

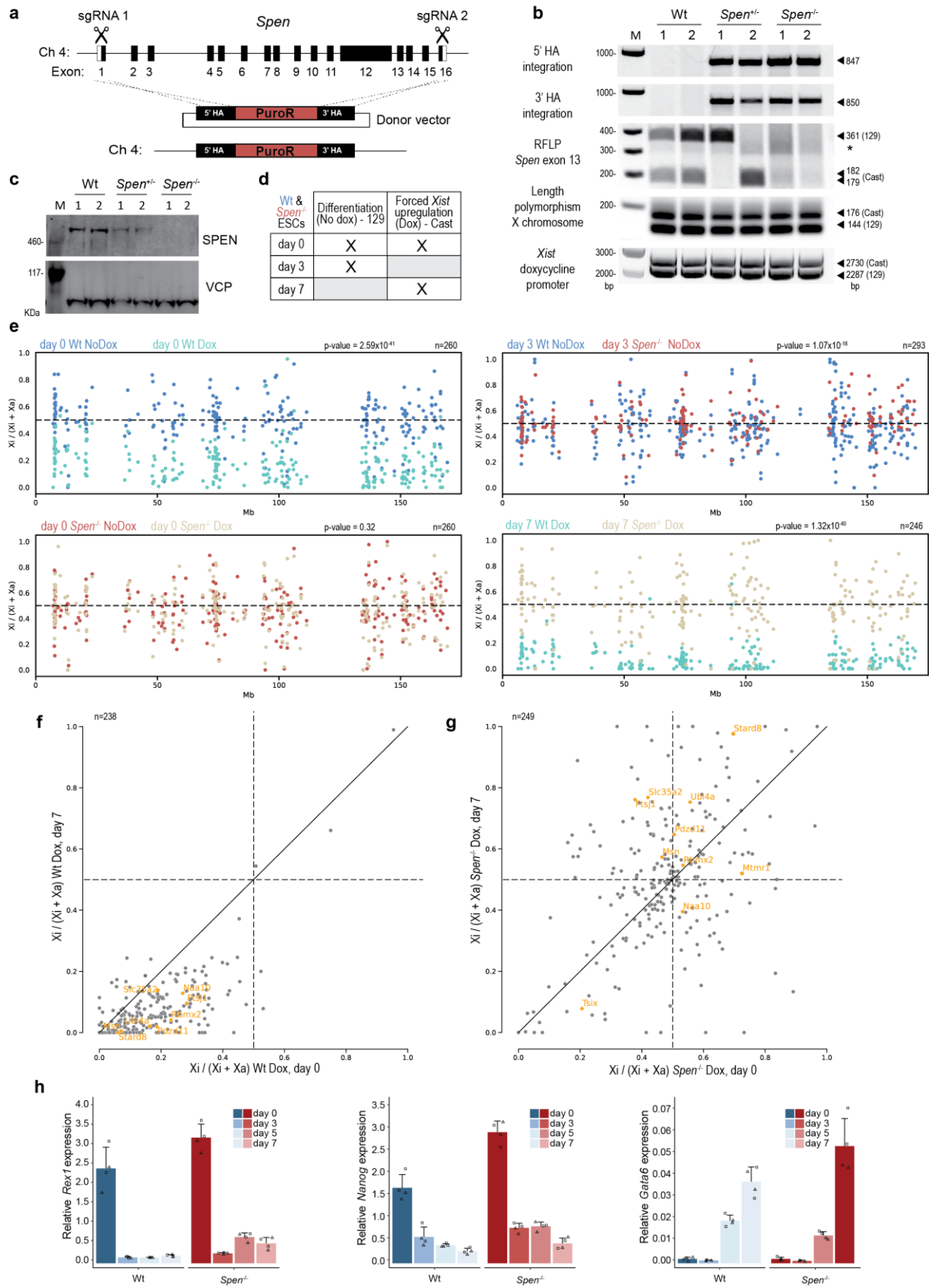
SPEN is Required for *Xist* Upregulation during Initiation of X Chromosome Inactivation

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Supplementary Information

Description: Supplementary Figures, Supplementary Tables and Supplementary References

