UNIVERSITÀ DEGLI STUDI DI TRIESTE

Dipartimento di Scienze della Vita

Article

nature

Targeting *Xist* with compounds that disrupt RNA structure and X inactivation

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Presented by Anne-Lou HYARDET and Lara EVERTS

December 5th 2025

- RNA-binding molecules screening techniques
- X-chromosome inactivation (XCI)

- ALIS Screening of RepA
- In vitro functional validation
- In vivo functional effects
- RepA conformational changes

- Conclusion
- Future outlook

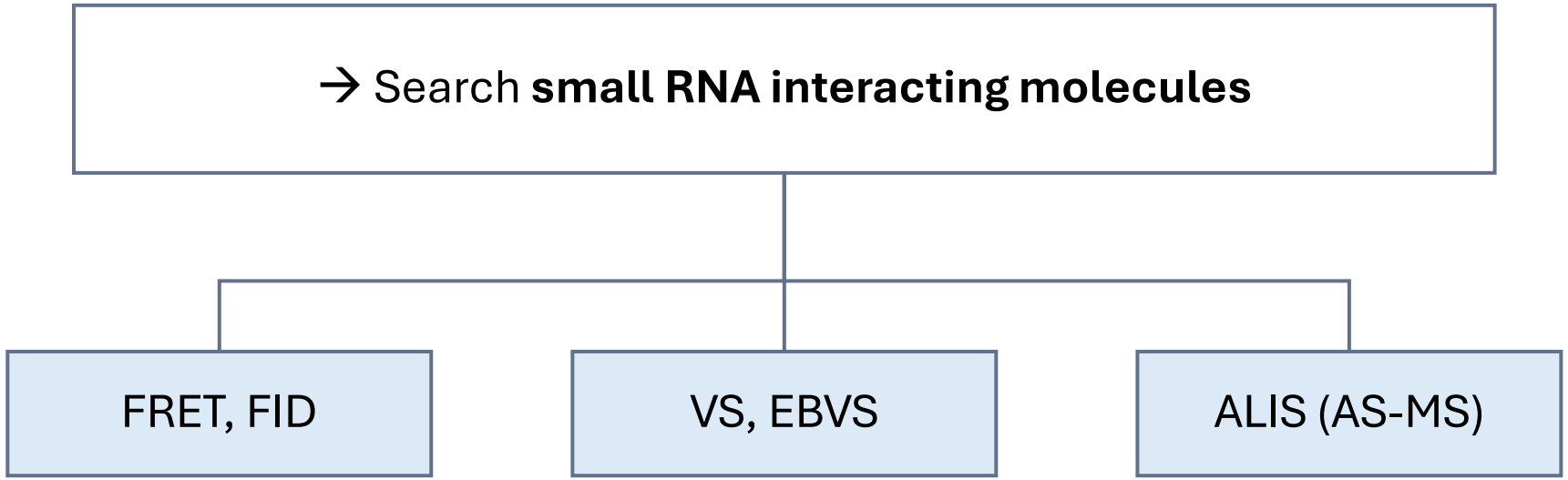
- RNA-binding molecules screening techniques
- X-chromosome inactivation (XCI)

How can we screen molecules interacting with RNA ?

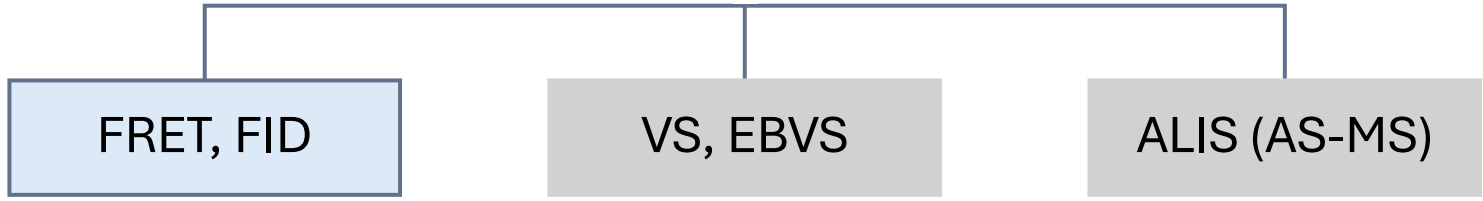
- Use of **phenotypic protein screening** to identify medicinal targets
- Enhance the target possibility: **RNA screening**

Difficult:

- Different RNA conformations possible
- Unstable 3D structure

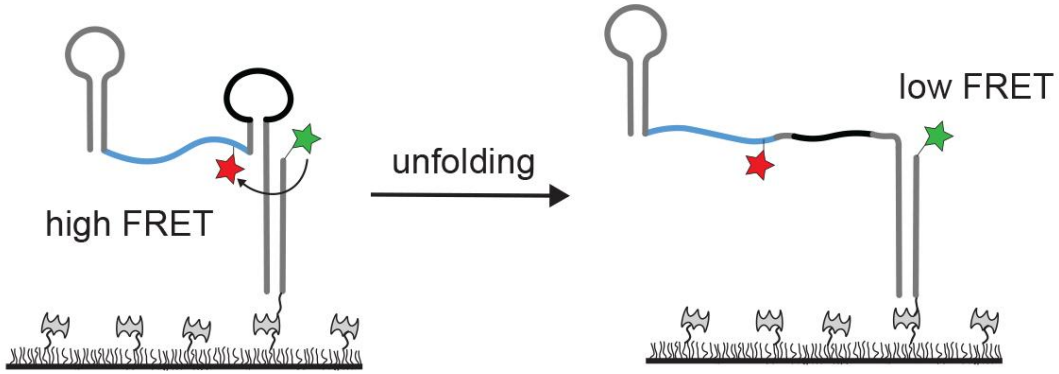


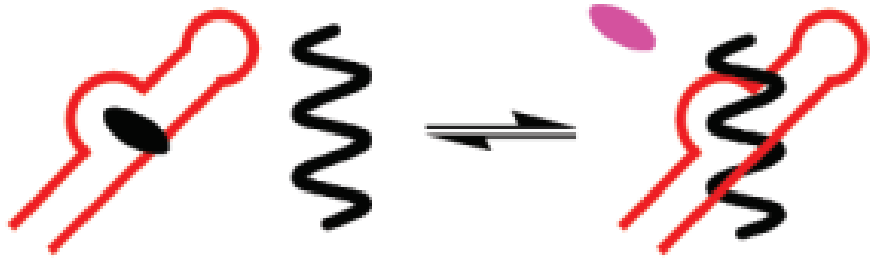
How can we screen molecules interacting with RNA ?



Fluorescent-based strategy: measure changes in a fluorescent signal to detect ligand binding, characterize conformational changes and the activity of an RNA

- **FRET**: Measure conformational changes in labelled RNA
- **FID**: Identifies hits by monitoring the displacement of a dye from the RNA





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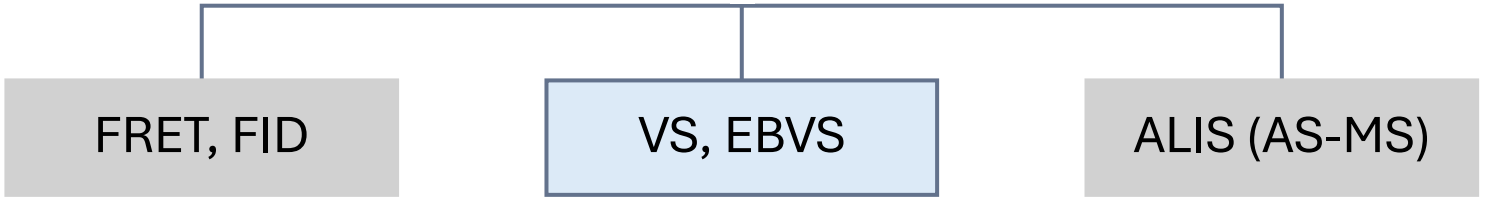
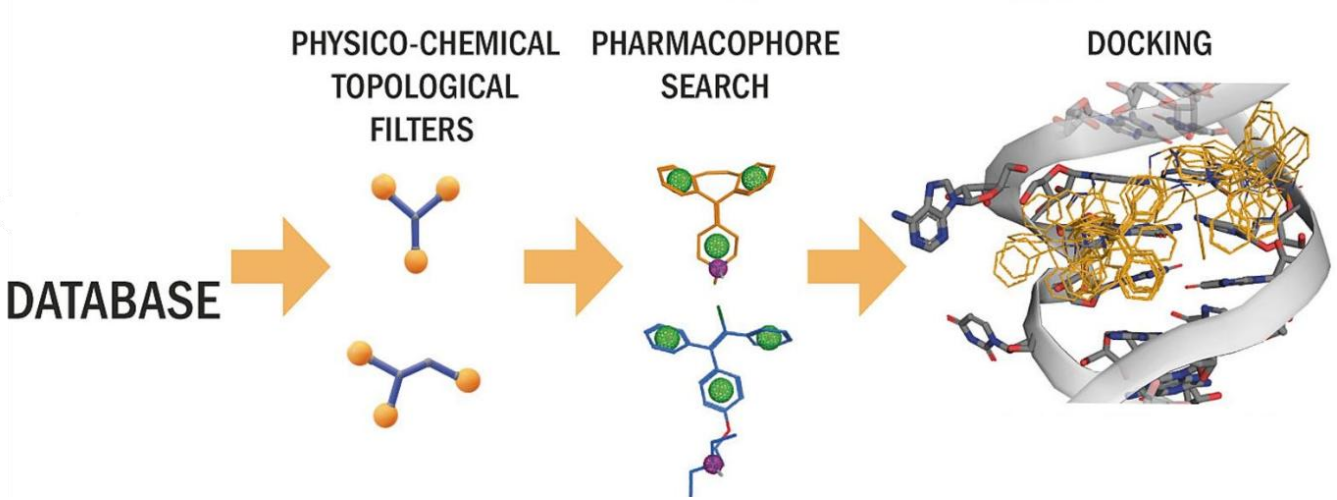
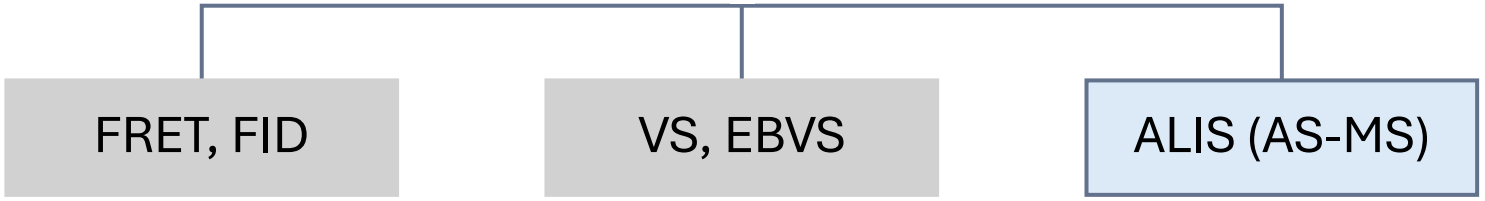


Computational screening: use 3D structural data to predict binding molecules

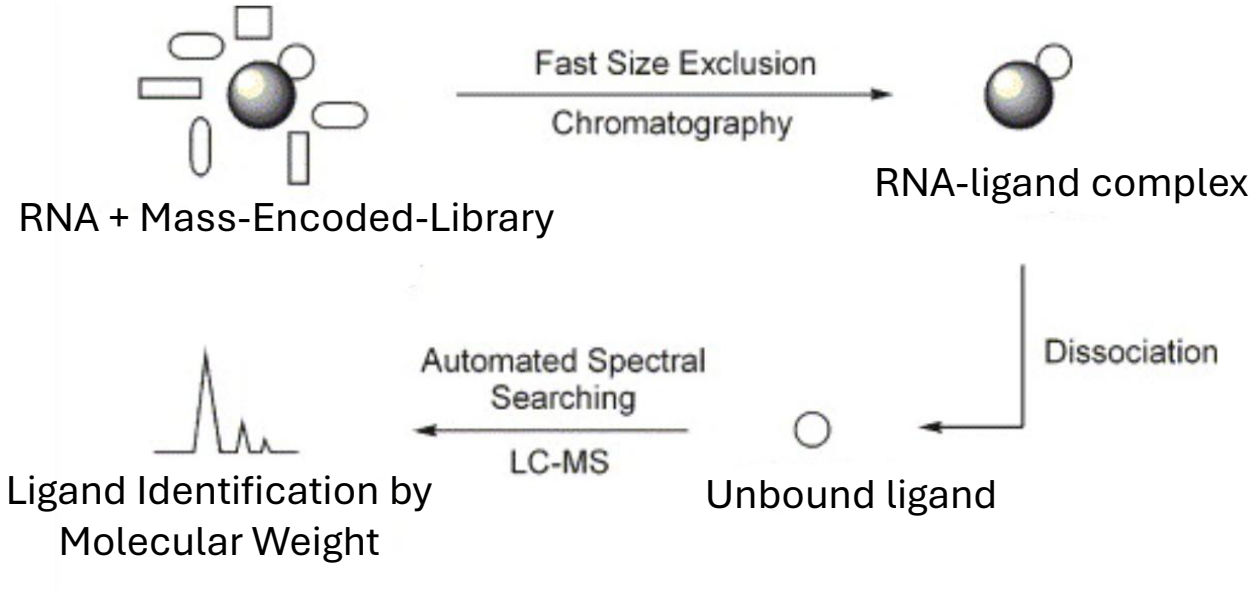
- **VS (virtual screening)**: Virtual small molecules chemical libraries and RNA binding pockets
- **EBVS (ensemble-based VS):** multiple dynamic conformations



How can we screen molecules interacting with RNA ?



ALIS (Automated Ligand Identification System): direct screening using physical binding
→ Use of Affinity Selection Mass Spectrometry (**AS-MS**) to identify ligands by size exclusion chromatography



How to determine the mode of action and functional validation ?

