

## Novel STAT3 Target Genes Exert Distinct Roles in the Inhibition of Mesoderm and Endoderm Differentiation in Cooperation with Nanog

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### ABSTRACT

Leukemia inhibitory factor (LIF) activates the transcription factor signal transducer and activator of transcription 3 (STAT3), which results in the maintenance of mouse embryonic stem cells in the pluripotent state by inhibiting both mesodermal and endodermal differentiation. How the LIF/STAT3 pathway inhibits commitment to both mesoderm and endoderm lineages is presently unknown. Using a hormone-dependent STAT3 and with microarray analysis, we identified 58 targets of STAT3 including 20 unknown genes. Functional analysis showed that 22 among the 23 STAT3 target genes analyzed contribute to the maintenance of the undifferentiated state, as evidenced by an increase in the frequency of differentiated colonies in a

self-renewal assay and a concomitant elevation of early differentiation markers upon knockdown. Fourteen of them, including *Dact1*, *Klf4*, *Klf5*, *Rgs16*, *Smad7*, *Ccrn4l*, *Cnnm1*, *Ocln*, *Ier3*, *Pim1*, *Cyr61*, and *Sgk*, were also regulated by Nanog. Analysis of lineage-specific markers showed that the STAT3 target genes fell into three distinct categories, depending on their capacity to inhibit either mesoderm or endoderm differentiation or both. The identification of genes that harness self-renewal and are downstream targets of both STAT3 and Nanog shed light on the mechanisms underlying functional redundancy between STAT3 and Nanog in mouse embryonic stem cells. *STEM CELLS* 2009;27:1760–1771

Disclosure of potential conflicts of interest is found at the end of this article.

### INTRODUCTION

Mouse embryonic stem (ES) cells can be easily propagated in defined medium lacking serum and feeder cells, under the sole action of recombinant leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP) 4, which together cooperate to suppress differentiation [1]. The LIF signaling pathway is involved in the inhibition of mesoderm and endoderm differentiation, whereas the BMP4 pathway is involved in the inhibition of the ectoderm pathway [2]. LIF engages a heterodimeric receptor complex consisting of LIF receptor and gp130. This complex recruits and phosphorylates a number of factors including the signal transducer and activator of transcription 3 (STAT3) [3], the phosphatidylinositol 3-kinase (PI3K) [4], Yes, a member of the Src family of tyrosine kinases [5], and the src-homology domain phosphatase 2 (SHP-2) [6]. Functional inactivation of STAT3, PI3K, and Yes, in the presence of LIF, induces spontaneous differentiation [4, 5, 7]. In contrast, functional inactivation of SHP-2 facilitates self-renewal [6], suggesting that LIF stimulates self-renewal by coactivating positive and negative signaling pathways in a

coordinated manner. Expression of a hormone-dependent STAT3 that can be activated directly by estradiol sustains self-renewal without the addition of LIF [8]. This finding suggests that the LIF/STAT3 pathway plays a preeminent role in the control of self-renewal and pluripotency of mouse ES cells.

Little is known about the effectors of STAT3-dependent self-renewal. The proto-oncogene *c-myc* is a direct target of STAT3. The forced expression of a mutated form of c-Myc temporarily inhibits the differentiation of mouse ES cells induced by withdrawal of LIF [9]. We recently identified two novel STAT3 target genes, *Pim1* and *Pim3*, that encode members of the family of Pim serine/threonine kinases. *Pim1* and *Pim3* are activated by a ligand-dependent STAT3 mutant in the presence of protein synthesis inhibitor, indicating that both are direct targets of the LIF/STAT3 pathway. Their activities are necessary for ES cell self-renewal, as shown by the increased rates of spontaneous differentiation after knockdown [10]. *Eed* was recently shown to maintain silencing of differentiation-associated genes upon direct activation by STAT3 in cooperation with the pluripotency gene *Oct4* in undifferentiated ES cells [11]. Contrasting with the four

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above-mentioned genes, *Socs3*, which encodes the suppressor of cytokine signaling 3 (SOCS3), functions as a feedback regulator of gp130 signaling [12] that blocks self-renewal when overexpressed [2]. *JunB*, *Jmjd1*, *Dax1*, and *Grg5* (the latter encoding the groucho-like protein Aes1) are also regulated by LIF/STAT3 in ES cells [8, 13–15], but evidence that they are effectors of self-renewal is lacking.

The propagation of ES cells in the undifferentiated state is also dependent on the activity of the homeodomain factor Nanog [16, 17]. Nanog is central, together with the POU domain factor OCT4 [18] and the HMG box factor SOX2 [19], to the transcriptional regulatory network that specifies ES cell identity [20–22]. Genome-wide location analysis using chromatin immunoprecipitation has shown that Nanog occupies, either alone or in association with Oct4 and Sox2, several hundred active genes encoding transcription factors, signal transduction components, and chromatin-modifying enzymes that promote ES cell self-renewal [23, 24]. Nanog target genes include *Zic3* and *Tcf3*, which have both been shown to contribute to the regulation of the undifferentiated state of mouse ES cells [25, 26].

Nanog was identified because of its ability to overcome differentiation induced by LIF starvation when overexpressed, thus suggesting a functional redundancy between STAT3 and Nanog [16]. How high levels of Nanog can bypass the requirement for LIF to maintain the undifferentiated state of ES cells is not known. Overexpression of Nanog does not increase the level of phosphorylated STAT3 significantly, and elevated STAT3 signaling seems not to affect Nanog expression. These findings suggest that Nanog is not a direct transcriptional target of STAT3 nor does Nanog regulate STAT3 activity [16]. Recently, chromatin immunoprecipitation coupled with massively parallel short tag-based sequencing (ChIP-seq) was used to map the locations of 13 transcription factors, including STAT3 and Nanog [27]. Of all genes analyzed in this study 16% harbored binding sites for both STAT3 and Nanog. Moreover, 55% of putative STAT3 target genes displayed binding sites for Nanog, and 41% of putative Nanog target genes displayed binding sites for STAT3. These results suggest that Nanog and STAT3 coregulate the expression of a large number of target genes whose expression is critical to the maintenance of the undifferentiated state.

To explore how STAT3 maintains mouse ES cells in the undifferentiated state, we characterized its transcriptome by microarray analysis. We identified a number of genes whose expression is regulated by both STAT3 and Nanog and that contribute to the inhibition of both mesoderm and endoderm differentiation.

## MATERIALS AND METHODS

### Plasmid Construction

The hormone-dependent STAT3-ER<sup>T2</sup> transcription factor was generated by fusing the coding sequence of the mouse STAT3 transcription factor to a 5'-XhoI/EcoRI-3' fragment containing the entire ER<sup>T2</sup> domain [28]. The resulting cDNA was subcloned into the EcoRI site in the episomal vector pPCAGIZ [7] to generate pPCAGIZ-STAT3-ER<sup>T2</sup>. Cre-ER<sup>T2</sup> was subcloned into the EcoRI site in pPCAGIZ to generate pPCAGIZCre-ER<sup>T2</sup>. The BLOCK-iT lentiviral RNAi Gateway vector kit (K4943-00; Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>) was used to generate lentiviral vectors expressing small hairpin (sh) RNAs according to the manufacturer's instructions. In the pLenti6/BLOCK-iT backbone vector, the blunted KpnI (blunt)/XhoI fragment was replaced by a 1.3-kilobase pair ClaI (blunt)/XhoI fragment con-

taining the PGK promoter and the *neo*<sup>R</sup> selectable gene to generate pLenti6/BLOCK-iT-PGKneo<sup>R</sup>. Similarly, the blunted KpnI/XhoI fragment was replaced by a 1.7-kilobase pair EcoRV/EcoRV fragment, containing the PGK promoter and the *hygro*<sup>R</sup> selectable gene, to generate pLenti6/BLOCK-iT-PGKhygro<sup>R</sup>. pGAE-CAG-Nanog/WPRE was generated from pGAE-CAG-eGFP/WPRE [29] by replacing the BamHI/SphI fragment containing eGFP with a BamHI/SphI polymerase chain reaction (PCR) fragment containing the full-length cDNA encoding mouse Nanog.

### shRNA Design

shRNA and small interfering (si) RNA sequences were designed using the siDESIGN Center application of Dharmacon (<http://www.dharmacon.com>). For unassigned expressed sequence tags, the sequences of the Affymetrix probe sets were used to design the sequence of the shRNA. For each targeted gene, five independent shRNAs were cloned into pENTRY (K4943-00; Invitrogen), and the resulting pENTRY-shRNA vectors were transfected into CGR8 ES cells to measure the interference by real-time PCR. For each targeted gene, the two shRNA sequences, which show the highest interference in the transient transfection assay were cloned into pLenti6/BLOCK-iT-PGKneo<sup>R</sup> or pLenti6/BLOCK-iT-PGKhygro<sup>R</sup>. The sequences of selected shRNAs and the interference resulting from transfection of pENTRY-shRNA vectors in CGR8 cells are given in supporting information online Table 1.

