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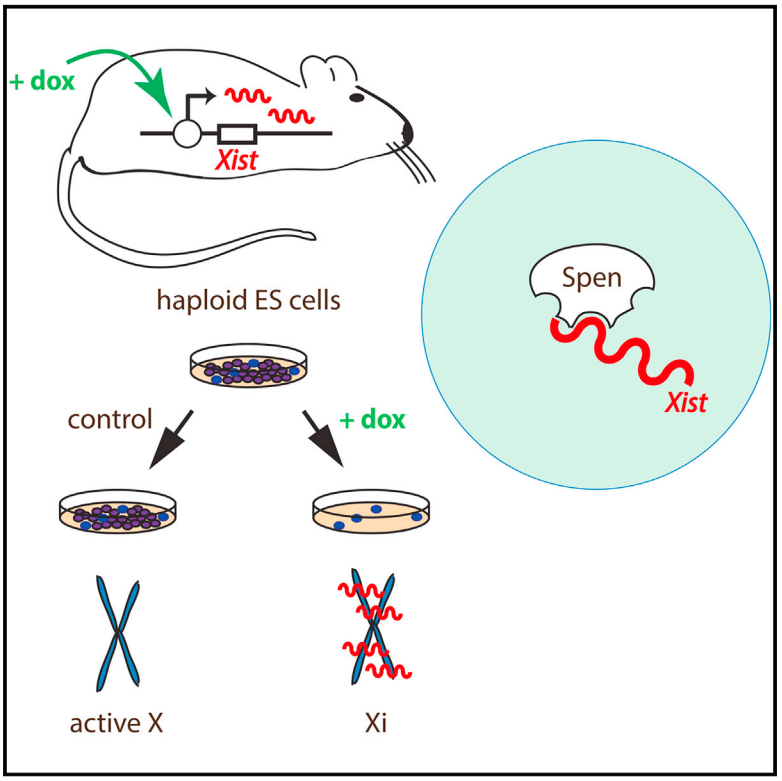
# Identification of *Spen* as a Crucial Factor for *Xist* Function through Forward Genetic Screening in Haploid Embryonic Stem Cells

## Graphical Abstract

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## In Brief

In mammals, one of the two X chromosomes is inactivated in female cells by the noncoding *Xist* RNA for dosage compensation. Monfort et al. use haploid embryonic stem cells to identify silencing factors and demonstrate that the RNA-binding protein SPEN is required for gene repression by *Xist*.

## Highlights

- A haploid embryonic stem cell screen identifies factors required for *Xist* function
- The RNA-binding protein Spen is required for gene repression by *Xist*
- Recruitment of Polycomb group proteins by *Xist* is affected in the absence of *Spen*
- Spen binds *Xist* A-repeat RNA but cannot discriminate functional from mutant motifs

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# Identification of *Spen* as a Crucial Factor for *Xist* Function through Forward Genetic Screening in Haploid Embryonic Stem Cells

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## SUMMARY

**In mammals, the noncoding *Xist* RNA triggers transcriptional silencing of one of the two X chromosomes in female cells. Here, we report a genetic screen for silencing factors in X chromosome inactivation using haploid mouse embryonic stem cells (ESCs) that carry an engineered selectable reporter system. This system was able to identify several candidate factors that are genetically required for chromosomal repression by *Xist*. Among the list of candidates, we identify the RNA-binding protein Spen, the homolog of *split ends*. Independent validation through gene deletion in ESCs confirms that *Spen* is required for gene repression by *Xist*. However, *Spen* is not required for *Xist* RNA localization and the recruitment of chromatin modifications, including Polycomb protein Ezh2. The identification of *Spen* opens avenues for further investigation into the gene-silencing pathway of *Xist* and shows the usefulness of haploid ESCs for genetic screening of epigenetic pathways.**

## INTRODUCTION

In mammals, one of the two X chromosomes in female cells is inactivated for dosage compensation between the sexes (Lyon, 1961); thereby, random chance determines which of the two chromosomes remains transcriptionally active and which is inactivated. X chromosome inactivation has become a paradigm for chromosome-wide gene repression in mammals. The human *XIST* (Brown et al., 1991a, 1991b) and mouse *Xist* (Borsani et al., 1991; Brockdorff et al., 1991) genes encode long noncoding RNAs that accumulate over the X chromosome from whence they are transcribed and initiate chromatin modifications and gene silencing. *Xist* is required for X chromosome inactivation and female mouse development (Marahrens et al., 1997; Penny et al., 1996). Conversely, transgenic expression of *Xist* is sufficient for initiation of repression in early embryonic cells (Jiang et al., 2013; Lee et al., 1996; Wutz and Jaenisch, 2000).

