

## TECHNICAL RESPONSE

## GENE EXPRESSION

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

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Wang *et al.* question whether Lamin B receptor is required for Xist-mediated silencing because they claim that our cells contain an inversion rather than a deletion. We present evidence that these cells contain a proper deletion and that the confusion is caused by DNA probes used in the experiment. Accordingly, the points raised have no effect on the conclusions in our paper.

We recently reported that Xist interacts with the Lamin B receptor (LBR) protein, leading to recruitment of the X chromosome to the nuclear lamina and transcriptional silencing (1). One of the many experiments we performed was to remove the sequence in Xist that corresponds to the LBR binding site ( $\Delta$ LBS-Xist) using clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 editing technologies. Wang *et al.* claim that our conclusions are not valid because they argue that the  $\Delta$ LBS-Xist cell line is not a deletion of the LBS region but rather an inversion (2). Their claim is incorrect.

There are several lines of evidence that the  $\Delta$ LBS-Xist line does contain a deletion of the LBR binding site in the Xist locus. (i) We selected single-cell colonies that contained a single polymerase chain reaction (PCR) product whose size corresponded to the expected size of the deletion (Fig. 1A). (ii) Sanger sequencing of this product showed that the LBS region is absent and the flanking regions in the adjacent genomic DNA are joined together (Fig. 1B). (iii) In the  $\Delta$ LBS-Xist RNA antisense purification (RAP)–DNA sequence data (3), we observe >550 proper read pairs that align to the flanking regions of the LBS deletion boundaries and skip the entire deleted region (Fig. 1, B and C). The genomic insert size of these spanning read pairs exceeds 99.9% of all read pairs in the genome and, accordingly, we would not expect to observe them if the region were indeed wild-type or contained an inversion (Fig. 1D). As expected, we do not observe even a single read pair that spans these breakpoints in the wild-type samples (Fig. 1C).

These data are also incompatible with an inversion because, in the case of an inversion, (i)

we should not observe a size shift in the amplified PCR fragment, (ii) we should observe the inverted sequence in the Sanger sequences, and (iii) we should not observe any reads spanning the deletion junction.

We understand why Wang *et al.* mistakenly concluded that the LBS region had not been deleted because the  $\Delta$ LBS cell line contains DNA sequencing reads originating from within the deleted region. However, these reads do not arise from the endogenous Xist locus because virtually none of the proper read pairs connect the LBS region and the remainder of the Xist locus (Fig. 1C). These results indicate that, although the LBS region was deleted from the Xist locus, it was likely integrated into a different location of the genome—an observation that we noted in our paper (4).

Despite the LBS integration into the genome, there are several lines of evidence that the LBS region is not expressed either as part of Xist or from anywhere else in either orientation. (i) In the  $\Delta$ LBS-Xist lines, we observe an ~97% reduction of quantitative reverse transcription PCR (RT-PCR) signal using primers that amplify the LBS region (transcribed in either orientation) relative to primers that amplify a distinct region of Xist (Fig. 1E). (ii) RNA sequencing performed in these cell lines shows no sequence reads overlapping the deleted region in either orientation. [Wang *et al.* incorrectly claim that there are no read pairs spanning this deletion, but there are actually four read pairs spanning this deletion (5), which is the median number of read pairs spanning any region of the Xist RNA in this sample. This discrepancy arises because, as they subsequently confirmed, they used the incorrect coordinates in their analysis (6)]. (iii) We observed no signal by single-molecule RNA fluorescence in situ hybridization (FISH) using probes against either orientation of the LBS region in any of the 80  $\Delta$ LBS cells analyzed, despite robust detec-

tion of other regions of Xist. These results clearly indicate that these cells express an Xist RNA with a deletion, not an inversion, of the LBS region.

