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Evidence of *Xist* RNA-independent initiation of mouse imprinted X-chromosome inactivation

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Summary

XX female mammals undergo transcriptional silencing of most genes on one of their two X-chromosomes to equalize X-linked gene dosage with XY males in a process referred to as X-chromosome inactivation (XCI). XCI is a paradigm of epigenetic regulation1. Once enacted in individual cells of the early female embryo, XCI is stably transmitted such that most descendant cells maintain silencing of that X-chromosome2. In eutherian mammals, XCI is thought to be triggered by the expression of the non-coding *Xist* RNA from the future inactive-X (Xi)3,4,5; *Xist* RNA in turn is proposed to recruit protein complexes that bring about heterochromatinization of the Xi6,7. Here we test whether imprinted XCI, which results in preferential inactivation of the paternal X-chromosome (Xp), occurs in mouse embryos inheriting an Xp lacking *Xist*. We find that silencing of Xp-linked genes can initiate in the absence of paternal *Xist; Xist* is, however, required to stabilize silencing of the Xp. Xp-linked gene silencing associated with mouse imprinted XCI, therefore, can initiate in the embryo independently of *Xist* RNA.

In the mouse, *Xist* RNA expression is first detected at the late two-cell stage of embryogenesis exclusively from the Xp8. During subsequent stages of preimplantation development *Xist* RNA spreads from its site of synthesis to eventually coat most of the Xp4,5,9, concomitant with transcriptional silencing of Xp-linked genes4,10,11. Imprinted XCI of the Xp is then maintained in extra-embryonic tissues of the post-implantation embryo12,13. The embryonic lineage, on the other hand, selectively reactivates the Xp during peri-implantation stages and subsequently undergoes random XCI11. As in imprinted

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Author Contributions

S.K. and T.M. conceived the study and designed the experimental strategy. S.K. conducted the experiments with assistance from S.P. in mouse genotyping, RNA FISH and RT-PCR experiments and from R.B.B. in RT-PCR experiments. S.K. and T.M. analyzed the data. J.S. performed the statistical evaluation of the RNA FISH and RT-PCR data. S.K. wrote the paper and T.M. edited the paper. All authors discussed the results and commented on the manuscript.

XCI, *Xist* expression is upregulated from the Xi-elect prior to X-linked gene silencing during random XCI14.

Despite the currently accepted role of *Xist* RNA in controlling XCI, two observations led us to question whether *Xist* is required to initiate imprinted XCI. In the course of a previous study we noticed that mouse trophoblast stem cells, which undergo imprinted XCI of the Xp15, do not proliferate if they reactivate their previously inactivated Xp16. We therefore reasoned that if *Xist* RNA were required for the initiation of imprinted XCI, then female embryos that inherit a paternal *Xist* mutation should lack trophoblast-derived structures. However, Xp-*Xist* mutant embryos can develop extensive trophectodermal-derivatives17. We thus hypothesized that *Xist* RNA may not be required for the initiation of imprinted XCI in early mouse embryos. In the present study, we provide several lines of evidence that substantiate the notion that imprinted XCI can initiate in the absence of *Xist*.

To determine when silencing of X-linked genes associated with imprinted XCI first occurs and if it's *Xist* RNA-dependent, we compared the expression of eleven X-linked genes by RNA fluorescence *in situ* hybridization (FISH) in multiple 2-, 4-, 8-, and 16-cell preimplantation stage female embryos that inherited either a wild-type (WT) Xp or an Xp harboring a null-allele of *Xist* (Xp-$Xist^{1lox}$; a deletion of the promoter and first three exons resulting in lack of *Xist* RNA expression)18(Fig. 1). If both the maternal (Xm) and Xp alleles are expressed, the expectation is that RNA FISH would yield biallelism; if one of the two alleles is silenced RNA FISH would result in monoallelism (a third category is 'no signal', if neither allele is detectably expressed). In WT 2-cell embryos, nine of the X-linked genes, *Ube1x*, *Fmr1*, *G6pdx*, *Chic1*, *Rnf12*, *Abcb7*, *Atrx*, *Atp7a*, and *Smcx* displayed a high level of biallelism (83–100%) and a low level of monoallelism (0–8%) (Fig. 1d and Supplementary Fig. 1). The remaining two genes, *Mecp2* and *Pgk1*, were expressed biallelically at a relatively lower level (59% and 46%, respectively) and monoallelically at a higher level (15% and 23%, respectively), but the embryos nevertheless harbored more biallelically-expressing nuclei. In Xp-$Xist^{1lox}$ embryos, the genes showed a similar distribution of nuclei (biallelism/monoallelism/no signal) to that observed in WT embryos. The paucity of monoallelism and a preponderance of biallelism in 2-cell embryos is consistent with previous reports of both alleles of X-linked genes being expressed at the 2-cell stage19,20, and argue against Xp-alleles being inherited as transcriptionally-inert due to silencing of the Xp during meiotic sex chromosome inactivation (MSCI) in the paternal germline4,21.

