Supplementary Note 1

In this study we focused on the roles of PTBP1, MATR3, TDP-43 and CELF1 in *Xist* localization, gene silencing and the formation of an epigenetic memory during the formation of the Xi in differentiating female ESCs. At the outset, we were interested in examining direct *Xist* interactors identified in our previous study[1] that had RNA binding activity but no additional annotated functional domains. In scanning the *Xist* repeat regions for potential binding sites for these factors, we identified multiple putative sites within the E-repeat (see Extended Data Fig. 3). Using a biotinylated *in vitro* transcribed E-repeat RNA, we were able to show that CELF1, PTBP1 and its well-known binding partner MATR3 bind the E-repeat, but not the A-repeat or other control regions of *Xist* (data not shown). In addition, we found that factors such as RALY, hnRNP-C and hnRNP-K (which is now known to bind the B-repeat[2]), do not bind the E-repeat (data not shown). We then expanded our candidate pool to additional *Xist*-associated proteins identified in other publications[3–6] and found iCLIP- and eCLIP-seq data in multiple cell types that supported direct binding of TDP-43 to the E-repeat of *Xist*[6–9]. We note that ELAVL1 has been suggested to bind the E-repeat[6], but current genomics data suggest that ELAV1 may also bind other regions of *Xist* with equal or higher affinity[6,10]. Thus, we limited our work to those factors whose binding was almost exclusively limited to the E-repeat sequence of *Xist*, which yielded PTBP1, MATR3, CELF1, and TDP-43.

Supplementary Note 2

Within the field of XCI, it is currently unclear how enrichment - as detected by immunostaining - of a factor within the *Xis*t-coated Xi relates to its direct/indirect interaction with *Xist* and its function during XCI. The nucleoplasmic levels of the E-repeat binding RBPs PTBP1, MATR3, CELF1 and TDP-43 are very high[11] due to their roles in RNA processing. Thus, an even higher enrichment in the Xi may not be necessary for function. Indeed, the SHARP/SPEN protein does not strongly enrich on the inactive X-chromosome when assessing the localization of the endogenous protein in cells inducibly expressing *Xist* by immunostaining[5], despite it now being well-recognized as the protein through which *Xist* mediates X-linked gene silencing[1,12,13]. Similarly, hnRNP-U which is recognized as a direct interaction partner of *Xist* and functionally important for *Xist* spreading and association with chromatin, does not strongly enrich within the Xi with immunostaining[14]. Conversely, chromatin complexes that do not directly interact with

Xist RNA, such as PRC2 and PRC1, enrich on the Xi[15–17]. Understanding the relationship between Xi-enrichment and function of a given factor in XCI is an area of interest in the XCI field and, perhaps, also the biomolecular condensate field, that is in need of further study.

Supplementary Note 3

The apparent decease in the intensity of H327me3 Xi-enrichment on the ΔE-*Xist* expressing Xi, when visualized with standard epifluorescence microscopy (Extended Data Fig.7a,b), indicated an overall loss of H3K27me3 enrichment. 3D-SIM super-resolution imaging (Extended Data Fig.7c) revealed a noticeably lower packaging density of super-resolved H3K27me3 segments around dispersed *Xist*-MS2 signal in the ΔE nucleus compared to WT, suggestive of chromatin decompaction as one cause of decreased H3K27me3 immunostaining intensity rather than just a decrease in the total amount of H3K27me3. In support of this, we found that the loss of ΔE *Xist* late in differentiation (day 7) is accompanied by the loss of intense DAPI staining, a hallmark of the compacted Xi (Extended Data Fig. 7d,e). In Extended Data Fig. 7e, the more intense DAPI staining colocalizing with the MS2 RNA FISH signal (marked by arrowheads) indicates the formation of the Barr Body at day 3 of differentiation in both WT and ΔE cells. Thus, chromatin compaction decreases from day 3 to day 7 of differentiation in cells expressing ΔE MS2+*Xist*.

