Comment on “Xist recruits the X chromosome to the nuclear lamina to enable chromosome-wide silencing”

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Chen et al. (Reports, 28 October 2016, p. 468) proposed that an interaction between Xist RNA and Lamin B receptor (LBR) is necessary and sufficient for Xist spreading during X-chromosome inactivation. We reanalyzed their data and found that reported genotypes of mutants are not supported by the sequencing data. These inconsistencies preclude assessment of the role of LBR in Xist spreading.

Mammalian X chromosome inactivation (XCI) is mediated by the noncoding Xist RNA, a transcript that spreads across the inactive X chromosome (Xi) to establish global silencing. The Xi resides preferentially at the nuclear periphery (1), with Xist interacting with components of the nuclear lamina, including Lamin B receptor (LBR) (2, 3). In a recent work, Chen et al. reported that spreading of Xist requires that interaction with LBR (4). By performing LBR Cross-linking immunoprecipitation sequencing (CLIP-seq), Chen et al. deduced three putative LBR-binding sites on Xist RNA. Upon deleting one site (ΔLBS), they reported that Xist-ΔLBS could not spread to active genes and that XCI was impaired. These defects were intriguingly rescued by synthetically tethering Xist–LBS rescue cells, with coverage densities being similar to those of flanking sequences (Fig. 1A). This observation indicated that the LBS region was not deleted. To exclude an inadvertent insertion into a different chromosome during clustered regularly interspaced short palindromic repeats (CRISPR) targeting, we looked for interchromosomal paired-end reads showing one end in LBS and the other in an ectopic location. As expected, the vast majority of read pairs aligned to ChrX within Xist. Interchromosomal pairs were also detected, but they were rare and no more likely to occur in LBS or LBS rescue relative to wild-type (WT) cells (Fig. 1B). We conclude that the ~800-bp (base pair) LBS region was not deleted in LBS.

Our analysis revealed another type of discordant read pair—one in which the two ends aligned to the same strand, rather than to opposite strands as would be expected in paired-end sequencing. In such discordant pairs, one read fell within the ΔLBS region and the other just outside (Fig. 1C, top and middle). This arrangement suggested creation of an inversion instead of a deletion. Indeed, read pairs crossing an inversion breakpoint would appear as two ends aligning to the same strand. This inversion signature occurred in both ΔLBS and LBS rescue cells. A small number of read pairs showed two ends that flanked the intended deletion (Fig. 1C, bottom), possibly signifying long inserts crossing the ~800-bp region or the existence of a small subpopulation with a deletion or more complex rearrangement. These data indicate that the authors created an inversion, not a deletion.

If so, the authors’ LBR CLIP-seq data should reveal RNA reads containing inverted sequences. However, there was a complete absence of CLIP tags in the ΔLBS region (Fig. 1D, red box). This finding seemingly supports creation of ΔLBS, but closer examination also revealed an absence of reads that cross over the intended deletion break-point (Fig. 1D). Such “crossover” reads should be present, as a deletion should directly juxtapose flanking regions. Failure to detect crossover reads was not due to sequencing depth, as there was high coverage over Xist. Nor was failure attributable to a bioinformatics pipeline issue, given our easy detection of crossover reads spanning comparable-length intron-exon junctions of spliced transcripts (e.g., Fig. 1E). Thus, our reanalysis of the CLIP data supported neither an inversion nor a deletion, revealing a major discrepancy between the RAP and CLIP data.

This discrepancy has implications for how LBR’s effect on Xist spreading can be deduced from the RAP data. RAP is a method of mapping an RNA’s (e.g., Xist’s) genomic binding pattern. Xist RNA coverage would depend on (i) how much Xist binds to the Xi, (ii) how well the RAP experiment worked, and (iii) the sequencing depth. Because deeper sequencing yields more reads per kilobase (which would simulate greater RNA coverage), RAP experiments require proper normalization. In the analysis of Chen et al., WT and ΔLBS rescue samples were sequenced most deeply (~16 and 20 million reads, respectively), with higher-quality reads that survived filtering of duplicates and unmappable reads (36 and 43%) than in other samples (Fig. 2A). Without appropriate normalization, the similarity to WT may explain why the authors concluded that ΔLBS was “rescued” by tethering Xist to the nuclear lamina (4). By contrast, the mutants, ΔLBS, ΔA, LBR, and SHARP knockdowns (KD) had only 9 to 12 million reads, with only 11 to 17% usable reads after filtering. The low sequencing depth and poorer-quality reads could have misled the authors to conclude that ΔLBS, ΔA, and LBR KD failed to spread Xist.

