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Satb1 and Satb2 Are Dispensable for X Chromosome Inactivation in Mice

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SUMMARY

Satb1 and Satb2 have been recently described as regulators of embryonic stem (ES) cell pluripotency and as silencing factors in X chromosome inactivation. The influence of the pluripotency machinery on X chromosome inactivation and the lack of an X chromosome inactivation defect in *Satb1*$^{-/-}$ and *Satb2*$^{-/-}$ mice raise the question of whether or not Satb proteins are directly and/or redundantly involved in this process. Here, we analyzed X chromosome inactivation in fibroblastic cells that were derived from female *Satb1*$^{-/-}$*Satb2*$^{-/-}$ embryos. By fluorescence in situ hybridization to visualize *Xist* RNA and by immunohistochemistry to detect H3K27me3 histone modifications, we found that female *Satb1*$^{-/-}$ *Satb2*$^{-/-}$ fibroblastic cells contain proper Barr bodies. Moreover, we did not detect an upregulation of X-linked genes, suggesting that Satb proteins are dispensable for X chromosome inactivation in mice.

INTRODUCTION

X chromosome inactivation (XCI) is the process by which female mammals achieve an equal ratio of X chromosomal to autosomal gene expression compared to male cells (Chow and Heard, 2009; Payer and Lee, 2008). Random XCI can be observed in early embryos (around E6.5) in vivo or in differentiating embryonic stem (ES) cells in vitro (Payer and Lee, 2008). Although pluripotent cells are not the only cells that can initiate XCI, they are the only cells that actually undergo endogenous X chromosome inactivation (Chow et al., 2007; Hall et al., 2002; Savarese et al., 2006). Notably, recent reports implicated the pluripotency machinery of ES cells in the regulation of key determinants of XCI, the noncoding RNA *Xist* and its antagonist *Tsix* (Donohoe et al., 2009; Navarro et al., 2008, 2010). *Xist* coats the inactive X chromosome (Xi) in *cis*, leading to a series of epigenetic modifications that are thought to act redundantly in the maintenance of a transcriptionally silent state (Brockdorff et al., 1992; Kohlmaier et al., 2004; Schoeftner et al., 2006).

Proteins required for the silencing of the inactive X chromosome were only recently identified. SmcHD1, a protein with a structural-maintenance-of-chromosomes (SCM) hinge domain characteristic of proteins involved in chromosome condensation and cohesion, was implicated in the maintenance of the silent state of the Xi (Blewitt et al., 2008). Embryos lacking SmcHD1 show defective XCI in vivo (Blewitt et al., 2008). However, SmcHD1-deficient female embryos display a rather late embryonic lethality, and hence it is thought that SmcHD1 plays a role in the maintenance of the silent state of the Xi, rather than in the initiation of silencing. Recently, the chromatin organizer Satb1 was identified as a critical regulator of *Xist*-mediated silencing (Agrelo et al., 2009; Cai et al., 2003). Differential gene expression profiling of *Xist*-resistant and *Xist*-sensitive T cell lymphomas identified *Satb1* among several genes that were specifically expressed in silencing-competent lymphomas (Agrelo et al., 2009). Moreover, Satb1 knockdown experiments in pluripotent ES cells and overexpression studies, in which Satb1 was found to confer upon fibroblasts the ability of initiating de novo XCI, suggested a role of Satb1 in the initiation of XCI (Agrelo et al., 2009). However, *Satb1*$^{-/-}$ female embryos do not display early lethality associated with aberrant XCI, raising the possibility of a functional redundancy with the closely related Satb2 protein (Dobreva et al., 2003, 2006). Both Satb1 and Satb2 are expressed at the onset of XCI in ES cell differentiation; however, the analysis of a potential functional redundancy of Satb1 and Satb2 was precluded by the inability to generate stable Satb1/Satb2 double knockdown ES cells (Agrelo et al., 2009).

Satb1 and Satb2 were also shown to regulate the pluripotency of ES cells, whereby Satb1 and Satb2 play an opposite role in the regulation of ES cell pluripotency and *Nanog* gene expression (Savarese et al., 2009). In particular, Satb1 was shown to repress *Nanog*, whereas Satb2 was found to activate *Nanog*. An antagonistic role of Satb proteins in the regulation of *Nanog* was also inferred from the analysis of *Satb1*$^{-/-}$*Satb2*$^{-/-}$ ES cells, which showed a less severe defect in self-renewal and differentiation than the corresponding single knockout ES cells (Savarese et al., 2009). Recently, these findings were independently confirmed by a small hairpin RNA (shRNA) screen (Kagey et al., 2010). Regulators of ES cell pluripotency, such as Oct4, have been found to indirectly regulate XCI (Donohoe et al., 2009; Navarro et al., 2008, 2010), raising the question of whether Satb1 and Satb2 modulate *Xist* expression via the pluripotency machinery, which restricts the potential of a cell to initiate *Xist*-mediated gene silencing.