We next assayed X-linked gene expression by RNA FISH in WT and Xp-$Xist^{1lox}$ 4-cell embryos, to assess the degree of silencing at this stage. As in 2-cell embryos, the percentage of biallelically-expressing nuclei in both genotypes was again greater than monoallelic nuclei for most genes (Fig. 1d and Supplementary Fig. 2). These data reiterate that imprinted XCI is not a consequence of MSCI-mediated silencing of Xp-linked genes.

Next, we surveyed expression of the X-linked genes in WT 8- and 16-cell morula-stage embryos by RNA FISH. In comparison to 2- and 4-cell embryos, a significantly higher percentage of WT 8-cell nuclei expressed the X-linked genes from only one allele (Fig. 1d and Supplementary Fig. 3). An examination of Xp-$Xist^{1lox}$ 8-cell embryos unexpectedly also

showed the presence of a significant percentage of monoallelic-expressing nuclei. If *Xist* RNA is required for the initiation of imprinted XCI, the prediction would be that in its absence Xp-linked gene silencing should not occur; this should result in a higher percentage of biallelism and a lower percentage of monoallelism in Xp-*Xist*^{1lox} embryos compared to WT embryos. However, nine of the eleven genes assayed (all except *Rnf12* and *Abcb7*) displayed statistically indistinguishable distributions of the three classes of nuclei (biallelism, monoallelism, and no signal) in 8-cell Xp-*Xist*^{1lox} from WT embryos (Supplementary Fig. 3). In 16-cell embryos, seven of the eleven genes (all except *Ube1x*, *Rnf12*, *Abcb7*, and *Atrx*) were expressed similarly in Xp-*Xist*^{1lox} and WT embryos (Fig. 1d and Supplementary Fig. 4). The parallel decrease in biallelism and increase in monoallelism of most genes in WT and Xp-*Xist*^{1lox} 8- and 16-cell embryos suggests that silencing of Xp-linked genes can occur in morulas in the absence Xp-*Xist*.

To confirm the silencing of Xp-linked genes in Xp-*Xist*^{1lox} 8-16-cell embryos observed by RNA FISH, we performed allele-specific RT-PCR in WT and Xp-*Xist*^{1lox} 8-16-cell stage morulae (Fig. 2a,c and Supplementary Table I). Silencing of Xp-linked genes during imprinted XCI contributes to the higher expression of maternal alleles of most X-linked genes relative to their paternal counterparts4,10,20. If *Xist* RNA is required for the initiation of silencing of Xp-alleles, the expression of both the Xm- and the Xp-alleles would be expected to be equal in Xp-*Xist*^{1lox} morulae. We therefore assayed and quantified the relative expression of Xm and Xp-alleles of seven X-linked genes distributed along the X-chromosome - *Ddx3x*, *Ube1x*, *Zfx*, *Rnf12*, *Atrx*, *Pdha1*, and *Utx* - in multiple individual F1 WT and Xp-*Xist*^{1lox} hybrid 8-16-cell female morulae. Although the relative levels of the two alleles varied between embryos, on average *Ddx3x*, *Ube1x*, *Zfx*, *Rnf12*, *Atrx*, and *Pdha1* were all expressed preferentially from the Xm-allele in WT female morulae. *Utx* appeared to escape XCI at this stage, as indicated by a lack of difference in the expression of the two alleles. Notably, in Xp-*Xist*^{1lox} female morulae the expression of *Ddx3x*, *Ube1x*, *Zfx*, and *Pdha1* also showed a maternal-allele bias, at levels similar to that in WT embryos; *Rnf12* was also predominantly expressed from the Xm, but the difference in expression was not as great as in WT morulae. *Atrx* appeared to require *Xist* for silencing of the Xp-allele; in contrast to WT embryos, *Atrx* expression, on average, did not favor the maternal allele in Xp-*Xist*^{1lox} females–although there clearly are morulae that do express the Xm *Atrx* allele more strongly than the Xp (Fig. 2a). Both *Rnf12* and *Atrx* also showed significant differences between WT and Xp-*Xist*^{1lox} 8-16-cell embryos by RNA FISH (Fig. 1d and Supplementary Figs. 3 and 4). As in WT embryos, *Utx* expression did not show an Xm-allele bias in Xp-*Xist*^{1lox} morulae. We excluded the possibility of strain-specific, as opposed to parent of origin-specific, transcriptional inhibition of the paternal *M. dom*. alleles by conducting allele-specific expression analysis of the seven X-linked genes in WT F1 hybrid morulae obtained from the reciprocal cross (Supplementary Fig. 5a,c and Supplementary Table III). In sum, of the six genes whose Xp-alleles are subject to transcriptional silencing in WT morulae, five also undergo silencing in the absence of Xp-*Xist*.