1- Functional mechanism

EMSAs (Electrophoretic Mobility Shift Assays): Determine RNA-bound molecules complexes and disruption of interaction

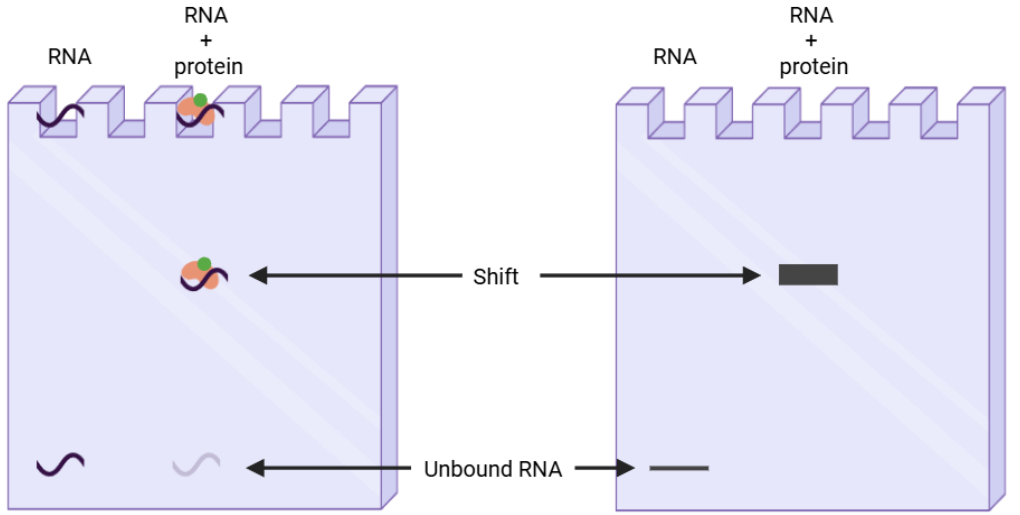


Figure 1. Schematic representation of a gel-shift assay

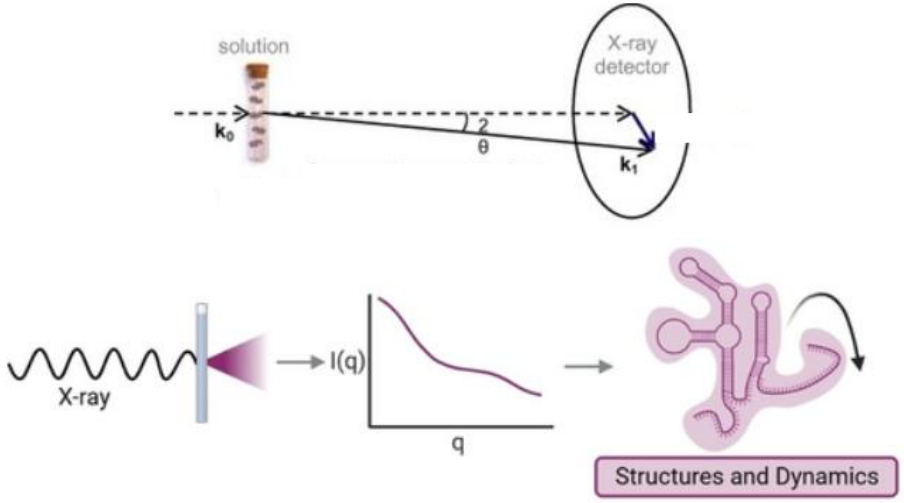


Figure 2, 3. Schematic representation of a SAXS experiment

2- Structural mechanism

SAXS (Small-Angle X-ray Scattering): Determine the size and shape of RNA in solution (can be combined with HPLC)

X-Chromosome Inactivation

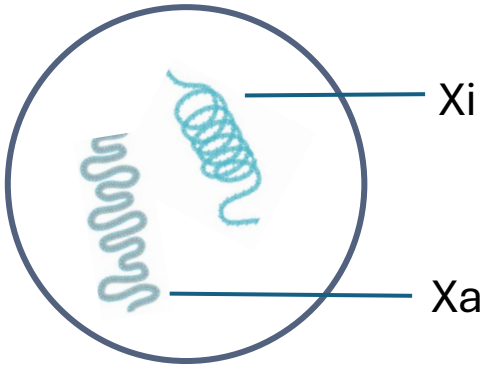
X-Chromosome Inactivation (XCI) is the process that **compensate the dosage difference of sex chromosomal genes** between female (XX) and male (XY) **by inactivating one X chromosome in female cells.**

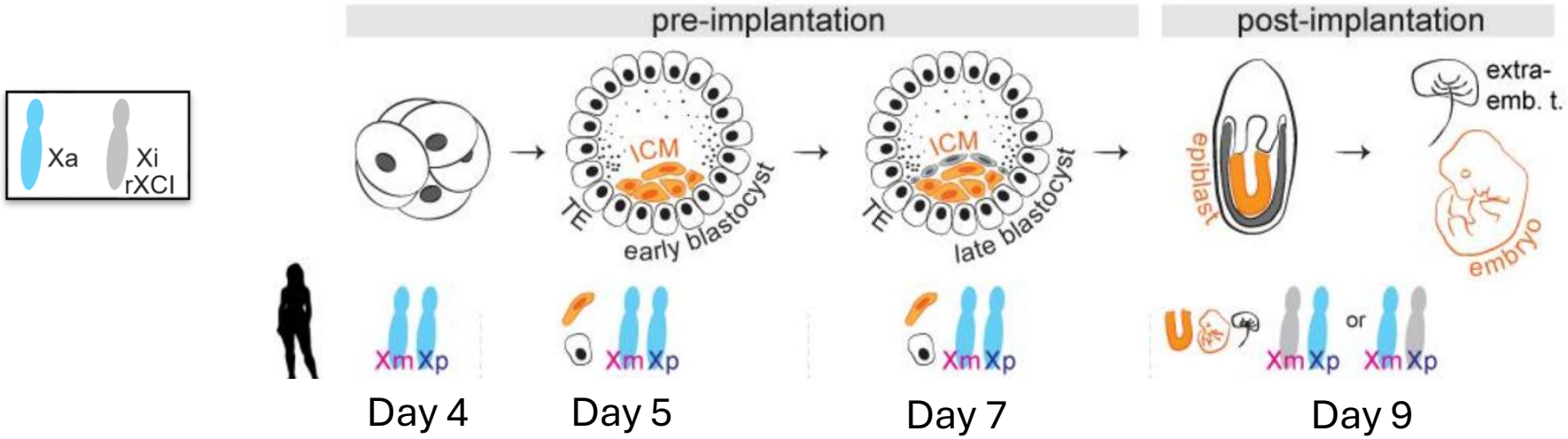
In mammals, **XCI** happen during early embryogenic state at a strictly temporal window.

On of the two X chromosome will be **randomly inactivated** it is the **Xi**.

Inactivated X (Xi) is silenced through heterochromatisation

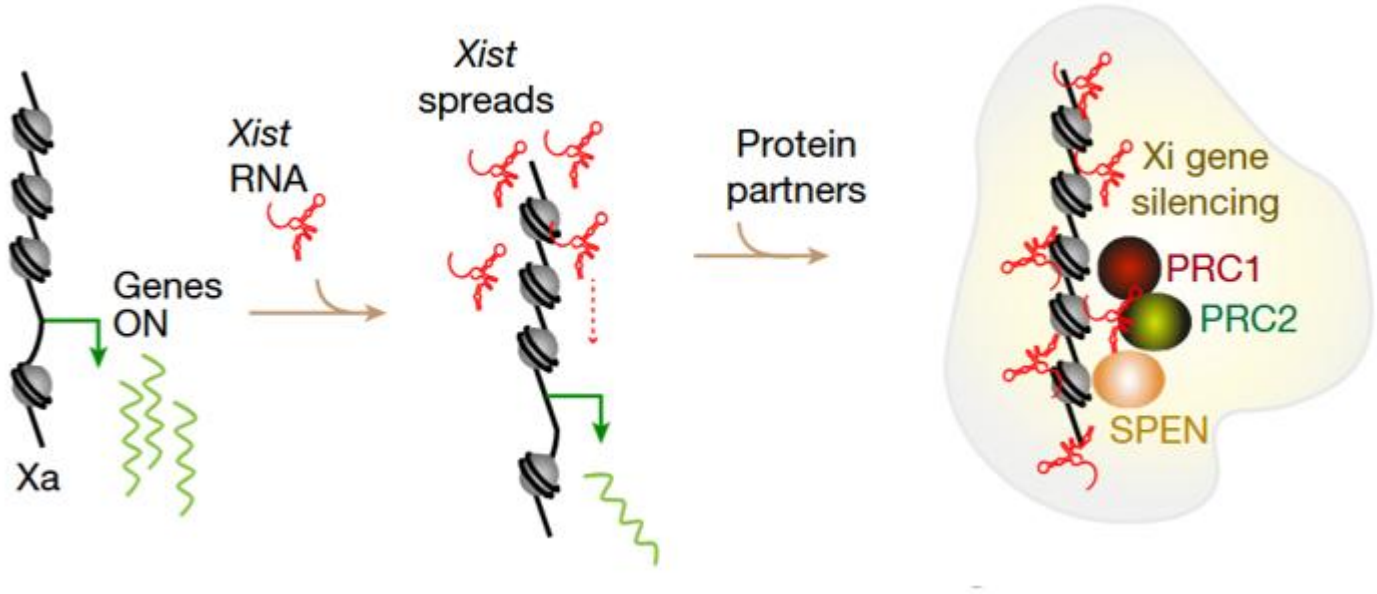
Activated X (Xa) is the chromosome that stays active





Xist a regulator of XCI

Xist is a **long non-coding RNA** that will be upregulated and coat the future inactivated X; by recruiting many protein it will allow the silencing of this chromosome.



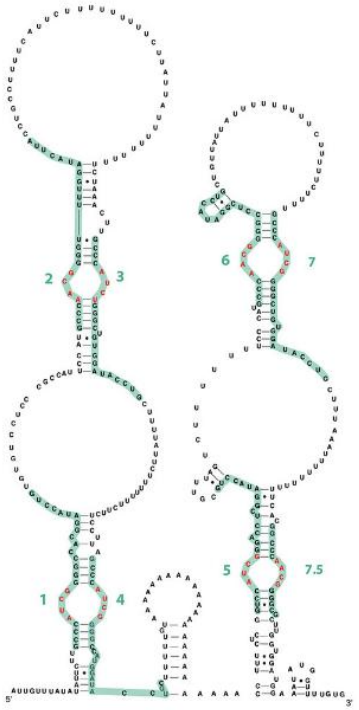
Xist is up-regulated in Xa and Xi
Tsix, its negative regulator, will prevent inactivation of Xa

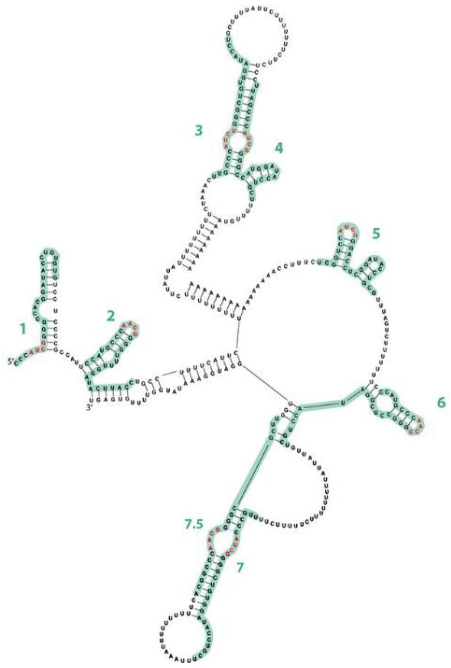
Xist a regulator of XCI – structure of Xist

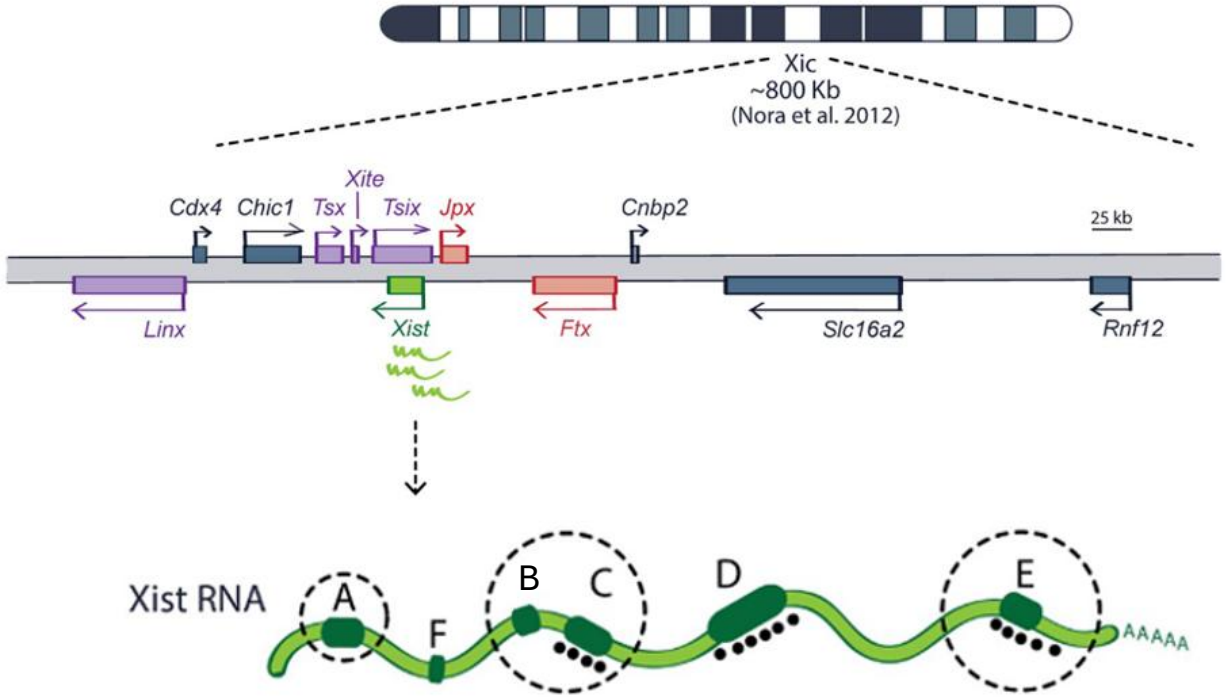
Xist possess **Tandem repeats** named A-F repeats, allowing the recruitment of proteins for XCI

The most **conserved repeat** among the mammals is **Repeat A** (RepA)

RepA is a necessary element of Xist to operate XCI





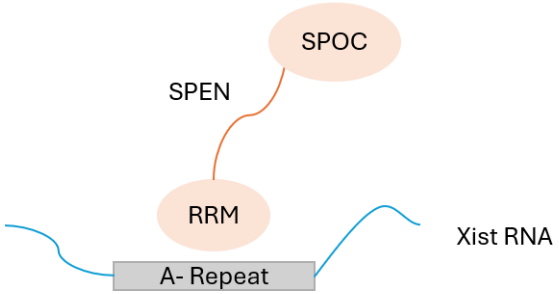


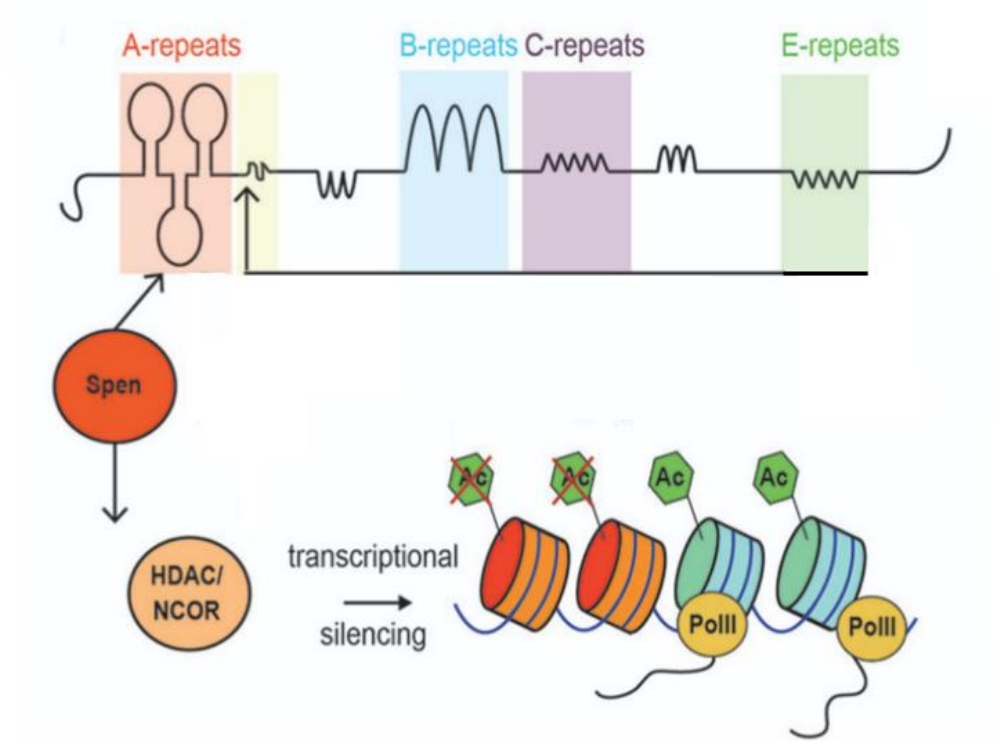
Proteins recruited by Xist - SPEN

SPEN : also known as SHARP, is a protein harbouring four **N-terminal RNA recognition motifs (RRMs),** and a highly conserved C-terminal Spen paralog and ortholog domain (SPOC).