To better understand the roles of Satb1 and Satb2 in XCI, we analyzed dosage compensation and the Xi in E13.5 mouse embryonic fibroblasts (MEFs) generated from *Satb1*$^{-/-}$, *Satb2*$^{-/-}$, and *Satb1*$^{-/-}$*Satb2*$^{-/-}$ female mice. We found that

Table 1. Statistics of the Genotypes of E13.5 Embryos Derived from *Satb1*$^{+/-}$*Satb2*$^{+/-}$ Intercrosses

Satb1	*Satb2*	n
+/+	+/+	4
+/+	+/−	24
+/+	−/−	15
+/−	+/+	17
+/−	+/−	53
+/−	−/−	34
−/−	+/+	12
−/−	+/−	30
−/−	−/−	7
total	196	

Of seven *Satb1*/*Satb2* double-deficient embryos, three were female and four were male. Most *Satb1*/*Satb2* double-mutant mice die independent of their sex after day 14.5, without showing any obvious phenotypic abnormalities at E13.5.

female *Satb1*$^{-/-}$*Satb2*$^{-/-}$ MEFs contained a normal Barr body and were properly dosage compensated, suggesting that Satb1 and Satb2 are dispensable for XCI in vivo.

RESULTS AND DISCUSSION

Female *Satb1/Satb2* Double-Deficient Embryos Are Viable

To examine a potential redundancy of Satb1 and Satb2 in the process of XCI, we generated E13.5 MEFs from intercrosses of *Satb1*$^{+/-}$*Satb2*$^{+/-}$ animals (Alvarez et al., 2000; Dobreva et al., 2006). Upon culture, we determined the genotype and sex of the embryos by PCR analysis (see Figure S1A available online). Because mutations in genes required for XCI result in early embryonic lethality around E6.5, we were surprised about the presence of female *Satb1*$^{-/-}$*Satb2*$^{-/-}$ embryos at day E13.5, from which we could derive MEFs (Table 1). Multiple rounds of genotyping confirmed that these cells were female and deficient for both *Satb1* and *Satb2* (Figure S1 and data not shown). Among 196 embryos, we detected three female and four male *Satb1*$^{-/-}$*Satb2*$^{-/-}$ embryos, slightly less than the expected six plus six embryos according to Mendelian inheritance. These findings indicate that XCI can be initiated during embryogenesis in the absence of both Satb1 and Satb2.

Satb1$^{-/-}$*Satb2*$^{-/-}$ MEFs Display Proper Cytological and Molecular Features of X Chromosome Inactivation

To examine whether the targeted inactivation of *Satb1* and *Satb2* influences the cytological manifestations of the Xi, we analyzed the Barr-body in female wild-type, *Satb1*$^{-/-}$ and *Satb2*$^{-/-}$ single-mutant MEFs, *Satb1*$^{-/-}$*Satb2*$^{-/-}$ double-mutant MEFs, and male wild-type MEFs, which served as a negative control. We performed *Xist* RNA fluorescence in situ hybridization (FISH) and immunfluorescence (IF) staining against H3K27me3, a well-described mark of the Xi (Kohlmaier et al., 2004; Plath et al., 2003). The localization of *Xist* and the pattern of H3K27me3 were normal not only in wild-type, *Satb1*$^{-/-}$, and *Satb2*$^{-/-}$ female MEFs, but also in *Satb1*$^{-/-}$*Satb2*$^{-/-}$ female MEFs (Figure 1A). Moreover, the analysis of the frequencies of cells with focal staining of *Xist* and H3K27me3 did not reveal significant differences between the various genotypes (Figure 1B). Thus, we failed to detect cytological manifestations of aberrant XCI, consistent with the similar growth rates of the wild-type, *Satb*$^{-/-}$, and *Satb1*$^{-/-}$*Satb2*$^{-/-}$ MEFs (data not shown). Because MEFs do not express Satb1 and Satb2 at a detectable level, these proteins do not appear to be required for *Xist* localization in these cells.

Focal *Xist* localization and H3K27me3 staining are cytological manifestations of the Xi but are not direct molecular readouts of a transcriptionally silent chromosome (Plath et al., 2003). Without proper dosage compensation, female embryos do not survive until E13.5, and they preclude the derivation of MEFs (Marahrens et al., 1997; Penny et al., 1996). To examine potential consequences of the Satb1 and Satb2 deficiencies on the silencing process itself, we analyzed gene expression of X-linked and autosomal genes in female wild-type, *Satb1*$^{-/-}$, *Satb2*$^{-/-}$, and two independent lines of *Satb1*$^{-/-}$*Satb2*$^{-/-}$ MEFs, as well as in male wild-type MEFs. Analysis of *Pgk1* and *Hprt*, genes normally silent on the Xi, revealed no dosage imbalance between these transcripts and those of the autosomal gene *Gapdh* in female wild-type, *Satb1*$^{-/-}$, *Satb2*$^{-/-}$, and *Satb1*$^{-/-}$*Satb2*$^{-/-}$ MEFs compared to male wild-type MEFs (Figure 2). *Pgk1* expression was slightly lower in *Satb2*$^{-/-}$ MEFs than in the other cells, which might be explained by the slightly slower growth of *Satb2*$^{-/-}$ MEFs. In addition, we analyzed the expression of *Xist* and found that it was normally expressed in female MEFs of all genotypes examined (Figure 2).

