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Supplemental Material to:

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Long non-coding RNAs as targets for cytosine methylation

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Supplementary Information

Supplementary Results and Discussion

Potential distinctive structural features of repeat 8 in XIST

To gain a first indication, whether the methylated cytosines that were detected in the A-region occupy a special position in the context of their structural surroundings, we undertook 3D structure modeling. In this analysis, we focused on XIST because the structural organization of its A-region has been studied in greater detail. For the modeling we used the solution structure of individual A-region repeats by Duszczyk *et al.* [1]. We first analyzed combinations of R8 and the structurally adjacent R5 repeat [1] as well as of the neighboring R6 and R7 repeats (supplementary Fig. S4A, B). The hairpin of R8 was often found to form a contiguous helix with the inter-repeat duplex region (46% of 3D models; Fig. S4A). In comparison, R6 and R7 were less frequently observed in stacking (Fig. S4B, D). Strikingly, when all 4 repeats were modeled together, the R8 hairpin was involved in helical stacking in 80% of the output 3D structures (Fig. S4C, D). In contrast, R6 and R7 hairpins were stacked in only 10% and 27% of the structures (Fig. S4D). The distinctive propensity of R8 to enter in helical stacking interactions may favor its recognition by cellular methylases. It is also possible that methylation of specific Cs in the hairpin stem may modulate the interaction surface between XIST and the PRC2 complex [2-5]. Of note, when the R8 hairpin is stacked on top of the inter-repeat helix, all the discovered methylated cytosines are positioned in close proximity on the external section of the helix (supplementary Fig. S5).

Supplementary References

1. Duszczyk MM, Wutz A, Rybin V, Sattler M. The Xist RNA A-repeat comprises a novel AUCG tetraloop fold and a platform for multimerization. RNA 2011; 17:1973-82.
2. Zhao J, Sun BK, Erwin JA, Song JJ, Lee JT. Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. Science 2008; 322:750-6.
3. Maenner S, Blaud M, Fouillen L, Savoye A, Marchand V, Dubois A, et al. 2-D structure of the A region of Xist RNA and its implication for PRC2 association. PLoS Biol 2010; 8:e1000276.
4. Kanhere A, Viiri K, Araujo CC, Rasaiyaah J, Bouwman RD, Whyte WA, et al. Short RNAs are transcribed from repressed polycomb target genes and interact with polycomb repressive complex-2. Mol Cell 2010; 38:675-88.
5. Kaneko S, Li G, Son J, Xu CF, Margueron R, Neubert TA, et al. Phosphorylation of the PRC2 component Ezh2 is cell cycle-regulated and up-regulates its binding to ncRNA. Genes Dev 2010; 24:2615-20.