For proper normalization, mutant samples should have been scaled up by 4- to 6-fold relative to WT and ΔLBS rescue (Fig. 2A). Once normalized, all RAP profiles looked similar to each other (Fig. 2B). The only exception was the WT sample, which showed the dominant RNA peak at the Xist locus and a characteristic wave pattern across the rest of the X (6, 7). In contrast, all mutants and knockdown patterns showed only a spike at Xist (Fig. 2B). This limited pattern typically stems from hybridization of capture probes to either the Xist DNA or nascent Xist transcripts. At a higher magnification, SHARP KD and ΔLBS rescue appeared to have greater Xist coverage, but this coverage fell far short of WT patterns (Fig. 2C). There were no biological replicates to exclude differences caused by experimental variability. We generated WT RAP profiles similar to those published by Engreitz et al. using the same cell line (6) (Fig. 2, B and C), arguing that the discrepancies did not stem from differences in our bioinformatics pipeline. Our reanalysis therefore suggests that the RAP experiments worked suboptimally, precluding any possible conclusion regarding the role of the LBS domain in Xist spreading.

Chen et al. also suggested that the spreading defect of ΔLBS phenocopies that of deleting Xist’s A-repeat (a domain critical for Xi silencing) (4, 6, 8). However, our reanalysis revealed that

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Fig. 1. ΔLBS harbors an inversion rather than a deletion of LBR-binding site 1.

(A) RAP coverage over the 5′ end of Xist in WT, ΔLBS, and ΔLBS rescue cell lines. The region between the guide RNAs (gRNAs) used to generate the ΔLBS deletion and the deletion of the Xist promoter in pSM33 are highlighted.

(B) Chromosome location for mates of discordant reads—discordant read pairs where one end maps to Xist but the other maps to a different chromosome or elsewhere on the X. We performed paired-end RAP alignments to the mm9 genome using Bowtie2 with default parameters; filtered out multiply mapped reads (sed ‘/XS:/d’), low-quality reads, and unmapped reads (samtools view -b -f 0x4 -q 1); removed polymerase chain reaction (PCR) duplicates (samtools rmdup); and visualized reads using Integrative Genomic Viewer (IGV) 2.3.81. We obtained similar results using BWA for alignment. We identified all reads flagged as “improperly paired” by BWA. Shown are these discordant paired reads where one end aligns to Xist and the other aligns elsewhere. Such events are counted by chromosome (chr). (samtools view -h -F14 * .bam chrX:100653712-100680572 |grep chrN |wc –l, where chrN is any chromosome).

(C) Read pairs at the intended deletion region (ΔLBS). A single read is depicted by a box and is linked to its paired-end sequencing mate by a line. Orientation of the reads is as indicated.

(D) Absence of crossover reads in the ΔLBS region in ΔLBS CLIP-seq data. Coverage tracks shown at top, followed by read pairs. Scale shown in brackets. We obtained the CLIP-seq data from GEO and aligned them to the mm9 genome using Bowtie2 with the parameters -k1–very-sensitive-local–maxins 2000. Reads were removed if they had mapping qualities less than 30. BAM files were loaded onto IGV to visualize regions of interest.

(E) By contrast, crossover reads are easily detected around splice junctions of other transcripts—e.g., Actb. Paired-end reads (boxes) are linked by lines. Coverage tracks are shown at top, followed by read-pairs. Scale shown in brackets.
the authors’ ‘ΔΔ’ line did not carry an appropriate A-repeat deletion, because reads could be detected from the ΔΔ region in the RAP data (Fig. 2D, blue-shaded region). The ΔA line was described as one previously used by Engreitz et al., one in which a mutant ΔA-Xist cDNA was knocked into the X-linked Hprt locus (‘Hprt-ΔΔ’) (6, 8). In such RNA-mapping experiments, dominant peaks usually occur at the site of transcription (6, 7). Indeed, in the ΔA RAP of Engreitz et al., the most prominent peak occurred at the knock-in Hprt locus, not at the silent endogenous Xist locus (Fig. 2, B and C). In contrast, an Hprt peak was missing in the ΔA RAP of Chen et al., where a peak was only seen at the endogenous

