

Xist Repeats B and C but not Repeat A mediate *de novo* recruitment of the Polycomb system in X chromosome inactivation.

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Recruitment of the Polycomb complexes PRC1 and PRC2 by Xist RNA is an important step in the process of X chromosome inactivation and has provided a valuable model for understanding the role of non-coding RNA in chromatin-based gene regulation. Xist-mediated Polycomb recruitment was originally attributed to direct binding of the PRC2 complex to the Xist RNA Repeat A element, triggering subsequent recruitment of PRC1 (Zhao et al., 2008), but more recent work found that hnRNPK bound to a sequence element encompassing Xist Repeats B/C initiates Polycomb recruitment via direct interaction with the PCGF3/5-PRC1 complex (Almeida et al., 2017; Pintacuda et al., 2017), findings that have been confirmed in subsequent studies (Bousard et al., 2019; Colognori et al., 2019; Nesterova et al., 2019). Colognori et al. (2020) in a recent paper in this journal revisit this question and reach the conclusion that Repeat A initiates low levels of Polycomb recruitment in early differentiation stages in mouse embryonic stem cells (mESCs), and that the Repeat B pathway subsequently enhances and stabilises high levels of Polycomb occupancy. We wish to point out that in reaching their conclusions (Colognori et al., 2020) appear to have overlooked the contributory role of Repeat C in Polycomb recruitment, and that taking this and related findings into account leads to a very different interpretation of their data.

Figure S1A illustrates the extent of Xist deletions used in the aforementioned studies. Note that an equivalent schematic in Colognori et al. (2020) mis-annotated the deletion used in Nesterova et al. (2019). The deletion described by Colognori et al. (2020) encompasses Repeat B and some 5' sequences, and is reported to strongly reduce, but not abolish, Polycomb recruitment. However, deletions that abolish Polycomb recruitment, as reported by ourselves and others, encompass some or all of the 3' located Repeat C region (Bousard et al., 2019; Nesterova et al., 2019; Pintacuda et al., 2017). Importantly, Bousard et al. (2019) also reported residual Polycomb recruitment following deletion of Repeat B alone, but went on to show that this is abolished when the deletion is extended to include Repeat C. Moreover, Pintacuda et al. (2017) highlighted that binding of hnRNPK to Xist RNA, determined by eCLIP analysis in an independent study (Cirillo et al., 2016), is strongest over Repeat B but also extends over Repeat C, and that consistent with this, Repeat C includes several cytosine tracts that are putative hnRNPK binding sites. We show the replotted hnRNPK eCLIP data and putative Repeat C hnRNPK binding sites in **Figures S1A,B** to illustrate this point. With these considerations in mind it is entirely expected that the deletion described by Colognori et al. (2020) retains residual Polycomb recruitment activity. Whilst there are variations in the mode of Xist expression (native promoter versus inducible doxycycline promoter) and the differentiation timepoints analysed in the aforementioned studies, the models used are generally equivalent and in our view cannot reasonably be argued to account for the absence of Polycomb on Xi in the Repeat B+C deletions

(Nesterova et al, 2019; Bousard et al., 2019). We note that Colognori et al. (2020) observed residual Polycomb recruitment using the Xist Repeat B deletion only at early stages of mESC differentiation. A plausible explanation is the progressive diminution of global levels of specific Polycomb proteins over differentiation, as reported in prior studies (see Nesterova et al. 2019, discussion and references therein). Indeed, the diminution of Polycomb-mediated histone modification levels can be seen on Xi in the parental XX mESCs expressing full length Xist in the Colognori et al. (2020) study, Figure 2E, WT-Xist. The aforementioned arguments are summarised in [Figure S1C](#).

In their study Colognori et al. (2020) attribute the residual Polycomb recruitment that they observe after deletion of Repeat B to a function of Repeat A. However, as noted above, the deletions encompassing Repeats B+C described by ourselves and others largely abolish Polycomb on Xi, despite Repeat A being present and intact (see also [Figure S1D](#)). Additionally, hnRNPK and associated PCGF3/5-PRC1 are absolutely required for Polycomb recruitment by Xist RNA (Almeida et al., 2017; Colognori et al., 2019; Pintacuda et al., 2017), but there is no hnRNPK binding evident at Repeat A (Cirillo et al., (2016) and see [Figure S1A](#)). We note that Bousard et al. (2019) reported a marginal increase in the level of Polycomb linked chromatin modifications on Xi after deletion of Repeat B+C, but this was only over the promoters and gene bodies of active genes, likely reflecting increased background levels of Polycomb-mediated histone modifications in the absence of antagonising effects from histone modifications linked to gene activity (see Zhang et al. (2015) for a review of this topic).

