Satb1 and Satb2 regulate embryonic stem cell differentiation and *Nanog* expression

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Satb1 and the closely related Satb2 proteins regulate gene expression and higher-order chromatin structure of multigene clusters in vivo. In examining the role of Satb proteins in murine embryonic stem (ES) cells, we find that *Satb1*$^{-/-}$ cells display an impaired differentiation potential and augmented expression of the pluripotency determinants *Nanog*, *Klf4*, and *Tbx3*. Metastable states of self-renewal and differentiation competence have been attributed to heterogeneity of ES cells in the expression of Nanog. *Satb1*$^{-/-}$ cultures have a higher proportion of Nanog^{high} cells, and an increased potential to reprogram human B lymphocytes in cell fusion experiments. Moreover, Satb1-deficient ES cells show an increased expression of Satb2, and we find that forced Satb2 expression in wild-type ES cells antagonizes differentiation-associated silencing of *Nanog* and enhances the induction of *NANOG* in cell fusions with human B lymphocytes. An antagonistic function of Satb1 and Satb2 is also supported by the almost normal differentiation potential of *Satb1*$^{-/-}$*Satb2*$^{-/-}$ ES cells. Taken together with the finding that both Satb1 and Satb2 bind the *Nanog* locus in vivo, our data suggest that the balance of Satb1 and Satb2 contributes to the plasticity of *Nanog* expression and ES cell pluripotency.

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Embryonic stem (ES) cells are pluripotent and self-renewing cell lines generated by cellular outgrowth of preimplantation embryos (for review, see Smith 2001; Niwa 2007; Murry and Keller 2008). Recently, the molecular basis of pluripotency has received great attention, due to the possibility of inducing a pluripotent state in human and murine somatic cells by gene transfer, providing new approaches to stem cell therapy (Takahashi and Yamanaka 2006; Yu et al. 2007; Daley and Scadden 2008; Jaenisch and Young 2008). A set of four transcription factors—Klf4, Oct4, Sox2, and c-myc—has been shown to reprogram somatic cells to pluripotency and generate cells, termed induced pluripotent stem cells (iPS), that resemble ES cells (Takahashi and Yamanaka 2006; Yu et al. 2007). A key regulator of ES cell pluripotency, Nanog, is particularly interesting, as ES cells are heterogeneous in the expression of Nanog and high versus low levels of Nanog expression correlate with the probability of self-renewal versus differentiation (Chambers et al. 2003, 2007; Mitsui et al. 2003; Graf and Stadtfeld 2008). Moreover, forced expression of Nanog is sufficient to prevent differentiation of ES cells even in the absence of Klf4 (Chambers et al. 2003; Mitsui et al. 2003; Niwa et al. 2009). Recently, the transcription factors Klf4 and Tbx3 were implicated in the regulation of *Nanog* gene expression, which may account for its role in keeping ES cells in a pluripotent state (Li et al. 2005; Jiang et al. 2008; Niwa et al. 2009). Various signaling pathways have been implicated in the maintenance of the pluripotent state and the exit of cells into differentiation (Chambers 2004). In particular, signaling by leukemia inhibitory factor (LIF) blocks differentiation of murine ES cells by two parallel pathways in which phosphorylation of Stat3 activates predominantly *Sox2* via Klf4 and Akt phosphorylation activates preferentially *Nanog* via Tbx3 (Niwa et al. 1998, 2009).

In addition to this core machinery of transcription factors, epigenetic mechanisms, particularly those mediated by polycomb proteins and Jmjd demethylases, are crucial for the self-renewal and differentiation of ES cells (Boyer et al. 2006; Loh et al. 2007; Spivakov and Fisher 2007). Like the early embryo, ES cells have not yet undergone X-chromosome inactivation (XCI), genomic imprinting,

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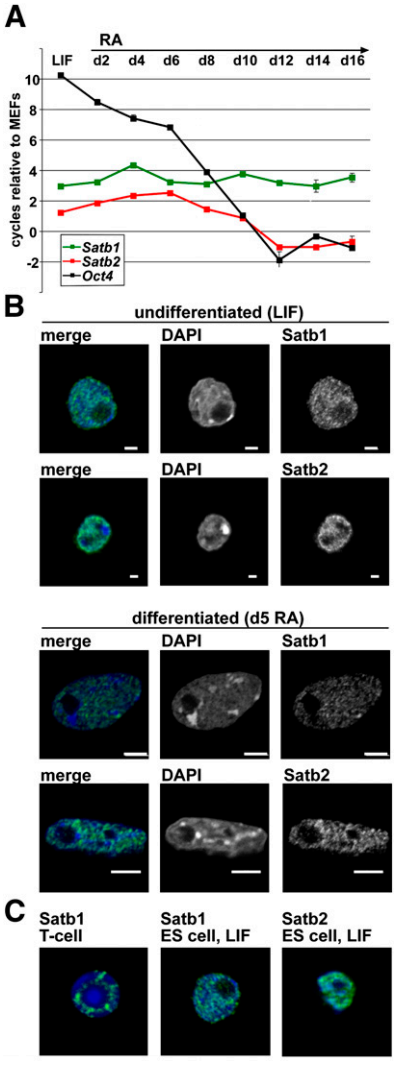
or *Hox* gene activation (Li 2002). These events can be triggered by differentiation of ES cells, which has made this cell type a model system for studying the molecular basis of these epigenetic events (Spivakov and Fisher 2007). In differentiating ES cells, the expression of *Hox* genes is induced in a colinear and temporally ordered manner, similar to the developmental regulation in the early embryo. *Hox* genes located near the 3′ end of the clusters are induced prior to the expression of genes near the 5′ end of the clusters (Kmita and Duboule 2003; Chambeyron and Bickmore 2004). In addition to *cis*-acting elements, the chromatin structure and the subnuclear organization of the *Hox* gene clusters, which involve a looping out of the chromosomal territories, contribute to the regulated expression of *Hox* genes in ES cells (Chambeyron and Bickmore 2004).

The special AT-rich sequence-binding protein Satb1 is one of the few proteins known to date that are involved in organizing higher-order chromatin structure, including the subnuclear organization of individual genes within multigene clusters (Yasui et al. 2002; Cai et al. 2003, 2006). One of the most prominent features of Satb1 is its unique nuclear distribution pattern in thymocytes in which Satb1 forms a so-called "cage-like" structure to which specific DNA sequences are tethered (Cai et al. 2003). Satb2 is closely related to Satb1 and has been shown to bind and activate the immunoglobulin heavy chain (IgH) enhancer in the *IgH* gene cluster (Dobreva et al. 2003). Recently, a loss-of-function study in the mouse has demonstrated that Satb2 is essential for proper facial patterning of the embryo and for normal bone development (Dobreva et al. 2006). These defects have been attributed to an increased expression of specific members of the *Hox* gene clusters and a decreased expression of osteoblast-specific genes, whereby Satb2 was shown to regulate these genes at the chromatin level (Dobreva et al. 2006). Therefore, the question arises as to whether Satb proteins play a role in the regulation of gene expression in ES cells.



Results

Expression of Satb1 and Satb2 in ES cells

To analyze the expression of *Satb1* and *Satb2* during the self-renewal and differentiation of ES cells, we performed a quantitative RT–PCR analysis (Fig. 1A). To ensure homogeneous differentiation and allow for the selection of undifferentiated or differentiated cells, we inserted, via homologous recombination, a hygromcycin resistance/HSV-thymdine kinase (HygroTK) fusion construct into the endogenous *Oct4* locus of wild-type ES cells (Chambeyron and Bickmore 2004). Normalizing the expression of *Satb1* and *Satb2* in ES cells relative to their expression in mouse embryonic fibroblasts (MEFs) in which these genes are transcribed at equally low levels (data not shown), we found that undifferentiated ES cells expressed *Satb1* at a higher level than *Satb2* (Fig. 1A). During retinoic acid (RA)-induced differentiation, which resulted in the efficient down-regulation of the pluripotency marker *Oct4*, we

Figure 1. Satb1 and Satb2 expression during ES cell differentiation. (*A*) Quantitative RT–PCR for *Satb1*, *Satb2*, and *Oct4* expression in *Oct4*-HygroTK wild-type ES cells grown for 3 d in the presence of hygromyin prior to differentiation. At day 6 of differentiation, gancyclovir was added in order to eliminate cells with active *Oct4* expression. cDNA was prepared from total RNA at the indicated time points, and the indicated transcript levels were normalized to *TBP* levels. Shown are differences in cycle numbers relative to expression levels in MEFs in which *Satb1* and *Satb2* are transcribed at equal levels. Primers for *Satb1* and *Satb2* were calibrated using the respective cDNAs. (*B*) Satb1 and Satb2 proteins do not display a defined subnuclear localization pattern in ES cells. In undifferentiated wild-type cultures, most of the cells express Satb1, but only ~20% express Satb2 at detectable levels, while MEFs do not stain for either Satb1 or Satb2. At day 5 of differentiation, most cells express Satb2, similar to Satb1. The white bar corresponds to 5 μm for LIF conditions and 10 μm at day 5, representing the nuclear expansion that accompanies ES cell differentiation. (*C*) Representative *Z*-stacks of a confocal immunofluorescence analysis demonstrate the differences in Satb1 (and Satb2) protein distribution in ES cells and T cells.

observed a transient up-regulation of *Satb2*, with a peak around day 6 and a subsequent down-regulation. *Satb1* was similarly induced, but its level of expression remained higher than in undifferentiated cells even after the addition of gancyclovir at day 6, which led to the elimination of *Oct4*-expressing cells.

By immunofluorescence analysis, Satb1 protein could be detected easily in most undifferentiated and differentiated cells (Fig. 1B). Satb2 was expressed only in a subset (~20%) of undifferentiated cells (Fig. 4C,D, below), but it was detectable in most cells at day 5 of differentiation. Notably, a punctate staining pattern of both Satb1 and Satb2 was found throughout the nucleus, excluding the nucleolus, which differs significantly from the cage-like pattern observed for Satb1 in thymocytes (Fig. 1C; Supplemental Fig. 1; Cai et al. 2003). Semiquantitative immunoblot analysis to detect Satb1 protein in T cells, ES cells, and MEFs indicated that T cells contain ~100 times more Satb1 protein than ES cells, indicating that the "Satb1 cage" might require very abundant protein expression (data not shown). Thus, both Satb1 and Satb2 are expressed in undifferentiated and differentiating ES cells, whereby the percentage of Satb2-expressing cells increases transiently during differentiation.

