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To assess the importance of SPEN during the initiation of XCI, we used an auxin-inducible degron (AID)[7] that enables controlled and acute depletion of the endogenous SPEN protein. We used our previously described female hybrid (*Mus musculus castaneus* × C57BL/6) TX1072[8] mouse embryonic stem cells (ES cells), in which a doxycycline (DOX)-inducible promoter upstream of the endogenous *Xist* locus enables conditional *Xist* RNA expression and XCI (Fig. 1a). In ES cells expressing the *Oryza sativa* TIR1 (OsTIR1) E3 ligase, we generated a homozygous knock-in that expressed the AID fused to a HaloTag at the C terminus of endogenous SPEN, in order to ensure auxin-dependent SPEN depletion (Extended Data Fig. 1a). Efficient degradation of SPEN occurred within 1 h of auxin treatment (Fig. 1b, Extended Data Fig. 1b, Supplementary Fig. 1) whereas the removal of auxin led to rapid recovery of SPEN (Fig. 1b), demonstrating potent AID-dependent modulation of SPEN levels.

To evaluate the immediate consequences of the loss of SPEN on the initiation of XCI, we acutely depleted SPEN for 4 h before inducing *Xist* expression for 24 h and performing RNA sequencing. Loss of SPEN had no effect on the formation of *Xist* RNA clouds (Extended Data Fig. 1c, e), confirming that SPEN is dispensable for *Xist* localization[2–5]. However, gene silencing was almost completely abolished along the entire X chromosome in the absence of SPEN (Fig. 1c, d, Supplementary Table 1), whereas auxin had no effect on XCI in wild-type cells (Extended Data Fig. 1d). Clustering analysis highlighted three groups of genes that differed in their silencing defects upon the loss of SPEN (Fig. 1e). Most X-linked genes (80% of 382) were found to be entirely dependent on SPEN for silencing, whereas only a small subset (6%) showed unaltered silencing in the absence of SPEN. This notable defect in XCI was confirmed by pyrosequencing (Fig. 1f) and nascent RNA fluorescence in situ hybridization (FISH) (Extended Data Fig. 1e).

We next assessed the requirement for SPEN in XCI in vivo during mouse early embryogenesis, using allele-specific RNA sequencing in embryonic day (E)3.5 *Spen*-knockout female embryos[9] harbouring hybrid X chromosomes (Fig. 1g, Extended Data Fig. 1f, g). At this stage in wild-type embryos, imprinted XCI has taken place[10] and only the paternal X chromosome is inactivated (Fig. 1h, Extended Data Fig. 1h).

