

ARTICLES

Dissecting self-renewal in stem cells with RNA interference

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We present an integrated approach to identify genetic mechanisms that control self-renewal in mouse embryonic stem cells. We use short hairpin RNA (shRNA) loss-of-function techniques to downregulate a set of gene products whose expression patterns suggest self-renewal regulatory functions. We focus on transcriptional regulators and identify seven genes for which shRNA-mediated depletion negatively affects self-renewal, including four genes with previously unrecognized roles in self-renewal. Perturbations of these gene products are combined with dynamic, global analyses of gene expression. Our studies suggest specific biological roles for these molecules and reveal the complexity of cell fate regulation in embryonic stem cells.

Mouse embryonic stem (ES) cells can differentiate into cells of all three germ layers, and self-renew extensively in culture¹. The transcription factors *Oct4* (also known as *Pou5f1*) and *Nanog*, as well as the LIF-gp130-Stat3, BMP-TGF-β-Smad, MAPK-ERK and possibly the WNT signalling pathways, all have important roles^{2–9}. Cell fate choices in ES cells are clearly regulated by a complex orchestration of multiple pathways. Because this complexity is poorly understood, it has been difficult to produce specific lineages from ES cells and to re-programme somatic cell genomes after nuclear transplantation^{10,11}. Here we dissect self-renewal using an integrated functional genomics strategy. We identify seven self-renewal regulators, suggest specific functional roles and provide a framework to analyse cell fate regulatory networks in ES cells.

Identifying gene products required for self-renewal

We reasoned that like *Nanog* and *Oct4*, other genes required for self-renewal are downregulated upon induction of differentiation. Microarray time-course analyses of ES cells (six time points at 1-day intervals) undergoing retinoic-acid-induced¹² differentiation identified 901 rapidly downregulated genes (Supplementary Fig. 1). Sixty-five genes encoding transcription factors/DNA-binding proteins and unassigned expressed sequence tags (ESTs), as well as five additional retinoic-acid-insensitive genes identified in our previous study¹³, were chosen and downregulated using shRNA delivered by lentiviral vectors (Fig. 1a and Supplementary Fig. 2). The shRNA is expressed from an H1 promoter, while a constitutive ubiquitin C promoter drives hygromycin-green fluorescent protein (GFP) expression. More than 90% ES cell transduction is routinely achieved without drug selection. For a complete description see Supplementary Information.

To measure self-renewal in shRNA-transduced ES cells, we devised a fluorescence-based competition assay (Fig. 1b). GFP-positive (GFP^+) shRNA-transduced ES cells were mixed in a 4-to-1 ratio with non-transduced GFP-negative (GFP^-) cells, and cultured in the presence of LIF. The GFP^+/GFP^- ratios were measured at every passage. We anticipated that if a given shRNA induces ES cell differentiation, GFP^+/GFP^- ratios will decrease with time. Among the possible causes for such decrease are: (1) changes in cell cycle kinetics¹⁴; (2) changes in cell adhesion; and (3) compromised cell

survival due to the lack of specific growth factors that may be required for the maintenance of differentiating cells.

Progressive decreases in GFP^+/GFP^- ratios were observed upon downregulation of 10 out of 70 genes (Fig. 1c). These are: transcription factors *Nanog*, *Oct4*, *Sox2*, *Tbx3*, *Esrrb*^{14,15,17}; a cofactor of the *Akt1* kinase *Tcl1* (ref. 18); an uncharacterized ES cell-specific gene *Dppa4* (ref. 11); and unassigned ESTs *Mm.343880*, *Mm.276044* and *Mm.219358*. Target messenger RNA levels were significantly decreased by all ten shRNAs. For detailed information see Supplementary Table 1.

Downregulation of some genes could retard the cell cycle without inducing differentiation. Accordingly, we evaluated cell morphology and alkaline phosphatase activity as indicators for undifferentiated cells. Downregulation of *Nanog*, *Oct4*, *Sox2*, *Tbx3*, *Esrrb*, *Tcl1*, *Dppa4* and *Mm.343880* resulted in morphological changes and loss of alkaline phosphatase activity (Supplementary Fig. 4). No colonies were obtained after downregulation of *Mm.276044* and *Mm.219358*, suggesting roles for these genes in the control of cell cycle or viability. In other cases, small alkaline-phosphatase-positive colonies were obtained and were not analysed further (data not shown). Morphological evaluation and alkaline phosphatase assays were performed for all 60 shRNA vectors that did not cause a change in GFP^+/GFP^- ratios. In no case was the morphology or alkaline phosphatase activity changed, indicating that the competition assay is a robust way to detect ES cell differentiation.