Studies of *Xist* have identified a number of factors that contribute to the structure and repression of the inactive X chromosome (Xi). The histone variant macroH2A (Costanzi and Pehrson, 1998) and the nuclear scaffold protein SAF-A/hnRNPU (Helbig and Fackelmayer, 2003) are enriched on the Xi. Polycomb/Trithorax group proteins, including Eed (Wang et al., 2001) and Ash2L (Pullirsch et al., 2010), have been implicated in X inactivation. In addition, multiple functions have been proposed for YY1 in X inactivation, including *Xist* RNA localization (Jeon and Lee, 2011) and *Xist* transcription (Makhlouf et al., 2014). Whereas *Xist* is not essential for maintenance of gene repression in somatic cells (Brown and Willard, 1994), loss of *Xist* in mice has recently been shown to lead to Xi reactivation in blood cells and leukemia (Yildirim et al., 2013). A genetic screen for epigenetic modifiers in mice has identified the *SmcHD1* gene, which is required for maintaining DNA methylation and repression of Xi-linked gene promoters (Blewitt et al., 2008). We have previously identified *SatB1* as a factor that is required for the initiation of gene repression in a mouse T-cell lymphoma model (Agrelo et al., 2009). However, *SatB1* is unlikely a key factor for gene silencing in the mouse embryo, as a mutation is compatible with female development (Nechanitzky et al., 2012). The mechanism that *Xist* uses for initiation of gene repression remains elusive.

Mouse embryonic stem cells (ESCs) provide a tractable culture system for recapitulating the different steps involved in X inactivation. To advance our understanding of the silencing pathway of *Xist*, we considered forward genetic screening using recently developed mouse haploid embryonic stem cells (ESCs) (Elling et al., 2011; Leeb and Wutz, 2011). Mutations in a haploid genome are hemizygous and efficiently reveal cellular phenotypes. Screens in haploid cells have been successful in uncovering toxin- and pathogen-related host factors (Jae et al., 2014). The maintenance of pluripotent characteristics of haploid ESCs makes them useful for addressing developmental questions (Leeb et al., 2014) and facilitates the genetic exploration of initiation of X inactivation by *Xist*.

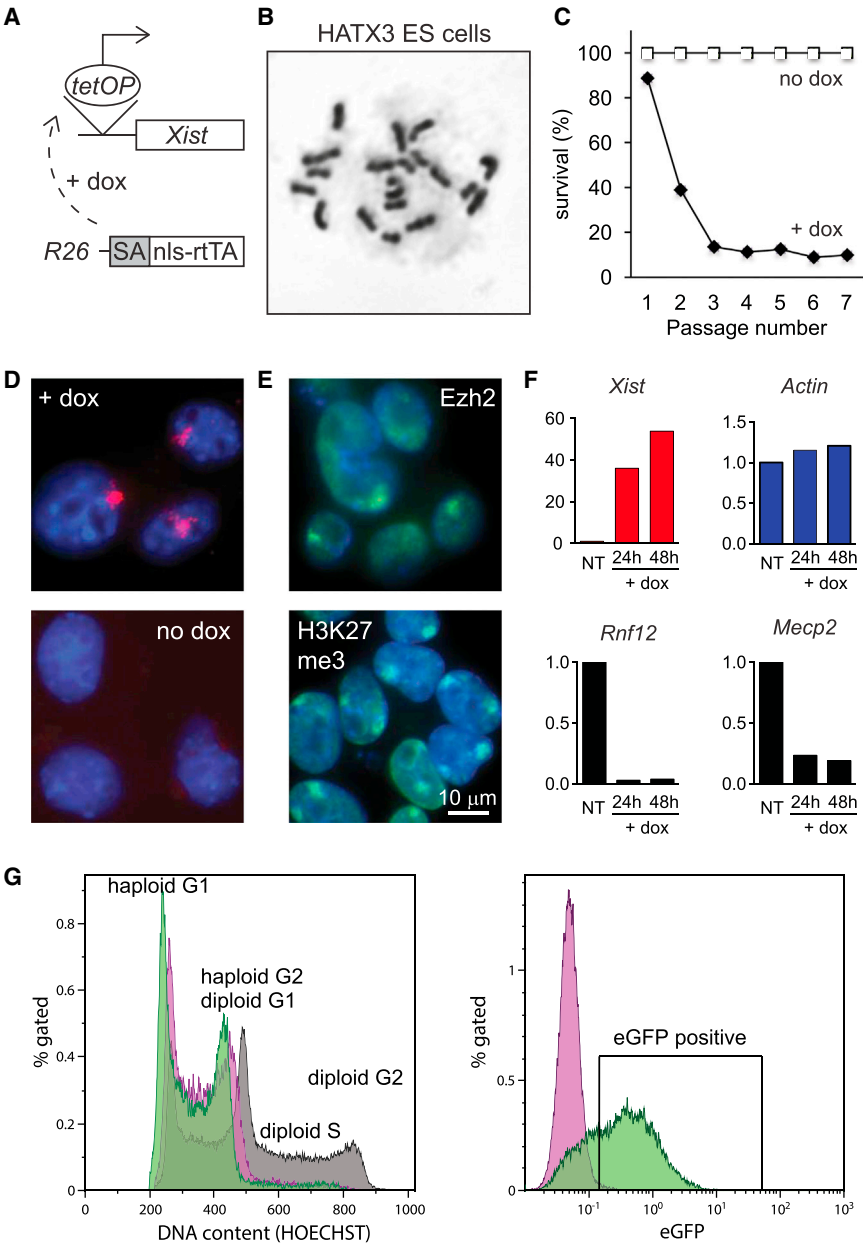


**Figure 1. Characterization of *Xist*-Inducible Mouse Haploid ESCs**

(A) Structure of the *Xist* inducible system.