Given all of the evidence that the  $\Delta$ LBS cell line contains a deletion, we reevaluated Wang *et al.*'s claim that these cells have an inversion. Their claim derives entirely from the observation that, although there are virtually no proper read pairs that connect the deleted fragment to the remainder of Xist, there are a large number of discordant read pairs that do so. Unlike proper read pairs where the two reads map to each of the two strands of genomic DNA, discordant read pairs occur when both reads map to the same strand.

In evaluating these discordant read pairs, we identified several problems with the interpretation that these represent an inversion. Specifically, if these discordant read pairs actually represent an inversion in the  $\Delta$ LBS cell line, then they should (i) only be present in the  $\Delta$ LBS cell line and not the unmodified wild-type cells and (ii) only be present flanking the LBS region but not other regions of Xist. Neither of these statements is correct. The discordant read pairs spanning the LBS region are also present in wild-type cells at a comparable proportion, and there are discordant read pairs present across all regions of Xist (Fig. 2, A and B). In fact, >75% of regions on Xist contain more discordant read pairs than overlap the LBS region in the  $\Delta$ LBS-Xist samples (Fig. 2, A and B).

In evaluating these discordant read pairs more carefully, it became clear that these reads do not represent genomic DNA because, unlike proper read pairs, they (i) align predominantly within exonic regions, (ii) pile up at specific regions on Xist in a highly nonuniform manner (Fig. 2C), and (iii) show an insert-size distribution significantly longer than the extreme tail of the genomic distribution (Fig. 2D).

Upon further examination, we noticed an almost perfect relationship between the location of the nonuniform pileups of the discordant read pairs and the location of the single-stranded DNA probes used to capture the Xist RNA (7) (Fig. 2C). In contrast, regions that did not overlap probe sequences did not contain discordant read pairs (Fig. 2C). This suggests that the discordant read pairs represent DNA probes. To confirm this, we looked at previously published RAP-DNA data sets for several distinct RNAs (8) and identified a large number of discordant read pairs overlapping only these targeted RNAs but not other regions in the genome (Fig. 2E). Consistent with this, we do not see discordant read pairs in RAP-DNA experiments performed with RNA probes (Fig. 2E).

Notwithstanding the clear evidence that  $\Delta$ LBS-Xist contains a deletion and the lack of evidence for an inversion, even if these lines contained an inversion, it would not alter our conclusions. There are many previous studies showing that inverting the sequence of a protein-binding site on an RNA, including on Xist (9, 10), affects protein binding and RNA function (9–14). Indeed,

