Supplementary Note

Results

To Figure 1 and Supplementary Figure 1

Localization of loci to the nuclear lamina reduces the transcriptional capacity of some genes in the *Xic*

In somatic cells, the nuclear lamina has been described as a repressive compartment[1-3]. To test whether in undifferentiated ESCs, the nuclear periphery is similarly repressive, we compared steady state transcript levels (using reverse transcription and quantitative PCR (RT-qPCR)) of X-linked genes in the vicinity of the TetO array, in bound versus control XX_{TetO} T-E-LaminB1 ESCs (**Figure 1A** and **1I**). Upon binding (ie minus dox), significant reduction in RNA levels was observed for some but not all endogenous genes in the *Xic*. Genes closest to the TetO array, such as *Chic1* and *Tsx* were more down-regulated than genes further away (**Figure 1I**). The neomycin resistance gene (*Neo*^{r}) situated immediately adjacent to the TetO array, showed the strongest repression, with expression levels being approximately 45-fold lower than in control cells (blue chart, **Figure 1I**). The degree of silencing of endogenous *Xic*-harbored genes located downstream of *Tsix* was, however, very minor and not linearly proportional to the distance from the TetO array (**Figure 1A** and **1I**). Of note, decrease in RNA levels was rather associated with location of the respective genes in the *Tsix* TAD, into which the TetO array was inserted. Importantly, the very low but nevertheless detectable levels of *Xist* transcript in ESCs were not significantly reduced upon T-E-LaminB1 binding to the *Xic-TetO* and relocalization to the nuclear lamina (**Figure 1A** and **1I)**.

As an important control, we also examined the effect of binding TetR-EGFP (without LaminB1) to the TetO array. This did not affect steady-state transcript levels of endogenous genes, although a slight but significant repression (2-fold) of the *Neo*^{r} gene adjacent to the TetO array could be observed (**Supplementary Figure 1D)**. Thus, as previously shown in

yeast[4], the binding of bacterial repressor proteins such as TetR to chromatin, can elicit proto-silencing effects in *cis* in mammalian cells. Nevertheless the effect was limited to the *Neo*^{r} gene, in immediate proximity of the TetO array locus, but not to the nearby *Chic1* or *Tsx* loci. The fact that *Neo*^{r} silencing was much greater upon relocalization of the locus to the lamina, and that nearby genes were also silenced to some extent, suggests that the nuclear periphery does indeed have a repressive effect, albeit mild, in ESCs (**Supplementary Figure 1E**).

Tsix, a direct negative regulator of *Xist*, was one of the few genes to show repression upon relocation to the lamina (**Figure 1I**). We therefore assessed the cell-to-cell variability of *Tsix* repression by performing RNA FISH for nascent *Tsix* transcripts in control versus bound XX_{TetO} T-E-LaminB1 ESCs. Surprisingly, even though steady state *Tsix* transcript levels were significantly reduced upon lamina relocalization (**Figure 1I**), the percentage of bound XX_{TetO} T-E-LaminB1 cells in which a nascent *Tsix* RNA FISH signal could be detected on both alleles was not substantially decreased, compared to control cells (**Supplementary Figure 1F** and **1G**). This suggested that reduced *Tsix* transcript levels might be due to a reduction in transcription levels (initiation or elongation), rather than to stable silencing in the lamina relocalized setting. If the latter were true the proportion of cells with relocalized *Xic* and monoallelic *Tsix* expression as detected by RNA FISH would be rather similar. We tested our hypothesis by semi-quantitative RNA FISH (as described in [5]) for *Tsix*. Although the number of transcribing alleles was indeed almost unaltered upon relocalization to the lamina (using standard RNA FISH), we found that the levels of nascent *Tsix* transcript in bound conditions were significantly reduced (**Supplementary Figure 1H**). Of note, *Tsix* transcript levels from the wildtype *Xic* allele were slightly lower than the *Xic-TetO* allele in control XX_{TetO} T-E-LaminB1 cells when compared to *Tsix* levels from the *Xic-TetO* allele (**Supplementary Figure 1H, I**). Relocalization of the *Xic-TetO* to the lamina merely "balanced" *Tsix* transcript levels between the two alleles.

Taken together our results imply that even though relocalization to the nuclear lamina in ESCs exerted some degree of repression on *Xic* genes, the transcriptional landscape of the *Xic* is not drastically altered, with the majority of relocalized *Xic* loci retaining their capacity to be transcribed, albeit at slightly lower levels. In particular, although steady state *Tsix* RNA levels were reduced upon *Xic* relocalization, antisense transcription (ie *Tsix*) across *Xist* was still detectable at the majority of loci relocalized to the nuclear lamina. Importantly, neither up-regulation nor repression of *Xist* could be observed in ESCs with relocalized *Xic-TetO*.

To Figure 2D

Controls

The *Xic-TetO* allele alone was shown not to interfere with the initiation of random XCI and *Xist* up-regulation[6]. Indeed, in control XX_{TetO} T-E-LaminB1 cells, *Xist* up-regulation was almost completely random after differentiation, with slight skewing (57%, n>500) towards the *Xic-TetO* allele (**Figure 2E**). This may be due to the different *Xce* alleles carried by the two X chromosomes in PGK12.1 cells (Xic^{129}/Xic^{PGK}) with the X^{129} chromosome being more prone to become inactivated than the X^{PGK} chromosome[7].

