Methods S1 file

Mathematical Appendices, Related to STAR Methods

Xist foci trajectories and effective confining potentials

Trajectories of individual Xist foci were extracted using single-particle tracking. After subtracting the overall translation due to developmental motion, three-dimensional coordinates of foci i, $(x_i(t_j), y_i(t_j), z_i(t_j))$ were collected at times t_j. Since each trajectory of about 10-15 sets of positions of foci are unsynchronized, we assumed that all foci are in a local equilibrium position at the start of the measurements. We thus aggregated all position points and assumed they are distributed according to an effective potential $U(\mathbf{r})$ where $\mathbf{r}$ is the position of a focus relative to its minimum-energy position.

Under this local equilibrium approximation, we evaluated whether or not the foci-confining effective potential changes over time. To further simplify matters, we assumed spherical symmetry in $U(\mathbf{r}) = U(r)$ and that each trajectory is represented by a particle in local thermodynamic equilibrium. After recentering each trajectory, the position vectors $r_i(t_j)\hat{\mathbf{r}}$ were defined via the magnitude

$$r_i(t_j) = \sqrt{(x_i(t_j) - X_i)^2 + (y_i(t_j) - Y_i)^2 + (z_i(t_j) - Z_i))^2}, \qquad \text{(Eq. S1)}$$

where (X_i, Y_i, Z_i) is the center-of-mass coordinate of trajectory i. Taking all positions within a trajectory and across all foci, we have a collection of N points with displacements r_k representing a sample from an underlying distribution $\rho(r)$. Associated with this distribution, we defined an effective energy potential $U(r)$ according to $\rho(r) \sim e^{-U(r)}$ (Berg and Harris, 2008).

To find $\rho(\mathbf{r})$ from our finite position data requires careful regularization or Bayesian approaches (Chang et al., 2015, 2014). As a first approximation, we defined the cumulative distribution function $\hat{C}(r)$ over the magnitude of displacements r (Berg and Harris, 2008; van Zon and Schofield, 2010),

$$\hat{C}(r) = \frac{1}{N}\sum_{k=1}^{N} \mathbb{1}(r_k, r), \qquad \text{(Eq. S2)}$$

where $\mathbb{1}(r_k, r) = 1$ if $r_k < r$ and zero otherwise. Mathematically, the cumulative distribution function (CDF) is related to the three-dimensional spherically symmetric density through

$$C(r) = 4\pi \int_0^r \rho(r')r'^2 \mathsf{d}r'. \qquad \text{(Eq. S3)}$$

The inference of $\rho(r)$ and ultimately $U(r)$ from discrete data $\hat{C}(r)$ can be quite ill-conditioned (sensitive to small variations in $\hat{C}(r)$) and some regularization, for example, through parametric fitting to a smooth function, should be applied. The parametric fitting to $\hat{C}(r)$ is further constrained by the symmetry and analytic properties we impose on the potential $U(r)$. For example, smoothness of $U(r)$ at $r \to 0$ precludes $n \leq 4$ (except for $n = 3$ which leads to a constant in $U(r = 0)$) in the small r property of $C(r) \sim r^n$. Finally, the cumulative needs to be normalized: $C(r \to \infty) \to 1$. We chose a quadratic potential $U \sim r^2$ as a "trial function" and added appropriate terms as corrections. Upon substituting $U(r) = kr^2$ into Eq. S3, we find the lowest order cumulative

$$\begin{aligned} C_0(r) &\propto \int_0^r e^{-kr'^2} r'^2 \mathsf{d}r' \propto k^{-3/2}\left[\sqrt{\pi}\mathrm{Erf}(\sqrt{k}r) - 2\sqrt{k}re^{-kr^2}\right] \\ &\approx \frac{r^3}{3k^{3/2}} - \frac{r^5}{5\sqrt{k}} + \frac{r^7\sqrt{k}}{14} - \ldots. \end{aligned} \qquad \text{(Eq. S4)}$$