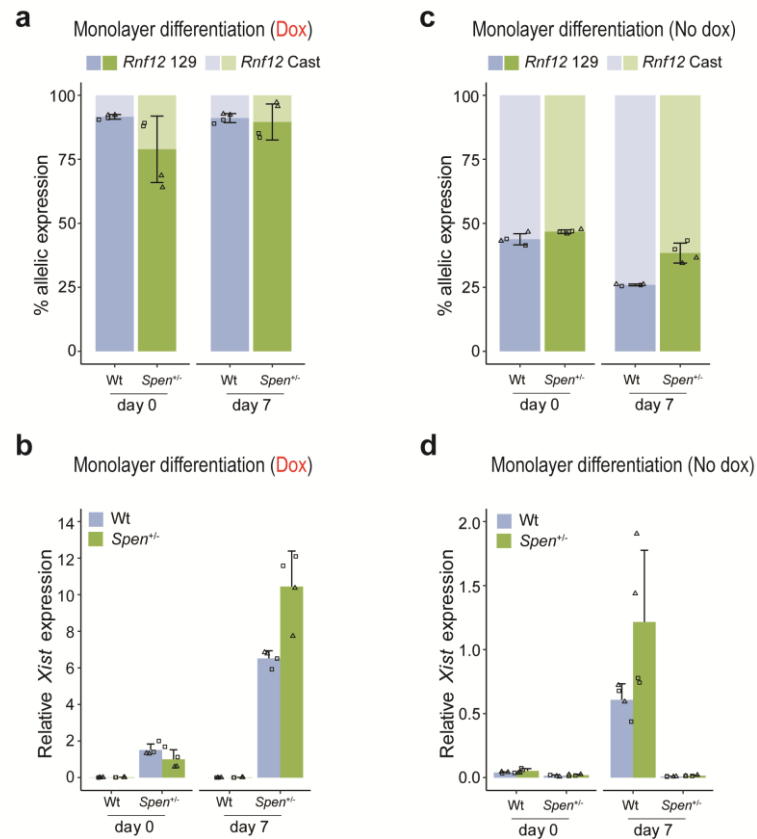
Supplementary Fig. 1



Supplementary Fig. 1 Generation and characterization of *Spn*^{+/-} and *Spn*^{-/-} ESC lines. RNA-seq analysis of Wt and *Spn*^{-/-} ESCs. | Related to **Fig. 1**.

(a) Targeting strategy used to generate *Spn* knockout ESC lines using the CRISPR/Cas9 system. Two single guide RNAs (sgRNA) targeting the 5' and 3' region of the *Spn* ORF were used to integrate a Puromycin resistance (PuroR) cassette. (b) PCR genotyping to identify heterozygote (+/-) and homozygote (-/-) *Spn* knockout ESC clones. Specific 5' and 3' integration of the PuroR cassette in the *Spn* locus. Restriction Fragment Length Polymorphism (RFLP) analysis on *Spn* exon 13 to determine the absence of *Spn* ORF in one or both alleles. Verification of the presence of two X chromosomes per line. Genotyping of the *Xist* endogenous doxycycline-inducible promoter. M = DNA ladder. (*) = unspecific band. (c) SPEN Western blot of two independent Wt, *Spn*^{+/-} and *Spn*^{-/-} ESC clones. VCP was used as a loading control. M = High molecular weight protein ladder. (b,c) Experiment performed at least two times with similar results. (d) Overview of the RNA-seq libraries generated in this study, summarizing the experimental conditions performed in Wt and *Spn*^{-/-} ESCs at different time points of differentiation, treated with or without doxycycline (X in the panel). Each condition includes two biological replicates, adding up to a total of 16 RNA-seq libraries. (e) Allelic ratio ((Xi)/(Xi+Xa)) of individual genes along the X chromosome of Wt (top-left) and *Spn*^{-/-} (bottom-left) undifferentiated (day 0) ESCs with and without doxycycline. Same analysis for Wt and *Spn*^{-/-} lines at day 3 of differentiation without doxycycline (top-right) and day 7 with doxycycline (bottom-right). Two-sided Wilcoxon signed-rank test corrected with Benjamini-Hochberg for multiple testing ($\alpha < 0.05$). (f) Scatter plot showing the allelic ratio (Xi/(Xi+Xa)) of X-linked genes in Wt ESCs treated with doxycycline at day 0 (x-axis) and day 7 (y-axis) of differentiation. Highlighted in orange are the lowly silenced genes in *Spn*^{-/-} ESC previously identified¹. Dashed lines = allelic ratio of 0.5; Diagonal solid line = equal ratio in both conditions. (g) Same as in (f), for *Spn*^{-/-} ESCs. (e-g) Only the genes (n) with sufficient reads in both conditions are shown. (h) *Rex1*, *Nanog* and *Gata6* relative expression of Wt and *Spn*^{-/-} ESC lines upon differentiation without doxycycline. Average expression $\pm$ SD, n=4 biological replicates including 2 independent clones (squares vs. circles) per condition. Reference gene: *Hist2h2aa1*.