### ES Cell Culture, Electroporation, Infection with Lentiviral Vectors, and Teratoma Formation

All ES cell lines were routinely cultured in Glasgow's modified Eagle's medium supplemented with 10% fetal calf serum (CRC0406; PerbioScience France, Berbieres, France, <http://www.perbio.com>) and 1,000 U/ml of LIF. For plasmid transfection and siRNA transfection, 15  $\mu$ g of supercoiled plasmid were mixed with 45  $\mu$ l of lipofectamine (11668; Invitrogen) in 3 ml of Opti-MEM (31985; Invitrogen). The resulting mixture was added to CGR8 ES cells at a density of  $2 \times 10^6$  cells per 10-cm dish in 15 ml of medium. Cells were cultured for 48 hours before analysis. For episomal supertransfection, E14/T cells [30] were electroporated with 20  $\mu$ g of supercoiled vectors at 200 V and 960  $\mu$ F in a 0.4-cm cuvette. Cells were plated at  $5 \times 10^4$  cells per 10-cm dish and cultured in the presence of 1  $\mu$ g/ml zeocine (Cayla, Toulouse, France, <http://www.cayla.com>) for 7 days. Resistant colonies were pooled and further propagated in selection medium for 1 week. E14/T ES cells expressing STAT3-ER<sup>T2</sup> were shifted to culture medium supplemented with 10 nM 4'-hydroxytamoxifen (4'-OHT) lacking LIF (579002; Calbiochem, San Diego, <http://www.emdbiosciences.com>). RCNHTK $\beta$  cells were electroporated with linearized pPCAGIZ-STAT3-ER<sup>T2</sup> at 260 V and 500  $\mu$ F in a 0.4-cm cuvette. Cells were plated at  $5 \times 10^6$  cells per 10-cm dish and cultured in the presence of 1  $\mu$ g/ml of zeocine for 7 days.

Lentiviral vectors were produced using the BLOCK-iT lentiviral RNAi expression system (K4944-00; Invitrogen) according to the manufacturer's instructions. For infection, CGR8 cells were plated at a density of  $10^4$  cells in 24-well plates in 1 ml of medium composed of 100  $\mu$ l of ES cell medium and 900  $\mu$ l of culture supernatant from virus-producer cells. After 48 hours, ES cells were trypsinized, replated at  $10^4$  cells per gelatin-coated 10-mm tissue culture dish and further cultured in complete ES cell medium supplemented with 250  $\mu$ g/ml of G418 [or with 100  $\mu$ g/ml of Hygromycin B (Roche Applied Science, Basel, Switzerland, <http://www.roche-applied-science.com>)] and 1,000 U/ml LIF for 6 days. For colony-forming assays, dishes were fixed in methanol for 15 minutes and then were stained for 15 minutes with a solution containing 1 mg/ml Fast Red TR salt (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) dissolved in 0.1 M Tris (pH 9.2) containing 200 mg/ml naphthol AS-MX phosphate. Teratomas were generated by injecting  $10^6$  ES cells into the testes of SCID mice.

### Semiquantitative and Real-Time PCR

RNA was extracted using RNeasy kits with on-column DNase digestion and reverse transcription was performed with M-MLV reverse transcriptase (M1701; Promega, Madison, WI, <http://www.promega.com>), according to the manufacturer's recommendations. Oligonucleotide sequences are given in supporting information Table 2. Quantitative PCR was performed using the LightCycler 1.5 system and the LightCycler Fast Start DNA Master SYBR Green I kit (Roche Applied Science) according to the manufacturer's instructions. All normalizations were carried out with  $\beta$ -actin.

### Microarrays and Bioinformatics

Total RNAs from ES cells were prepared with the Qiagen column kit (Qiagen, Hilden, Germany, <http://www1.qiagen.com>) and treated with DNase (5 U/100 mg of RNA, Sigma-Aldrich). Biotinylated cRNA was prepared according to the standard Affymetrix protocol (Expression Analysis Technical Manual, 1999; Affymetrix, Santa Clara, CA, <http://www.affymetrix.com>). In brief, double-stranded cDNA was synthesized from 10  $\mu$ g of total RNA using the SuperScript Choice System from Invitrogen and the Affymetrix T7-(dT)<sub>24</sub> primer that contains a T7 RNA polymerase promoter attached to a poly(dT) sequence. The cDNA was transcribed into biotin-labeled cRNA using the Retic Lysate IVT kit (Ambion, Austin, TX, <http://www.ambion.com>). The cRNAs produced were purified using an RNeasy kit (Qiagen) and fragmented to an average size of 30-50 bases according to Affymetrix recommendations. Then 15  $\mu$ g of fragmented cRNA was hybridized for 16 hours at 45°C on the Mouse Genome 430 2.0 Array. Arrays were washed and stained in the Affymetrix Fluidics Station 450 and further scanned using the Affymetrix GeneChip Scanner 3000. The image data were analyzed with the GeneChip Operating Software using Affymetrix default analysis settings. After passing quality control, assays were commonly normalized by the log scale robust multiarray analysis [31]. After outlier removal using the Nalimov test at  $p < .001$  a parametric analysis of variance (ANOVA) (F test) was applied to obtain global expression differences between the different conditions. Hierarchical clustering of expression values of probe sets differentially expressed in the ANOVA was performed by using Cluster version 2.11 [32], applying mean-centering and normalization of genes and arrays before average linkage clustering with uncentered correlation. Information on quality control of microarray data is available in supporting information Table 3.

### Accession Numbers

Details about the microarray deposition can be found at <http://www.ebi.ac.uk/arrayexpress/experiments/E-TABM-562>.

## RESULTS

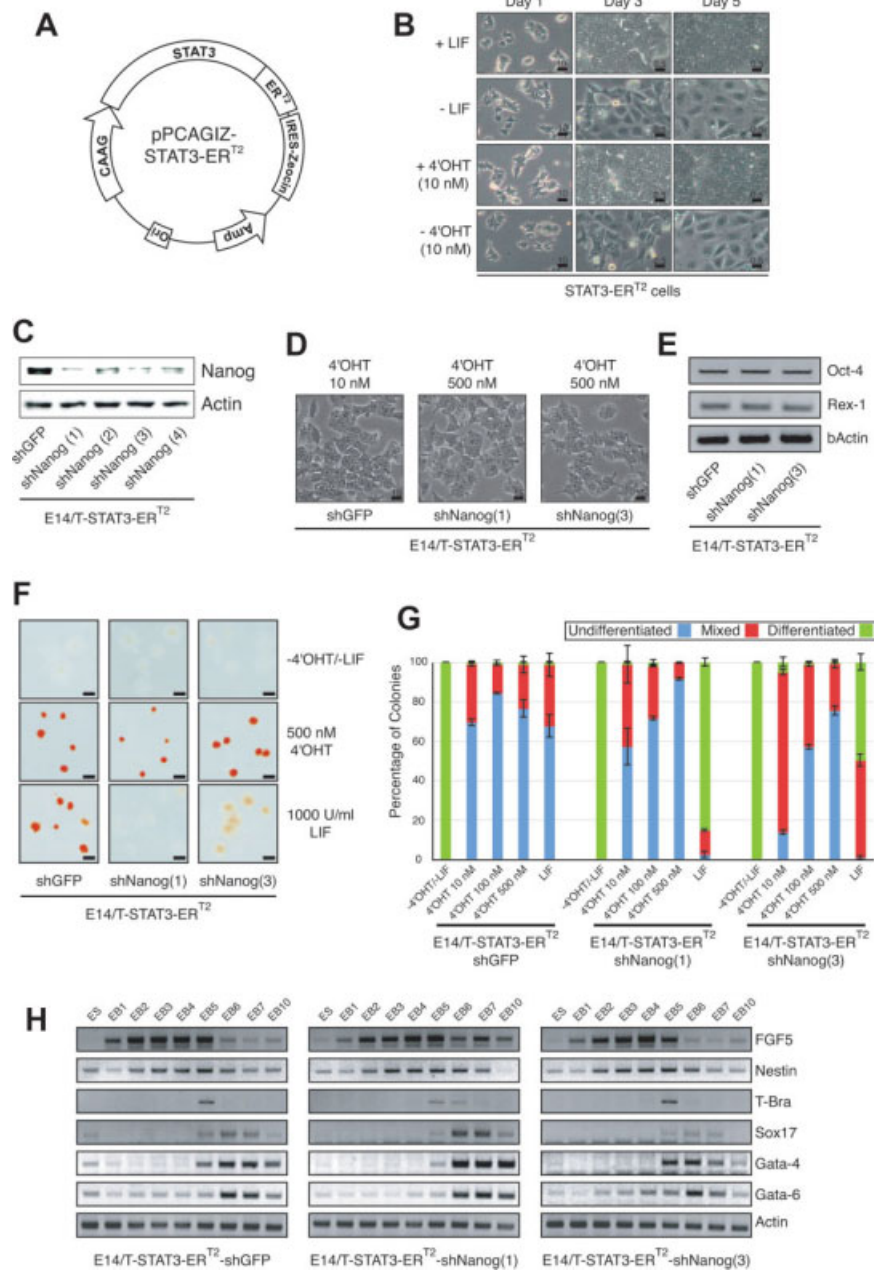
### Episomally Expressed STAT3-ER<sup>T2</sup> Sustains Self-Renewal in the Presence of a Reduced Amount of Nanog