Roles of Satb1 and Satb2 in ES cell differentiation

To examine a potential role of Satb proteins in the regulation of *Hox* genes and/or differentiation of ES cells, we generated ES cells from blastocysts that were derived from crosses of $Satb1^{+/-}Satb2^{+/-}$ mice (Alvarez et al. 2000; Dobreva et al. 2006). In 23 derived ES cell lines, all genotypes were present with the exception of $Satb1^{+/+}Satb2^{-/-}$ ES cells, which were also not represented in 10 ES cell lines that were derived from blastocysts of $Satb1^{+/+}/Satb2^{+/-}$ intercrosses (Supplemental Fig. 2a,b).

In a comparison of wild-type and $Satb1^{-/-}$ ES cells, both of which have been stimulated with RA to induce neural differentiation and colinear *Hox* gene expression, we observed an impaired differentiation of $Satb1^{-/-}$ ES cells. ES cell-like colonies were present in the $Satb1^{-/-}$ culture even after 6 d of RA stimulation (Fig. 2A). To

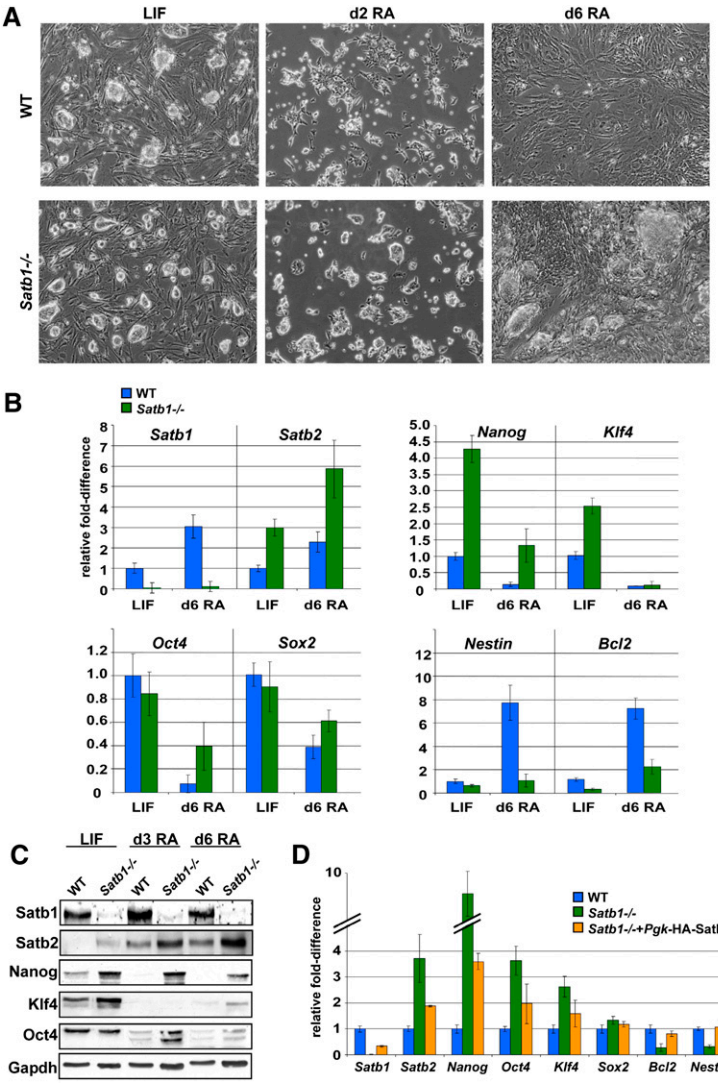


Figure 2. Satb1 is required for the differentiation of pluripotent cells. (*A*) Phase contrast microscopy of wild-type and $Satb1^{-/-}$ ES cells at the indicated time points of RA-mediated differentiation. Satb1-deficient cells retain morphologies of undifferentiated cultures even after prolonged exposure to RA. (*B*) Quantitative RT–PCR analysis was performed on cDNA generated from cultures at the indicated time points. Expression levels were normalized to wild-type levels in undifferentiated cells (=1) for each individual transcript. The *Y*-axis denotes fold changes of mRNA expression. Satb1-deficient ES cells display abnormally high *Nanog* and *Klf4* levels under LIF conditions and maintain high *Nanog* levels upon RA-mediated differentiation, while bulk cultures show severely impaired *Bcl2* and *Nestin* induction. (*C*) Immunoblot analysis of the indicated proteins in wild-type and $Satb1^{-/-}$ ES cells at the indicated time points demonstrates high Nanog levels at all times of culture in $Satb1^{-/-}$ cells. (*D*) Analysis of gene expression in wild-type, $Satb1^{-/-}$, and $Satb1^{-/-}$ cells re-expressing Satb1. Shown is a quantitative RT–PCR analysis of the indicated transcripts in cells of the indicated genotypes differentiated for 6 d in the presence of RA. Expression values were normalized to expression in wild-type ES cells for every transcript (=1), and differences in expression are shown as fold changes.

better understand this finding, we analyzed the expression of *Nestin* and *Bcl2* as differentiation markers, and that of *Nanog, Oct4*, *Sox2*, and *Klf4* as pluripotency markers. RA-stimulated *Satb1*$^{-/-}$ cells showed an impaired expression of the differentiation-associated genes *Nestin* and *Bcl2* and an increase in the expression of the pluripotency markers, in particular of *Nanog* (Fig. 2B). However, no significant change was detected in the expression of *Oct4* and *Sox2*. The expression of *Nanog* and *Klf4* was also increased in undifferentiated mutant ES cells, suggesting that the deregulation of these two genes may contribute to the impaired differentiation potential of *Satb1*$^{-/-}$ cells. We also noticed that *Satb2* expression was elevated in undifferentiated and differentiated *Satb1*$^{-/-}$ ES cells, as compared with wild-type cells. In an immunoblot analysis, we found a similar alteration in protein expression, whereby Nanog protein expression was most clearly up-regulated in both undifferentiated cells and RA-stimulated cells (Fig. 2C). To exclude the possibility of an artifact due to clonal variation, we included in this analysis an independently derived *Satb1*$^{-/-}$ ES cell clone that showed similar defects in differentiation and *Nanog* expression (Supplemental Fig. 2c). In addition, we examined whether the re-expression of Satb1 rescues the differentiation defect of *Satb1*$^{-/-}$ cells by stably transfecting the mutant cells with a Satb1 expression construct under the control of the *Pgk* promoter. In a stably transfected cell line in which exogenous *Satb1* was expressed at ~40% of wild-type levels, we detected a down-regulation of *Satb2*, *Nanog*, and *Klf4* and an up-regulation of *Bcl2* and *Nestin*, relative to the parental *Satb1*$^{-/-}$ cells (Fig. 2D). Taken together, these results provide evidence for the role of Satb1 in the deregulation of the pluripotency genes *Nanog* and *Klf4*.

To further investigate the differentiation defect of *Satb1*$^{-/-}$ ES cells, we removed only LIF from the culture medium, resulting in spontaneous differentiation of ES cells. Wild-type cells differentiated normally, whereas many *Satb1*$^{-/-}$ cells continued to grow in colonies (Fig. 3A). Quantitative RT–PCR analysis of differentiation- and pluripotency markers indicated that Satb1-deficient ES cells, cultured without LIF, display a pattern of marker gene expression similar to that of undifferentiated wild-type cells, including a low expression of the mesoderm marker brachyury (*T*) (Fig. 3B). To analyze p-STAT3 levels as a readout for self-renewal and LIF signaling (Niwa et al. 1998, 2009), we performed an immunoblot analysis with lysates of wild-type and Satb1-deficient cultures. After the withdrawal of LIF from the culture for 21 h, LIF, either alone or in combination with a JAK inhibitor, was added back to the culture for different time periods (Fig. 3C). We did not detect any significant differences between wild-type and *Satb1*$^{-/-}$ cells in this experimental setup, suggesting that the Satb1 deficiency does not affect LIF signaling.

Finally, we examined whether *Satb1*$^{-/-}$ ES cells can self-renew in the absence of LIF by culturing cells on gelatine-coated dishes and passaging them every 2 d. Immunoblot and immunofluorescence analysis demonstrated that Nanog protein expression was up-regulated in mutant ES cell cultures relative to wild-type cells, even after six passages, consistent with the enhanced self-renewal of Satb1-deficient cells in the absence of LIF and feeder cells (Fig. 3D–F). Under these conditions, an up-regulation of Klf4 expression was detected only up to one passage, and no significant change of Oct4 expression was observed.

Satb1 and Satb2 expression is related to the heterogeneity of Nanog expression

The ability of ES cells to both respond to differentiation signals and retain a self-renewal potential has been attributed to heterogeneity in the expression of transcription factors associated with pluripotency (for review, see Graf and Stadtfeld 2008). Specifically, the expression of Nanog, Dppa3, and Rex1 is heterogeneous, whereby ES cells expressing these proteins at a high level have a preference for self-renewal (Chambers et al. 2007; Singh et al. 2007; Hayashi et al. 2008; Toyooka et al. 2008). To examine whether the elevated Nanog levels in Satb1-deficient ES cells are reflected in an altered heterogeneity of Nanog expression, we performed immunofluorescence analysis of ES cell colonies cultured for four passages in the presence of LIF, but on gelatine without feeders (Fig. 4A; Chambers et al. 2007). In wild-type colonies, few cells were found to express Nanog at high levels, whereas in Satb1-deficient colonies a marked increase in the frequency of Nanog^{high}- expressing cells could be observed. In contrast, no significant difference in Oct4 expression was detected. To quantitate the frequencies of Nanog^{high}- and Nanog^{low}-expressing cells, we used the ImageJ software to determine the relative fluorescence intensities of individual cells, using DAPI staining as an internal control (Fig. 4B). In wild-type colonies, 70% of the cells expressed Nanog at a low level (<50 arbitrary units), and ~30% at a medium (50–100 arbitrary units) and high level (>100 arbitrary units). In Satb1-deficient colonies, the frequencies of Nanog^{low}-expressing cells were reduced to ~30%. Since Satb2 is overexpressed in Satb1-deficient ES cells, we also examined whether a higher percentage of *Satb1*$^{-/-}$ cells expresses Satb2. Indeed, we found that almost 50% of *Satb1*$^{-/-}$ ES cells express Satb2 protein, corresponding to an approximately threefold increase in the frequency of Satb2-expressing cells relative to wild-type cells (Fig. 4C,D). Therefore, we examined whether a high level of Nanog expression correlates with the expression of Satb2. Most of the Nanog^{high} cells in *Satb1*$^{-/-}$ colonies also expressed Satb2, and this correlation was also observed in wild-type cultures (Fig. 4E,F; Supplemental Fig. 3a). Moreover, we found that the doxycycline-induced expression of ectopic HA-tagged Satb2 correlates with an increased expression of endogenous Nanog after four passages of the cells on gelatine (Supplemental Fig. 3b–e). Finally, we incubated *Satb1*$^{-/-}$ cells with RA for 6 d and examined the expression of *Satb2* and *Nanog* in self-renewing and differentiated cells that have been separated by flow cytometry, due their small and large sizes, respectively (Fig. 4G). Population R1, consisting of smaller cells, expressed *Nanog*, *Klf4*,

