# Escape from X inactivation is directly modulated by levels of Xist non-coding RNA

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## Abstract

In placental females, one of the two X chromosomes is silenced during a narrow developmental time window, in a process mediated by the non-coding RNA Xist. Here, we demonstrate that Xist can initiate X-chromosome inactivation (XCI) well beyond early embryogenesis. By modifying its endogenous level, we show that Xist has the capacity to actively silence genes that escape XCI both in neuronal progenitor cells (NPCs) and in vivo, in mouse embryos. We also show that Xist plays a direct role in eliminating TAD-like structures associated with clusters of escapees on the inactive X chromosome, and that this is dependent on Xist's XCI initiation partner, SPEN. We further demonstrate that Xist's function in suppressing gene expression of escapees and topological domains is reversible for up to seven days post-induction, but that sustained Xist up-regulation leads to irreversible silencing. Thus, the distinctive transcriptional and regulatory topologies of the silenced X chromosome is actively, directly and reversibly controlled by Xist RNA throughout life.

## Increased levels of Xist RNA silence Xi escapees in NPCs

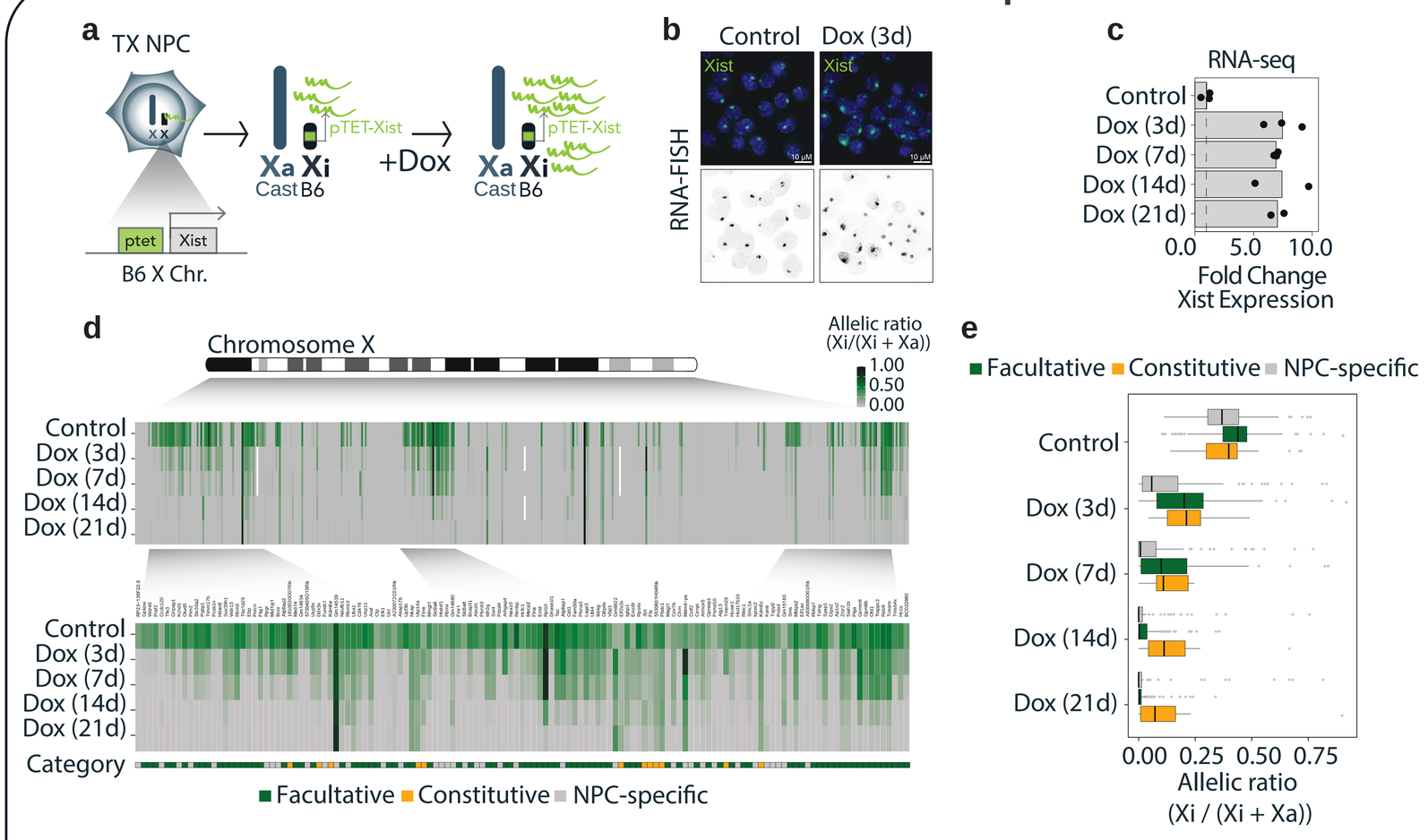