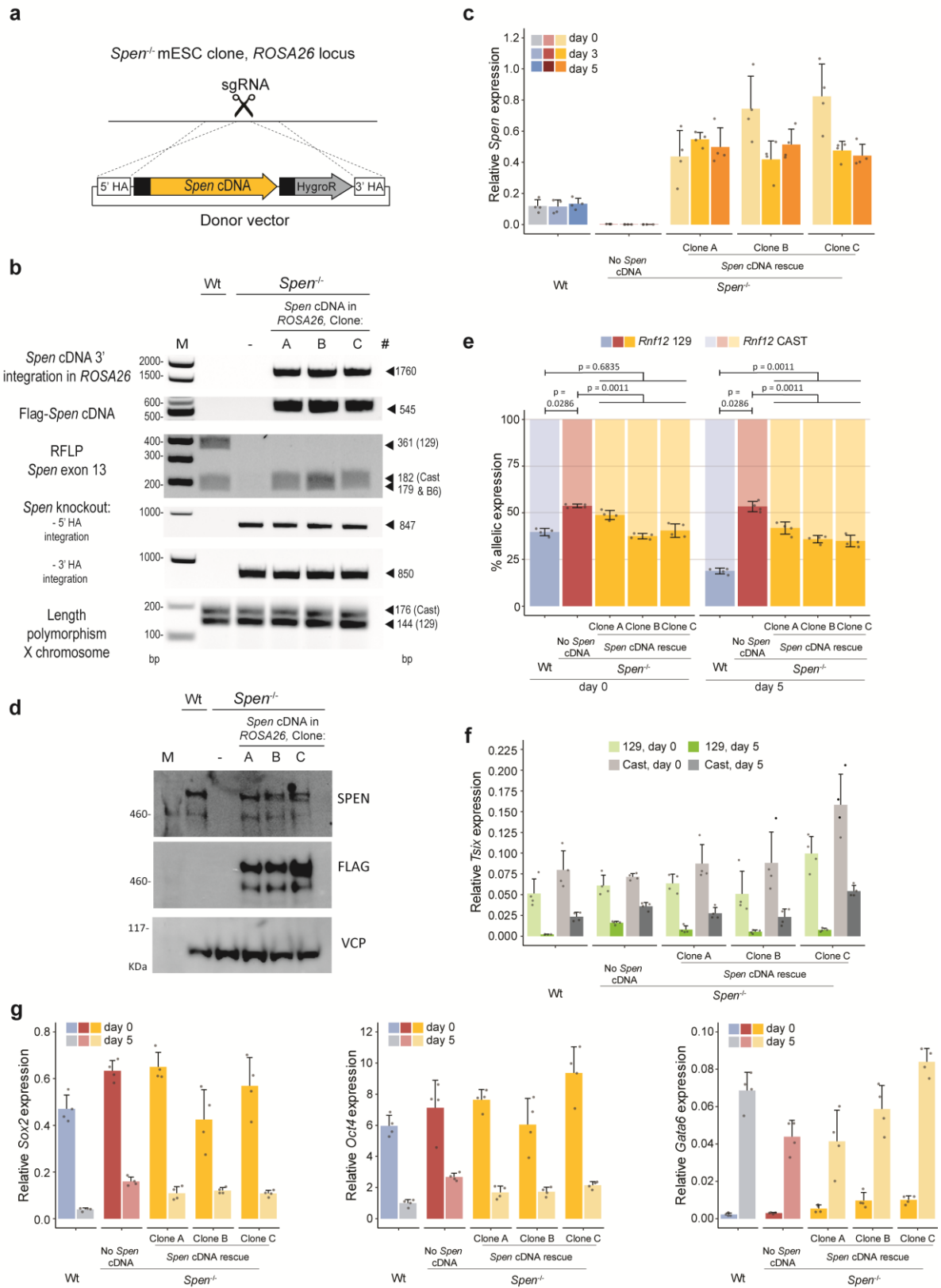
Supplementary Fig. 2



Supplementary Fig. 2 $Spn^{+/-}$ ESCs lines are able to upregulate *Xist*, but show less X-linked gene silencing, compared to Wt ESCs upon monolayer differentiation. | Related to **Fig. 1**.

(a) *Rnf12* percentage of allelic expression of Wt and $Spn^{+/-}$ lines treated with doxycycline at day 0 and 7 of differentiation. Wt samples are the same as in Fig. 1c. Relative *Rnf12* allelic (129 and Cast) expression was normalized to *Rnf12* total expression and averaged $\pm$ SD, $n=4$ biological replicates including 2 independent clones (squares vs. circles) per condition. Reference gene: *Hist2h2aa1*. **(b)** Relative allele-specific *Xist* expression of two Wt and $Spn^{+/-}$ ESC clones treated with doxycycline at day 0 and 7 of monolayer differentiation, determined by RT-qPCR. Wt samples are the same as in Fig. 1d. Average expression $\pm$ SD, $n=4$ biological replicates including 2 independent clones (squares vs. circles) per condition. Reference gene: *Hist2h2aa1*. **(c)** Same as displayed in **(a)** without doxycycline. Wt samples are the same as in Fig. 1e. **(d)** Same as displayed in **(b)** without doxycycline. Wt samples are the same as in Fig. 1f.