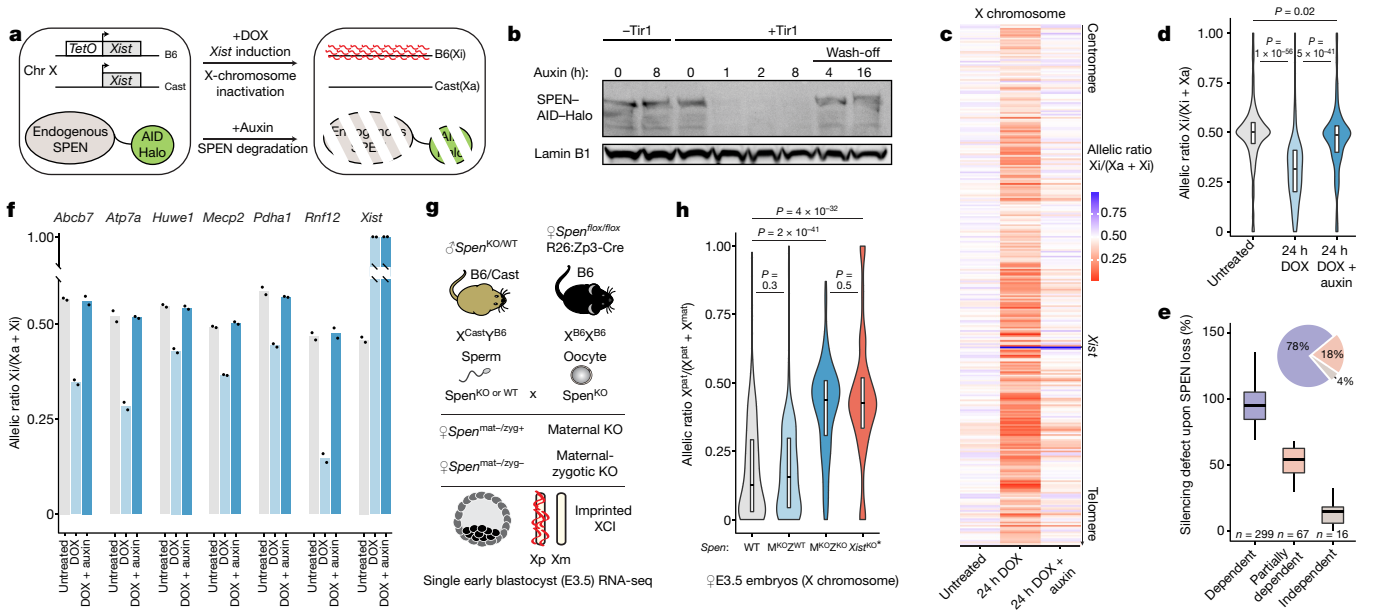

Fig. 1 | SPEN mediates gene silencing across the entire X chromosome in vitro and in vivo. a, Schematic of SPEN-degron *Xist*-inducible mouse ES cells. Xa, active X chromosome; Xi, inactive X chromosome. **b**, Western blot showing auxin-induced degradation of endogenous HaloTagged SPEN. This experiment was repeated at least twice with similar results. **c**, **d**, Heat map (**c**) and violin plots (**d**) showing X-chromosomal transcript allelic ratios after 0 h, 24 h DOX or 24 h DOX + auxin treatment in SPEN-degron mouse ES cells ($n = 434$ genes, two-sided Student's *t*-test). **e**, Box plot representation of gene-silencing defect upon SPEN loss in three groups of genes differing by their level of dependence on SPEN for *Xist*-mediated silencing. The pie chart shows the relative number of genes in each group. **f**, Pyrosequencing assay of seven X-linked transcripts in mouse ES cells after 0 h, 24 h DOX or 24 h DOX + auxin treatment. Data in **c**–**f** are averages of two independent clones; in **f**, individual data points are shown. **g**, The mouse crossbreeding scheme for the *Spen*-knockout experiment. KO, knockout; WT, wild type; mat., maternal; zyg., zygotic; Xm, maternal X chromosome; Xp, paternal X chromosome. **h**, X-chromosomal transcript allelic ratio distribution ($n = 256$ genes) in wild-type ($n = 2$), maternal-only (M) *Spen*-knockout ($n = 3$), maternal-zygotic (Z) *Spen*-knockout ($n = 5$), and *Xist*-knockout E3.5 embryos ($n = 30$ single cells, two-sided Wilcoxon rank-sum test. For * refer to ref. [10]). In **d**, **e**, **h**, horizontal lines denote the median, box limits correspond to the upper and lower quartiles.

In maternal-zygotic *Spen* knockouts, imprinted XCI is severely hindered although paternal *Xist* is expressed. Both maternal and paternal X chromosomes are expressed equally, phenocopying *Xist*-knockout E3.5 embryos[10] (Fig. 1h, Extended Data Fig. 1g, h, Supplementary Table 2). A maternal-only *Spen* knockout has no effect on imprinted XCI (Fig. 1h), suggesting that the zygotic pool of SPEN is necessary and sufficient for this process. Therefore, the early gene-silencing mechanism(s) involved in imprinted and random XCI are dependent on SPEN.

We next assessed precisely when SPEN is recruited during XCI. HaloTag labelling[11] of SPEN combined with *Xist* RNA FISH revealed that SPEN associates with *Xist* RNA rapidly upon *Xist* coating and throughout XCI (Fig. 2a). To capture early *Xist*–SPEN dynamics during the short time window in which *Xist* becomes upregulated, we followed both *Xist* and SPEN in living cells. We tagged endogenous SPEN with GFP in a background in which *Xist* RNA is visualized via a BglG–mCherry fusion protein binding to Bgl stem–loops inserted within *Xist*[12] (Extended Data Fig. 2a, b). Live-cell imaging revealed that SPEN colocalizes with *Xist* from the very onset of *Xist* upregulation (Extended Data Fig. 2c, d, Supplementary Video 1). Therefore, SPEN can initiate gene silencing immediately upon *Xist* coating.

We also found that SPEN robustly accumulated on the inactive X chromosome after differentiation into neural progenitor cells (NPCs, Fig. 2b), in which XCI is epigenetically maintained. The depletion of SPEN for up to two days in independent NPC clones (Fig. 2c) did not lead to reactivation of fully silenced genes (Fig. 2d, Supplementary Table 3); however, we observed moderate but significant upregulation of genes escaping XCI (Fig. 2e, f), which suggests that SPEN buffers the overexpression of X-linked escapee genes in female cells.

Chromosome conformation capture has revealed that, in differentiated cells, the inactive X chromosome is folded into megadomains[13–15] and is globally depleted of topologically associating domains except in regions that contain clusters of escapee genes[13]. *Xist* RNA has been found to have a role in the conformation of the inactive X chromosome[1,14]. To assess whether SPEN is involved, we performed allele-specific Hi-C in NPCs after 48 h of SPEN depletion. No notable conformational changes were observed on the inactive X chromosome (Extended Data Fig. 2e–g); we therefore conclude that the structural effects mediated by *Xist* RNA in differentiated cells occur independently of SPEN.

In summary, our data suggest that SPEN exerts its role by actively promoting gene silencing during the earliest stages of XCI. However, it has no major role in stabilizing the transcriptionally inactive state of the inactive X chromosome, or in ensuring the maintenance of its conformation.

We next sought to identify which parts of SPEN ensure its function during XCI. SPEN is a very large protein (around 400 kDa) that contains four RNA recognition motifs (RRMs), a nuclear receptor interaction domain (RID) and a SPEN paralogue/orthologue C-terminal (SPOC) domain (Fig. 3a). We overexpressed a series of SPEN complementary DNA truncations, stably targeted into the *Rosa26* locus in the SPEN-degron mouse ES cell line (Extended Data Fig. 3a, b, Fig. 3a). We then induced *Xist* expression for 24 h and assessed which SPEN fragments could rescue XCI-initiation function in the context of auxin-mediated depletion of endogenous SPEN. We found that the RRM1 domain and the RID are dispensable for SPEN accumulation on the inactive X chromosome, as well as for X-linked gene silencing (Fig. 3b, c). By contrast, a SPEN truncation lacking the RRM2–4 domains failed to accumulate on the inactive X chromosome and failed to rescue XCI (Fig. 3b, c). SPEN recruitment to the inactive X chromosome is therefore mediated by the RRM2–4 domains and is necessary for gene silencing. This is consistent with studies showing that RRM2–4 directly bind the A-repeat of *Xist*