We also examined expression of the X-linked genes in extra-embryonic (ExEmb) tissues of embryonic day 6.5 (E6.5) WT and Xp-*Xist*^{1lox} embryos, to determine the profile of allele-specific expression of the seven genes during post-implantation development (Fig. 2b,d and

Supplementary 5b,d and Supplementary Table II). ExEmb tissues of post-implantation embryos maintain imprinted XCI that initiates during preimplantation development12,13. In agreement, in WT E6.5 ExEmb tissues the Xm-alleles of all seven genes were expressed at higher levels than the Xp-alleles, some almost exclusively. However, of the five genes that displayed preferential expression of the Xm-allele in Xp-*Xist*^{1lox} morulae (*Ddx3x, Ube1x, Zfx*, *Rnf12*, and *Pdha1*), *Ddx3x, Ube1x*, and *Pdha1* lacked biased expression of the Xm-allele in Xp-*Xist*^{1lox} E6.5 ExEmb tissues. *Zfx* and *Rnf12* displayed preferential expression of the Xm-allele in Xp-*Xist*^{1lox} E6.5 ExEmb tissues, at levels similar to or more than in Xp-*Xist*^{1lox} morulae, but not as high a degree as in WT E6.5 ExEmb samples. We again ruled out strain-dependent silencing of the Xp-alleles in E6.5 ExEmb through a reciprocal cross (Supplementary Fig. 5b,d and Supplementary Table IV). Thus, in the absence of Xp-*Xist*, the silencing of paternal alleles of some, but not all, X-linked genes observed in morulae is not maintained in E6.5 ExEmb tissues. The silencing of *Zfx* and *Rnf12* paternal alleles in Xp-*Xist*^{1lox} E6.5 ExEmb samples, however, suggests that even in the absence of Xp-*Xist* long-term silencing of Xp-alleles of some genes can be maintained.

We also tracked the expression of an Xp-linked *green fluorescent protein* transgene (Xp-*GFP*) to visualize the dynamics of X-linked gene expression in pre-, peri-, and post-implantation stage WT and Xp-*Xist*^{1lox} embryos (Fig. 3). The Xp-*GFP* transgene is normally subject to imprinted XCI and is silenced in ExEmb tissues of post-implantation embryos but is expressed in the embryonic lineage due to random XCI16,22,23. If *Xist* RNA is required for the initiation of imprinted XCI, then Xp-*Xist*^{1lox} ExEmb cells should uniformly express the Xp-*GFP* transgene. In preimplantation stage E3.5 embryos, the Xp-*GFP* transgene is expressed in both the embryonic and ExEmb lineages (the inner cell mass [ICM] and the trophectoderm [TE], respectively) in both WT & Xp-*Xist*^{1lox} embryos (Fig. 3a–b). At peri-implantation stage E4.5 embryos, the transgene appears silenced in a majority of the cells of the ExEmb TE lineage in both genotypes (Fig. 3c–d). In post-implantation stage E5.75, E6.25, and E7.5 WT embryos, green fluorescence due to transgene expression is undetectable in the ExEmb compartment (Fig. 3e,g,i). In corresponding Xp-*Xist*^{1lox} embryos, the transgene appears to be expressed in an increasing number of ExEmb cells, correlating with the increasing severity of the mutant phenotype (Fig. 3f,h,j). We confirmed the capability of the transgene to be expressed throughout the ExEmb compartment, by generating embryos that inherited the transgene on the maternal-X (Xm-*GFP*); the Xm-*GFP* transgene in fact appears to be expressed in most, if not all, of the ExEmb cells of pre- and post-implantation embryos (Supplementary Fig. 6). The gradual increase in the number of ExEmb cells expressing the Xp-*GFP* transgene during post-implantation development, thus, reiterates a requirement of Xp-*Xist* in maintaining silencing of Xp-genes during imprinted XCI. Nevertheless, the presence of ExEmb cells that do not express Xp-*GFP* in peri- and post-implantation Xp-*Xist*^{1lox} embryos indicates that silencing of Xp-linked genes can initiate and persist well into development in the absence of *Xist* RNA.

Our data are consistent with the hypothesis that X-linked genes have evolved to become dependent on *Xist* RNA for their silencing; however, silencing can initially occur independently of *Xist* RNA and is a consequence of differentiation of the mammalian sex chromosomes (see below). Of the X-linked genes whose expression we have analyzed, three

(*Rnf12*, *Abcb7*, and *Atrx*) appear to undergo silencing to a lesser degree in Xp-*Xist* mutants compared to WT morulas. Interestingly, these genes map to the region of the X-chromosome that is thought to have undergone dosage compensation first during the evolution of the mammalian sex chromosomes24. Thus, the degree to which a gene requires *Xist* RNA during initiation of silencing is dependent on the length of time that that gene has been subject to dosage compensation: the longer a gene has been dosage compensated, the more it is dependent on *Xist* RNA for its silencing.

