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 (Dobreira 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
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 absence of both Satb1 and Satb2, we analyzed both protein and RNA expression in wild-type and double-mutant MEFs. In addition, we analyzed the expression of X-linked and autosomal genes in female wild-type, Satb1–/–, Satb2–/–, and two independent lines of Satb1–/– Satb2–/– MEFs compared to 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 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.

Table 1. Statistics of the Genotypes of E13.5 Embryos Derived from Satb1+/– Satb2+/– Intercrosses

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
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<tbody>
<tr>
<td>Satb1+/+</td>
<td>4</td>
</tr>
<tr>
<td>Satb1+/-</td>
<td>24</td>
</tr>
<tr>
<td>Satb1-/-</td>
<td>15</td>
</tr>
<tr>
<td>Satb2+/+</td>
<td>17</td>
</tr>
<tr>
<td>Satb2+/-</td>
<td>53</td>
</tr>
<tr>
<td>Satb2-/-</td>
<td>34</td>
</tr>
<tr>
<td>total</td>
<td>196</td>
</tr>
</tbody>
</table>

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.

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 X, 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 immunofluorescence (IF) staining against H3K27me3, a well-described mark of the X (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, Satb1–/–, 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 X 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 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.
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 germ-line-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 down-regulated in Xist-resistant thymic lymphoma cells relative to
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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−/− neurons? 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 XCI 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., 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 (Hasegawa et al., 2011; Pullirsch et al., 2010). Results from MEFs of two Satb1/Satb2−/− 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).

**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% formaldehyde/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% formaldehyde 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 (Zimmer 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:** ttctctcagacccgctttt, Hprt reverse: cctggttcatcatcgctaatc
- **Pgk1 forward:** taccctcgctcttgattgagt, Pgk1 reverse: cacagccggctcatttct
- **Gapdh forward:** acacgccgacatttctggc, Gapdh reverse: catttggcgatctgaaatg

**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 (Satb1/Satb2−/−). Results from MEFs of two Satb1/Satb2−/− 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).
Proper XCI in the Absence of Satb Proteins

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 that shows 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., 2009). Satb1 defines the developmental context for gene silencing by Xist in lymphoma and embryonic cells. WT, 507–516.

(C) Immunoblot analysis of Satb1 expression demonstrates that Satb1 is abundantly expressed in both silencing-incorrect CD4^+CD8^- and 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-incorrect 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 DNA-binding domain forward: TGGGATGCAAGCTTGGGAGC
Satb1 PDZ domain forward: TTTGGGCTTGGGAGCAGAGCTG
Satb1 DNA-binding domain reverse: CAGCTCTCATGCGCATCA
Gapdh forward: GCCAGCCTCCTTGCCTGAGACAAA
Gapdh reverse: TGGGTCGACGTGATGCCATG

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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