(B) Chromosome spread of haploid HATX ESCs.

(C) *Xist* induction (+ dox) leads to loss of cells compared to growth in absence of doxycycline (no dox).

(D) *Xist* RNA FISH in HATX3 ESCs grown with and without doxycycline.

(E) Immunofluorescence image showing focal Ezh2 and H3K27me3 staining in HATX3 ESCs after *Xist* induction.

(F) qRT-PCR analysis showing induction of *Xist* and repression of X-linked genes in HATX3 ESCs after doxycycline addition. The autosomal β-actin gene is shown as a control. NT, nontreated.

(G) Flow cytometry profile of HATX3 ESCs before (red) and after (green) infection with gene trap viruses. DNA content (left) and GFP fluorescence (right) of a gene trap encoded reporter are shown. A mixed haploid/diploid DNA content profile is shown for reference in gray.

## RESULTS

### A Genetic Screen for Silencing Factors

We derived haploid ESC lines from *Xist*$^{TX/TX}$ *R26*$^{nlsrtTA/nlsrtTA}$ mice that carry an inducible promoter inserted within the *Xist* locus and a tetracycline-regulated transactivator targeted to the ROSA 26 locus on chromosome 6 (Savarese et al., 2006) (Figure 1A). We obtained 11 haploid ESC lines (HATX-1 to HATX-11) from a total of 170 activated oocytes (Figure 1B). Addition of doxycycline to HATX cultures induced *Xist* expression from the single X chromosome and caused cell loss (Figures 1C and 1D). After *Xist* induction, we observed focal recruitment of the Polycomb protein Ezh2 and the associated histone modification H3K27me3 (Figure 1E). qRT-PCR analysis further showed the repression of X-linked genes, but not of an autosomal control gene, after *Xist* induction in HATX ESCs (Figure 1F). These observations are consistent with earlier findings that *Xist* expression initiates X inactivation in ESCs (Wutz and Jaenisch, 2000; Wutz et al., 2002). In haploid cells, loss of gene expression from the single-X chromosome is not compatible with cell survival. The *Xist*-induced lethality then provides an efficient selection strategy for recovering mutations abrogating the silencing pathway of *Xist* and, therefore, preventing *Xist*-dependent cell death.

For generating a genome-wide set of mutations, we adopted a viral gene trap mutagenesis strategy that has been previously devised for screening in haploid human tumor cells (Carette et al., 2009). We infected 80 million haploid HATX ESCs, which were sorted for a haploid genome content one passage earlier, with a high-titer gene trap vector retrovirus preparation. We confirmed efficient infection by analyzing the fluorescence of a promoter-less EGFP reporter that is encoded by the gene trap virus (Jae et al., 2014) (Figure 1G). The cell pools were subsequently divided into two samples that were either subjected to selection by inducing *Xist* expression or cultured without doxycycline for obtaining control samples. DNA from both control and selected samples was prepared and used for identification of viral insertion sites through next-generation sequencing (NGS) (Carette et al., 2009). We calculated the number of independent insertions for each gene in selected and control data sets and ranked candidates according to the fold increase in the number of insertions