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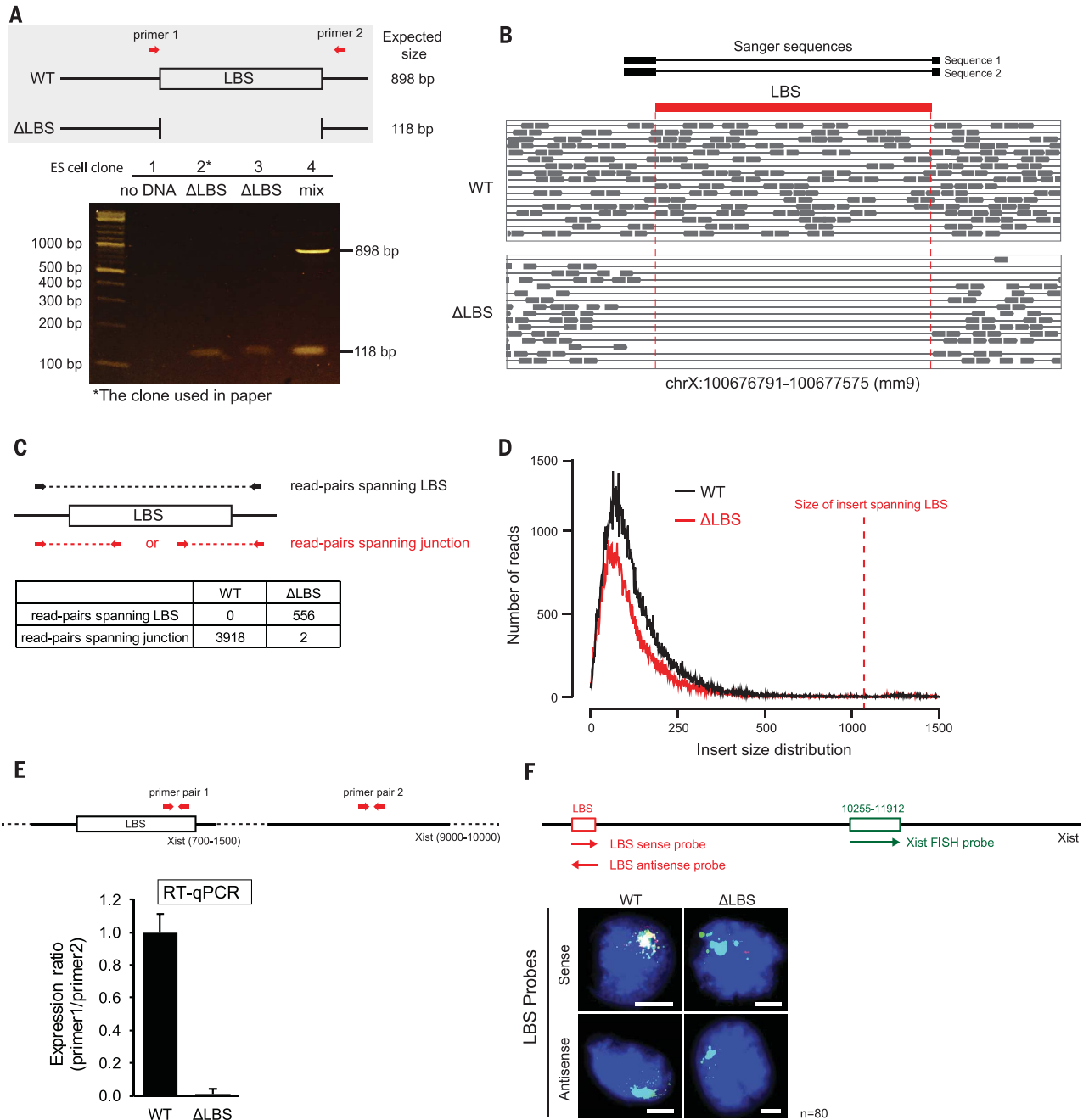
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in our paper, we presented several lines of evidence that Xist expressed in these cells fails to bind to LBR and therefore represents an appropriate cell line in which to study the function of LBR binding on Xist. In addition to the disruption

of LBR binding, the conclusions derived in our paper are supported by LBR knockdown, knockout, and rescue experiments.