Complementation rescue of compromised self-renewal activity

To rule out nonspecific effects of shRNAs we designed three additional shRNAs for each gene (Supplementary Table 1). At least one additional shRNA was effective for *Nanog*, *Oct4*, *Sox2*, *Esrrb* and *Tcl1*, showing that off-target effects are not responsible for the observed phenotypes. We also devised a genetic complementation (rescue) strategy where the original shRNA vectors were modified to include tetracycline-inducible versions of the shRNA-targeted gene products (Fig. 2a). To facilitate this rescue strategy the original shRNA targets were chosen to be in the 3' untranslated region (with the exception of *Oct4* and *Tcl1*). The shRNA-immune versions contain only the protein-coding sequences. Transactivator (rtTA)-expressing ES cells were transduced¹⁹. Self-renewal in transduced cells

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is doxycycline-dependent, as shown in Fig. 2b for *Nanog*. *Nanog* rescue-vector-transduced GFP⁺ cells (*NanogR*) were cloned in the presence of doxycycline and compared to clones transduced with empty vector (ControlR). Endogenous *Nanog* transcript was permanently downregulated, whereas vector-derived transcript was induced in the presence of doxycycline. The latter was undetectable 2 days after doxycycline removal. By morphological and alkaline phosphatase criteria, *NanogR* clones were undifferentiated in the presence of doxycycline. Upon doxycycline removal *NanogR* clones underwent morphological changes and lost alkaline phosphatase activity. Morphology and alkaline phosphatase activity of ControlR clones were unaffected by doxycycline (Fig. 2c). Robust doxycycline-dependent self-renewal was observed in *Sox2*, *Esrrb*, *Tbx3* and *Tcl1* rescue experiments. In the case of *Oct4*, we were not able to fully restore endogenous *Oct4* levels. *Oct4R* clones grow slowly but retain ES cell morphology, alkaline phosphatase activity and express high levels of *Nanog*. However, further analysis showed that several markers of trophectoderm stem (TS) cells, such as *Hand1*, *Cdx2*, *Eomes* and *Mash2* (referenced in Supplementary Table 2), were upregulated in the presence of doxycycline (Supplementary Fig. 6). These cells probably represent an intermediate stage between ES and TS cells. After removal of doxycycline, *Oct4R* cells become negative for alkaline phosphatase, further upregulate TS markers and acquire characteristic trophoblast morphology. In the case of *Dppa4*, all rescue clones showed strong downregulation of endogenous mRNA but yielded a significant number of undifferentiated colonies (data not shown). Therefore, *Dppa4* may not be absolutely required for self-renewal. No rescue clones were obtained for *Mm.343880*, suggesting that the morphological changes observed in the initial shRNA experiments were potentially due to the off-target effects or technical

complications associated with vector packaging (Supplementary Fig. 5). This gene was excluded from most subsequent analyses. To confirm further the differentiated state of cells maintained without doxycycline we measured the expression levels of pluripotency markers *Nanog* and *Oct4*. After 8 days without doxycycline both markers were significantly reduced in *NanogR*, *Sox2R*, *EsrrbR*, *Tbx3R* and *Tcl1R* cells (Fig. 2e).

Self-renewal regulators suppress differentiation

Loss of individual self-renewal regulators may initiate distinct differentiation programmes. We measured expression of early trophectodermal, mesodermal, ectodermal and endodermal markers in the rescue clones during an 8-day period after doxycycline removal. In addition, the expression of markers for more mature cell types that originate from each germ layer was measured at day 8. In total, a set of 35 markers was analysed (Supplementary Table 2). All polymerase chain reaction with reverse transcription (RT-PCR) analyses were performed using two independent rescue clones. Observed marker patterns are shown in Supplementary Figs 6–9.

Trophectodermal markers *Hand1*, *Cdx2*, *PL1*, *Mash2* and *Ehx* were induced in *Oct4R*, *Sox2R* and *NanogR* cells during the 8-day period after doxycycline removal. These markers were not induced when *NanogR* and *Sox2R* cells were maintained with doxycycline. The TS cell marker *Eomes* was upregulated only in *Oct4R* cells (Supplementary Fig. 6).

Goosecoid (*Gsc*) and brachury (*T*), markers for primitive streak and early mesoderm, were transiently expressed in *NanogR*, *Sox2R*, *EsrrbR* and *Tbx3R* cells. In addition, *Mixl1*, expressed in cells of the organizer region that will become mesoderm, was induced in *NanogR* and *Sox2R* cells. After the expression of early mesodermal markers, expression of genes specific to mesoderm-derived cell lineages was detected. These are: *Cd34* and β -globin (extraembryonic mesoderm); *Bmp5*, *Shh* and *Tbx5* (dorsal mesoderm); *Gata5* and *Isl1* (cardiac mesoderm and definitive endoderm); *Meox1* and *Cart1* (lateral mesoderm). The induction of these markers was not observed when cells were maintained with doxycycline (Supplementary Fig. 7).

