Live-Cell Imaging and Functional Dissection of Xist RNA Reveal Mechanisms of X Chromosome Inactivation and Reactivation

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HIGHLIGHTS
A Pumilio-based system allows efficient double-tagging of Xist RNA in live cells

Induced XCI in undifferentiated ES cells reveals the roles of Kat8 and Msl2 in XCR

Live-cell imaging reveals the undersized "ΔA mutant" Xist signals

Tethering proteins onto "ΔA mutant" reveals their role in Xist-mediated silencing

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Article

Live-Cell Imaging and Functional Dissection of Xist RNA Reveal Mechanisms of X Chromosome Inactivation and Reactivation

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SUMMARY

We double-tagged Xist (inactivated X chromosome-specific transcript), a prototype long non-coding RNA pivotal for X chromosome inactivation (XCI), using the programmable RNA sequence binding domain of Pumilio protein, one tag for live-cell imaging and the other replacing A-repeat (a critical domain of Xist) to generate “ΔA mutant” and to tether effector proteins for dissecting Xist functionality. Based on the observation in live cells that the induced XCI in undifferentiated embryonic stem (ES) cells is counteracted by the intrinsic X chromosome reactivation (XCR), we identified Kat8 and Msl2, homologs of Drosophila dosage compensation proteins, as players involved in mammalian XCR. Furthermore, live-cell imaging revealed the obviously undersized ΔA Xist cloud signals, clarifying an issue regarding the previous RNA fluorescence in situ hybridization results. Tethering candidate proteins onto the ΔA mutant reveals the significant roles of Ythdc1, Ezh2, and SPOC (Spen) in Xist-mediated gene silencing and the significant role of Ezh2 in Xist RNA spreading.

INTRODUCTION

In mammalian female somatic cells, one of the two X chromosomes is silenced to compensate the X-linked gene dosage between males and females. This phenomenon is known as X chromosome inactivation (XCI) (Payer and Lee, 2008). Inactivated X chromosome-specific transcript (Xist) plays an essential role in XCI (Payer and Lee, 2008). Upon the initiation of XCI, Xist transcription is up-regulated from the future inactive X chromosome (Xi), and the RNA transcripts spread out to paint the entire chromosome territory to establish chromosome-wide gene silencing. Coating of the Xi by Xist transcripts produces an interesting Xist “cloud” signal in RNA fluorescence in situ hybridization (FISH) (Clemson et al., 1996). To date, labeling of Xist RNA in the cellular context is nearly exclusively achieved by RNA FISH. Visualizing the spatial distribution and dynamics of Xist RNA in live cells may provide important insights into the functional mechanism of Xist.

However, although various approaches have been proposed and developed (Urbanek et al., 2014), live-cell imaging of RNA remains technically challenging. A previous study reported that based on the interaction between an RNA motif (MS2 motif) from the MS2 phage and the MS2 RNA-binding protein (MCP), Xist RNA fused to a tandem array of MS2 motifs can be visualized by GFP-tagged MCP (MCP-GFP) (Ng et al., 2011). An inducible Xist cDNA transgene fused with 24 MS2 motifs at its 3’ end was constructed, and a transgenic cell line carrying 7 copies of the Xist cDNA transgene on chromosome 7 was established for live-cell imaging. Possibly due to technical limitations, the report did not provide any time-lapse video file to illustrate the RNA’s behavior in live cells.

RESULTS

The Experimental System

In this study, we took advantage of programmable sequence-specific RNA binding by the Pumilio homology domain (PUF) to visualize Xist RNA in live cells (Wang et al., 2002; Cheong and Hall, 2006). A total of 25 copies of PUF binding sites (PBSb) (Cheng et al., 2016) were fused to the 5’ end of a full-length Xist transgene. An inducible Xist cell line was then generated from Ainv15 cells (Kyba et al., 2002), a male mouse embryonic stem (ES) cell line carrying an engineered cassette upstream of the X-linked Hprt gene (Figure 1A). Through Cre-mediated gene targeting, the transgene was inserted downstream of the tetracycline response element (TRE) of Ainv15 cells, restoring neomycin resistance (Figure 1A). Moreover, a red
fluorescent protein (tdTomato) was included as a reporter gene (Figure 1A). The resulting cell line is a male mouse ES cell line carrying an inducible, single-copy and full-length \(\text{Xist}\) transgene on its X chromosome (Figure 1A). Both neomycin resistance and tdTomato were used as reporters to assess the functionality of the inducible \(\text{Xist}\) transgene. Ectopic expression of PUFb-GFP fusion protein resulted in a cell line (GFP-iXist) that permits the spatiotemporal analysis of \(\text{Xist}\) RNA distribution and dynamics in live cells (Figure 1B).

To take advantage of the Pumilio system, which provides multiple PUFs and PBSs, we engineered an inducible \(\text{Xist}\) ES cell line in which the A-repeat of \(\text{Xist}\) was replaced by 10 copies of PBSa (Cheng et al., 2016) (GFP-PBSa-iXist) (Figure 1B). A-repeat is a conserved region of \(\text{Xist}\), which is functionally important for \(\text{Xist}\)-mediated gene silencing (Wutz et al., 2002). GFP-PBSa-iXist can be regarded as a “\(\Delta\)A Mutant” of the inducible \(\text{Xist}\) transgene. Ectopic expression of PUFb-GFP fusion protein resulted in a cell line (GFP-PBSa-iXist) that permits the spatiotemporal analysis of \(\text{Xist}\) RNA distribution and dynamics in live cells (Figure 1B).

Figure 1. The Experimental System and the Inducible \(\text{Xist}\) Cell Lines

(A) Schemes of the iXist cell line and the inducible \(\text{Xist}\) allele. TRE, tetracycline response element; \(\Delta\)Neo, the coding region of neomycin resistance gene without the start codon; pPGK-ATG: PGK promoter and a start codon.

(B) Diagrams of the live-cell imaging system and the different engineered inducible \(\text{Xist}\) alleles used in this study. PUF, Pumilio homology domain; PBS: PUF binding site.

(C) RNA FISH to validate live-cell labeling of \(\text{Xist}\). The RNA FISH probe was Cy3-labeled (red). DNA was counterstained with DAPI (blue). The cell was imaged in high resolution by Airyscan Super-resolution Confocal Microscope (Carl Zeiss). The images shown are from a single Z-section. Note: although the emission spectrums of Cy3 and tdTomato are overlapping, the \(\text{Xist}\) RNA FISH signals were clearly detected. This is possibly due to the RNA FISH signal intensity and/or \(\text{Xist}\)-mediated gene silencing on the tdTomato reporter. Scale bar: 5 \(\mu\)m.

See also Figure S1.