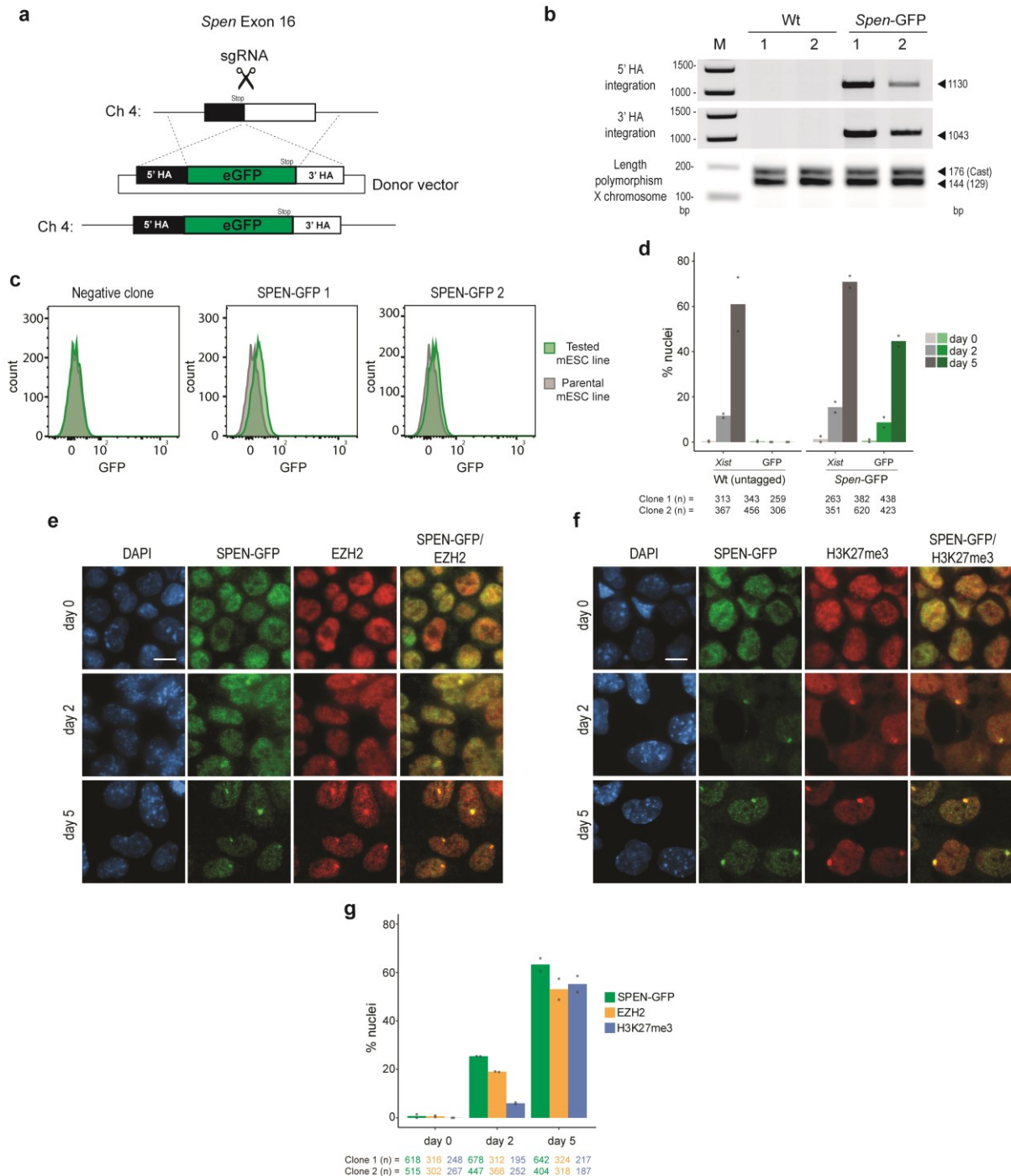
Supplementary Fig. 3



Supplementary Fig. 3 *Spen* cDNA rescue ESC lines characterization. | Related to **Fig. 2**.

(a) Targeting strategy to stably express the *Spen* cDNA from the *ROSA26* locus in *Spen*^{-/-} ESCs, making use of a sgRNA targeting the *ROSA26* locus, and a vector coding for the *Spen* cDNA and a hygromycin resistance (HygroR) cassette². (b) Strategy to identify correct *Spen* cDNA rescue clones by PCR on gDNA. Specific 3' *Spen* cDNA integration in the *ROSA26* locus. Primer on the Flag-tag present in the 5' *Spen* cDNA end to identify those clones containing the *Spen* cDNA vector. Specific 5' and 3' integration of the PuroR cassette in the *Spen* locus to identify the *Spen*^{-/-} line. RFLP analysis on *Spen* exon 13 to determine the presence of *Spen* in the *ROSA26* locus and its absence in the *Spen*^{-/-} cells; the *Spen* rescue cDNA sequence is C57BL/6 (B6). Verification of the presence of two X chromosomes per line. Experiment performed at least two times with similar results. M = DNA ladder. (c) Relative *Spen* expression in Wt, *Spen*^{-/-} and *Spen* cDNA rescue ESC lines (Clone A, B and C) upon monolayer differentiation, determined by RT-qPCR. Average expression $\pm$ SD, n=4 biological replicates per condition. Reference gene: *Hist2h2aa1*. (d) SPEN and FLAG Western blot of Wt, *Spen*^{-/-} and three *Spen* cDNA rescue clones. VCP was used as a loading control. Experiment performed at least two times with similar results. M = High molecular weight protein ladder. (e) Percentage of *Rnf12* allelic expression of Wt, *Spen*^{-/-} and three *Spen* cDNA rescue clone upon differentiation. Relative *Rnf12* allelic (129 and Cast) expression was normalized to *Rnf12* total expression and averaged $\pm$ SD, n=4 biological replicates per condition. Two-sided Mann-Whitney test ($\alpha < 0.05$). Reference gene: *Hist2h2aa1*. (f) Relative allele-specific *Tsix* expression of Wt, *Spen*^{-/-} and three *Spen* cDNA rescue clones upon differentiation. Average expression $\pm$ SD, n=4 biological replicates per condition. Reference gene: *Hist2h2aa1*. (g) *Sox2*, *Oct4* and *Gata6* relative expression of Wt, *Spen*^{-/-} and *Spen* cDNA rescue clones upon differentiation. Average expression $\pm$ SD, n=4 biological replicates per condition. Reference gene: *Hist2h2aa1*.