Expression of the primitive ectoderm marker *Fgf5* was transiently induced in *NanogR*, *Sox2R*, *EsrrbR*, *Tbx3R* and *Tcl1R* cells, and was followed by upregulation of the ectodermal marker *Cxcl12*. *Mash1* and *Pax3* but not *Sox1* were also induced, indicating that these cells differentiate into neural crest derivatives. Other neural crest markers such as *Ednra*, *Eya2*, *Ngn2*, *Phox2b* and *Slug* were also detected in these cells. Only a subset of these markers was induced in *Tcl1R* cells after doxycycline removal, suggesting that additional signals are required to sustain differentiation induced by downregulation of *Tcl1* (Supplementary Fig. 8).

We found that ControlR cells spontaneously upregulated endodermal markers when maintained without passaging for more than 6 days. Therefore, endodermal commitment was analysed 4 days after doxycycline removal. At this time endodermal markers (*Gata4*, *Gata6*, *Foxa2*, *Sox17*, *Nr2f1*, *Nr2f2* and *Bmp2*) were induced in *NanogR*, *Sox2R* and *EsrrbR* cells (Supplementary Fig. 9). The markers were induced at the same time or after the induction of the organizer gene *Gsc*. Therefore, these cells may be differentiating into definitive endoderm from *Gsc*⁺/*Foxa2*⁺ precursors²⁰. Collectively, our results demonstrate that in addition to both *Oct4* and *Nanog*, *Sox2*, *Esrrb*, *Tbx3* and *Tcl1* function to suppress ES cell differentiation *in vitro*. None of the differentiation programmes was induced after downregulation of *Dppa4*. We also confirmed that removal of doxycycline does not induce apoptosis or cell cycle arrest in any of the rescue cells (Supplementary Figs 17 and 18).

To explore possible interactions between the identified gene products and known signalling pathways implicated in the control of self-renewal, we asked whether the activation status of LIF, ERK1/2, BMP and WNT signalling pathways is altered by the shRNA treatments (Supplementary Fig. 12). Stat3, Smad1/5/8 and Smad2/3 phosphorylation as well as total levels of β -catenin were unaffected by any shRNAs.

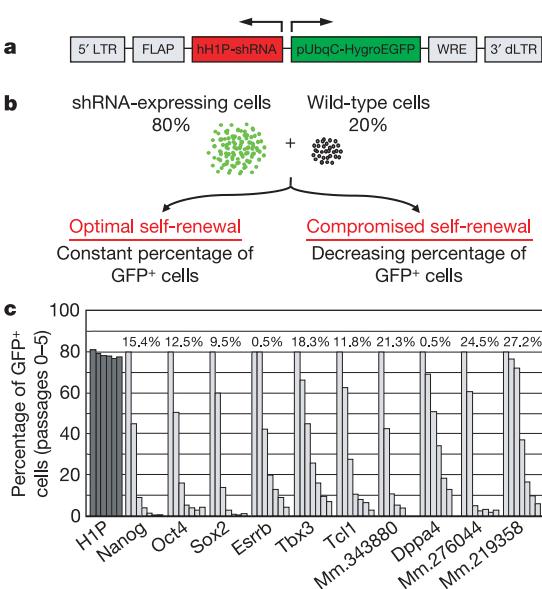


Figure 1 | Identification of self-renewal regulators in ES cells. **a**, Lentiviral vectors expressing both a shRNA and a hygromycin-EGFP fusion protein were constructed for each of the selected genes. FLAP, nucleotide segment that improves transduction efficiency; dLTR, deleted long-terminal repeat; WRE, woodchuck hepatitis virus post-transcriptional regulatory element. **b**, A competition strategy was designed to identify gene products whose depletion compromises ES cell self-renewal. GFP⁺ (shRNA-expressing) cells were mixed in a 4-to-1 ratio with untransduced (GFP⁻) cells, and cultured in the presence of LIF. With each passage the ratio of GFP⁺/total cells was measured by flow cytometry. **c**, Depletion of Nanog and Oct4 as well as eight additional gene products results in impaired self-renewal. The proportions of GFP⁺ cells transduced with the empty H1P vector do not change (representative experiment shown). In all cases Real-Time PCR (RT-PCR) confirms the reduction of mRNA levels (residual amounts relative to wild-type levels are shown as percentages above each time course).

ERK1/2 was hyper-phosphorylated after downregulation of *Nanog*, *Oct4*, *Sox2* and *Mm.343880*. The ERK1/2 pathway is activated in trophectoderm; therefore the increased phosphorylation of ERK1/2 correlates with the induction of the trophectodermal markers by *Nanog*, *Oct4* and *Sox2* shRNAs^{21,22}.

