Supplementary File

Exploring the role of Polycomb recruitment in *Xist*-mediated silencing of the X chromosome in ES cells

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***Xist* mutant**	**Clone Number**	**Size of deletion**	**Deleted region**	**% of cells with *Xist* domains**	***Xist* coating**	**Nº of experiments**	**Total cell counted**
Xist FL	n.a.	n.a.	n.a.	45 ± 6 %	+++++	3	956
Xist ΔA	n.a.	917 bp	Δ1-917	53 ± 9 %	++++	3	990
Xist ΔF+B+C	1	3,908 bp	Δ890-4796	77 ± 4 %	+++	3	811
	2	3,908 bp	Δ890-4796	n.d.	+++	n.d.	n.a.
Xist ΔF+B	1	2,119 bp	Δ875-2982	76 ± 3 %	+++	3	869
	2	2,130 bp	Δ862-2990	87%	+++	1	272
Xist ΔB+C	1	2,109 bp	Δ2,703-4810	60 ± 8 %	+++++	3	952
	2	2,049 bp	Δ2,699-4744 (+ 45 bp insertion)	7%	+++++	1	269
Xist ΔB+1/2C*	1	1,383 bp	Δ2,703-4,084	60 ± 3 %	+++++	3	921
	2	454 bp & 810 bp	Δ2,705-3,158 & Δ3,558-4,367	52%	+++++	1	298
Xist ΔB	1	335 bp	Δ2,707-3,041	67 ± 4 %	+++++	3	978
	2	337 bp	Δ2,705-3,041	44%	+++++	1	365
Xist ΔC	1	1,716 bp	Δ3,041-4,752	71 ± 2 %	+++++	3	863
	2	1,716 bp	Δ3,041-4,752	34%	+++++	1	298

Figure 1 - source data 1 – Full set of *Xist*-TetOP mutants generated

Summary table of the analysis of full set of the *Xist*-TetOP mutants generated (including the second clone per type of mutation) in terms of: deletion size, coordinates of the deleted regions (based on Ensembl *Xist* exonic sequence); number of cells with *Xist* domains (in % ± S.E.M.) and capacity of *Xist* coating (+++++ indicates *Xist* cloud signals similar to *Xist* FL; ++++ and +++ indicate *Xist* cloud signals of decreased size compared to *Xist* FL); n.d. – not done; n. a. – not applied; * To generate *Xist* ΔB+1/2C mutants, we use a 3'end gRNA which recognized a unique region within repeat C, however, several other sequences with only 1 or 2 mismatched can be found within this repeat and were likely to be targeted, creating distinct deletions as can be seen for the two clones of this type of mutant.

Xist mutant	Clone	JARID2	EZH2	H3K27me3	RING1B	H2AK119ub
Xist FL	n.a.	88 ± 4 % (4 exp, n = 275)	85 ± 7 % (3 exp, n = 242)	92 ± 7 % (3 exp, n = 248)	48 ± 14 % (4 exp, n = 313)	96 ± 1 % (3 exp, n = 223)
Xist ΔA	n.a.	78 ± 4 % (3 exp, n = 261)	78 ± 11 % (3 exp, n = 243)	97 ± 2 % (3 exp, n = 228)	50 ± 10 % (4 exp, n = 302)	97 ± 2 % (3 exp, n = 269)
Xist ΔF+B+C	1	1 ± 1 % (3 exp, n = 257)	1 ± 1 % (3 exp, n = 232)	3 ± 2 % (3 exp, n = 236)	0 ± 0 % (2 exp, n = 143)	1 ± 0 % (3 exp, n = 227)
	2	n.d.	n.d.	0% (1 exp, n = 85)	n.d.	2% (1 exp, n =83)
Xist ΔF+B	1	1 ± 1 % (2 exp, n = 204)	2 ± 2 % (2 exp, n = 180)	18 ± 15 % (2 exp, n = 198)	0 ± 0 % (3 exp, n = 218)	28 ± 7 % (2 exp, n = 154)
	2	14% (1 exp, n = 93)	18% (1 exp, n = 180)	32% (1 exp, n = 66)	0% (1 exp, n = 57)	32% (1 exp, n = 57)
Xist ΔB+C	1	0 ± 0 % (3 exp, n = 237)	0 ± 0 % (3 exp, n = 216)	1 ± 0 % (3 exp, n = 208)	0 ± 0 % (4 exp, n = 292)	1 ± 1 % (2 exp, n = 181)
	2	0% (1 exp, n = 68)	0% (1 exp, n = 63)	5% (1 exp, n = 63)	0 ± 0 % (2 exp, n = 98)	3% (1 exp, n = 65)
Xist ΔB+1/2C	1	0 ± 0 % (2 exp, n = 202)	0 ± 0 % (2 exp, n = 165)	5 ± 0 % (2 exp, n = 182)	0% (1 exp, n = 54)	17 ± 2 % (3 exp, n = 262)
	2	1% (1 exp, n = 87)	n.d.	5% (1 exp, n = 63)	0% (1 exp, n = 100)	14% (1 exp, n = 100)
Xist ΔB	1	6 ± 3 % (3 exp, n = 265)	18 ± 5 % (3 exp, n = 270)	49 ± 7 % (3 exp, n = 240)	1 ± 1 % (3 exp, n = 217)	61 ± 8 % (3 exp, n = 257)
	2	n.d.	n.d.	38% (1 exp, n = 90)	n.d.	63 ± 16 % (2 exp, n = 93)
Xist ΔC	1	89 ± 4 % (3 exp, n = 255)	81 ± 9% (2 exp, n = 172)	78 ± 2 % (3 exp, n = 195)	37 ± 4 % (3 exp, n = 240)	99 ± 0 % (3 exp, n = 288)
	2	89% (1 exp, n = 72)	n.d.	65% (1 exp, n = 63)	n.d.	97% (1 exp, n = 72)

Figure 1 - source data 2 - Summary table of IF/*Xist* RNA FISH experiments in the full set of *Xist*-TetOP mutants generated

The table displays the percentage (± S.E.M.) of *Xist*-coated chromosomes exhibiting enrichment of the PcG proteins (JARID2, EZH2 and RING1B) and histone marks (H3K27me3 and H2AK119ub); a minimum of 50 *Xist*-coated chromosomes were counted per experiment (exp); n – number of cells counted in at least one or more biological replicates; n.a. – not applied; n.d. – not done.