Supplementary Fig. 4

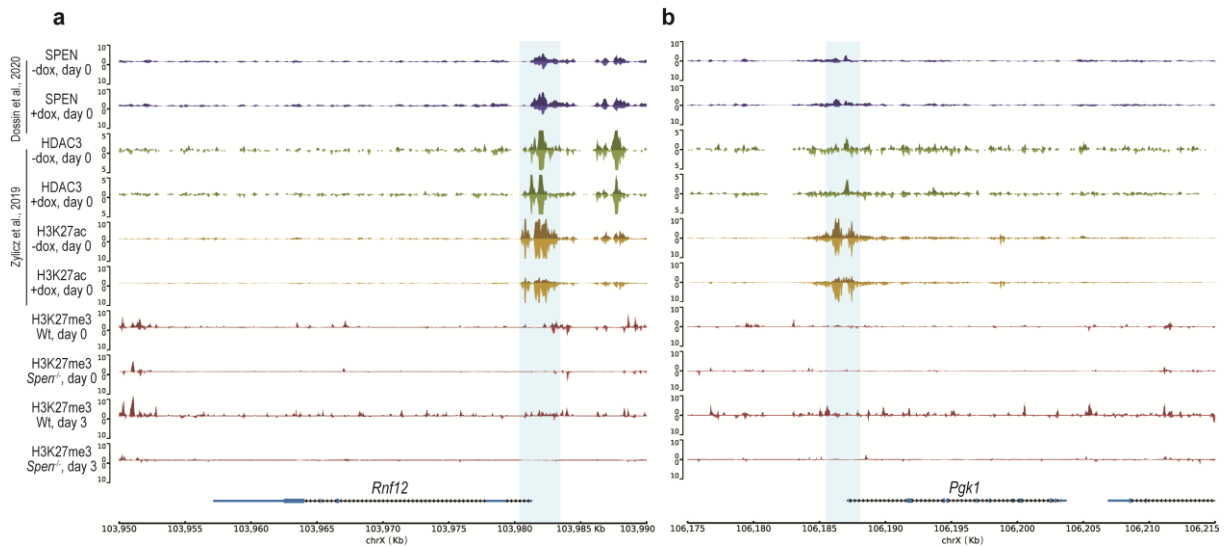


Supplementary Fig. 4 Generation and characterization of a SPEN-GFP C-terminal tag line. | Related to Fig. 3.

(a) eGFP knock-in strategy in *Spn* exon 16 using the CRISPR/Cas9 system. (b) Genotyping of the *Spn*-GFP knock-in clones by PCR on gDNA to determine the specific integration of the 5' and 3' HA and the presence of two different X chromosomes making use of a length polymorphism. Experiment performed at least two times with similar results. M = DNA ladder. (c) Flow cytometry histograms comparing the GFP fluorescence in the parental line (gray) with a negative clone (left) and two

independent SPEN-GFP-tagged ESC clones (middle and right). **(d)** Percentage of nuclei with *Xist* clouds and SPEN accumulation in Wt (untagged) and *Spn*-GFP ESCs. Quantification of Fig. 3d. Average percentage $\pm$ SD, n=2 independent clones, the total number of counted nuclei per clone is indicated. **(e)** Double IF staining of SPEN-GFP (α GFP, green) and EZH2 (red) at day 0, 2 and 5 of differentiation of a *Spn*-GFP ESC line. DNA is stained with DAPI (blue). Scale bar: 10 μ m. **(f)** Double IF staining of SPEN-GFP (α GFP, green) and H3K27me3 (red) at day 0, 2 and 5 of differentiation of a *Spn*-GFP ESC line. DNA is stained with DAPI (blue). Scale bar: 10 μ m. **(g)** Quantification of **(e and f)**, showing the percentage of nuclei with SPEN, EZH2 and H3K27me3 accumulation upon monolayer differentiation (day 0, 2 and 5). Average percentage $\pm$ SD, n=2 independent clones, the total number of counted nuclei per clone is indicated.