No Evidence for Residual Satb1 or Satb2 Expression in Satb1/Satb2 Double-Deficient MEFs

To examine whether or not the targeted mutations of *Satb1* and *Satb2* allow for the generation of protein with residual activity, we analyzed both protein and RNA expression in wild-type and mutant cells. The mutation of the *Satb1* allele removes exons 1–5, encoding the PDZ dimerization domain (Alvarez et al., 2000). A truncated protein could theoretically be produced by translation from an in-frame ATG in exon 6 (Figure S2A). However, such a putative truncated form of Satb1 would not dimerize and would be severely impaired in its DNA-binding efficiency (Purbey et al., 2008). By immunoblot analysis with a previously published polyclonal anti-Satb1 antiserum that also detects epitopes in the C-terminal half of Satb1 and has some cross-reactivity toward the closely related Satb2 protein (Figures S2B–S2D), no truncated form of Satb1 was detected in *Satb1*$^{-/-}$ and *Satb1*$^{-/-}$*Satb2*$^{-/-}$ ES cells (Figure S2B). In undifferentiated and differentiating *Satb1*$^{-/-}$*Satb2*$^{-/-}$ cells, grown in LIF- and retinoic acid-containing medium, respectively, we also failed to detect *Satb1* transcripts encoding the PDZ domain or the DNA-binding domain (Figure S2E). Likewise, the *Satb2* allele, used for the generation of *Satb1*$^{-/-}$*Satb2*$^{-/-}$ mice, is most likely a null allele, because it generates virtually the same mutant phenotype as a different mutant *Satb2* allele that was generated by a different targeting strategy (Alcamo et al., 2008; Britanova et al., 2008). Taken together, these data suggest that the lack of a defect of X inactivation in *Satb1*$^{-/-}$*Satb2*$^{-/-}$ mice cannot be explained by residual Satb1 and/or Satb2 activity.

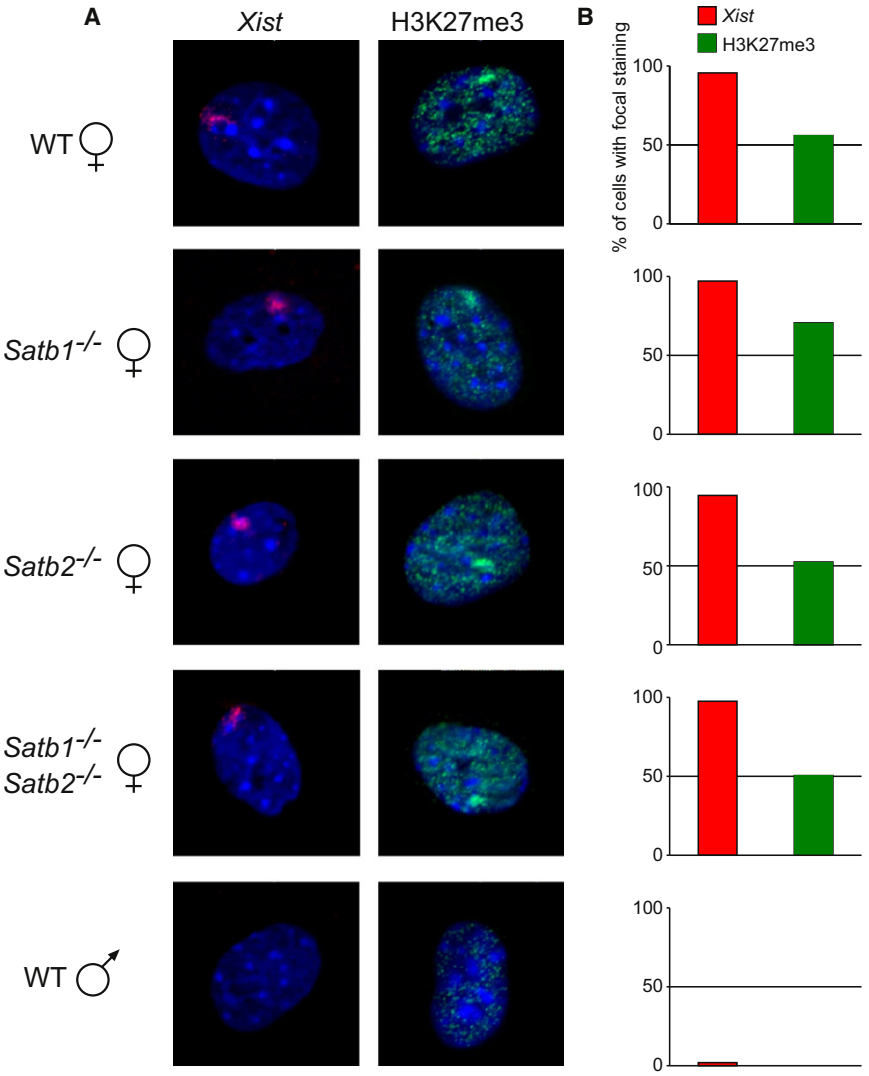

Figure 1. Satb1 and Satb2 Are Dispensable for Barr Body Formation

(A) *Xist* RNA FISH and H3K27me3 immunofluorescence stainings reveal normal focal *Xist* and H3K27me3 signals in female *Satb1*$^{-/-}$, *Satb2*$^{-/-}$, and *Satb1*$^{-/-}$*Satb2*$^{-/-}$ MEFs.

(B) Statistical analysis of cells displaying focal *Xist* or focal H3K27me3 staining. For each cell type and staining, more than 100 individual nuclei were analyzed.