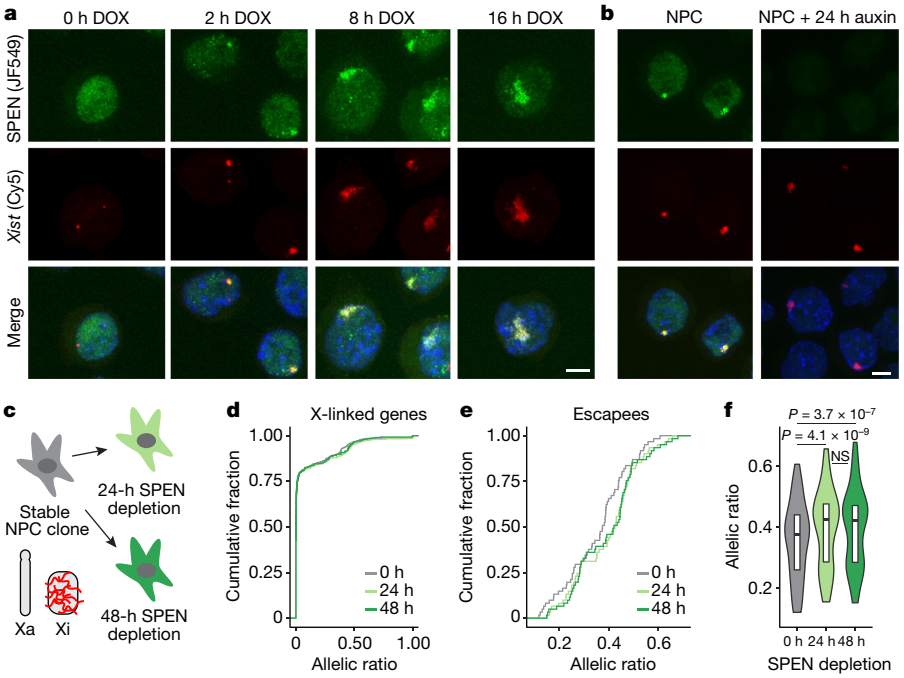

Fig. 2 | SPEN localizes to the X chromosome immediately upon *Xist* upregulation and throughout the stages of XCI, but is dispensable for the maintenance of X-linked gene silencing. a, **b**, Images from combined HaloTag labelling of SPEN (green) and FISH for *Xist* RNA (red) in mouse ES cells during a time course of *Xist* induction (**a**) and in NPCs (**b**). Scale bars, 5 µm. **c**, Schematic of the SPEN-degron experiment in NPCs. **d**, Cumulative distribution of transcript allelic ratios across the X chromosome ($n = 387$ genes) after SPEN depletion in NPCs. **e**, **f**, Cumulative distribution (**e**) and violin plot representation (**f**) of the transcript allelic ratio of escapees after SPEN depletion in NPCs ($n = 65$, two-sided Wilcoxon signed-rank test. NS, not significant. Horizontal lines denote the median, box limits correspond to upper and lower quartiles). Data in **d**–**f** are the average of two independent NPC clones. The experiments in **a**, **b** were repeated at least twice with similar results.

RNA in vitro[4,16]—a region of *Xist* that is necessary for gene silencing[17]. Conversely, a truncation of the SPOC domain enabled efficient SPEN accumulation on the inactive X chromosome, but failed to rescue XCI (Fig. 3b, c). To validate this observation, we performed homozygous deletion of the SPOC domain at the endogenous *Spen* locus in mouse ES cells (Extended Data Fig. 3c). Deletion of the SPOC domain had no effect either on SPEN recruitment to the inactive X chromosome or on *Xist* RNA clouds (Extended Data Fig. 3d–f), but resulted in strongly deficient XCI, albeit milder than that in SPEN-depleted cells (Extended Data Fig. 3g–j). Collectively, these results demonstrate that the SPOC domain is essential for XCI. However, other uncharacterized regions of SPEN contribute—albeit to a lesser extent—to ensure its full silencing potential.

To test whether the SPOC domain alone could mediate X-linked gene silencing, we used SPEN-degron ES cells to introduce an array of BglI stem–loops at the *Xist* locus (identical to the live-imaging strategy). In this background, we generated several independent ES cell lines expressing a BglG–GFP–SPOC protein fusion (or BglG–GFP as a control) targeted into *Rosa26*. These proteins would become tethered to *Xist*–BglI stem–loop RNA via BglG (Fig. 3d). Notably, upon induction of *Xist* RNA in the absence of endogenous SPEN, tethering of BglG–GFP–SPOC (but not of BglG–GFP alone) resulted in substantial gene silencing across the X chromosome, with over half of the genes being silenced by more than 50% (Fig. 3e, f). SPOC-specific rescue was confirmed using pyrosequencing (Fig. 3g). Consistent with previous studies[18–20], our results reveal SPOC as a key domain of SPEN that enables gene silencing once recruited to the X chromosome by *Xist* RNA.

The SPOC domain of SPEN was originally identified as an interactor of the NCoR and SMRT corepressors in human cells[18,21,22]. Given that NCoR and SMRT interact with and activate HDAC3[23], it was proposed that SPEN triggers XCI via HDAC3[2], the activity of which is important for *Xist*-mediated silencing[2,24]. However, XCI is more markedly affected upon the loss of SPEN and SPOC than upon the loss of HDAC3 (Extended Data Fig. 3j, k). These observations suggest that a model involving HDAC3 only partially explains the function of SPEN, and that SPOC must exert its key role in gene silencing also through other, HDAC3-independent pathways. To identify such pathways, we characterized the protein interactome of the SPOC domain by performing GFP pull-downs from mouse ES cells that stably expressed BglG–GFP–SPOC (or BglG–GFP as a control, Fig. 3h, Supplementary Table 4), followed by mass spectrometry analysis.