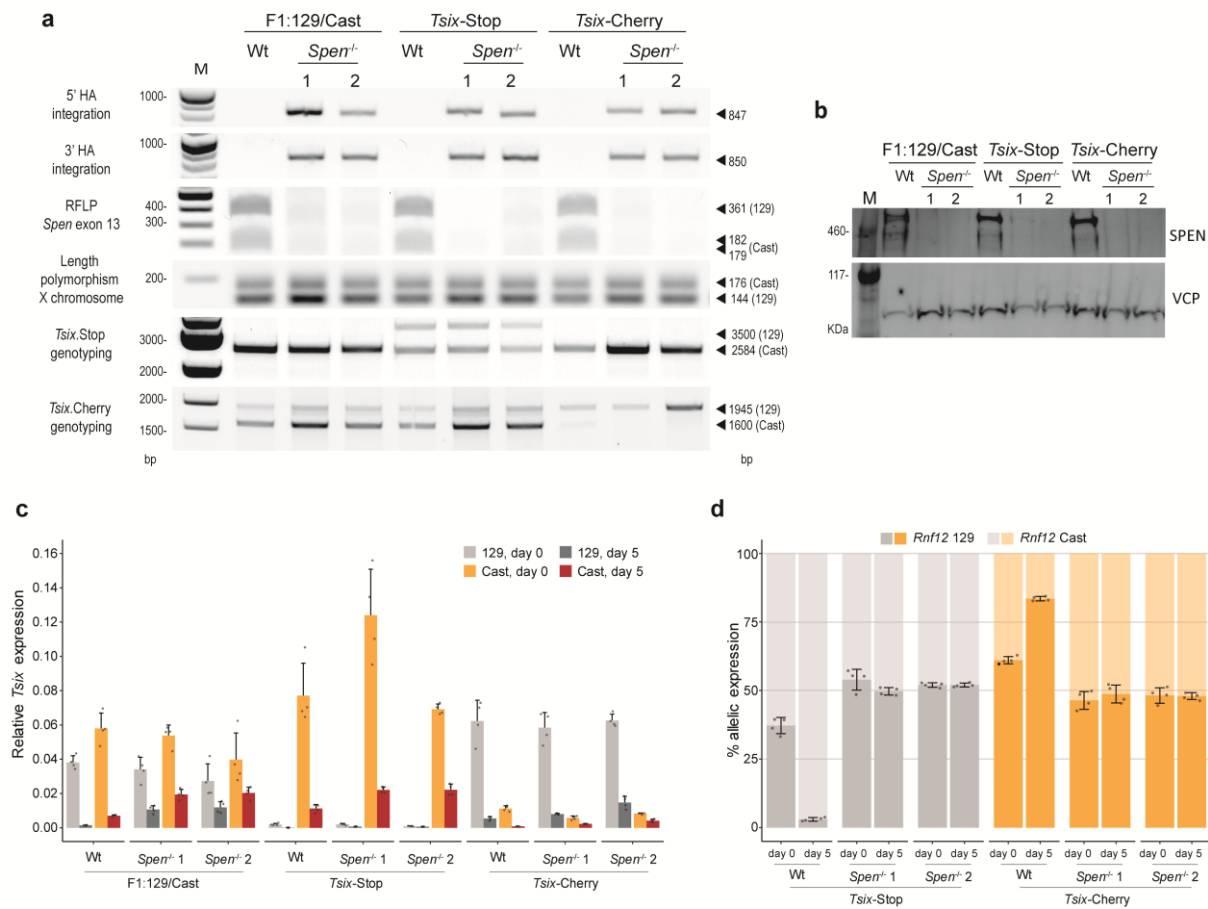
Supplementary Fig. 5



Supplementary Fig. 5 Chromatin features of X-linked genes. | Related to **Fig. 5**.

(a-b) Genome browser tracks showing the allele-specific SPEN, HDAC3, H3K27ac and H3K27me3 binding for two X-linked genes: **(a)** *Rnf12* and **(b)** *Pgk1*. The top part (dark colour) of each track represents the Xi and the bottom (light colour) the Xa. SPEN CUT&RUN (blue, top) profile in *Xist*-inducible undifferentiated ESCs (day 0) untreated or treated with doxycycline (24h)². HDAC3 (green, top-middle) and H3K27ac ChIP-seq (yellow, bottom-middle) in *Xist*-inducible undifferentiated ESCs (day 0) untreated or treated with doxycycline (24h)³. H3K27me3 ChIP-seq (red, bottom) in Wt and *Spen*^{-/-} ESCs at day 0 and 3 of monolayer differentiation. The light-blue square highlights the promoter region of *Rnf12* and *Pgk1*.

Supplementary Fig. 6



Supplementary Fig. 6 Generation and characterization of *Sp^{en}^{-/-}* in *Tsix* defective ESC lines. | Related to Fig. 6.

(a) Genotyping strategy to identify optimal *Sp^{en}^{-/-}* clones in F1:129/Cast, *Tsix*-Stop and *Tsix*-Cherry ESC lines, done by PCR on gDNA. Specific 5' and 3' integrations of the PuroR cassette in the *Sp^{en}* locus to identify *Sp^{en}* knockout lines. RFLP analysis on *Sp^{en}* exon 13 to determine the absence of the *Sp^{en}* 129 and/or Cast allele. Verification of the presence of two X chromosomes per ESC line, making use of a length polymorphism on the X chromosome. *Tsix*-Stop line genotyping, using a primer pair across the triple poly(A) signal blocking *Tsix* transcription. *Tsix*-Cherry line genotyping by determining the loss of the Cast band of a specific length polymorphism, indicating proper mCherry integration downstream of the *Tsix* promoter. Experiment performed at least two times with similar results. M = DNA ladder.