Constitutive expression of self-renewal regulators

We next asked whether enforced expression of identified self-renewal regulators is sufficient to block the commitment to specific lineages. Rescue clones were used to produce embryoid bodies²³ in the presence of doxycycline. Temporal profiles of markers for neuro-ectoderm, mesoderm and endoderm were analysed during a 12-day period (Supplementary Fig. 10). Markers for all three lineages were induced in NanogR-, Tcf1R- and Dppa4R-derived embryoid bodies. Mesodermal commitment was affected in both Sox2R and Tbx3R cells. Marker patterns suggest that mesodermal development is blocked after the induction of *Gsc* in Sox2R cells (no *Mixl1* and no *T* expression) and slightly later in Tbx3R cells (normal *Mixl1* and no *T* expression). A complete block of both mesodermal and neuro-ectodermal differentiation was observed in EsrrbR cultures, where neuroectodermal differentiation was arrested after the induction of *Fgf5*, and mesodermal differentiation was blocked before the induction of *Gsc*. EsrrbR cells retained the capacity to form endoderm. In addition, expression of *Sox1* was increased in Sox2R cells and probably reflects the importance of *Sox2* in the neuroectodermal lineage²⁴. Enforced expression of *Tcf1* may also promote the expansion of

neuroectodermal precursors and/or prevent their differentiation.

We also analysed the capacity of rescue cells to contribute to different tissues (brain, liver, muscle and coat hair) *in vivo*. ControlR cells contributed efficiently to all tissues, including the germ line, demonstrating that the rescue clone derivation procedures do not compromise pluripotency. NanogR, SoxR, Tbx3R and Tcl1R cells maintained without doxycycline for three passages did not contribute to any tissue, whereas EsrrbR cells retained limited capacity to contribute to neural crest derivatives such as coat melanocytes (see Supplementary Information for a detailed description).

shRNA-induced gene expression dynamics

We next analysed transcriptome dynamics after downregulating the expression of each of the seven genes identified in the screen. shRNA-transduced GFP⁺ cells were purified by fluorescence-activated cell sorting (FACS) daily during a 7-day interval and were used to interrogate Affymetrix microarrays. We identified a total of 3,109 non-redundant genes whose expression was perturbed after treatment with at least one shRNA (see Supplementary Information). Several distinct patterns of gene expression were observed. The first pattern (Fig. 3a) contains 771 genes that are up- or downregulated in response to most shRNA treatments. The second pattern (Fig. 3b) contains 474 genes perturbed in response to *Nanog*, *Oct4* and *Sox2* but not to *Esrrb*, *Tbx3*, *Tcl1* or *Dppa4* shRNA treatments. Recent chromatin studies have shown that *Nanog*, *Oct4* and *Sox2* often co-occupy the promoters of target genes²⁵. The third pattern (Fig. 3c)