Supplementary Note 4

Immunostaining of CIZ1 in MS2-probed WT and ΔE MCP-PTBP1, -MATR3 and -TDP-43 rescue cell lines revealed that CIZ1 does not accumulate on the ΔE-*Xist* coated Xi in these rescue cell lines (Extended Data Fig.10f). The CIZ1 Xi-enrichment seen in WT and ΔE cells expressing MCP-PTBP1, MCP-MATR3 or MCP-TDP43 derives from the $X_{Cas}^{Xist\ WT}$ -Xi that expresses the wild-type *Xist* allele (indicated by arrow in Extended Data Fig.10f), and does not co-localize with the MS2+ signal. This is in contrast to CELF1, which *does* accumulate on the ΔE-*Xist* coated Xi upon MCP-PTBP1, -MATR3 or -TDP-43 expression (Fig. 3g,h).

Supplementary Note 5

The droplets produced by 60μM rPTBP1 with lower amounts of the E-repeat RNA (0.1 - 0.5μM) presented as phase-separated by multiple parameters: (1) upon addition of RNA, the PTBP1

solution became turbid (data not shown), (2) spherical, transparent droplets accumulated over time, (3) these droplets fused with other droplets, and (4) higher concentrations (0.3-0.5uM) of E-repeat RNA led to larger droplets than lower concentrations (0.1uM) (Fig. 4a-c and Extended Data Fig. 11a-c). We found that in the absence of RNA, a solution of rPTBP1 (60uM) remained uniformly clear as assessed by light microscopy (Extended Data Fig. 11a). Together, these findings indicate that, compared to a control RNA containing 5 PTBP1 sites, the multivalent E-repeat has a high propensity for condensation with PTBP1.

Supplementary Note 6

Our results suggest a new model for how *Xist* establishes the Xi-domain that involves the formation of a higher-order condensate. Upon induction of differentiation, *Xist* is upregulated, binds various diffusible proteins across the RNA (Fig. 4h) and spreads along the X-chromosome (Fig. 4i, top). Previous studies have elucidated functions for several of the *Xist*-interacting proteins. For instance, SAF-A/hnRNP-U mediates the chromatin attachment of *Xist*[14]. SHARP, bound at the A-repeat of *Xist*[1,3,13], and the PRC1 complex containing PCGF3 or PCGF5 recruited via hnRNP-K binding to the B-repeat of *Xist*[2], silence transcription of X-linked genes (Fig. 4h, i-middle WT *Xist*). We now show that the E-repeat directly binds the RBPs PTBP1, MATR3, TDP-43 and CELF1, bringing many molecules of these proteins into the *Xist* domain. These factors each carry multiple RRMs that potentially allow for the simultaneous engagement of distinct repeat motifs within in the E-repeat on or between individual transcripts, potentially increasing the avidity of binding to *Xist.*

Our results define homo- and heterotypic interactions between PTBP1, MATR3, TDP-43 and CELF1 that are required for faithful XCI. Together, these multivalent RNA-protein and protein-protein interactions induce the formation of a higher-order *Xist*–protein network that forms the Xi-compartment (Fig. 4i middle, bottom of WT *Xist*). The direct binding of RBPs to *Xist* leads to the recruitment of additional protein molecules into the X-chromosome territory via protein-protein interactions. We postulate that at a critical concentration threshold, self-aggregation properties and weak, multivalent protein-protein interactions promote condensation of the factors, establishing the Xi-compartment. Intriguingly, the features of the Xi-compartment change over time, exemplified by the increasing Xi-accumulation of CELF1 from day 3 to day 7

of differentiation and by manifestation of the phenotype upon E-repeat loss only after day 3 of differentiation (Fig. 4i middle, bottom). We propose that increasing *Xist* abundance with differentiation, coupled with gene silencing, loss of transcriptional activators, chromatin compaction and changes in chromatin state, overall protein availability, post-translational protein modifications, or modifications to *Xist*, alter the interactions between *Xist* and its bound proteins and, more importantly, between the proteins recruited into the compartment through protein-protein interactions.

Our results also reveal that, by binding and concentrating PTBP1, CELF1, MATR3, and TDP-43, *Xist* enforces its own *cis*-limited spread in addition to enforcing gene silencing. In this way, the protein condensate contributes to the sequestration of *Xist* in a defined nuclear territory close to the *Xist* transcription locus.