SPEN is required to achieved correct XCI, as it **might initiate transcriptional silencing** by actively recruiting HDAC3

Through its **RRM,** SPEN **binds Xist** RNA on its **RepA** region





Proteins recruited by Xist – PRC1/PRC2

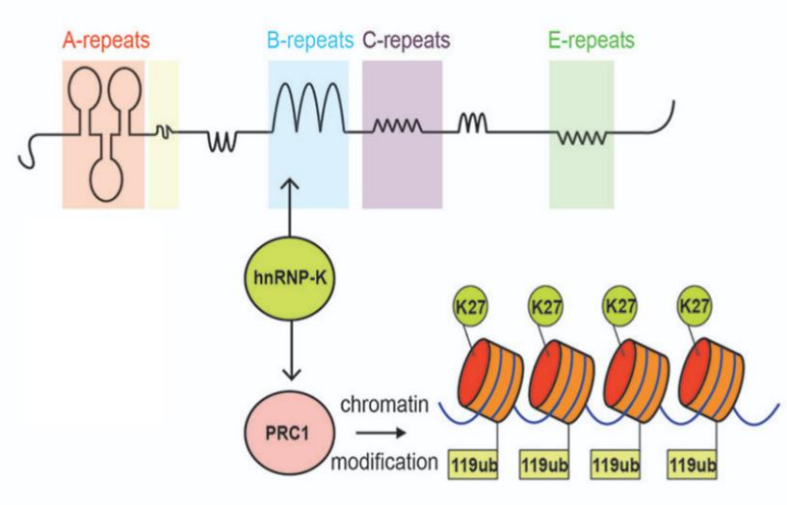
The Polycomb Repressive Complexes **PRC1** and **PRC2** are epigenic regulators recruited by Xist.

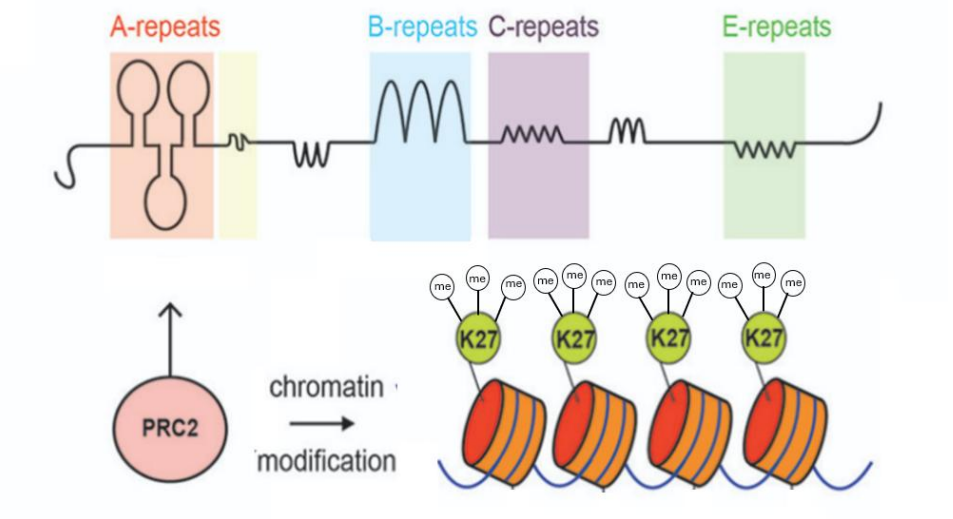
PRC1

- Recruited by repeats B and C of Xist
- H2AK119 ubiquitination (H2AK119ub)

PRC2

- Recruited by repeat A of Xist
- H3K27 trimethylation (H3K27me3)



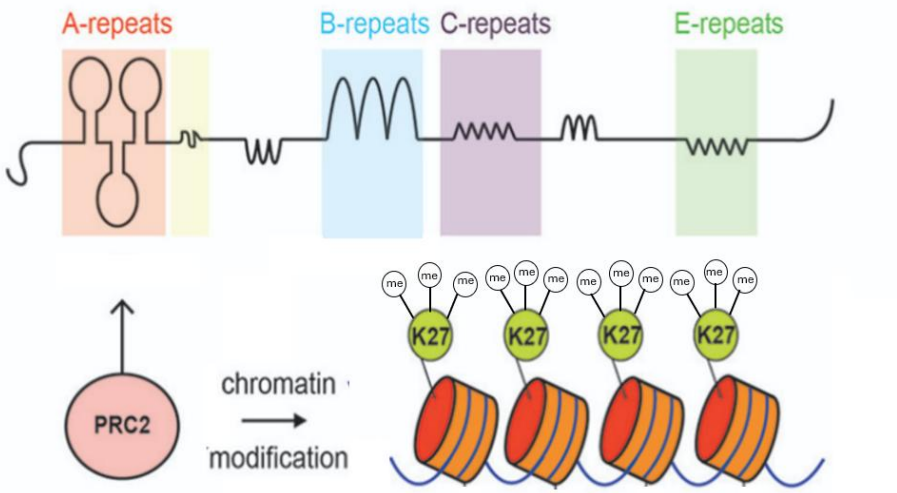


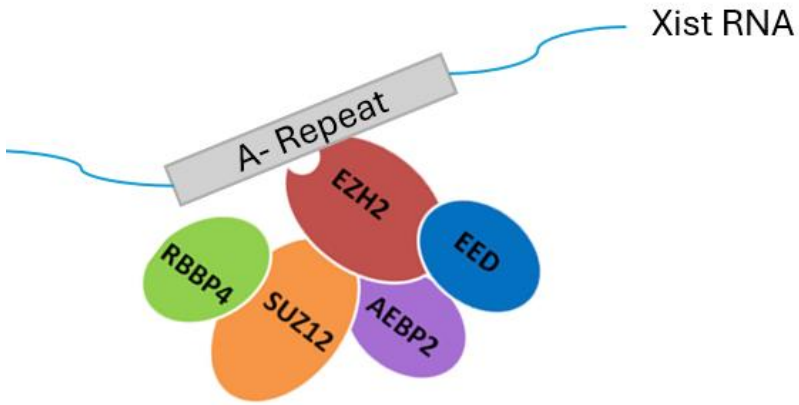
Proteins recruited by Xist – PRC1/PRC2

The Polycomb Repressive Complexe **PRC2** is an epigenic regulators recruited by Xist.

PRC2

- Recruited by repeat A of Xist
- H3K27 trimethylation (H3K27me3)
- **EZH2**: allow the **H3K27me3 modification, binds Rep A**
- **SUZ12**: a modulator of EZH2



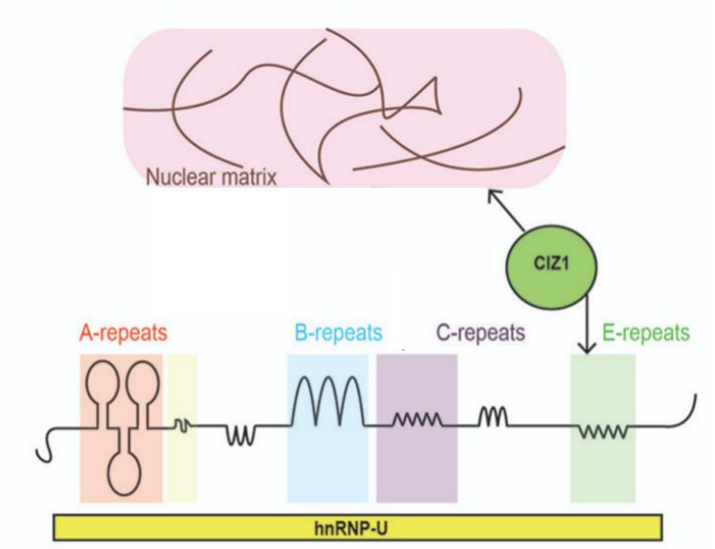


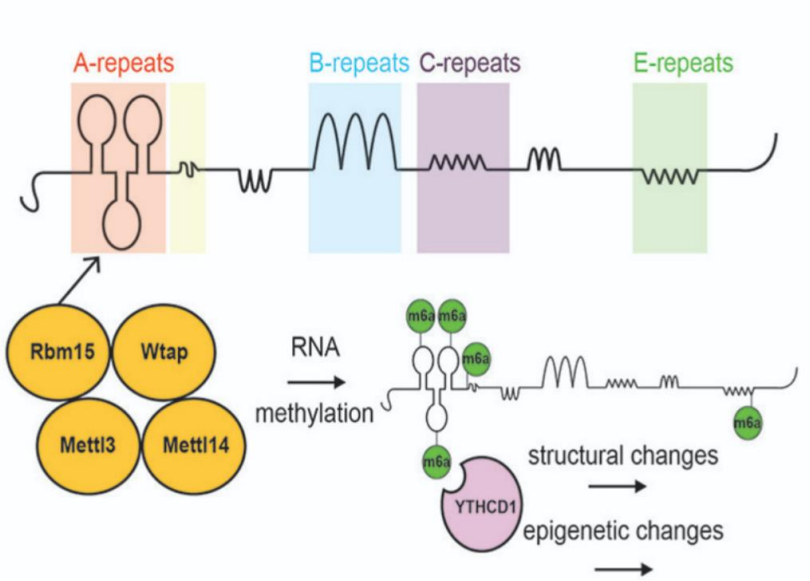
Proteins recruited by Xist – RBM15/CIZ1

- **RBM15**: RNA-binding motif protein 15 recruit and bind the N6-methyladenosine (m6A) RNA methylation machinery, it **binds near RepA on Xist**

  m6A RNA methylation machinery : adenosine methylation, a reversible RNA-modification pathway that forms m^6A

- **CIZ1**: Cip1-interacting zinc finger protein 1, contribute to Xist localization, it **binds to Repeat E**





Can the well-known Xist model validate a general strategy for finding small molecules that target disease-causing ncRNA ?

Article

Targeting *Xist* with compounds that disrupt RNA structure and X inactivation

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Although more than 98% of the human genome is non-coding[1], nearly all of the drugs on the market target one of about 700 disease-related proteins. The historical reluctance to invest in non-coding RNA stems partly from requirements for drug targets to adopt a single stable conformation[2]. Most RNAs can adopt several conformations of similar stabilities. RNA structures also remain challenging to determine[3]. Nonetheless, an increasing number of diseases are now being attributed to non-coding RNA[4] and the ability to target them would vastly expand the chemical space for drug development. Here we devise a screening strategy and identify small molecules that bind the non-coding RNA prototype *Xist*[5]. The X1 compound has drug-like properties and binds specifically the RepA motif[6] of *Xist* in vitro and in vivo. Small-angle X-ray scattering analysis reveals that RepA can adopt multiple conformations but favours one structure in solution. X1 binding reduces the conformational space of RepA, displaces cognate interacting protein factors (PRC2 and SPEN), suppresses histone H3K27 trimethylation, and blocks initiation of X-chromosome inactivation. X1 inhibits cell differentiation and growth in a female-specific manner. Thus, RNA can be systematically targeted by drug-like compounds that disrupt RNA structure and epigenetic function.

- **ALIS Screening of RepA**
- In vitro functional validation
- In vivo functional effects
- RepA conformational changes

Which compounds can bind to RepA ?

ALIS (Automated Ligand Identification System) performed on 50 000 compounds from synthetised RepA

- **Confirmation screening** gave only one positive hit: **X22**, a molecule with pharmacological properties

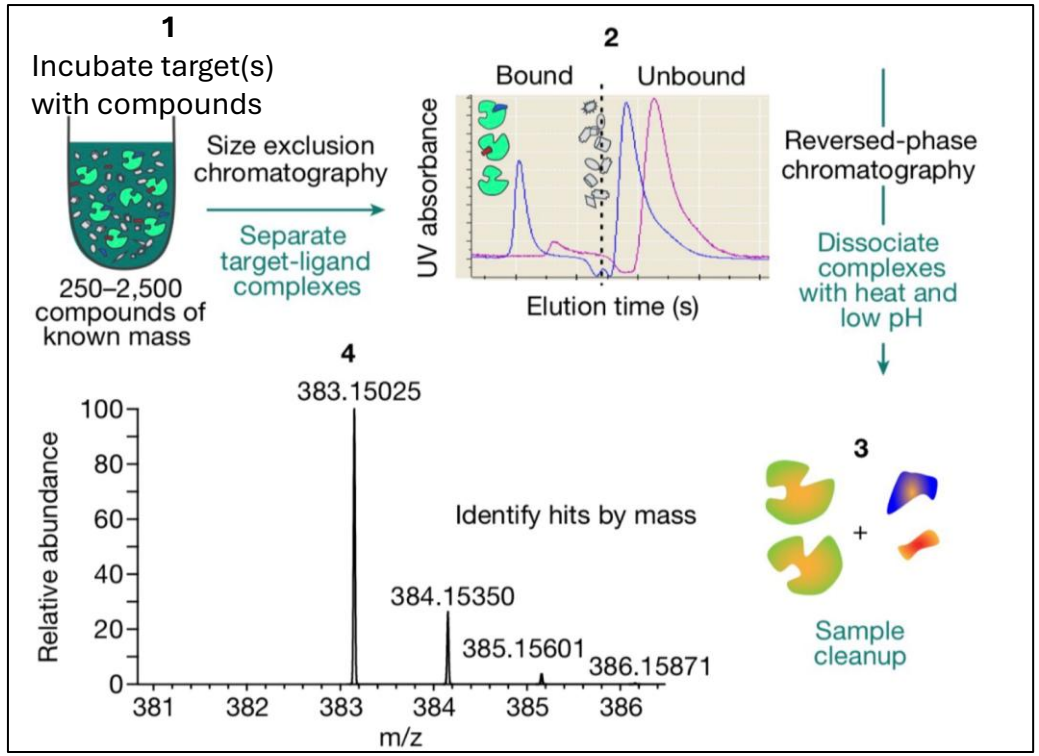


Figure 4. ALIS performed steps

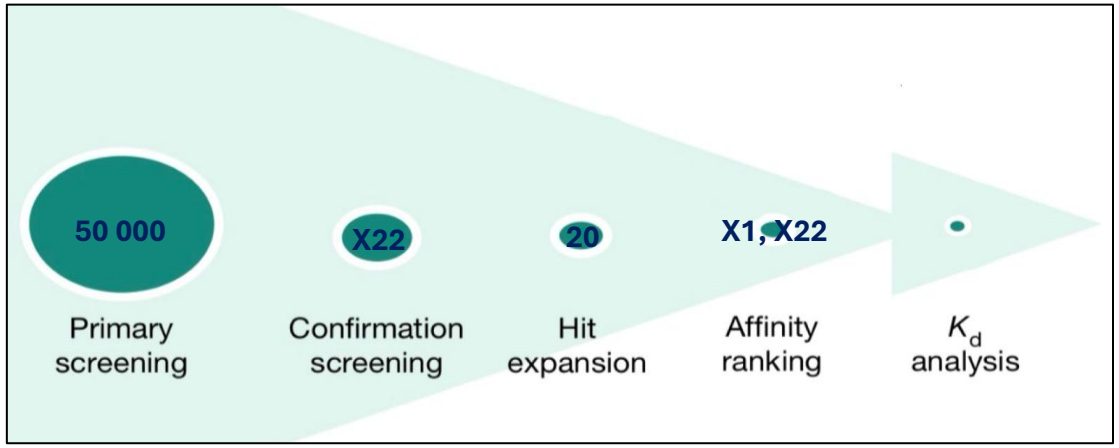


Figure 5. ALIS performed steps

Which compounds can bind to RepA ?