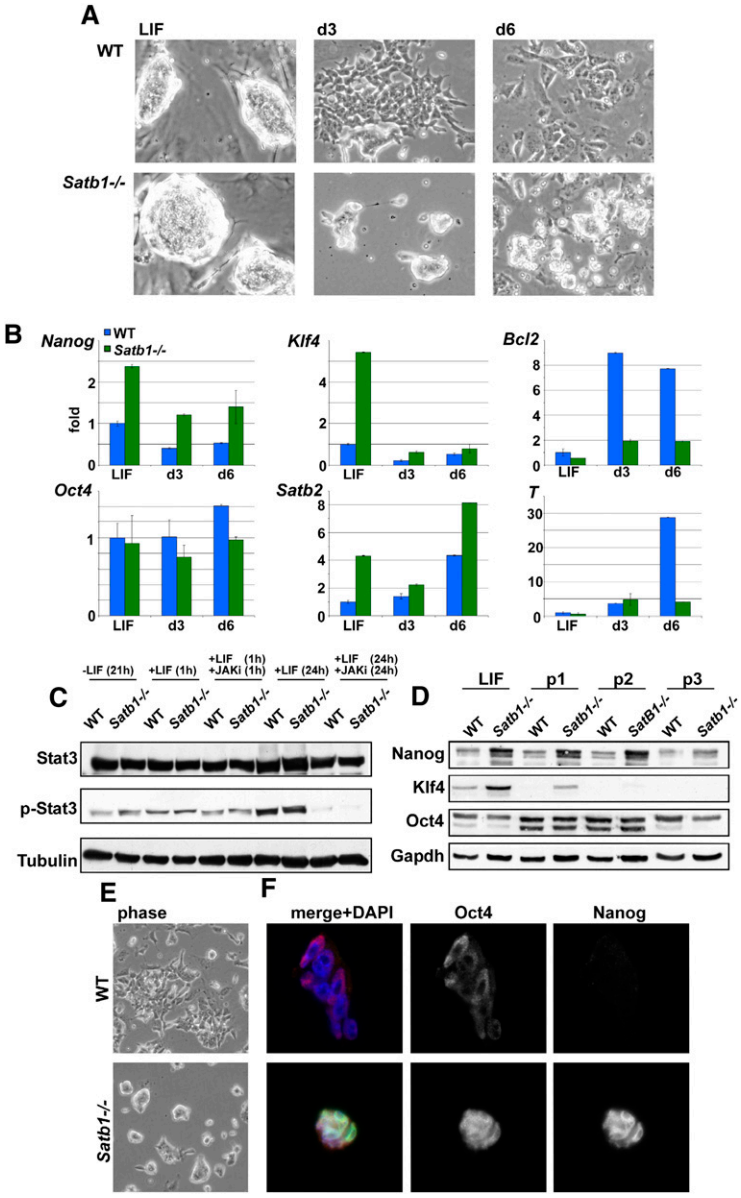


Figure 3. *Satb1*$^{-/-}$ ES cells display a general defect in differentiation that is not specific for RA induction and can self-renew in the absence of feeders and LIF. (*A*) Phase contrast microscopy of wild-type and *Satb1*$^{-/-}$ ES cells at the indicated time points of differentiation triggered by LIF withdrawal. Satb1-deficient cells retain morphologies of undifferentiated cultures in the absence of LIF. (*B*) Quantitative RT–PCR analysis of the indicated transcripts in wild-type and *Satb1*$^{-/-}$ ES cells differentiated by LIF removal. Values were normalized to the expression levels in undifferentiated wild-type cells (value = 1). (*C*) Immunoblot analysis of total STAT3 and p-STAT3 levels in wild-type and *Satb1*$^{-/-}$ ES cells. LIF was removed from proliferating cultures for 21 h and then added back, either alone or in combination with a JAK inhibitor for 1 h or 24 h. (*D*) Immunoblot analysis of the indicated proteins in wild-type and *Satb1*$^{-/-}$ ES cells passaged every 2 d in the absence of LIF (on gelatine, without feeders). (*E*,*F*) Phase contrast microscopy (*E*) and immunofluorescence analysis (*F*) of Oct4 (568 nm) and Nanog (488 nm) in wild-type and *Satb1*$^{-/-}$ ES cells at passage 6 (on gelatine, without feeders and LIF). The phase and fluorescent channels do not show the same cells.

Oct4, and *Satb2* at higher levels than population R2, which included larger differentiated cells and expressed higher levels of *Bcl2* and *Nestin* (Fig. 4H). Taken together, this analysis suggests that the expression of Satb2 marks the self-renewing Nanog^{high} fraction of ES cells.

Modest differentiation defects of Satb1$^{+/-}$Satb2$^{-/-}$ *and* Satb1$^{-/-}$Satb2$^{-/-}$ *ES cells*

The analysis of the role of Satb2 in ES cells was hampered by our inability to derive stable *Satb2*$^{-/-}$ ES cell clones. Therefore, we used *Satb1*$^{+/-}$*Satb2*$^{-/-}$ cells to assess the role of Satb2 in differentiation. At early passages (passages 4–5), *Satb1*$^{+/-}$*Satb2*$^{-/-}$ cells displayed an abnormal cellular morphology, accelerated silencing of *Oct4* and *Nanog* during RA-induced differentiation, and augmented expression of *Satb1* relative to wild-type cells (Supplemental Fig. 4a,b). During continuous culture in LIF, both *Satb1*$^{+/-}$*Satb2*$^{-/-}$ ES cell lines changed their growth characteristics and gene expression profile, resulting in cells that express *Satb1* at very low levels and *Nanog* and *Klf4* at higher levels (Supplemental Figs. 4c, 5). The later passages of *Satb1*$^{+/-}$*Satb2*$^{-/-}$ cells differentiated like wild-type cells, and only a few cells displaying an undifferentiated morphology were detected after 2 d of differentiation (Fig. 5A). During RA-mediated differentiation, these cells induced the expression of *Bcl2*, *Nestin*, and *Satb1* and silenced the expression of *Klf4* and *Nanog* (Fig. 5B; Supplemental Fig. 5a). Wild-type, *Satb1*$^{-/-}$, and *Satb1*$^{+/-}$*Satb2*$^{-/-}$ cells divided at a similar rate in LIF conditions (Fig. 5C), but *Satb1*$^{+/-}$*Satb2*$^{-/-}$ cultures displayed an impaired proliferation during RA-mediated differentiation (Fig. 5D). These observations raised the possibility that ES cells cannot sustain high levels of

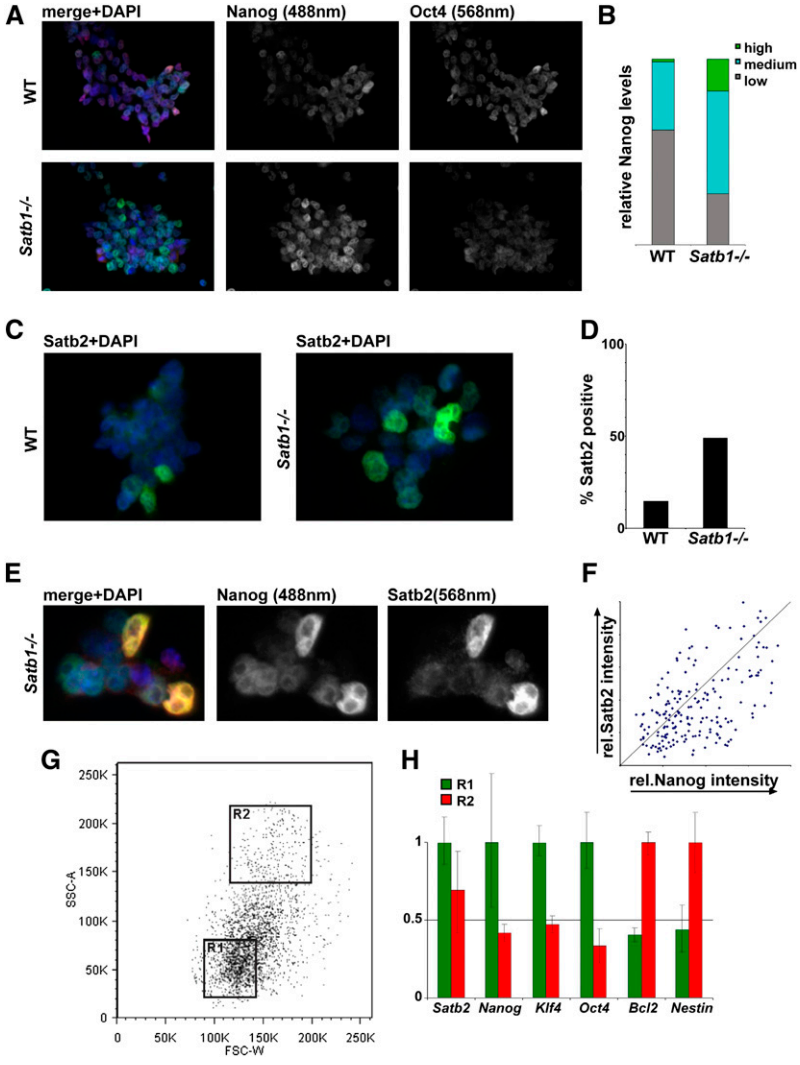


Figure 4. High levels of Nanog expression in Satb1-deficient cultures are reflected by an increase in the frequencies of Nanog^{high} cells. (*A*) Immunofluorescence analysis of Nanog and Oct4 expression in wild-type and *Satb1*$^{-/-}$ ES cells. (*B*) Quantification of Nanog levels by a software tool (ImageJ) reveals a drastic shift from Nanog^{low} to Nanog^{high} cells in Satb1-deficient cultures. Cells are grouped as high-, medium-, and low-expressing, relative to the Nanog signal intensity of the specific cell. DAPI was used as an internal control and the bars indicate percentage of the total population. ($n \geq 150$ per genotype). (*C*) Immunofluorescence analysis of Satb2 expression in wild-type and *Satb1*$^{-/-}$ cells, using an anti-Satb2 antibody. The specificity of the anti-Satb2 antibody was confirmed by the increase of Satb2 staining in cells in which ectopic Satb2 has been induced with doxycycline (data not shown). (*D*) Percentage of wild-type and *Satb1*$^{-/-}$ cells displaying detectable Satb2 expression. ($n \geq 250$ per genotype). (*E*) Correlation between Nanog and Satb2 expression in Satb1-deficient cells by immunofluorescence. (*F*) Quantitative correlation of Satb2 and Nanog levels determined by measuring signal intensities in *Satb1*$^{-/-}$ ES cells using ImageJ demonstrates that cells expressing Nanog at high levels are almost always also expressing Satb2 at high levels. Measurements were performed analogous to the experiments shown in *B*. Each spot of the graph indicates a single cell. (*G*) Sorting scheme for small, undifferentiated (R1) and larger, differentiating (R2) *Satb1*$^{-/-}$ ES cells (day 6 of RA stimulation). (*H*) Analysis of gene expression in R1 and R2 fractions. Shown is a quantitative RT–PCR analysis of the indicated transcripts. For each transcript, the higher value was set as "1" and differences in expression are shown as fold changes.