Our results show that although silencing of Xp-linked genes can initiate in the absence of *Xist*, Xp-*Xist* is clearly required for imprinted XCI; in its absence, after initial silencing Xp-linked genes become expressed in progressively higher numbers of cells and/or at increased levels. Thus, *Xist* RNA appears to be required to stabilize imprinted XCI. *Xist* RNA preferentially decorates the fraction of the X-chromosome that is rich in repetitive sequences14,25. The silencing of these regions may serve to form a heterochromatin scaffold that ensures long-term silencing of the X-chromosome as a whole25. By participating in the stable silencing of repetitive sequences, *Xist* RNA may thus bring about enduring silencing of X-linked genes.

XCI is part of a dosage-compensation mechanism that is thought to be a by-product of sexual differentiation of a pair of homologous chromosomes into an X- and a Y-chromosome26. The gradual loss of genes from the proto-Y during X-Y differentiation is believed to have driven silencing of homologous X-linked genes in a piecemeal fashion24,26. Furthermore, evidence suggests that *Xist* has evolved following the advent of X-linked gene silencing24. Metatherian mammals (marsupials), which diverged from eutherians (placental mammals) about 150 million years ago27, undergo imprinted XCI in both embryonic and extra-embryonic tissues without the aid of *Xist* RNA, albeit the silencing appears to not be stringent28,29. Combined with the results of this study, the evolution of XCI to encompass individual segments and the relatively recent appearance of *Xist* RNA argue that the initial silencing of X-linked genes may be a local or regional, rather than a chromosome-wide, event; multiple mechanisms may, therefore, mediate the initiation of imprinted XCI.

Methods

RNA FISH

Embryos were permeabilized by sequential transfers into ice-cold cytoskeletal extraction buffer (CSK; 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, and 10 mM PIPES ph 6.8) for 30 sec, ice-cold CSK containing 0.4% Triton X-100 for 1.5–5 mins, followed twice with ice-cold CSK for 30 sec each. All CSK buffers were supplemented with 400 units/mL of RNase-inhibitor RNAsin (Promega). Embryos were then mounted on a glass coverslip coated with 1X Denhardt's solution in a small drop of ice-cold solution containing 1% paraformaldehyde and 20% CSK in 1X PBS. Excess solution was aspirated off, embryos air-dried for 15 mins, and fixed in cold 3% paraformaldehyde for 10 mins in humid chamber. After fixation, the coverslips were submerged in 70% ethanol. After three changes of 70% ethanol, the cells or embryos were either stored in 70% ethanol at −20°C or processed for RNA FISH (details included in Supplementary Methods).