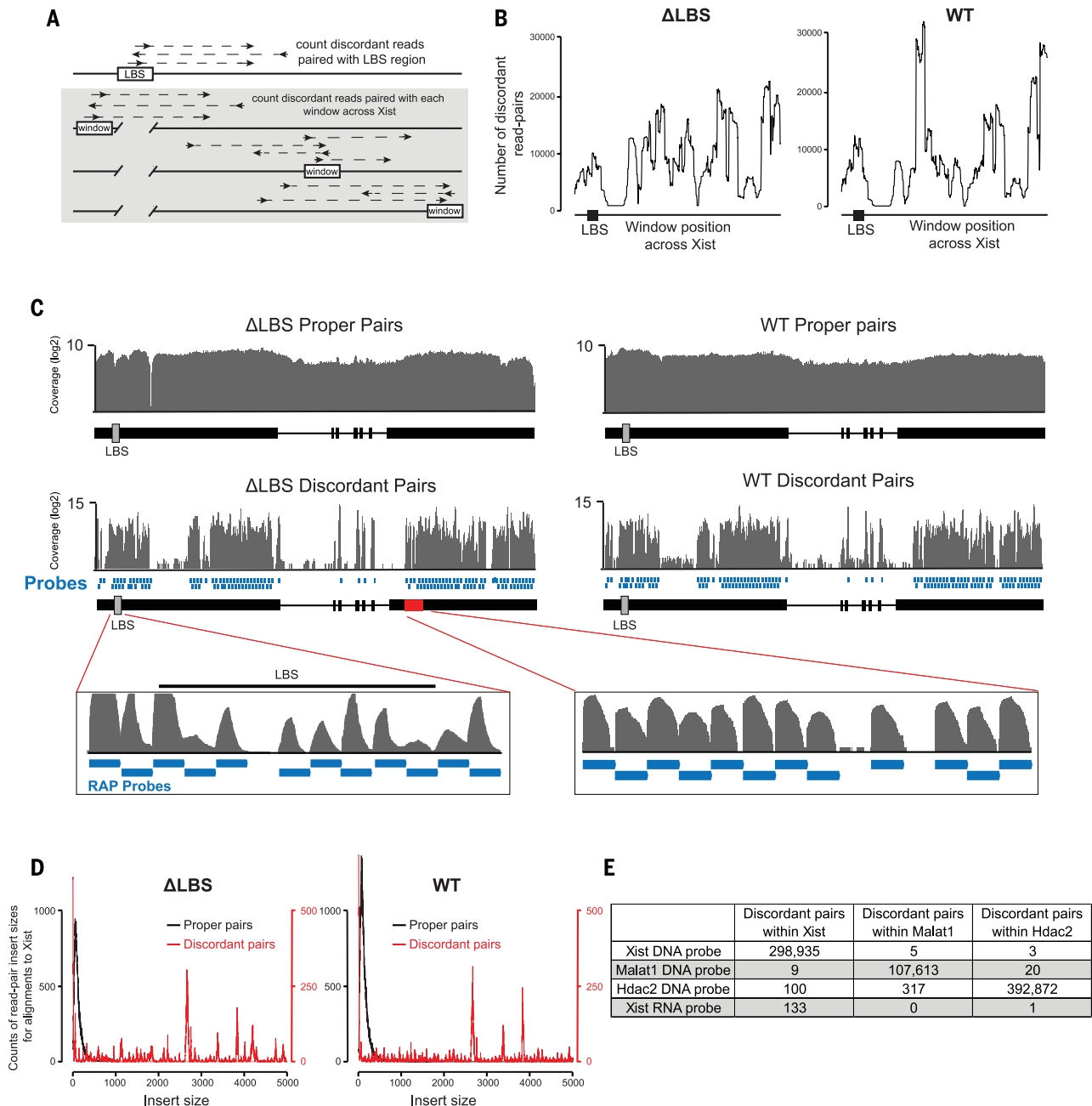
In our paper, we showed that disruption of the Xist-LBR interaction leads to exclusion of

active genes from the Xist-coated nuclear compartment. Wang *et al.* claim that this conclusion is derived from a single experiment and may simply be explained by noise. To the contrary, we performed numerous independent experiments to



**Fig. 1. ΔLBS-Xist contains a deletion of the LBR binding site on the Xist RNA.** (A) PCR of the genomic DNA of individual ΔLBS embryonic stem cell clones using primers flanking the LBS region. (B) The ΔLBS region defined by Sanger-sequenced PCR product of ΔLBS (top, red bar). A subset of sequence read pairs from RAP-DNA is shown. There are many read pairs that connect the LBS region and the rest of Xist in wild-type cells (top panel) and many read pairs that skip the entire LBS region in ΔLBS cells (bottom panel). (C) The number of read pairs connecting the LBS region and Xist as well as read pairs that skip the LBS junction in wild-type and ΔLBS cells, respectively. (D) Distribution plot of the genomic insert size for wild-type (black) and ΔLBS cells (red). Dashed line represents

the average fragment size of the read pairs that skip the LBS region. (E) Quantitative RT-PCR of wild-type and ΔLBS cells using the primer set inside or outside of the LBS region, respectively. The graph represents the ratio of relative expression level of primer set 1 to primer set 2. The numbers represent the coordinates of the primers on the Xist cDNA. (F) Two-color single-molecule RNA FISH using probes against a region on Xist outside of the LBS region along with the probes against either the sense strand (top) or the antisense strand (bottom) of the LBS region. Although Xist RNA is colocalized with sense LBS RNA in wild-type cells, no sense LBS RNA signal was observed in ΔLBS cells. Neither wild-type cells nor ΔLBS cells show antisense LBS RNA signal in 80 single cells imaged for each.