Satb1 in the absence of Satb2. Analysis of ES cells carrying conditional *Satb2*$^{fl/fl}$ alleles, which can be inactivated by the expression of Cre-recombinase, indicated that only two out of 100 clones expressing a transfected Cre-construct showed recombination at the *Satb2* locus, although even these clones retained cells that have not undergone recombination (Supplemental Fig. 4d). After two passages of these cell clones, virtually no recombined alleles were detected, suggesting that Satb2-deficient ES cells have a growth and/or survival disadvantage relative to wild-type ES cells.

To gain further insight into the functional circuitry of Satb1 and Satb2, we analyzed the phenotype of *Satb1*$^{-/-}$*Satb2*$^{-/-}$ ES cells. Morphological analysis of Satb1 and Satb2 double-deficient ES cells indicated that the cells undergo virtually normal differentiation in response to stimulation with RA (Fig. 5A). Quantitative RT–PCR analysis indicated that expression of *Klf4* and *Nanog* was slightly elevated, whereas the expression of *Sox2* and *Oct4* was unchanged (Fig. 5B; Supplemental Fig. 5a). Moreover, immunofluorescence analysis of RA-differentiated double-mutant cells revealed that ~52% and 11% of the cells were positive for the expression of Nestin and Nanog, respectively, whereas *Satb1*$^{-/-}$ cultures contained only ~6% Nestin-positive and ~75% Nanog-positive cells (Fig. 5E; Supplemental Fig. 5). However, undifferentiated *Satb1*$^{-/-}$*Satb2*$^{-/-}$ cultures contained a higher percentage of Nanog^{high} cells, relative to wild-type cells (Supplemental Fig. 5c,d).

Finally, we examined the phenotype of *Satb1*$^{-/-}$ *Satb2*$^{-/-}$ cells that express exogenous *Satb1* under the control of the *Pgk* promoter (Fig. 5B; Supplemental Fig. 5a,e,f). RA-induced differentiation of these cells augmented the expression of *Bcl2* and *Nestin*, but repressed *Nanog* and *Klf4* levels in both LIF and RA conditions (Fig. 5B; Supplemental Fig. 5a). *Satb1*$^{-/-}$*Satb2*$^{-/-}$ cells re-expressing Satb1 showed morphologies similar to early passages of *Satb1*$^{+/-}$*Satb2*$^{-/-}$ cells and a gradual decrease of exogenous Satb1 expression (data not shown). Taken together, these data suggest that *Satb1*$^{-/-}$*Satb2*$^{-/-}$ ES cells have an almost normal phenotype and do not sustain Satb1 expression in the absence of Satb2.

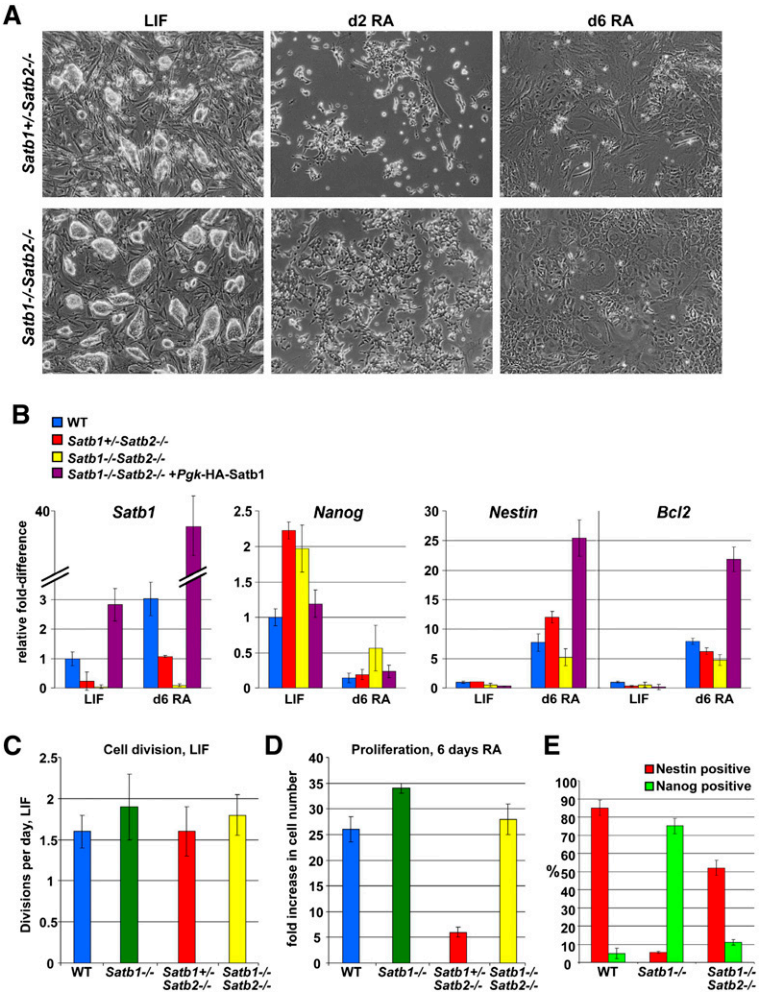


Figure 5. $Satb1^{+/-}Satb2^{-/-}$ and $Satb1^{-/-}Satb2^{-/-}$ display a modest differentiation defect, which is rescued by the re-expression of Satb1. (*A*) Phase contrast microscopy of $Satb1^{+/-}Satb2^{-/-}$ and $Satb1^{-/-}Satb2^{-/-}$ ES cells at the indicated time points of RA-mediated differentiation. (*B*) Analysis of gene expression in $Satb1^{+/-}Satb2^{-/-}$, $Satb1^{-/-}Satb2^{-/-}$, and $Satb1^{-/-}Satb2^{-/-}$ cells re-expressing Satb1. Shown is a quantitative RT–PCR analysis of the indicated transcripts in undifferentiated and differentiated cells of the indicated genotypes. Expression values were normalized to expression in undifferentiated wild-type ES cells for every transcript (=1), and differences in expression are shown as fold changes. (*C*) Satb1 or Satb2 deficiency does not affect cell division of cells cultured with LIF-containing medium. Identical numbers of cells were seeded on feeders and grown in duplicates for 3 d for two passages, total numbers were counted, and the cell division index was calculated and depicted as divisions per 24 h. (*D*) $Satb1^{+/-}Satb2^{-/-}$ cells display reduced proliferation during RA-mediated differentiation. Cells (100,000) were seeded on gelatine-coated dishes and grown in triplicate for 6 d under RA conditions. The *Y*-axis indicates cell numbers as multiplicities of 10^5. (*E*) Immunofluorescence analysis of Nanog and Nestin in the indicated cell types after 6 d of RA-mediated differentiation demonstrates that $Satb1^{-/-}Satb2^{-/-}$ cells differentiate with an efficiency similar to wild-type cells. The graph depicts the percentage of Nanog- and Nestin-positive cells at day 6 of differentiation ($n = 2 \times 100$ cells per genotype).

Satb1 and Satb2 are dispensable for Hox *gene regulation in ES cells*

To determine whether Satb proteins regulate the expression of *Hox* genes during ES cell differentiation, we analyzed RNA from wild-type, $Satb1^{-/-}$, and $Satb1^{+/-}Satb2^{-/-}$ ES cells, as well as doxycycline-inducible tetO-HA-Satb2 ES cells. RT–PCR analysis in wild-type ES cells demonstrated the correct colinear induction of *HoxB* genes accompanied by *Oct4* and *Nanog* repression in our ES cell cultures (Supplemental Fig. 6a). Comparing the expression of various *Hox* genes between undifferentiated and differentiating wild-type, $Satb1^{-/-}$, and $Satb1^{+/-}Satb2^{-/-}$ cells, or in cells with forced expression of Satb2, we found no major differences between the levels of *Hox* genes in these different genetic settings (Supplemental Fig. 6b,c). Thus, the colinear expression of *Hox* gene is normal in a genetic background associated with impaired cellular differentiation.

Identification of Satb1 and Satb2 target genes

To identify genes that are regulated by Satb1, we performed an Affymetrix microarray analysis using RNA from undifferentiated wild-type and $Satb1^{-/-}$ ES cells. Three independent cultures were used for the generation of RNA for both genotypes. After hybridization, we found the expression of >50 genes significantly altered between wild-type and $Satb1^{-/-}$ cells (Fig. 6A). Among the transcripts overexpressed in $Satb1^{-/-}$ cells, we found several regulators of pluripotency, including *Nanog*, *Tbx3*, and *Klf4*. Additionally, we identified *Dppa3*, *Cdkn1b*, and *Jarid1a*, which are also associated with ES cell differentiation. Among the genes expressed at lower levels in $Satb1^{-/-}$ cells compared with wild-type cells, we found *H19* and *Dnmt3a*, pointing at potential roles of Satb1 in genomic imprinting. We used independently generated RNA for the verification of the hybridization results using quantitative RT–PCR (Fig. 6B).