Satb1 and Satb2 Are Dispensable for X Chromosome Inactivation In Vivo

Our findings that female embryos are properly dosage compensated in the absence of both Satb1 and Satb2 in vivo raise issues about the roles of these proteins in XCI in ES cells. Although the targeted inactivation of both *Satb1* and *Satb2* does not significantly affect cell viability, the double knockdown of both genes by small interfering RNA (siRNA) results in cell lethality (Agrelo et al., 2009; Savarese et al., 2009). Differences in the effects of siRNA-mediated downregulation and targeted gene inactivation on ES cell pluripotency were reported for the *REST* gene (Buckley et al., 2009; Jørgensen et al., 2009; Jørgensen and Fisher, 2010; Singh et al., 2008). To date, no gene has been identified that is required for XCI in ES cells but not in mice, which is not surprising because ES cells serve as a bona fide model for the molecular mechanism of XCI in early embryos (Lee et al., 1996; Wutz and Jaenisch, 2000). However, the influence of the pluripotency machinery on XCI and the functional relationship between Nanog, Oct4, and the expression of *Xist* in ES cells have not yet been addressed in embryos (Donohoe et al., 2009; Navarro et al., 2008, 2010). Moreover, the developmental window in which pluripotent cells are present in the embryo is temporally limited, whereas the maintenance of the pluripotent state is the defining hallmark of ES cells (Jaenisch and Young, 2008; Silva and Smith, 2008).

Satb1 and Satb2 Are Dynamically Expressed during ES Cell Differentiation

The finding that the ability for *Xist* to initiate chromosomal silencing is limited to the first few days of ES cell differentiation provided an understanding of the cellular basis of XCI and explained the regulation of this process by factors like Nanog and Oct4 (Donohoe et al., 2009; Navarro et al., 2008, 2010; Wutz and Jaenisch, 2000; Wutz et al., 2002). Therefore, the expression and/or activity of a factor that is solely involved in regulating XCI would have to be limited to undifferentiated ES cells and the earliest stages of ES cell differentiation (Brockdorff, 2009). Although Satb1 and Satb2 were reported to be expressed in this way (Agrelo et al., 2009), we failed to observe a decrease of *Satb1* RNA and protein expression under various differentiation conditions (Savarese et al., 2009). Immunoblot analysis with a newly available monoclonal anti-Satb1 antibody indicated that Satb1 protein expression is augmented at the onset of retinoic acid-induced differentiation of ES cells and is not significantly altered during further differentiation (Figure 3A). In this experiment, two different wild-type ES cell lines, including the germline-competent cell line W4 and an ES cell line that allows for selection of undifferentiated or differentiated cells (Savarese et al., 2009), were used to monitor Satb1 protein levels during differentiation. The maintenance of Satb1 expression during ES cell differentiation was also observed with the polyclonal anti-Satb1 antiserum (Figure S2D).

Moreover, Satb1 expression does not simply correlate with the Xi silencing competence of developing thymocytes, which is found in CD4^{+}CD8^{+} cells but not in earlier-stage CD4^{-}CD8^{-} cells or in later-stage CD4^{-}CD8^{+} and CD4^{+}CD8^{-} cells (Savarese et al., 2006). *Satb1* was identified as a gene that is downregulated in *Xist*-resistant thymic lymphoma cells relative to

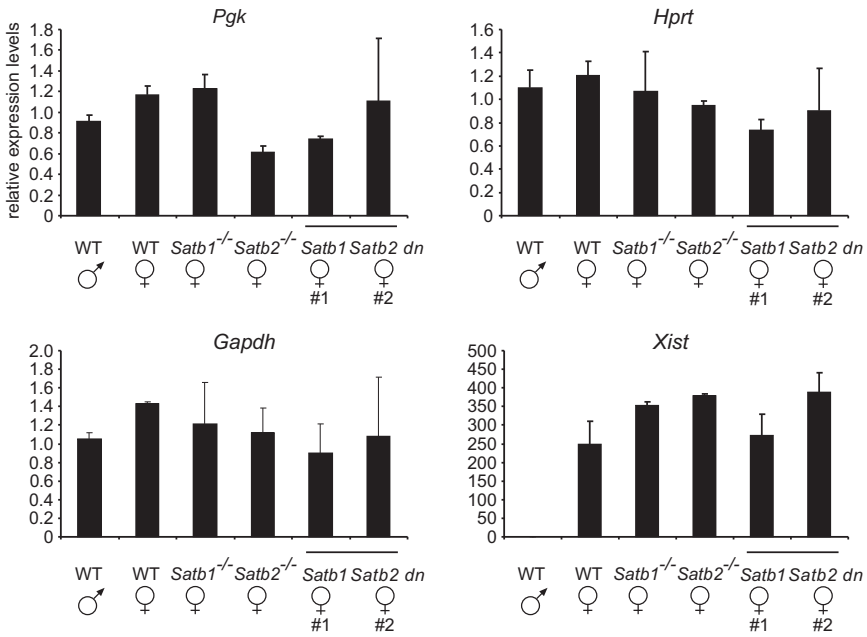

Figure 2. X Chromosomal Gene Silencing Is Normal in the Absence of Satb1 and Satb2

Quantitative RT-PCR analysis of the indicated transcripts reveals that the X-linked genes *Pgk1* and *Hprt* are not de-repressed in MEFs from female embryos lacking either Satb1(*Satb1*$^{-/-}$), Satb2 (*Satb2*$^{-/-}$), or both Satb1 and Satb2 (*Satb1Satb2dn*). Results from MEFs of two *Satb1Satb2dn* embryos are shown. The autosomal gene *Gapdh* serves as a control, demonstrating no dosage imbalance between X-linked and autosomal transcripts. Quantitative RT-PCR analysis of *Xist* demonstrates that Satb1 and Satb2 are not required for proper *Xist* expression. Error bars indicate standard deviation (SD).