Figure 2 - source data 1 – Full list of ChIRP-MS peptide counts for *Xist* FL (noDOX and DOX) and *Xist* ΔB+C (DOX) – supplementary excel file

Sheet 1 – Selection of peptide counts for the 81 *Xist* hits [according to (Chu et al., 2015)] found in *Xist* FL and *Xist* ΔB+C interactome (with a minimum of 2.5 DOX/noDOX fold-change in *Xist* FL or *Xist* ΔB+C; Weakly annotated protein isoforms with an Annotation score in UniprotKB < 3 (out of 5) were excluded.
Sheet 2 – Filtered list of total number of peptides counts for in *Xist* FL (noDOX and DOX) and *Xist* ΔB+C; In this list, only peptides with a minimum of 2.5 DOX/noDOX fold-change in *Xist* FL were selected; Weakly annotated protein isoforms with an Annotation score in UniprotKB < 3 (out of 5) were excluded; this was the case of two poorly annotated isoforms of hnRNPK: tr|Q3TL71, less bound to *Xist* ΔB+C and tr|Q3U6X2, equally bound to *Xist* FL and ΔB+C.
Sheet 3 – Total number of peptide counts in *Xist* FL (noDOX and DOX) and *Xist* ΔB+C (DOX).

***Xist* mutant**	**Clone Number**	***Lamp2* (D2)**	***Pgk1* (D2)**	***Pgk1* (D4)**	***Rnf12* (D4)**
Xist FL (noDOX)	n.a.	58 ± 9% (2 exp, n = 245)	53 ± 7% (3 exp, n = 308)	68 ± 8% (2 exp, n =353)	73% (1 exp, n = 106)
Xist FL	n.a.	10 ± 1 % (3 exp, n = 345)	9 ± 3% (3 exp, n = 375)	11 ± 2 % (4 exp, n = 363)	19 ± 9 % (2 exp, n = 211)
Xist ΔA	n.a.	75 ± 2 % (3 exp, n = 344)	68 ± 6% (3 exp, n = 352)	64 ± 7 % (3 exp, n = 332)	85 ± 5 % (2 exp, n = 228)
Xist ΔF+B+C	1	21 ±1 % (3 exp, n = 351)	15 ± 1% (2 exp, n = 246)	14 ± 2 % (3 exp, n = 353)	n.d.
	2	10% (1 exp, n = 81)	n.d.	n.d.	n.d.
Xist ΔF+B	1	5% (1 exp, n = 107)	12 ± 2 % (2 exp, n = 216)	21 ± 8 % (3 exp, n = 327)	23 ± 4 % (2 exp, n = 210)
	2	n.d.	n.d.	9% (1 exp, n =111)	12% (1 exp, n = 105)
Xist ΔB+C	1	17 ± 1 % (3 exp, n = 331)	11 ± 1 % (3 exp, n = 367)	17 ± 4 % (4 exp, n = 377)	17% (1 exp, n = 97)
	2	20% (1 exp, n = 66)	16% (1 exp, n = 64)	n.d.	n.d.
Xist ΔB+1/2C	1	18% (1 exp, n = 99)	11 ± 2% (2 exp, n = 260)	19 ± 1 % (2 exp, n = 246)	5% (1 exp, n = 111)
	2	n.d.	n.d.	6% (1 exp, n = 112)	n.d.
Xist ΔB	1	13 ± 1% (3 exp, n = 330)	11 ± 1% (2 exp, n = 220)	14 ± 1 % (2 exp, n = 205)	n.d.
	2	n.d.	n.d.	23% (1 exp, n = 93)	n.d.
Xist ΔC	1	8± 2% (3 exp, n = 292)	5 ± 2 % (2 exp, n = 198)	5 ± 1 % (2 exp, n = 327)	n.d.
	2	n.d.	0% (1 exp, n = 59)	n.d.	n.d.

Figure 4 - source data 1 - Summary table of combined *Xist* with X-linked nascent-transcript RNA FISH for *Pgk1*, *Lamp2* and *Rnf12* in the full set of *Xist*-TetOP mutants

The table displays the percentage (± S.E.M.) of *Xist*-coated chromosomes exhibiting an active *Pgk1* (at D2 and D4), *Lamp2* (at D2) and *Rnf12* gene (at D4); a minimum of 50 *Xist*-coated chromosomes were counted per experiment (exp); for *Xist* FL noDOX a minimum of 100 cells (which do not have *Xist*-coated chromosome) were counted; n – number of cells counted in at least one or more biological replicate (exp); n.d. – not done; n.a. – not applied.