Our findings uncover a crucial role for these ubiquitously expressed RBPs in the formation of a nuclear compartment that regulates gene expression. It remains possible that additional factors contribute to the condensate seeded by the E-repeat. Among the *Xist*-interactors, NONO and FUS are of interest given their propensity to phase separate[18] as well as ELAVL1, which is predicted to bind the E- and A/F-repeats at high density[6]. Our observation that CIZ1, another E-repeat interacting protein[19,20], does not support the formation of the E-repeat-dependent condensate suggests, however, that the formation of this Xi-condensate requires specific proteins, and not simply the ability to form weak protein-protein interactions.

In the absence of the E-repeat, loss of gene silencing and dissociation of *Xist* from the X-chromosome domain occur, but only after transcriptional shutoff and heterochromatin formation have initiated (Fig. 4i, ΔE *Xist*). This explains why previous studies examining the role of PTBP1, CELF1, and MATR3 during the earliest, *Xist*-dependent phase of XCI failed to detect mutant phenotypes[1,21], and uncovers a transition from an E-repeat-independent mode of XCI to one that is E-repeat-dependent (Fig. 4i). Intriguingly, the condensate formed by PTBP1, CELF1, MATR3 and TDP-43 via binding to the E-repeat is critical for sustained silencing of X-linked genes, during a period of XCI that has previously shown to be *Xist*-independent[22] (Fig. 4i). Our results therefore suggest a previously undescribed mechanism of epigenetic inheritance that

perpetuates the silent state after the inducing molecule (*Xist*) has been deleted. We propose that continued gene silencing upon deletion of *Xist* after day 3 of differentiation, is mediated by the E-repeat-seeded protein condensate. This is consistent with our finding that CELF1 enrichment on the Xi can be maintained in the absence of *Xist* and recent work showing that SHARP remains within in the Xi but disengages from chromatin once transcriptional shutoff has completed[12]. To explain how the E-repeat-dependent condensate can maintain silencing in the absence of *Xist*, we hypothesize that the condensate integrates additional *Xist*-interacting proteins, for instance SHARP, via specific protein interactions (Fig. 4i, bottom, WT *Xist*). In this way, SHARP and other *Xist*-interactors could maintain association with the multi-molecular assembly independently of direct *Xist* interaction (Fig. 4i, bottom, WT *Xist*).

It has been proposed that about 50-100 *Xist* granules (foci)[23,24] comprise the *Xist* cloud. A condensate containing the silencing protein SHARP as well as other *Xist*-interactors could also explain how this limited number of *Xist* granules can induce the silencing of >1000 genes across 167Mb of X chromosome DNA. We posit that even at differentiation day 3, *Xist*-mediated condensation events that largely rely on *Xist*-interacting proteins that do not bind to the E-repeat, may be critical for the initiation of gene silencing.

A full understanding of how the Xi-condensate controls gene silencing and *Xist* localization and how it changes over time will involve determining all of its components and their stoichiometry within the Xi as well as further biophysical characterization of the condensate. For instance, an interesting question will be whether liquid-liquid phase separation is a feature of the Xi-condensate *in vivo*[25]. These studies may also reveal why certain *Xist*-interacting proteins enrich on the Xi and others do not. It will also be interesting to examine whether the small subset of genes that escape inactivation exhibit different interactions with the condensate or reside at distinct locations within it when compared to inactivated loci. Taken together, our work provides a new way of thinking about the mechanism of XCI, where the condensation of *Xist*-interacting proteins drives the formation of a functional nuclear compartment. Our results also reveal how RBPs, known for their roles in RNA processing, mediate lncRNA localization and exert control over gene regulation via mechanisms independent of their previously described RNA processing activities.