RT-PCR

mRNA was prepped using the Dynabeads mRNA DIRECT Micro Kit (Invitrogen, Cat. #610.21). Individual morula-stage embryos and extra-embryonic tissues from E6.5 embryos were lysed in 100 ul of lysis/binding buffer. SuperScript III One-Step RT-PCR Platinum *Taq* enzyme mixture (Invitrogen, Cat. #12574-035) was used to prepare and amplify the cDNA. A portion of the final RT-PCR product was used to perform one round of amplification in the presence of a trace amount of ^{32}P-dCTP to minimize heteroduplexes and to radioactively-label the RT-PCR product. The final PCR product was digested with the appropriate restriction enzyme (see Supplementary Table VI), and run on a 7.5% acrylamide gel, vacuum dried, and exposed to film. Band intensities were quantified using NIH ImageJ software. In case of multiple maternal- or paternal-specific bands, the quantified band intensities were added to yield a single value for each allele. Additional details are included in Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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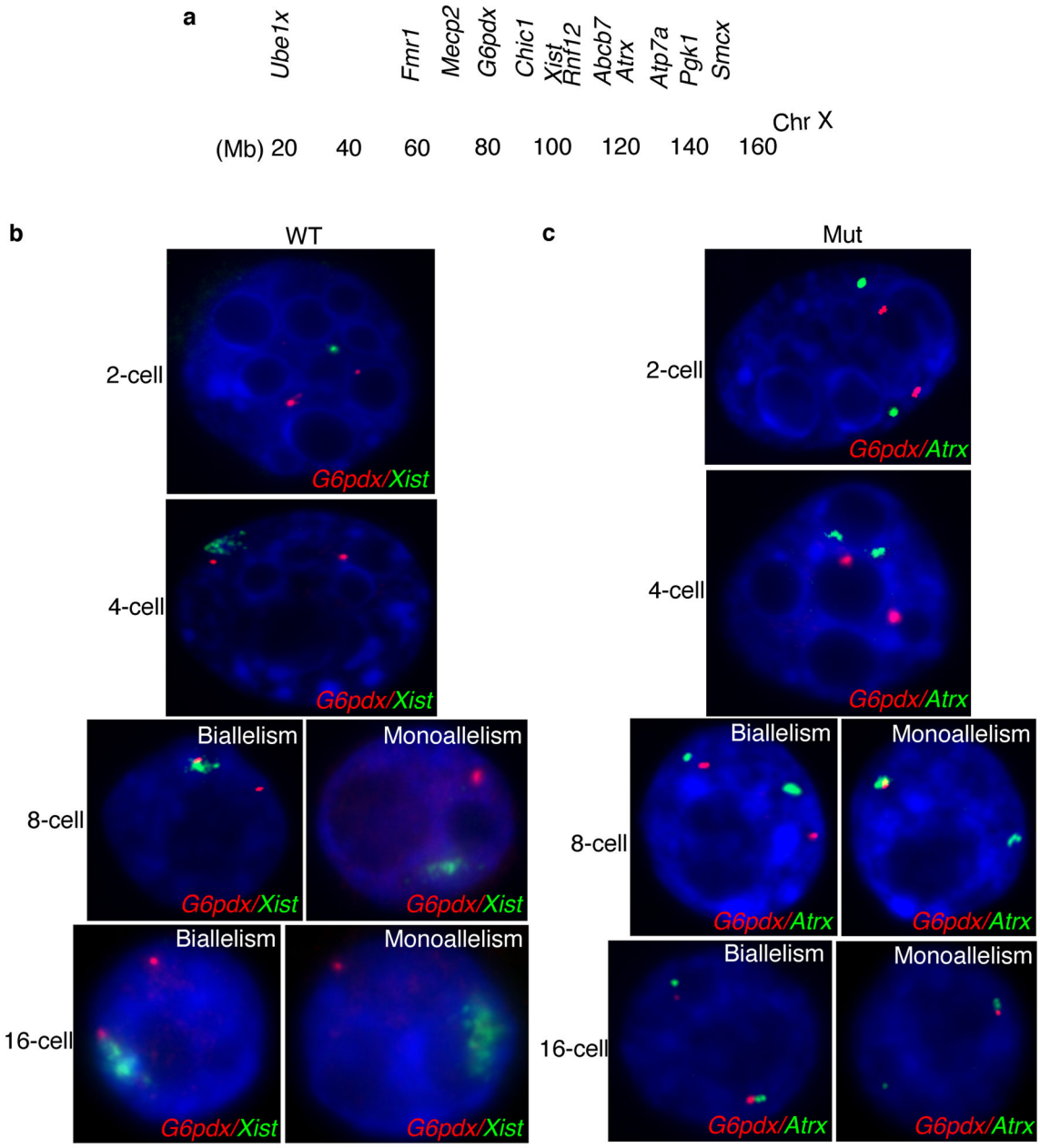