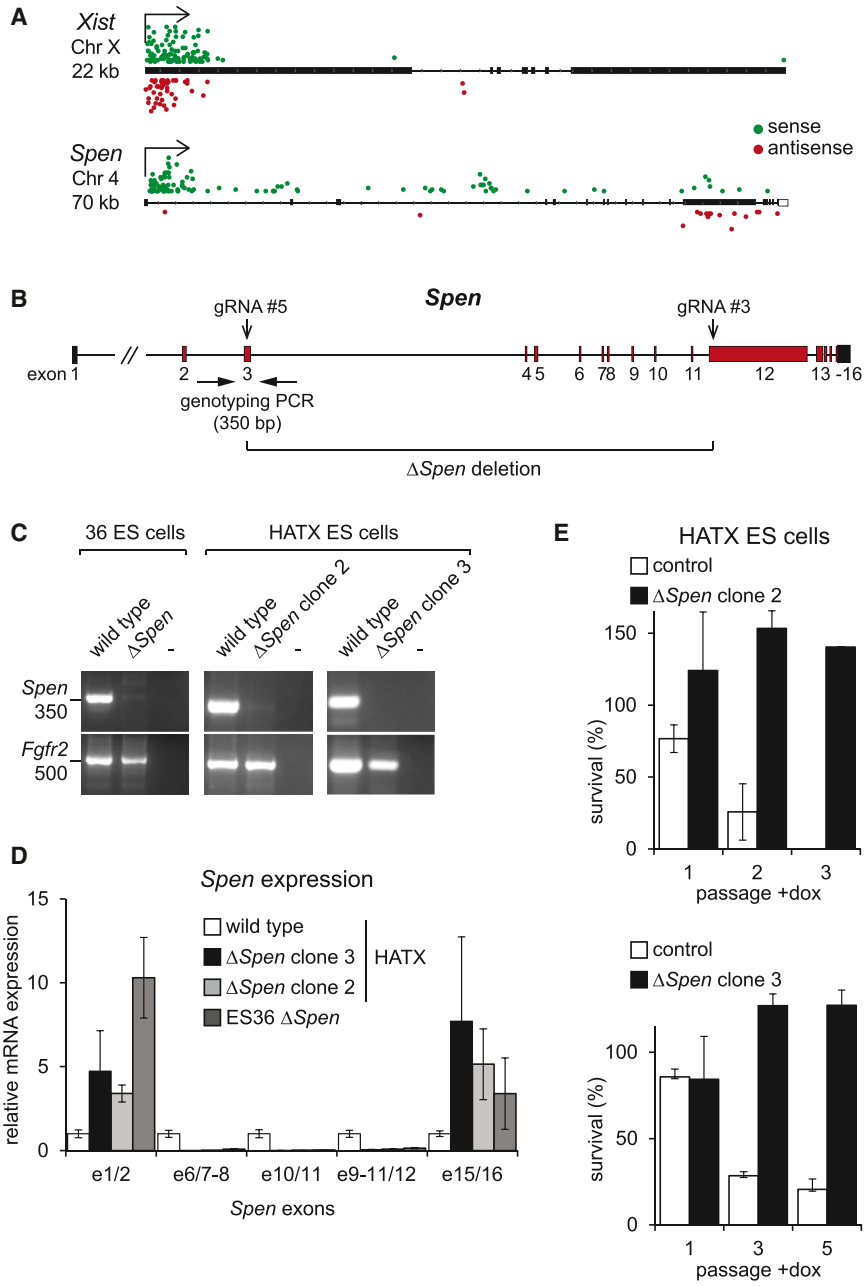


**Figure 2. Mutation of *Spen* in ESCs**

(A) Schematic representation of viral gene trap insertions in *Xist* and *Spen* locus (not to scale, gene size is indicated). Chr, chromosome.

(B) Schematic representation of the *Spen* gene locus showing the location of CRISPR/Cas9 guide RNAs (gRNAs) used for engineering a deletion and genotyping PCR primers.

(C) Genomic PCR confirming the absence of wild-type *Spen* fragment in *Spen* mutant ESCs.

(D) Quantitative expression analysis of *Spen* using primer sets spanning exons as indicated. Δ*Spen* ESCs lack transcript from the deleted region. Error bars represent SD (n = 3).

(E) Cell survival of control HATX3 ESCs and derived Δ*Spen* ESCs clone 2 (top; n = 2) and clone 3 (bottom; n = 3) after *Xist* induction (+ dox). Survival was calculated relative to uninduced cells. Error bars represent SD.

See also Figure S1.

in selected samples over controls. As anticipated, *Xist* was found on top of the list of candidates (Tables S1 and S2). Insertions were preferentially observed within the 5′-most region of *Xist* and in an orientation that aligns the poly-adenylation sequences of the gene trap with the transcription unit consistent with the potential termination of *Xist* transcription (Figure 2A). The ROSA26 locus from which the tetracycline responsive transactivator nls-rtTA is expressed was also among the selected candidates. A large number of insertions further mapped within the nls-rtTA cDNA (Figure S1), demonstrating that our screen robustly recovered factors that were anticipated from the technical setup of the screen.

The list of candidates included the chromatin assembly factors Hira and Ubn2, the kinase Cdk8, a subunit of the mediator complex Med25, the RNA-binding protein Spen, and one hypothetical gene *Gm3139* (Figure S1; Tables S1–S3). For all candidates except *Gm3139*, we could observe a strong bias for gene trap insertions in the orientation of the transcription of the gene in selected but not control data sets, which suggests that potential loss-of-function mutations had been enriched. These observations suggest that the selected candidates are involved in *Xist*-mediated gene silencing. However, different mechanisms can be considered: (1) direct interaction with *Xist*, (2) affecting *Xist* expression, and (3) indirect effects from regulating gene expression and signal transduction in pluripotent cells. Indirect effects likely explain the recovery of general regulators of gene expression such as *Hira* and *Med25*. In contrast, *Spen* (also called *Mint* and *Sharp*) possesses RNA-binding domains and has been previously implicated in gene repression (Arieti et al., 2014; Kuroda et al., 2003; Shi et al., 2001), which suggests it as a potential silencing factor of *Xist*. Therefore, we selected *Spen* for further characterization.

## *Spen* Is Required for *Xist*-Mediated Gene Repression in ESCs

*Spen* was enriched in all seven selected samples over controls, indicating that mutations were recovered with high reproducibility. Distribution and orientation of gene trap virus insertions were, furthermore, consistent with a high likelihood of inducing loss-of-function mutations (Figure 2A). To independently validate

*Spen* as a silencing factor, we used CRISPR/Cas9 nucleases for engineering a deletion within the gene (Figure 2B). We deleted the *Spen* exons encoding the RNA-binding domains and nuclear localization signals in HATX ESCs and clone 36 ESCs, which carry an inducible *Xist* transgene on chromosome 11 (Wutz and Jaenisch, 2000). CRISPR/Cas9 nucleases have previously been shown to be very efficient and can yield homozygous mutations (Doudna and Charpentier, 2014). Our strategy involved the simultaneous use of two guide RNAs for deleting a large fragment of the transcription unit of *Spen*. Using this strategy, we were successful in obtaining two independent ESC clones with *Spen* deletions in HATX3 ESCs and one clone with a homozygous deletion of *Spen* in clone 36 ESCs. We confirmed the deletion by genomic PCR and Southern blot analysis (Figure 2C; Figures S2A and S2B). qRT-PCR showed an absence of transcript over the deleted region, but the remaining exons appeared to give rise to a transcript of considerably higher abundance than wild-type *Spen* message (Figure 2D).