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and its binding proteins. Ectopic expression of PUFa-effector fusion proteins resulted in additional transgenic cell lines (Figure 1B).

We validated the live-cell labeling of Xist in the established transgenic cell lines. With a 24-hr doxycycline (dox) treatment, GFP-labeled Xist clouds could be clearly detected in ~70%–90% of nuclei in all established cell lines (data not shown). Xist RNA FISH on fixed cells confirmed that the Xist signals labeled by GFP overlap with the RNA signal detected by the RNA FISH probe (Figure 1C). Since two PUFs are involved in this study and they are highly homologous to each other, we then tested the binding specificity of a PUF with its PBS. When PUFa-EGFP was expressed in a cell line carrying PBSb-Xist fusion, no Xist signals were detected after a 24-hr dox treatment (Figure S1A). This result confirms that there is no cross-reactivity between PUFa and PBSb. Furthermore, this result also helps to address the concern on the PBSa sites located within the endogenous sequence of Xist. We analyzed the sequence of the Xist gene body, including introns, and identified two PBSa sites and two antisense PBSa sites (Figure S1B). Meanwhile, no PBSb sites were identified (Figure S1B). It is a concern that the endogenous PBSa sites may interact with PUFa. It is also a probability in theory that the endogenous antisense PBSa sites may interact with the PBSa tag and in consequence affect the secondary structure of Xist. We performed multiple experiments to address these concerns. Here, the result that no Xist signals were observed in the induced cells overexpressing PUFa-EGFP (Figure S1A) shows that the endogenous PBSa sites did not result in “unspecific” signals in live-cell imaging.

The Difficulties of “Sunrise” in Undifferentiated ES Cells

With the established cell lines, we first examined the emergence and growth of Xist RNA signals upon dox treatment (the “sunrise” process). Live-cell imaging was performed 1 hr after dox treatment, which allowed us to monitor the sunrise process in most of the differentiating cells within the 2-hr time span of live-cell imaging. Interestingly, the sunrise behavior was markedly different between differentiating and undifferentiated ES cells. In differentiating cells, the Xist RNA signals emerged quickly (Figure 2A) and in a synchronized manner (Video S1). Most Xist RNA signals first appeared as small puncta that then gradually grew into ~2-μm-large Xist RNA clouds within 60–90 min (Figures 2B and S2). In undifferentiated cells, the onset of sunrise occurred later and was more heterogeneous (Figure 2A and Videos S2, S3, and S4). Some Xist RNA puncta gradually developed into clouds, very much like the Xist RNA signals in differentiating cells (we call this behavior “blossom”). However, a large fraction of Xist RNA puncta never showed significant growth within ~60 min (Figure 2C) (we call this behavior “star”). Even after a prolonged treatment with dox (2 hr) before live cell imaging, a significant fraction of undifferentiated cells did not develop Xist RNA clouds during the subsequent 2-hr time span of live-cell imaging (Videos S2, S3, and S4). In general, Xist RNA growth in undifferentiated cells was significantly slower than in differentiating cells (Figures 2D and 2E).

The “Tug of War” between the Induced XCI and the Intrinsic XCR

To further study the special sunrise behaviors, we examined the effects of induced Xist expression on X-linked genes. The induced Xist expression clearly repressed the transcription of X-linked genes in both undifferentiated (Figure S3) and differentiating cells (Figure 3A). However, Xist-mediated gene silencing is much weaker or inefficient in undifferentiated cells (Figure 3A). In the 6 X-linked genes tested, the induced Xist expression levels are comparable between undifferentiated and differentiating cells. Hprt showed a slightly higher expression level in differentiating cells than in undifferentiated cells, which can be explained by the close head-to-head orientation of the Hprt promoter and the TRE (Figure 1A). The strong transcriptional activity of TRE may cause hypermorphic expression of Hprt. The rest of the 4 X-linked genes tested all showed significantly lower expression levels in differentiating cells (Figure 3A). Therefore, the difficulties of sunrise in undifferentiated cells are associated with weaker Xist-mediated silencing.

The inducible Xist transgene is X-linked. Therefore, inducible Xist expression causes cell death and growth arrest due to Xist-mediated gene silencing along the single X chromosome in male cells. This “killing effect” of induced XCI can be used to assess the functionality of Xist-mediated gene silencing. Indeed, dox treatment showed a stronger killing effect in differentiating cells, which is consistent with the more efficient Xist-mediated gene silencing in differentiating cells (Figures 3B and 3C).

Moreover, it is known that the undifferentiated ES cells are maintained in culture in metastable pluripotent states with a heterogeneous population of Nanog-high and Nanog-low cells (Chambers et al., 2007). We observed that during the early hours of dox treatment, Xist clouds appear more frequently in
Nanog-low cells (Figures 3D and S4). Taken together, these results argue for the biological significance underlying the difficulties of sunrise observed in undifferentiated cells.

It is well known that an undifferentiated ES cell is able to reprogram or dedifferentiate a somatic cell back to pluripotency, if the two cells are fused (Ohhata and Wutz, 2013). During this process, the Xi is also reactivated (Ohhata and Wutz, 2013). Therefore, a pluripotent mouse ES cell possesses a built-in capacity for X chromosome reactivation (XCR). We speculated that the induced XCI status in undifferentiated iXist cells is a balanced outcome of two counteracting forces, the induced XCI and the intrinsic XCR (Figure 3E). Thus,
Figure 3. The “Tug of War” between Induced XCI and Intrinsic XCR

(A) Quantitative RT-PCR results to show the Xist-mediated silencing effect on X-linked genes between undifferentiated and differentiating cells. Doxycycline treatment was carried out for 24 hr. The results are shown in relative fold expression. Normalization was performed using Actb and Gapdh. The expression level of each gene in undifferentiated cells is set as 1. Error bars indicate SEM (n = 3). The statistical analysis used is Student’s t test. *p Value was calculated between the pair of datasets of each gene. *p < 0.05.

(B and C) (B) Doxycycline-induced cell death assay on iXist cells. Cells were cultured either as undifferentiated or as differentiating ES cells. Doxycycline treatment was carried out for 4 days. (C) Cell survival rate for differentiating cells was calculated by measuring the area stained by crystal violet staining. Cell survival rate for undifferentiated cells was calculated by alkaline phosphatase-stained colony counts. Data are shown as mean ± SEM of biological triplicate. The statistical analysis used is Student’s t test. *p < 0.05.