E14/T-STAT3-ER<sup>T2</sup> ES cells [10], referred to as E14-S3ER, express the hormone-dependent STAT3-ER<sup>T2</sup> from an episomal vector (Fig. 1A). They could self-renew with 10 nM 4'-OHT in the absence of LIF and differentiate after withdrawal of hormone (Fig. 1B). E14-S3ER ES cells propagated in medium supplemented with 10 nM 4'-OHT were infected with pLenti6/BLOCK-iT-PGKhygro<sup>R</sup>-shNanog, an interfering lentiviral vector expressing a Nanog shRNA (*shNanog*) and a *hygro<sup>R</sup>* selectable gene. The resulting *hygro<sup>R</sup>* colonies were stained to reveal alkaline phosphatase (AP) activity, a marker of undifferentiated cells. Few AP<sup>+</sup> *hygro<sup>R</sup>* colonies were observed after selection (supporting information Fig. 1), and

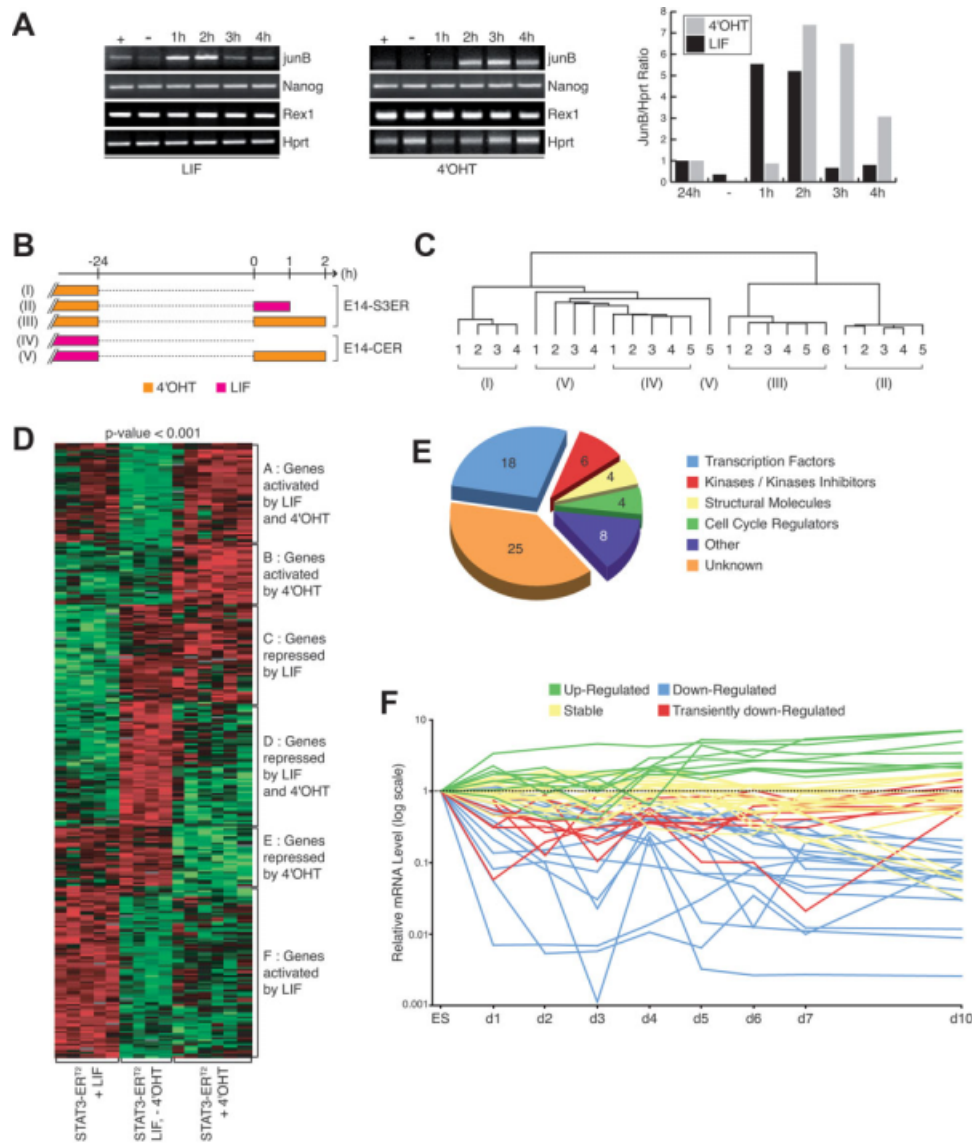
none of those that were found showed knockdown of Nanog expression (data not shown). In contrast, when E14-S3ER ES cells were supplemented with 500 nM 4'-OHT during infection with the interfering vector and subsequent selection of infected cells, many AP<sup>+</sup> *hygro<sup>R</sup>* colonies were observed, some of them expressing low Nanog levels (Fig. 1C). Two clones, E14-S3ER-shNanog [1] and E14-S3ER-shNanog [3], showed a strong reduction in Nanog level. E14-S3ER-shNanog clones propagated in 500 nM 4'-OHT and E14-S3ER-shGFP control cells propagated in 10 nM 4'-OHT showed the characteristic morphology of undifferentiated ES cells (Fig. 1D), and all three expressed *Rex1* and *Oct4* pluripotency markers at comparable levels (Fig. 1E). This observation suggests that increased STAT3 activity can compensate for the reduction in the Nanog expression level and hence sustain pluripotency. To confirm this hypothesis, E14-S3ER-shNanog [1], E14-S3ER-shNanog [3], and E14-S3ER-shGFP ES cells were plated at clonal density and further grown for 6 days in medium supplemented with 0, 10, 100, and 500 nM 4'-OHT or with 1000 U/ml of LIF. The resulting colonies were stained for AP activity (Fig. 1F), and the percentages of undifferentiated, mixed, and differentiated colonies were calculated (Fig. 1G). E14-S3ER-shGFP ES cells formed undifferentiated colonies in 10 nM 4'-OHT as well as in LIF. The proportion of undifferentiated colonies was only marginally augmented as a result of increasing the concentration of 4'-OHT up to 100 nM. In contrast, the two E14-S3ER-shNanog clones showed reduced capacity to form undifferentiated colonies in 10 nM 4'-OHT and in LIF compared with control cells. This capacity was partially restored in 100 nM and fully restored in 500 nM 4'-OHT. E14-S3ER-shNanog [1] and E14-S3ER-shNanog [3] ES cells propagated in 500 nM 4'-OHT were induced to differentiate by formation of embryoid bodies (Fig. 1H). Analysis of differentiation markers (*Fgf5*, *nestin*, *T-Bra*, *Sox17*, *GATA-4*, and *GATA-6*) revealed similar kinetics of ectoderm-, mesoderm- and endoderm-associated gene expression between E14-S3ER-shNanog (+500 nM 4'-OHT) and E14-S3ER-shGFP (+10 nM 4'-OHT) control cells. Injection of E14-S3ER-shNanog cells in the testes of SCID mice consistently resulted in teratoma formation. Immunohistochemical analysis showed that tumors derived from E14-S3ER-shNanog cells included derivatives of the ectoderm (astrocytes and neural rosettes), mesoderm (bone, adipocytes, smooth muscle, and cartilage), and endoderm (hepatocytes and gut epithelium). The range of differentiation observed within the teratomas made from E14-S3ER-shNanog cells was comparable to that with E14-S3ER-shGFP control cells (supporting information Fig. 2). Together, these results show that elevation of STAT3-ER<sup>T2</sup> activity compensates for Nanog deficiency to induce and maintain the undifferentiated state and further demonstrates the preeminent role played by STAT3 in the maintenance of pluripotency.

### Identification of STAT3 Target Genes

Stimulation of E14-S3ER ES cells, either with 10,000 U/ml of LIF to activate endogenous STAT3 or with 100 nM 4'-OHT to activate ligand-dependent STAT3-ER<sup>T2</sup>, induced expression of the STAT3 target gene *JunB* with comparable efficiencies (Fig. 2A). The kinetics of *JunB* activation were, however, different between LIF- and 4'-OHT-stimulated cells. *JunB* expression peaked 1-2 hours after stimulation with LIF and 2-4 hours after stimulation with 4'-OHT. This difference is possibly explained by the lack of a negative feedback mechanism dependent upon inactivation of JAK kinases by SOCS3 after activation of STAT3-ER<sup>T2</sup> with 4'-OHT [12, 33]. Note that withdrawal of LIF and 4'-OHT for 24 hours (as was applied to E14-S3ER ES cells to increase the magnitude