Next, we investigated whether mutation of *Spen* abrogates the ability of *Xist* to induce X inactivation. For this, we measured cell survival after *Xist* induction. Whereas induction of *Xist* caused cell loss in parental HATX3 ESCs, cells with a deletion of *Spen* appeared unaffected (Figure 2E). To further confirm that *Xist* was induced and repression of X-linked genes was abrogated, we performed qRT-PCR analysis. *Xist* induction was observed 24 and 48 hr after doxycycline addition in *Spen* mutant and control cells (Figure 3A). Repression of X-linked genes was observed in HATX3 control cells but not in cells carrying a *Spen* deletion. Autosomal control genes remained unaffected by *Xist* in both *Spen* mutant and wild-type cells (Figure 3B). Taken together, these observations showed that a mutation in *Spen* abrogated the ability of *Xist* for initiating gene repression. We further analyzed the effect of the *Spen* deletion in clone 36 ESCs, in which *Xist* induction represses a puromycin-resistance gene that was co-integrated with the *Xist* transgene into chromosome 11 and the imprinted *Meg1* gene. *Xist* induction caused efficient repression of the puromycin marker after 48 hr in control cells but less efficient repression in cells lacking *Spen* (Figure S2C). Consistent with this observation, *Spen* mutant cells showed an increased survival in the presence of puromycin and less efficient silencing of *Meg1* when *Xist* was induced (Figures S2D–S2F), indicating that loss of *Spen* also led to a reduced efficiency of *Xist*-mediated repression in 36 ESCs. Taken together, our results demonstrate that *Spen* is required for gene repression by *Xist* in ESCs.

To further explore whether the RNA-binding domains of *Spen* could bind to *Xist*, we focused on the A-repeat motif that has been shown previously to be required for initiation of silencing (Wutz et al., 2002). The interaction of three *Spen* RRM domains with the noncoding *steroid receptor RNA activator* (SRA) has been recently investigated in detail (Arieti et al., 2014). We performed electrophoretic mobility shift experiments with purified recombinant *Spen* RRM domains, using the H12-H13 substructure of SRA and a dimer of *Xist* A-repeat motifs (XCR), which has a similar length as that of SRA, as radiolabeled probe. We found that *Spen* RRMs can bind XCR (Figure 3C) and that XCR also competes for SRA binding (Figures S2G and S2H). Experiments using in-vitro, T7-transcribed, as well as fully synthetic RNAs indicate that XCR can bind to *Spen* RRMs similarly to SRA, whereas tRNA does not compete for binding. We also observed that two mutant XCR RNAs (XS1 and XNX) that are not active in initiating gene silencing (Wutz et al., 2002), nonetheless, can compete for binding to *Spen* RRMs (Figure 3D). These results show that *Spen* can bind to *Xist* A-repeat sequences in vitro. However, under our conditions, the binding specificity cannot fully explain the silencing function of *Xist* A-repeat, potentially reflecting technical limitations of our assay or indicating that other factors cooperate with *Spen* in binding to *Xist* A-repeat in vivo.

### Mutation of *Spen* Does Not Affect *Xist* Localization but Reduces Recruitment of Polycomb Proteins

To further explore whether loss of *Spen* affected *Xist*-mediated chromatin modifications, we performed *Xist* RNA fluorescence in situ hybridization (FISH) and immunofluorescence staining. *Xist* clusters in control and *Spen* mutant cells appeared with similar efficiency (Figures S2F, S3A, and S3B), suggesting that *Xist* localization was not affected by the deletion of *Spen*. The Polycomb proteins Ezh2 and Ring1b were also recruited by *Xist*, and focal enrichment of H3K27me3 could be observed in the absence of *Spen* (Figures 4A and 4C; Figures S3 and S4). Similarly, *Xist* clusters appeared in a volume that was characterized by low signals of acetylated histone H4 and RNA polymerase II, consistent with the presence of a repressive compartment (Figures 4E and 4F). However, Ezh2 and H3K27me3 foci were less prominent in *Spen* mutant cells compared to control cells. Combined immunofluorescence staining and RNA FISH showed that the efficiency of Ring1b and Ezh2 recruitment to the *Xist* cluster was reduced in *Spen* mutant cells (Figures 4B, 4D, and S4). Taken together, these data demonstrate that loss of *Spen* resulted in a decreased efficiency of recruitment of chromatin modifications by *Xist* in ESCs. These results are consistent with previous observations that a mutant *Xist* RNA lacking A repeat can localize and recruit chromatin modifications but does not initiate gene repression (Wutz et al., 2002). The A-repeat mutant *Xist* RNA also displays a strong deficit in Polycomb recruitment in ESCs, but recruitment increases upon entry of ESCs into differentiation (Kohlmaier et al., 2004). However, we did not see an increase in efficiency of recruitment of Ring1b and Ezh2 in *Spen* mutant cells upon entry in differentiation (Figures 4B and 4D).