ALIS (Automated Ligand Identification System) performed on 50 000 compounds from synthetised RepA

- **Confirmation screening** gave only one positive hit: **X22**, a molecule with pharmalogical properties
- **Hit expansion** of X22 to find **analogues** with more than 70% structure similarity gave **20 analogues**
- **Affinity ranking** performed: **X1 has the best affinity**

X1 and X22 are compounds with high affinity for RepA

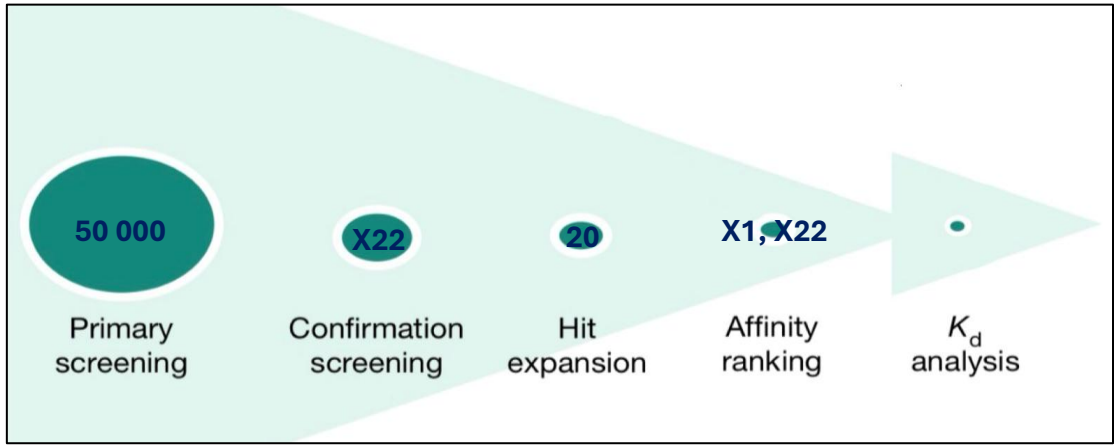


Figure 5. ALIS performed steps

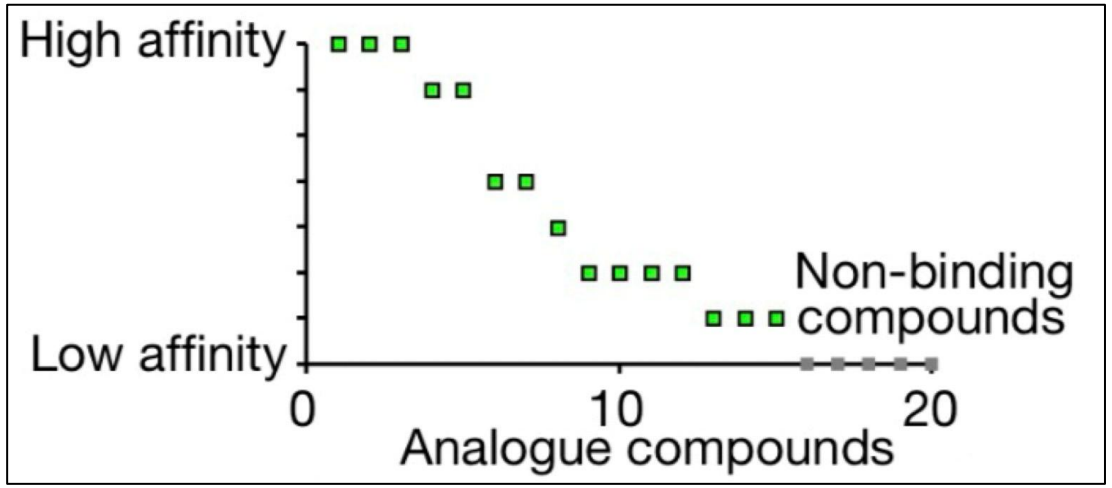


Figure 6. Affinity ranking

- ALIS Screening of RepA
- **In vitro functional validation**
- In vivo functional effects
- RepA conformational changes

Which of X1 and X22 has better properties ?

Kd = Dissociation constant

- **X1 and X22 have similar pharmacological properties**
- **Affinity assay on Xist**: X1 has the highest affinity

  → X1 might be a DNA intercalator ?
- **Binding assay** with other RNA: X1 do not or weakly bound with other RNA

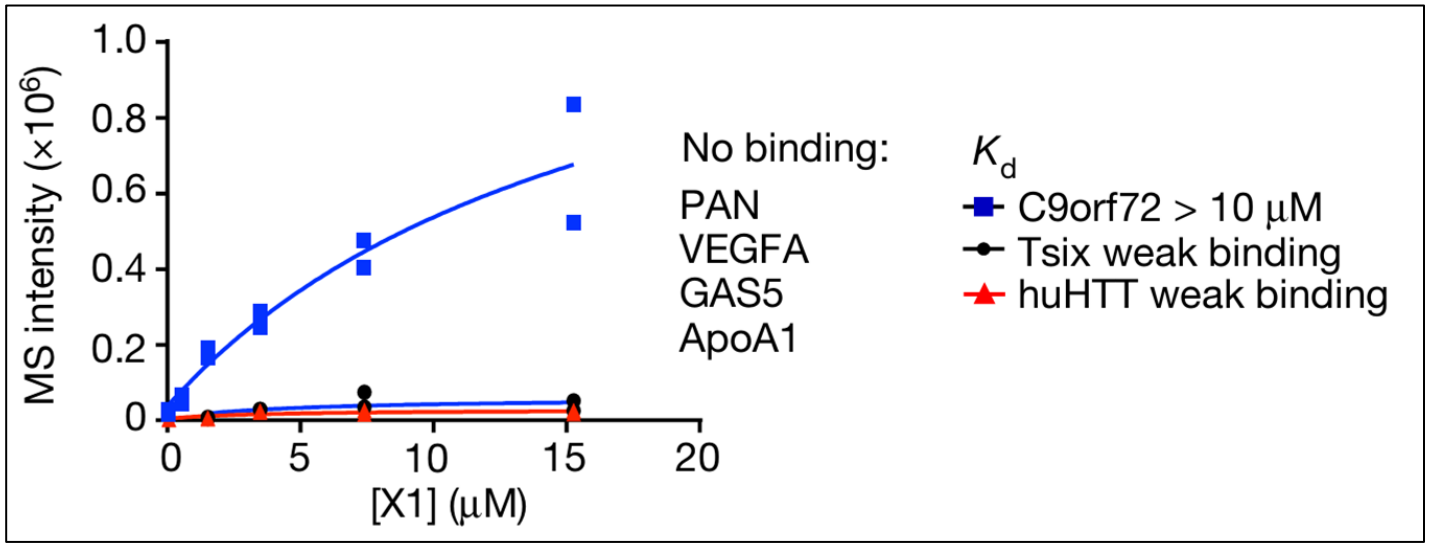


Figure 7. Affinity assay of X1 with Tsix, PAN, VEGFA, GAS5, ApoA1, huHTT and C9orf72

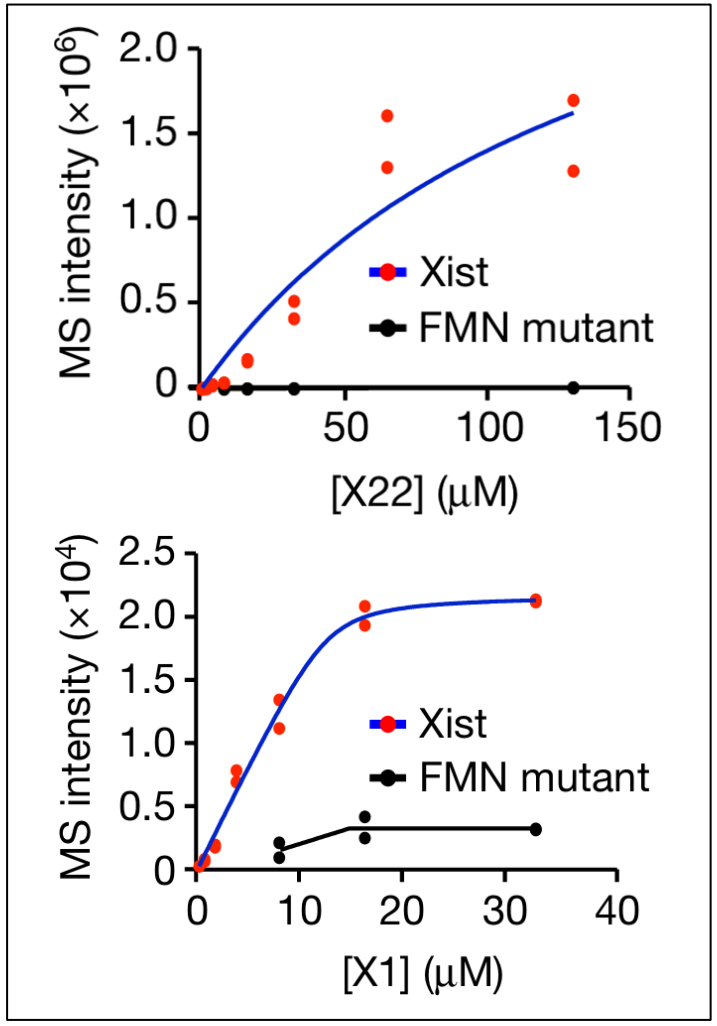


Figure 8. Affinity assay of X22 and X1 to Xist and bacterial FMN riboswitch, as negative control

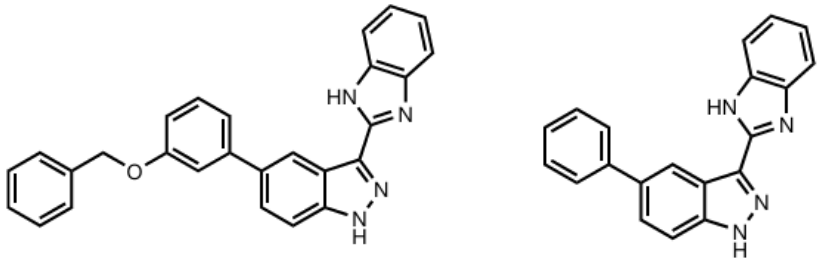
X1 has a higher affinity to Xist and do not bind any other RNA

Does X1 affects RepA function ?

EMSA (Electrophoretic Mobility Shift Assay)

Titration of X1, X16 and X-negative (0-100µM) with fixed concentrations of RepA-PRC2 and RepA-SPEN

- **X16:** analogue of X1, different structure



X1
Lead compound

X16
X1 analogue

- **X-negative:** negative control

→ **Progressive disruptive effects** on RepA-PRC2 and RepA-SPEN interactions

- RepA has a **higher affinity for PRC2** than SPEN

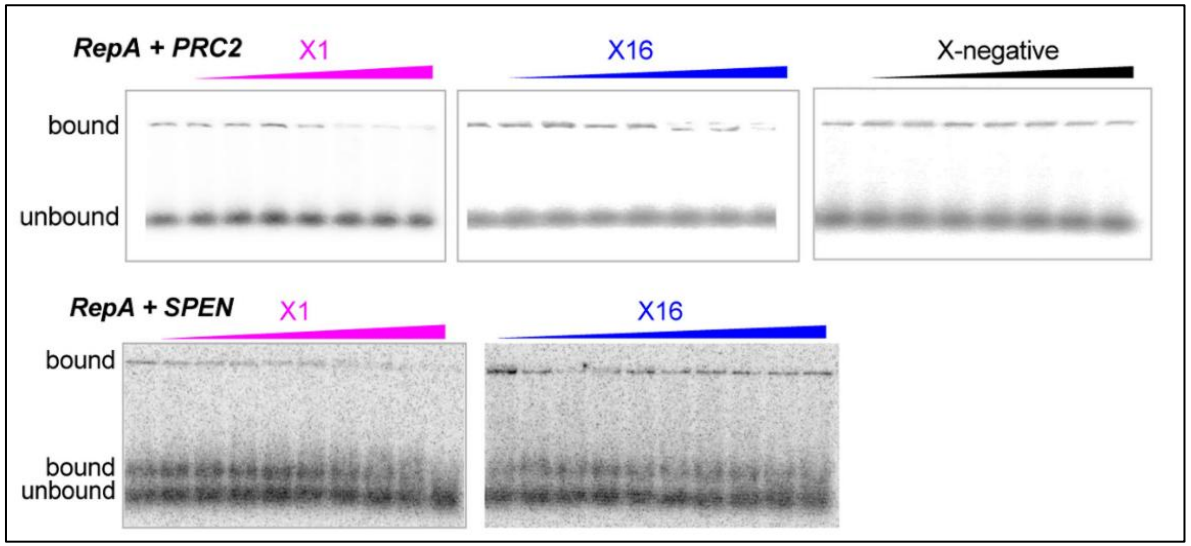


Figure 9. EMSA of RepA-PRC2 and RepA-SPEN interaction with increasing concentrations of X1, 16 and X-neg

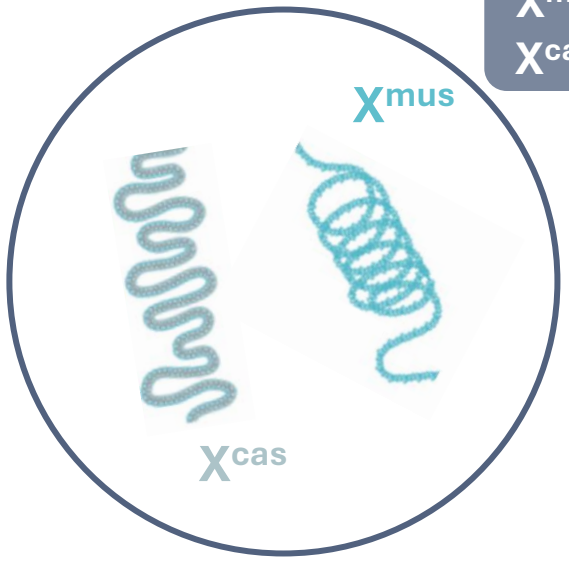
X1 weakens RepA-SPEN and RepA-PRC2 interactions. The structure impacts its activity.