We identified NCoR and SMRT as expected, but we also found HDAC3 (Fig. 3h, Extended Data Fig. 3l), which further supports the proposed model for the function of SPEN in XCI[2]. Notably, we identified the m^{6}A methyltransferase complex and the m^{6}A reader YTHDC1 (Fig. 3h, Extended Data Fig. 3l), which have been proposed to play a role in XCI[5,20,25]. One of these factors, WTAP, co-purified with *Xist* RNA in an A-repeat-dependent manner[3]—although, contrary to the case of SPEN, a direct interaction between WTAP and *Xist* A-repeat has not been reported. Our results therefore suggest that SPOC may participate in the recruitment of m^{6}A machinery to *Xist* RNA. We also identified the NuRD complex—a potent repressor that displaces RNA polymerase II (RNAPII) from transcription start sites through chromatin remodelling[26]—and RNAPII, together with factors that are involved in the regulation of transcription initiation and elongation (Fig. 3h, Extended Data Fig. 3l). Together these findings show that, through its SPOC domain, SPEN bridges *Xist* to multiple factors that are involved in transcription and chromatin regulation, and together they mediate efficient gene silencing. Given that SPOC immunoprecipitation was performed in the absence of *Xist* induction, the identified interactions are not mediated by *Xist* RNA.

We also investigated where SPEN binds to the X chromosome during XCI, and whether it has distinct binding sites or whether it associates with chromatin diffusely across the entire chromosome, as anticipated

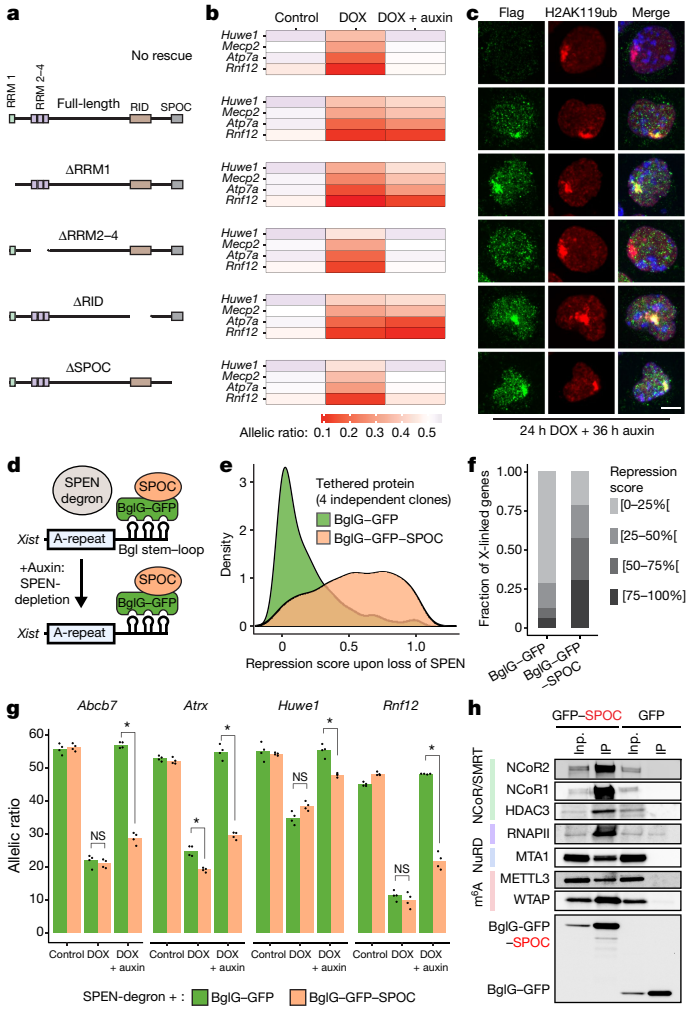

Fig. 3 | The SPOC domain of SPEN mediates gene silencing and interacts with multiple molecular pathways. a, *Spen* cDNA fragments used for the rescue experiment. **b**, Heat map representation of four X-linked transcript allelic ratios (obtained by pyrosequencing) in control, 24 h DOX- and 24 h DOX + 36 h auxin-treated SPEN-degron mouse ES cells overexpressing each cDNA construct. Data represent averages of two to three independent clones. **c**, Immunofluorescence detection of Flag-tagged SPEN truncations (green) and H2AK119ub1 (red), a marker of the inactive X chromosome, in SPEN-degron mouse ES cells treated with DOX and auxin. Scale bar, 5 µm. **d**, Schematic showing the tethering of BglG–SPOC to *Xist*. **e**, Distribution of gene-repression scores observed across the X chromosome upon the depletion of endogenous SPEN and the tethering of BglG–GFP (green) or BglG–GFP–SPOC (orange) to *Xist*. **f**, Bar graphs showing the fraction of X-linked genes within four windows of repression score. **g**, Transcript allelic ratio (obtained by pyrosequencing) for four X-linked genes upon the depletion of endogenous SPEN and the tethering of BglG–GFP or BglG–GFP–SPOC to *Xist* (*$P < 0.01$, two-sided Student's *t*-test). **h**, Western blot showing co-immunoprecipitated proteins in BglG–GFP and BglG–GFP–SPOC immunoprecipitation experiments. One per cent of the input was loaded (0.1% for RNAPII), and 10% of the pull-down. The experiments in **c**, **h** were repeated at least twice with similar results. The data in **e**–**g** are the average of four independent clones.

from our imaging results. We performed allele-specific, cross-linked CUT&RUN[27] experiments on SPEN during a time course of *Xist* induction (0 h, 4 h, 8 h, 24 h DOX, or 8 h DOX + auxin as a negative control).