## DISCUSSION

The mechanism of X chromosome inactivation has been subject to many studies, but the genetic requirements for initiation of gene repression have remained elusive. To advance the genetic basis of the initiation of X inactivation in a developmentally relevant cell system, our study combines haploid ESCs that carry a developmentally validated selection system with saturation mutagenesis as a statistically robust method for the identification of candidate genes.

Our data demonstrate that *Spen* is required for *Xist*-mediated silencing in ESCs. Spen possesses four RNA-recognition motif (RRM) domains and is a predominantly nuclear protein. Mutation of *Spen* in mice causes embryonic lethality (Kuroda et al., 2003),

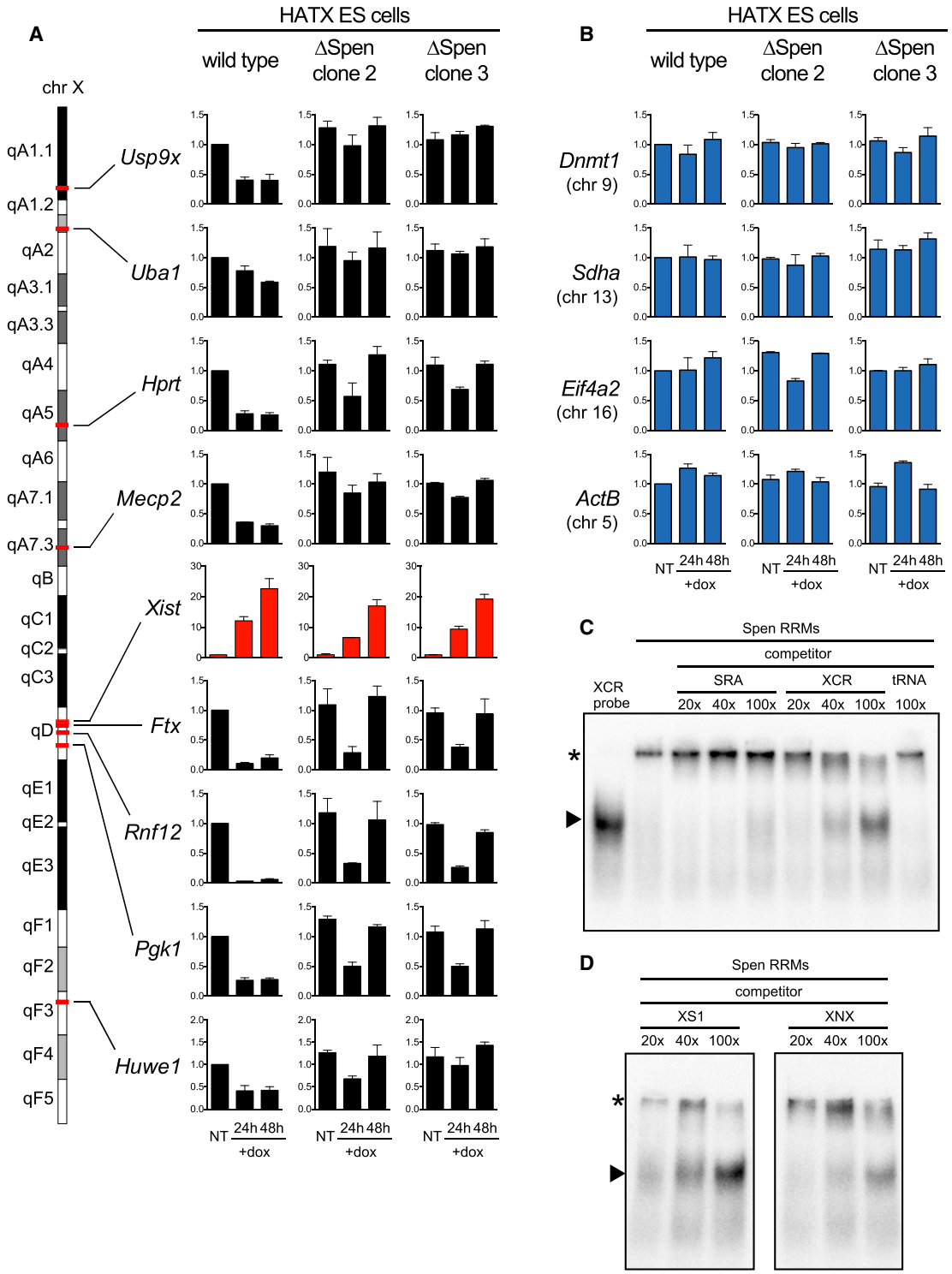


**Figure 3. *Spen* Is Required for *Xist* Function in ESCs and Binds *Xist* A-Repeat Sequences In Vitro**

(A and B) qRT-PCR analysis of the X-linked genes (A) and the autosomal control genes (B) as indicated in HATX and *Spen* mutant ESCs. Error bars represent SD (n = 3). The locations of the genes tested on the X chromosome (chr) is shown at the left. q represents the region of the chromosome. +dox, *Xist* induction; NT, nontreated.