(D) Immuno-RNA FISH to detect Nanog and Xist in undifferentiated iXist cells treated with dox for 3 hr. DNA was counterstained with DAPI (blue). Cells were cultured in the conventional Lif-containing ES culture medium without 2i. The nuclei in the image relevant for the discussion are labeled as #1–6. The RNA FISH probe was Cy3-labeled (red). White arrows indicate the three Xist cloud signals detected in nuclei #3–5. Nanog was detected by immunostaining (green). The Nanog-high and Nanog-low cells were identified by ImageJ with a selected signal intensity threshold. Nuclei #1 and #2 are Nanog-high, because the entire nucleus or a large part of it was recognized as a single “particle” (the yellow outline highlighting the perimeter) above the selected signal intensity threshold. Nuclei #6 could be grouped into either Nanog-high cells or undetermined cells depending on the subjectivity of the data analyzer. Nuclei #3–5 belong to Nanog-low cells.

(E) The counteracting forces of XCI and XCR determine the X inactivation status in undifferentiated iXist cells upon dox induction.

(F) The rate of Xist RNA signal disappearance upon doxycycline removal. See also Figures S3–S5.
we examined the “sunset” process (disappearance of Xist signals upon dox removal), which reflects XCR. The results show that sunset proceeds at a faster rate in undifferentiated cells than in differentiating cells, indicating higher activity of XCR in undifferentiated cells (Figure 3F). Here, one concern is that the Xist clouds may dissipate more rapidly in fast dividing cells. To find out whether the faster sunset rate observed in undifferentiated cells is related to a faster cell division rate, we examined the cell division rates of the two samples during the 3-hr time window of the live-cell imaging experiment (Figure S5). Although undifferentiated ES cells divide faster than differentiating cells under normal tissue culture conditions, the cell growth rates may differ under the live-cell imaging experimental conditions. The cells (both undifferentiated and differentiating cells) were trypsinized shortly before the experiments. Cell colony formation and feeder cells were avoided for imaging purposes. Therefore, the trypsinized cells were directly attached to the fibronectin-coated glass surface of the MatTec dish in low cell density without feeder cells. The live-cell imaging experiments were then carried out within a 3-hr time window. Based on our measurements, the undifferentiated cells and the differentiating cells showed nearly identical cell division rates under these conditions (Figure S5). More importantly, limited amount of cell division was detected during the 3-hr time window of live-cell imaging. Taken together, the result of the sunset experiment rules out the technical concern that the difficulties of sunrise in undifferentiated cells are due to their insensitivity to dox treatment. Most importantly, these results explain the heterogeneous, late onset and slow sunrise observed in undifferentiated cells and confirm that the induced XCI status in undifferentiated cells is determined by the two counteracting forces of XCI and XCR. Therefore, the established experimental system can be used as a unique tool to study both XCI and XCR. Manipulating genes involved in either XCI or XCR can tip the balance toward a predictable side.

The Roles of Histone Acetyltransferase Kat8 Protein Complex in XCR

Spen, a transcriptional repressor associated with histone deacetylase (HDAC) activities, was recently identified as a Xist-binding protein functionally important for Xist-mediated gene silencing (McHugh et al., 2015, Chu et al., 2015, Minagaki et al., 2015, Monfort et al., 2015, Moindrot et al., 2015). Therefore, we hypothesized that histone acetyltransferases (HATs) may be involved in XCR. To test this, we selected a few candidate HATs and their protein partners, which are expressed in undifferentiated ES cells (Figure S6). Short hairpin RNA (shRNA)-mediated knockdown of lysine acetyltransferase 8 (Kat8) and its protein partner male-specific lethal 2 (Msl2) significantly increased the killing effect of induced XCI in undifferentiated iXist cells (Figures 4A, 4B, and S6). To rule out the off-targeting effect of shRNAs, we used a second shRNA against Kat8 and Msl2 and obtained similar results (Figure S7). A previous study showed that homozygous deletion of Kat8 disrupts pluripotency, whereas the effect of heterozygous deletion was moderate and the mutant animals were largely normal (Li et al., 2012). To confirm that Kat8 and Msl2 are directly involved in XCR, we confirmed the shRNA knockdown of Kat8 and Msl2 (Figure S6) and showed that knocking down the candidate genes did not affect the expression of key pluripotency-related genes in the established cell lines (Figure S8). To further investigate the involvement of Kat8 and Msl2 in XCR, we examined Xist signals in live cells. shRNA against Kat8 and Msl2 helped to correct the late-onset and slow-growth sunrise behaviors in undifferentiated cells (Figures 4C and 4D). Meanwhile, shRNA Msl2 also helped to slow down sunset in undifferentiated cells (Figure 4E). These results suggest that Kat8 and Msl2 are involved in XCR.

The Small Size of ΔA Mutant Xist Clouds

The sunrise behavior in undifferentiated cells during early hours of dox treatment is the outcome of a tug of war between XCI and XCR. Long hours of dox treatment unavoidably tip the balance toward XCI. Xist clouds could be observed in ~70%-90% of cells in all the established inducible Xist cell lines after a 24-hr dox treatment. We consider the Xist clouds observed at this stage as full-sized Xist signals. In wild-type cells, the Xist clouds are large and often associated with speckles scattered around a defined main territory covered by the cloud (Figure 5A). Interestingly, we noticed that the full-sized Xist clouds in ΔA mutants are significantly smaller and more compact compared with the wild-type clouds (Figures 5A–5C). We name this phenomenon “stopped budding.” This observation holds true whether or not the cells were cultured as undifferentiated or differentiating cells (Figures 5B, 5C, and S9). We also generated cell lines in which A-repeat was deleted but not replaced with any exogenous sequences and obtained similar results (data not shown).

This result clarifies an issue regarding the ΔA Xist RNA signals. Based on RNA and DNA FISH techniques, it has been observed that, at the onset of XCI, the Xist RNA transcripts first form a silencing compartment (the core), which excludes the Pol II transcription machinery. At a later step, actively transcribed X-linked genes,
Figure 4. The Putative Roles of Histone Acetyltransferase Kat8 Protein Complex in XCR

(A and B) (A) Doxycycline-induced cell death assay on different inducible Xist cell lines. Cells were cultured as undifferentiated ES cells. Doxycycline treatment was carried out for 4 days. (B) Cell survival rate was calculated by alkaline phosphatase-stained colony counts. Data are shown as mean ± SEM of biological triplicate. The statistical analysis used is Student’s t test. *p < 0.05.

(C) The growth curves of Xist RNA signals. The size (area) of a signal when it was first detected is defined as “size one” for the signal. Xist RNA signals were analyzed in 20 cells randomly selected from each sample. Data are shown as mean ± SEM with a trend line.

(D) Xist signals in live cells treated with dox for 2 hr. Representative images of Xist RNA signals in live cells after 2-hr dox treatment are shown as maximum intensity z projections. Scale bars, 5 µm.