Whilst the above arguments strongly imply that Repeat A has no direct role in recruiting the Polycomb system, deletion of Repeat A does result in reduced Polycomb levels on Xi, likely due to a combination of indirect effects: First, levels of Xist RNA and/or the size of Xist RNA domains are reduced following deletion of Repeat A (Nesterova et al., 2019), and this is referred to in Colognori et al. (2020). Second, deletion of Repeat A results in a more diffuse chromosomal localisation of Xist RNA (Nesterova et al, 2019), also reported in Colognori et al. (2020), Figure 3E. Finally, widespread gene activity on the X chromosome in cells with a Repeat A deletion, or following knockout of the gene encoding HDAC3, a downstream silencing factor in the Repeat A pathway, directly antagonises spread of Polycomb from surrounding regions into expressed gene bodies (Zylicz et al., 2019), presumably due to inhibitory effects of histone modifications linked to gene activity. Together these effects lead to a marked reduction in Polycomb levels on Xi (Zylicz et al., 2019), and this provides a likely explanation for why Colognori et al. (2020), Figure 3 find loss of the already low levels of Repeat C-mediated Xi Polycomb following deletion of Repeat B together with Repeat A.

In summary our alternative interpretation of findings on Polycomb recruitment by Xist RNA reported in Colognori et al. (2020) is fully consistent with previous reports showing that Xist Repeat B+C solely account for RNA directed Polycomb recruitment in X inactivation at all developmental stages.

Acknowledgements

We thank members of the Brockdorff lab for useful input and discussion. Work in the Brockdorff lab is supported by the Wellcome Trust, grant number 215513.

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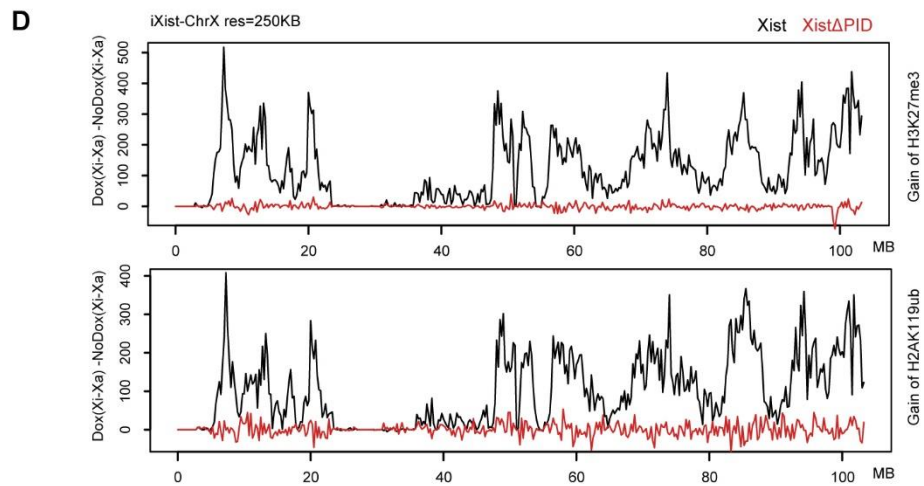
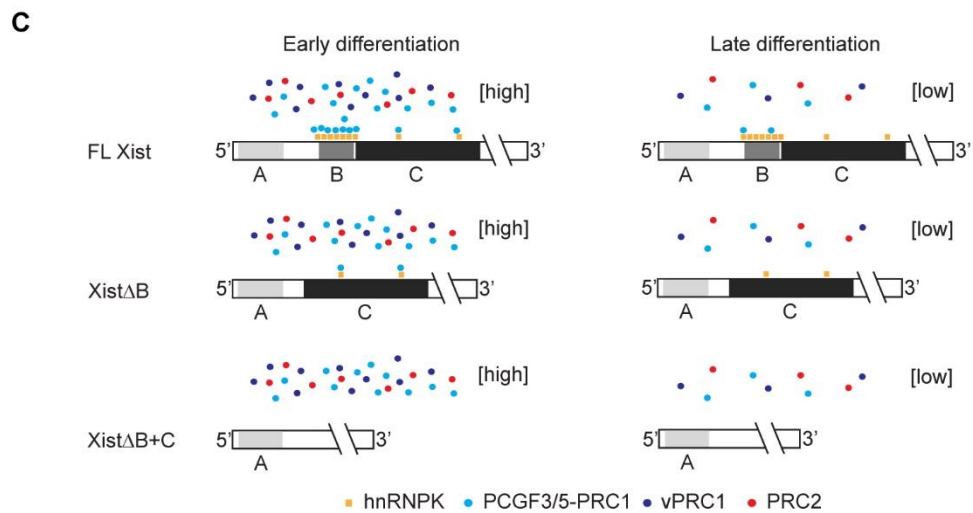
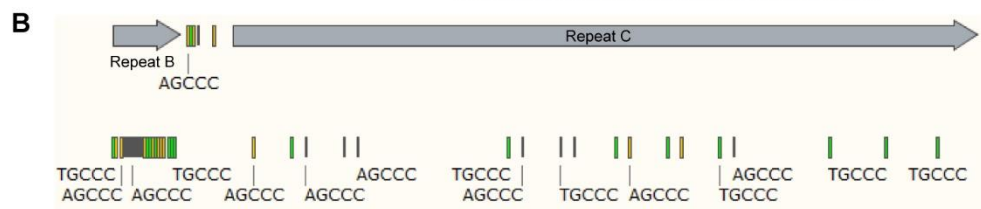
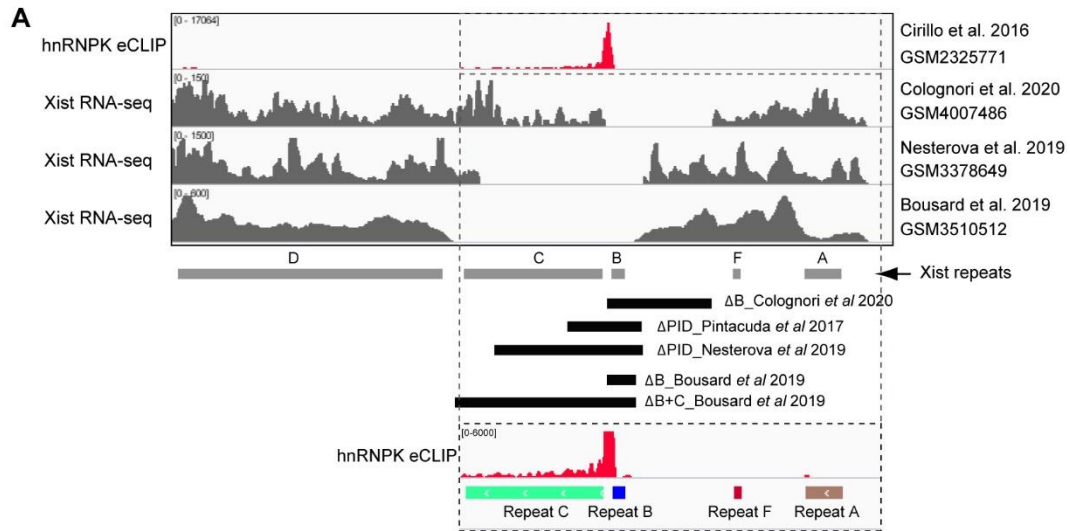