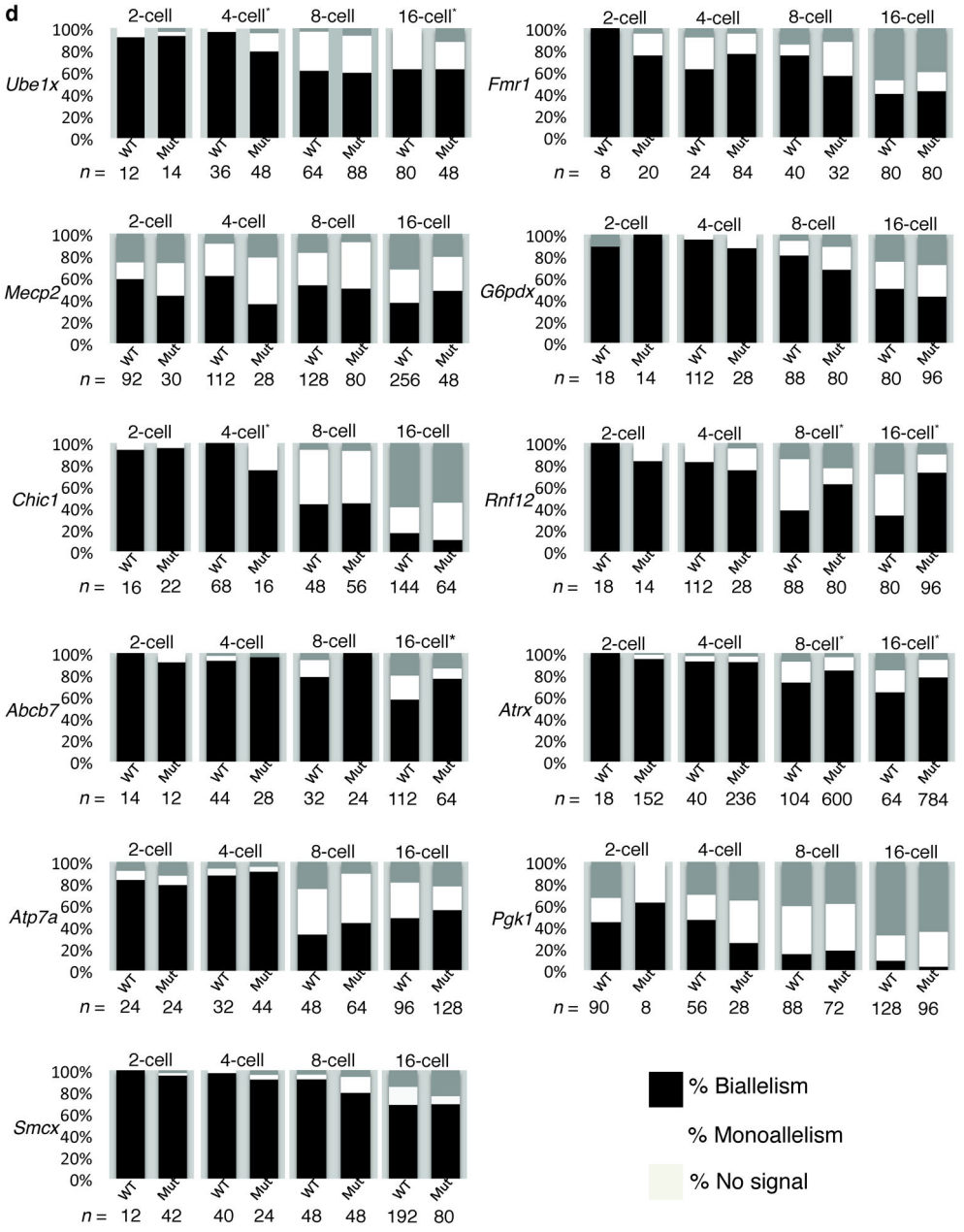
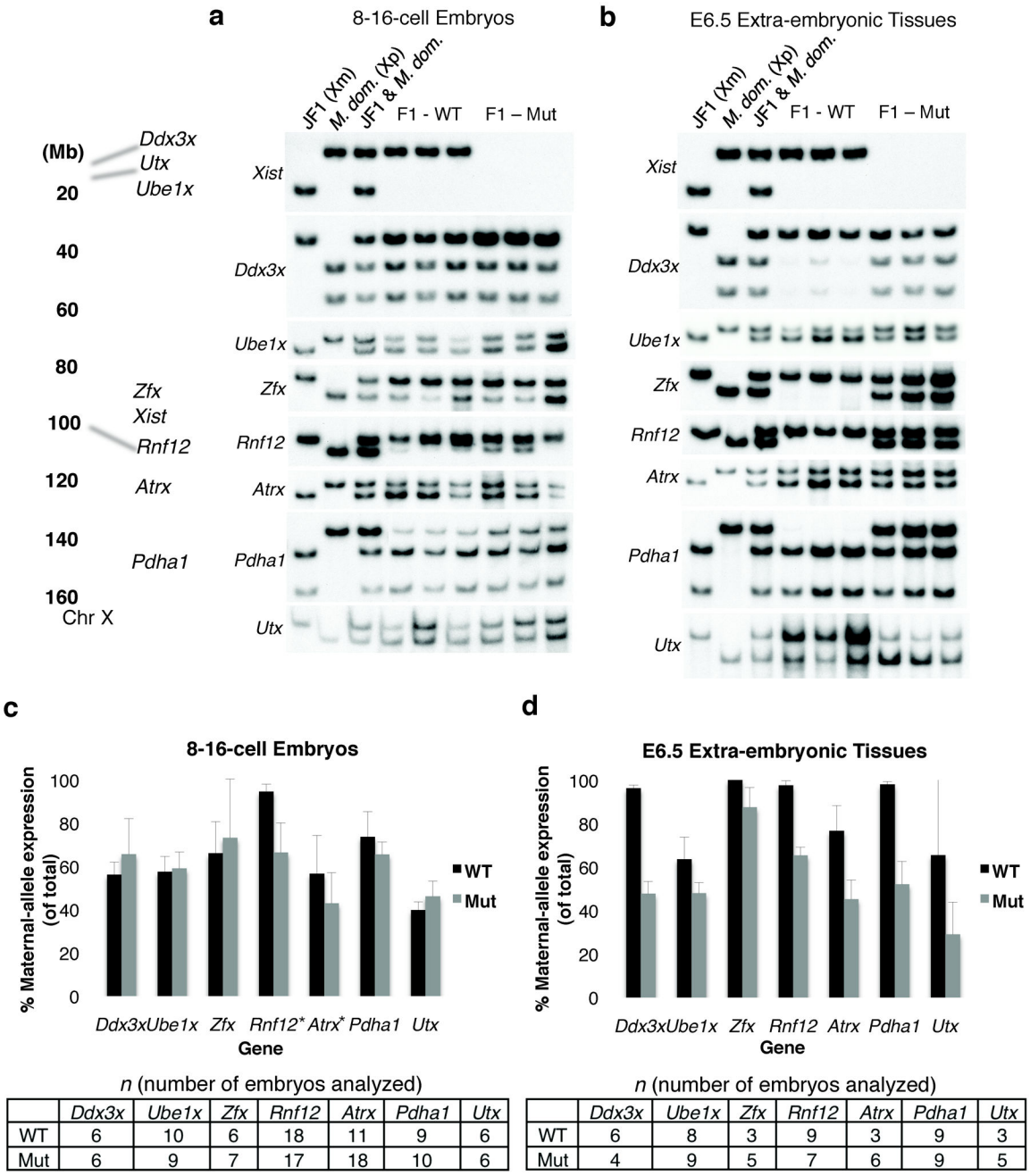