(b) SPEN Western blot of Wt and *Sp^{en}^{-/-}* F1:129/Cast, *Tsix*-Stop and *Tsix*-Cherry ESC lines. VCP was used as a loading control. Experiment performed at least two times with similar results. M = High molecular weight protein ladder.

(c) Relative allele-specific *Tsix* expression at day 0 and 5 of monolayer differentiation of Wt and *Sp^{en}^{-/-}* F1:129/Cast, *Tsix*-Stop and *Tsix*-Cherry ESC lines, determined by RT-qPCR. Average expression $\pm$ SD, n=4 biological replicates per condition. Reference gene: *Hist2h2aa1*.

(d) Percentage of *Rnf12* allelic expression at day 0 and 5 of monolayer differentiation of Wt and *Sp^{en}^{-/-}*

^{-/-} F1:129/Cast, *Tsix*-Stop and *Tsix*-Cherry ESC lines, determined by RT-qPCR. Relative *Rnf12* allelic (129 and Cast) expression was normalized to *Rnf12* total expression and averaged $\pm$ SD, n=4 biological replicates per condition. Reference gene: *Hist2h2aa1*.

Supplementary Table 1. Overview of publications that studied *Spen* in relation to XCI. KO = knockout, KD = knockdown, n.d. = not-determined.

Reference	Doxycycline-inducible <i>Xist</i> or physiological XCI	<i>Spen</i> KO or KD	<i>Xist</i> upregulation	XCI
4	Doxycycline-inducible <i>Xist</i> / physiological XCI	KD, siRNA	+	-
5	Doxycycline-inducible <i>Xist</i>	KD, siRNA	+	-
6	n.d.	n.d.	n.d.	n.d.
7	Doxycycline-inducible <i>Xist</i> / physiological XCI	KD, shRNA	+	-
8	Doxycycline-inducible <i>Xist</i>	KO	+	-
1	Doxycycline-inducible <i>Xist</i>	KO	+	-
2	Doxycycline-inducible <i>Xist</i>	KD, Auxin-inducible degron	+	-
9	Doxycycline-inducible <i>Xist</i> / physiological XCI	KO	+ / ?	-
10	Doxycycline-inducible <i>Xist</i>	KO	+	-
This study	Doxycycline-inducible <i>Xist</i> / physiological XCI	KO	+/-	-

Supplementary Table 2. Genetically modified ESC lines generated in this study.

Genetic modification	Donor vector, sgRNA(s)	Transfected ESC line	ESC line source	Genotype	Clone names	Used in
<i>Spn</i> homozygous and heterozygous knockout	5'HA – PuroR – 3'HA, 5'+ 3' sgRNA	Doxycycline responsive endogenous <i>Xist</i> promoter (Clone 87)	¹¹	Wt	Wt 1: parental Wt2: C1 (transfected, no deletion)	Fig.1, Fig. 4, Fig. 5, Supplementary Fig. 1, Supplementary Fig. 2 and Supplementary Fig. 5
				<i>Spn</i> ^{+/-}	<i>Spn</i> ^{+/-} 1: D7 <i>Spn</i> ^{+/-} 2: E7	
				<i>Spn</i> ^{-/-}	<i>Spn</i> ^{-/-} 1: B3 <i>Spn</i> ^{-/-} 2: G4	
		F1:129/Cast	¹²	Wt	Parental	Fig. 6 and Supplementary Fig. 6
				<i>Spn</i> ^{-/-}	<i>Spn</i> ^{-/-} 1: A8 <i>Spn</i> ^{-/-} 2: A10	
		<i>Tsix</i> -Stop	¹³	Wt	Parental	
				<i>Spn</i> ^{-/-}	<i>Spn</i> ^{-/-} 1: A5 <i>Spn</i> ^{-/-} 2: B6	
		<i>Tsix</i> -Cherry	¹⁴	Wt	Parental	
				<i>Spn</i> ^{-/-}	<i>Spn</i> ^{-/-} 1: A4 <i>Spn</i> ^{-/-} 2: H2	
<i>Spn</i> cDNA rescue in <i>Spn</i> homozygous knockout line	pFD46 vector ²	<i>Spn</i> ^{-/-} (Clone B3)	This study	Wt	Clone 87	Fig. 2 and Supplementary Fig. 3
				<i>Spn</i> ^{-/-}	parental	
				<i>Spn</i> ^{-/-} + <i>Spn</i> cDNA in <i>ROSA26</i> locus	Clone A: B6 Clone B: E4 Clone C: F3	
<i>Spn</i> C-terminal eGFP tag	5'HA – eGFP – 3'HA, 3' sgRNA	Doxycycline responsive endogenous <i>Xist</i> promoter (Clone 87)	¹¹	Wt	Wt1: parental Wt2: G5 (transfected, no integration)	Fig. 3 and Supplementary Fig. 4
				<i>Spn</i> -eGFP	<i>Spn</i> -GFP 1: B2 <i>Spn</i> -GFP 2: D7	

Supplementary Table 3. List of genotyping primers.

