

Polycomb repressive complexes 1 and 2 are each essential for maintenance of X inactivation in extra-embryonic lineages

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Additional information

Supplementary Table 1

The table includes allelic ratio, the ratio of the number of paternal reads to the number of total allele specific reads for each sample. X-linked genes with at least one informative sample in either experimental set (Ring1A/B experimental set; 3 Ring1A/B control and 3 Δ Ring1A/B, or Eed experimental set; 3 Eed control and 3 Δ Eed) are shown.

Supplementary Table 2

The table includes fold change of allelic ratio upon *Ring1A/B* deletion and *P*-value for each sample. X-linked genes with at least one informative sample in either experimental set (Ring1A/B experimental set; 3 Ring1A/B control and 3 Δ Ring1A/B, or Eed experimental set; 3 Eed control and 3 Δ Eed) are shown. *P*-values were calculated using one-sided Student's t-test.

Supplementary Table 3

The table includes fold change of allelic ratio upon *Eed* deletion and *P*-value for each sample. X-linked genes with at least one informative sample in either experimental set (Ring1A/B experimental set; 3 Ring1A/B control and 3 Δ Ring1A/B, or Eed experimental set; 3 Eed control and 3 Δ Eed) are shown. *P*-values were calculated using one-sided Student's t-test.

Supplementary Table 4

The table includes fold change of allelic ratio and *P* values of informative genes identified with allele-specific RNA-seq analyses. Presence of CpG island $\pm$ 2 kb from TSS is also indicated. *P*-values were calculated using one-sided Student's t-test.

Supplementary Table 5

Upper part: Genotyping primers with genomic DNA used in this study.

Lower part: Genotyping primers with cDNA used in this study.

Supplementary information

Allele-specific RNA-seq analysis

In allele-specific RNA-seq analysis, we noticed that two genes, *Tmsb4x* and *Tsix*, were incorrectly assigned in the allele-specific mapping for the following reasons and were therefore omitted from further analyses in this study.

During our initial analysis of allele-specific RNA-seq, a certain number of *Tmsb4x* transcripts from inactive B6-ChrXT^{MSM} allele was detected in both control and Δ Ring1A/B EPC samples, indicating that *Tmsb4x* is a potential escapee from XCI. In a more detailed analysis, however, we noticed that *Tmsb4x* contains only one informative indel in its 3'UTR, i.e. 9 sequential adenines in B6 allele and 10 sequential adenines in MSM/Ms allele. This homopolymer could be incorrectly amplified and/or processed during the RNA-seq library preparation and finally cause the misalignment in the analysis. Moreover, we analyzed pure B6 E7.5 transcripts prepared with the same allele-specific RNA-seq library preparation method, and found that some reads were annotated as MSM-derived transcripts. These results strongly suggest that *Tmsb4x* transcripts from B6 allele can be converted to those of MSM allele and cause misalignment in the allele-specific RNA-seq analysis. *Tmsb4x* has not been reported to escape from XCI. Therefore, *Tmsb4x* was removed from further allele-specific analysis.

Tsix, the antisense of *Xist*, is biallelically expressed in undifferentiated cells, monoallelically expressed from active X chromosome in early differentiated cells, and completely silenced in terminally differentiated cells⁵⁴. Thus, it is anticipated that *Tsix* is either not expressed or hardly expressed in EPCs and only expressed from the maternal active B6 allele in our allele-specific RNA-seq analysis, if any. We indeed detected a small number of transcripts (less than 60 reads per each EPC) annotated to *Tsix* in both control and Δ Ring1A/B EPC samples. Surprisingly, however, many of these *Tsix* transcripts were aligned to paternal inactive MSM/Ms *Tsix* allele. This prompted us to investigate these *Tsix* reads more carefully. We analyzed all *Tsix* transcripts regardless of SNPs/indels and found that *Xist*-intron-spanning reads were contained in these *Tsix*-annotated reads. *Xist*-intron-spanning reads such as *Xist* exon1-exon2 fusion reads should be strictly *Xist*-specific and never happened in *Tsix* transcripts. Approximately 1% of these *Xist*-intron spanning reads were aligned to *Tsix*-orientation, which could be mistakenly recognized during strand-specific RNA-seq library preparation with unknown reason. This is consistent with the number of allele-specific reads of *Xist* transcripts, which were between 5000 to 10000 reads in each EPC sample. *Tsix* was, therefore, removed from further allele-specific analysis.

Although it is not known how this incompleteness of strand specificity occurs, because of its likely small probability (~1%) and the other X-linked genes do not have their overlapping antisense counterpart, we conclude that this does not affect the overall analysis.

Supplementary Information Reference

54. Lee, J. T., Davidow, L. S. & Warshawsky, D. *Tsix*, a gene antisense to *Xist* at the X-inactivation centre. *Nat. Genet.* **21**, 400-404 (1999)

Supplementary discussion

In a recent study that examined the impact of a zygotic knockout of *Ring1B/Rnf2* (PRC1 hypomorphic) and *Eed* (PRC2-null) embryos, a substantial number of X-linked genes were found to be expressed from the Xi in E6.5 extraembryonic ectoderm (ExE) in these mutant females³⁷. The authors found that all PRC1-dependent genes (n = 35) were included in the fraction of PRC2-dependent escapees (n = 74). This situation is very different to our study, where we found many X-linked genes are more sensitive to PRC1 depletion but unaffected by PRC2 depletion in E7.5 EPCs (Fig. 7, 8). This discrepancy could be attributable to the rather different experimental setup used in the two studies. First, Andergassen et al. examined a PRC1-hypomorph (only *Ring1B/Rnf2* was mutated, not *Ring1A*) and second, they looked at zygotic knockout embryos where Polycomb functions were depleted much earlier, during the initiation rather than maintenance of XCI, and probably impacting many pathways. Indeed, imprinted XCI initiates at the 2-4 cell stage and is established at E3.5, thereafter being in maintenance phase^{26,27}. In our study, we induced *Ring1A/B* deletion at E5.5 and *Eed* deletion at E3.5, when imprinted XCI is already established. Thus, our study has focused specifically on maintenance of XCI²⁷ and can evaluate the roles of PRC1 and PRC2 for the maintenance of XCI, whereas the zygotic knockout for *Ring1B/Rnf2* or *Eed* used by Andergassen et al. would affect initiation and maintenance.