(C) Electrophoretic mobility shift analysis of Spen RRM domains using a $^{32}$P-labeled synthetic XCR RNA probe and cold XCR, SRA, and tRNA competitors as indicated. Asterisk and black triangle indicate position of the RNA-protein complex and free probe, respectively.

(D) Competition with cold XS1 and XNX competitors as in (C).

See also Figure S2.

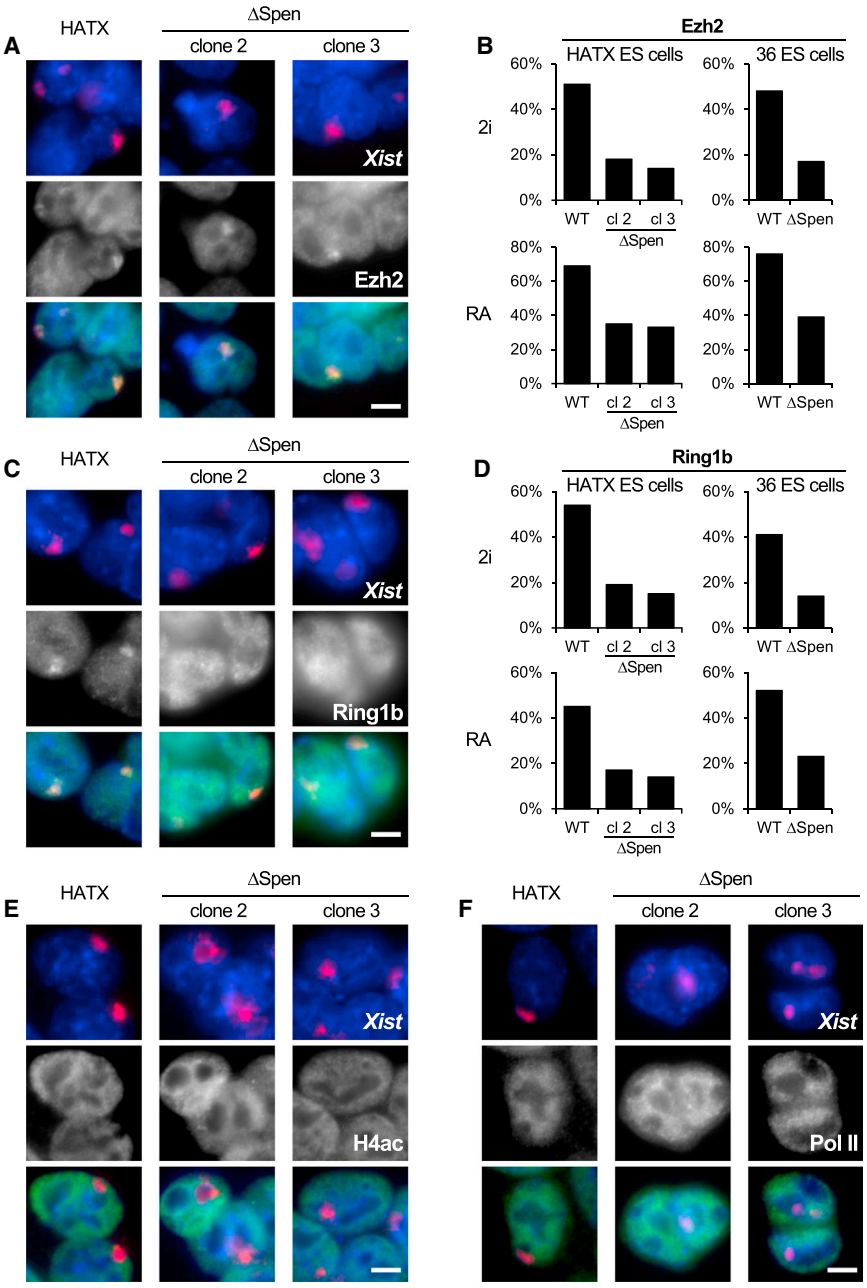


**Figure 4. Mutation of *Spen* Reduces Recruitment of Polycomb Proteins**

(A, C, E, and F) Combined *Xist* RNA FISH and Ezh2 (A), Ring1b (C), acetylated histone H4 (E), and RNA polymerase II (F) immunofluorescence analysis of *Spen* mutant and control ESCs after 24 hr of induction with doxycycline. Scale bar, 5 μm.

(B and D) Quantification of focal recruitment of Ezh2 (B) and Ring1b (D) by *Xist* expression in 2i-cultured (2i) and 24-hr-differentiated (retinoic acid; RA) ESCs (n = 100). cl 2 and cl 3 represent clones 2 and 3, respectively.