Figure 1. a, Experimental outline: single NPC clones carrying the inactivated B6 allele were picked and expanded and Xist RNA levels were increased by adding doxycycline to the culture media. b, FISH for Xist RNA (green) in NPC clone E6 in untreated conditions (Control) and after 3 days of doxycycline treatment (Dox 3d). DNA is stained with DAPI. c, RNA-seq data showing the fold change in Xist expression (normalised CPM) compared to untreated cells across the time course of doxycycline treatment. d, Heatmap showing X-linked transcript allelic ratios in untreated clone E6 and after 3, 7, 14 and 21 days of doxycycline treatment. Allelic ratio indicates the fraction of reads from the Xi (ratio=1: Xi monoallelic expression; ratio=0: Xa monoallelic expression; ratio=0.5: biallelic expression; ratio > 0.1: escape). e, Box plot showing the changes in allelic ratios for different escape categories across the time course of doxycycline treatment.

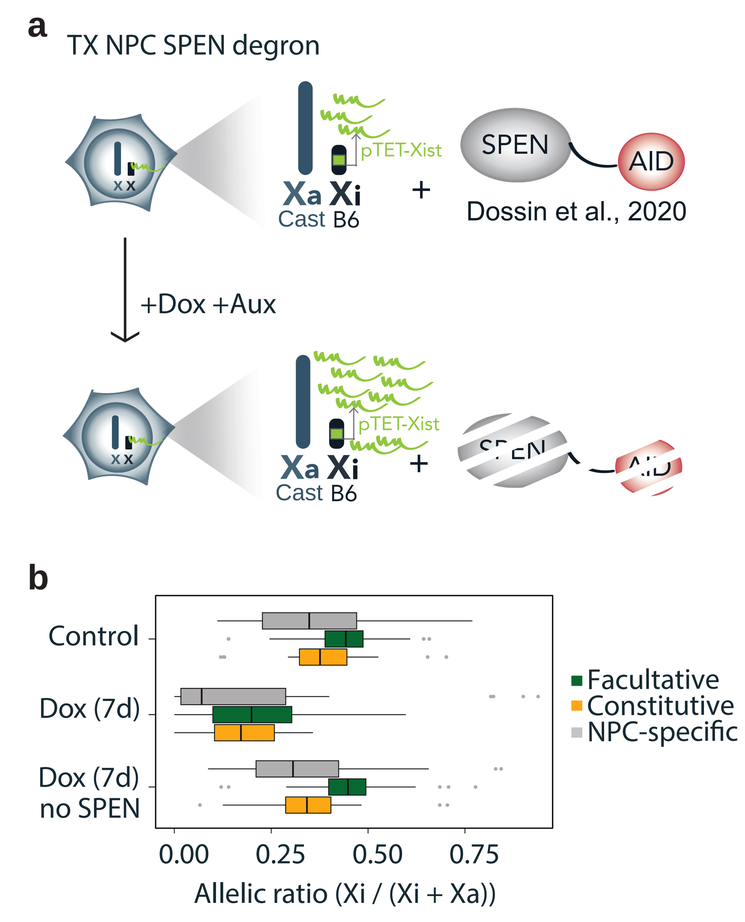


Figure 2. a, Experimental outline: Xist RNA levels were increased in NPC clones carrying a SPEN-AID degron (Dossin at al. 2020). SPEN was depleted by adding auxin (aux) to the culture media for 2 days before inducing Xist upregulation with doxycycline (Dox) for 7 days in the presence of auxin. b, Box plot showing the changes in allelic ratios for different escape categories upon doxycyclin/auxin treatment.