**Figure 1.** A ligand-activated STAT3-ER<sup>T2</sup> sustains self-renewal in the absence of LIF and with a reduced Nanog level. (A): pPCAGIZ-STAT3-ER<sup>T2</sup> episomal expression vector used to express STAT3-ER<sup>T2</sup> in E14/T embryonic stem (ES) cells. The plasmid carries a polyoma origin (*ori*) with the F101 mutation, allowing episomal replication in ES cells. STAT3-ER<sup>T2</sup> is cloned directionally within a transcription unit linked to the zeocine resistance gene (*zeo*) through an IRES and driven by the CAAG promoter. (B): Morphology of E14-S3ER ES cells after propagation for 1 month in medium supplemented with 1,000 U/ml LIF (+LIF) or with 10 nM 4'-OHT (+4'-OHT). -LIF and -4'-OHT indicate withdrawal of LIF and 4'-OHT, respectively, to induce differentiation. (C): E14-S3ER ES cells were infected with an interfering lentiviral vector expressing a Nanog shRNA (*shNanog*) and a *hygro<sup>R</sup>* selectable gene. The concentration of 4'-OHT was shifted from low (10 nM) to high concentration (500 nM) at the time of infection. *Hygro<sup>R</sup>*-resistant colonies were picked and analyzed for the expression of *Nanog* and  $\beta$ -actin by reverse transcriptase-polymerase chain reaction (RT-PCR). A vector expressing a GFP shRNA was used as a control. One shGFP clone (E14-S3ER-shGFP) and two shNanog clones (E14-S3ER-shN) were selected for further analysis. (D): Morphology of the E14-S3ER-shGFP clone propagated in 10 nM 4'-OHT and of the two E14-S3ER-shN clones propagated in 500 nM 4'-OHT. (E): RT-PCR analysis of *Oct4*, *Rex-1*, and  $\beta$ -actin expression in E14-S3ER-shGFP (100 nM 4'-OHT) and E14-S3ER-shN clones (500 nM 4'-OHT). (F): Self-renewal assay of the E14-S3ER-shGFP clone and of the two E14-S3ER-shN clones. Photographs of representative colonies were taken on day 7 after plating. (G): Histogram shows the percentages of undifferentiated (blue), mixed (red), and differentiated (yellow) colonies after clonal growth in 0, 10, 100, and 500 nM 4'-OHT and in 1,000 U/ml LIF. Means and SEs were calculated from three replicates. (H): RT-PCR analysis of *Fgf5*, *nestin*, *T-Bra*, *Sox17*, *GATA-4*, *GATA-6*, and  $\beta$ -actin during differentiation of E14-S3ER-shGFP and E14-S3ER-shN clones induced by formation of embryoid bodies. Abbreviations: GFP, green fluorescent protein; IRES, internal ribosome entry site; LIF, leukemia inhibitory factor; OHT, 4'-hydroxytamoxifen; sh, short hairpin; STAT3, signal transducer and activator of transcription 3.



**Figure 2.** Gene expression profiles of E14-S3ER and E14-CER embryonic stem (ES) cells and identification of STAT3 target genes. **(A):** Semi-quantitative reverse transcriptase-polymerase chain reaction (PCR) analysis of *Nanog*, *Rex1*, and *JunB* expression in E14-S3ER ES cells after stimulation for 1-4 hours with 10,000 U/ml LIF to activate endogenous STAT3, or with 100 nM 4'-OHT to activate the ligand-dependent STAT3. +, E14-S3ER ES cells propagated for 1 month in medium supplemented either with 1,000 U/ml LIF or with 10 nM 4'-OHT before the experiment. -, deprivation of LIF or 4'-OHT for 24 hours before restimulation. Histogram shows the quantification of *JunB* expression normalized to *hprt*. **(B):** Schematic representation of the experimental strategy to identify STAT3 response genes. **(C):** Dendrogram of hierarchical clustering of probe sets differentially expressed between the five experimental conditions (analysis of variance [ANOVA],  $p < .05$ ). **(D):** K-means clustering of differentially expressed probe sets (ANOVA,  $p < .001$ ) in E14-S3ER ES cells after withdrawal of both LIF and 4'-OHT for 24 hours (E14-S3ER -LIF, -4'-OHT), followed by stimulation with LIF for 1 hour (E14-S3ER +LIF) or with 4'-OHT for 2 hours (E14-S3ER +4'-OHT). Clusters C and F contain the probe sets that were up- and downregulated upon stimulation with LIF only. Clusters B and E contain the probe sets that were up- and downregulated after stimulation with 4'-OHT only. Clusters A and D contain the probe sets that were up- and downregulated upon stimulation with both LIF and 4'-OHT. **(E):** Chart pie representing the functional annotation of the selected probe sets according to the DAVID 2007 Bioinformatics Gene Functional Classification tool. **(F):** Expression of 59 unique probe sets measured by real time-PCR at different days after differentiation induced by the formation of embryoid bodies. Abbreviations: LIF, leukemia inhibitory factor; OHT, 4'-hydroxy-tamoxifen; STAT3, signal transducer and activator of transcription-3.

of STAT3 target gene response after stimulation) did not alter expression of *Nanog* and *Rex1*. This result indicates that withdrawal of both ligands for 24 hours does not induce observable differentiation. In contrast, E14/T ES cells expressing the hormone-dependent Cre-ER<sup>T2</sup> recombinase [28], referred to as E14-CER, failed to activate *JunB* expression in response to 4'-OHT. E14-CER ES cells were strictly dependent on LIF for propagation in the undifferentiated state (not shown). E14-

CER ES cells were used as control cells in all subsequent experiments.

E14-S3ER ES cells were used to identify the genes regulated by STAT3 activity. The gene expression profiles of E14-S3ER and E14-CER cells before and after stimulation with LIF or 4'-OHT were compared using Mouse Genome 430 2.0 arrays. The following five conditions were examined (Fig. 2B): (I) E14-S3ER cells deprived of LIF and 4'-OHT

for 24 hours ( $n = 4$ ); (II) E14-S3ER cells treated as in (I) and restimulated with 10,000 U/ml of LIF for 1 hour to reactivate endogenous STAT3 ( $n = 5$ ); (III) E14-S3ER cells treated as in (I) and restimulated with 100 nM 4'-OHT for 2 hours to activate STAT3-ER<sup>T2</sup> ( $n = 6$ ); (IV) E14-CER cells deprived of LIF for 24 hours ( $n = 5$ ); and (V) E14-CER cells treated as in (IV) and restimulated with 100 nM tamoxifen for 2 hours ( $n = 5$ ). E14-CER cells were used to identify any 4'-OHT-response genes, irrespective of STAT3 activity. Hierarchical clustering analysis of the relative expression of probe sets (ANOVA,  $p < .05$ ) showed that E14-S3ER cells stimulated with LIF and 4'-OHT appeared more similar to each other than to E14-S3ER and E14-CER cells after deprivation of LIF and 4'-OHT (Fig. 2C). This observation indicates that stimulation of STAT3 activity is the primary cause of the observed differences in gene expression signatures in these five ES cell populations. K-means clustering of probe sets differentially expressed in the E14-S3ER cells before and after stimulation with LIF and 4'-OHT resulted in six independent clusters (Fig. 2D). We reasoned that STAT3 target genes playing important roles in self-renewal should be up-regulated by endogenous STAT3 as a result of LIF stimulation and by STAT3-ER<sup>T2</sup> as a result of 4'-OHT stimulation. Thus, all subsequent analyses were focused on cluster I, which contains the probe sets that were up-regulated by the two ligands. After elimination of redundant probe sets, elimination of probe sets activated by 4'-OHT in the E14-CER control cells, and use of filtering of 1.3-fold change in expression, 77 probe sets were selected. Sixty-five were confirmed by quantitative reverse transcriptase (RT)-PCR with a high degree of correlation ( $r^2 = 0.95$ ), confirming the validity of the microarray data (Table 1; supporting information Table 4; supporting information Fig. 3). Note that a 1.3-fold change in expression was used as a cutoff criterion to include *myc* in the list of STAT3 target genes [9]. Gene ontology analysis showed that the annotated probe sets correspond to genes encoding transcription factors, kinases, kinase inhibitors, structural molecules, and cell cycle regulators. Thirteen are Rikens (Fig. 2E; Table 1).

The expression pattern of the 65 probe sets was analyzed during differentiation induced by suspension culture (embryoid bodies) (Fig. 2F; Table 1). Fifty-nine of the 65 genes fell into four categories according to their expression pattern in undifferentiated versus differentiated cells: (a) 17 genes had their expression down-regulated as early as the first day of differentiation (blue); (b) eight genes had their expression down-regulated at the onset of differentiation and reactivated at later stages (red); (c) 13 genes had their expression up-regulated during differentiation (green); and (d) 21 genes had stable expression during differentiation (yellow). To determine whether the 65 probe sets were direct targets of STAT3, their expression was analyzed in E14-S3ER cells after stimulation with 100 nM 4'-OHT in the presence of cycloheximide. Seven STAT3 probe sets were not activated by 4'-OHT, indicating that their transcriptional regulation by STAT3-ER<sup>T2</sup> required de novo protein synthesis. In contrast, 58 probe sets were found to be resistant to cycloheximide treatment, strongly suggesting that they are direct transcriptional targets of STAT3-ER<sup>T2</sup> (Table 1).