(E) The rate of Xist RNA signal disappearance upon doxycycline removal. The cells were cultured as undifferentiated ES cells and treated with dox overnight before dox removal (n > 175 for the samples of each time point).

See also Figures S6–S8.
Figure 5. The Small Size of ΔA Mutant Xist Clouds

(A) Representative images of Xist RNA signals in live cells. Images are shown as maximum intensity z projections.

(B and C) (B) Xist cloud size measurement (n ≥ 50 for each dataset). Cells were cultured as differentiating ES cells. Doxycycline treatment was carried out for 24 hr. (C) Statistical analysis (Student’s t test) was carried out to compare the Xist cloud size between all sample pairs within the experimental panel. p Values greater than 0.05 are labeled in red. See also Figure S9.
which are located outside or at the periphery of the compartment, are silenced (Chaumeil et al., 2006). The formation of silencing core does not depend on A-repeat, but the later step does. Recently, genomic mapping results further provided direct evidence that the ∆A mutant Xist transcripts could not efficiently cover actively transcribed genes (Engreitz et al., 2013). Based on these results, it is straightforward to assume that the ∆A Xist cloud should have a smaller size than the wild-type, because it only covers the silencing core but has difficulties to reach out to efficiently cover the actively transcribed genes located at the peripheral region. However, possibly because of the technical limitations of RNA FISH, it was concluded that the appearances of Xist clouds were “comparable” between the wild-type and the ∆A mutant (Chaumeil et al., 2006; Wutz et al., 2002; Engreitz et al., 2013), even though undersized ∆A mutant Xist clouds can be seen in the published data (Engreitz et al., 2013). Our live-cell imaging experiments clarify this issue, and the results fit in more comfortably with the previous observations.

Artificial Tethering of Ezh2 Restored the Attenuated PRC Activities along the ∆A Mutant Xist RNA

In our experimental system, PUFa can be fused with individual candidate proteins and tethers the protein back onto the ∆A mutant Xist transcripts to dissect the Xist functionality. Three candidate proteins (Ezh2, Spen, and Ythdc1) were selected for this study. Unfortunately, we encountered difficulties in generating the large DNA constructs of SPEN (∼3,700 amino acids). Spen (split end), as its name suggests, contains two functional domains separated by a large linker region (Aniyoshi and Schwabe, 2003). The two functional domains are the N-terminal RNA binding domain and the SPOC domain (Spen paralog and ortholog C terminal). It is a reasonable assumption that SPOC is the critical functional domain directly involved in XCI. We, therefore, tethered the SPOC domain onto the ∆A mutant transcripts. Artificial tethering of Ezh2, SPOC, and Ythdc1 all partially rescued the “stopped budding” mutant phenotype, whereas Ezh2 clearly showed the most significant rescue effect among the three (Figures 5A–5C). This result shows that Ezh2 is functionally related to Xist RNA spreading. To assess the functionality of Xist-mediated gene silencing, we measured the killing effect of induced XCI (Figure 6A). The results show that GFP-iXist is functionally equivalent to the wild-type iXist. Replacing of A-repeat with PBSa in GFP-PBSa-iXist (∆A mutant) clearly disrupted the Xist-mediated gene silencing (Figure 6A). This mutant phenotype was significantly rescued by artificially tethering any of the three candidate proteins onto the mutant Xist transcripts (Figure 6A). Other than the killing effect, we also examined the direct effect of Ezh2 tethering on X-linked gene expression. The results confirm that Ezh2 tethering helped to restore the capacity of Xist-mediated gene silencing (Figure S10). To confirm that the observed rescue effect is due to the tethering of the candidate proteins onto the deleted A-repeat region but not the two endogenous PBSa sites along Xist, we overexpressed Ezh2-PUFa in cells carrying a Xist transgene untagged with PBSa (Figure S11A). Induced Xist expression in these cells showed a killing effect comparable to the iXist control cells. This result shows that the possible interaction between Ezh2-PUFa and the two endogenous PBSa sites within Xist did not generate a detectable effect in the cell killing assay (Figure S11A). Moreover, when Ezh2-PUFa overexpression was uncoupled from the induced Xist expression, Ezh2 overexpression alone was not able to silence the neomycin resistance gene and generate a detectable effect on cell growth (Figure S11B). Taken together, these results show that Ezh2 is important for both Xist RNA spreading and Xist-mediated gene silencing. Ythdc1 and SPOC play more specialized roles in Xist-mediated gene silencing.

It is known that PRC proteins play important roles in establishing the repressive chromatin states in XCI (Brockdorff, 2017). There are two major PRCs, PRC1 and PRC2. PRC1 catalyzes mono-ubiquitylation of histone H2A lysine 119 (H2AK119u1). Ezh2 is the enzyme of the PRC2 complex, which methylates histone H3 lysine 27 (H3K27me3). Currently, how PRC activities are recruited in XCI is under debate. One hypothesis is that PRC2 is recruited through the direct interaction between Ezh2 and Xist (Zhao et al., 2008). The other hypothesis is that the PRC1 activity recruits PRC2 (Almeida et al., 2017). To study the PRC activities in XCI, we performed immunostaining to detect H3K27me3 and H2AK119ub enrichment along Xist. Interestingly, different from what is reported in the supplementary section of a previous literature (Almeida et al., 2017), we observed that the PRC activities (H3K27me3 and H2AK119ub enrichment) were significantly attenuated in ∆A mutant (Figure 6B). This result shows that the A-repeat is involved in recruiting or spreading PRC activities. Artificial tethering of Ezh2 onto the ∆A mutant Xist transcripts significantly restored not only the H3K27me3 enrichment but also the H2AK119ub enrichment (Figure 6B). This result confirms, regardless of whether PRC2 is recruited in XCI through PRC1 in the first place, the synergetic mechanism between PRC1 and PRC2: one helps to recruit the other (Almeida et al., 2017).
DISCUSSION
The Pumilio-Based System Provides Both a Label and an Effector in Studying Long Non-coding RNAs

In summary, the Pumilio-based labeling system we report here enables high-quality live-cell imaging of Xist RNA and provides insightful details into the RNA’s behavior in live cells. Utilizing a second PBS to artificially tether candidate proteins onto Xist provides a “second handle” in the system, which helps to dissect the functional mechanisms underlying XCI. This approach can be applied to study other RNAs, in particular long non-coding RNAs.