Xist-responsive thymic lymphoma cells (Agrelo et al., 2009). However, all stages of differentiating T cells express significantly higher *Satb1* levels than ES cells, and abundant Satb1 expression is detected in the silencing-incompetent mature CD4^{+}CD8^{-} and CD4^{-}CD8^{+} thymocytes (Figures 3B–3D).

Molecular Mediators of XCI: Pluripotency Genes, Nuclear Matrix, or Factor "X"?

Although the induction of *Xist* responsiveness in Satb1-overexpressing cells supports a role of Satb1 in the initiation of X inactivation, what could account for the absence of an X inactivation defect in MEFs derived from female *Satb1*$^{-/-}$*Satb2*$^{-/-}$ embryos and the development of female *Satb1*$^{-/-}$*Satb2*$^{-/-}$ embryos? Our data do not favor the simplest possibility that *Satb1*$^{-/-}$*Satb2*$^{-/-}$ embryos produce residual, possibly truncated or alternative forms of Satb1 and Satb2. However, Satb proteins could influence XCI indirectly via the activation of the pluripotency machinery. The pluripotency marker and transcription factor Rex-1 is induced by Satb2 expression in cell fusions of ES cells and human B lymphocytes (Savarese et al., 2009). Rex1 binds and activates the *Tsix* gene (Navarro et al., 2010), and therefore an indirect regulation of *Tsix* expression by Satb proteins may influence XCI. Another possible explanation for the dispensable function of Satb1 and Satb2 in XCI and the lack of a simple correlation of the Xi silencing competence and Satb1 expression is a redundancy with a yet-unidentified determinant of X inactivation. A potential candidate for a protein that may compensate for the combined loss of Satb1 and Satb2 is SAF-A (hnRNP-U), which was demonstrated to mark the inactive X (Hasegawa et al., 2010; Helbig and Fackelmayer, 2003; Nakagawa and Prasanth, 2011; Pullirsch et al., 2010). Similar to Satb1 and Satb2, SAF-A has been previously identified as a protein that binds to nuclear matrix attachment regions (Hart and Laemmli, 1998; Scheuermann and Garrard, 1999). Proteins that bind to nuclear matrix regions have been proposed to mediate long-range chromosomal interactions; despite gene-specific functions of Satb proteins, the common property of binding to nuclear matrix attachment regions may account for a potential redundancy of Satb proteins and SAF-A in X chromosome inactivation (Nakagawa and Prasanth, 2011). However, the expression of SAF-A does not also mirror Xi silencing competence, suggesting that another yet-unidentified protein may be involved in X chromosome inactivation.

EXPERIMENTAL PROCEDURES

RNA FISH and Immunofluorescence

MEFs were plated the previous day on gelatinized slides for immunofluorescence and RNA FISH experiments. RNA FISH was performed as described previously (Gribnau et al., 1998). In brief, cells were fixed with 4% formamide/5% acetic acid/0.9% NaCl at room temperature for 18 min, washed with PBS, and permeabilized with 0.01% pepsin digestion in 0.01 M HCl for 5 min at 37°C. Cells were postfixed with 3.7% formamide at room temperature for 5 min. The slides were washed with PBS and dehydrated with ethanol baths prior to hybridization. Hybridization was performed overnight at 37°C using a *Xist* cDNA probe Cy3-labeled by random priming with a Prime-It II kit (Stratagene). Immunofluorescence was performed as described previously (Zinner et al., 2006) using a rabbit antibody specific for H3K27me3 (kindly provided by Thomas Jenuwein), which was detected by making use of an anti-rabbit IgG antibody coupled to Alexa-488 or Alexa-568 (Molecular Probes). Nuclear counterstaining was performed with DAPI (4',6'-diamidino-2-phenylindole).

Immunoblot Analysis and Quantitative PCR

Immunoblotting was essentially performed as described (Dobreva et al., 2006), using a polyclonal anti-Satb1 antiserum (Agrelo et al., 2009) or a monoclonal anti-Satb1 antibody (Abcam, ab92307). Antibody dilutions were always prepared freshly. RNA isolation was performed by TRIZOL extraction (Invitrogen) following manufacture's instructions. We used 1 μg RNA for subsequent reverse transcription. For the cDNA synthesis, 200 U SuperscriptII (Invitrogen) was used with random hexamers. Real-time PCR was performed with SYBR GREEN PCR master mix (Applied Biosystems) using the ABI PRISM 7000 sequence detection system. The cycle numbers were normalized to *Tbp*. The following primers were used for detection of cDNA transcripts:

Hprt forward: ttcttctcagaccgctttt, *Hprt* reverse: cctggttcatcatcgctaatc
Pgk1 forward: tacctgctggctggatgg, *Pgk1* reverse: cacagcctcggcatatttct
Gapdh forward: acagccgcatcttcttgtgc, *Gapdh* reverse: cactttgccactgc aaatgg
Tbp forward: ggggagctgtgatgtgaagt, *Tbp* reverse: ccaggaaataattctggctcat
Xist: forward: catcgcccatcggtgctttttatgg, *Xist* reverse: ctaagccgagttatgc ggcaagtct

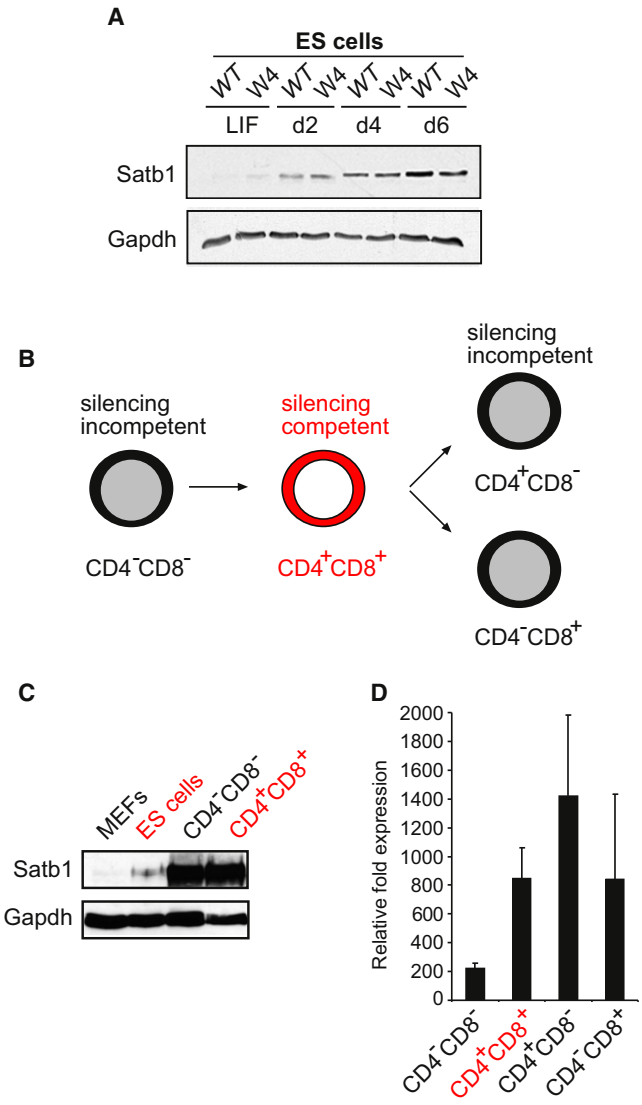

Figure 3. Dynamics of Satb Protein Expression in Differentiating ES Cells and Lymphoid Cells

(A) Immunoblot analysis to detect Satb1 in wild-type (WT) *Oct4-HygTK* ES cells (Savarese et al., 2009) and in W4 ES cells shows that Satb1 expression is induced and maintained upon differentiation.

(B) Scheme displaying the transient appearance of *Xist*-responsive cells during T cell development (Savarese et al., 2006).

(C) Immunoblot analysis of Satb1 expression demonstrates that Satb1 is abundantly expressed in both silencing-incompetent $CD4^{-}CD8^{-}$ cells and in silencing-competent $CD4^{+}CD8^{+}$ cells, indicating that Satb1 expression does not distinguish these two cell types. $CD4^{-}CD8^{-}$ cells express even higher levels of Satb1 than silencing-competent ES cells.

(D) Quantitative RT-PCR analysis of *Satb1* expression during T cell differentiation reveals that *Satb1* is abundantly expressed at all stages of T cell development. Notably, silencing-incompetent single positive T cells contain more *Satb1* transcripts than $CD4^{+}CD8^{+}$ cells. Error bars refer to standard deviation.

The following RT PCR primers were used:

Satb1 PDZ domain forward: AAGATTGCCCGCCTGGAGCA
Satb1 PDZ domain reverse: TTTGGCTTGGGCAGCAGAGCTG
Satb1 DNA-binding domain forward: CAGCTCTCTCACGGCAGTCA
Satb1 DNA-binding domain reverse: TGGGATGCAGTCTTGGGGTC
Gapdh forward: GCCAGCCTCGTCCCGTAGACAAAA
Gapdh reverse: TGGGTGGCAGTGATGGCATG

Genotyping

Genotype determination of embryos and MEFs was performed by PCR as described previously (Dobreva et al., 2006) on DNA isolated from cultured cells or embryo tails.

Sly forward: tgcagggcaggggcgtatga, *Sly* reverse: cctgctgccacacctccagc
Satb1^{wt} forward: tgatctgtaagacagtgactgagt, *Satb1*^{wt} reverse: cctaaggtt ggttttcatgagatggcc
Satb1^{mut} forward: ccaagggaggaaggacaccaaaac, *Satb1*^{mut} reverse: gttgg cgcctaccggtggatgtg
Satb2 forward: cggtggggactttgtctcca, *Satb2*^{wt} reverse: gccaccctctgggta aaccac,
Satb2^{mut} reverse: cgggaatcttcgctattacg

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2012.09.018.