***Xist* mutants**	**5'end /3'end sgRNAs**	**Primers to confirm deletion (F/R)**	**Primers to confirm loss of WT sequence (F/R)**
Xist ΔF+B+C	TCACGCAGAAGCCATAATGG/ CTTGAGAGATGATACCTCCA	CTGCTGATCGTTTGGTGCTG/ CAGACCTGTGTTTGCCCCTT	CTGCTGATCGTTTGGTGCTG/ ATCAAGGCGAATCCCGCAAC
Xist ΔF+B	TCACGCAGAAGCCATAATGG/ AGGGCTGGACTGGATTGGGT	TGGTGCTGTGTGAGTGAACC/ TTAGCACTGAATCAATGAAGA	CTGCTGATCGTTTGGTGCTG/ ATCAAGGCGAATCCCGCAAC
Xist ΔB+C	TATAACAGTAAGTCTGATAG/ GTGTATCTTGATTAACATGA	ATGACTGGATGTCAGGAGTA/ CAGACCTGTGTTTGCCCCTT	ATGACTGGATGTCAGGAGTA/ CTGAGTCTTGAGGAGAATCT
Xist ΔB+1/2C	TATAACAGTAAGTCTGATAG/ CATACTGACTTCTAGAGTCA*	ATGACTGGATGTCAGGAGTA/ CAGACCTGTGTTTGCCCCTT	ATGACTGGATGTCAGGAGTA/ CTGAGTCTTGAGGAGAATCT
Xist ΔB	TATAACAGTAAGTCTGATAG/ CTCTAAGTAGAAGTGGGCTT	ATGACTGGATGTCAGGAGTA/ TTAGCACTGAATCAATGAAGA	ATGACTGGATGTCAGGAGTA/ CTGAGTCTTGAGGAGAATCT
Xist ΔC	CTCTAAGTAGAAGTGGGCTT/ GTGTATCTTGATTAACATGA	CCAGGCCCAGATACTTTCAG/ CAGACCTGTGTTTGCCCCTT	TCCATGGACAAGTAAACAAAGAA/ TGTTTGCCCCTTTGCTAAAT

Supplementary File 1 – List of gRNA sequences and primers used for *CRISPR/Cas9* editing of the different *Xist*-TetOP mutants

Primer sequences to confirm deletion and loss of wild-type (WT) allele are displayed for each *Xist* mutant; ***** highlights for the fact that the 3'end gRNA used to generate ΔB+1/2C was designed to a unique region within the repeat C, but several sequences with only 1 or 2 mismatched can be found within this repeat and are likely to be targeted to generate different types of ΔB+1/2C mutants.

RT-PCR analysis	Sequences (F/R)
Xist exon 1-exon 3	GCTGGTTCGTCTATCTTGTGGG / CAGAGTAGCGAGGACTTGAAGAG
Xist before repeat B	ATGACTGGATGTCAGGAGTA / CTGAGTCTTGAGGAGAATCT
Xist before repeat C	TCCATGGACAAGTAAACAAAGAA / TGTTTGCCCCTTTGCTAAAT
Gapdh	AACTTTGGCATTGTGGAAGG / ACACATTGGGGGTAGGAACA

Supplementary File 2- Primer sequences for RT-PCR analysis of *Xist* mutants (used in Figure 1 – supplement figure 1B)

Figure 1 - figure supplement 1 – Characterization of the novel *Xist*-TetOP mutants

A. Deletion mapping by Sanger sequencing and expression analysis across deleted region in the novel *Xist* ΔF+B+C, ΔB+F, ΔB+C, ΔB+1/2C, ΔB and ΔC mutants (this analysis is for clone 1 of each mutant type); red arrows indicate forward primer and green arrows represent reverse primers; the primer of the left is the sequenced primer for each mutant; B means PCR blank.
B. RT-PCR analysis of the splicing pattern and expression across the repeat B and C regions of the different *Xist*-TetOP mutants; Primer pairs indicated in the scheme in green (see Materials and Methods); B means PCR blank.
C. *Xist* RNA FISH analysis upon D4 of differentiation in the presence of DOX (also noDOX for *Xist* FL) in the *Xist* ΔF+B+C, ΔB+F, ΔB+C, ΔB+1/2C, ΔB and ΔC mutants (this analysis is for clone 1 of each mutant type); values represent the % ± standard error (S.E.M.) of cells with a *Xist*-coated chromosome (at least 3 biological replicates with a minimum of 250 cells counted per replicate); Scale bar: 10 μm.

Figure 1 - figure supplement 2 – Recruitment of a PRC2 member (EZH2), a PRC2 co-factor (JARID2) and PRC1 member (RING1B) in the different *Xist*-TetOP mutants

A. Representative images of combined IF for JARID2 (green) with RNA FISH for *Xist* (red) in *Xist*-TetOP lines (for clone 1 of each mutant type) upon D2 in the presence of DOX; DAPI in blue.
B. Representative images of combined IF for RING1B (green) with RNA FISH for *Xist* (red) in *Xist*-TetOP lines (for clone 1 of each mutant type) upon D2 in the presence of DOX; DAPI in blue; Scale bar: 10 μm.
C. Graph representing the % of *Xist*-coated chromosomes enriched for JARID2, EZH2 and RING1B in the different *Xist*-TetOP mutants (for clone 1 of each mutant type) from to 2-to-4 independent experiments. A minimum of 50 *Xist*-coated chromosomes were counted per experiment. Significant differences from unpaired Student's *t*-test, comparing mutants to *Xist* FL are indicated as * p-value < 0.05.