Supplementary Figures

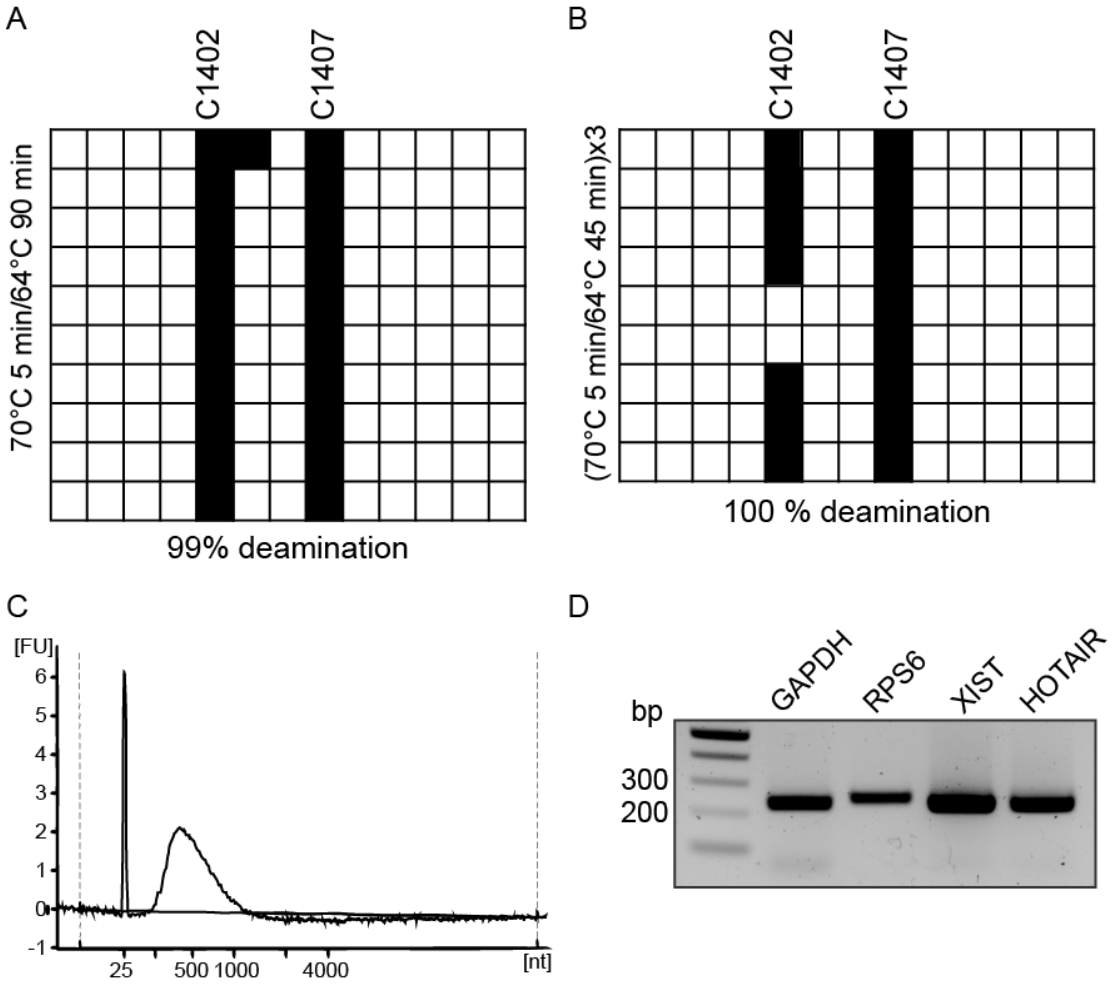


Fig. S1 Modification of BS sequencing protocol for the use with poly(A)RNA. (A), (B) BS sequencing results of *E. coli* 16S rRNA clones. RNA was subjected to different conditions of BS treatment as indicated at the left in each panel. (C) Absorbance profile of BS treated poly(A)RNA obtained with an Agilent 2100 Bioanalyzer. FU, fluorescence units; nt, nucleotides. (D) PCR amplification of the indicated transcripts using cDNA derived from BS treated poly(A)RNA.

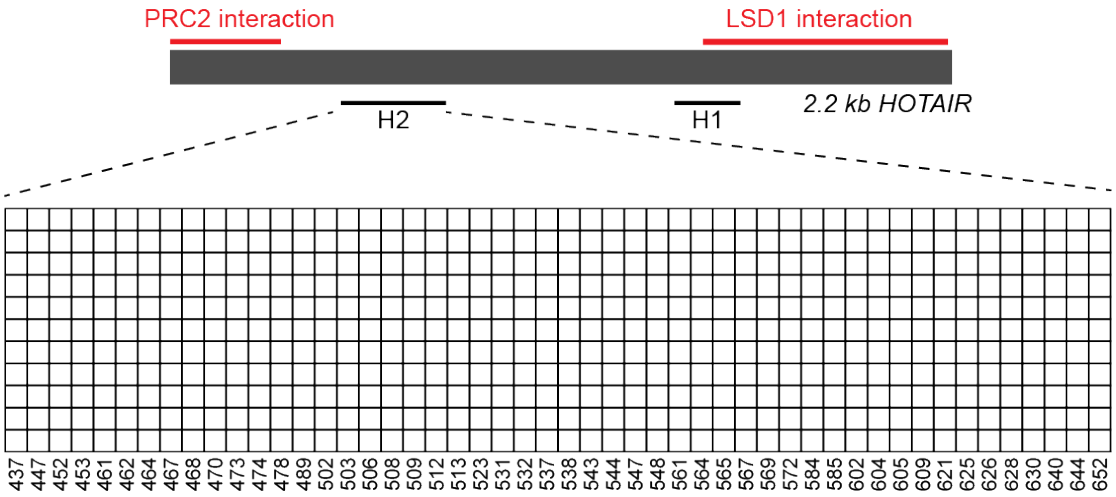


Fig. S2 BS sequencing results for the internal region H2 of HOTAIR RNA. The location of the analyzed region is indicated at the scematic representation at the top. human XIST. White squares, deaminated C.