Figure 6. Artificial Tethering of Ezh2 Restored the Attenuated PRC Activities along the ΔA Mutant Xist RNA
(A) Functional validation of the inducible Xist alleles by doxycycline-induced cell death. Cells were cultured as undifferentiated ES cells. Doxycycline treatment was carried out for 5 days. Cell survival rate was calculated by measuring area stained by alkaline phosphatase staining. Data are shown as mean ± SEM of biological triplicate. The statistical analysis used is Student’s t test. The cell survival rate of ΔA mutant was compared with the other 4 samples. *p < 0.05.
(B) Immuno-RNA FISH to detect H3K27me3, H2AK119ub, and Xist in cells, which were differentiated and treated with dox for 48 hr. The immunostains were performed before the RNA FISH. DNA was counterstained with DAPI. Data are shown as mean ± SD of biological triplicate (n = 63–153 for each sample). Scale bar, 5 μM.

See also Figures S10 and S11.
Inducible XCI in Undifferentiated ES Cells Provides a Tool to Study Both XCI and XCR

XCI and XCR are two excellent biological events for studying epigenetic mechanisms. As opposed to XCI, XCR is poorly investigated. Two waves of XCR occur during the natural life cycle of a mouse, blastocyst formation and primordial germ cell specification (Ohhata and Wutz, 2013), which are two embryonic developmental events difficult to be recapitulated in vitro. XCR also occurs in the artificial experimental system of induced pluripotent stem cell (iPSC) formation (Ohhata and Wutz, 2013), which is a slow, sporadic, and heterogeneous event less favorable for detailed studies on XCR. In this study, we show that the induced XCI status in undifferentiated cells is the balanced outcome of two counteracting forces, XCI and XCR. Genetic manipulation on related genes may tip the balance toward a predictable direction. Using this unique experimental system, we identify Kat8 and Msl2, two proteins of Drosophila dosage compensation responsible for enhancing the gene transcription from the single X chromosome in males (Lucchesi and Kuroda, 2015), as two players involved in mammalian XCR.

The observed “star” behavior of sunrise in undifferentiated cells indicates an underlying mechanism preventing Xist cloud formation. Whether Kat8 and Msl2 are directly involved in Xist cloud formation remains to be elucidated by future studies. It should also be noted that Kat8 expression may not be perfectly restricted in undifferentiated ES cells. Although Western blot results have shown that the protein level of Kat8 is sharply down-regulated during in vitro differentiation of ES cells (Li et al., 2012), our quantitative RT-PCR data did not detect the similar pattern at the RNA level (data not shown). However, somatic expression of a gene cannot rule out the gene’s role in XCR. We believe XCI is achieved through multiple layers of epigenetic modifications. In theory, genes involved in reactivating the “inner” layers of epigenetic silencing may still be expressed in somatic cells without reactivating the silenced X chromosome. Meanwhile, it would be interesting to manipulate the expression level of Kat8 and Msl2 in female cells (ES cells, somatic cells, or iPSCs) to study their effect on XCI.

A previous study showed that Kat8-associated protein complexes positively regulate Tsix expression and, as a consequence, repress Xist expression (Chelmicki et al., 2014). It is known that Tsix counteracts Xist through the action of antisense transcription across the gene body of Xist (Shibata and Lee, 2004; Luikenhuis et al., 2001). The inducible Xist transgene involved in this study does not contain the 5′ region of Tsix, including the gene’s promoter (Figure S12). Moreover, the Xist transgene is controlled by a doxycycline-inducible promoter and located ~50 Mb away from the endogenous Tsix gene. We performed RT-PCR and confirmed that no antisense transcription occurs along the 5′ region of the inducible Xist transgene (Figure S12).

Other than the intrinsic XCR, the difficulties of sunrise in undifferentiated cells could also be explained by the shortage of resources required in XCI, which are only up-regulated upon differentiation. The two possibilities are not mutually exclusive. The results of sunset experiments help to confirm that the XCR is one reason behind the difficulties of sunrise in undifferentiated cells, but do not rule out the other possibility.

Functional Dissection of Xist: Spreading versus Silencing

Artificially recruiting Ezh2, SPOC, and Ythdc1 onto the ΔA mutant Xist transcripts showed the effects of all three proteins on both Xist RNA spreading and Xist-mediated gene silencing, whereas Ezh2 showed the most significant role in Xist RNA spreading among the three. With little effect on rescuing the Xist RNA spreading defect, artificially recruiting Ythdc1 alone or SPOC alone significantly restored the killing effect. This result can be explained by the neomycin resistance gene located close to the inducible Xist transgene. A significant fraction of cell death caused by induced XCI is due to Xist-mediated silencing of the nearby neomycin-resistant gene. We carried out control experiments and confirmed that the silencing of the nearby neomycin-resistant gene was mediated by Xist, as the Ezh2 transgene showed no detectable effect when its expression was uncoupled from the induced Xist expression (Figure S11).

Technical Limitation of DNA/RNA FISH: Sensitivity versus Specificity

A variety of approaches are used in RNA or DNA FISH to increase the signal specificity. For example, a high concentration of Cot-1 DNA is mixed with the FISH probes to block unspecific hybridization. As junk DNA, the unlabeled Cot-1 DNA helps to increase the detection specificity, but it unavoidably decreases the detection resolution and sensitivity. In this study, our live-cell imaging experiments clearly revealed the small size of the ΔA mutant Xist clouds. We believe the small and compact Xist clouds observed in the ΔA mutant are Xist RNAs covering the silencing core. For the wild-type Xist clouds, we believe many
speculles, if not all, that scattered from the main territory of an Xist cloud are Xist RNAs associated with actively transcribed genes positioned further away from the main territory. Indeed, the results of RNA polymerase II (Pol II) immunostaining show that the silencing core territory devoid of Pol II staining is smaller than the territory marked by Xist-GFP (Figure S13). In fact, the scattered appearance of the wild-type Xist clouds is occasionally seen in RNA FISH (data not shown). Similarly, DNA FISH on X-linked genes occasionally detects a gene located “far away” from a Xist cloud detected in RNA FISH or a “chromosome territory” detected by chromosome paint probes in DNA FISH (Chen et al., 2016). However, no specific interpretation has been made on these observations. We believe many actively transcribed X-linked genes are located outside of the “chromosome territory” defined by the Xist RNA FISH signals or the chromosome paint DNA FISH signals. The scattered appearance of Xist clouds clearly detected in live-cell imaging indicates Xist RNAs spreading out to cover the actively transcribed genes, which are located not only at the periphery of the main territory of a Xist cloud but also farther away from it. How Xist RNA spreads out and precisely covers X-linked genes that are actively transcribed from its host chromosome and located “far away” from the main chromosome territory awaits future studies.

Technical Limitations of the Study

It is noticeable that the experimental system of this study has its own technical limitations. The “wild-type” Xist in this study is an induced transgene in male cells located at the Hprt locus carrying PBSb tags on its 5’ end. Its behavior may not be identical to that of the endogenous Xist in female cells. However, all the foregoing artificial features are commonly shared by the “wild-type” and the mutant Xist, in undifferentiated and differentiating cells. The comparison between the “wild-type” and the mutant and the comparison between undifferentiated and differentiating cells help to reveal the biological insights, as the observed differences cannot be attributed to the artificial features commonly shared by all parties.