## Silencing of escapees is SPEN dependent

## Xist-mediated silencing of escapees leads to loss of TAD-like domains on the Xi

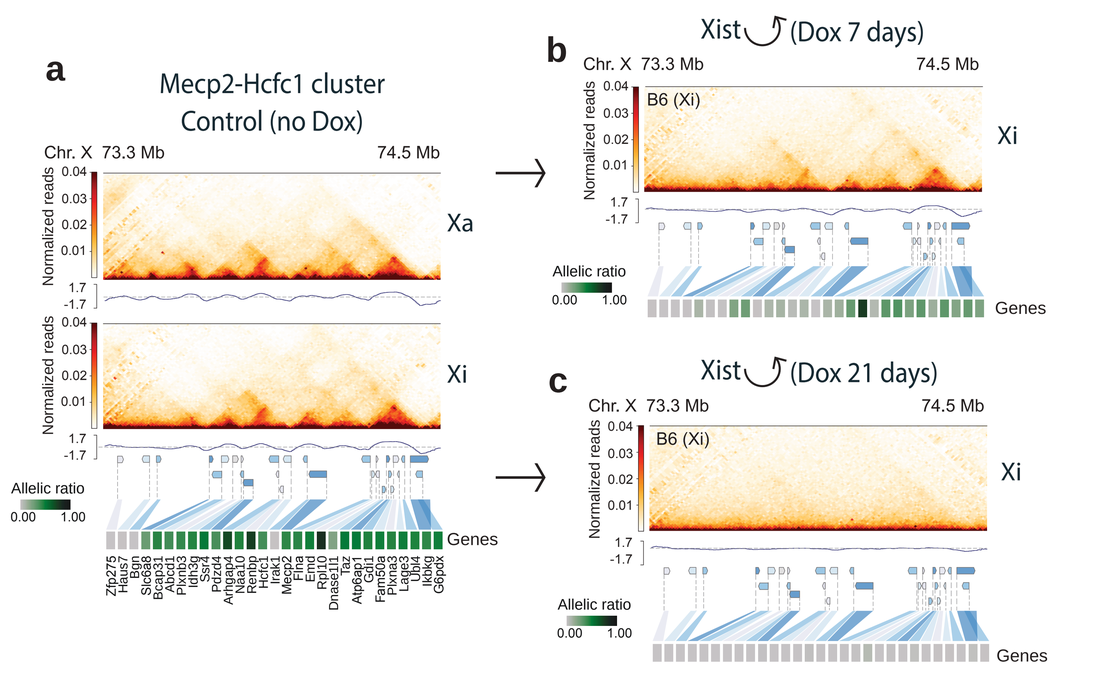


Figure 3. a-c, Capture Hi-C interactions and insulation score at the *Mecp2-Hcfc1* cluster prior to and upon doxycycline treatment. Capture Hi-C interactions are shown for the active (Xa) and the inactive (Xi) X chromosomes in untreated conditions (a) and for the Xi upon doxycycline treatment for 7 days (b) and 21 days (c). Data is shown at 10-kb resolution. Heatmaps of allelic ratios for 29 X-linked genes included in the captured regions are shown.