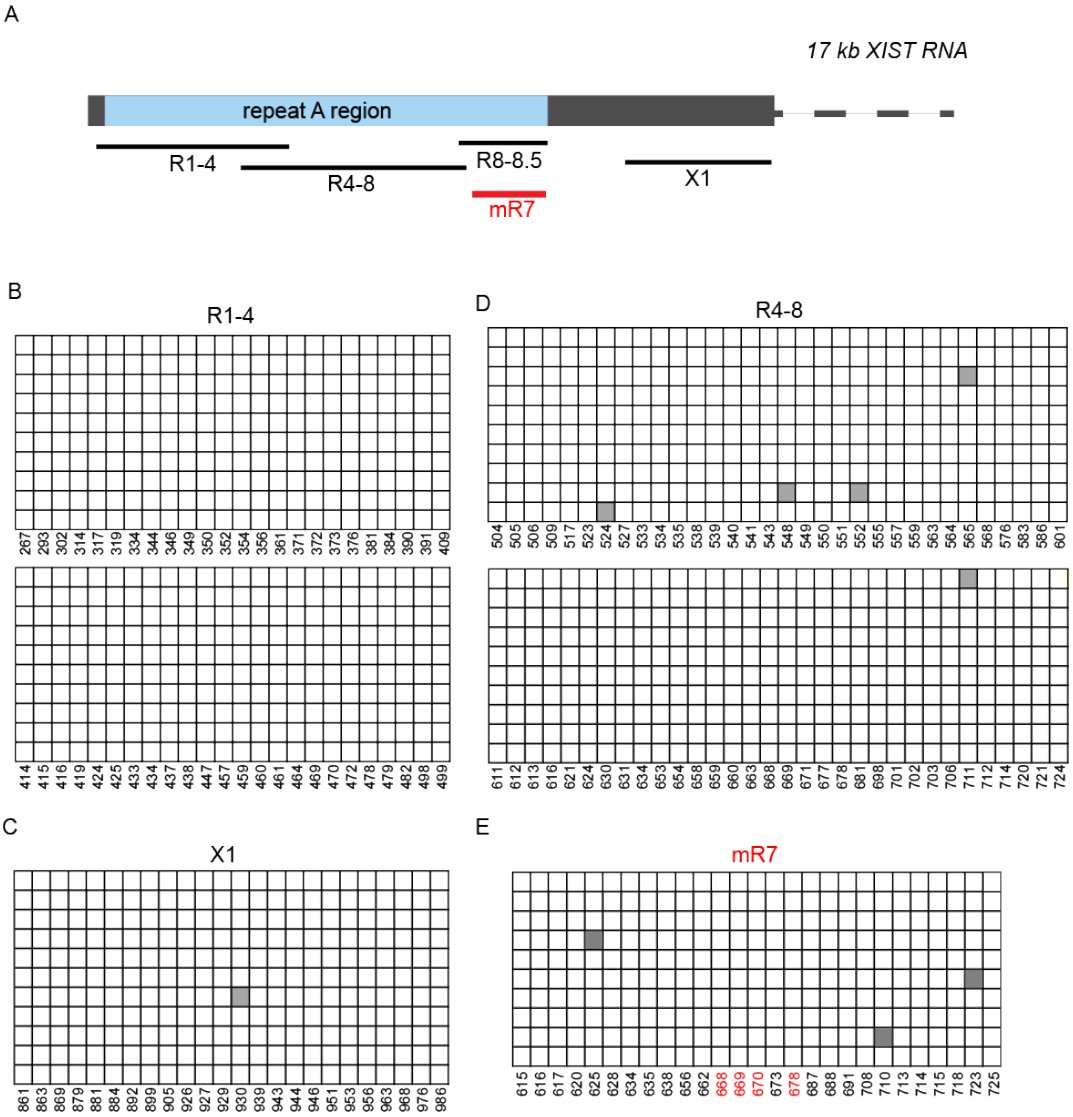
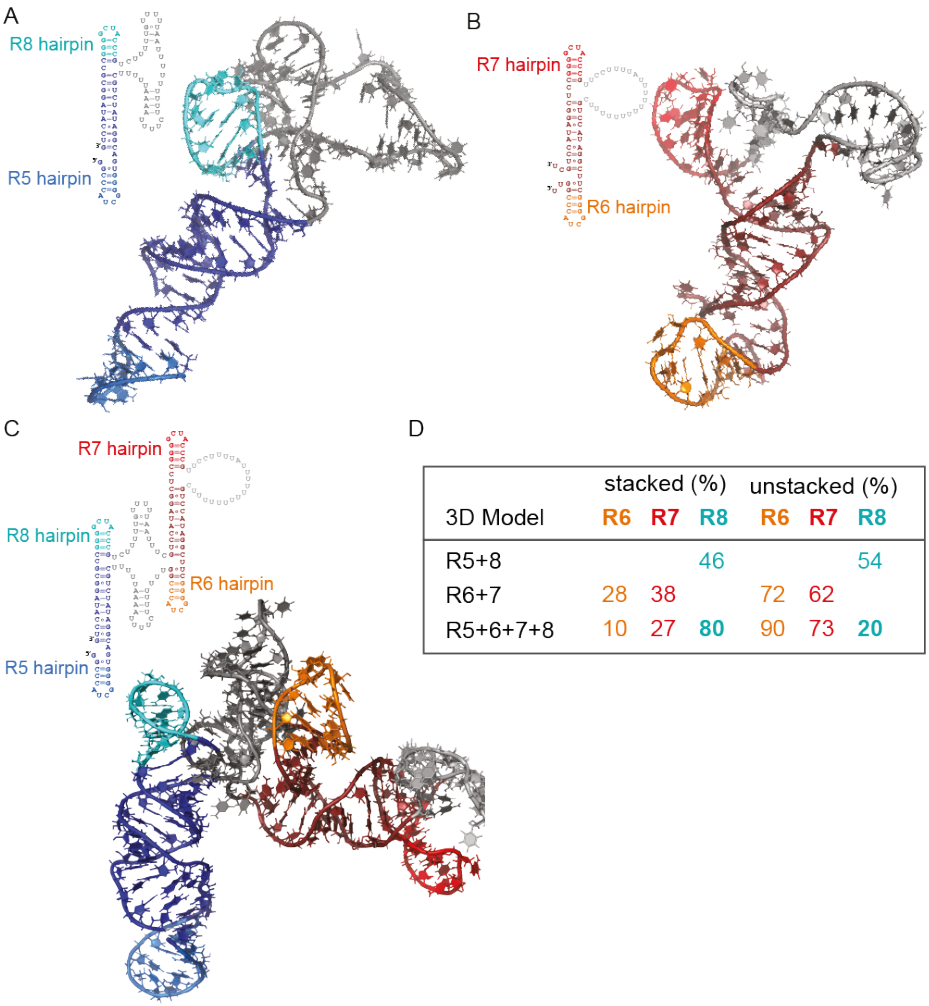


