenous Nanog is expressed. In the absence of dox, *Nanog* rescue clones show a differentiated morphology and lose GFP expression gradually (**Fig. 3a**). Immunoblotting confirms that the Nanog protein is downregulated upon withdrawal of dox (**Fig. 3b**). The use of specific primers to detect endogenous and exogenous Nanog mRNA expression in *Nanog* rescue clones shows that endogenous Nanog mRNA is constantly downregulated, but exogenous Nanog mRNA is expressed only in the presence of dox (D0) and lost in the absence of dox (D1, D3 and D5; **Fig. 3c**). These results strongly suggest that the expression of Nanog is tightly controlled by the *TRE* promoter in the *Nanog* rescue clone. Dynamic studies of the effect of Nanog knockdown are approachable in this system. To monitor the dynamic perturbation of the pluripotency network upon Nanog knockdown, the expression of *Nanog* downstream targets are integrated into the *Nanog*-associated pluripotency network[13–17,19–20]. In the presence of dox, Nanog and its pluripotency targets are highly expressed in mESCs,

which are able to sustain mESC self-renewal and pluripotency. Upon dox withdrawal, Nanog is rapidly downregulated, but its downstream targets are not substantially altered by day 1 (**Fig. 3d**, upper panel). The Nanog-associated pluripotency network starts to be disrupted at day 3 (**Fig. 3d**, lower panel), showing a substantial decrease in the gene expression of pluripotency factors Oct4, Esrrb and Klf4. Importantly, the expression of all Nanog downstream genes is markedly downregulated, causing a 'collapse' of the whole network (**Fig. 3d**) and supporting the fully differentiated morphology of the *Nanog* rescue clones at day 5 (**Fig. 3a**).

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