We found that there are few binding sites for SPEN across the genome of uninduced ES cells (Extended Data Fig. 4a). Conversely, hundreds of SPEN-binding sites appeared specifically on the X chromosome as early as 4 h after *Xist* induction (Fig. 4a, Extended Data Fig. 4a). This is consistent with imaging data (Extended Data Fig. 2). We note that SPEN accumulation is seen across the gene body of *Xist* (Fig. 4b), suggesting that SPEN binds *Xist* RNA while it is transcribed. In sharp contrast to the *Xist* locus, SPEN shows focal binding on the rest of the genome, with peaks falling almost exclusively on promoters and enhancers (Fig. 4c, d, Extended Data Figs. 4b, 5a–g).

After *Xist* induction, recruitment of SPEN to the inactive X chromosome reaches a maximum at 4 h (Fig. 4a, Extended Data Fig. 4c), showing the highest enrichment within regions that were coated earliest by *Xist*[28] (entry sites, Fig. 4e). SPEN accumulation thus follows the spatial dynamics of *Xist* spreading. Among promoter targets on the X chromosome, SPEN preferentially binds those of actively expressed genes (Fig. 4f, Extended Data Fig. 4d), which suggests that the ability of SPEN to target chromatin depends on transcriptional activity. Consistently, genes that are classified as fully dependent on SPEN for silencing (Fig. 1e)—which show a greater degree of SPEN binding at their promoters within 4 h of *Xist* coating than less-dependent genes (Extended Data Fig. 4e)—also show initially higher transcription levels (Fig. 4g).

Furthermore, within 4 h of *Xist* induction, SPEN binding is greater at the promoters of efficiently silenced genes than at the promoters of less-efficiently silenced genes (Fig. 4h). Similarly, upon *Xist* coating, efficiently deacetylated enhancers[24] show a higher enrichment of SPEN than less-efficiently deacetylated enhancers (Fig. 4i). Finally, genes that are subject to very little silencing—or those that completely escape XCI in our *Xist*-inducible system—show a significantly lower SPEN signal at their promoters (Extended Data Figs. 4f, g, 5h–n). This pattern of SPEN recruitment at discrete sites to the X chromosome that is undergoing XCI indicates that transcriptional silencing is caused by the binding of SPEN to active promoters and enhancers.

To understand how SPEN might function at enhancers and promoters, we integrated CUT&RUN profiles with publicly available data from chromatin immunoprecipitation followed by sequencing (ChIP–seq) experiments for transcription and chromatin-associated factors identified in our mass spectrometry analysis. We included HDAC3[24], RNAPII[26] and two members of the NuRD complex (MBD3 and CHD4)[26]. SPEN binding strongly overlaps with HDAC3 at enhancers but not at promoters (Extended Data Fig. 4h). Our recent findings revealed that HDAC3 is pre-bound predominantly at enhancers on the X chromosome[24]. Therefore, *Xist*-mediated recruitment of SPEN to enhancers may activate HDAC3. Conversely, a strong overlap with SPEN binding is observed for the NuRD complex specifically at promoters but not at enhancers (Extended Data Fig. 4h). Furthermore, SPEN peaks extensively overlap with RNAPII phosphorylated on serine 5, which is associated with transcription initiation (Extended Data Fig. 4h). This analysis suggests that SPEN may operate at enhancers and promoters through distinct pathways to promote gene silencing.

Notably, the binding of SPEN to chromatin decreases across the whole X chromosome after 24 h of *Xist* induction (Fig. 4a, Extended Data Fig. 4c). Clustering of CUT&RUN profiles at SPEN-bound promoters (Extended Data Fig. 4i, Supplementary Table 5) revealed distinct groups of promoters, grouped on the basis of how efficiently SPEN was lost within 24 h of XCI (Fig. 4j). In the 'strong SPEN loss' group, binding was maximal by 4 h but decreased after 8 h, and even more markedly after 24 h (Fig. 4j). Conversely, the 'mild SPEN loss' group showed maximal and persistent SPEN binding at 4 h and 8 h of *Xist* induction, respectively, with only a mild reduction of SPEN binding by 24 h (Fig. 4j). Finally, a third group—comprising fewer promoters—showed both mild SPEN enrichment at 4 h and low loss at 24 h (Fig. 4j). The group that lost SPEN most efficiently also showed the most pronounced gene silencing by 24 h when compared with the groups that significantly retained SPEN (Fig. 4k, Extended Data Fig. 4j). Altogether this analysis suggests that, once recruited to the X chromosome by *Xist* RNA, SPEN associates with enhancers and promoters in a transcription-dependent manner. This recruitment leads to gene silencing, after which the favourable transcriptional context for SPEN binding is lost, and SPEN binding to chromatin decreases. Despite loss of the chromatin-bound SPEN