Name	Sequence	Source
9.5'-PuroR.integration.Fw	GTGTCCTCATGCAAAGTGG	
12.5'-PuroR.integration.Rv	TTAATTGTAGCCGCGTTCTAAC	
14.3'-PuroR.integration.Fw	AGACTGCCTTGGGAAAAGCG	
16.3'-PuroR.integration.Rv	CTGTACCCGAAGCACCATTT	
250.RFLP1.Exon13.BlpI.Fw	GACTTCAGCGTGAGGCAGAG	
250.RFLP1.Exon13.BlpI.Rv	CACGCAGCCTATACCACCTG	
X-LP-Dxmit65.Fw	ATATTAAGGGAGGTAACAAAGACCC	Whitehead Institute at MIT; Center for Genome Research
X-LP-Dxmit65.Rv	GGTTTCTGTGATTGCTATAGGACA	
2.Across.Xist.Dox.Promoter.Fw	CCCAGATGGGCAAGTTTAGA	
4.Across.Xist.Dox.Promoter.Rv	CAGGACATCTGGGGCTATACA	
48.Spen.Rosa26.integration.Right.Fw	TTTGCATTCCAAAGGAACC	
49.Spen.Rosa26.integration.Right.Rv	ATACGAGGTCGCCAACATCT	
55.SpenRRM1.Fw	CGCTCCCTGTTATCTGAAGC	
56.Spen.Flag.Rv	AAGGACCACGACGGAGACTA	
1.3'-eGFP.integration.Fw	TCCTTGAAGTCGATGCCCTT	
2.3'-eGFP.integration.Rv	GTGGAAACCGACTACTGCCT	
4.5'-eGFP.integration.Fw	GTTCTGCACATCCGACCAAG	
7.5'-eGFP.integration.Rv	CACATGAAGCAGCACGACTT	
350-36. Fw Tsix LP, for DNA	AGTGCAGCGCTTGTGTCA	¹⁴
351-41. Rv Tsix LP, for DNA	TATTACCCACGCCAGGCTTA	
356.Tsix Stop genotyping - 3F	CTTTGGTTTTGATGCGGATT	
357.Tsix Stop genotyping - 3R	GCCTCTGTCACTCCATCTCC	

Supplementary Table 4. List of expression and allele-specific primers.

Name	Sequence	Source
Xist_129_F4	GGAAGAAGGTAGGATTCTACCTCTTC	
Xist_cast_F4	GGAAGAAGGTAGGATTCTACCTCATG	
Xist_R4	GCCAGCACTGATCTCAAGC	
358.Xist_Ex1-2_F	GGATCCTGCTTGAAGTACTGC	15
359.Xist_Ex1-2_R	CAGGCAATCCTTCTTCTTGAG	
rnf12_Ex5_129F	CAGAACGGGAAAAGGTACGC	
rnf12_Ex5_129R	ATACCGGCAGAGAGATAGTATAGCTTGC	
rnf12_Ex5_Cast_F	TTCAGAACGGGAAAAGGTACGT	
rnf12_Ex5_Cast_R	GAATACCGGCAGAGAGATAGTATAGCTTGT	
Tsix_In3_129_R	CAGGGCTACCCTGGAAAACT	
Tsix_In3_Cast_R	CCAGGGCTATCCTGGAAAAATC	
Tsix_In3_F	TGCATTAGCTGCTCCTCTT	
297.Hist2h2aa1.qPCR.Ctl.Fw	GTTTGCGCTTTCGTGATGTC	
298.Hist2h2aa1.qPCR.Ctl.Rv	CCCCACCGGGAAGTGTAG	
295.Rex1_F	CTAAGCAAGACGAGGCAAG	16
296.Rex1_R	AGAATGGGTTCGGAAAACTC	
Nanog qPCR_FW	AGGATGAAGTGCAAGCGGTG	17
Nanog qPCR_RV	TGCTGAGCCCTTCTGAATCAG	
Gata6.Fw	GAGCTGGTGCTACCAAGAGG	18
Gata6.Rv	TGCAAAAGCCCATCTCTTCT	
372_β-Actin qPCR_FW	ACTATTGGCAACGAGCGGTTC	19
373_β-Actin qPCR_RV	AGAGGTCTTTACGGATGTCAACG	
94. SPEN Exon10. qPCR Fw	GCAAATCGGGAAAGCCAACT	8
95. SPEN Exon11. qPCR Rv	CTGCACTCCAGTCTTCATGC	
489.Klf4 qPCR_FW	CCAGCAAGTCAGCTTGTA	16
490.Klf4 qPCR_RV	GGGCATGTTCAAGTTGGATT	
485.Oct4 qPCR_FW	CCCAATGCCGTGAAGTTG	16
486.Oct4 qPCR_RV	TCAGCAGCTTGGCAAAGTGT	

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