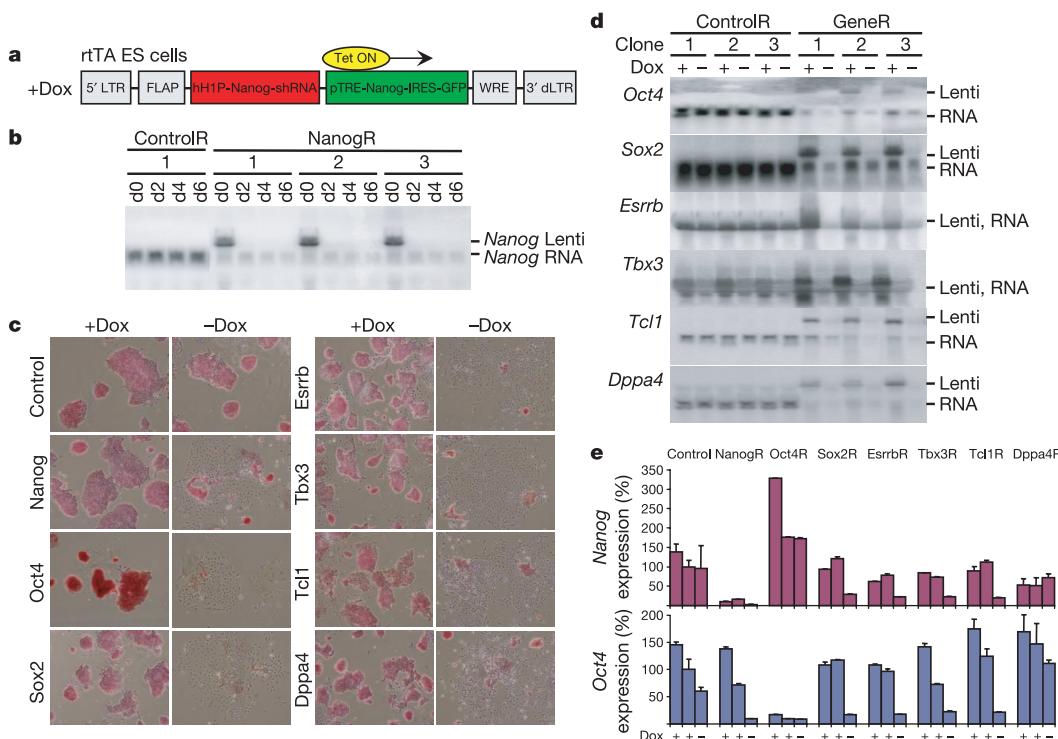


Figure 2 | Genetic complementation to rescue shRNA-induced self-renewal defects. **a**, The shRNA vectors were modified to produce tetracycline-inducible versions of endogenous targets. EGFP is coupled to the overexpressed gene via an internal ribosome entry sequence (IRES) (*Nanog* rescue vector is shown as an example). **b**, This approach was tested using *Nanog*. The rescue vectors were introduced into rtTA transactivator expressing ES cells in the presence of doxycycline (Dox); individual clones were isolated and expanded. Three clones (labelled 1–3) were chosen for further analysis. Control (ControlR) clones transduced with empty rescue vector (H1P-pTRE-IRES-EGFP) are also shown. Levels of endogenous *Nanog* (*Nanog* RNA) and vector derived *Nanog* (*Nanog* Lenti) were measured by northern blotting at 2-day intervals for 6 days (d0–d6) after withdrawal of doxycycline. In all three NanogR clones endogenous *Nanog*

mRNA was permanently decreased. Vector-derived transcripts were expressed in the presence of doxycycline and rapidly reduced after doxycycline withdrawal. **c**, Self-renewal was rescued in the case of Nanog and for six other gene products. **d**, Levels of endogenous (RNA) and vector-derived (Lenti) transcripts were measured for three independent clones with and without doxycycline for the six additional genes. In all cases, the levels of endogenous transcript were decreased, and expression of the vector-derived transcripts was dependent on doxycycline. **e**, Expression of self-renewal markers *Nanog* and *Oct4* was reduced in the rescue cells maintained without doxycycline for 8 days. Expression level before the removal of doxycycline is shown as d0. Expression in d8 ControlR cells maintained with doxycycline is set as 100%. Each bar represents the average expression in three independent rescue clones corresponding to each gene; standard deviations are shown as error bars.

contains 272 gene products that are responsive to *Esrrb*, *Tbx3*, *Tcl1* and *Dppa4* shRNAs, but not to *Nanog*, *Oct4* or *Sox2* shRNAs. Patterns 2 and 3 suggest the existence of at least two distinct pathways that are necessary for self-renewal. Interestingly, whereas pattern 2 contains equal numbers of up- and downregulated gene products, pattern 3 contains gene products that are preferentially upregulated by shRNA treatments. This suggests that the corresponding pathway functions to repress differentiation-promoting genes. We have identified a number of smaller gene clusters that were perturbed by single shRNA treatments or by pairs of shRNA treatments (Supplementary Fig. 13).

Identification of gene products that promote differentiation

A number of transcriptional regulators are present in the gene expression patterns defined above. Some of these are upregulated after shRNA depletion of the self-renewal regulatory genes and may act as master positive regulators of differentiation. To identify such regulators, 160 gene products for which expression profiles suggest differentiation-inducing activity were overexpressed in ES cells. In 18 cases differentiation was suggested by changes in cell morphology,

loss of alkaline phosphatase activity and downregulation of *Nanog* (Fig. 3d and Supplementary Fig. 14). Temporal expression profiles of these 18 genes upon shRNA-mediated inactivation of self-renewal regulators (Fig. 3e) suggest that many differentiation inducers are under the control of multiple self-renewal regulators.

Nanog compensates for loss of several self-renewal regulators

Most of the differentiation regulators identified in the overexpression screen are upregulated by *Nanog* shRNA treatment. *Nanog* is a strong positive regulator of self-renewal and counteracts removal of LIF and BMP^{4,7}. We asked whether *Nanog* could block the differentiation induced by downregulation of the other genes identified in our studies. *Nanog* was overexpressed in ES cells as shown in Fig. 4a. This provides an approximately threefold increase in total *Nanog* levels and confers LIF independence (Supplementary Fig. 15). These cells were transduced with shRNA vectors, maintained in the presence of LIF, and evaluated using morphological criteria and alkaline phosphatase staining. *Nanog* overexpression restored undifferentiated morphology and alkaline phosphatase activity levels in *Esrrb*, *Tbx3*,