References cited in Supplementary Notes

1. McHugh, C. A. *et al.* The Xist lncRNA interacts directly with SHARP to silence transcription through HDAC3. *Nature* **521**, 232–236 (2015).
2. Pintacuda, G. *et al.* hnRNPK Recruits PCGF3/5-PRC1 to the Xist RNA B-Repeat to Establish Polycomb-Mediated Chromosomal Silencing. *Mol. Cell* **68**, 955–969.e10 (2017).
3. Chu, C. *et al.* Systematic discovery of Xist RNA binding proteins. *Cell* **161**, 404–416 (2015).
4. Minajigi, A. *et al.* Chromosomes. A comprehensive Xist interactome reveals cohesin repulsion and an RNA-directed chromosome conformation. *Science* **349**, (2015).
5. Moindrot, B. *et al.* A Pooled shRNA Screen Identifies Rbm15, Spen, and Wtap as Factors Required for Xist RNA-Mediated Silencing. *Cell Rep.* **12**, 562–572 (2015).
6. Smola, M. J. *et al.* SHAPE reveals transcript-wide interactions, complex structural domains, and protein interactions across the *Xist* lncRNA in living cells. *Proc. Natl. Acad. Sci.* **113**, 10322–10327 (2016).
7. Van Nostrand, E. L. *et al.* Robust transcriptome-wide discovery of RNA-binding protein binding sites with enhanced CLIP (eCLIP). *Nat. Methods* **13**, 508–514 (2016).
8. Vuong, J. K. *et al.* PTBP1 and PTBP2 Serve Both Specific and Redundant Functions in Neuronal Pre-mRNA Splicing. *Cell Rep.* **17**, 2766–2775 (2016).
9. Rogelj, B. *et al.* Widespread binding of FUS along nascent RNA regulates alternative splicing in the brain. *Sci. Rep.* **2**, 603 (2012).
10. ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. *Nature* **489**, 57–74 (2012).
11. Hein, M. Y. *et al.* A human interactome in three quantitative dimensions organized by stoichiometries and abundances. *Cell* **163**, 712–723 (2015).
12. Dossin, F. *et al.* SPEN integrates transcriptional and epigenetic control of X-inactivation. *Nature* (2020) doi:10.1038/s41586-020-1974-9.
13. Żylicz, J. J. *et al.* The Implication of Early Chromatin Changes in X Chromosome Inactivation. *Cell* **176**, 182–197.e23 (2019).
14. Hasegawa, Y. *et al.* The matrix protein hnRNP U is required for chromosomal localization of Xist RNA. *Dev. Cell* **19**, 469–476 (2010).
15. Plath, K. Role of Histone H3 Lysine 27 Methylation in X Inactivation. *Science* **300**, 131–135 (2003).
16. Plath, K. *et al.* Developmentally regulated alterations in Polycomb repressive complex 1 proteins on the inactive X chromosome. *J. Cell Biol.* **167**, 1025–1035 (2004).
17. Silva, J. *et al.* Establishment of histone h3 methylation on the inactive X chromosome requires transient recruitment of Eed-Enx1 polycomb group complexes. *Dev. Cell* **4**, 481–495 (2003).
18. Hirose, T., Yamazaki, T. & Nakagawa, S. Molecular anatomy of the architectural NEAT1 noncoding RNA: The domains, interactors, and biogenesis pathway required to build phase-separated nuclear paraspeckles. *Wiley Interdiscip. Rev. RNA* **10**, e1545 (2019).
19. Ridings-Figueroa, R. *et al.* The nuclear matrix protein CIZ1 facilitates localization of Xist RNA to the inactive X-chromosome territory. *Genes Dev.* **31**, 876–888 (2017).
20. Sunwoo, H., Colognori, D., Froberg, J. E., Jeon, Y. & Lee, J. T. Repeat E anchors Xist RNA to the inactive X chromosomal compartment through CDKN1A-interacting protein (CIZ1). *Proc. Natl. Acad. Sci. U. S. A.* **114**, 10654–10659 (2017).
21. Wutz, A., Rasmussen, T. P. & Jaenisch, R. Chromosomal silencing and localization are

mediated by different domains of Xist RNA. *Nat. Genet.* **30**, 167–174 (2002).
22. Wutz, A. & Jaenisch, R. A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. *Mol. Cell* **5**, 695–705 (2000).
23. Sunwoo, H., Wu, J. Y. & Lee, J. T. The Xist RNA-PRC2 complex at 20-nm resolution reveals a low Xist stoichiometry and suggests a hit-and-run mechanism in mouse cells. *Proc. Natl. Acad. Sci. U. S. A.* **112**, E4216-4225 (2015).
24. Smeets, D. *et al.* Three-dimensional super-resolution microscopy of the inactive X chromosome territory reveals a collapse of its active nuclear compartment harboring distinct Xist RNA foci. *Epigenetics Chromatin* **7**, 8 (2014).
25. Cerase, A. *et al.* Phase separation drives X-chromosome inactivation: a hypothesis. *Nat. Struct. Mol. Biol.* **26**, 331–334 (2019).