Figure 2 - figure supplement 1 – Quality check of ChIRP procedure in *Xist* FL and *Xist* ΔB+C cells

A. RT-qPCR with three primer pairs along *Xist* to evaluate RNA retrieval after ChIRP procedure for *Xist* FL (in noDOX and DOX conditions) and *Xist* ΔB+C (DOX) at D3.
B. Table showing the percentage of cells exhibiting a *Xist*-coated X chromosome for *Xist* FL (both noDOX and DOX) and *Xist* ΔB+C (DOX) as determined by *Xist* RNA FISH used for ChIRP-MS; A minimum of 500 cells were counted.
C. Blot visualized with Coomassie blue staining showing the band pattern of proteins displayed by *Xist* FL (both noDOX and DOX) and *Xist* ΔB+C (DOX) after ChIRP.

Figure 3 - figure supplement 1 – nChIP-seq confirms residual enrichment of H3K27m3 and H2AK119ub marks at X-linked active genes

A. Normalized signal of H3K27me3 and H2AK119ub around *HoxC* cluster; shown is the signal of each sample around these cluster, normalized by the size of the library.
B. Barplot representing percentages of H3K27me3 and H2AK119ub reads mapping on X chromosome (chrX) in each sample.
C. Violin plots quantifying H3K27me3 and H2AK119ub enrichment over intergenic regions, active promoters and active gene bodies on X chromosome and on autosomes in *Xist* FL and *Xist* ΔB+C cell lines upon DOX induction at day of differentiation; Shown is the calculated log2 fold change of DOX vs noDOX conditions; n = indicates the number of genes analyzed; p-values were calculated using unilateral Wilcoxon test, comparing X chromosome (chrX) and autosomal enrichment of PcG marks for each genomic region.

Figure 3 - figure supplement 2 – Normalization of H3K27me3 and H2AK119ub enrichment over the X chromosome in *Xist* FL and *Xist* ΔB+C based on *Xist* induction levels

A. Table showing the percentage of cells exhibiting a *Xist*-coated X chromosome (chrX) for the different duplicates of *Xist* FL and *Xist* ΔB+C in DOX and noDOX conditions as determined by *Xist* RNA FISH; A minimum of 500 cells were counted to calculate the percentage of cells with a *Xist*-coated chrX.
B. Violin plots quantifying H3K27me3 and H2AK119ub enrichment over intergenic regions, active promoters and gene bodies on chrX in *Xist* FL and *Xist* ΔB+C upon DOX induction at day 2 of differentiation after normalization for the percentage of

cells with *Xist*-coated chromosomes. Shown is the log2 fold change of DOX vs noDOX conditions; n = indicates the number of genes analyzed; p-values were calculated using paired Wilcoxon test, comparing *Xist* FL and *Xist* ΔB+C cell lines.

Figure 4 - figure supplement 1 –Assessment of transcriptional changes by RNA-seq in *Xist* FL, *Xist* ΔA and *Xist* ΔB+C induced cells

A. Genome browsers plots showing RNA-seq reads on *Xist/Tsix* genes for *Xist* FL, *Xist* ΔA and *Xist* ΔB+C mutants in DOX and noDOX conditions at D2 of differentiation; yellow boxes display the deleted regions in both *Xist* ΔA and *Xist* ΔB+C.
B. Barplot representing percentages of RNA-seq reads mapping on X-chromosome (chrX) in each sample.
C. Table showing the percentage of cells exhibiting an *Xist*-coated chrX for the different duplicates of *Xist* FL, *Xist* ΔA and *Xist* ΔB+C in DOX and noDOX conditions as determined by *Xist* RNA FISH; at least 500 cells were counted to estimate the percentage of cells with a *Xist*-coated chrX.
D. Violin plots displaying the average log2(fold-change) in gene expression between DOX and noDOX conditions on chrX and autosomes in *Xist* FL, *Xist* ΔA and *Xist* ΔB+C at D2 after normalization for the percentage of cells with a *Xist*-coated chrX; n = indicates the number of genes analyzed; p-values for chrX were calculated using paired Wilcoxon test; n = indicates the number of genes analyzed.
E. Plots displays the comparison of log2(fold-change) in X-linked gene silencing upon DOX induction between *Xist* FL and *Xist* ΔB+C at D2 of differentiation; Limma *t*-test did not find any gene differentially expressed between *Xist* FL and *Xist* ΔB+C.
F. Boxplots displaying the normalized read enrichment at promoters for H3K27me3 and H2AK119ub upon DOX induction for distinct categories of X-linked genes with different degrees of gene silencing between DOX and noDOX conditions in both *Xist* FL and *Xist* ΔB+C at D2; p-values were calculated using Wilcoxon test; numbers inside the boxplots indicate the number of genes analyzed.
G. Boxplots displaying H3K27me3 and H2AK119ub normalized enrichment levels at promoters upon induction in two categories of X-linked genes: with no or little accumulation versus with accumulation of these PcG marks in induced *Xist* ΔB+C cells; p-values were calculated using Wilcoxon test; n = indicates the number of genes analyzed

H. CpG content in promoters of X-linked genes with no or little accumulation versus with accumulation of PcG marks in induced *Xist* ΔB+C cells; p-values were calculated using Wilcoxon test; Numbers inside the boxplots indicate the number of genes analyzed.