To Figure 5 and Supplementary Figure 4

Biallelic *Tsix* expression is not required for pairing of the *Xist/Tsix* locus

In order to explore further the possible links between *Xist* and *Tsix* expression and *Xic* pairing, we examined *Xic* pairing frequencies in a female ESC line (TX1072) harboring a doxycycline-inducible *Xist* promoter on one of the two X chromosomes, which enables monoallelic overexpression of *Xist* from the endogenous locus and repression of *Tsix* in *cis*[8]. This system gives rise to a population of cells with monoallelic *Xist* expression and lack of biallelic *Tsix* expression without affecting the underlying *Tsix* and *Xist* DNA sequences (**Figure**

5A). We induced *Xist*, either one day prior to differentiation (+Dox D-1) or at the same time as differentiation (+Dox D0) and compared these cells to ones where *Xist* overexpression was not induced (control) (**Figure 5B**). We analyzed *Xist* RNA accumulation, *Xist* expression and *Tsix* expression by RNA FISH (**Figure 5C**). Already in undifferentiated cells induction of *Xist* expression (+dox) for one day resulted in considerable overexpression and accumulation of *Xist* with more than 50% of the cells displaying nascent *Xist* expression and 70% with fully formed Xist RNA domains on one X chromosome. In uninduced control cells only approximately 5% of the cells contained small clusters of Xist RNA (**Figure 5C** and **Supplementary Figure 4A**, n>100). During differentiation, TX1072 cells kept in doxycycline containing medium displayed much higher frequencies of monoallelic *Xist* accumulation compared to control cells (**Figure 5D** and **Supplementary Figure 4A**, n>100; compare also the fraction of non-inducible cells displaying *Xist* expression undergoing differentiation in Figures 2B,C and 3F,G). The fraction of cells with monoallelic rather than biallelic *Tsix* expression was also substantially higher under these conditions (**Figure 5D**) with *Tsix* being repressed on the allele overexpressing *Xist*.

We assessed whether these conditions of induced monoallelic *Xist* and *Tsix* expression influenced the frequency of pairing events, by DNA FISH for the *Xist*/*Tsix* region (**Figure 5E**) and 3D distance measurement. Under all tested conditions (control, +Dox D-1, +Dox D0) the proportion of *Xic* pairing cells (*Xic*-*Xic* d<2 µm) increased during early differentiation and peaked at day 1.5 (**Figure 5F**, n>190). Thus *Xic* pairing frequencies were similar in the three conditions at all time points during early differentiation (**Figure 5F**).

These results lead to several conclusions. First, biallelic *Tsix* expression is not an exclusive facilitator of *Xic* pairing; second, increased up-regulation of *Xist* from one allele does not influence the frequency of pairing during differentiation, nor does it induce pairing of the *Xist*/*Tsix* region in undifferentiated cells. Thus, homologous *Xic* pairing can occur in populations of differentiating female ESCs in which the majority of cells express *Xist* and *Tsix*

in a monoallelic and reciprocal manner. *Xic* pairing at the level of the *Tsix* locus, does not correlate specifically with either biallelic *Tsix* or *Xist* expression. This suggests that it may not have a direct role in transforming biallelic expression into monoallelic expression states, at least at this stage of ESC differentiation. Third, the timing of pairing events appears to depend on the kinetics of differentiation rather than on the fraction of cells in which *Xist* has already been up-regulated (a choice has been made), or in which *Tsix* is expressed in a biallelic manner (no choice has been made) under the tested conditions.

Taken together, our results confirm that homologous *Xic* pairing is a frequent and reproducible process, but we also demonstrate that it is unlikely to be directly involved in the monoallelic regulation of *Xist* during XCI, as it still occurs in cells in which initiation and choice-making have already occurred by forced *Xist* overexpression.

To Figure 6 and Supplementary Figure 5

Xist expression is not required _per se_ for _Xic_ pairing to occur

Although the above results show that *Xist* induction does not influence frequencies of pairing, the question of whether *Xist* expression or Xist RNA accumulation are necessary for *Xic* pairing during differentiation was not addressed. We therefore generated a CRISPR/Cas9-mediated homozygous deletion of *Xist* in PGK12.1 XX_{TetO} cells (**Figure 6A, Supplementary Figure 5A**). These XX_{TetO} Δ*Xist* DKO (double knock-out) cells were differentiated by LIF withdrawal, alongside control XX_{TetO} cells. *Xist*/*Tsix* expression together with *Xic* pairing were assessed by FISH and 3D distance measurements. RNA FISH confirmed the complete absence of *Xist* expression as expected in XX_{TetO} *Xist* DKO cells during differentiation (**Figure 6B** and **6C,** n>250). In control XX_{TetO} cells, the fraction of cells with monoallelic accumulation of Xist RNA as well as the fraction of cells with monoallelic expression of *Tsix* increased during differentiation (**Figure 6B** and **6C**, n>250). However, in differentiating XX_{TetO} *Xist* DKO cells, the proportion of cells with monoallelic or no *Tsix*

expression did not substantially increase; nor did the proportion of cells with biallelic *Tsix* expression decrease (**Figure 6B** and **6C,** n>250). A slight delay in down-regulation of several pluripotency factor genes was observed in XX_{TetO} *Xist* DKO cells after day 2 of differentiation compared to wild type XX_{TetO} cells (**Supplementary Figure 5B**). This is consistent with results of our previous study showing that initiation of XCI is required to enable the efficient differentiation of female ESCs[8].

Supplementary Note References

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