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Supplemental Information

Satb1 and Satb2 Are Dispensable for X Chromosome Inactivation in Mice

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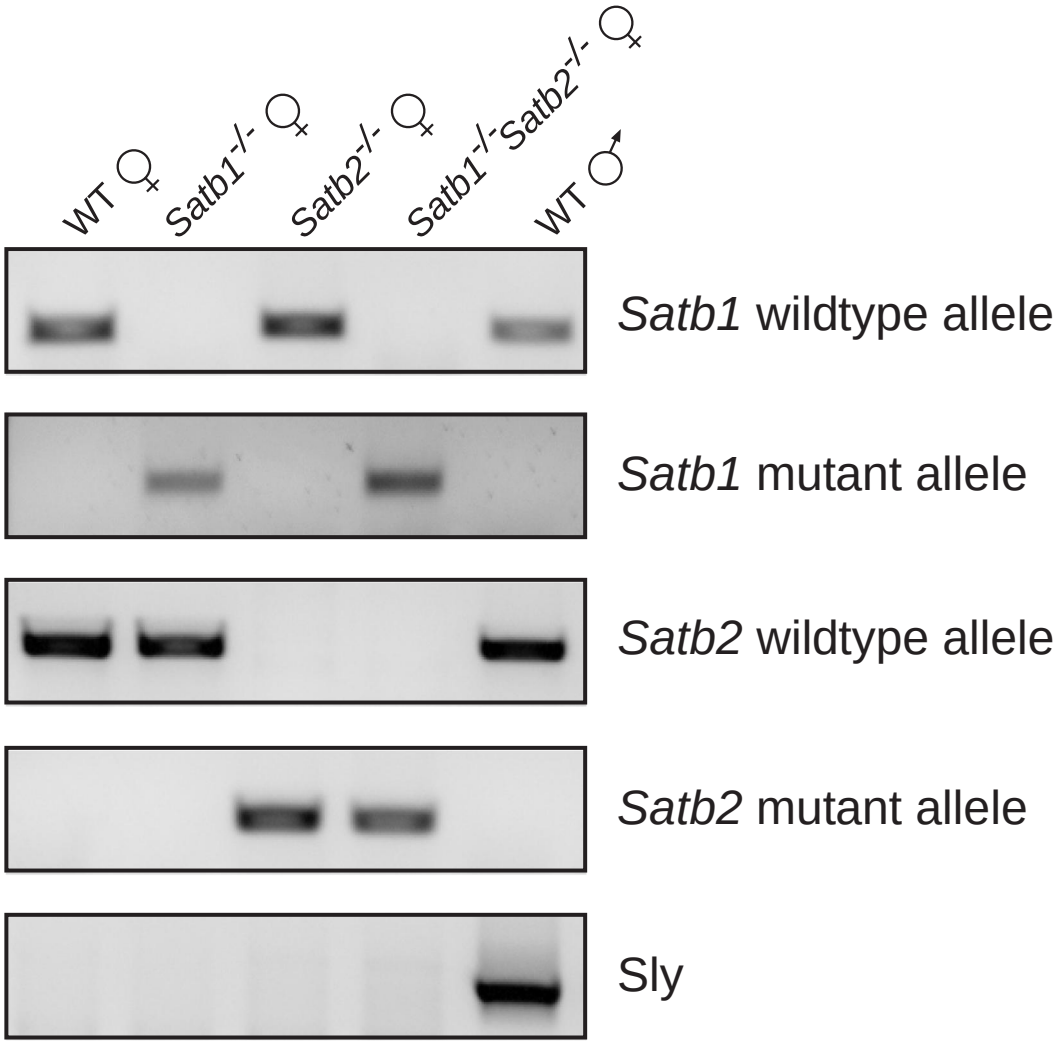
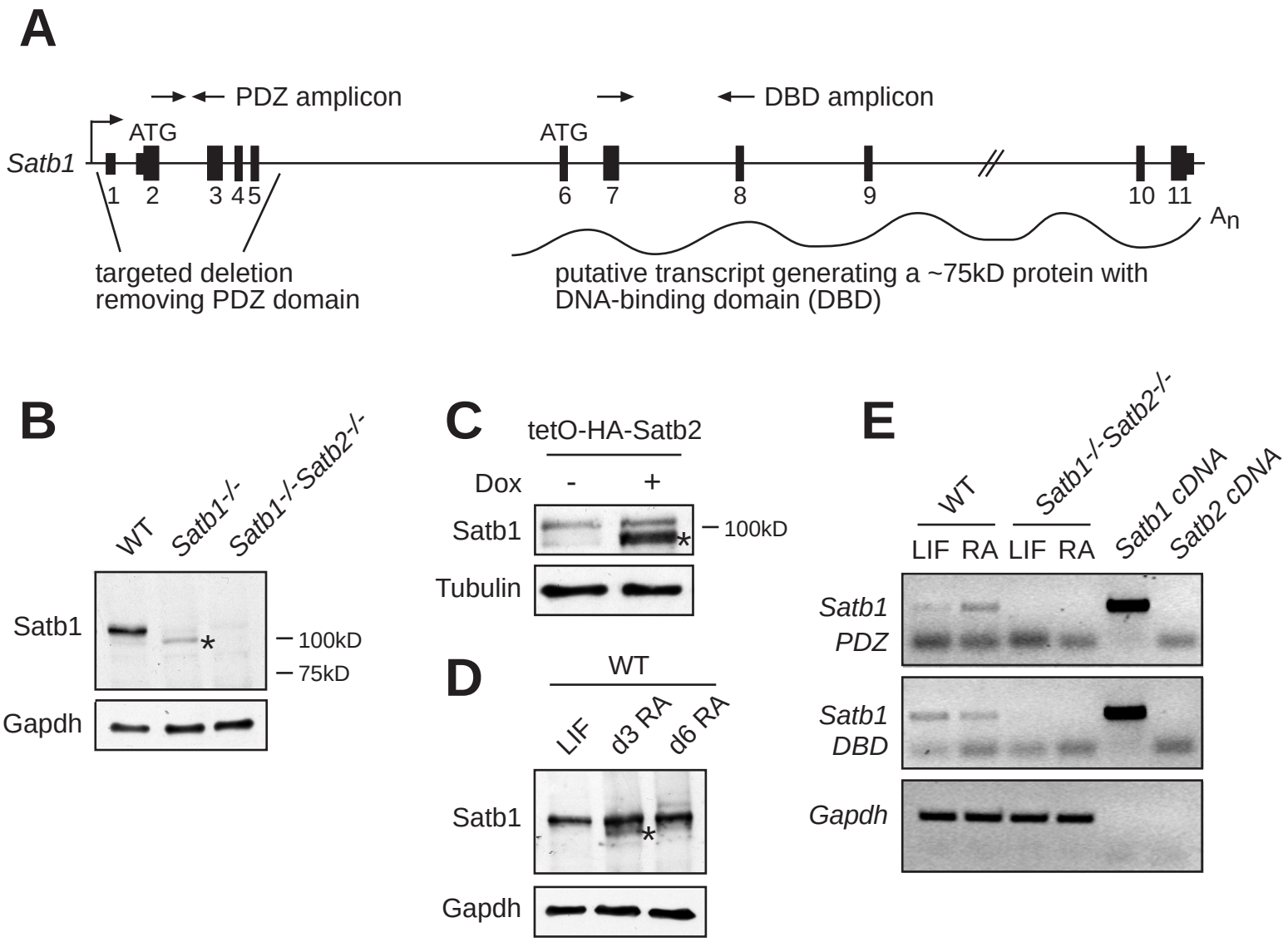
Contents:



Nechanitzky et al. Figure S1



Nechanitzky et al. Figure S2

Figure S1. Both Satb1 and Satb2 are dispensable for X-chromosome inactivation *in vivo.* PCR analysis demonstrating the presence of female *Satb1*$^{-/-}$ *Satb2*$^{-/-}$ embryos at E13.5 indicates that Satb1 and Satb2 are not required for XCI *in vivo*. Primers specific for the wild type (WT) or mutant alleles of *Satb1* and *Satb2* were used in combination with primers specific for *Sly*, a Y-chromosomal transcript, to allow for the sex-determination of embryos.

Figure S2. The *Satb1* mutant allele does not produce transcripts that encode the DNA-binding domain but lack the PDZ domain. **A)** Schematic representation of the *Satb1* locus indicating the targeting strategy used to delete the five first exons which encode the PDZ domain (Alvarez et al., 2000). An alternative form of Satb1 which might be still able to bind DNA could be produced if an alternative or truncated form of the mRNA were translated starting with an in frame ATG encoded by exon 6. Short arrows indicate the primers used to amplify transcripts containing the PDZ domain or DNA-binding domain. **B)** Immunoblot analysis of lysates from wild type (WT), *Satb1*$^{-/-}$ and *Satb1*$^{-/-}$*Satb2*$^{-/-}$ ES cells reveals that only the expected 100kD form of Satb1 is generated in WT cells. No alternative 75kD form is produced in WT and mutant cells. The asterisk denotes a band corresponding to Satb2, which is abundantly expressed in Satb1-deficient cells and cross-reacts with the anti-Satb1 antiserum (Savarese et al., 2009). **C)** Immunoblot analysis demonstrating the Satb2 cross-reactivity of the anti-Satb1 antiserum. Ectopic Satb2 expression was induced in tetO-HA-Satb2 cells (Savarese et al., 2009) and subsequently probed with the anti-Satb1 antiserum.

A band, corresponding to the size of HA-Satb2 and denoted by the asterisk, is detected only under induced conditions. **D)** Immunoblot analysis using the polyclonal anti-Satb1-antiserum (Agrelo et al., 2009) shows that the level of Satb1 expression remains high after retinoic acid (RA)-induced differentiation of wild type (WT) ES cells at days 3 and 6. The asterisk indicates the detection of Satb2. **E)** RT-PCR analysis to detect *Satb1* transcripts containing the PDZ domain or DNA-binding domain (DBD) in undifferentiated (LIF) or RA-differentiated WT and *Satb1*$^{-/-}$*Satb2*$^{-/-}$ ES cells. The positions of the amplicons are shown in panel A. No transcripts containing the PDZ- or DNA-binding domain are present in *Satb1*$^{-/-}$*Satb2*$^{-/-}$ *cells*.

Supplemental References

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