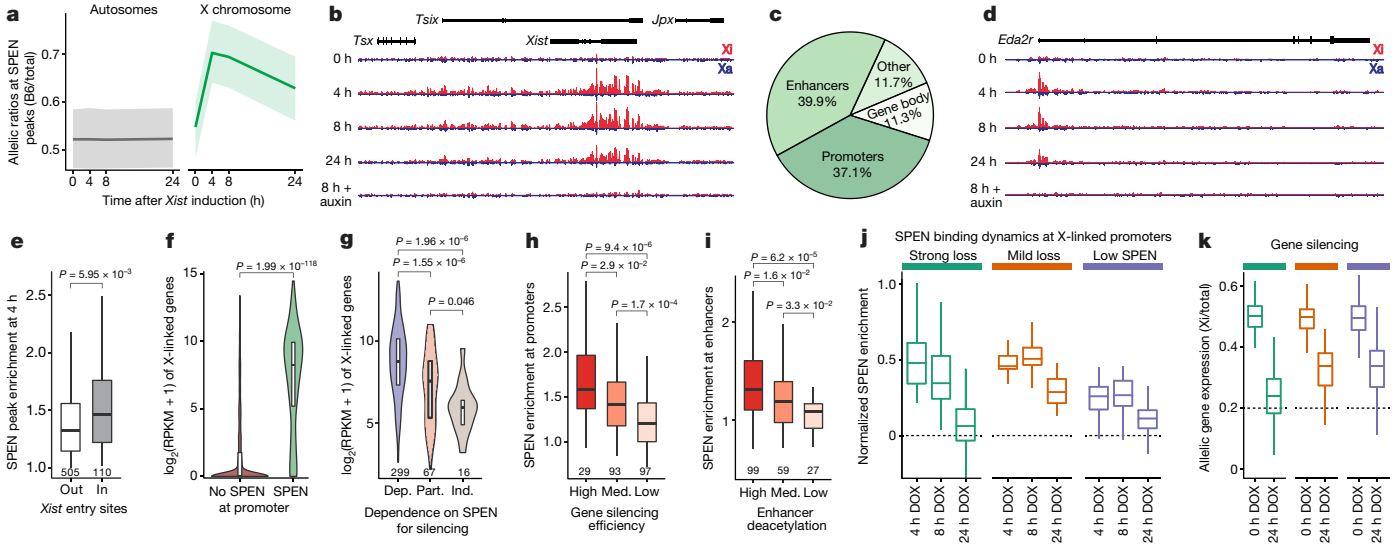

Fig. 4 | SPEN is recruited by *Xist* to active gene promoters and enhancers, where it silences transcription and subsequently disengages from chromatin. a, SPEN allele-specific accumulation (obtained from CUT&RUN experiments) on peaks at autosomes (grey, $n = 948$) and on the X chromosome (green, $n = 635$) after 0 h, 4 h, 8 h and 24 h of *Xist* induction in mouse ES cells. Shown are average allelic-ratios (shading is the interquartile range) of all peaks. **b**, UCSC Genome Browser allele-specific track showing SPEN binding around *Xist*. **c**, Annotation of SPEN peaks on the X chromosome. **d**, UCSC Genome Browser allele-specific track showing SPEN binding around *Eda2r*, an X-linked gene. In **b**, **d**, blue denotes Cast-Xa; red denotes B6-Xi; tracks are scaled identically. **e**, Box plot showing SPEN enrichment at 4 h in peaks outside or within *Xist* entry sites. **f**, Violin plot showing gene expression (reads per kilobase per million reads, RPKM) of genes accumulating SPEN ($n = 289$) or not accumulating SPEN ($n = 2,325$) at their promoters. **g**, Violin plot showing gene expression levels (RPKM in control conditions) of genes grouped on the basis of their level of dependence on SPEN for gene silencing (see Fig. 1e). **h**, **i**, Box plots showing SPEN enrichment after 4 h of *Xist* induction within peaks at promoters grouped on the basis of how efficiently their respective genes are silenced (**h**) or at enhancers grouped on the basis of how efficiently they are deacetylated during XCI (**i**). **j**, **k**, Box plots showing normalized SPEN enrichment at promoters (**j**) and gene silencing (transcript allelic ratio) during XCI (**k**) within 3 groups of X-linked genes showing different dynamics of SPEN accumulation and loss ($n = 86$ strong loss, $n = 92$ mild loss, $n = 39$ low SPEN). In **e**–**i**, the two-sided Wilcoxon rank-sum test was used; in **e**–**k**, horizontal lines denote the median, box limits correspond to upper and lower quartiles.

fraction, persistent *Xist* RNA expression and coating ensure that SPEN remains strongly accumulated around the inactive X chromosome (Fig. 2a, b).

Our study demonstrates that SPEN is a crucial factor that collaborates with *Xist* RNA to initiate gene silencing across the X chromosome, both during XCI in vitro and imprinted XCI in vivo. SPEN becomes dispensable for maintaining gene silencing after XCI has been established, but partially represses escapees, which suggests that *Xist* may have a silencing role even in somatic cells. Although SPEN coats the X chromosome immediately upon *Xist* induction, it contacts chromatin only at active promoters and enhancers, which serve as substrates for SPEN-mediated gene silencing. SPEN association with chromatin is favoured by active transcription, as SPEN disengages from chromatin when X-linked genes become silenced. We identify the SPOC domain of SPEN as a potent transcriptional repressor, which is crucial for SPEN-dependent XCI. On the basis of our mass spectrometry analysis, we propose that the SPOC domain is key for bridging *Xist* with other factors implicated in XCI—such as HDAC3—which we find to be present at most X-linked enhancers to which SPEN is recruited. In particular, the interaction of the SPOC domain with the NuRD complex and the transcription machinery points to a role for SPEN in direct transcriptional repression. We also identify SPOC as an interactor of the m^{6}A methyltransferase complex, which has a role in *Xist* RNA methylation, a modification that is important for *Xist*-dependent silencing[25]. Methylation of *Xist* is mediated by RBM15[25], which interacts with the m^{6}A machinery directly through ZC3H13[29]—the most highly enriched m^{6}A machinery factor identified in our mass spectrometry experiments. Because RBM15 also carries a SPOC domain, our study raises the possibility that the interaction with the RNA methylation machinery is not restricted solely to the SPOC domain of SPEN, but may instead be a feature that is shared across SPOC-containing proteins.