It should be also noticed that, with the secondary structure of Xist largely unknown, any tagging of the RNA has the potential risk of disrupting the RNA’s putative secondary structure. However, it is unlikely that the long RNA forms a single rigid secondary structure. In a study using a serial deletion approach to functionally dissect Xist, A-repeat was identified as the single most critical domain for the Xist functionality (Wutz et al., 2002). This result argues against the possibility that the full-length Xist RNA forms a scaffold with a rigid structure. Indeed, it is hypothesized that Xist functions as a malleable scaffold with modular and swappable functional domains (Guttman and Rinn, 2012). Based on current research, A-repeat may form its own structural domain independent from the remaining parts of the long RNA (Liu et al., 2017). Therefore, replacing A-repeat with PBS may not affect the global secondary structure of the long Xist RNA. Sequence wise, the A-repeat region consists of 7.5 sequence repeats (Broedonf et al., 1992). Structure wise, the region may form an elaborated secondary structure in which the sequence repeats behave differently in forming different structural units (Liu et al., 2017). How the structural units recruit proteins and how many copies of each protein are recruited by the structural units remain unknown. In this study, we assumed that each sequence repeat recruits one copy of the candidate protein and used 10 copies of PBSa to replace the A-repeat region. In theory, 10 copies of each target protein can be tethered onto each RNA transcript maximally. The actual amount of each candidate protein recruited onto each RNA transcript in this study may differ from the endogenous situation and can be further optimized in future studies.

Xist is a long RNA with the critical functional domain near its 5’ end. In theory, the live-cell imaging tag can be placed either at the 5’ or 3’ end of the RNA. Tagging at the 3’ end may prevent the imaging tag from interfering with the functional domain of Xist. On the other hand, 3’ tagging may not allow the system to closely monitor the RNA’s behavior in live cells. Labeling at the 5’ end provides the opposite advantage and disadvantage. In this study, we chose 5’ tagging, because we encountered technical difficulties in generating the DNA constructs of 3’ tagging. Our data show that the Xist functionality remains largely intact with 5’ tagging.

In live-cell imaging, the length of the observation time window is limited to 2–3 hr due to technical issues, such as photobleaching, cell tracing, re-focusing, and stage drifting.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods, 13 figures, and 4 videos and can be found with this article online at https://doi.org/10.1016/j.isci.2018.09.007.

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AUTHOR CONTRIBUTIONS

L-F.Z. conceived the idea. L.C. and L-F.Z. designed the experiments. N.H. performed the majority of the experiments. S.P.Y.V., Y.Z., and L-T.L. performed shRNA knockdown experiments. R.C. established the iXist cell line. N.H., L.C., and L-F.Z. analyzed and interpreted the data. S-K.L. and H-Y.L. provided technical assistance on Airyscan microscopy. A.L. and S.S. provided technical support for spinning disk confocal microscopy. L.C. and L-F.Z. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Live-Cell Imaging and Functional Dissection of Xist RNA Reveal Mechanisms of X Chromosome Inactivation and Reactivation

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Figure S1: The cross-reactivity of PUFa / PBSb and the PBSa sites identified in the endogenous sequence of *Xist* (Related to Figure 1). (A) The inducible *Xist* transgene carries 25 copies of PBSb at its 5’ end. The cells were transiently transfected with a plasmid carrying a PUFa-EGFP fusion gene. The transfected cells were treated with dox for 24 hours. A live-cell image of the GFP channel is shown. The image was taken using a wide-field fluorescent microscope. No *Xist* signals were detected from the entire cell population, which confirms no cross-reactivity between PUFa and PBSb. (B) A map of *Xist* gene with the PBSa sites identified in its endogenous sequence.
Figure S2: The emergence of an $Xist$ RNA signal upon dox induction

(Related to Figure 2). The emergence of an $Xist$ RNA signal in a differentiating ES cell. Images are shown in a 90-min time span with a 10-min time interval. The time point when the signal was first detected is defined as time zero. Maximum intensity z-projections are shown.
Figure S3: Quantitative RT-PCR results to show the effect of induced $Xist$ expression on X-linked genes in undifferentiated cells (Related to Figure 3).

Dox treatment was carried out for 24 hours. The results are shown in relative fold expression. Normalization was performed using Actb and Gapdh. Error bars indicate SEM ($n = 3$). The statistical analysis used is the Student’s t-test. One asterisk indicates $P$-value smaller than 0.05.
Figure S4: Measurement of Nanog signal intensity (Related to Figure 3).

Immuno-RNA FISH to detect Nanog and Xist in undifferentiated iXist cells treated with dox for 3 hours. Cells were cultured in the conventional Lif-containing ES culture medium without 2i. Nanog was detected by immunostain. The mean Nanog signal intensity in each cell was measure by ImageJ. To normalize the signal intensity variance among difference microscope images, the cells within one image were ranked according to the mean Nanog signal intensity within each cell. The cell with the highest ranking was scored 1 for its “Relative Nanog Signal Intensity”. The cell with the lowest ranking was scored 0. The rest of the cells from the same image were scored between 1 and 0 proportionally according to each cell’s mean Nanog signal intensity. The relative Nanog signal intensity value 0.5 is marked by a dashed line.
Figure S5: The growth curves of GFP-iXist cells during the 6-hour time window after Dox removal (Related to Figure 3). Data are shown as mean ± S.D. of biological triplicate.
Figure S6: Test on the involvement of histone acetyltransferase candidate proteins in XCR (Related to Figure 4). (a) Doxycycline-induced cell death assay was carried out on iXist cells. The cells were either treated with chemical inhibitors for p300 or transfected with shRNAs against different candidate...
proteins. Dox treatment was carried out for 4 days. Data are shown as mean ± S.E.M of biological triplicate. Note: The cell survival rates were calculated based on hemocytometer cell count, which has more experimental error than alkaline phosphatase staining. (b) Quantitative RT-PCR to validate the effects of shRNA knocking-down. Data are shown in relative fold expression. Normalization was performed using Actb and Gapdh. Error bars indicate SEM (n = 3).
Figure S7: Using a 2nd shRNA against Kat8 and Msl2 to rule out the shRNA off-targeting effect (Related to Figure 4). (A, B) Doxycycline-induced cell death assay on different inducible Xist cell lines. Cells were cultured as undifferentiated ES cells. Dox treatment was carried out for 4 days. Cell survival rate was calculated by AP stained colony counts. Data are shown as mean ± S.E.M of biological triplicate. The statistical analysis used is the Student’s t-test. One asterisk indicates P-value smaller than 0.05. (C) Quantitative RT-PCR to validate the effects of shRNA knocking-down. Data are shown in relative fold expression. Normalization was performed using Actb and Gapdh. Error bars indicate SEM (n = 3).
Figure S8: Expression profile of pluripotency-related genes in iXist and cells carrying shRNAs against Kat8 and Msl2 (Related to Figure 4).