### Knockdown of STAT3 Target Genes Is Detrimental to Self-Renewal

Twenty-four STAT3 target genes were selected for functional analysis, based on the presence of binding sites for STAT3 and/or Nanog in their 5' upstream region as determined by previous reports [23, 27] (Fig. 3A). They encode transcription

factors (*Klf4*, *Klf5*, *Smad7*, *Zfp36*, *Zfp36L1*, *Spry2*, *Sall4*, *Gbx2*, and *Stat3*), kinases (*Pim1*, *Pim3*, and *Sgk*), regulators of signaling (*Rgs16* and *Dact1*), a cyclin (*Cnmm1*), adhesion molecules (*ocln* and *Icam1*), a stress-response gene (*Ier3*), an extracellular matrix-associated protein (*Cyr61*), a deadenylase (*Ccrn4l*), a sulfatase (*Sulf1*), and a protein of the cytoskeleton (*vim*). Two are Rikens (*5230400M03Rik* and *1700051K13Rik*) (Table 1). To determine their role in the inhibition of ES cell differentiation, their expression was knocked down by means of interfering lentiviral vectors, and the engineered cells were analyzed in a colony-forming assay. For all genes studied, a 35%–87% reduction in transcript levels was observed in transient transfection experiments with the shRNA sequences selected (supporting information Table 1). Forty-eight hours after infection with interfering lentiviral vectors, CGR8 ES cells were dissociated, replated at clonal density, and further grown for 6 days in LIF-supportive conditions, in the presence of G418 to kill the noninfected cells. The resulting colonies were stained for AP activity and the percentages of undifferentiated, mixed, and differentiated colonies were calculated (Fig. 3B, upper panel). Expression of control shRNA-1 (recognizing GFP) resulted in 77.5% of G418-resistant cells forming undifferentiated AP<sup>+</sup> stem cell colonies, whereas knockdown of Nanog and STAT3 (positive controls) resulted in only 13.7 and 0.3%, respectively. Knockdown of 22 of the 23 target genes analyzed resulted in a significant increase in the percentage of mixed/differentiated colonies at the expense of undifferentiated colonies, relative to the GFP control. The increase in the frequency of mixed/differentiated colonies ranged from 30% (*Sall4*) to 68% (*Sgk*). Comparable results were obtained for each gene with an independent shRNA sequence (shRNA-2) (Fig. 3B, bottom panel). Interestingly, there was a significant correlation of the percentages of undifferentiated colonies after knockdown with shRNA-1 and shRNA-2 ( $r^2 = 0.57$ ,  $p < .01$ ). This correlation strongly suggests that the differences observed between the 22 genes in the frequency of undifferentiated colonies resulted primarily from the relative contribution of each gene to the suppression of differentiation, rather than from gene-to-gene variations in the level of interference. The effect of *Dact1*, *Sall4*, *E030031B1Rik*, and *1700051K13Rik* knockdown on the frequency of undifferentiated, mixed, and differentiated colonies was confirmed using scrambled shRNA sequences (supporting information Fig. 4).

Undifferentiated, mixed, and differentiated colonies obtained in colony-forming assays were pooled and subsequently analyzed by real-time PCR to quantify the expression of pluripotency and differentiation markers. Expression of the pluripotency marker *Rex1* (*Zfp42*) was reduced relative to control after knockdown of all genes examined (1.1- to 2.2-fold) (Fig. 3C, upper panel). Expression of the early ectodermal marker *Fgf5* was induced after knockdown of all genes examined (1.5- to 3.5-fold) (Fig. 3C, bottom panel).

Together, these results show that the knockdown of 23 of the 24 genes analyzed (including *Stat3*) induced differentiation in LIF-supportive conditions. We conclude that all 23 genes contribute to the maintenance of the undifferentiated state.

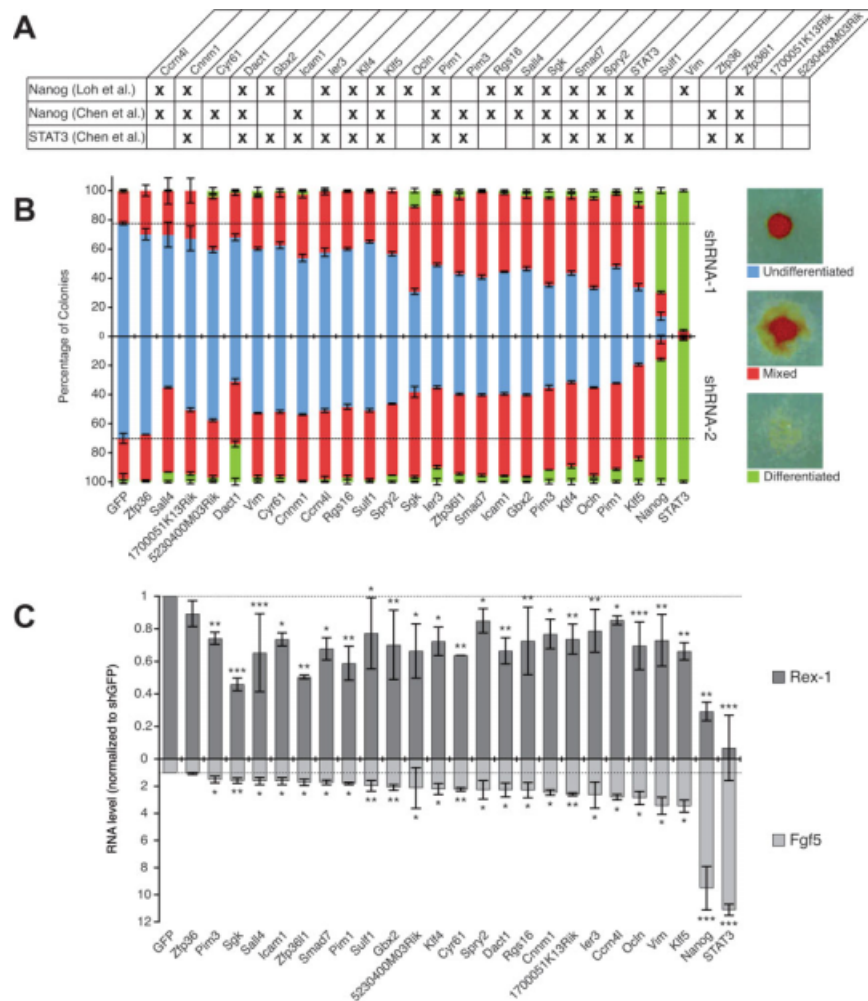
### Knockdown of STAT3 Target Genes Leads to Three Distinct Patterns of Differentiation

The expression of endodermal and mesodermal markers was analyzed after knockdown of each STAT3 target gene. Knockdown of most genes except *Icam1*, *Pim1*, *Gbx2*, *Zfp36*, and *Klf5* induced expression of the endodermal markers *Sox17* and *FoxA2* (Fig. 4A, upper panel). In contrast,

**Table 1.** Genes regulated by leukemia inhibitory factor and ligand-dependent STAT3-ER<sup>T2</sup> in E14-S3ER embryonic stem cells