SPEN binds other non-coding RNAs, including *SRA*[18], which is involved in steroid-receptor regulation. Furthermore, another *SRA*-binding protein—SLIRP—has been shown to bind promoters in an *SRA*-dependent manner[30]; this raises the possibility that, similarly to *Xist*, *SRA* could guide SPEN to target gene regulatory elements.

In conclusion, our study suggests that RNA-mediated recruitment of SPEN and other SPOC-containing proteins—which are found across fungi, plants and animals—may be a widespread means by which to acutely repress transcription by co-ordinately engaging several lay-ers of epigenetic and transcriptional control. We propose that SPEN bridges *Xist* to the transcription machinery, histone deacetylases and chromosome remodelling factors to ensure robust and efficient XCI (Extended Data Fig. 4k).

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Multiple choice:

1. The authors use a doxycycline (D0X)-controlled transcriptional activation system. What is doxycycline?
    a. Broad-spectrum tetracycline-class antibiotic that inhibits protein synthesis
    b. Broad-spectrum antibiotic that inhibits cell wall biosynthesis
    c. A hormone
    d. An anti-tumor drug

2. How do you use an auxin-inducible degron? (Note: Auxin, a plant hormone, works by binding to the TIR1 E3 ubiquitin ligase. Also read the text, look at Fig. 1 and read the reference list)

A. Auxin binds to the auxin-binding domain tagged onto protein X, and addition of auxin to cells that also contain TIR1 leads to the rapid destruction of protein X
B. Auxin binds to the auxin-binding domain tagged onto protein X, e leads to the destruction of the auxin-binding domain by cell's E3 ligases
C. Auxin induces the degradation of all proteins recognized by E3 ligases
D. auxin leads to the degradation of TIR1, and protein X tagged with the auxin-inducible degron is no longer degraded. Withdrawal of auxin causes the destruction of the tagged protein X.

Fig. 1c shows a heat map which represents the ratio of the allelic transcripts in SPEN-degron ES cells? Which genes escape inactivation after auxin dox addition in this dataset?
    a. The one represented by blue lines
    b. The ones represented by red lines
    c. All
    d. none

3. Which one of the following statements define embryonic stem cells (ES cells)?
    a. Totipotent stem cell derived from the i.nner cell mass of the blastocyst
    b. Totipotent stem cell derived from a single blastomere
    c. Pluripotent stem cell derived from the inner cell mass of the blastocyst
    d. Multipotent stem cell derived from the inner cell mass of the blastocyst

4. Which description fits best paralogue/orthologue genes?
    a. Paralogues genes are non-homologous sequences that code for proteins of related function
    b. Orthologues genes are homologous sequences in related organisms that were also present in a common ancestor organism
    c. Paralogues are homologous sequences in related organisms that were also present in a common ancestor organism
    d. Orthologues are homologous sequences that have a common ancestral gene that underwent duplication event

5. Fig 1d shows the same data as Fig 1c, in a different visual format. What is the meaning of the p values indicated in the panel?
    a. The probability that two groups of allelic ratios are equal

b. The probability that the 3 groups of allelic ratios are equal
c. The probability that the means of the 3 groups are different
d. The probability that the means of the 3 groups are equal

6. Which description fits best long noncoding RNAs like *Xist* ?
    a. The length of the transcript is around 100-200 nt
    b. The length of the transcript exceeds 200 nt
    c. Long noncoding RNAs only mediate chromatin remodeling
    d. Long noncoding RNAs only mediate transcriptional and post-transcriptional events

7. Fig 1F and 3G show the data collected from pyrosequencing. What is pyrosequencing?
    a. DNA sequencing based on “sequencing by synthesis”
    b. DNA sequencing based on “sequencing by ligation”
    c. DNA sequencing based on incorporation of chain-terminating dideoxynucleotides
    d. DNA sequencing based on the detection of changes of the magnitude of the electric current density

8. How does SPEN work in neural progenitor cells?
    a. It is dispensable for maintenance of XCI
    b. It accumulates on the X chromosome and therefore it is important for XCI
    c. Depletion of SPEN leads to the reactivation of silenced genes
    d. Presence of SPEN does not decrease the expression of genes that escape XCI

9. What is a topologically associated domain ?
    a. A portion of the chromosome where DNA segments are in close spatial vicinity among themselves, and not with other portions of the chromosome
    b. A specific part of the nucleus where the chromosome is confined
    c. A part of the chromosome where the supercoiling is similar
    d. A portion of the chromosome where enhancers and promoters interact

10. What is the *Rosa26* locus ?
    a. A mouse gene which is expressed in all cells at all times
    b. A mouse gene that can be induced after exposure of cells to tamoxifen
    c. A mouse gene that is expressed only in ES cells
    d. A mouse gene that is not expressed, and thus not essential, in ES cells

11. SPEN can either bind enhancers or promoters. Which of the following statements about enhancers in mammals is correct?
    a. May be located upstream or downstream, but in close proximity to promoters
    b. May be located upstream or downstream, within several hundred thousand base pairs to the cognate promoter
    c. May be located upstream or downstream, within several hundred thousand base pairs to the cognate promoter, but not within transcribed regions
    d. May be located upstream or downstream, within several hundred thousand base pair to the cognate promoter, or even on a different chromosome