See also Figures S3 and S4.

which might be partly attributable to a repressive function in the Notch signaling pathway (Yabe et al., 2007). *Spen* is further implicated in nuclear hormone receptor signaling and interacts with the regulatory noncoding SRA RNA through its RRM domains (Arieti et al., 2014; Shi et al., 2001). Its repressor function and the binding of *Spen* RRM domains to *Xist* A-repeat are consistent with the expected properties of silencing factors, but we cannot exclude additional factors that regulate the interaction with *Xist*. Two recent studies have also identified *Spen* as an interactor of *Xist*, using biochemical strategies (Chu et al., 2015; McHugh et al., 2015). Both studies implicate *Spen* in X inactivation, with slightly different mechanisms. The finding that the Spen protein is detected more abundantly when the *Xist* A-repeat is present (Chu et al., 2015) is consistent with our observation that *Spen* RRMs have binding activity for *Xist* A-repeat core motifs in vitro. However, our data also suggest that this RNA-binding specificity does not fully explain the silencing function of *Xist* A-repeat. Notably, depletion of *Spen* by RNAi has been reported to abrogate Polycomb recruitment by *Xist* by preventing deacetylation of histones (McHugh et al., 2015). We do observe a reduction, but not a complete block, of Polycomb recruitment in the absence of *Spen*. To reconcile the different findings, we consider technical differences between RNAi-mediated depletion and our engineered genetic deletion of *Spen* exons encoding its RNA-binding domains. Whereas our mutation abrogates gene repression by *Xist* to a large extent, we observe a moderate and transient reduction of genes close to *Xist*, including *Ftx*, *Rnf12*, and *Pgk1* in HATX Δ*Spen* ESCs (Figure 3A). We find that *Xist* can recruit Polycomb proteins and form a repressive compartment characterized by low RNA polymerase II and acetylated histone H4 in Δ*Spen* ESCs, suggesting that multiple interactions between Spen and *Xist* might exist. The genetic implication of *Spen* will facilitate further advances on the gene-silencing pathways that operate during X inactivation.

## EXPERIMENTAL PROCEDURES

### Cell Culture and Generation of Transgenic Cell Lines

Derivation of haploid ESCs from Xist$^{TX/TX}$ R26$^{nlsrtTA/nlsrtTA}$ mice was performed as previously published (Leeb and Wutz, 2011). For introducing a deletion into the *Spen* gene locus (Figure 2A) using the CRISPR/Cas system, previously published guide RNA sequences (Koike-Yusa et al., 2014) against *Spen* exons (gRNA3 Fw: GGGGTGTCTCCTGCGCATT; gRNA5 Fw: CGGACAAGACA TTACGATC) were inserted into the pX330-U6-Chimeric_BB-CBh-hSpCas9 vector (Addgene, #42230). Briefly, the vectors were lipofected into HATX3

cells, together with a GFP reporter vector in a 5:1 ratio. 48 hr later, cells were sorted for green fluorescence and replated to a very low density for isolating individual clones. *Spen* deletions were identified by PCR on genomic DNA, using the primers 5′-GTCGTCCCTGTAGATATCCCTGTG-3′ and 5′-CAGA AAGAGGCGAGGCGTAAAG-3′ (Figure S2A). Fgfr2 primers were used as controls (5′-ATTTCTTGGCACCAGACAGG-3′ and 5′-TGCCAACTCCAGAG GACTTT-3′).

### Screening in Haploid ESCs

$10^7$ sorted haploid ESCs were grown in 3 × 145 $cm^2$ dishes for 3 days and harvested. Haploid cells were infected with gene trap virus as previously described (Jae et al., 2014) and plated on 10 × 145 $cm^2$ dishes. After 3 days, each dish was split into two 145-$cm^2$ dishes, one used as control and one for selection. For selection, *Xist* expression was induced by addition of 1 μg/ml doxycycline. Every 3 days, half of the selected cells were passaged and half of the cell pool was used for DNA extraction up to passage 5. Library preparation and computational analysis of sequencing datasets are detailed in the Supplemental Experimental Procedures. The sequencing datasets are deposited in the NCBI Short-Read Archive (http://www.ncbi.nlm.nih.gov/sra) and can be accessed using accession numbers SRX1060416 and SRX1060407.

### Immunofluorescence and RNA Analysis

Immunofluorescence staining was performed as previously described (Pullirsch et al., 2010). For further details, see Supplemental Experimental Procedures. For gene expression analysis, RNA was isolated using the QiAshredder (QIAGEN, #79656), purified using the RNeasy Mini Kit (QIAGEN, #74104) and reverse transcribed into cDNA. Real-time PCR was performed using SYBR Green and a LightCycler 480 System (Roche). *Eif4a2* was used for normalization. For details, see Supplemental Experimental Procedures.

### Electrophoretic Mobility Shift Assay

Binding studies were performed using purified recombinant Spen RRM domain protein and either chemically synthesized or T7 in-vitro-transcribed RNA as previously described (Arieti et al., 2014). For experimental details, see Supplemental Experimental Procedures.

## ACCESSION NUMBERS

The sequencing datasets are deposited in the NCBI Short-Read Archive (http://www.ncbi.nlm.nih.gov/sra) and can be accessed using accession numbers SRX1060416 and SRX1060407.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.06.067.

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