Probeset	Gene Symbol	UniGene ID	Description	LIF	4'OHT	4'OHT + Cyclo	Expression during differentiation													
							RT-PCR							Quantification						
							ES 1	2	3	4	5	6	7	10	ES 1	2	3	4	5	6
1455899	Socs3	Mm.3468	Suppressor of cytokine signaling 3	117.2	59.6	58.4														
1415899	Junb	Mm.1167	Jun-B oncogene	22.05	4.31	7.6														
1452519	Zfp36	Mm.333723	Zinc finger protein 36	6.1	4.1	6														
1459961	H3077C12-3 NIA	---	---	3.61	3.55	12.6														
1440682	7030408E20Rik	---	---	3.36	4.07	1.98														
1417394	Klf4	Mm.4325	Kruppel-like factor 4 (gut)	2.65	1.73	2.1														
1444598	1700051K13Rik	Mm.360277	---	2.21	2.83	1.8														
1420337	Gbx2	Mm.204730	Gastrulation brain homeobox 2	1.87	2.72	1.7														
1446525	---	---	---	1.47	3.03	1.4														
1416613	Cyp1b1	Mm.214016	Cytochrome P450, family 1, subfamily b, polypeptide 1	5.52	2.66	15.9														
1423100	Fos	Mm.246513	FBJ osteosarcoma oncogene	17.84	3.23	14.2														
1424067	Icam1	Mm.90364	Intercellular adhesion molecule	3.13	2.33	2.9														
1441718	4432411E13Rik	Mm.275426	---	2.85	2.21	1.5														
1441843	5290400M03Rik	Mm.274277	---	2.15	2.18	1.3														
1439311	B830012L14Rik	Mm.136993	---	1.48	1.67	1.5														
1428055	Rlan	Mm.293263	RNA imprinted and accumulated in nucleus	1.2	1.67	1														
1451021	Klf5	Mm.30262	Kruppel-like factor 5	2.82	2.52	1.2														
1422528	Zfp361	Mm.235132	Zinc finger protein 36, C3H type-like 1	2.39	1.92	2.5														
1422914	Sp5	Mm.300809	Trans-acting transcription factor 5	6.35	10.76	24.8														
1448873	Ocln	Mm.4807	occludin	1.33	1.35	1.5														
1460700	Stat3	Mm.249934	Signal transducer and activator of transcription 3	2.25	2.52	2.2														
1431094	1110006E14Rik	Mm.151018	---	3.33	1.69	1.4														
1441231	BCO19575	Mm.216313	---	1.48	2.2	1.9														
1456160	---	---	---	2.7	2.52	1.4														
1437880	Lbxcor1	Mm.36349	Ladybird homeobox 1 homolog (Drosophila) corepressor 1	2.11	1.55	1.6														
1434025	---	---	---	2.41	1.69	1.4														
1426037	Rgs 16	Mm.181709	Regulator of G-protein signaling 16	1.67	3.86	4.4														
1443161	E330040F24Rik	---	---	1.77	8.71	2.2														
1437100	Pim3	Mm.273436	Proviral integration site 3	2.45	1.41	1.7														
1428765	Gtl2	Mm.289645	Imprinted maternally expressed untranslated mRNA	1.59	2.19	1.6														
1454288	5830468K08Rik	---	---	1.23	1.67	1.2														
1429600	C630010D07Rik	Mm.153030	---	1.31	2.04	1.6														
1417937	Dact1	Mm.46662	Dapper homolog 1, antagonist of beta-catenin (xenopus)	1.32	2.78	3.6														
1416039	Cyr61	Mm.1231	Cysteine rich protein 61	2.84	4	4.1														
1458075	B930079L03Rik	---	---	1.72	2.12	1.9														
1419647	Ier3	Mm.25613	Immediate early response 3	2.32	1.71	1.8														
1436584	Spry2	Mm.89982	Sprouty homolog 2 (Drosophila)	2.53	2.22	1.5														
1425922	Nmyc1	Mm.16469	Neuroblastoma myc-related oncogene 1	2.98	1.42	1.4														
1449009	Tgtp	Mm.15793	T-cell specific GTPase	1.92	1.93	2.6														
1418756	Trh	Mm.1363	Thyrotropin releasing hormone	1.27	2.04	1.4														
1418025	Bhlhb2	Mm.2436	Basic helix-loop-helix domain containing, class B2	1.71	1.69	1.6														
1456677	---	---	---	1.47	1.63	1.1														
1418041	Sgk	Mm.28405	Serum/glucocorticoid regulated kinase	1.78	1.85	1.5														
1446550	Gspt1	Mm.325827	G1 to S phase transition 1	1.29	1.33	1.3														
1429438	Bcor	Mm.196328	Bcl6 interacting corepressor	1.2	1.69	2.6														
1425837	Ccrn4l	Mm.86541	CCR4 carbon catabolite repression 4-like (S. cerevisiae)	5.16	3.57	2.1														
1440752	Sall 4	---	Sal-like 4 (Drosophila)	1.5	2.13	1.8														
1435284	Rtn4	Mm.192580	Reticulon 4	1.64	2.33	1.3														
1449109	Socs2	Mm.4132	Suppressor of cytokine signaling 2	4.58	2.94	1.6														
1420354	Cnrm1	Mm.39388	Cyclin M1	1.34	2.56	3.5														
1446426	---	---	---	1.34	1.75	1														
1424942	Myc	Mm.2444	Myelocytomatosis oncogene	1.33	1.59	2														
1425503	Gcnt2	Mm.314757	Glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	1.41	2.13	1.6														
1456993	---	---	---	1.27	1.89	1.6														
1457402	Sulf1	---	---	1.53	1.24	1.9														
1442484	---	Mm.260545	---	1.8	2.04	0.9														
1423389	Smad7	Mm.34407	MAD homolog 7 (Drosophila)	2.1	1.65	4.3														
1438814	---	---	---	1.33	1.32	1														
1436766	A130042L18Rik	---	---	1.56	1.53	1.8														
1447286	---	---	---	1.86	1.86	1.1														
1423006	Pim1	Mm.328931	Proviral integration site 1	1.35	1.35	1.7														
1457944	---	---	---	1.93	1.39	1														
1430448	6720418B01Rik	---	---	1.39	1.39	2.3														
1456292	Vim	Mm.268000	Vimentin	1.23	2.17	1.3														
1443721	Stn	Mm.262102	Strawberry notch homolog (Drosophila)	1.44	1.32	1.9														

Shading corresponds to genes whose expression is up-regulated 4'OHT in the presence of 4'OHT, therefore are putative primary targets of the LIF/STAT3 pathway. 4'OHT + Cyclo, stimulation of cells with 4'OHT in the presence of cycloheximide. Abbreviations: Cyclo, cycloheximide; ES, embryonic stem; LIF, leukemia inhibitory factor; 4'-OHT, 4'-hydroxytamoxifen; PCR, polymerase chain reaction; RT, reverse transcriptase.



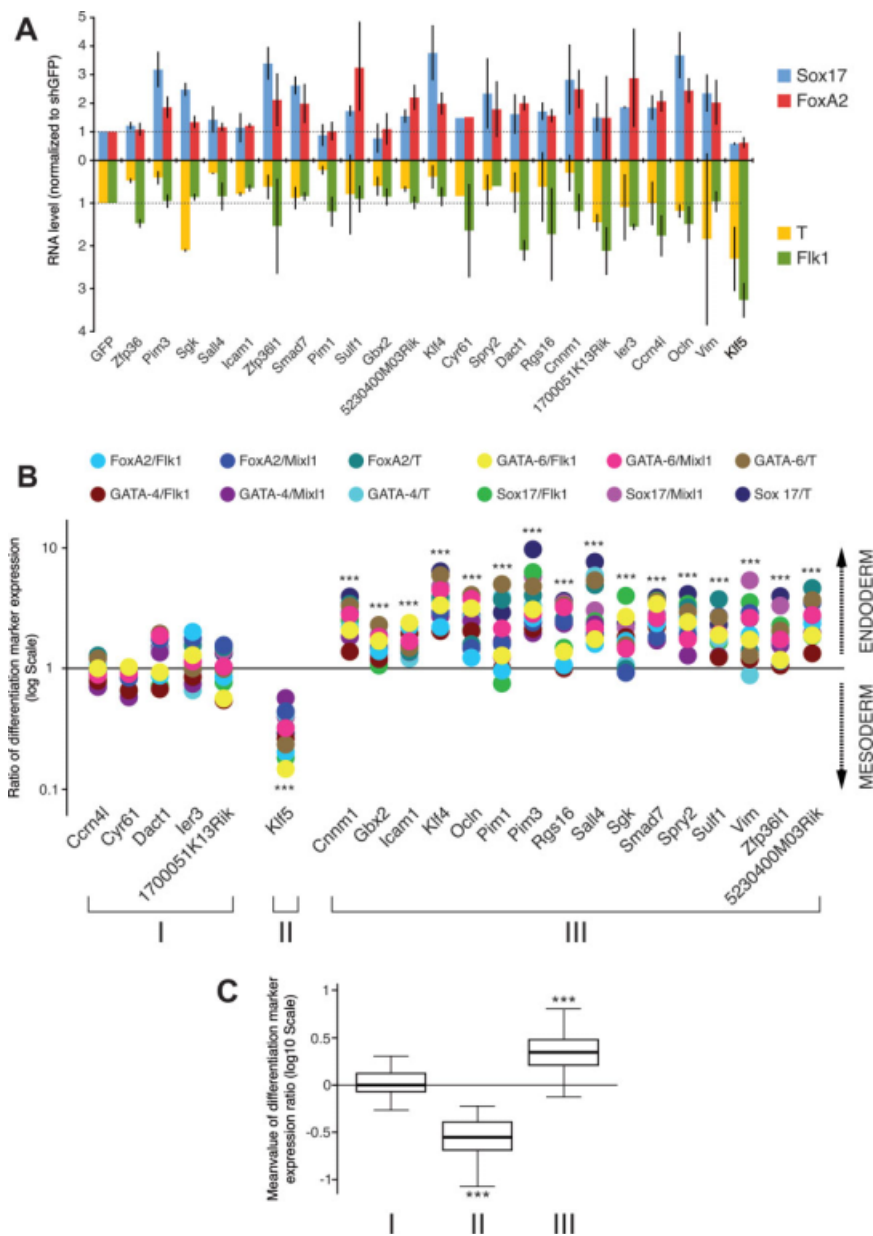
**Figure 3.** Knockdown of signal transducer and activator of transcription-3 (STAT3) target genes with lentiviral vectors expressing shRNA. **(A):** Selection of STAT3 target genes for functional studies according to Chen et al. [27] and Loh et al. [23]. **(B):** Histogram showing the percentages of undifferentiated (blue), mixed (red), and differentiated (green) colonies observed in clonal assays after knockdown of STAT3/Nanog response genes by means of lentiviral infection with the pLenti6/BLOCK-iT-PGKneo<sup>R</sup> interfering vector expressing shRNA-1 (upper panel) and shRNA-2 (lower panel). CGR8 embryonic stem cells were infected with viral supernatants. Forty-eight hours after infection, cells were dissociated and replated at clonal density (100 cells/cm<sup>2</sup>). They were further grown for 6 days in medium supplemented with LIF in the presence of G418 to kill noninfected cells. The resulting colonies were stained for alkaline phosphatase activity. Means and SEs were calculated from analysis of variance (ANOVA) for three replicates. Genes are shown according to the *p* value in the ANOVA (from highest [left] to lowest [right]). **(C):** Real-time polymerase chain reaction analysis of *Rex1* (upper panel) and *Fgf5* (lower panel) expression in the colonies harvested in **(A)**. Means and SEs were calculated from three replicates using Student's *t* test (\*, *p* < .05; \*\*, *p* < .01; \*\*\*, *p* < .001). Abbreviations: GFP, green fluorescent protein; sh, short hairpin