To identify additional genes regulated by Satb1 and/or Satb2, we performed an Affymetrix microarray analysis using tetO-HA-Satb1 and tetO-HA-Satb2 ES cells. To favor the identification of primary targets, we induced Satb1 or Satb2 expression for 24 h and subsequently generated cDNA from three induced cultures and two uninduced control cultures. Upon Satb1 induction, we identified four genes that were up-regulated and 12 genes that were down-regulated (Supplemental Fig. 7a). Some of these targets were validated by quantitative RT–PCR

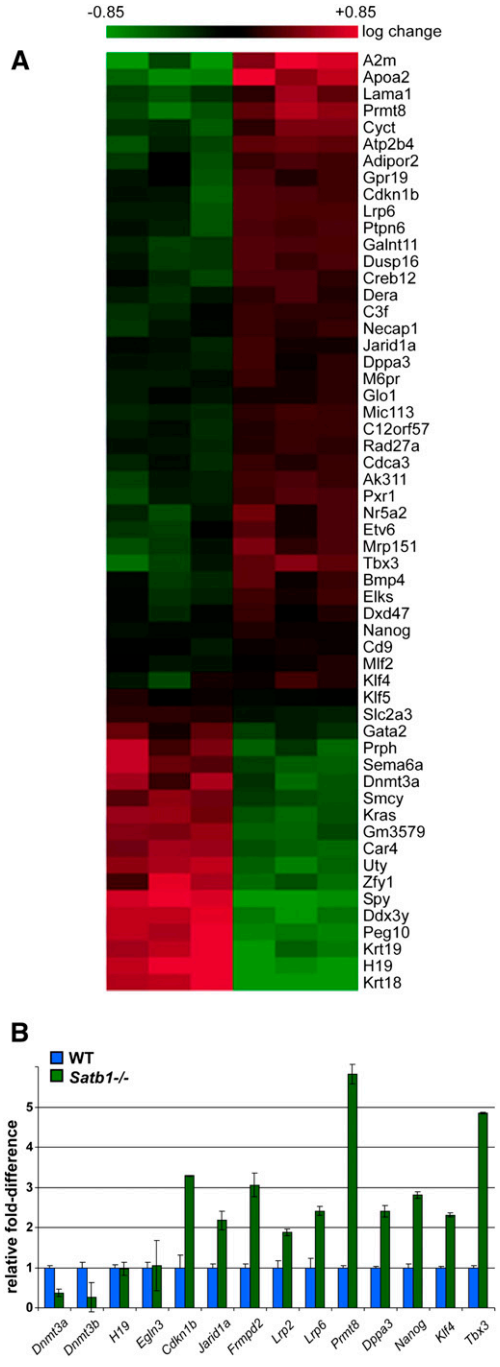


Figure 6. Identification of Satb1 target genes by Affymetrix microarrays. (*A*) Heat map of three individual hybridizations using RNA from undifferentiated wild-type and *Satb1*$^{-/-}$ cultures. Shown are the 38 most significantly up-regulated and the 17 most significantly down-regulated genes (in *Satb1*$^{-/-}$ cells). (*B*) Quantitative RT–PCR verification of the results obtained by an Affymetrix GeneChip array hybridization using mRNA from wild-type and *Satb1*$^{-/-}$. Some genes identified as Satb1 targets using the tetO-HA-Satb1 cells were also included.

(Supplemental Fig. 7b). In this analysis, we included *Bcl2*, a SATB1 target in human cells, and found an increase in *Bcl2* expression in induced tetO-HA-Satb1 ES cells. In tetO-HA-Satb2 cells, we found more deregulated genes that included *Cspp1* and *Otx2*, which were validated by quantitative RT–PCR (Supplemental Fig. 7c,d). Notably, we found some overlapping target genes for Satb1 and Satb2, such as *Lrp2*, which was activated by both Satb1 and Satb2, whereas other genes, such as *Frmpd2*, were differentially regulated by these proteins. Thus, Satb proteins may regulate genes in both a redundant and antagonistic manner, reminiscent of the redundant and antagonistic regulation of ES cell differentiation and proliferation by Klf4 and Klf5 (Ema et al. 2008).

We further confirmed the microarray experiment by an immunoblot analysis of tetO-HA-Satb1 cells in which Satb1 had been induced under LIF conditions for 48 h. Upon Satb1 induction, Klf4 levels were reduced, whereas Nanog levels were only modestly affected and Oct4 levels remained unchanged (Supplemental Fig. 8a). In this experiment, we observed a marked reduction of induced Satb1 expression after 3 d of culture (Supplemental Fig. 8b).

Overexpression of Satb2, which could be sustained for >2 wk, was found to impair cellular differentiation, in part resembling the differentiation phenotype of *Satb1*$^{-/-}$ cells (Supplemental Fig. 8c–g). In induced tetO-HA-Satb2 cultures, >100 Oct4-positive colonies per 100,000 plated cells were detectable without a microscope, whereas on average only one colony per 100,000 cells appeared without ectopic HA-Satb2 expression (Supplemental Fig. 8e,f). Control cultures in which the transcription factor Ebf1 was induced via the "tet-ON" system showed no differences between induced and uninduced conditions (data not shown). Immunoblot and RT–PCR analysis revealed that Satb2 overexpression specifically impairs RA-mediated silencing of Nanog, but not of Klf4 and Oct4 (Supplemental Fig. 8c,d). Notably, ectopic Satb2 was found to repress the expression of *Satb1* after 2 wk of RA-mediated differentiation (Supplemental Fig. 8d).

Satb1 and Satb2 bind to the Nanog *locus in vivo*

The altered expression of *Klf4*, *Nanog*, and *Bcl2* in *Satb1*$^{-/-}$ cells, and the identification of *Klf4* and *Bcl2* as deregulated genes in our microarray analysis, raised the possibility that these genes are direct targets of Satb1 and/or Satb2. We used the bioinformatics software Genomatix to identify potential Satb1-binding sequences in these three gene loci. Indeed, the program predicted several potential Satb1-binding sites (Fig. 7A), which were further examined for in vivo occupancy by chromatin immunoprecipitations (ChIPs) of ES cells. Due to the strong Satb1–Satb2 cross-reactivity of a noncommercial Satb1 antiserum and the inability of commercial Satb1 and Satb2 antibodies to work in ChIP experiments, we used our cell lines in which HA-Satb1 or HA-Satb2 could be induced by doxycycline treatment (Fig. 7B; Supplemental Fig. S9a). Quantitative PCR amplification of chromatin fragments that were immunoprecipitated with an anti-HA antibody from uninduced and induced undifferentiated cells indicated that an intragenic sequence in the second intron of *Klf4* can be bound by HA-Satb1 and HA-Satb2 in vivo (Fig. 7B; Supplemental Fig. 9a). In the *Nanog* locus, a region ~10 kb upstream of the

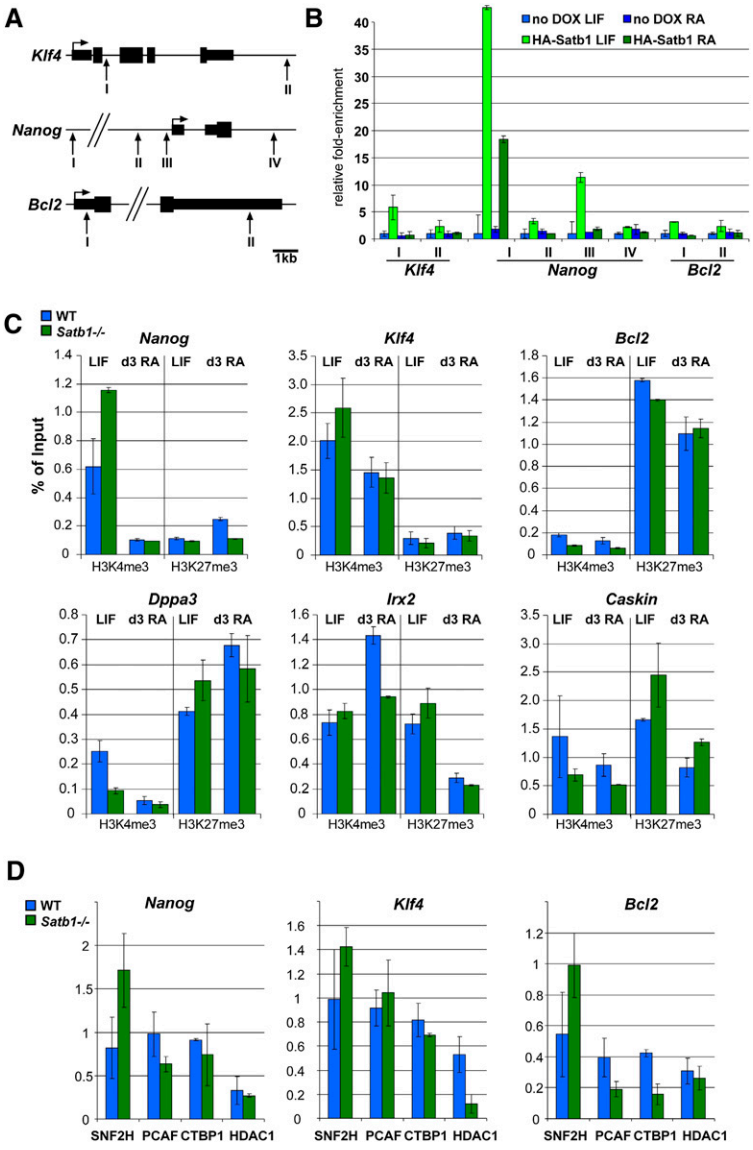


Figure 7. Analysis of Satb1 target gene regulation at the chromatin level. (*A*) Identification of potential Satb1-binding sites in the *Klf4*, *Nanog*, and *Bcl2* loci discovered by software analysis (Genomatix). (*B*) Anti-HA ChIP assay to detect HA-Satb1 at the sites represented in *A* in undifferentiated and differentiating (d3 RA) ES cells. Quantitative RT–PCR analysis of immunoprecipitated material obtained from uninduced and induced tetO-HA-Satb1 ES cells using primers designed to detect the potential Satb1-binding sites. Shown is the fold enrichment correlated to uninduced cells (value = 1). (*C*) Chromatin analysis of wild-type and Satb1-deficient ES cells. Quantitative RT–PCR analysis of sequences from the indicated promoters performed on material immunoprecipitated with an antibody specific for either H3K4me3 or H3K27me3 in undifferentiated and differentiating wild-type and *Satb1*$^{-/-}$ ES cells. The *Y*-axis represents the percentage of immunoprecipitated DNA compared with the individual input values. *Nanog*, *Klf4*, and *Bcl2* are direct Satb1 target genes. The analysis of *Dppa3* suggests that *Nanog*, but not other genes residing in the extended *Nanog* locus, is regulated by Satb1. The bivalent genes *Irx2* and *Caskin* are equally enriched for H3K4me3 and H3K27me3 in undifferentiated wild-type and mutant cells. (*D*) Chromatin analysis of wild-type and Satb1-deficient ES cells. Quantitative RT–PCR analysis of sequences from the indicated promoters performed on material immunoprecipitated with an antibody specific for SNFH2, PCAF, CTBP1, or HDAC1 in undifferentiated wild-type and *Satb1*$^{-/-}$ ES cells. The analysis was performed analogous to that shown in *C*.

transcription initiation site, containing a cluster of 11 potential Satb1-binding sites, was found to be highly enriched in the immunoprecipitated chromatin fragments. Another region close to the transcription start site was also occupied by HA-Satb1 and HA-Satb2. In the *Bcl2* locus, however, we did not detect any significant amplification of the region encompassing the predicted Satb-binding sites. We also examined whether Satb1 remains bound to these genomic sequences in cells that were differentiated by RA for 3 d. Satb1 remained bound to the *Nanog* locus, but only at the ~10-kb upstream element (Fig. 8A). The *Nanong* and *Klf4* promoters were not occupied in differentiated cells, suggesting a dynamic association of Satb1 with its target genes during the process of ES cell differentiation.