To this single-parameter (k) function, we added the parametric correction function

$$\delta C(r) = \left[a_3 r^3 + \sum_{j=5} a_j r^j \right] e^{-br^2}, \qquad \text{(Eq. S5)}$$

and fitted the total function $C(r|\theta) = C_0(r|k) + \delta C(r|a_j, b)$ to the experimental data $\hat{C}(r)$. For our analysis, we used the odd coefficients $j = 5, 7, 9, 11$ and found the best-fit parameters $\theta^* = \{k^*, a_j^*, b^*\}$ to construct the best-fit density

$$\rho(r|\theta^*) \equiv \frac{1}{4\pi r^2} \frac{\mathrm{d}C(r|\theta^*)}{\mathrm{d}r}, \qquad \text{(Eq. S6)}$$

from which we extracted the potential $U^*(r) \sim -\log \rho(r|\theta^*)$. We have also tested the robustness of our potential reconstruction by using kernel density estimation to find similar potentials.

Mass-action binding and dissociation model of FRAP dynamics

We propose a simple model describing photobleached and fluorescing molecules binding to and detaching from specific sites. The fluorescence of Xist in our experiments relies on the fast and strong binding of freely diffusing MCP-GFP to newly produced Xist. An abundance of MCP-GFP ensures that newly produced Xist "instantaneously" fluoresces, and that once photobleached, stays photobleached. We assume that before photobleaching, the concentration of either Xist or a specific Xist-effector protein is at their specific steady-state value C_{ss} in the photobleaching region of interest. Binding sites are also assumed to be uniformly randomly distributed in the photobleaching region and can contain different classes of binding sites, with each class having different molecular attachment and detachment rates. A schematic of the FRAP process is shown in **Figure 4C**. Additionally, we assumed that molecules bind to these different sites in a parallel manner, not relying on the occupancy of the other types of sites.

In steady state, the overall concentrations of molecules bound to the i-type sites are denoted by $C_i(t)$. After photobleaching, the bulk free molecule concentration is replaced by fluorescing protein or Xist that diffuses into the evaluation region. This diffusion process, including binding and unbinding to sites, has been modeled using mass-action diffusion-reaction equations with complex solutions (Braga et al., 2007). Here, we made the approximation that the replacement is fast and that the concentration of fluorescing molecules reaches $C_{\mathrm{ss}}^* \approx C_{\mathrm{ss}}$ very shortly after photobleaching, but before appreciable dissociation has occurred. Thus, at very short times, the fluorescence recovery curves may appear to start at finite levels corresponding to the fluorescence of the free, newly replaced molecules. After this fast replacement, the bulk concentration of fluorescing Xist or protein will be denoted $C_{\mathrm{ss}}^* = C_{\mathrm{ss}}$, where the $*$ indicates a concentration of fluorescing molecules. The time-dependent concentration of fluorescing molecules bound to a type-i binding site is correspondingly denoted by $C_i^*(t)$. The mass-action equations for the photobleached and fluorescing protein concentrations are:

$$\begin{aligned} \frac{\mathrm{d}C_i(t)}{\mathrm{d}t} &= -d_i C_i(t) + k_i (C_i^{(0)} - C_i(t) - C_i^*(t)), \\ \frac{\mathrm{d}C_i^*(t)}{\mathrm{d}t} &= -d_i C_i^* + k_i^* (C_i^{(0)} - C_i(t) - C_i^*(t)), \end{aligned} \qquad \text{(Eq. S7)}$$

where $C_i^{(0)}$ is the total concentration of available i-type sites, d_i are dissociation rates from i-type sites, and $k_i \equiv b_i C_{\mathrm{ss}}$ and $k_i^* \equiv b_i C_{\mathrm{ss}}^*$ are the bulk concentration-weighted attachment rates. The initial condition immediately after photobleaching is $C_i^*(0) = 0$. The initial steady-state fraction of all available sites containing photobleached molecules, $C_i(0) \lesssim C_i^{(0)}$, should be close to the total number of available sites and can be found by considering the steady state before photobleaching (when $C_i^* = 0$) $\mathrm{d}C_i/\mathrm{d}t = -d_i C_i + k_i (C_i^{(0)} - C_i) = 0$, leading to the initial condition $\bar{C}_i = k_i C_i^{(0)} / (k_i + d_i)$.