Figure 1. Dynamics of X-linked gene expression assayed by RNA FISH in 2-, 4-, 8-, and 16-cell wild-type (WT) and Xp-*Xist*^{1lox} (Mut) female mouse embryos

a, Physical map of the eleven X-linked genes assayed. **b**, **c**, Single representative nuclei from WT and Xp-*Xist*^{1lox} embryos probed for expression of *G6pdx* (red punctate signal) and *Xist* (green; in WT embryos) or *Atrx* (green; in Xp-*Xist*^{1lox} embryos). In WT embryos, *Xist* is expressed exclusively from and marks the paternal X-chromosome (Xp)19,30. In Xp-*Xist*^{1lox} embryos, *Atrx* expression is used to mark the two Xs. In nuclei from 2- and 4-cell embryos, most genes are expressed predominantly biallelically. DNA is stained blue with 4′, 6-diamidino-2-phenylindole (DAPI). **d**, Comparison of the distribution of nuclei displaying biallelism, monoallelism, and no signal in WT and Xp-*Xist*^{1lox} embryos. *n*, numbers of

nuclei. Only those genes/stages denoted by a '*' show significant differences in the distribution of the three classes of nuclei (see Supplementary Figures 1–4).



n (number of embryos analyzed)

	Ddx3x	*Ube1x*	*Zfx*	*Rnf12*	*Atrx*	*Pdha1*	*Utx*
WT	6	10	6	18	11	9	6
Mut	6	9	7	17	18	10	6

n (number of embryos analyzed)

	Ddx3x	*Ube1x*	*Zfx*	*Rnf12*	*Atrx*	*Pdha1*	*Utx*
WT	6	8	3	9	3	9	3
Mut	4	9	5	7	6	9	5

Figure 2. Dynamics of X-linked gene expression assayed by allele-specific RT-PCR in wild-type (WT) and Xp-*Xist*^{1lox} (Mut) 8-16-cell mouse embryos and embryonic day 6.5 (E6.5) extra-embryonic tissues

Allele-specific RT-PCR expression analysis of the X-linked genes *Xist, Ddx3x, Ube1x, Zfx, Rnf12, Atrx, Pdha1*, and *Utx* in individual F1 hybrid WT & Xp-*Xist*^{1lox} (**a,c**) 8-16-cell embryos (morulas) and (**b,d**) embryonic day 6.5 (E6.5) extra-embryonic tissues. F1 hybrid embryos were generated by a cross of the *Mus molossinus* (*M. mol.*) strain JF1 females to largely *Mus domesticus* (*M. dom.*)-derived males from wild-type and Xp-*Xist*^{1lox} laboratory mice strains. Expression of the two alleles (maternal, Xm; paternal, Xp) was distinguished

by single nucleotide polymorphisms between the strains resulting in a strain-specific pattern of fragments after restriction enzyme digestion (see Supplementary Methods and Supplementary Table VI). Lane 1, *M. mol*. allele (Xm); lane 2, *M. dom.* allele (Xp); lane 3, equal amounts of *M. mol.* and *M. dom*; lanes 4–6, representative F1 hybrid WT samples; lanes 7–9 representative F1 hybrid Xp-*Xist*^{1lox} samples. *Xist* is expressed exclusively from the Xp in WT morulas and in WT E6.5 extra-embryonic tissues and is absent in Xp-*Xist*^{1lox} morulas and E6.5 extra-embryonic tissues. Only those genes denoted by a '*' show differences in the degree of silencing of the paternal alleles in Xp-*Xist*^{1lox} morulas compared to WT embryos (see Supplementary Table I); all genes show differences in E6.5 extra-embryonic tissues (see Supplementary Table II).