Chromatin analysis of Satb1 target genes

Satb1 and Satb2 regulate gene expression at the chromatin level (Cai et al. 2006; Dobreva et al. 2006; Kumar et al. 2007). Therefore, we analyzed H3K4me3 and H3K9 acetylation, two marks of active chromatin, and H3K27me3, a repressive histone mark, at the *Nanog*, *Klf4*, and *Bcl2* promoters in wild-type and *Satb1*$^{-/-}$ cells. In ChIP experiments, we found that the *Nanog* promoter has higher H3K4me3 levels in undifferentiated Satb1-deficient cells relative to wild-type cells, consistent with the increased expression in *Satb1*$^{-/-}$ cells (Fig. 7C; Supplemental Fig. 9b,c). RA-mediated differentiation reduced H3K4me3 levels both in wild-type and *Satb1*$^{-/-}$ cells. In wild-type, but not in *Satb1*$^{-/-}$ cells, H3K27me3 levels started to increase by day 3 of differentiation, which may explain the higher *Nanog* levels in *Satb1*$^{-/-}$ cells at this and later stages of differentiation. *Klf4* displays slightly higher H3K4me3 levels at the promoter in undifferentiated *Satb1*$^{-/-}$ cells, but we did not detect differences between wild-type and mutant cells for H3K4me3 in differentiated cells, consistent with the

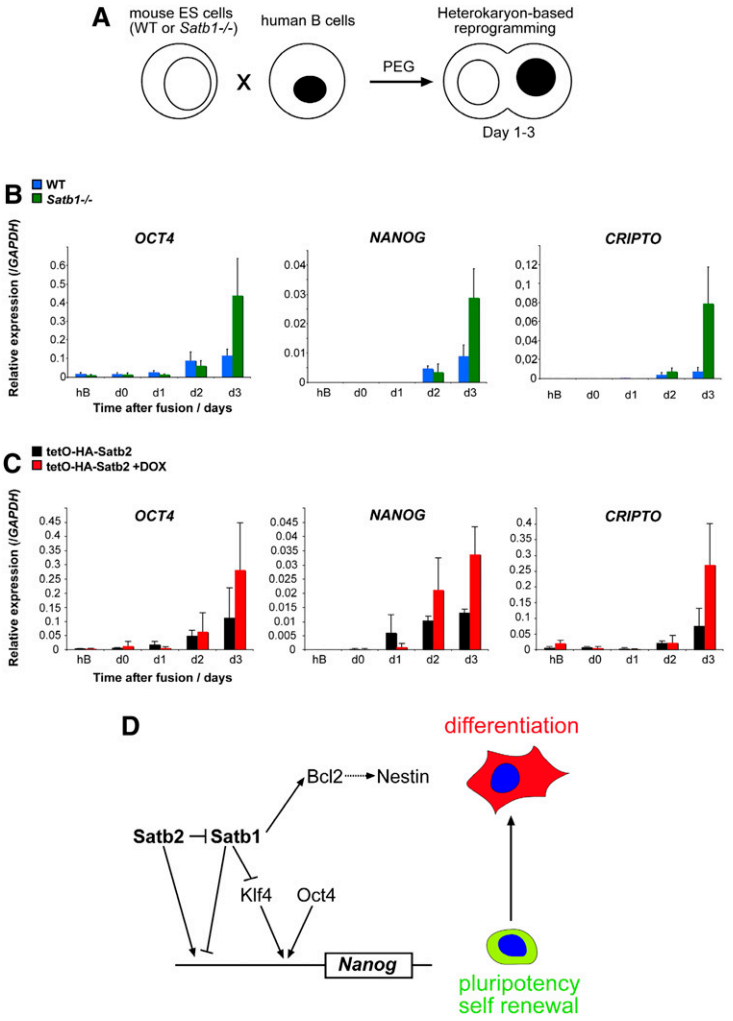


Figure 8. *Satb1*$^{-/-}$ ES cells efficiently reprogram human B cells in heterokaryon fusion experiments. (*A*) Schematic representation of a fusion experiment between mouse ES cells and human B cells. (*B*) Quantitative RT–PCR analysis of transcripts of human pluripotency genes in human B cells and heterokaryons between *Satb1*$^{-/-}$ ES cells and human B cells at the indicated time points after fusion. The fold of activation of pluripotency genes was calculated relative to the *GAPDH* levels for each indicated time point. (*C*) Quantitative RT–PCR analysis of transcripts of human pluripotency genes in human B cells and heterokaryons between human B cells and ES cells in which ectopic Satb2 expression was induced for 30 or 48 h. (*D*) Model for a transcription factor network in which Satb1 regulates the balance between self-renewal and differentiation of ES cells by repressing pluripotency factors, such as *Nanog* and *Klf4,* and inducing genes involved in differentiation, such as *Bcl2*. Satb2 activates *Nanog* expression by direct binding to the *Nanog* locus and indirectly by antagonizing the expression and/or the activity of *Satb1*. *Oct4* is not regulated by either Satb1 or Satb2, and mediates proper *Nanog* expression in the absence of Satb proteins.

deregulation of *Klf4* expression in cells cultured in LIF medium. As expected, we found higher levels of H3K27me3 than H3K4me3 at the *Bcl2* promoter. H3K4me3 levels were generally very low, suggesting that *Bcl2* may be induced by the loss of repressive histone marks. The *Nanog* gene is coregulated with its neighboring genes, *Dppa3* and *GDF3*, in an Oct-4-dependent manner that involves the formation of a higher-order chromatin structure (Levasseur et al. 2008). However, neither the H3K4me3 nor the H3K27me3 levels at the *Dppa3* and *GDF3* genes were found to be altered in *Satb1*$^{-/-}$ cells like the *Nanog* promoter, suggesting a gene-specific regulation of *Nanog* by Satb proteins (Fig. 7C; Supplemental Fig. 9b). We also analyzed the "bivalent" genes *Itx*2 (Bernstein et al. 2006) and *Caskin* and found no significant differences between *Satb1*$^{-/-}$ and wild-type cells, arguing against a role of Satb1 in the regulation of the global chromatin configuration in ES cells (Fig. 7C). Analysis of *Brd2*, a gene highly expressed in undifferentiated cells and highly enriched for H3K4me3, and analysis of *Tpsg1*, an inactive gene marked by H3K27me3, served as technical controls, and they revealed no changes in the levels of these two modifications between wild-type and *Satb1*$^{-/-}$ cells (Supplemental Fig. 9b,c).

To gain insight into which protein complexes might be associated with Satb1 at target promoters in undifferentiated wild-type and *Satb1*$^{-/-}$ cells, we performed ChIP experiments with antibodies against SNF2H, PCAF, CTBP1, and HDAC1, which have been reported to bind Satb1 (Yasui et al. 2002; Kumar et al. 2005; Pavan Kumar et al. 2006; Purbey et al. 2009). In *Satb1*$^{-/-}$ cells, we detected a modest increase of SNF2H at the *Nanog*, *Klf4*, and *Bcl2* promoters (Fig. 7D). At the *Klf4* promoter, we also detected reduced HDAC1 levels in *Satb1*$^{-/-}$ cells, which may suggest an involvement of histone acetylation in the deregulation of *Klf4* expression (Fig. 7D; Supplemental Fig. 9d).

Increased 'reprogramming' efficiency of Satb1-deficient and Satb2-overexpressing ES cells in cell fusions with human B lymphocytes

ES cells have been shown to reprogram differentiated B lymphocytes, whereby pluripotency-associated markers are induced in the hybrid cells (Ying et al. 2002; Pereira

et al. 2008). Moreover, the overexpression of Nanog in ES cells has been shown to augment the expression of pluripotency genes in undifferentiated neural stem cells (Silva et al. 2006). Since *Satb1*$^{-/-}$ ES cells display an impaired differentiation and deregulation of pluripotency factors, such as *Nanog,* we examined whether the lack of Satb1 had an effect on the reprogramming potential after heterokaryon formation with human B cells (Fig. 8A). After 3 d, which is the time period required for nuclei to merge after cell fusion, heterokaryons of human B cells and *Satb1*$^{-/-}$ ES cells were found to express human *NANOG* and *OCT4* at significantly higher levels than heterokaryons of human B cells and wild-type ES cells (Fig. 8B). The effects on markers of human ES cells such as *CRIPTO* and *REX1* were even more pronounced (Fig. 8B; Supplemental Fig. S10a), demonstrating the more efficient reactivation of the pluripotency machinery in heterokaryons of human B cells and Satb1-deficient ES cells. Similar results were obtained with the second *Satb1*$^{-/-}$ clone (data not shown). As a control, *HPRT* expression was not changed by fusions with *Satb1*$^{-/-}$ cells, demonstrating that the effect of Satb1 deficiency specifically affects pluripotency genes (Supplemental Fig. 10b). This difference in the reprogramming efficiency became even more pronounced 4 d after cell fusion (Supplemental Fig. 10d). Importantly, the frequency of cell fusions using *Satb1*$^{-/-}$ ES cells was similar to that observed with wild-type ES cells (Supplemental Fig. 10e,f).

Since the overexpression of Satb2 interferes with differentiation associates silencing of *Nanog*, and since Satb2 is overexpressed in *Satb1*$^{-/-}$ cultures, we used tetO-HA-Satb2 ES cells in fusion experiments. Induction of ectopic Satb2 expression during the time course of the experiments augmented the reactivation of *NANOG*, *OCT4*, and *CRIPTO,* similar to *Satb1*$^{-/-}$ cells (Fig. 8C; Supplemental Fig. 10c). Thus, the reprogramming capacity of these three ES cell clones further supports the model in which Satb1 negatively regulates various pluripotency factors and Satb2 activates *Nanog* expression, possibly by antagonizing the function of Satb1 (Fig. 8D).