knockdown of *Klf5* and *1700051K13Rik* induced expression of the mesodermal markers *T* (Brachyury) and *Flk1* (Fig. 4A, bottom panel). These results suggest that STAT3 target genes are not equally involved in the inhibition of mesoderm and endoderm differentiation. To address this issue further, expression of the mesoderm (*Flk1*, *Mixl1*, and *T*)- and endoderm (*FoxA2*, *GATA-4*, *GATA-6*, and *Sox17*)-associated genes was analyzed by quantitative RT-PCR in pools of *neo*<sup>R</sup> colonies resulting from the infection of ES cells with the interfering vectors (supporting information Table 5). To reveal a bias toward mesoderm or endoderm differentiation, endoderm/mesoderm ratios (R) were calculated for all 12 combinations of endoderm and mesoderm markers. In Figure 4B, each of the 22 genes is represented by a set of 12 R scores, which together reflect the bias toward endoderm or mesoderm differentiation induced by gene knockdown. Of the 22 genes analyzed, 16 showed a bias toward endoderm differentiation

(*Cnnm1*, *Gbx2*, *Icam1*, *Klf4*, *Ocln*, *Pim1*, *Pim3*, *Rgs16*, *Sall4*, *Sgk*, *Smad7*, *Spry2*, *Sulf1*, *Vim*, *Zfp36L1*, and *5230400M03Rik*), 1 showed a bias toward mesoderm differentiation (*Klf5*), and 5 did not show any significant bias toward either lineages (*Ccrn4l*, *Cyr61*, *Dact1*, *Ier3*, and *1700051K13Rik*). These results strongly suggest that some STAT3 target genes are involved in suppression of both endoderm and mesoderm differentiation, whereas others show lineage specificity (Fig. 4C). Of note, expression of the neuroectodermal markers *Nestin* and *Musashi* was not induced in this assay (data not shown).

### Nanog Regulates a Subset of STAT3 Target Genes

Of the 24 STAT3 target genes analyzed in our study, 21 were found to contain Nanog binding sites in their 5' upstream sequences [23, 27] (Fig. 3A), suggesting that Nanog, in addition to LIF, regulates their transcriptional activity. To explore

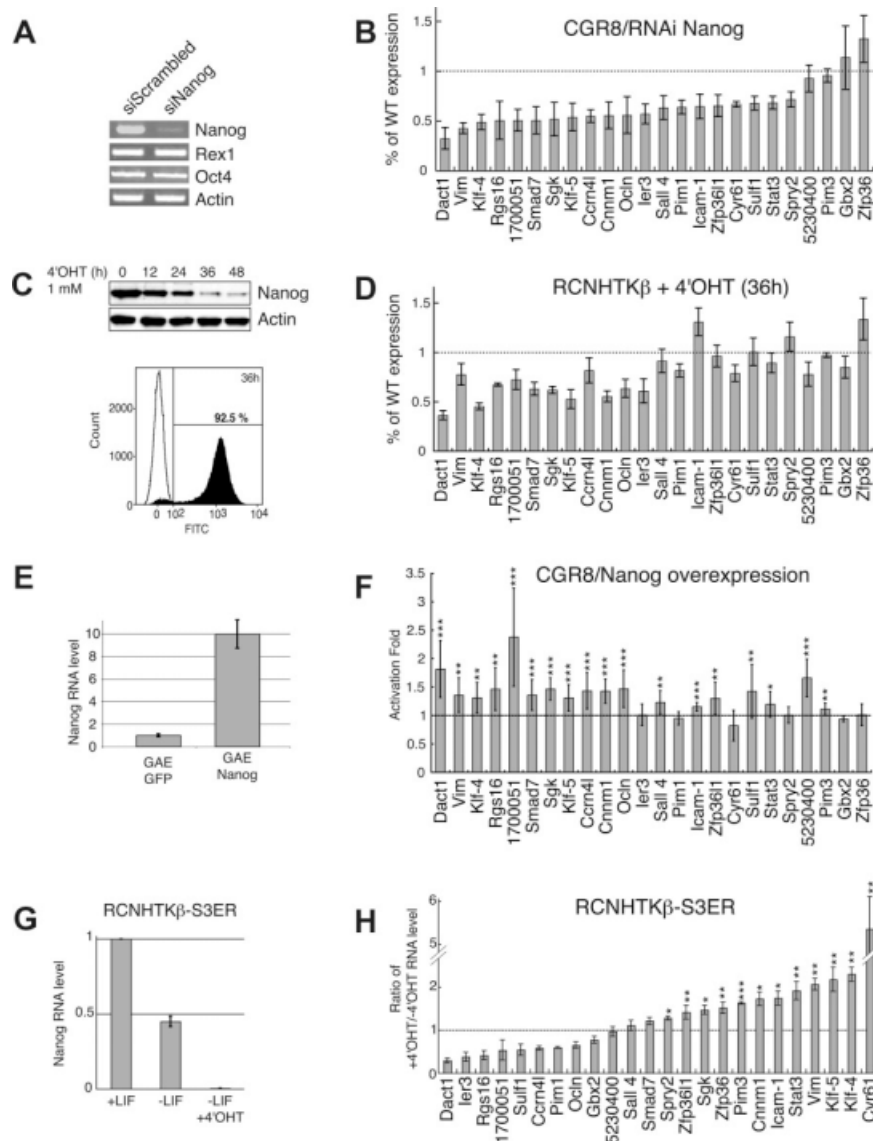


**Figure 4.** Expression of mesoderm and endoderm markers after knockdown of STAT3 target genes. **(A):** Real-time polymerase chain reaction (PCR) analysis of *Sox17* and *FoxA2* (upper panel) and *T* and *Flk1* (lower panel) expression in the colony-forming assay. All normalizations were performed with  $\beta$ -actin. **(B):** Color dots represent ratios between endoderm (*FoxA2*, *GATA-4*, *GATA-6*, and *Sox17*) and mesoderm (*Flk1*, *Mix1*, and *T*) markers measured by real-time PCR. *R* scores were calculated for each of the 22 STAT3 target genes after knockdown. *R* scores are represented on a log scale.  $R > 1$  indicates a bias toward endoderm differentiation, whereas  $R < 1$  indicates a bias toward mesoderm differentiation. The 22 genes fall into three distinct categories. The difference of the log *R* scores from 1 in the three groups was tested with a one-way analysis of variance (cell means model) (\*\*\*,  $p < .001$ ). **(C):** The box-and-whisker plots show the median values as solid lines with the interquartile range indicated by the box limits. The whiskers indicate approximately 1.5 times the distance away from the box. Group I shows no significant difference from 0 whereas the other two are highly significant ( $p < 10^{-4}$ ). Abbreviation: GFP, green fluorescent protein.

this issue further, expression of the 24 STAT3 target genes was analyzed in CGR8 cells after knockdown of *Nanog* expression by siRNA transfection (Fig. 5A). Nineteen of them displayed a significant reduction in their expression level after *Nanog* knockdown (Fig. 5B). Only one of them (*Sulf1*) has not been shown to contain Nanog binding sites in its regulatory sequences [23, 27]. Note that expression of *Oct4* and *Rex1* pluripotency markers was not changed, indicating that *Nanog* knockdown did not induce observable differentiation within the time frame of the experiment (Fig. 5A). Of these 19 genes, 14 also exhibited a significant reduction of their expression level after 4'-OHT-induced *Nanog* inactivation in RCNHTK $\beta$  cells [21] (Fig. 5C, 5D). Of the 24 STAT3 target genes analyzed, 17 exhibited increased expression after transfection of a Nanog expression vector (Fig. 5E, 5F). Together, these results indicate that the vast majority of the STAT3 target genes identified in our study (*Dact1*, *Klf4*, *Klf5*, *Rgs16*, *Smad7*, *Ccm4l*, *Cnmm1*, *Ocln*, *Ier3*, *Pim1*, *Cyr61*, *Sgk*, *Stat3*, *1700051K13Rik*, and

*5230400M03Rik*) are also transcriptionally regulated by Nanog, in agreement with ChIP-seq data (Fig. 3A) [27].

We showed that increasing STAT3-ER<sup>T2</sup> activity prevented differentiation in E14-S3ER cells induced by knockdown of *Nanog* expression (Fig. 1C–1H). We therefore asked whether increasing STAT3 activity could compensate for the decreased expression of STAT3 target genes after *Nanog* knockdown. To address this question, RCNHTK $\beta$  cells were engineered to overexpress STAT3-ER<sup>T2</sup>. Treatment of RCNHTK $\beta$ -S3ER cells with 4'-OHT resulted in inactivation of *Nanog* (Fig. 5G) and concomitant activation of STAT3-ER<sup>T2</sup>. Of the 24 genes analyzed, 9 had their expression level reduced after 4'-OHT treatment, indicating that STAT3-ER<sup>T2</sup> activity was unable to compensate for Nanog inactivation (Fig. 5H). In contrast, 15 genes had their expression level either maintained or increased after treatment with 4'-OHT, indicating that STAT3-ER<sup>T2</sup> activity was able to restore their expression after *Nanog* inactivation.