The concentration $C_{\rm ss}$ of photobleached molecules near binding sites decreases in time as they diffuse away and degrade. Rather than formulate the full diffusion-degradation equation for $C_{\rm ss}$, as was done in Braga *et al.* 2007, we assumed that once photobleached molecules dissociate, they are lost to the bulk (instantaneously replaced by unbleached molecules) and do not rebind. Thus, we approximated $k_i = b_i C_{\rm ss}(t) \approx 0$ and solved Eqs. S7 to find $C_i(t) \approx C_i(0) e^{-(k_i + d_i)t}$ and

$$C_i^*(t) = \frac{k_i^* C_i^{(0)}}{k_i^* + d_i} \left(1 - e^{-d_i t}\right). \qquad \text{(Eq. S8)}$$

By including the steady-state bulk fluorescence $C_{\rm ss}^*$ and all possible site types i, we constructed the total fractional fluorescence recovery,

$$\begin{aligned} F(t) &= \frac{C_{\rm ss}^* + \sum_i \left(\frac{k_i^* C_i^{(0)}}{k_i^* + d_i}\right)(1 - e^{-d_i t})}{C_{\rm ss}^* + \sum_i \left(\frac{k_i^* C_i^{(0)}}{k_i^* + d_i}\right)} \\ &= 1 - \frac{\sum_i \bar{C}_i^* e^{-d_i t}}{C_{\rm ss}^* + \sum_j \bar{C}_j^*}, \quad \bar{C}_i^* \equiv \frac{k_i^* C_i^{(0)}}{k_i^* + d_i}, \\ &\equiv 1 - \sum_i f_i e^{-d_i t}, \end{aligned} \qquad \text{(Eq. S9)}$$

where

$$f_i \equiv \frac{\bar{C}_i^*}{C_{\rm ss}^* + \sum_{j=1} \bar{C}_j^*} \qquad \text{(Eq. S10)}$$

is related to the fractional contribution to the fluorescence from molecules that bind to type i sites. These fitting formulae mirror previous standard FRAP models (McNally, 2008; Braga et al., 2007; Kang et al., 2010).

As a consequence of the initial steady-state concentrations and the neglect of $C_{\rm ss}(t)$, the FRAP recovery dynamics depend only on the detachment rates d_i. Thus, each type of site leads to a simple exponential rise in FRAP recovery. To allow for a biphasic recovery, we also considered a two binding site model $i = 1, 2$. The slower recovery, ($d_2 < d_1$) would describe the dissociation from stronger binding sites and may appear as non-recovering populations in FRAP curves (immobile fractions), especially if the FRAP experiment does not extend to long times.

We fitted all FRAP curves with a two-rate model (using f_1, f_2, d_1, d_2 as the four fitting parameters) and found that for Xist and CIZ1 recovery, only one dissociation rate $d_1 \equiv d$ is observable. The lifetimes $\sim 1/d_1$ for these two components are on the order of many minutes.

Since Xist and CIZ1 recovery is not complete by the end of the experiment at time T, any other types of possible binding sites must be strongly binding and have extremely slow dissociation rates $d_2 \ll 1/T$. However, the fraction f_2 of slow binding sites can be estimated from the final recovery plateau at the end of the experiment ($T \approx$30min). The Xist and CIZ1 recovery curves clearly have three identifiable features, the "initial" recovery at short times, the exponential rise, and the final recovery level. These three features determine the three parameters $1 - f_1 - f_2$, d_1, and $1 - f_2$, respectively.

For all other proteins examined, there is initially a much faster recovery process on the order of seconds and the FRAP curves exhibit two time scales of recovery. In these cases, a biphasic model provides stable best-fits. This indicates that there is a complex distribution of binding sites for each protein in the Xi and that these binding sites either have different binding strengths or dissociate sequentially (Brackley and Marenduzzo, 2020). We approximated the heterogeneity in binding sites by two effective populations.