- ALIS Screening of RepA
- In vitro functional validation
- **In vivo functional effects**
- RepA conformational changes

What are the effects of X1 in vivo

Model used: Embryonic Stem cell of female mouse (ESC) model $Tsix^{TST/+}$ (TST)

X^{mus} : Xi
X^{cas} : Xa



Two different fixed X alleles:

- **X^{mus}: X inactive** (from *Mus musculus* origin)
- **X^{cas}: X active** (from *Mus castaneus* origin)

TST carries **two active Xa** but go over XCI when they are induced to differentiate into embryoid bodies (EB) after 4 days

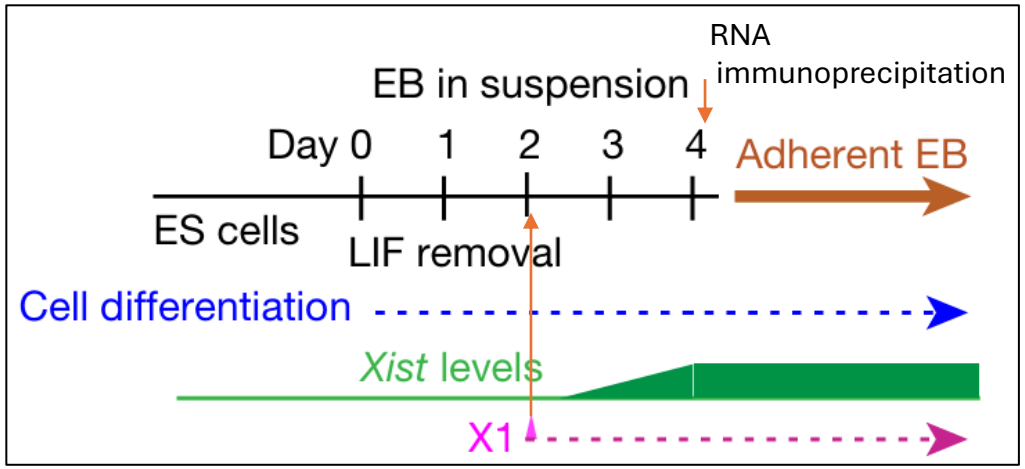


Figure 10. Schematic for analysis of XCI effects, X1 (10 µM) was added at day 2 and replenished daily with medium change.

X^{mus} : Xi
X^{cas} : Xa

What are the effects of X1 in vivo

RNA immunoprecipitation (RIP):

- anti-SUZ12 or anti-EZH2 antibodies to pull down PRC2-interacting RNAs

quantitative PCR with reverse transcription (RT-qPCR) to quantify co-eluted RNAs

→**No interference** of X1 with other compounds than PRC2 and SPEN

→X1 **reduce more** RepA-SPEN interaction than RepA-PRC2 interaction

X1 can selectively disrupt interaction of Xist in vivo.

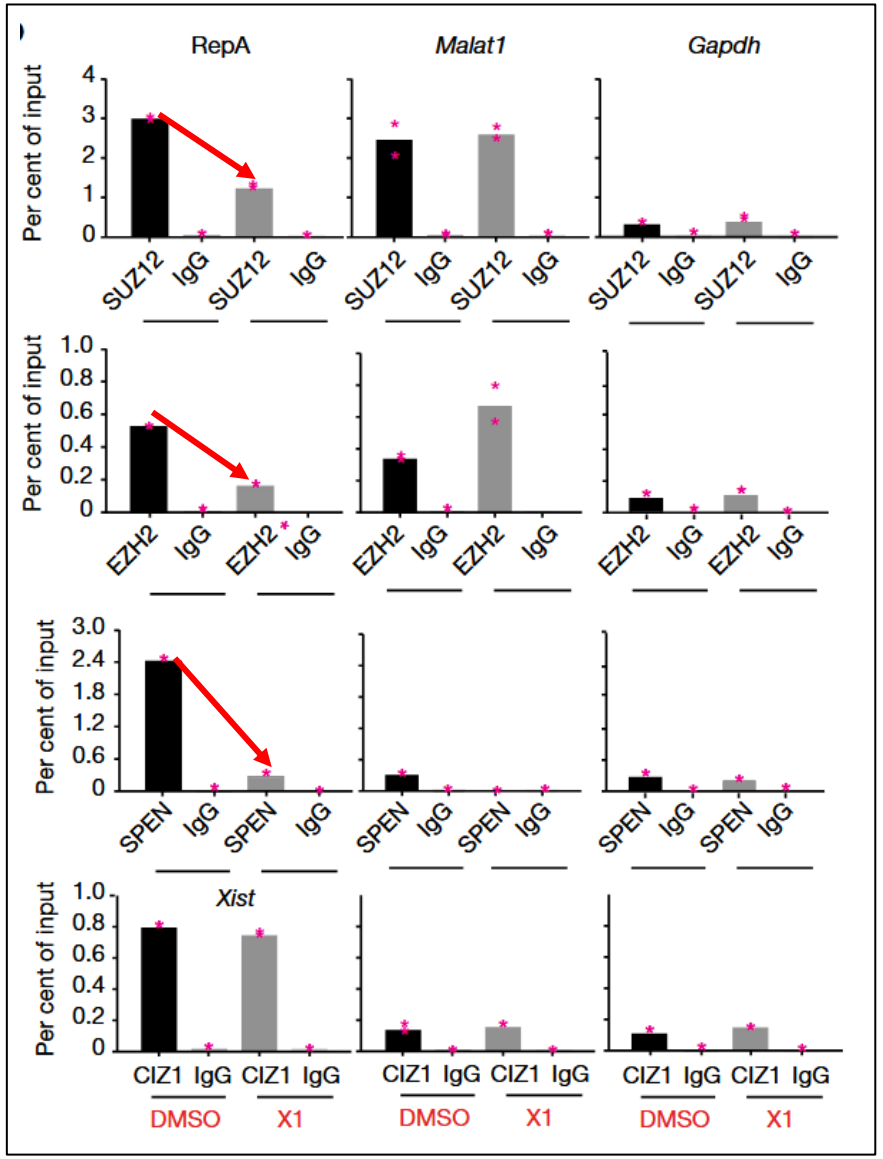


Figure 11. RIP with RT–qPCR in day 4 female TST ES cells to evaluate Xist binding to SUZ12, EZH2, SPEN and CIZ

Does X1 target RepA ?

X-A: -RepA
X+P: iXist

Model used:

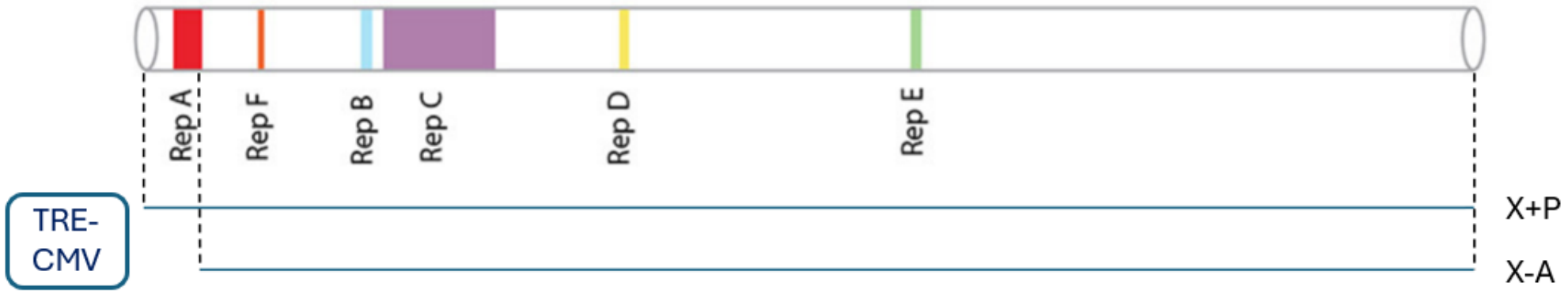
-Mouse embryonic fibroblasts X-A or X+P

X+P: full-length doxycycline inducible Xist transgene in mouse embryonic fibroblast
X-A: Depletion of Rep A in MEF

TST X-A

-Embryonic Stem cell (TST X-A)

TST X-A: Depletion of Rep A in the TST model



Does X1 target RepA ?

RIP in fibroblast expressing X+P cells or X-A cells.

→ When there is **no RepA**, there is **no** more **inhibitory effect** of X1

RNA pulldown assay on a tritiated analogue of X1

→ X1 is recovered only in wild type cells

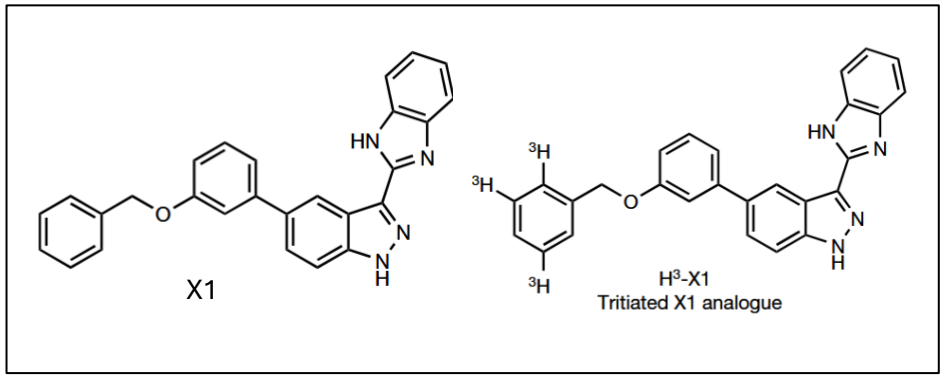


Figure 13. Structure of X1 and tritiated X1 analogue

X1 selectivity binds RepA inside cells.

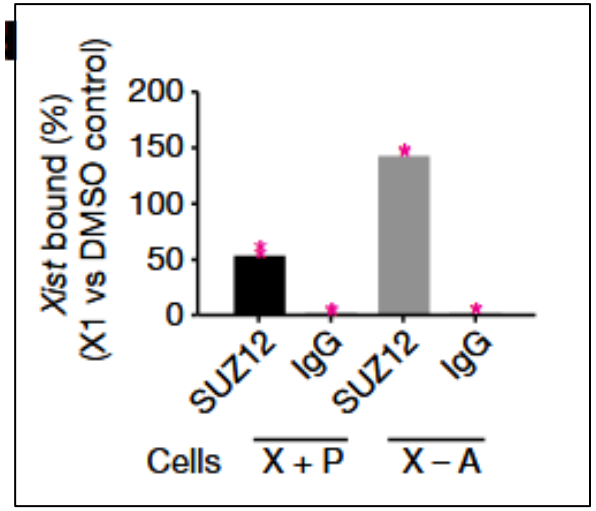


Figure 12. RIP with RT–qPC in X-P or X-A cells

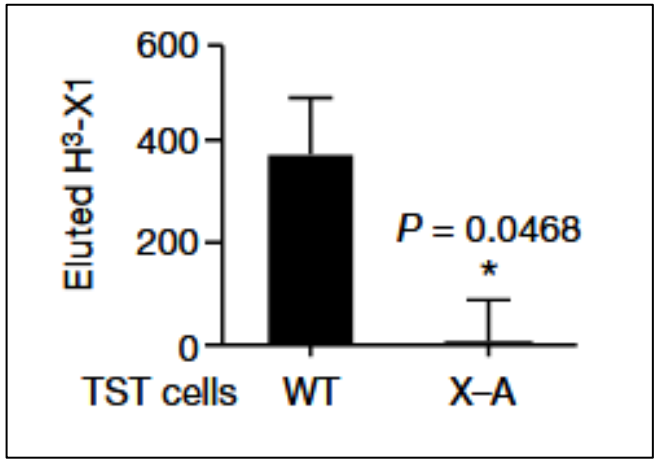
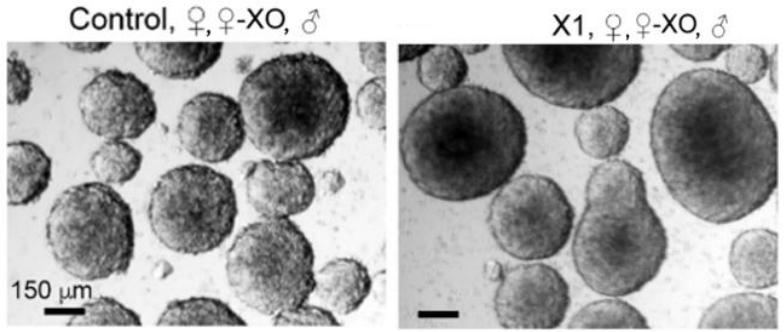


Figure 14. RNA pulldown assays using biotinylated probes to capture Xist in TST

Does the interaction of X1 with Xist affect XCI

Phenotypic assays to assess X1 effects on Embryoid bodies (EB) outgrowth

Embryoid bodies Day 3



Embryoid bodies Day 5

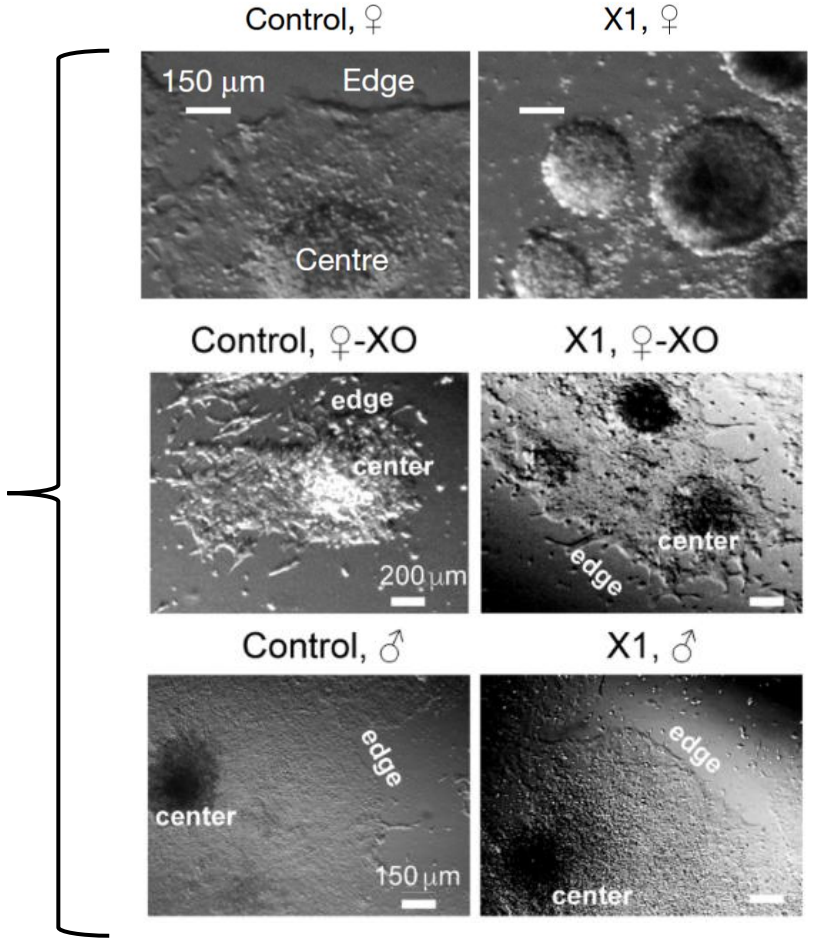


Figure 15. X1 effects on EB outgrowth in ♀- TST-XX, ♀-XO, and ♂-XY EB cells

X1 exerts a phenotypic effect on embryoid body outgrowth. This effect is Xist-dependent and is observed only in XX female ES cells.

Does the female-specific effect results from aberrant XCI?

RNA fluorescence in situ hybridization (FISH)

In both control and X1-treated cells

→ **Day 3**: no impact of X1 on Xist, Tsix and H3K27me3

→ **Day 5**: In X1 treated cells H3K27me3 was severely blunt

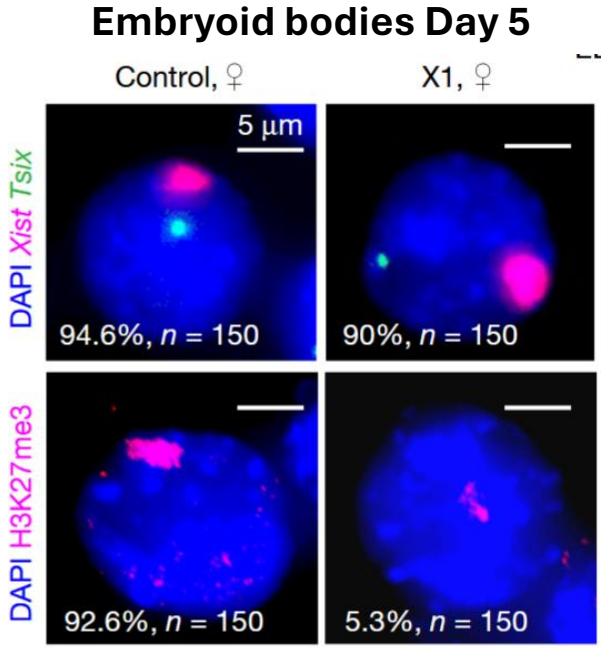


Figure 16. Xist and Tsix RNA FISH and immunostaining for H3K27me3.