Quantitative RT-PCR results are shown in relative fold expression. Normalization was performed using Actb and Gapdh. Error bars indicate SEM (n = 3). Cells were cultured as undifferentiated cells. Nanog, Sox2, Oct4, Klf4 and Fut9 are supposed to be expressed at high levels in undifferentiated cells. Gata4 and Fgf5 are not supposed to be expressed or expressed at very low levels in undifferentiated cells. To show the low expression levels of Gata4 and Fgf5, Nanog expression level in iXist was included in the plots for direct comparison.
**Figure S9:** The Repeat A region of *Xist* is involved in the spreading of *Xist* RNA along the chromosome territory (Related to Figure 5). Cells were cultured as undifferentiated ES cells. Dox treatment was carried out for 24 hr. Representative images of *Xist* RNA signals in live cells are shown as maximum intensity z-projections.
Figure S10: Quantitative RT-PCR results to show the effect of Ezh2-tethering on X-linked gene expression (Related to Figure 6). Cells were cultured as undifferentiated cells. Dox treatment was carried out for 24 hours. The results are shown in relative fold expression. Normalization was performed using Actb. Error bars indicate SEM (n = 3).
Figure S11: The side effects of Ezh2-PUFa overexpression without the induced Xist expression (Related to Figure 6). (A) Doxycycline-induced cell death assay was carried out for 6 days and alkaline phosphatase staining was performed. Data are shown as mean ± S.D. of biological triplicate. (B) The growth curves of Ezh2-PUFa overexpressing ΔA cells cultured with or without G418. Data are shown as mean ± S.D. of biological triplicate.
Figure S12: RT-PCR to detect the endogenous *Tsix* expression and the antisense transcription along the inducible *Xist* transgene (Related to Figure 4). Total RNA was isolated from undifferentiated iXist cells. Reverse transcription reactions were carried out using either a gene-specific primer for the endogenous *Tsix* (the blue arrowhead) or a gene-specific primer designed for the antisense transcription across the inducible *Xist* transgene (the orange arrowhead). cDNA samples were PCR amplified using a primer pair (the two black arrowheads) targeting the 5’ region of *Xist* exon 1. The black arrow points to the PCR amplicon with the expected size.
Figure S13: ImmunoRNA FISH of RNA polymerase II (Pol II) and Xist

(Related to Figure 5). (A) Immunostain of Pol II on an undifferentiated GFP-iXist cell treated with dox for 24 hours. 3D localization of Pol II (Alexa 647), Xist (GFP) and DAPI were examined using Airyscan Super-resolution Confocal Microscope (Carl Zeiss) (X-Y: 36nm per pixel; Z: 140nm per pixel). Red dotted lines illustrate the silencing core territory of the Xist cloud without Pol II staining. Yellow dotted lines illustrate the main territory of the Xist cloud. Scale Bar: 2 µm. (B) Graphical illustration showing the Pol II staining pattern and the Xist cloud in 3D.
**Transparent Methods**

**Cell lines and culture**

If not explicitly stated otherwise, mouse ES cells were cultured in 2i medium with Lif (Ying et al., 2008). For in vitro differentiation, cells were cultured in differentiating medium containing 50 μg/ml L-ascorbic acid (Sigma). Doxycycline treatment of 1 μg/ml was used throughout the study. G418 (ThermoFisher) treatment was carried out at 400 μg/ml. Inhibitors for p300, L002 (Sigma) and C646 (Sigma), were used at 10 μM and 25 μM respectively.

Alkaline phosphatase (AP) staining (Vector Laboratories) and Crystal Violet staining (Merck) were used to quantify undifferentiated ES cells and differentiating ES cells respectively. CellProfiler (Carpenter et al., 2006) and ImageJ (Schneider et al., 2012) were used to analyze AP staining data and Crystal Violet staining data respectively.

**RNA FISH, immunostaining and immuno-RNA FISH**

RNA FISH, immunostaining and immuno-RNA FISH were carried out as previously described (Zhang et al., 2007). Immunostaining for Nanog was performed using a rabbit polyclonal antibody against Nanog (Abcam; ab80892; 1:200) with a secondary antibody conjugated with Alexa-488 (ThermoFisher; A-11008; 1:1000). Immunostaining for Pol II was performed using a mouse monoclonal antibody against Pol II (Santa Cruz; sc-47701; 1:500) with a secondary antibody conjugated with Alexa-647 (ThermoFisher; A-21236; 1:500).
Immunostaining for H3K27me3 was performed using a mouse monoclonal antibody (Abcam; ab6002; 1:1000) with a secondary antibody conjugated with Alexa-647 (ThermoFisher; A-21236; 1:1000). Immunostaining for H2AK119ub was performed using a rabbit monoclonal antibody (Cell Signaling Technology; D27C4; 1:2000) with a secondary antibody conjugated with Alexa-647 (Abcam; ab150075; 1:1000). Immunostaining was followed by RNA FISH. The Xist RNA was detected with Sx9 probe, a P1 DNA construct containing a 40 kb genomic fragment covering the Xist gene. Nucleotide analogs used in probe labeling were Cy3-dUTP (Amersham, Cat# PA53022).