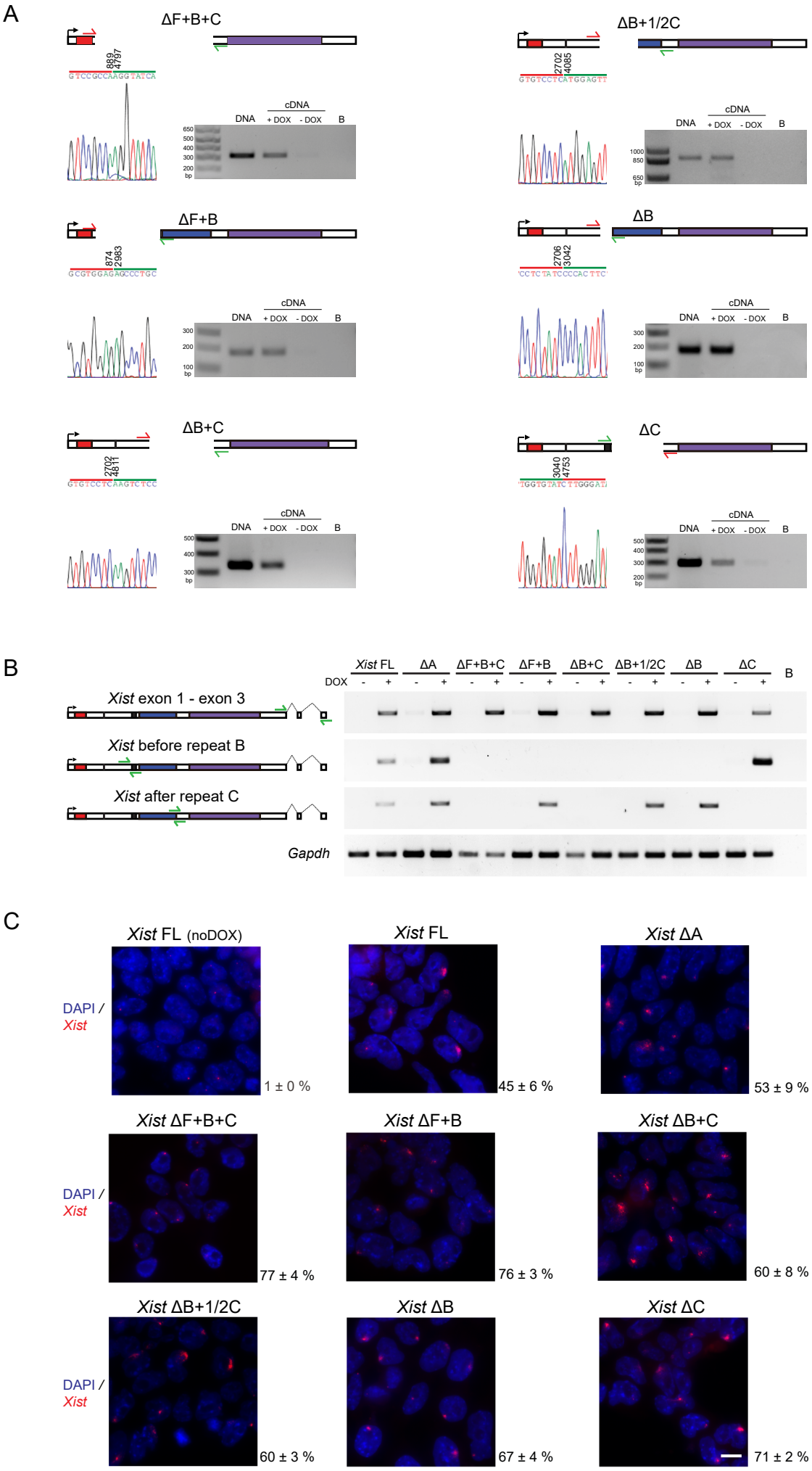


Figure 1 - figure supplement 1

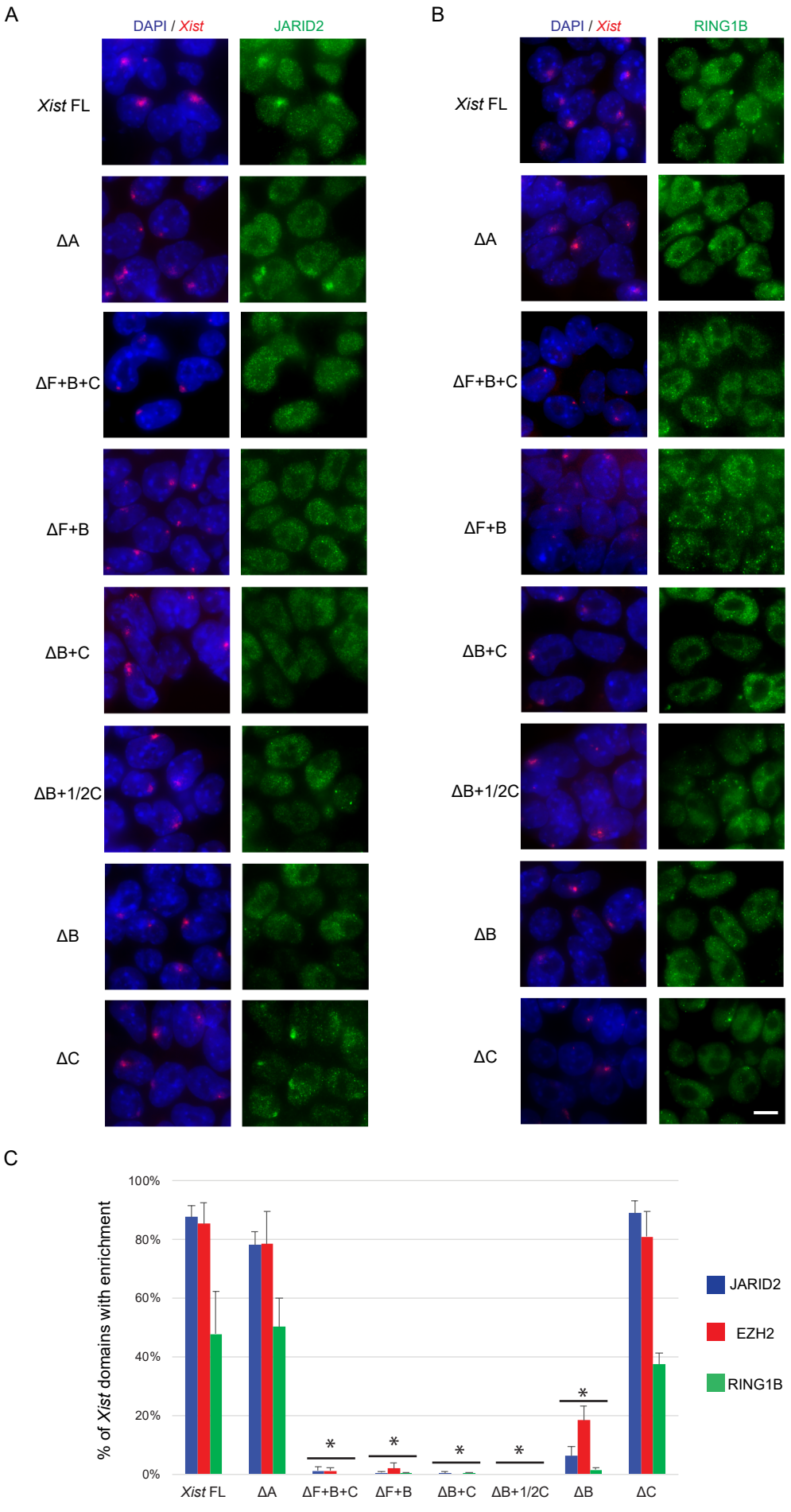
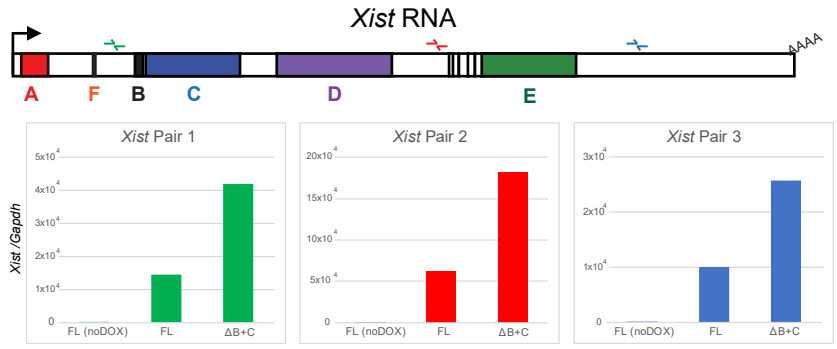


Figure 1 - figure supplement 2

A



B

Samples	% of cells with *Xist* domains
Xist FL noDOX	0%
Xist FL DOX	24.0%
Xist ΔB+C DOX	50.9%