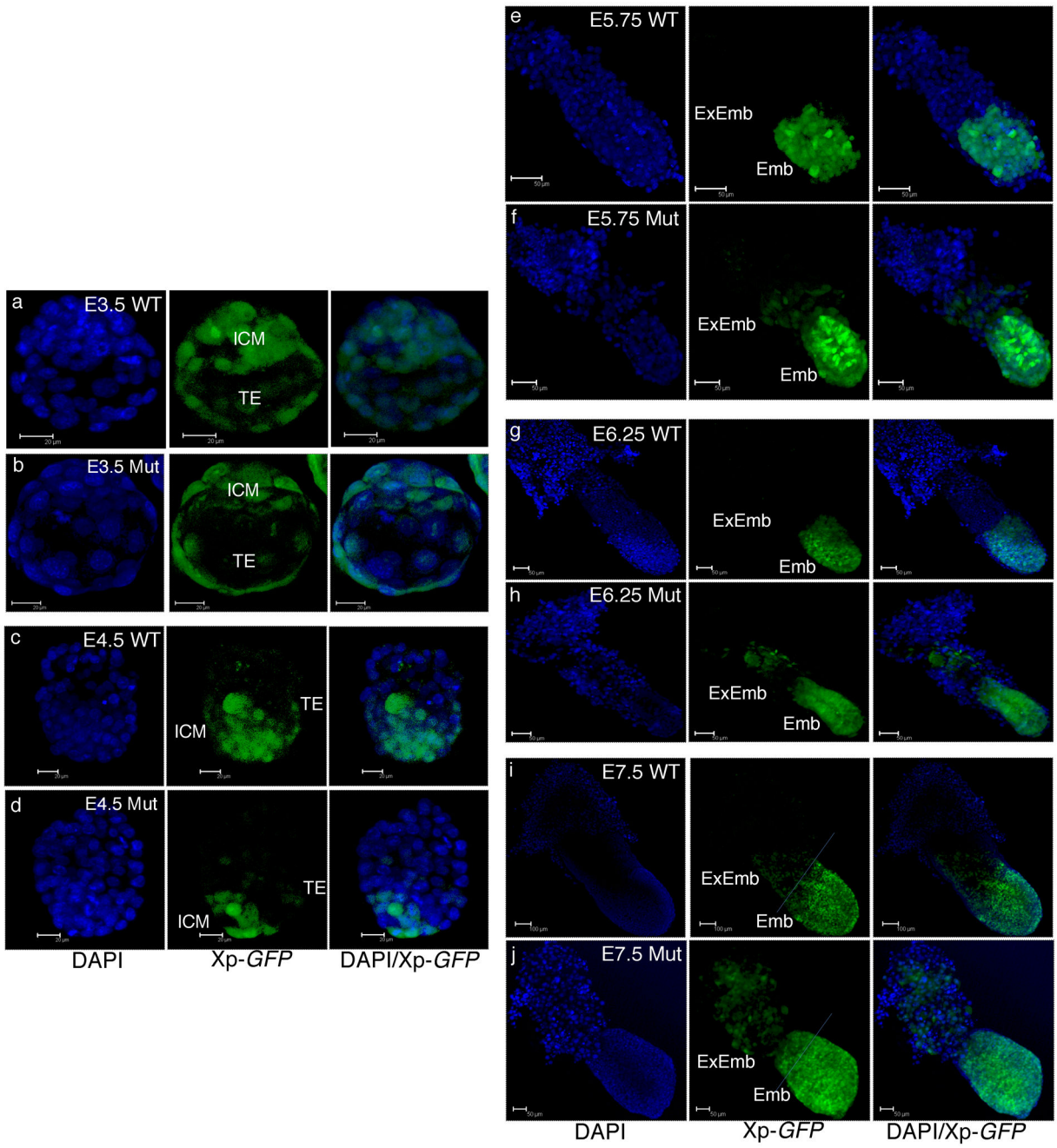


Figure 3. Expression of paternal X-linked green fluorescent protein (Xp-*GFP*) transgene in WT and Xp-*Xist*^{1lox} (Mut) pre- and post-implantation stage female mouse embryos

a–b, Wild-type (WT) and Xp-*Xist*^{1lox} blastocyst (embryonic day 3.5 [E3.5]) female embryos expressing Xp-*GFP* in both the trophectoderm (TE) and inner cell mass (ICM) lineages. **c–d,** WT and Xp-*Xist*^{1lox} E4.5 female embryos silencing Xp-*GFP* in the TE but showing continued expression of the Xp-*GFP* in the ICM-derived cells. The hatched line demarcates the boundary between the TE and the ICM-derivatives. **e,g,i,** WT E5.75, E6.25, and E7.5 female embryos devoid of Xp-*GFP* expression in the extra-embryonic ectoderm and its derivatives (ExEmb; derived from the TE) due to imprinted XCI. The embryonic ectoderm

(Emb), descended from the ICM, fluoresces green because of Xp-*GFP* expression due to random XCI that results in Xp activity in approximately half of the cells. The hatched line demarcates the boundary between the ExEmb and the Emb. **f,h,j,** Xp-*Xist*^{1lox} E5.75, E6.25, and E7.5 female embryos with a mosaic extra-embryonic compartment that is comprised of both cells that silence and express the Xp-*GFP*. DNA is stained blue with DAPI.