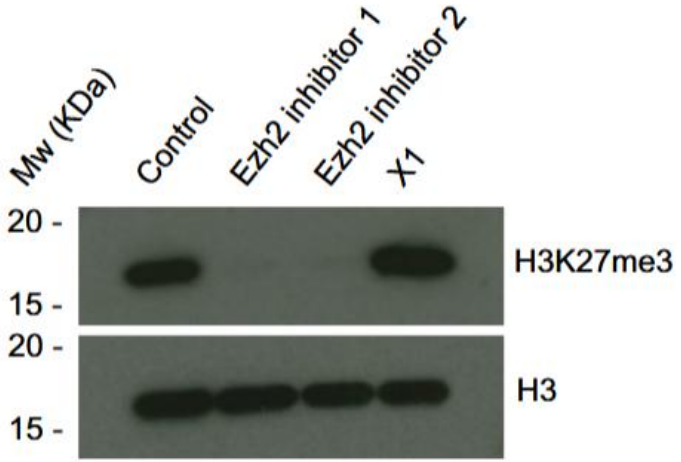


Figure 17. Western blot using H3K27me3 and total histone H3 antibodies. Total cell extracts were obtained from day 7 female EB cells

On **Day 7**: Western blot of H3K27me3 and H3 on cells treated with X1 or EZH2 inhibitor 1 or 2

→ **X1 do not change H3K27me3** in bulk

X1 uncouples Xist expression from polycomb function in cells.

How to confirm X1's target specificity ?

X^{mus} : Xi
X^{cas} : Xa

Allele specific ChIP-seq experiment

Map the epigenetic modification on two genetically distinct chromosomes within the same cell, using SNPs

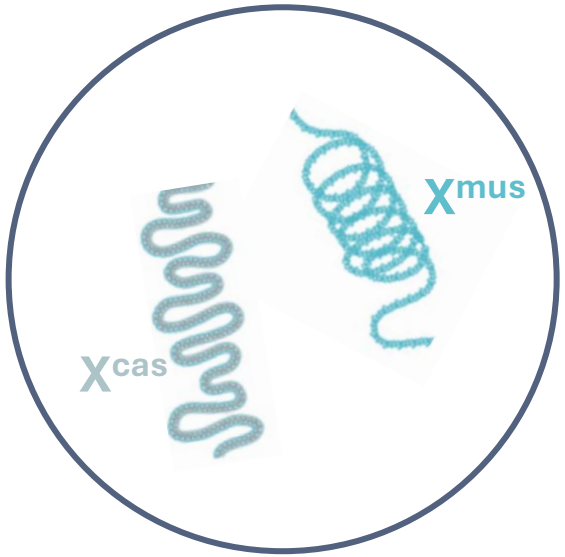
⊕ Can **distinguish two different alleles** in a single nucleus ⊕ High resolution view

Model used:

Hybrid TST embryonic stem cells (ES) from **female mice (XX)**

Two different fixed X alleles:

- **X^{mus}: X inactive** (from *Mus musculus* origin)
- **X^{cas}: X active** (from *Mus castaneus* origin)



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X^{mus} : Xi
X^{cas} : Xa

Allele specific ChIP-seq experiment

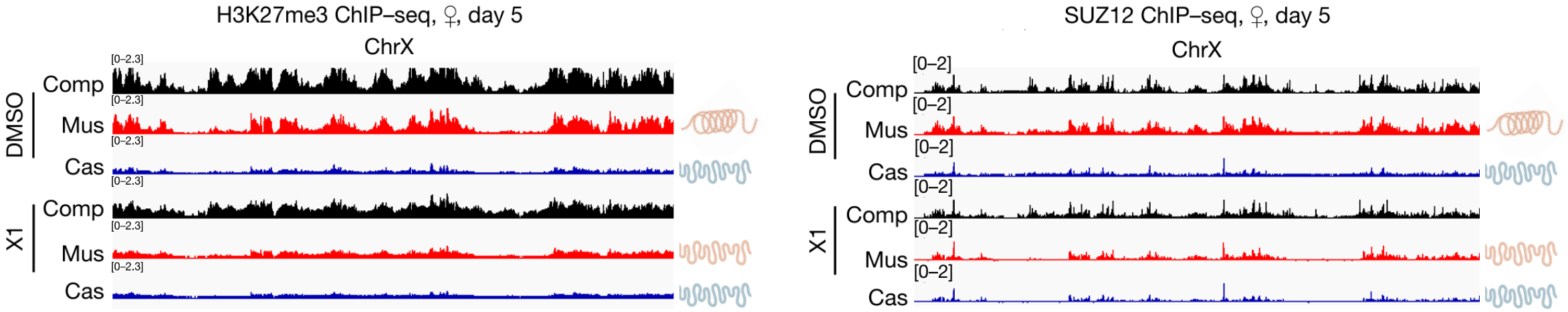


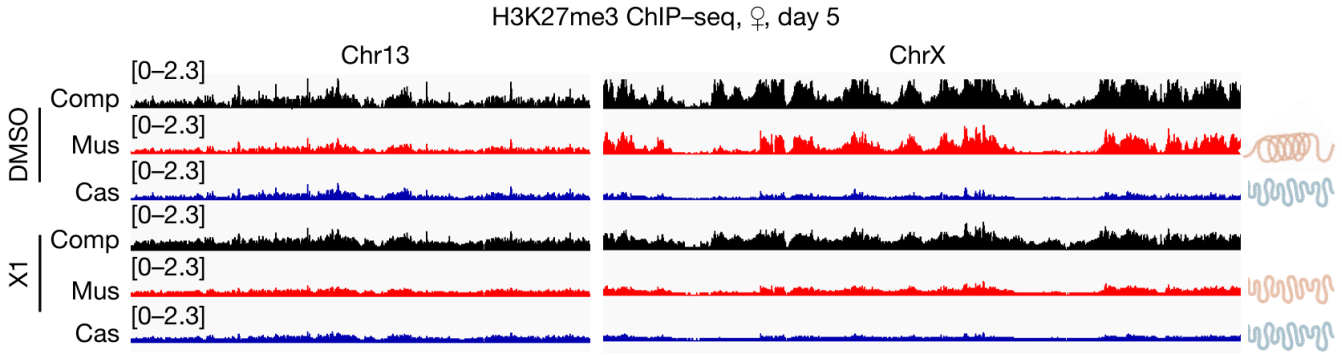
Figure 18. Allele-specific H3K27me3 and SUZ12 CHIP-seq analysis of day 5 female embryoid bodies treated with 10 μM X1 or DMSO for 72 hours.

- **DMSO control**: X^{mus} shows strong **PRC2 and H3K27me3 enrichment** → X inactivation
- **X1 addition**: X^{mus} loses **PRC2 interaction and H3K27me3 enrichment** on ChrX

How to confirm X1's target specificity ?

X^{mus} : Xi
X^{cas} : Xa

Allele specific ChIP-seq experiment



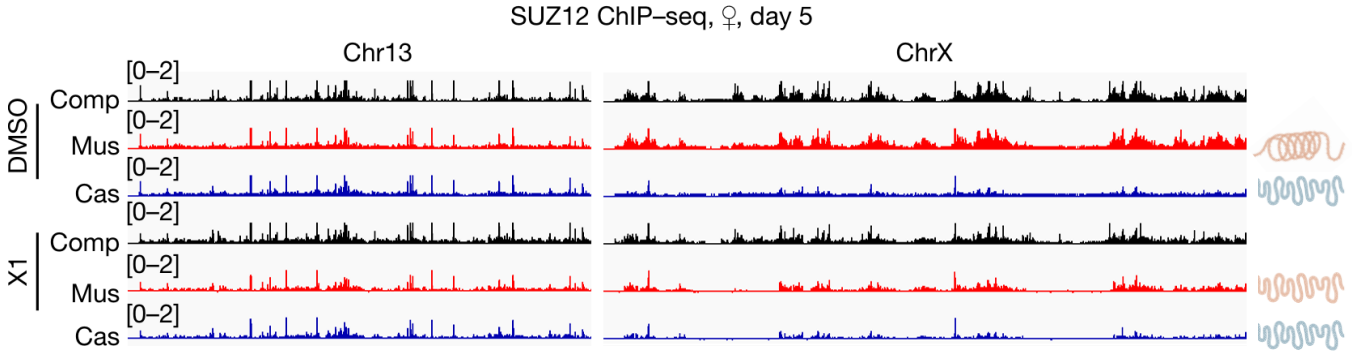


Figure 18: Allele-specific H3K27me3 and SUZ12 CHIP-seq analysis of day 5 female embryoid bodies treated with 10 μM X1 or DMSO for 72 hours.

- **DMSO control**: X^{mus} shows strong **PRC2 and H3K27me3 enrichment** → X inactivation
- **X1 addition**: X^{mus} loses **PRC2 interaction and H3K27me3 enrichment** on ChrX
- X-linked genes and Xist gene have reduced H3K27me3
- **Autosomal and escapee genes are not affected**

How to confirm X1's target specificity ?

X^{mus} : Xi
X^{cas} : Xa

Metagene analysis (same model used)

- **X1 addition**: inhibition of H3K27me3 enrichment and PRC2 accumulation on Xi
- Xa and autosomes are less affected

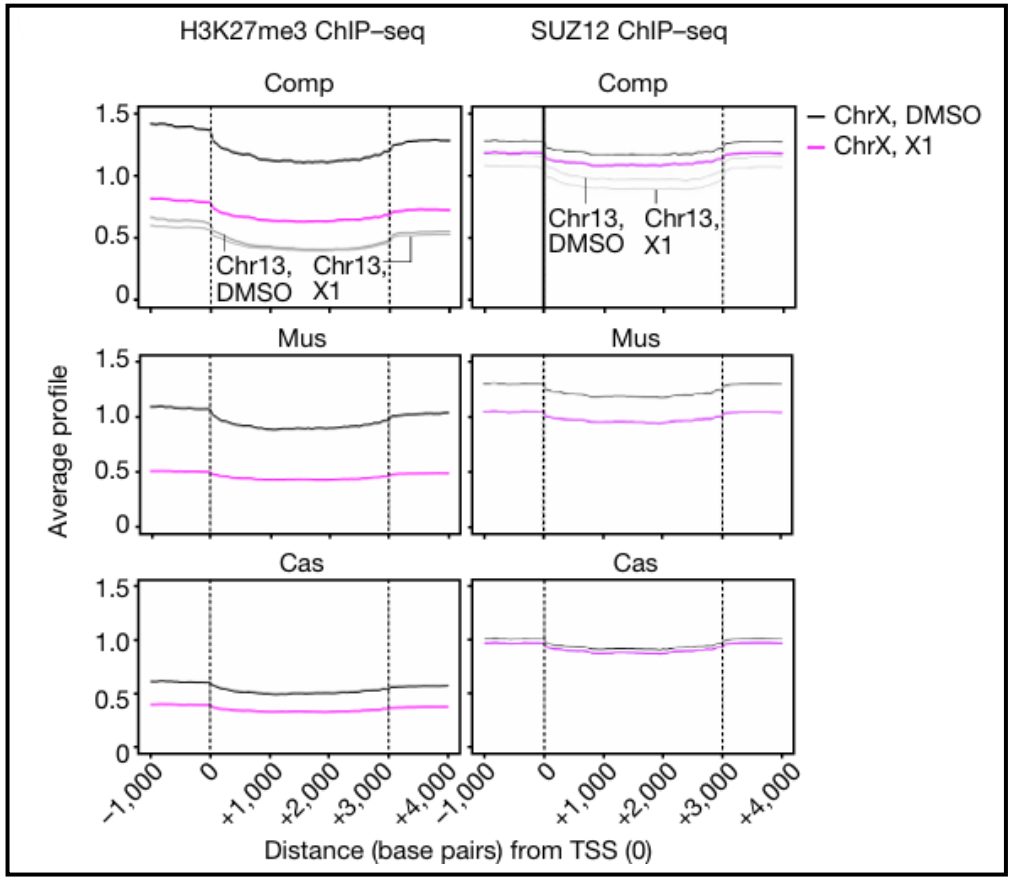


Figure 19. Metagene analysis of the average Xi gene, Xa gene and Chr13 gene with or without X1 treatment

In cells:

- X1 has an effect at 5 µM whereas in vitro it needs 6-fold more X1 to disrupt RepA-PRC2 interaction

→ Cells can **concentrate X1** at the locus

X1 shows strong on-target effects on PRC2 recruitment to the Xi

How is Xist expression affected with X1 blocking Xi ?

Allele-specific RT-qPCR: the effect of X1 addition (same model used)

X^{mus} : Xi
X^{cas} : Xa

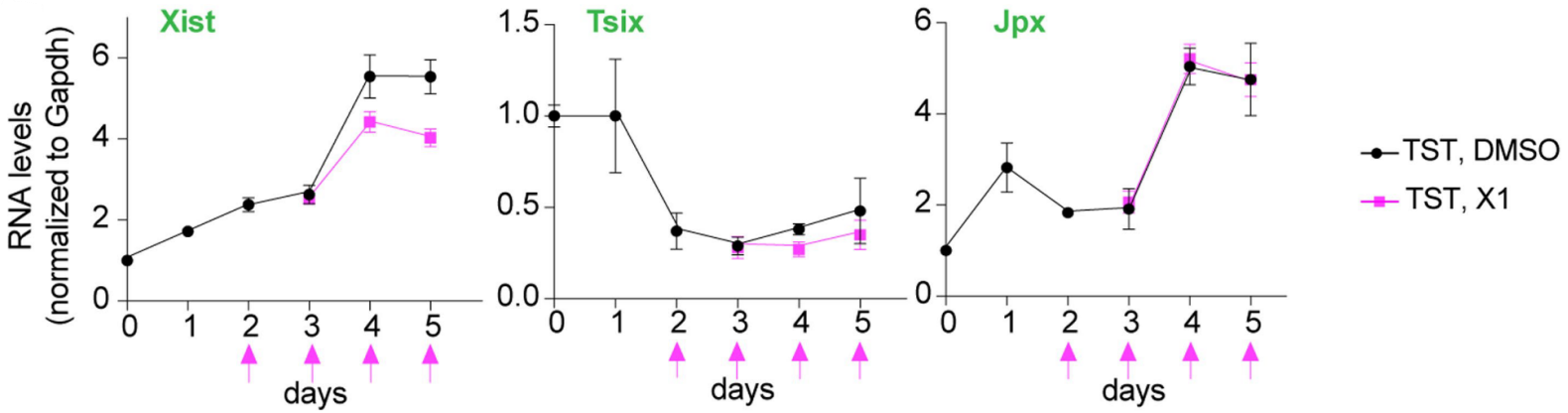


Figure 20. RT-qPCR of control genes in DMSO- or X-treated female embryoid bodies, Tsix and Jpx are Xist regulators.