**Fig. 2. Discordant read pairs represent RAP-DNA probe sequences, not an inversion.** (A) A schematic illustration of how we compute coverage of discordant read pairs across Xist. A window the size of the LBS region (784 base pairs) was slid base by base along Xist, and, for each window, the number of discordant read pairs that had one read fully contained within the window was counted. For all windows other than those overlapping the LBS, we did not count any read pair where one of the two reads mapped within the LBS region. (B) The numbers of discordant

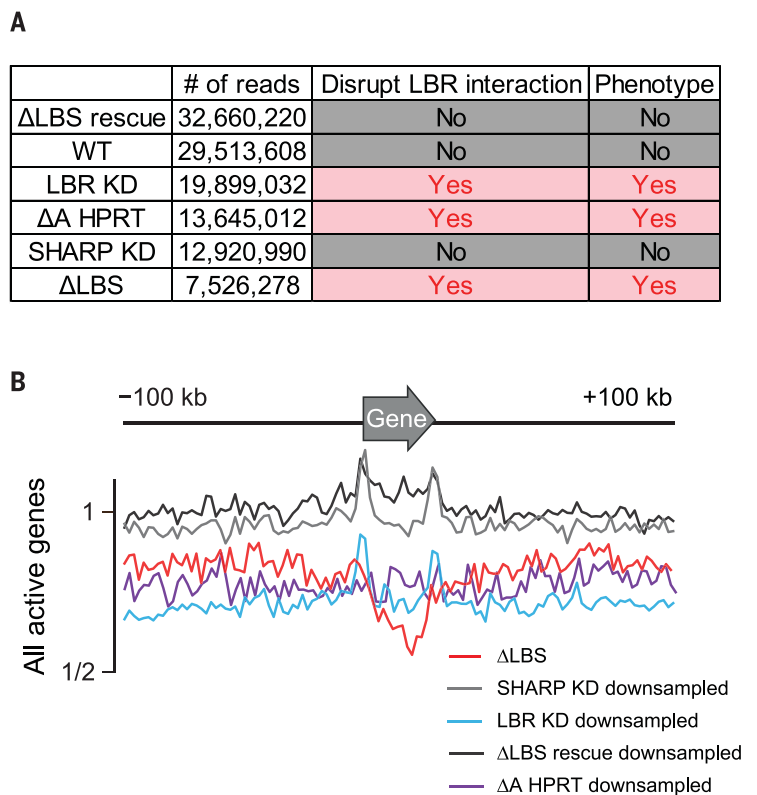
read pairs as described in (A) are plotted relative to the 5' position of the window for  $\Delta$ LBS and wild-type cells. (C) Coverage of proper and discordant read pairs in  $\Delta$ LBS and wild-type cells plotted along with the probes used for RAP. (D) Distribution of insert size for proper (black) and discordant (red) read pairs for  $\Delta$ LBS and wild-type cells. (E) Total number of discordant read pairs within Xist, Malat1(GSE55914), and Hdac2 (GSE55914) locus for RAP-DNA samples using DNA probes against Xist, Malat1, and Hdac2 RNA, respectively, and RNA probes against Xist RNA.

demonstrate this result, including (i) microscopy using four different active X chromosome loci across multiple independent replicates, (ii) a microscopy time course measuring gene position relative to Xist, and (iii) RAP-DNA followed by sequencing of all sites of the X chromosome, including active and inactive DNA regions. Furthermore, we performed several biological replicate