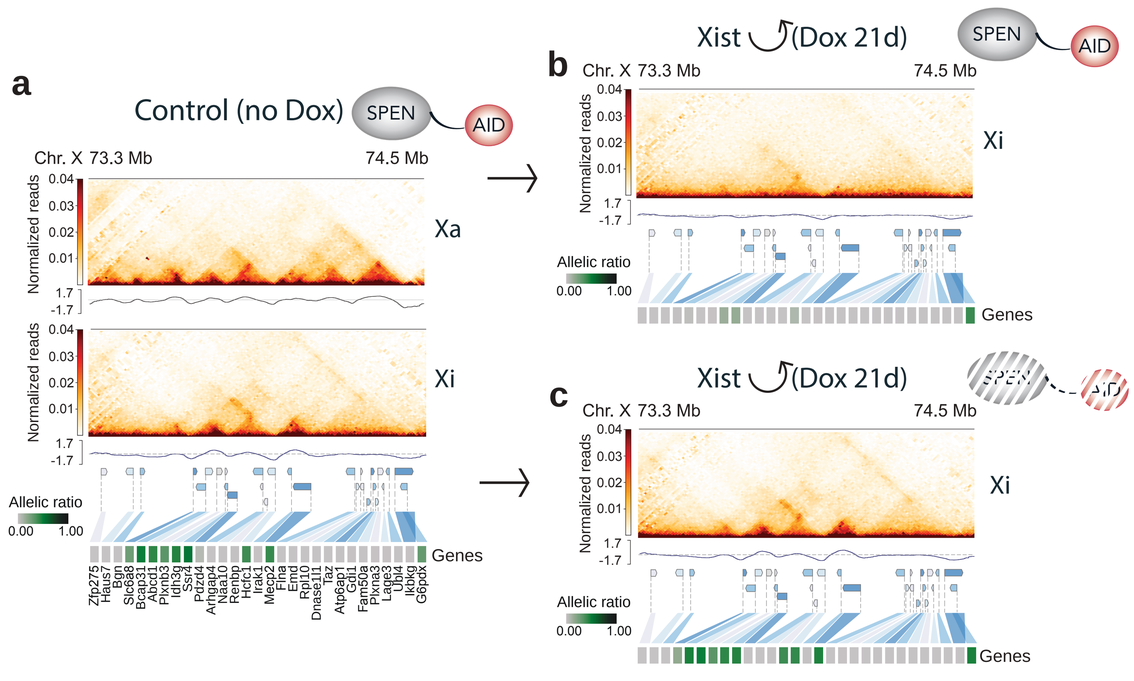


Figure 4. a-c, Capture Hi-C interactions and insulation score at the *Mecp2-Hcfc1* cluster for Xi in clone CL30.7. (a) untreated cells (control no Dox) (b) 21 days of doxycycline induction; (c) 23 days of auxin treatment in combination with 21 days of doxycycline induction. Capture Hi-C data is shown at 10-kb resolution. Heatmaps showing allelic ratios for 29 X-linked genes included in the captured region are shown.

## Xist levels modulate X-linked dosage in vivo

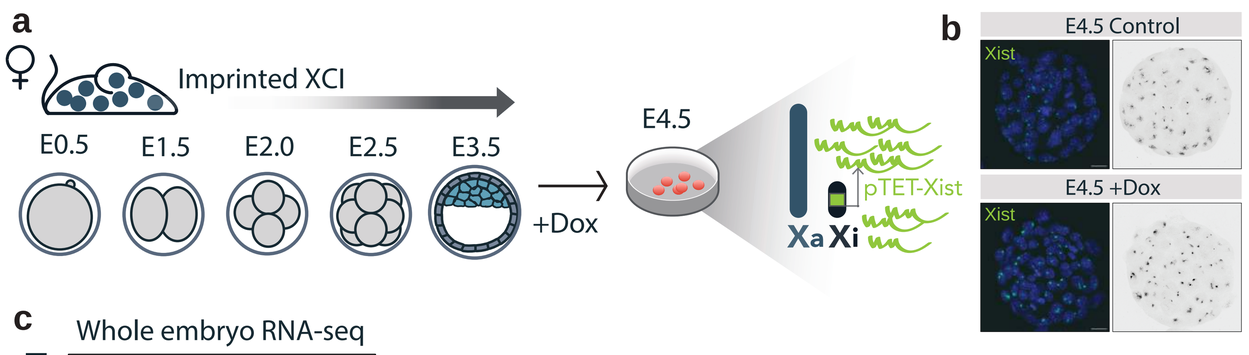


Figure 5. a, experimental outline: B6/JF1 F1 embryos were collected at E3.5 and cultured for 24 hours while adding doxycycline to the culture media. RNA-seq was performed at E4.5. b, FISH for Xist RNA (green) in E4.5 XX embryos obtained by crossing males TX B6 mice (XptetY; R26rtTA/rtTA) with WT JF1 females. DNA is stained with DAPI. c, Box plots showing mean allelic ratios for treated and untreated embryos for the different escapee categories.

## Conclusions

1. Xist RNA regulates X-linked gene dosage far beyond embryogenesis potentially throughout life
2. TAD-like domains on the Xi are the consequences of XCI escape
3. Facultative but not constitutive escapees can be fully (and irreversibly) silenced by Xist in post XCI cells
4. Natural fluctuations in Xist RNA levels may impact X-linked protein dosage

! Small changes in X-linked dosage can have key phenotypic consequences (e.g. cancer)

*Richart et al., 2022 Cell*