Expression-diffusion-degradation model of Xist confinement

While our research uncovered mechanisms of Xist and Xist-effector protein accumulation in the X chromosome, the proposed mechanistic picture ultimately relies on the confinement of Xist to the X chromosome throughout the XCI process. Xist has been shown to be constitutively expressed throughout the inactivation process (Hendrich et al., 1997) (also see **Figure S2E**) and its concentration will continue to increase, unless its degradation is sufficiently active. Here, we provided a qualitative mechanistic argument for Xist confinement. Although the Xist RNA will have many complex interactions within the X, conservation of their overall numbers requires a balanced degradation of Xist to prevent its continual spread out of the X.

First, we assumed that Xist molecules do not strongly interact with each other or other proteins. The spread of independent Xist molecules can then be described by an effective, simple diffusion process. Along with degradation and a localized source (from the Xist expression site), a rough description of Xist distribution is a mass-action expression-diffusion-degradation model

$$\frac{\partial C(\mathbf{r},t)}{\partial t} = \nabla \cdot (D(\mathbf{r},t)\nabla C(\mathbf{r},t)) - \mu(\mathbf{r},t)C(\mathbf{r},t) + S(\mathbf{r},t), \qquad \text{(Eq. S11)}$$

where $C(\mathbf{r},t)$ is the concentration of Xist at position $\mathbf{r}$ and time t, $D(\mathbf{r},t)$ is the effective diffusivity of Xist in the chromatin environment, $\mu(\mathbf{r},t)$ is the Xist degradation rate, and $S(\mathbf{r},t)$ is the source of Xist. A simplifying approximation is to assume spherical symmetry, that D and μ are constants, and that the source $S(\mathbf{r},t) = S_0\delta(\mathbf{r})$ derives from a "δ-function" source representing a single expression site at position $\mathbf{r} = 0$ within the X chromosome. Here, and in the preceding FRAP model, we assumed that the Xist cloud is quickly formed and considered the 3D steady state solution

$$C_{\rm ss}(r) = \frac{S_0}{r}\exp\left[-\sqrt{\frac{\mu}{D}}r\right]. \qquad \text{(Eq. S12)}$$

This simple diffusion-degradation model yields an Xist concentration profile that is confined within a radius of $L \approx \sqrt{D/\mu}$. Thus, an X-chromosome size of $L \sim 2\mu$m places a constraint on the ratio D/μ. Xist RNA has high affinity to chromatin through its binding to proteins that contain DNA binding domains (Mor et al., 2010) which can be considered as a distribution of association sites. Transport across these association sites can thus be described by a small effective diffusivity that has been measured to be with a range $D \approx 0.005 - 0.02\mu$m^2/s or $D \sim 5\times10^{-11} - 2\times10^{-10}$cm^2/s (Eliscovich et al., 2013; Braga et al., 2007; Mor et al., 2010). This effective diffusivity is measured in the presence of strong interactions and the real "free" effective diffusion in the "non-strongly-binding" regions of the chromatin are typically larger. If $D \sim 10^{-9}$cm^2/s, a free-Xist lifetime of $1/\mu \lesssim 1$min would be necessary for it to remain confined within distance L. In the preceding FRAP analysis (**Mass-action binding and dissociation model of FRAP dynamics**), the uniform steady-state concentration $C_{\rm ss}$ can be identified as the X-region-average of $C_{\rm ss}(\mathbf{r})$ from solving Eq. S12.

This simple conservation argument provides a qualitative mechanism for Xist localization. From this starting point, other processes such as the binding of Xist to itself, to specific sequences, or to other factors can be incorporated. Such additional interactions, especially self-binding, could generate sharper confinement of Xist molecules or foci to the X-chromosome and may be invoked to explain the asymmetric distribution of Xist about transcription site at $\mathbf{r} = 0$, which is typically not in at the center of the X, but at its periphery (see **Figure S2E**). More complex interactions also lead to higher order structures such as multimeric foci or supramolecular complexes of RNA and proteins within which Xist may be very stable.