C

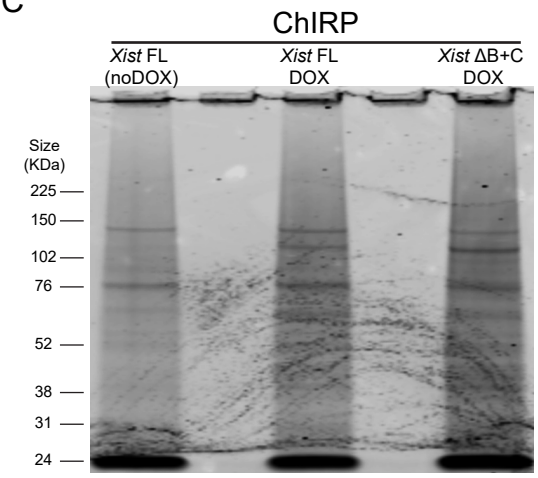


Figure 2 - figure supplement 1

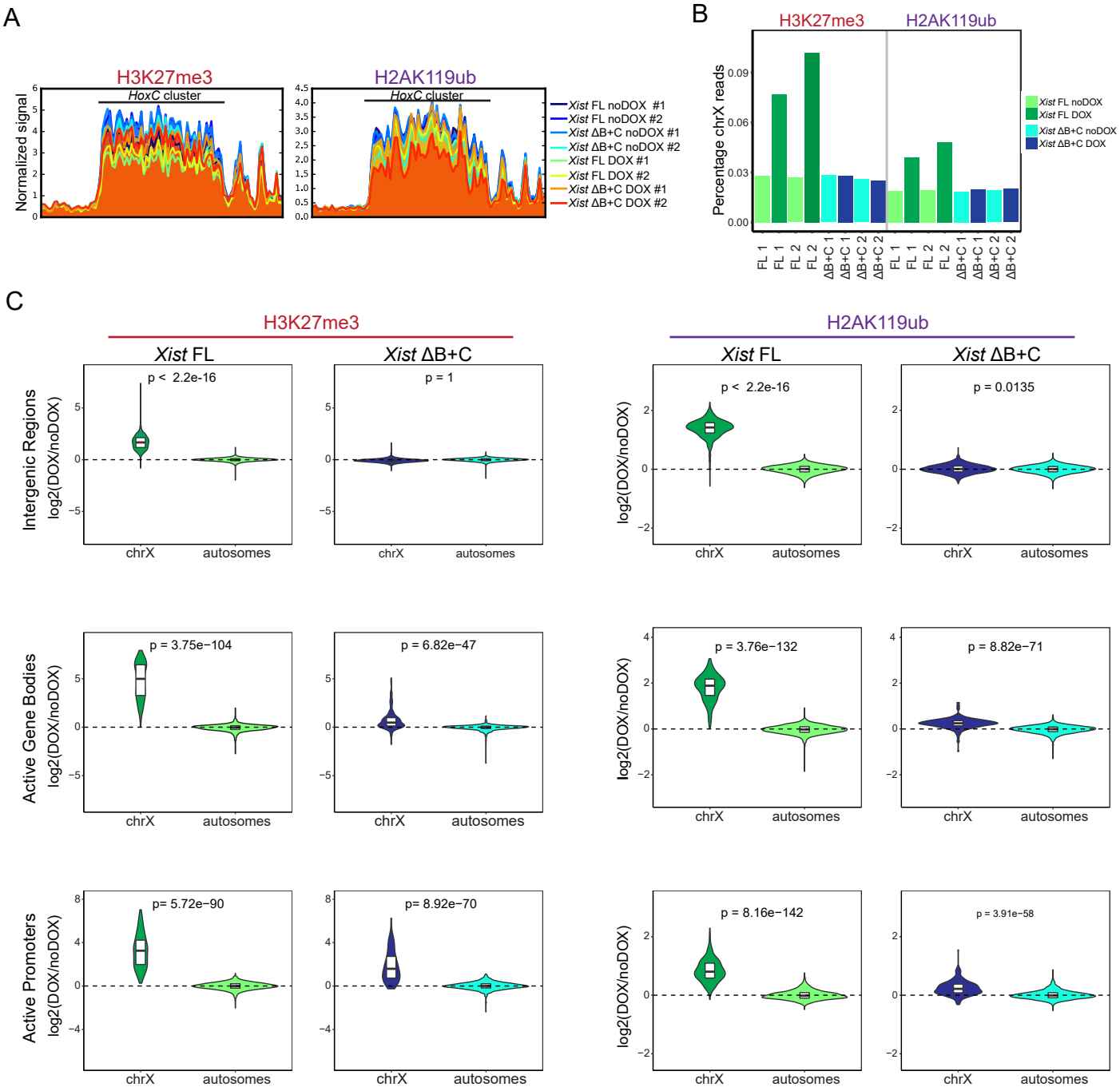


Figure 3 - figure supplement 1

A

Samples	% of cells with *Xist* domains
Xist FL noDOX #1	0%
Xist FL noDOX #2	0%
Xist FL DOX #1	46.6%
Xist FL DOX #2	59.6%
Xist ΔB+C noDOX #1	0%
Xist ΔB+C noDOX #2	0%
Xist ΔB+C DOX #1	66.3%
Xist ΔB+C DOX #2	56.3%

B

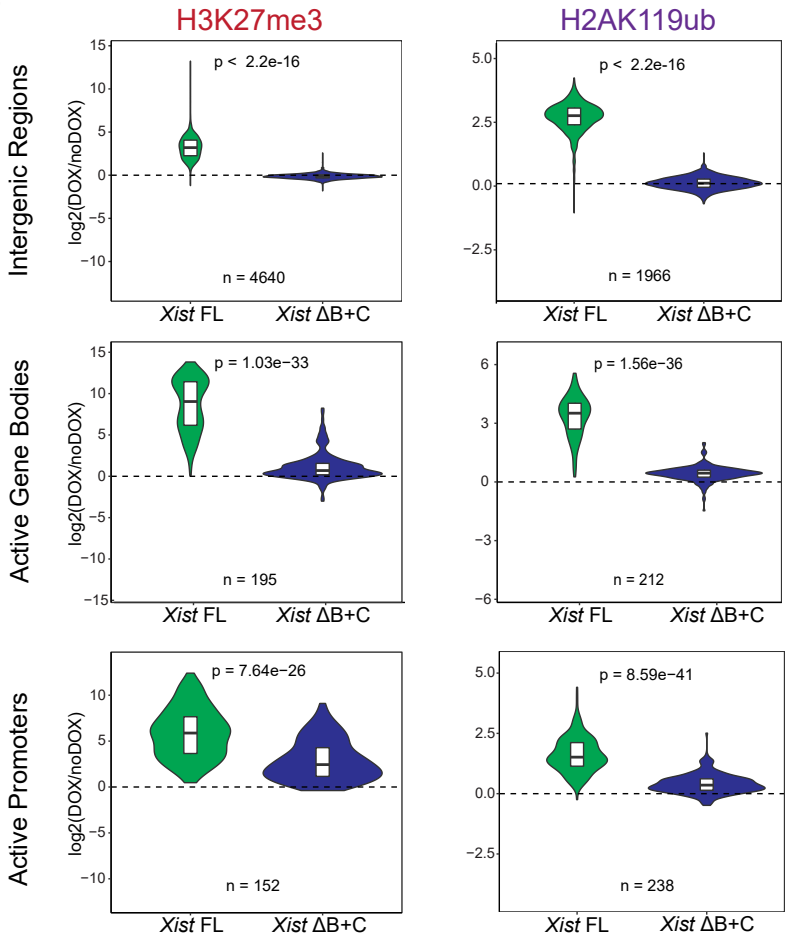
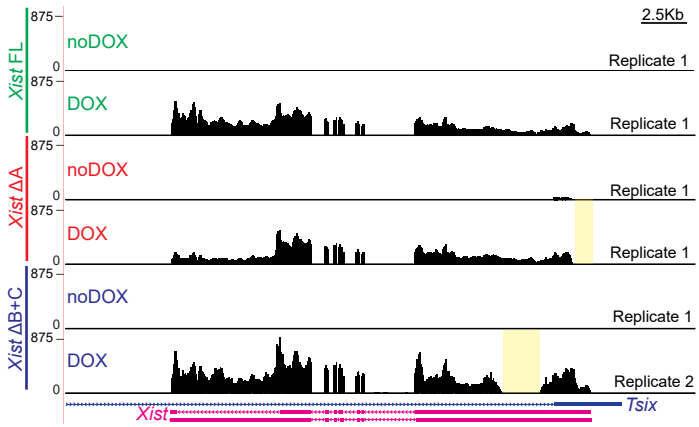