**Fig. 2. Reanalysis of the Xist RAP and CLIP data in ΔLBS and ΔA cells.** (A) WT and mutant RAP statistics. (B) Xist enrichment profiles across the X chromosome. We obtained the RAP data of Engreitz et al. from GEO (GSE46918) and applied the same pipeline, with a quality filter to remove reads with multiple alignments, unmapped reads, PCR duplicates, discordant reads, and singletons. Xist enrichment profiles were generated by dividing RAP with input (scaled by read count, 1-kb bin size, and pseudocount 0.1). Enrichment profiles were visualized with IGV using a mean windowing function and 0 to 300 scale. (C) Magnified view of (B) at 0 to 100 scale. (D) Molecular signatures of a true Hprt-ΔΔ are missing in the ΔΔ RAP of Chen et al. FPM (fragments per million)–normalized input and RAP coverage tracks of indicated cell lines at the Xist locus. Read pairs passing the quality filter were used to generate the FPM-normalized coverage profile. Green bar, signature deletion at the Xist promoter of pSM33; blue bar, location of the A-repeat. In Hprt-WT and Hprt-ΔΔ RAP, chromatin of Xist cDNA (Hprt locus) was pulled down by nascent Xist transcripts at Hprt, but the reads aligned to the endogenous Xist locus (note enrichment of exonic, not intronic, reads). (E) Signature Hprt promoter deletion in Hprt-ΔΔ (brown bar) is absent in ΔΔ used by Chen et al. Shown are FPM-normalized coverage tracts at the Hprt locus. (F and G) Strand-specific CLIP analysis at the Xist (F) or Hprt (G) locus suggests that Chen et al. used a cell line carrying a ΔA transgene inserted elsewhere in the genome, not at Hprt or Xist, which remain intact. Tsix RNA, + strand; Xist RNA, – strand. Green box, pSM33 deletion signature; blue box, A-repeat; brown box, Hprt deletion in the proper Hprt-ΔΔ knock-in. Hprt is intact and expressed (+ strand) in the ΔΔ cell line used for CLIP in Chen et al. Absence of RNA reads in the A-repeat region of Xist but not Tsix implies expression of a ΔΔ transgene elsewhere in the genome.
Xist locus. These data suggest that the “ΔA line” of Chen et al. is of different provenance—likely one related to the parental cell line (pSM33) used to create the presumptive ΔLBS line. In pSM33, an inducible promoter replaced 1 kb of the endogenous Xist promoter, and this deletion signature was present in all mutants of Chen et al., including the presumptive ΔA line (Fig. 2D, bottom tracks, green-shaded region). On the other hand, the Hprt deletion created by the Xist cDNA knock-in was missing in the ΔA cells of Chen et al., while being present in those of Engreitz et al. (Fig. 2E).

Because RAP was not performed in a true ΔAc cell line, the role of the A-repeat in Xist spreading cannot be determined from the data. To deduce the genotype of the presumptive ΔA line, we examined the authors’ CLIP data and found that the ΔA used for RAP and CLIP were not the same. The pSM33 deletion seen in the ΔA RAP was missing in the ΔA CLIP (Fig. 2F). Furthermore, the ΔA CLIP lacked reads over the A-repeat of the expressed Xist RNA (Fig. 2F), whereas these reads were present in the ΔA RAP (Fig. 2D). The endogenous Xist locus in this cell line is intact, given clear Tsix reads on the antisense strand (Fig. 2F). Hprt is also intact (Fig. 2G). Thus, the presumptive ΔA cells used for CLIP likely carried a ΔA Xist transgene inserted elsewhere in the genome. The location of the ΔA Xist transgene is relevant, because Xist RNA is cis-acting and, if produced from an autosomal site in embryonic stem cells, would not bind the X-chromosome. These findings further caution against interpreting data from the ΔA data sets.

The evidence herein suggests that the conclusions of Chen et al. should be reconsidered in light of major technical issues. Most problematic is the high likelihood that the intended deletion cell lines (ΔLBS, ΔLBS rescue, and ΔA)—on which the entire study was to be based—were not properly created. Finally, if the deletions could be reconstructed, the authors’ model must recognize the fact that the intended ΔLBS deletion overlaps Xist’s nucleation site and consider the possibility that any spreading defect could be secondary to a failure of nucleation.
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