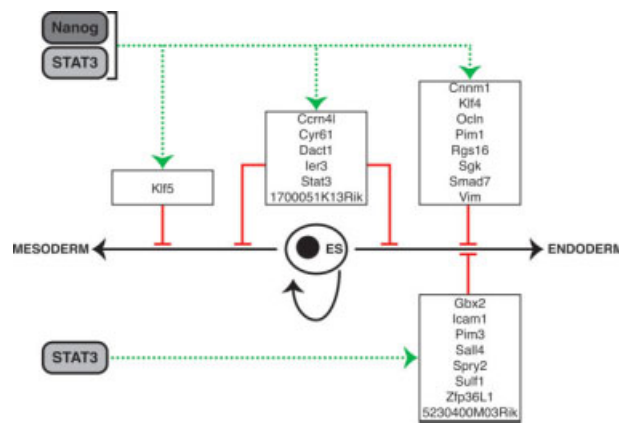
**Figure 5.** Regulation of STAT3 target genes by Nanog. **(A):** Reverse transcriptase-polymerase chain reaction (PCR) analysis of *Nanog*, *Oct4*, and *Rex1* expression in CGR8 embryonic stem (ES) cells 48 hours after transfection of siRNA Nanog and siRNA scrambled. **(B):** Real-time PCR analysis of the expression of STAT3 target genes in CGR8 ES cells after transfection of siRNA *Nanog*, normalized to control siRNA. **(C):** Top panel: Western blot analysis of Nanog expression in RCNHTK $\beta$  cells after treatment with 1 mM 4'-OHT for the time indicated. Bottom panel: flow cytometry analysis of GFP expression in RCNHTK $\beta$  cells before (white histogram) and after (black histogram) inactivation of *Nanog* by 4'-OHT. **(D):** Real-time PCR analysis of the expression of STAT3 target genes in RCNHTK $\beta$  cells after treatment with 4'-OHT for 36 hours (values are normalized to expression levels measured in untreated cells). **(E):** Real-time PCR analysis of *Nanog* expression after transient transfection of *Nanog* expression vector (pGAE-CAG-Nanog/WPRE). **(F):** Real-time PCR analysis of the expression of STAT3 target genes in CGR8 ES cells after *Nanog* overexpression (values are normalized to expression levels measured in control cells transfected with the pGAE-CAG-eGFP/WPRE vector). **(G):** Real-time PCR analysis of *Nanog* expression in RCNHTK $\beta$ -S3ER cells after treatment with 1 mM 4'-OHT for 36 hours. **(H):** Real-time PCR analysis of the expression of STAT3 target genes in RCNHTK $\beta$ -S3ER cells after treatment with 1 mM 4'-OHT (values are normalized to expression levels measured in control cells before treatment with 4'-OHT). **(B, D, F, H):** All histograms represent means and SDs calculated from three replicates (Student's *t* test: \*,  $p < .05$ ; \*\*,  $p < .01$ ; \*\*\*,  $p < .001$ ). Abbreviations: FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; LIF, leukemia inhibitory factor; OHT, 4'-hydroxytamoxifen; si, small interfering; WT, wild type.

We propose a model in which STAT3 and Nanog coregulate the expression of common target genes involved in the inhibition of mesoderm and endoderm differentiation (Fig. 6).

## DISCUSSION

STAT3 is an important regulator of mouse ES cell self-renewal [7, 8], which inhibits differentiation into both meso-

derm and endoderm lineages [2]. Its mechanism of action remains to be elucidated. To this aim, we identified 58 genes (35 known genes, 13 Rikens, and 10 unidentified probe sets) whose expression is activated by a conditional ligand-dependent STAT3 mutant (in addition to activation by LIF) in the presence of a protein synthesis inhibitor. Of the 35 novel STAT3 target genes identified, 23 display STAT3 binding sites in their regulatory sequences [27], confirming the validity of our strategy to identify primary targets of the LIF/STAT3 pathway. Of note, 258 other genes are regulated by



**Figure 6.** Schematic diagram illustrating the role of STAT3 and Nanog in the activation of target genes and the suppression of mesoderm and endoderm differentiation. Abbreviation: STAT3, signal transducer and activator of transcription 3.

LIF but not by ligand-dependent STAT3, that is, by a STAT3-independent mechanism. PI3K [4], cYes [5], and SHP-2 [6] are candidate signaling molecules to functionally link the activated LIF receptor to these 258 genes.

We identified 22 STAT3 target genes whose knockdown of expression induces spontaneous differentiation measured in a colony-forming assay. Whereas the effect of knockdown of individual genes on ES cell differentiation is sometimes rather modest, which may indicate a marginal contribution to the inhibition of differentiation, the sum of the individual effects results in the maintenance of the undifferentiated state. The 22 identified genes encode transcription factors, various types of proteins involved in protein and nucleic acid modification (serine/threonine kinases, sulfatase, and deadenylase), adhesion molecules, extracellular matrix- and cytoskeleton-associated proteins, and a stress response factor. Such a variety of molecules indicates that LIF/STAT3-dependent self-renewal results from the concomitant regulation of a wide range of cellular activities, including the control of cell-cell and cell-matrix interactions. Eight genes appear to have a greater contribution to the inhibition of differentiation, evidenced by a stronger increase in the number of mixed/differentiated colonies and by a higher induction of differentiation marker expression after knockdown. They include *Pim1* and *Pim3* whose regulation by STAT3 and role in self-renewal have been reported elsewhere [10], *Klf4* and *Klf5*, which are known to play a pivotal role in the maintenance of the undifferentiated state [33–36], *Sgk*, *Smad7*, *Ocln*, and *Icam1*. Of note, these eight genes are highly expressed in undifferentiated ES cells and decline between day 1 and 4 of differentiation, suggesting that all eight contribute to ES cell identity. *Sgk* encodes the protein kinase B (PKB)-related protein serum and glucocorticoid-inducible kinase (SGK). SGK is a downstream effector of the PI3K/3-phosphoinositide-dependent protein kinase signaling pathway [37, 38], which contributes to the maintenance of the undifferentiated state [4]. The LIF/STAT3 and LIF/PI3K pathways may act synergistically to activate SGK and promote self-renewal. SGK, like PKB, inactivates glycogen synthase kinase (GSK)-3 in vitro [38]. Inhibition of GSK-3 activity is known to facilitate self-renewal of mouse ES cells [39], providing a possible explanatory mechanism for SGK action.

*Klf4* and *Klf5*, together with *Klf2*, were shown to form a *Klf*-based molecular circuitry that regulates ES cell self-

renewal by activating *Nanog* expression [35]. Our observation that both *Klf4* and *Klf5* are regulated by STAT3 activity functionally links the LIF/STAT3 pathway to the regulation of *Nanog* expression. In addition, we observed that *Sall4* is also regulated by STAT3. *Sall4* transcription factor forms complexes with *Nanog* and enhances its transcriptional activity [40]. We thus propose that LIF/STAT3 signaling contributes to the regulation of *Nanog* transcriptional activity and indirectly feeds the *Nanog/Oct4/Sox2* core regulatory network [20] through activation of *Klf4*, *Klf5*, and *Sall4*.

Our results demonstrate that STAT3 and *Nanog* coregulate the expression of 14 genes involved in pluripotency control, including *Klf4* and *Klf5*. Of these 14 genes, 12 contain both STAT3 and *Nanog* binding sites identified by ChIP-seq [27], indicating that they are all direct targets of both transcription factors. The two other target genes are unidentified Rikens. The identification of pluripotency genes coregulated by STAT3 and *Nanog* helps explain how *Nanog* overcomes differentiation induced by LIF starvation when overexpressed [16, 17]. Several of these target genes (*Klf4*, *Klf5*, *Sgk*, *Vim*, *Smad7*, *Pim3*, *Cnnm1*, and *Cyr61*) are highly expressed after both *Nanog* inactivation and STAT3-ER<sup>T2</sup> activation induced by 4'-OHT. This finding may explain why elevation of STAT3-ER<sup>T2</sup> activity is able to sustain self-renewal when expression of *Nanog* is partially inactivated (this work). We thus hypothesize that *Nanog* and STAT3 have the capacity to activate the expression of common target genes and, thereby, to regulate pluripotency via partially redundant mechanisms. Of note, 13 genes were found to be downregulated by LIF and 4'-OHT in the microarray experiment (Fig. 2D). Of the seven known genes, four displayed binding sites for *Nanog* (supporting information Table 6) [23, 27].

The LIF/STAT3 signaling pathway is known to suppress differentiation to both endomesoderm and extraembryonic endoderm lineages [2, 7]. Our lineage marker analysis demonstrates that STAT3 target genes are not equally involved in the inhibition of mesoderm and endoderm differentiation. Most of them show lineage specificity, suggesting that they inhibit differentiation by preventing the activation of lineage-specific differentiation programs.

## ACKNOWLEDGMENTS

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## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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