12. One of the statistical tests used by the authors is the two-side Wilcoxon rank sum test; how can this test be described?
    a. Nonparametric test used on dependent samples
    b. Nonparametric test used on independent samples
    c. Parametric test used on dependent samples
    d. Parametric test used on independent samples

13. The paper introduces the concept of embryonic development. Which are the phases of embryonic development (from early to late)?
    a. Zygote, blastomere, morula, blastula, gastrula
    b. Zygote, blastomere, blastula, morula, gastrula
    c. Zygote, blastomere, blastula, gastrula, morula
    d. Zygote, blastomere, gastrula, morula, blastula

14. The authors introduce an array of Bgl stem-loops into Xist in order to:
    a. Inactivate XCI induced by Xist
    b. Make Xist visible in living cells
    c. Block the binding of SPEN
    d. Allow the recruitment to Xist of the SPOC domain alone

15. Fig 3h shows co-ImmunoPrecipitations. From the data shown, which antibody was used to generate the immunoprecipitate ?
    a. Anti-GFP
    b. Anti-SPOC
    c. Anti-PolII
    d. Anti-BglG

16. In Fig. 4, which panel demonstrates directly that SPEN binds to promoters?
    a. panel a
    b. panel b
    c. panel d
    d. panel f

17. The RNA FISH protocol implies that:
    a. Radioactive probes are used to bind the nucleic acid sequence and are detected with a microscope
    b. Fluorescent probes are used to bind the nucleic acid sequence and are detected with a microscope
    c. A fluorescent protein binds a specific RNA
    d. Fluorescent probes are used to bind the nucleic acid sequence and are detected by flow citometry

18. The Student t-test, see for example Fig 3g, can be used to compare:
    a. The same group at two different times
    b. Two independent groups, where the variable is continuous (for example, mass)
    c. Three independent groups, where the variable is continuous (for example, mass)
    d. Two independent groups, where the variable is binary (for example: dead or alive)

19. Western blot analysis is an analytical technique used to detect which kind of samples?
    a. RNA
    b. DNA
    c. Proteins
    d. antibodies

20. Which of the following proteins cited in the paper mediate deacetylation?
    a. NCoR
    b. SMRT
    c. HDAC3
    d. RBM15

21. Based on this paper, which statement about the SPOC domain is true:
    a. It is present only in the C-terminal of SPEN
    b. It is important for SPEN recruitment on the X-chromosome
    c. Loss of SPOC leads to a more severe XCI than the loss of SPEN
    d. SPOC interacts with the m^6A methyltransferase complex

22. Neural Progenitor cells (NPCs) are:
    a. Cells that give rise to glia, microglia and neuronal cell types
    b. Cells that give rise to glia and neuronal cell types
    c. Cells that give rise to microglia and neuronal cell types
    d. Cells that give rise to glia, microglia cell types

23. The authors suggest that SPEN reduces gene expression by:
    a. Binding to Xist and then to highly expressed genes close to where Xist localizes
    b. Binding to all highly expressed genes, and then silencing the ones close to where Xist localizes
    c. Staying bound to genes that need dosage compensation
    d. Binding to Xist and repressing all genes close to where Xist localizes

24. The authors suggest that SPEN is implicated in RNA methylation because
    a. It interacts directly with METTL3 and WTAP
    b. Xist is methylated
    c. XCI requires Xist methylation
    d. It contains the SPOC domain

25. Fig 4b shows a result obtained by:
    a. Chromatin immunoprecipitation
    b. Transcriptomics
    c. Chromosome capture
    d. Mass spectrometry

26. The authors suggest that genes bound by SPEN are eventually silenced because
    a. SPEN is a silencing protein that remains bound to enhancers
    b. SPEN is a silencing protein that remains bound to promoters

c. SPEN brings about the deacetylation of nucleosomes
d. SPEN brings about the acetylation of nucleosomes

27. The inactive X chromosome is
    a. More condensed than the active one
    b. Equally condensed
    c. Less condensed than the active one
    d. Its condensation state can fluctuate

28. On the inactive X chromosome
    a. All genes are silenced
    b. A specific subset of genes are silenced, and some are not silenced
    c. The list of genes that are silenced or not silenced is somewhat variable, as a result of stochasticity in inactivation
    d. The silenced genes are different in different cell types

29. In ES cells, X chromosomes
    a. Are both actively transcribed
    b. Are both silenced
    c. One is transcribed and one is silenced, and the active one is the same in all cells in the population
    d. One is transcribed in 50% of the cell population and the other one is transcribed in 50% of the cell population

30. The authors use ES cells from a cross of *Mus musculus castaneus* with the C57BL/6 mouse because:
    a. They can distinguish which X chromosome is being transcribed because the sequence of the transcript is somewhat different in the X chromosome from *Mus musculus castaneus* and the X chromosome from *Mus musculus castaneus*
    b. They must use ES cells from a cross that yield infertile progeny
    c. This cross produces ES cells that can be manipulated easily
    d. ES cells from this specific cross are used widely for gene modification and regeneration of genetically modified mice

Open questions:

1. The paper introduces the concept of epigenetic regulation. Describe briefly what is epigenetic regulation
2. X-inactivation is a mode of dosage compensation between males and females in mammals. Describe briefly one alternative mode of sex-linked dosage compensation in another group of organisms

Answer

1A 2Ab 3C 4B 5A 6B 7A 8A 9A 10A 11B 12B 13A 14D 15A 16C 17B 18B 19C 20C 21D 22B 23A 24A 25A 26C 27A 28C 29A 30A