**Quantitative RT-PCR**

Total RNA was isolated by TRIzol (Life technologies). cDNA was synthesized using iScript reverse transcription kit (170-8840, Bio-Rad). The real-time PCR was carried out on the CFX Connect real-time PCR system (Bio-Rad) using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). The following PCR primers were used: Actb (F:5’- ACTGCCGCATCCTCTTCTCTC-3’, R: 5’-CCGCTCGTTGCCAATAGTGA-3’); Gapdh (F:5’-CCAATGTGTCCGTCGTGGAT-3’, R: 5’-TGCTCTGCTTCACCACCTTCT-3’); Nanog (F:5’-TCGAATTCTGGGAACGCCTC-3’, R: 5’-GTCTTCAGAGGAAGGGCGAG-3’); Sox2 (F:5’-TTCGAGGAAAGGGTTCTTGCTG-3’, R: 5’-TCCTTCCTTGTGATACCGGTCCT-3’); Oct4 (F:5’-TGTTCCCGTCACTGCTCTGG-3’, R: 5’-TTGCTTTGGGCACAGCATC-3’); Klf4
(F:5’-GTGCCCGACTAACCCTTTG-3’, R: 5’-GTGGTTGAACTCCTCGGTCT-3’); Fut9 (F:5’-CAGTCAATGAGCTGCAA-3’, R: 5’-
CCATACCCAAACCAGAATGG-3’); Gata4 (F:5’-
CCTGGAAGACACCACATCTC-3’, R: 5’-GGTAGTGTCCCCGTCCCATC-3’); Fgf5 (F:5’-CCTTGCGACCCAGGAGCTTA-
CCGTCTGTGGTTTCTGTTGAGG-3’); Kat8 (F:5’-
AACCAGAAGTCCAAGGACGCCT-3’, R: 5’-TCCTGGTCATTCGAGAC-3’); Msl2 (F:5’-CCGGGTGACTCTCTTTTGCT-
GCTTCCAAGTTTGGCTGCAA-3’); MeCP2 (F:5’-
CAGGGAGGAAAAGTCACCGTGGAG-3’); Hprt (F:5’-GATTAGCGATGATGAACCAGTT-3’, R: 5’-
CCTCCCATCTCTTTCATGACA-3’); Gpc4 (F:5’-GGCAGCTGGCACTAGTTTG-3’, R: 5’-
AACGGTGCTTGGGAGAGAG-3’); Neomycin (F:5’-
GGCTATTCGGCTATGACTGGGC-3’, R: 5’-
GCAGTTCATTCAGGGCACCG-3’); tdTomato (F:5’-CCGACATCCCCGATTAAAAGCT-3’, R: 5’-
TTGTAGATCGGTCCGCT-3’); Xist (E1_F: 5’-
CGGCCCTCTAGTTTGTCCATT-3’, E1_R: 5’-GATGGCATGATGGAATTGTA-3’).

**Plasmid Constructs**

PBS and PUF plasmids were obtained as gifts from Dr. Wang Haoyi. *galK*-mediated recombinering system was used to generate all the DNA constructs of inducible Xist transgenes (Warming et al., 2005).
A human SPEN cDNA (~11kb) clone was purchased from OriGene (RC213922). A frame shift mutation (3351_3352insA) within the coding region of SPEN was found in the original cDNA clone. The coding region of the SPOC domain is intact and was PCR amplified. cDNA of Ezh2 and Ythdc1 were generated using SuperScript III reverse transcriptase kit (ThermoFisher) followed by PCR amplification using Herculase II Fusion Enzyme (Agilent Technologies). The sequences of the cloned cDNA fragments of SPOC, Ezh2 and Ythdc1 were confirmed by Sanger sequencing (data not shown). The following PCR primers were used: Ezh2 (F:5'-ATGGGCCAGACTGGGAAGAAATC-3', R:5'-CATTTCTCGTTCCATGCCCACATA-3'); Ythdc1 (F:5'-ATGGCGGCCGACAGC-3', R:5'-AACGACCTCTCTCCCTCCG-3'); SPOC (F:5'-ATGCTCTCAAGTGTCCAGGAG-3', R:5'-AATGACAATCATGAGGTGGGGAGAG-3'). Plasmid constructs for fusion proteins, PUF-EGFP and PUF-effector, were established using Gibson Assembly (NEB).

An shRNA system (OligoEngine, pSUPER RNAi System) was used. The following shRNA sequences were designed against Msl1 (5'-GTACCTTTCACCACAGAAT-3'), Msl2 (5'-CCCATGCTCTTAGCCATAATG-3'), Msl2-2 (5'-CTGACCTCAAGCTAGCTTAT-3'), Msl3 (5'-GCTGCGTTCAAGAAAGGAAAT-3'), Kat7 (5'-CCTCGAACTCCAACCGGAAAT-3') and Kat8 (5'-GTGATCCAGTCTCGAGTGA-3'), Kat8-2 (5'-GCGAAAGCATGAGATCAA-3').
Microscopy

Wide-field fluorescent microscopy work was carried out on an Eclipse Ti microscope (Nikon) with a digital camera (Clara Series model C01, Andor). Airyscan microscopy was carried out on a Zeiss LSM 710 inverted confocal microscope with Airyscan detector and an oil immersion alpha Plan-Apochromat 100x/1.4 Oil M27 objective lens.

Live-cell imaging was carried out on a CorrSight spinning disk confocal system (FEI Company) equipped with an Orca R2 CCD camera (Hamamatsu). 1 day before imaging, 800K feeder-free ES cells were seeded on fibronectin-coated glass-bottom dishes (MatTec Corp). Prior to live-cell imaging, cells were washed with 1x PBS and replaced with imaging medium composing complete medium with DMEM substituted with FluoroBrite DMEM (ThermoFisher). For sunrise experiments, 1 μg/ml of doxycycline was supplemented to the imaging medium to induce Xist expression. For sunset experiments, Xist was induced by 1 μg/ml of doxycycline for at least 16 hr. The cells were washed a few times using 1x PBS. The culture medium was then switched to the imaging medium free of doxycycline. For live-cell time-lapse video recording, cells were placed into the microscope cell culture chamber heated to 37 °C at least 1 hr before imaging. Imaging was carried out in a closed chamber maintained at 37 °C with 5% CO2 and 90% humidity. DNA dyes, such as Hoechst, was avoided due to its toxicity in long-term live-cell imaging. A 488-nm laser line (iChrome MLE-LFA) was set at 100% laser power. Images were acquired using a PlanApo 63x/1.4
N.A. oil-immersion objective (Zeiss) (heated to 37 °C) with standard filter sets. The exposure time was set at 200 ms. All live-cell time-lapse video recording, unless explicitly stated otherwise, was carried out in a 2-hr time span with a 2-min time interval. For each time point, a 10-μm thick Z-stack with a 1-μm interval was collected. Autofocus system (Focus Clamp) was used to minimize out-of-focus throughout the recordings. Time-lapse imaging was started 2 hr after the addition of doxycycline for undifferentiated cells and 1 hr after the addition of doxycycline for differentiating cells. Other than time-lapse video recording, the snap-shot images of Xist signals in live cells were captured with an 800-ms exposure time at 100% laser power in 10-μm Z-stacks at 1-μm intervals. Maximum intensity Z-projection was used to represent each Z-stack. All acquired images were processed and analyzed using ImageJ (Schneider et al., 2012). Drift correction was applied to all time-lapse recordings.

**Statistical analyses**

All data were analyzed by one-tailed Student’s t-test for equal variances and were conducted in Excel. A $P$-value that is less than 0.05 is considered statistically significant. Statistical tests used for all experiments were described in detail in the relevant figure legends of the Results and Supplemental Information.
Supplemental References

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