Discussion

Recently, transcription factor networks underlying pluripotency have begun to be unraveled (for review, see Niwa 2007; Pan and Thomson 2007; Kim et al. 2008). In particular, Oct4, Nanog, and Sox2 have been shown to form interconnected autoregulatory loops that regulate ES cell identity (Boyer et al. 2005; Loh et al. 2006). In addition, Klf4 and Tbx3 have been found to regulate *Nanog* expression (Ema et al. 2008; Jiang et al. 2008; Niwa et al. 2009). In this study, we provide evidence that the DNA-binding protein Satb1 negatively regulates the expression of *Nanog*, *Klf4*, and *Tbx3*. The up-regulation of these pluripotency factors allows Satb1-deficient ES cells to self-renew in the absence of LIF and feeder cells for multiple passages, and these cells maintain high levels of Nanog expression even upon RA-mediated differentiation. Thus, *Satb1*$^{-/-}$ ES cells resemble ES cells in which Nanog is ectopically expressed and self-renewal is strongly favored over differentiation (Chambers et al. 2003; Mitsui et al. 2003; Niwa et al. 2009). Recently, heterogeneity of ES cells in their expression of Nanog has been attributed to differences in the self-renewal potential of individual cells (Chambers et al. 2007; Graf and Stadtfeld 2008). Nanog^{high} cells are self-renewing and differentiation-incompetent, whereas Nanog^{low} cells exit the pluripotent state and can differentiate, indicating that the down-regulation of Nanog is critically required for lineage commitment of ES cells (Chambers et al. 2007; Singh et al. 2007). Notably, in Satb1-deficient ES cells, the up-regulation of Nanog expression in the bulk culture can be accounted for by a marked increase in the frequency of Nanog^{high} cells. A recent study describes Oct4-mediated higher-order chromatin regulation of the *Nanog* and its flanking genes, *Apobec1*, *GDF3*, and *Dppa3* (Levasseur et al. 2008). In particular, chromosome conformation capture (3C) analysis indicated that all genes in the extended 160-kb *Nanog* locus are regulated by Oct4. Although Satb1 is a higher-order chromatin organizer that has been shown to regulate multiple genes in the major histocompatibility complex (MHC) class I gene cluster and the T_H2 cytokine gene cluster in T lymphocytes (Cai et al. 2006; Kumar et al. 2007), we found that Satb1 regulates predominantly *Nanog* and not the other genes in the cluster at the chromatin level. This gene-specific action of Satb1 in the *Nanog* locus is reminiscent of the gene-specific regulation of *Hoxa2* by Satb2 in osteoblasts (Dobreva et al. 2006). In ES cells, Satb1 is expressed at a level that is significantly lower than that found in T cells, raising the interesting possibility that an abundant expression and a cage-like subnuclear distribution of Satb1 may be required for the long-range action of Satb1, which has been proposed to act as a protein scaffold to which specific DNA sequences are tethered (Cai et al. 2003, 2006).

The regulation of *Nanog* also involves Satb2, which is expressed in the Nanog^{high} fraction of both wild-type and Satb1-deficient cultures. Moreover, the ectopic overexpression of Satb2 results in an increase of *Nanog* expression upon differentiation and down-regulation of *Satb1*, which suggests that these genes form a regulatory network. Notably, ectopic expression of Satb2 efficiently induces the reactivation of *NANOG* in heterokaryon fusion experiments. The similar effects of Satb1 deficiency and Satb2 overexpression raise the question of whether these genes regulate each other and whether Satb2 regulates the expression of *Nanog* indirectly via the repression of *Satb1*. A reciprocal cross-regulation of the *Satb1* and *Satb2* genes is unlikely, because both genes are expressed in ES cells and the expression of *Satb2* is unchanged upon Satb1 overexpression. Moreover, we detected only a twofold down-regulation of *Satb1* in Satb2-overexpressing ES cells. Finally, the ChIP experiments suggest that both Satb1 and Satb2 can bind to 5′-flanking sequences of the endogenous *Nanog* gene. Therefore, we favor an alternative model that is based on a differential activity of Satb1 and Satb2 homodimers and Satb1/Satb2 heterodimers. Satb proteins have a similar

domain structure, but differ in their interaction with other proteins. Satb1 and Satb2 can form both homodimers and heterodimers via a highly conserved PDZ domain (Galande et al. 2001; T Treiber and R Grosschedl, unpubl.). Interestingly, the PDZ domain of Satb1, but not that of Satb2, harbors a short peptide sequence that mediates association with the corepressor CtBP1 (C-terminal binding protein 1) (Purbey et al. 2009). In addition, Satb2 contains two SUMO acceptor sites that are not present in Satb1 (Dobreva et al. 2003). Thus, changes in the compositions of Satb dimers could alter the transcriptional activation of Satb target genes, which would be reminiscent of the well-studied example of the AP1 family of transcription factors (Wagner 2002). According to this view, the heterogeneity of wild-type ES cells in their level of Nanog expression could reflect differences in the dimer composition of Satb proteins.

Loss-of-function mutations in genes encoding pluripotency factors, such as Nanog, Oct4, and Sox2, typically preclude the derivation of self-renewing stem cell lines from mutant embryos (Nichols et al. 1998; Avilion et al. 2003; Chambers et al. 2003). For example, Nanog is critically required for the establishment of ES cell cultures from the early embryo, but not for the maintenance of these cells in vitro (Chambers et al. 2007). The inability to derive Satb2-deficient ES cell lines by cellular outgrowth of preimplantation embryos may be accounted for by the lack of Satb2-mediated repression of Satb1, which may result in differentiation at the expense of self-renewal. This view is strengthened by the progressive adaptation of *Satb1*$^{+/-}$*Satb2*$^{-/-}$ cells to in vitro culture, evidenced by a change in cellular morphology and reduction of *Satb1* expression. Moreover, we were not able to generate continuously growing Satb2-deficient lines from ES cells carrying two floxed *Satb2* alleles by Cre-mediated deletion in vitro. Likewise, we were not able to generate subclones of *Satb1*$^{-/-}$ ES cells re-expressing Satb1 at high levels, most likely due to a proliferative disadvantage of such cells compared with Satb1-deficient ES cells maintained under LIF conditions. In fact, *Satb1*$^{-/-}$ *Satb2*$^{-/-}$ cells in which Satb1 is re-expressed display rapid loss of *Satb1* expression during continuous culture. Both Satb1 and Satb2 are dispensable for proper embryonic development, indicating that the early embryo is an environment permissive for the deregulated *Nanog* expression caused by the lack of Satb1 and Satb2 observed in ES cell cultures. Our data, however, demonstrate that the function of Satb1 and Satb2 is critically required for the proper differentiation of ES cells into somatic cells in vitro, most likely by regulating the heterogeneity of Nanog expression in ES cell cultures—a process not present in vivo that is, however, indispensable for the application of pluripotent stem cells in regenerative medicine (Daley and Scadden 2008).

Apart from its role in mediating proper *Nanog*, *Klf4*, *and Tbx3* expression in undifferentiated cells, Satb1 may also function in the process of ES cell differentiation by regulating the expression of *Bcl2,* which is required for neural commitment of ES cells (Trouillas et al. 2008). *BCL2* is a SATB1 target in human cells (Ramakrishnan et al. 2000) and, in agreement with this observation, we found that *Bcl2* is also a Satb1 target in murine ES cells.

In contrast to the regulation of pluripotency genes by Satb1 and Satb2, the colinear expression of *Hox* genes does not require Satb proteins and can be efficiently induced in *Satb1*$^{-/-}$ ES cells. This finding separates the activation of *Hox* genes from other epigenetic processes such as XCI and genomic imprinting, which depend on a specific cellular context and are linked to pluripotency (Wutz and Jaenisch 2000; Navarro et al. 2008). Interestingly, Satb1 and Satb2 were implicated recently in the synergistic regulation of XCI (Agrelo et al. 2009). As XCI has been shown to be tightly controlled by the pluripotency machinery of the cells (Navarro et al. 2008; Donohoe et al. 2009), we suggest that the effect of Satb1 and/or Satb2 in this process might be indirect, through regulation of *Nanog* and *Klf4*.

In conclusion, our results demonstrate novel functions for Satb proteins. Satb1 represses the expression of genes encoding the pluripotency factors *Nanog, Klf4*, and *Tbx3*. Satb2 is required for the efficient derivation and survival of pluripotent cell lines with functional *Satb1* alleles, and its expression marks the self-renewing Nanog^{high} fraction of ES cell cultures. Our data also suggest antagonistic activities of Satb1 and Satb2 that may involve potential changes in dimer composition and protein activity. Thus, the relative levels of Satb1 and Satb2 may regulate the balance of self-renewal versus differentiation.

Materials and methods

Derivation of ES cell lines

Single embryonic day 3.5 (E3.5) blastocysts were cultured on irradiated MEFs in ES cell medium (DMEM, 15% FCS, LIF, PSG, NEAA, sodium pyruvate) supplemented with the MEK inhibitor PD98059. We noticed that the inhibition of the MAPK/Erk pathway during the derivation of ES cells may influence their dependence on Satb proteins. After 7 d, the medium was changed; after 10 d, cells were split on new feeders and culture was continued without PD98059. Genotypes of the individual lines were tested by PCR on genomic DNA. We tested two clones per genotype to rule out clonal variations, except for the *Satb1*/*Satb2* double-deficient cells as only one cell line was available.

ES cell culture

ES cells were generally passaged on feeders every 3 d. After trypsinization, cells were resuspended in ES medium and the suspension was allowed to settle for 5 min in falcon tubes in order to get rid of feeders and cellular aggregates. For random differentiation, cells were plated on gelatine-coated dishes in ES cell medium without LIF. For neural differentiation and *Hox* gene induction, RA was added at a 5 μM concentration 1 d after LIF withdrawal, as published (Chambeyron and Bickmore 2004). In general, cells were differentiated for 6 d after plating. At day 6, cells were trypsinized and put on new gelatine-coated dishes. In all differentiation experiments in which the *Oct4*-HygroTK selection marker was used, undifferentiated cells were cultured for one passage in ES cell medium supplemented with 100 μg/mL hygromycin. Counterselection with 2.5 μM gancyclovir was initiated after 6 d of differentiation, if applicable.

Generation of tetO-HA-Satb1 and tetO-HA-Satb2 cells

Wild-type ES cells were electroporated with a *Rosa26*-nlsrtTA-puromycinR targeting vector (Wutz and Jaenisch 2000), underwent selection, and were subsequently genotyped by Southern blotting. Positive clones were coelectroporated with a hygromycin resistance cDNA under the control of the *Pgk* promoter, and a with plasmid encoding either HA-tagged Satb1 or Satb2 under the control of the tet-operator (tetO-HA-Satb1/2). After electroporation, cells were kept on ES medium supplemented with 100 μg/mL hygromycin for 1 wk. After 10 d, individual clones were picked and analyzed by immunoblotting both under uninduced and induced conditions (ES medium supplemented with 1 μg/mL doxycycline for 24 h).