Fig. S3 Analysis of cytosine methylation within the A-region of XIST. (A) Schematic representation of the 5' region of human XIST RNA. The A-region is shown in blue. Black lines, regions analyzed by BS sequencing. The red line indicates the region covering repeat 7 of mouse XIST, which corresponds to the human R8 region (B-D) BS sequencing results for regions R1-4 (B), X1 (C) and R4-8 (D) of human XIST. (E) BS sequencing results for region mR7 of mouse XIST. White squares, deaminated C; grey squares, non-deaminated C considered artifacts.



	stacked (%)			unstacked (%)		
3D Model	R6	R7	R8	R6	R7	R8
R5+8			46			54
R6+7	28	38		72	62	
R5+6+7+8	10	27	**80**	90	73	**20**

Fig. S4 3D modeling predicts distinct structural features of XIST A-region repeat 8. 2D-structures for models of R5+8 (A), and R6+7 (B) as well as R5+6+7+8 (C) are shown as small insets. (A, B) Examples of MC-Sym-generated 3D structures for R5+8 (A) and R6+7 (B) duplexes, in which the repeats are in an unstacked conformation. (C) Example for a 3D structure of R5+6+7+8 in which R8 is in a stacked conformation while repeats 6+7 are unstacked. (D) Table shows the frequencies (in % of all computed 3D models) with which the indicated repeats are predicted to adopt a stacked or unstacked conformation . Data summarize results from the modeling of R5+8, R6+7 and of R5+6+7+8.