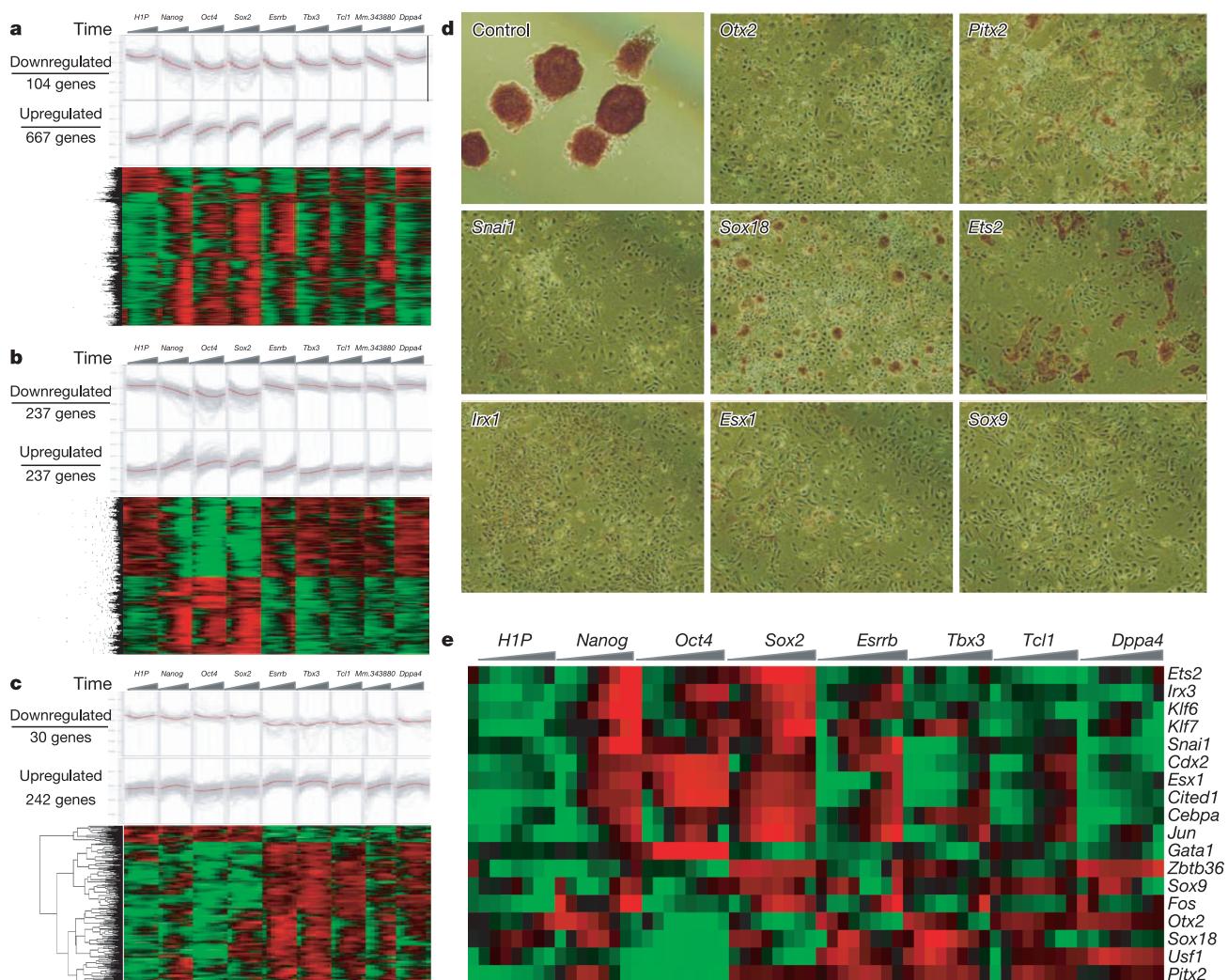


Figure 3 | Global gene expression changes after downregulation of individual self-renewal regulators. Microarray analysis time courses were performed to measure gene expression changes that accompany differentiation. **a–c**, Clustering revealed distinct patterns of gene expression. A number of transcriptional regulators present in the gene expression patterns that are upregulated upon shRNA-mediated depletion of self-renewal genes can induce differentiation when overexpressed in wild-type ES cells. **d**, Differentiation was apparent from the morphologies of the cells

as well as the loss of *Nanog* expression (data not shown). **e**, Temporal expression profiles of identified differentiation inducers upon shRNA-mediated inactivation of individual self-renewal regulators show that the genes (listed on the right side of the panel) are upregulated in at least one, and usually more than one, shRNA time course. Expression levels of the genes were centred and scaled to one standard deviation, and are indicated by colour, from green (low expression) to red (high expression).

Tcl1 and *Dppa4* shRNA-transduced cells. Differentiation induced by depletion of *Oct4* and *Sox2* was unaffected (Fig. 4b and Supplementary Fig. 16). Therefore, the overexpression of *Nanog* may block differentiation into mesodermal, ectodermal and neural crest cell lineages. *Nanog* may block differentiation by simply upregulating the endogenous targeted gene to levels that are insensitive to shRNA depletion. However, in all cases the endogenous genes were similarly depleted both in *Nanog* overexpressing (*NanogIP*) and control (ControlIP) cells (Fig. 4c). Instead, the expression levels of other self-renewal regulators that are normally downregulated by shRNA treatments were fully or partially restored. Specifically, *Oct4* and *Sox2* were downregulated by *Esrrb* shRNA in ControlIP cells, but were maintained in *NanogIP* cells. *Nanog* overexpression restored the levels of *Oct4*, *Sox2* and *Esrrb* in *Tbx3* shRNA-treated cells and the levels of *Esrrb* and *Tbx3* in *Tcl1* shRNA-treated cells. Overexpression of *Nanog* was sufficient to prevent the induction of several differentiation inducers. *Cited1*, *Fos* and *Irx3* genes are shown in Fig. 4c as examples of such regulation.