Figure 3 - figure supplement 2

A



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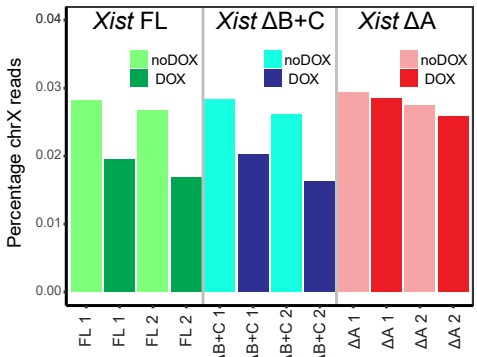
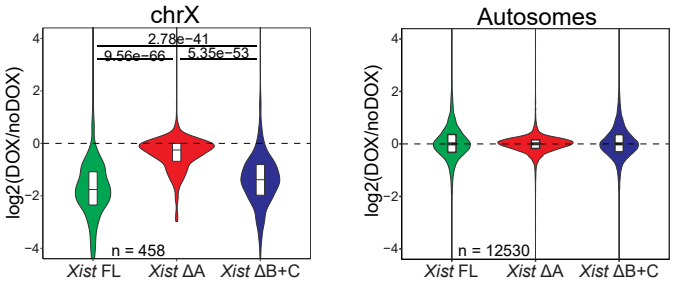


C

Samples	Cells with *Xist*-coated chrX
Xist FL noDOX #1	0.00%
Xist FL noDOX #2	0.00%
Xist FL DOX #1	46.83%
Xist FL DOX #2	49.35%
Xist ΔA noDOX #1	0.00%
Xist ΔA noDOX #2	0.00%
Xist ΔA DOX #1	50.98%
Xist ΔA DOX #2	54.03%
Xist ΔB+C noDOX #1	0.00%
Xist ΔB+C noDOX #2	0.00%
Xist ΔB+C DOX #1	55.38%
Xist ΔB+C DOX #2	56.69%

D



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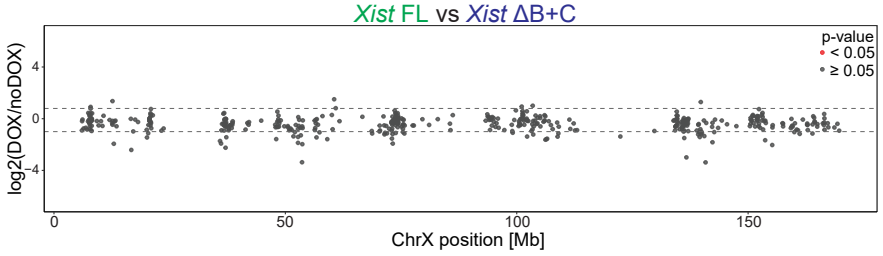

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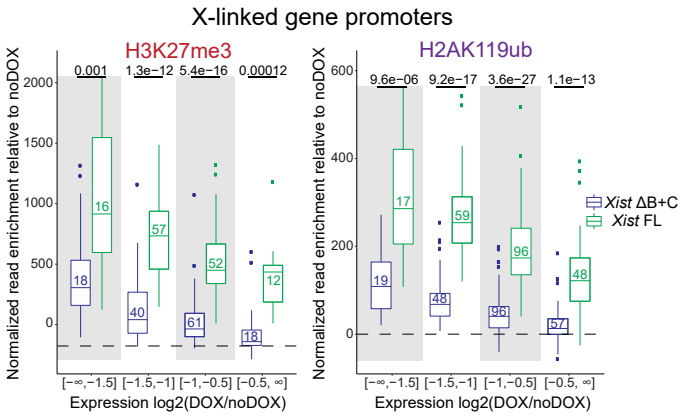

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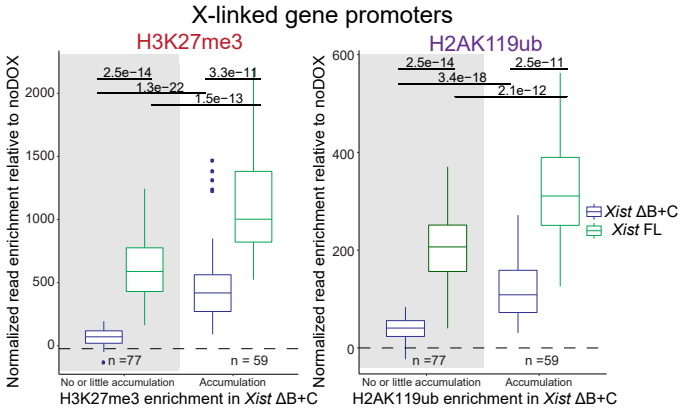

H

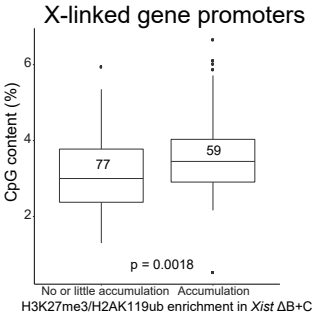

Figure 4 - figure supplement 1