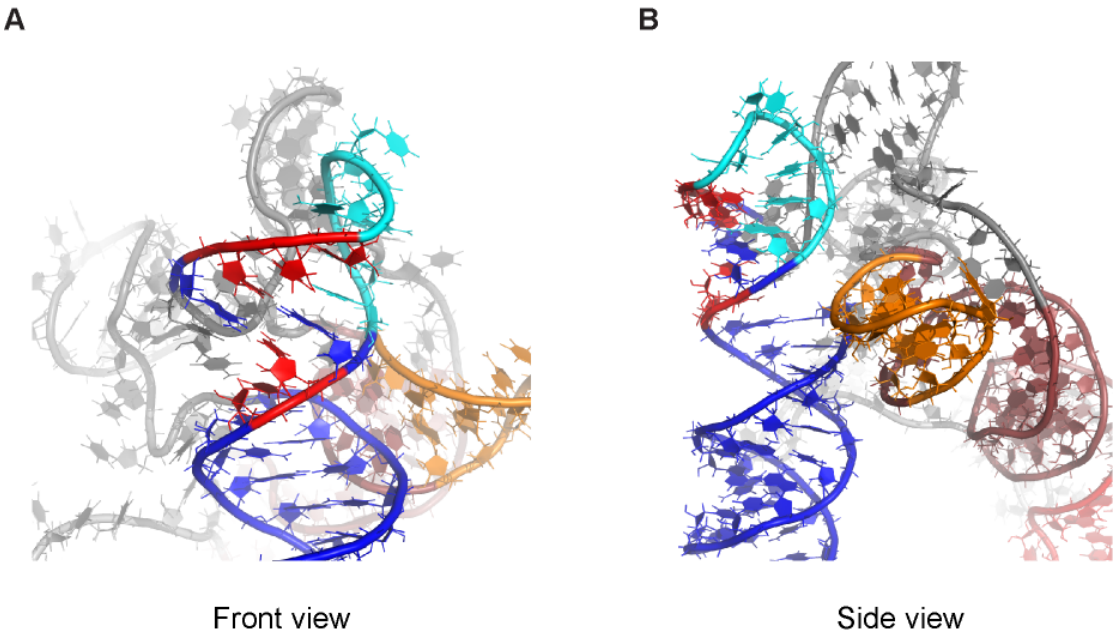


Fig. S5 3D Model of R8 in stacked helical conformation with adjacent inter-repeat duplex region. (A) Front view. (B) Side view. The methylatable cytosines of R8 (red) face the surface of the folded RNA. R8 nucleotides are shown in cyan and inter-repeat nucleotides are shown in blue.

Supplementary Table S1: Sequences of utilized primers

Primer	Sequence (5' - 3')
XIST *PCR primers*	
XIST 1 forw.	GAA AGT ATT TGG AAT ATT TTG AGG
XIST 1 rev.	CAA ATA TCC AAT ACC CCA ATA AAC
XIST 2 forw.	TAT TTG TTT TTT ATT TTT TTT TTT TTA G
XIST 2 rev.	ACC AAT AAA CAA TAA AAA AAA AAA ATC
XIST 2 forw_n	TAT TGG GGT TGT GGA TAT TTG
XIST 2 rev_n	ATC CAT AAA AAA CAC CAA TAA AC
XIST 3 forw.	GTT TAT TGG GGT TTT GGA TAT TTG
XIST 3 rev.	CTT AAC TAC AAA ATC ATT CTC TAC C
XIST downstream forw.	TAT TTG GTG GTG TGT GAG TGT ATT TAT
XIST downstream rev.	ACC TAA CCT ACT ATC ATC CTA CTT ACC T
XIST mouse forw.	TGT TTA ATG GGG TTT TGG ATA TTT G
XIST mouse rev.	CCA CAC CCT AAA CAC CCA CTC
XIST *in vitro transcription primers (underlined T7 promoter sequence)*	
XIST_forw_T7	GAA ATT AAT ACG ACT CAC TAT AGG GTC TTC TTG ACA CGT CCT CCA
XIST_rev.	GAG AGT GCA ACA ACC CAC AA
HOTAIR *PCR primers*	
HOXdeamin646Forw1	TTT TTG GAA GTT TTT GAA GGT TT
HOXdeamin646Rev1	CCA TTT ACA ACA AAA TCC CAC T
Hotair_m_forw.	AAG AGT TTG ATG TTT ATA AGA
Hotair_m_rev.	CTT ACT CCC TTA CCT ACA TT
HOTAIR *qRT-PCR primers*	
Forward	TGT AAT TGC TGG TTT AGG TTG CA
Reverse	CTG GCA GAG AAA AGG CTG AAA T
HOTAIR TaqMan probe	FAM-TTC TCT CGC CAA TGT GCA TAC TTA TAA G-BHQ1
HOTAIR *Barcode*	
cDNA RT-Primer with **Barcode**	TGG CAC AGT CCA CCA AAT TA**N NNN** CCA TTT ACA ACA AAA TCC CAC T
PCR Primer forw.	TTT TTG GAA GTT TTT GAA GGT TT
PCR Primer rev.	ACC GTG TCA GGT GGT TTA AT