RAP-DNA experiments aimed at testing the same Xist-LBR interaction. For example, knockdown of LBR, deletion of the LBR binding site on Xist, and deletion of the A-repeat all disrupt the Xist-LBR interaction and all show the same phenotype in the RAP-DNA assay (Fig. 3A). These independent results highlight the robustness of our observations. [Although we had intended to include

the  $\Delta$ A-HPRT data, due to a miscommunication when performing this analysis, we mistakenly included RAP-DNA data from a different A-repeat mutant cell line that was generated for a distinct purpose but that shows the same phenotype. The  $\Delta$ A-HPRT results are already presented in our previous paper (15) and included here for comparison (Fig. 3B).]

**Fig. 3. Xist-LBR phenotypes observed by RAP-DNA are not affected by differences in read depth. (A)** Total number of reads sorted in descending order and its corresponding observed phenotype. **(B)** Fold change of Xist enrichment across the X chromosome as measured by RAP-DNA averaged across all the actively transcribed genes and inactive genes on the X chromosome for  $\Delta$ LBS, SHARP knockdown, LBR knockdown, and  $\Delta$ LBS-BoxB + LMNB1- $\lambda$ N ( $\Delta$ LBS rescue) and  $\Delta$ A-HPRT, where each sample is randomly down-sampled to match the number of total reads of the  $\Delta$ LBS sample.



Wang *et al.* claim that the decrease in Xist localization over actively transcribed regions by RAP-DNA may simply reflect a difference in the number of sequencing reads that were generated for each sample. This is incorrect. (i) There is no relationship between the number of sequencing reads generated for each sample and the phenotype observed (Fig. 3A). (ii) Our calculations already normalize for sequencing depth directly. [Wang *et al.* claim that the “normalized” Xist localization patterns look different, but this is only because they do not properly account for probe sequences. When excluding probes, as we did in our paper, the normalized plots are as already reported in our paper (1).] (iii) Our analysis compares Xist localization on active versus inactive genes within the same sample. Therefore, any differences in sequencing coverage will affect the entire sample consistently and not affect our results. (iv) We randomly down-sampled the reads such that they all contain the same number of reads, and we observed the same results: Xist localization is depleted over actively transcribed genes in all three samples that disrupt the Xist-LBR interaction

but not in any of the three samples that do not (Fig. 3B).

In conclusion,  $\Delta$ LBS-Xist is an appropriate cell line for studying the disruption of the Xist-LBR interaction and is not the only result that is inconsistent with the “nucleation defect” reported by Lee and colleagues (16). Indeed, previous studies that have also deleted this region of Xist (9) or the reported YY1 protein (17, 18) similarly fail to identify this nucleation defect. Based on all of the evidence presented here and in our paper, we are confident that none of the points raised by Wang *et al.* have any effect on the results or conclusions reported in our paper.

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3. For simplicity of presentation, we merged all of the reads generated in  $\Delta$ LBS cell lines and all of the reads generated from the unedited wild-type cell line and refer to this as the  $\Delta$ LBS and WT samples, respectively.
4. See the “Generating  $\Delta$ LBS and  $\Delta$ LBS-BoxB Xist” section in the supplement of our paper. We performed inverse PCR followed by Sanger sequencing to map this integration and found that, in all cases, it mapped to a long terminal repeat element

- that we were able to map in a few cases to a specific region on chromosome 12 at position 19.81 megabases.
5. The read pairs spanning the LBS deletion are SRR4246936.1928051, SRR4246936.1928049, SRR4246937.4677231, and SRR4246937.4677228.
6. The coordinates of the LBS deletion region are position 898-1682 (pSM33 Xist cDNA), position 998-1782 (NR\_001463), chromosome X 100676791-100677575 (mm9), and chromosome X 103481452-103482236 (mm10).
7. RAP-DNA uses single-stranded 90-nucleotide DNA probes to hybridize to the RNA of interest for capture. These probes are designed across the expressed RNA region and thus would only be present overlapping specific exonic regions.
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