Discussion

Using an integrated functional genomics approach we have demonstrated that *Esrrb*, *Tbx3* and *Tcl1*, as well as previously identified *Nanog*, *Oct4* and *Sox2*, are required for efficient self-renewal of ES cells *in vitro*. Downregulation of each gene induces differentiation of ES cells along specific lineages. The data obtained from our studies are summarized in Fig. 5.

According to current models *Oct4* is required to prevent trophectodermal differentiation of ES cells³, whereas *Nanog* is required to block differentiation to the endodermal lineage⁵. Whereas *Oct4*

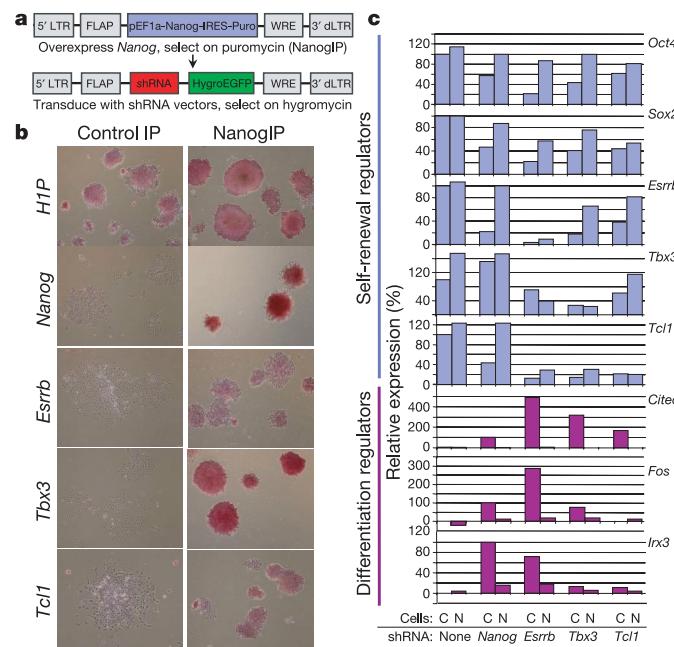


Figure 4 | Nanog rescue of phenotypes caused by shRNA directed against other self-renewal regulators. **a**, ES cells were transduced with a *Nanog* overexpressing vector. *Nanog* overexpressing (*NanogIP*, N) and control (ControlIP, C) cells were challenged with the entire set of shRNA vectors. **b**, The cells were propagated in the presence of LIF, and analysed by morphology and alkaline phosphatase staining. **c**, Enforced *Nanog* activity does not simply upregulate the targeted endogenous genes to levels insensitive to shRNA inhibition but may also compensate for the loss of function of the targeted gene by maintaining the expression of other self-renewal regulators (blue) and by preventing the induction of key differentiation inducers (purple) that normally occurs upon shRNA-mediated downregulation of *Esrrb*, *Tbx3* or *Tcl1*. Gene expression levels were measured by microarrays.

downregulation exclusively induces the set of trophectodermal genes, we found that, in addition to endoderm, *Nanog* downregulation induces the expression of markers for trophectoderm and epiblast-derived lineages, namely mesoderm, ectoderm and neural crest cells. Therefore, *Nanog* seems to be a global regulator that represses multiple differentiation programmes. *Sox2* functions to repress the development of trophectoderm and epiblast-derived lineages, whereas *Esrrb* and *Tbx3* are necessary to block the differentiation into mesoderm, ectoderm and neural crest cells but are not required to repress trophectoderm differentiation. The function of *Tcl1* seems to be even more restricted as it appears to repress only a subset of neural crest genes. In addition, enforced expression of *Sox2* and *Tbx3* is sufficient to repress the commitment to mesodermal lineage and *Esrrb* is capable of blocking both mesodermal and neuroectodermal commitment in embryoid body conditions.

We identify several patterns of transcriptional regulation. These patterns suggest the existence of two global pathways required for self-renewal and will be useful in efforts to further elucidate epistatic and other relationships. A number of genes that are upregulated after shRNA depletion of the self-renewal regulatory genes can act as positive regulators of differentiation programmes. Downregulation of *Nanog*, *Sox2*, *Esrrb*, *Tbx3* or *Tcl1* leads to the immediate induction of *Otx2*, *Pitx2*, *Sox18* and probably additional genes. All three genes are expressed in the epiblast; *Otx2* and *Pitx2* are important for mesodermal and neuroectodermal development *in vivo*^{26–29}. *Otx2* seems to be a direct transcriptional target of both *Nanog* and *Oct4*, whereas *Pitx2* may be directly regulated by *Nanog* and *Sox2*, as suggested by recent chromatin-precipitation studies^{25,30}. The activation of early-induced differentiation regulators is followed by the induction of the next wave of differentiation genes. Combinatorial action of these later-acting genes specifies the precise developmental outcome, such as mesoderm, ectoderm or neural-crest-like cells observed in our experimental settings. Notably, some of the late-induced genes seem to be direct transcriptional targets of self-renewal regulators. For instance, *Nanog* and *Oct4* are directly bound to the promoter regions of *Snai1*, *Klf6* and *Klf7* genes³⁰.