Generation of Oct4-*HygroTK cells*

ES cells of all genotypes were electroporated with the *Oct4*-IRES-HygTKpA plasmid (generously provided by A. Smith). Cells underwent selection for 7 d in ES cell medium supplemented with 100 μg/mL hygromycin. Two clones were picked per genotype and analyzed under differentiating conditions. Cells were differentiated by the addition of RA for 5 d; subsequently, cells were plated on gelatine-coated dishes and exposed to gancyclovir starting at day 6 for 4 d. At day 10 of differentiation, RNA was harvested and it was confirmed that all surviving clones had virtually undetectable *Oct4* levels compared with control cultures, which were not exposed to gancyclovir. Two clones per genotype were analyzed to rule out clonal effects. In addition, a PCR specific for the homologous recombination into the *Oct4* locus was performed. $Satb1^{-/-}Satb2^{-/-}$ cells were subsequently electroporated with a vector carrying an HA-Satb1 cDNA under the control of the *Pgk* promoter and analyzed for Satb1 expression by immunoblotting after selection and subcloning.

Additional methods are described in the Supplemental Material

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References

Agrelo R, Souabni A, Novatchkova M, Haslinger C, Leeb M, Komnenovic V, Kishimoto H, Gresh L, Kohwi-Shigematsu T, Kenner L, et al. 2009. SATB1 defines the developmental context for gene silencing by Xist in lymphoma and embryonic cells. *Dev Cell* **16:** 507–516.

Alvarez JD, Yasui DH, Niida H, Joh T, Loh DY, Kohwi-Shigematsu T. 2000. The MAR-binding protein SATB1 orchestrates temporal and spatial expression of multiple genes during T-cell development. *Genes & Dev* **14:** 521–535.

Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R. 2003. Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes & Dev* **17:** 126–140.

Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, et al. 2006. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* **125:** 315–326.

Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, et al. 2005. Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* **122:** 947–956.

Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, Levine SS, Wernig M, Tajonar A, Ray MK, et al. 2006. Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* **441:** 349–353.

Cai S, Han HJ, Kohwi-Shigematsu T. 2003. Tissue-specific nuclear architecture and gene expression regulated by SATB1. *Nat Genet* **34:** 42–51.

Cai S, Lee CC, Kohwi-Shigematsu T. 2006. SATB1 packages densely looped, transcriptionally active chromatin for coordinated expression of cytokine genes. *Nat Genet* **38:** 1278–1288.

Chambers I. 2004. The molecular basis of pluripotency in mouse embryonic stem cells. *Cloning Stem Cells* **6:** 386–391.

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. 2003. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113:** 643–655.

Chambers I, Silva J, Colby D, Nichols J, Nijmeijer B, Robertson M, Vrana J, Jones K, Grotewold L, Smith A. 2007. Nanog safeguards pluripotency and mediates germline development. *Nature* **450:** 1230–1234.

Chambeyron S, Bickmore WA. 2004. Chromatin decondensation and nuclear reorganization of the HoxB locus upon induction of transcription. *Genes & Dev* **18:** 1119–1130.

Daley GQ, Scadden DT. 2008. Prospects for stem cell-based therapy. *Cell* **132:** 544–548.

Dobreva G, Dambacher J, Grosschedl R. 2003. SUMO modification of a novel MAR-binding protein, SATB2, modulates immunoglobulin μ gene expression. *Genes & Dev* **17:** 3048–3061.

Dobreva G, Chahrour M, Dautzenberg M, Chirivella L, Kanzler B, Farinas I, Karsenty G, Grosschedl R. 2006. SATB2 is a multifunctional determinant of craniofacial patterning and osteoblast differentiation. *Cell* **125:** 971–986.

Donohoe ME, Silva SS, Pinter SF, Xu N, Lee JT. 2009. The pluripotency factor Oct4 interacts with Ctcf and also controls X-chromosome pairing and counting. *Nature* **460:** 128–132.

Ema M, Mori D, Niwa H, Hasegawa Y, Yamanaka Y, Hitoshi S, Mimura J, Kawabe Y, Hosoya T, Morita M, et al. 2008. Kruppel-like factor 5 is essential for blastocyst development and the normal self-renewal of mouse ESCs. *Cell Stem Cell* **3:** 555–567.

Galande S, Dickinson LA, Mian IS, Sikorska M, Kohwi-Shigematsu T. 2001. SATB1 cleavage by caspase 6 disrupts PDZ domain-mediated dimerization, causing detachment from chromatin early in T-cell apoptosis. *Mol Cell Biol* **21:** 5591–5604.

Graf T, Stadtfeld M. 2008. Heterogeneity of embryonic and adult stem cells. *Cell Stem Cell* **3:** 480–483.

Hayashi K, Lopes SM, Tang F, Surani MA. 2008. Dynamic equilibrium and heterogeneity of mouse pluripotent stem cells with distinct functional and epigenetic states. *Cell Stem Cell* **3:** 391–401.

Jaenisch R, Young R. 2008. Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell* **132:** 567–582.

Jiang J, Chan YS, Loh YH, Cai J, Tong GQ, Lim CA, Robson P, Zhong S, Ng HH. 2008. A core Klf circuitry regulates self-renewal of embryonic stem cells. *Nat Cell Biol* **10:** 353–360.

Kim J, Chu J, Shen X, Wang J, Orkin SH. 2008. An extended transcriptional network for pluripotency of embryonic stem cells. *Cell* **132:** 1049–1061.

Kmita M, Duboule D. 2003. Organizing axes in time and space; 25 years of colinear tinkering. *Science* **301:** 331–333.

Kumar PP, Purbey PK, Ravi DS, Mitra D, Galande S. 2005. Displacement of SATB1-bound histone deacetylase 1 corepressor by the human immunodeficiency virus type 1 transactivator induces expression of interleukin-2 and its receptor in T cells. *Mol Cell Biol* **25:** 1620–1633.

Kumar PP, Bischof O, Purbey PK, Notani D, Urlaub H, Dejean A, Galande S. 2007. Functional interaction between PML and SATB1 regulates chromatin-loop architecture and transcription of the MHC class I locus. *Nat Cell Biol* **9:** 45–56.

Levasseur DN, Wang J, Dorschner MO, Stamatoyannopoulos JA, Orkin SH. 2008. Oct4 dependence of chromatin structure within the extended Nanog locus in ES cells. *Genes & Dev* **22:** 575–580.

Li E. 2002. Chromatin modification and epigenetic reprogramming in mammalian development. *Nat Rev Genet* **3:** 662–673.

Li Y, McClintick J, Zhong L, Edenberg HJ, Yoder MC, Chan RJ. 2005. Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Klf4. *Blood* **105:** 635–637.

Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J, et al. 2006. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet* **38:** 431–440.

Loh YH, Zhang W, Chen X, George J, Ng HH. 2007. Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. *Genes & Dev* **21:** 2545–2557.

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S. 2003. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* **113:** 631–642.

Murry CE, Keller G. 2008. Differentiation of embryonic stem cells to clinically relevant populations: Lessons from embryonic development. *Cell* **132:** 661–680.

Navarro P, Chambers I, Karwacki-Neisius V, Chureau C, Morey C, Rougeulle C, Avner P. 2008. Molecular coupling of Xist regulation and pluripotency. *Science* **321:** 1693–1695.

Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H, Smith A. 1998. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95:** 379–391.

Niwa H. 2007. How is pluripotency determined and maintained? *Development* **134:** 635–646.

Niwa H, Burdon T, Chambers I, Smith A. 1998. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes & Dev* **12:** 2048–2060.

Niwa H, Ogawa K, Shimosato D, Adachi K. 2009. A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. *Nature* **460:** 118–122.

Pan G, Thomson JA. 2007. Nanog and transcriptional networks in embryonic stem cell pluripotency. *Cell Res* **17:** 42–49.

Pavan Kumar P, Purbey PK, Sinha CK, Notani D, Limaye A, Jayani RS, Galande S. 2006. Phosphorylation of SATB1, a global gene regulator, acts as a molecular switch regulating its transcriptional activity in vivo. *Mol Cell* **22:** 231–243.

Pereira CF, Terranova R, Ryan NK, Santos J, Morris KJ, Cui W, Merkenschlager M, Fisher AG. 2008. Heterokaryon-based reprogramming of human B lymphocytes for pluripotency requires Oct4 but not Sox2. *PLoS Genet* **4:** e1000170. doi: 10.1371/journal.pgen.1000170.

Purbey PK, Singh S, Notani D, Kumar PP, Limaye AS, Galande S. 2009. Acetylation-dependent interaction of SATB1 and CtBP1 mediates transcriptional repression by SATB1. *Mol Cell Biol* **29:** 1321–1337.

Ramakrishnan M, Liu WM, DiCroce PA, Posner A, Zheng J, Kohwi-Shigematsu T, Krontiris TG. 2000. Modulated binding of SATB1, a matrix attachment region protein, to the AT-rich sequence flanking the major breakpoint region of BCL2. *Mol Cell Biol* **20:** 868–877.

Silva J, Chambers I, Pollard S, Smith A. 2006. Nanog promotes transfer of pluripotency after cell fusion. *Nature* **441:** 997–1001.

Singh AM, Hamazaki T, Hankowski KE, Terada N. 2007. A heterogeneous expression pattern for Nanog in embryonic stem cells. *Stem Cells* **25:** 2534–2542.

Smith AG. 2001. Embryo-derived stem cells: Of mice and men. *Annu Rev Cell Dev Biol* **17:** 435–462.

Spivakov M, Fisher AG. 2007. Epigenetic signatures of stem-cell identity. *Nat Rev Genet* **8:** 263–271.

Takahashi K, Yamanaka S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126:** 663–676.

Toyooka Y, Shimosato D, Murakami K, Takahashi K, Niwa H. 2008. Identification and characterization of subpopulations in undifferentiated ES cell culture. *Development* **135:** 909–918.

Trouillas M, Saucourt C, Duval D, Gauthereau X, Thibault C, Dembele D, Feraud O, Menager J, Rallu M, Pradier L, et al. 2008. Bcl2, a transcriptional target of p38α, is critical for neuronal commitment of mouse embryonic stem cells. *Cell Death Differ* **15:** 1450–1459.

Wagner EF. 2002. Functions of AP1 (Fos/Jun) in bone development. *Ann Rheum Dis* **61:** ii40–ii42. doi: 10.1136/ard.61.suppl_2.ii40.

Wutz A, Jaenisch R. 2000. A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. *Mol Cell* **5:** 695–705.

Yasui D, Miyano M, Cai S, Varga-Weisz P, Kohwi-Shigematsu T. 2002. SATB1 targets chromatin remodelling to regulate genes over long distances. *Nature* **419:** 641–645.

Ying QL, Nichols J, Evans EP, Smith AG. 2002. Changing potency by spontaneous fusion. *Nature* **416:** 545–548.

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, et al. 2007. Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318:** 1917–1920.