- No effect on Xist regulators or its own expression

How is Xist expression affected with X1 blocking Xi ?

X^{mus} : Xi
X^{cas} : Xa

Allele-specific RT-qPCR: the effect of X1 addition (same model used)

- **Dose-dependant failure of Xi**, high X-linked RNA levels maintained on X^{mus}
- **No effect** on **X-linked gene expression** for X^{cas}

RepA deletion (X-A): same effect as X1 treatment
→ Reenforce that **X1 is mediated through RepA**

→ Further studies detected a **reversible effect** if X1 is removed after its addition

X1 blocks PRC2 recruitment and X inactivation in a RepA dependant manner

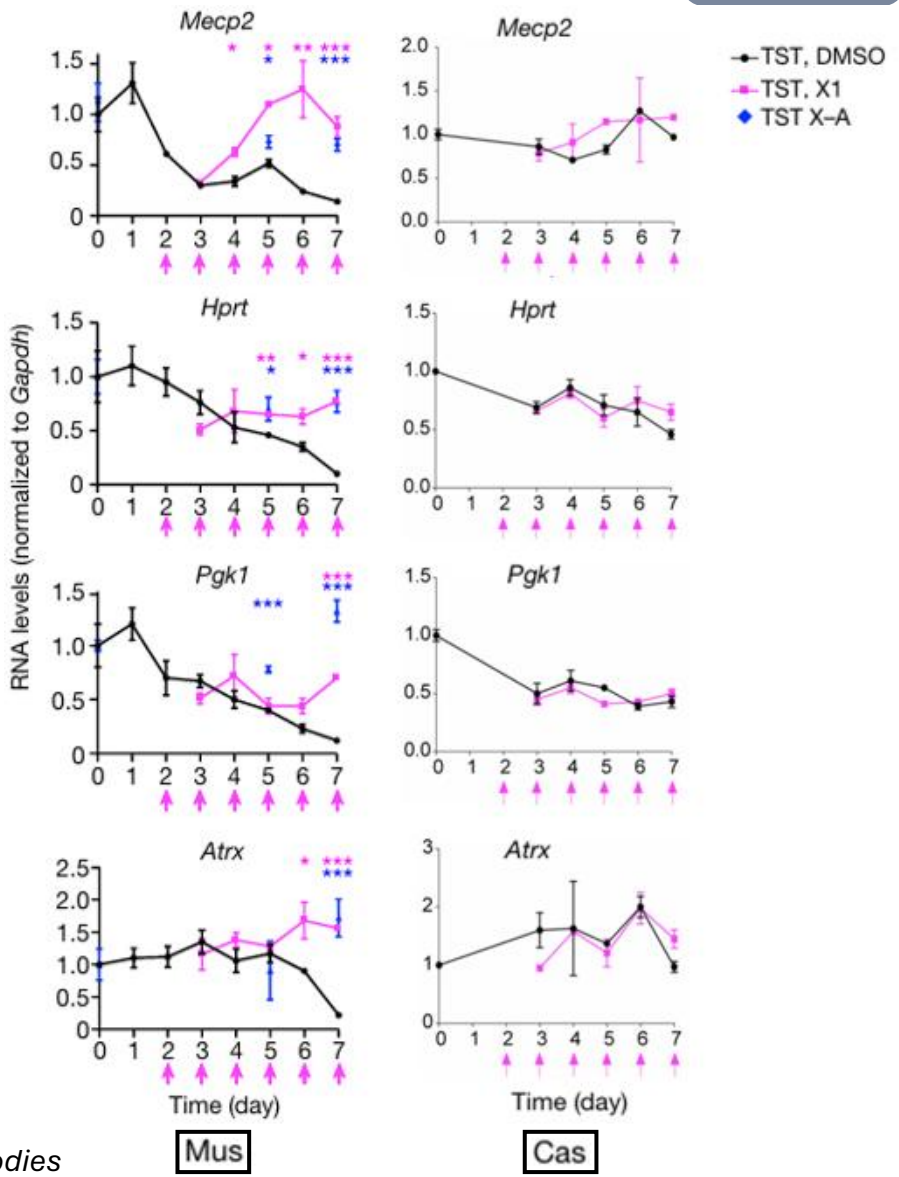
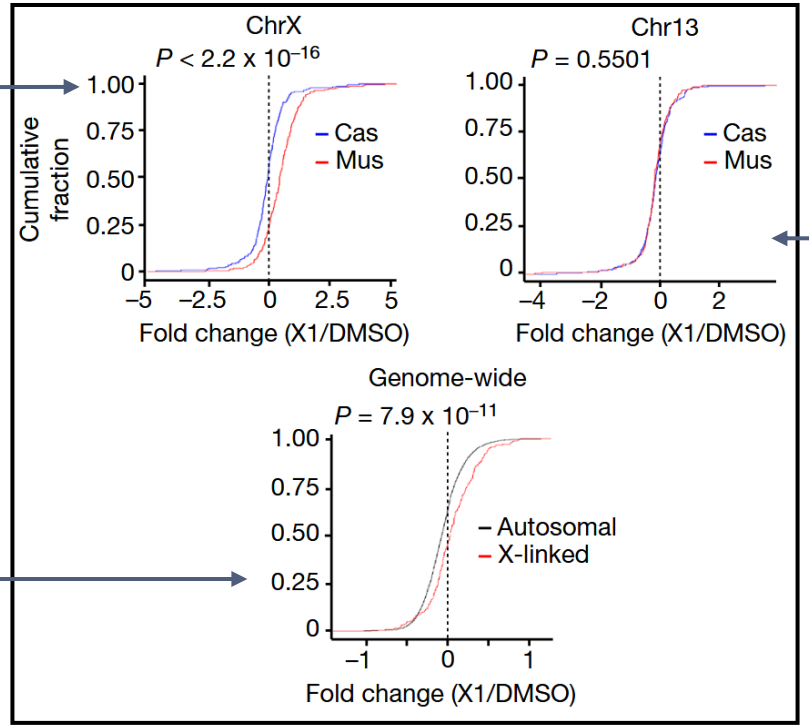


Figure 21. Xi and Xa allele-specific RT-qPCR of X-linked genes in DMSO- or X-treated female embryoid bodies

How to confirm on-target effect of X1 on Xi genes ?

X^{mus} : Xi
X^{cas} : Xa

Shift in Xi expression compared to **Xa expression** in X1-treated cells



On an **autosome**, there was **no-allele specific effect**

Increased expression of Xi genes for X-linked genes compared to autosomal genes

Figure 22. Cumulative distribution plots of fold changes in gene expression in X1- versus DMSO-treated embryoid bodies.

There is an on-target effect of X1 on Xi genes

Can X1 cause off-target effects on individual autosomal genes ?

Differential expression analysis

- X1 induce **changes on 197 autosomal genes**: 141 upregulated and 56 downregulated.

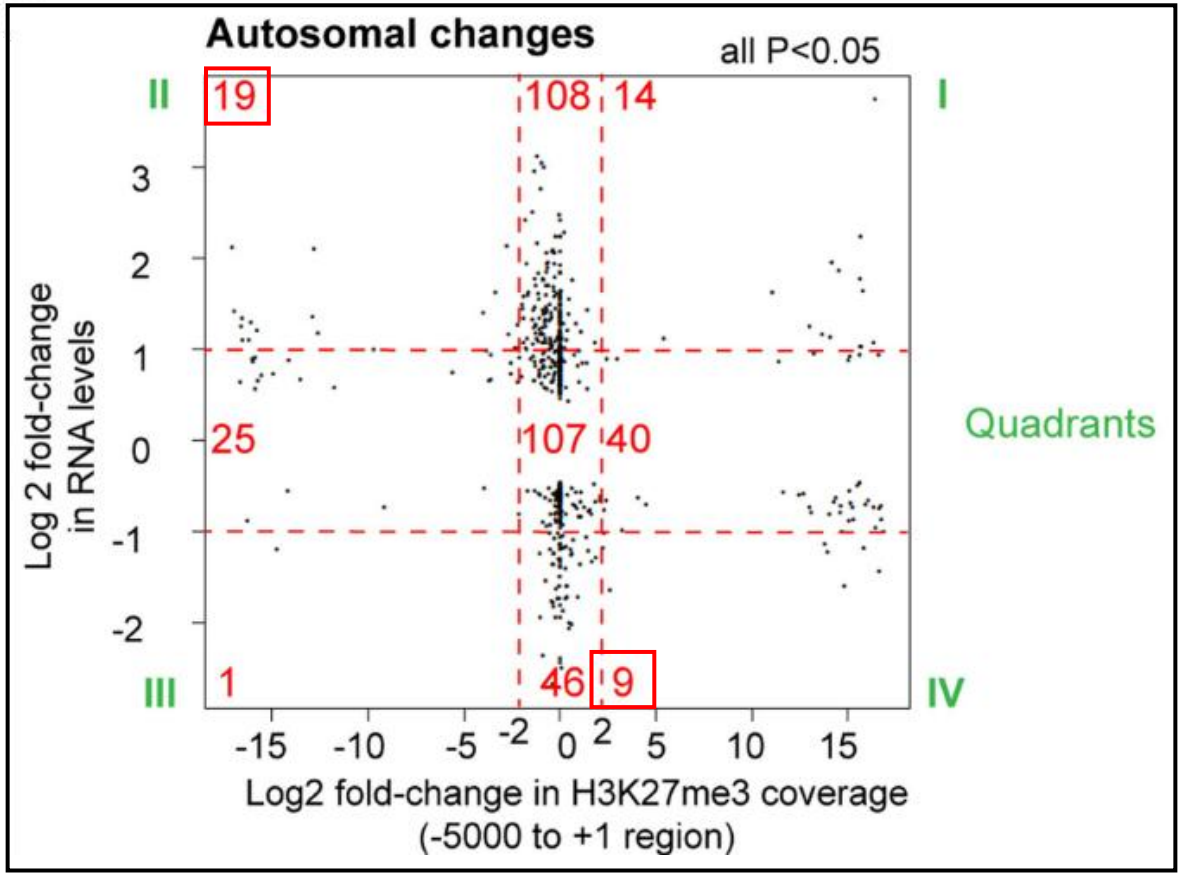


Figure 23. Differentially expressed autosomal genes (y axis) and their corresponding changes in H3K27me3 enrichment (x axis)

Cause can be

Directly due to X1 treatment = **true off-target effect**

Indirectly caused from X-linked changes

Would cause **H3K27me3 decreasing**

- Quadrant II: direct effect → 19 activated genes
- Quadrant IV: H3K27me3 gain → 9 genes inhibited

Due to the small changes, autosomal effects were probably secondary effects

- ALIS Screening of RepA
- In vitro functional validation
- In vivo functional effects
- **RepA conformational changes**

How does X1 blocks Xist RNA function through RepA ?

2 possibilities

Change in RNA conformation

Steric blockade

→ **X1 disrupt both PRC2 and SPEN binding from RepA**
This suggests a **global conformational change** in the RNA**.**

- Free RepA and X1-bound RepA have **similar dimensions**

→ against a larger occupied space, no steric blockade

- Internal change: **increased maximum distance in X1-treated RepA** molecule

→ **Reorganisation of the RNA structure**

SAXS/HPLC: **exclude steric blockade**

X1 acts by changing the conformation of RepA

How is RepA structure determined in solution ±X1 ?

Ab initio modelling package DAMMIN

- Generation of 100 model, followed by clustering using DAMCLUST

RepA without X1:

→ 16 conformational clusters

C13 is dominant

RepA with X1:

→ 6 conformational clusters only

C6’ is dominant

X1 induce reduced conformational heterogenicity

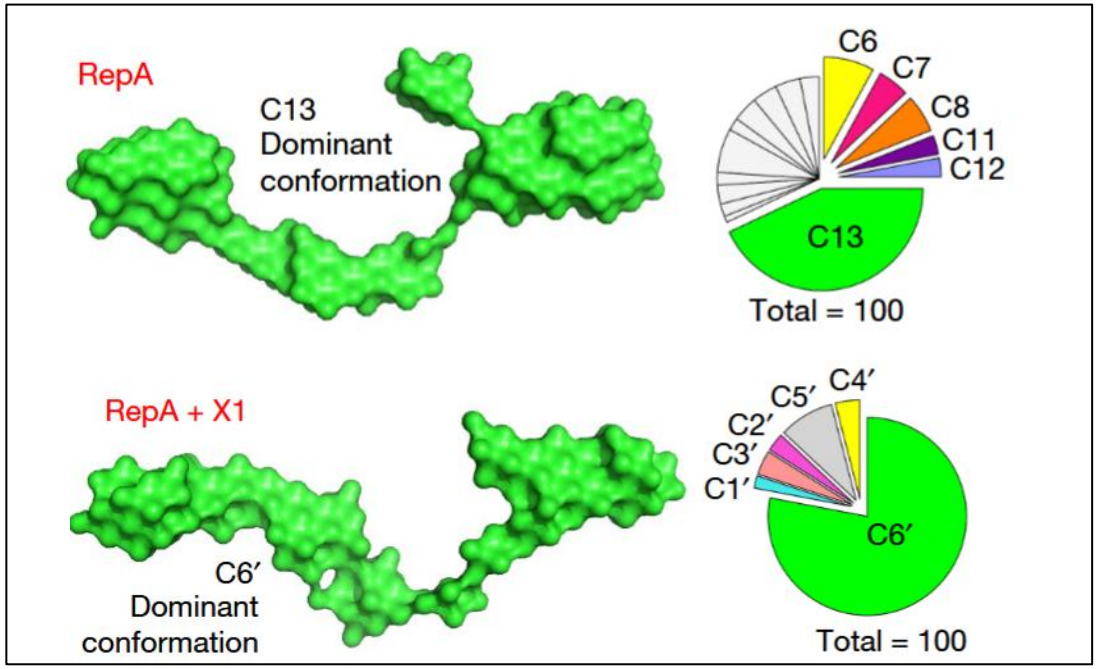


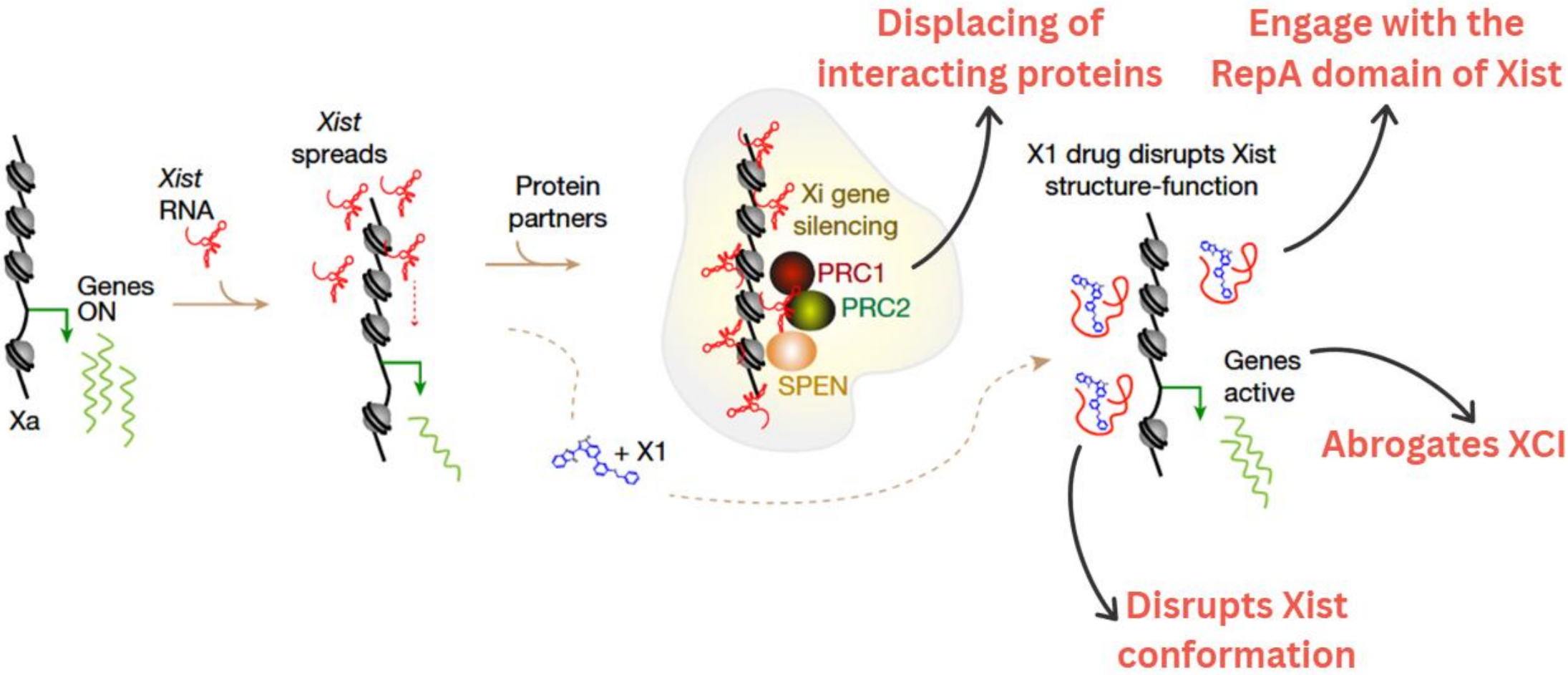
Figure 24. Main conformations of RepA alone and Rep A with X1

X1 stabilize RepA in a new conformation, decreasing affinity for other cognate factors.