Figure S1. Repeats B+C accounts for Polycomb recruitment by Xist RNA

A. Top panel shows hnRNPK eCLIP and Xist RNA-seq data from indicated references replotted in relation to the 5' region of Xist exon 1. The schematic below indicates the deletions analysed in different studies, as indicated, with a rescaled view of hnRNPK eCLIP data illustrating that whilst its distribution is concentrated over Repeat B, it extends also across Repeat C. Little or no hnRNPK is found over Repeat A.

B. Schematic showing cytosine rich tracts in Repeat B and Repeat C that are candidate hnRNPK binding sites.

C. Schematic summarising arguments for how full-length (FL) Xist RNA and Repeat B/Repeat B+C deletions affect *de novo* recruitment of Polycomb complexes to Xi at early and late differentiation stages. Relevant tandem repeat regions are indicated with different shading. Proposals are based on published observations showing hnRNPK binding sites are concentrated in Xist Repeat B and present to a lesser extent in Repeat C. The initiating Polycomb complex PCGF3/5-PRC1 is recruited via interaction with bound hnRNPK and catalyses H2AK119ub1 which is then recognised by other variant (v) PRC1 complexes and by PRC2 which catalyses H3K27me3 (see references herein). The predicted effect of diminishing levels of Polycomb complexes ([low] or [high]) over differentiation time courses is also illustrated.

D. Reshowing of Figure S6 from Nesterova et al, (2019) illustrating gain of Polycomb-mediated histone modifications H3K27me3 (PRC2) and H2AK119ub1 (PRC1) on the inactive X chromosome over the proximal 105 Mb of chromosome X following Xist induction for 24h in mESCs (black traces). Gain of both modifications is abolished in mESCs expressing Xist RNA with the Xist Δ PID deletion (red traces). For further detail and explanation see Nesterova et al, (2019).