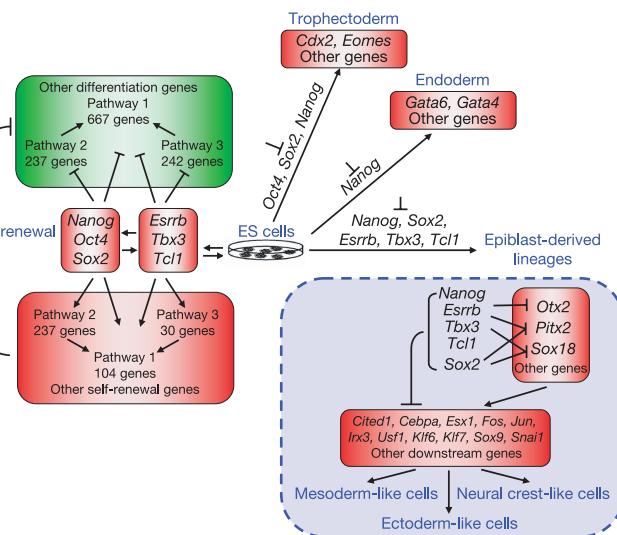


Figure 5 | Provisional model of cell fate regulatory interactions in ES cells in vitro. *Esrrb*, *Tbx3* and *Tcl1*, as well as previously identified *Nanog*, *Oct4* and *Sox2*, are required for efficient self-renewal of ES cells *in vitro*. *Oct4* is required to prevent trophectodermal differentiation, *Nanog* and *Sox2* appear to be global regulators that repress multiple differentiation programmes, whereas *Esrrb*, *Tbx3* and *Tcl1* are necessary to block the differentiation into epiblast-derived lineages. Self-renewal regulators are integrated into an interconnected transcriptional network and control the expression of downstream target genes through distinct molecular pathways.

Nanog can substitute for *Esrrb*, *Tbx3* and *Tcl1* when overexpressed, probably through compensatory changes in the levels of other self-renewal regulators. This suggests that the self-renewal regulators are integrated into an interconnected transcriptional network and that the loss of function of one regulator can, in some cases, be compensated by adjusting the expression levels of other network components. Such a network would explain why knockouts of *Esrrb*, *Tbx3* and *Tcl1* show phenotypes at fairly advanced embryonic stages^{17,31–34}. It will also be interesting to check whether *Sox2*, *Esrrb* and *Tbx3* can rescue differentiation into the epiblast-derived lineages induced by *Nanog* shRNA treatment. The function of *Dppa4* in ES cells remains unknown. None of the differentiation programmes analysed was induced in *Dppa4R* cells after removal of doxycycline, and the enforced expression of *Dppa4* did not affect differentiation in the embryoid body assay.

The screening strategy developed in this study can be extended to genome-scale shRNA libraries to identify additional gene products that have important roles in ES cell self-renewal^{35,36}. It can also be applied to other systems, such as haematopoietic and neural stem cells, where developmental read-outs can be readily measured.

Regulatory similarities in mouse and human ES cells are unclear. *Oct4* and *Nanog* are essential; however, the LIF-gp130-Stat3 pathway appears not to be required in human ES cells³⁷. It will be important to evaluate the panel of genes described herein as candidate regulators in the human ES system.

METHODS

A detailed description of the methods used in these studies can be found in Supplementary Information.

Cell lines. Retinoic-acid-induced differentiation, competition assays, western blot analyses, microarray analyses of shRNA-induced differentiation and rescue of shRNA-induced phenotypes by *Nanog* overexpression were performed using mouse cell line CCE. Overexpression of shRNA-induced genes was performed using E14/T21 cells. E14/T21 cells were made by introducing EGFP under the control of the 6-kb *Nanog* promoter region into E14/T cells (a gift from A. Smith) using BAC transgenic technology (I.R.L. laboratory, unpublished data). CCE and E14/T21 ES cells were maintained on gelatin-coated dishes in DMEM media supplemented with 15% FBS (Hyclone), 100 mM MEM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 1 mM L-glutamine (Invitrogen) and 10³ U ml⁻¹ of LIF (Chemicon).

rtTA-expressing ES cells Aniv15 (a gift from G. Daley) were used for derivation of rescue clones, and were maintained on primary mouse embryonic fibroblasts in the above-described media supplemented with doxycycline (1 µg ml⁻¹). For differentiation assays the cells were trypsinized and plated for 30 min on standard tissue culture dishes in order to remove primary mouse embryonic fibroblasts, and floating ES cells were collected and plated on gelatin-coated dishes.

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