- Conclusion
- Future outlook

Conclusion

- ncRNA can be **targeted** by **small molecules** for **phenotypic impact**
- **X1** has different **effects:**



Future outlook

- Innovation and therapeutic potential :
  - ncRNA can be targeted by small molecules → **X1 can be optimized for Xi reactivation and treatment of X-linked diseases** (ex: Rett syndrome)

- ALIS: X1 comes from **direct RNA-binding screen** → advanced conception approach.
  - **Agnostic to the mechanism of action**: applicable to any RNA
  - Can overcome structure complexity and target Xist for example

- Future perspectives:
  - Studies in **medical chemistry** to increase X1's potency and specificity
  - **Develop chemical libraries** with different RNA-binding properties

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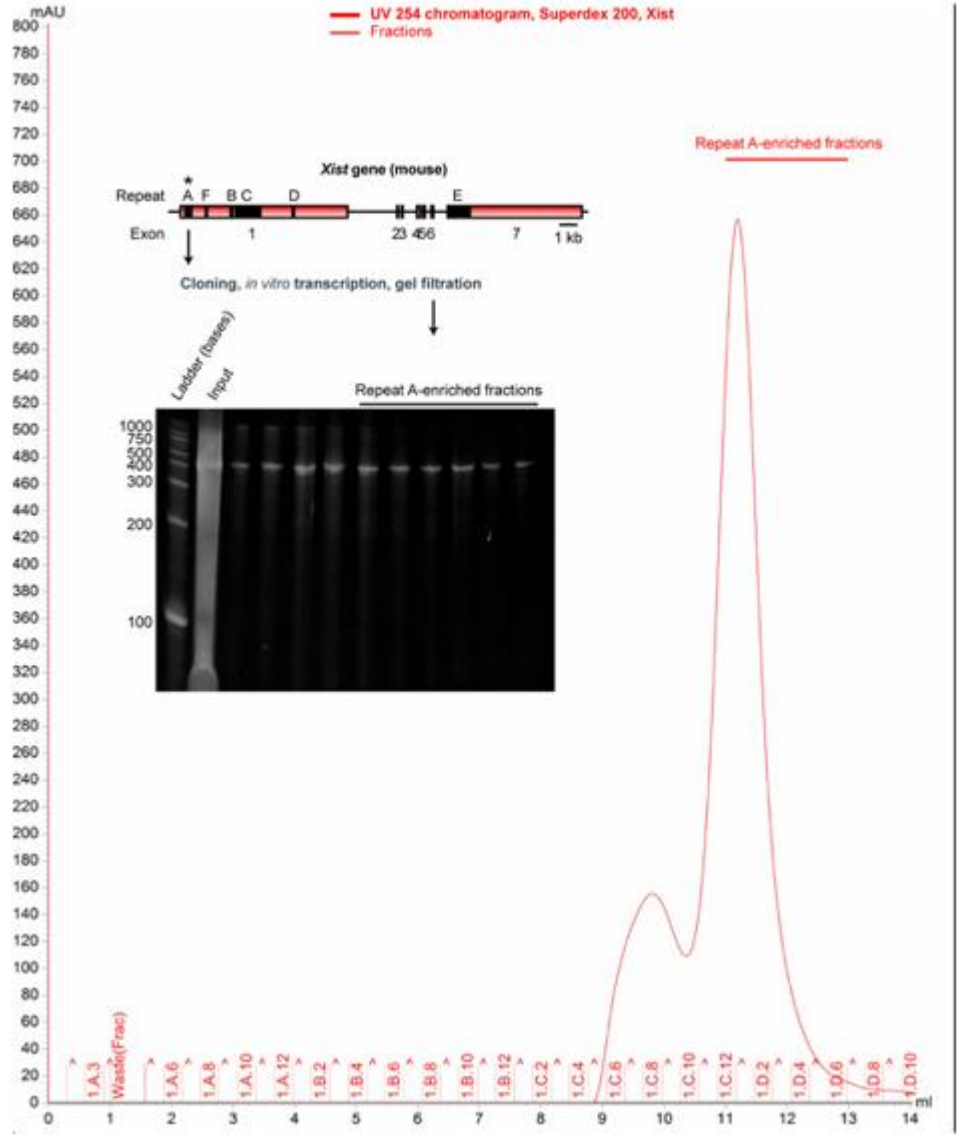
Thank you for your attention!
Let’s discuss together

Supplementary data

How is the mouse RepA synthetised ?

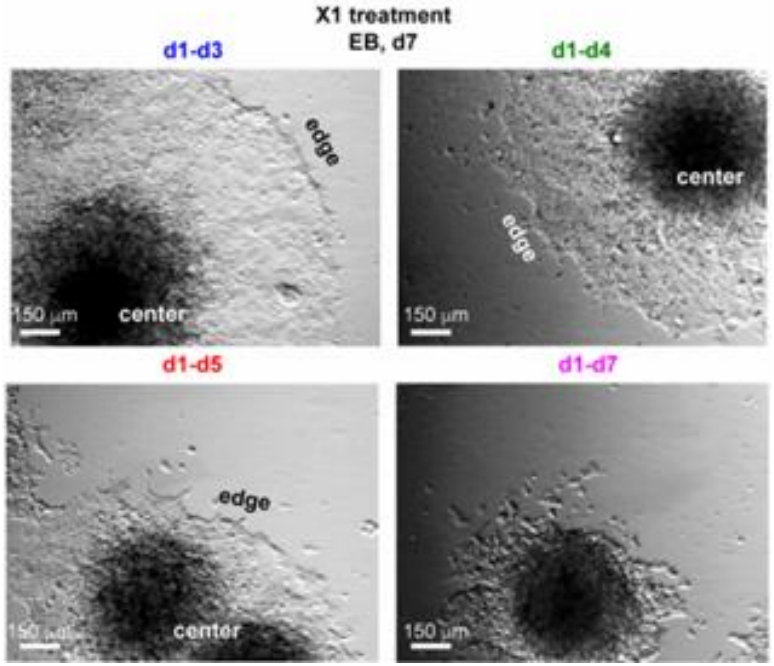
Purified under native conditions to retain the secondary structure after synthetising it.



Purification of Xist RepA RNA. A 431 Repeat A fragment of Xist RNA was in vitro transcribed and purified under native conditions by FPLC . A representative chromatogram is shown. To confirm size and stability of the sample just prior to ALIS, we visualized the RNA in a denaturing urea-PAGE

What do you mean about withdrawing of X1?

Embryoid body growth resumed after withdrawing of X1.



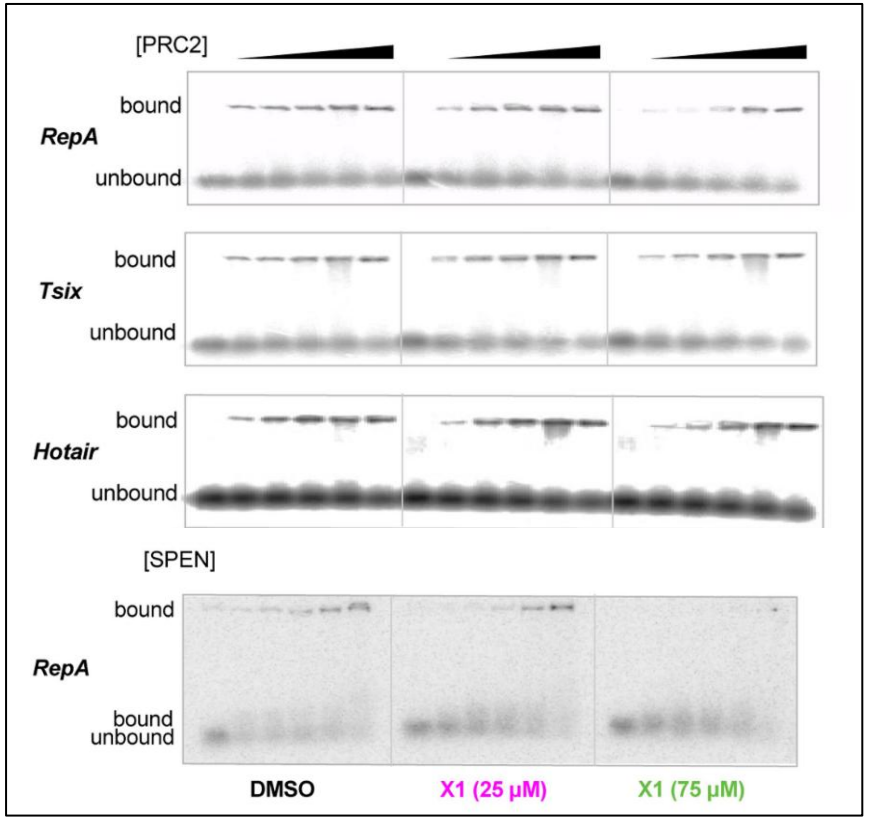
Female EB were grown from d1 in 10 μM X1 and the treatment was suspended on day 3, 4, 5, or maintained up to day 7. This figure shows they growth morphology.

How much X1 is needed to interfere with RepA binding ?

Measure of the dissociation constant (Kd) change to quantify X1 disrupted effect.

- Input of 0.25 or 75 µM of X1 against Xist, Tsix and Hotair

→ An input of **75µM of X1** has a huge **disrupting effect**
→ X1 has **no effect** on Tsix and Hotair interactions
→ RepA as a **higher affinity** for PRC2 than SPEN



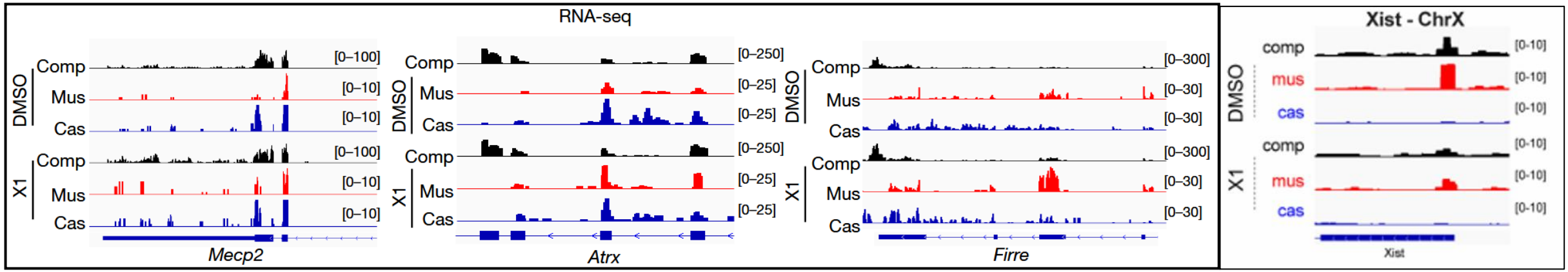
EMSAs titrating PRC2 or SPEN-RRM against a fixed concentration of X1 and RepA, Tsix, or Hotair RNA

X1 selectively inhibits interactions of RepA with its binding partners in vivo

How to confirm on-target effect of X1 on Xi genes ?

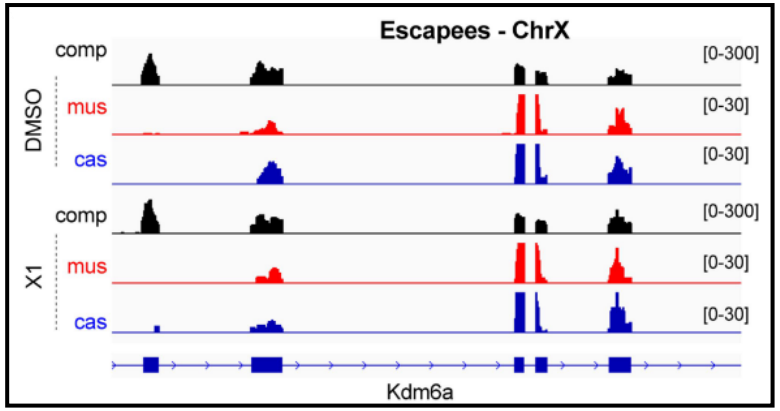
X^{mus} : Xi
X^{cas} : Xa

Allele-specific RNA-sequencing: verify loss of Xi silencing



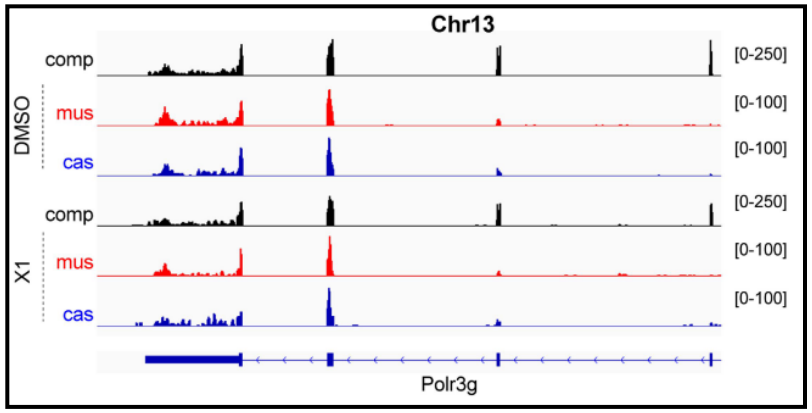
RNA-seq analyses of H3K27me3 on day 5 DMSO- or X1-treated female embryoid bodies

- X1 absence → **Xi silencing** after 5 days
- X1 addition → **Xi genes activation** after 5 days, even with Xist expression



RNA-seq analyses of day 5 DMSO- or X1-treated female embryoid bodies

Escapee genes: not affected by X1



RNA-seq analyses of day 5 DMSO- or X1-treated female embryoid bodies

Autosomal genes: not affected by X1