Xist imprinting is promoted by the hemizygous (unpaired) state in the male germ line

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The long noncoding X-inactivation-specific transcript (*Xist* gene) is responsible for mammalian X-chromosome dosage compensation between the sexes, the process by which one of the two X chromosomes is inactivated in the female soma. *Xist* is essential for both the random and imprinted forms of X-chromosome inactivation. In the imprinted form, *Xist* is paternally marked to be expressed in female embryos. To investigate the mechanism of *Xist* imprinting, we introduce *Xist* transgenes (Tg) into the male germ line. Although ectopic high-level *Xist* expression on autosomes can be compatible with viability, transgenic animals demonstrate reduced fitness, subfertility, defective meiotic pairing, and other germ-cell abnormalities. In the progeny, paternal-specific expression is recapitulated by the 200-kb *Xist* Tg. However, *Xist* imprinting occurs efficiently only when it is in an unpaired or unpartnered state during male meiosis. When transmitted from a hemizygous father (+/Tg), the *Xist* Tg demonstrates paternal-specific expression in the early embryo. When transmitted by a homozygous father (Tg/Tg), the Tg fails to show imprinted expression. Thus, *Xist* imprinting is directed by sequences within a 200-kb X-linked region, and the hemizygous (unpaired) state of the *Xist* region promotes its imprinting in the male germ line.

Xist | imprinting | X inactivation | transgenerational inheritance | meiotic silencing by unpaired DNA

In mammals, the balance of X-linked gene dosage between XX females and XY males is achieved through X-chromosome inactivation (XCI) (1–4). Although marsupial mammals exhibit imprinted XCI and thereby inactivate only the paternally derived X chromosome (X^P), placental mammals demonstrate two forms of XCI: Imprinted X^P inactivation in the early embryo and trophoblast and random X inactivation in embryonic (epiblast) tissues (5–7). In placental mammals, both imprinted and random XCI are controlled by the X-inactivation center (*Xic*), which is located on the X chromosome and is necessary and sufficient to direct the silencing for an entire X chromosome in the female mammal (8, 9). As a master regulatory locus, the *Xic* contains multiple long noncoding RNAs (lncRNAs) including *Xist* X-inactivation-specific transcript (*Xist*), a 17-kb noncoding transcript which coats the inactive X chromosome (Xi) and initiates silencing (3, 10).

Xist is required for both random XCI and imprinted X^P inactivation (11–14). During random XCI, *Xist* is up-regulated exclusively from the future Xi at the same time that expression of its antisense partner, *Tsix* (15), is down-regulated *in cis*. During imprinted XCI, *Xist* is expressed for the first time from the X^P at the time of zygotic gene activation in the two-cell embryo (16, 17). In the paternal germ line, however, *Xist* RNA is expressed only at low levels, suggesting that it is not required for spermatogenesis (18, 19). Consistent with this notion, male mice with an *Xist* deletion are fertile (12, 20).

Although significant progress has been made in understanding the control of *Xist* expression for random XCI, the control of

imprinted XCI is still poorly understood (2, 4, 16, 21–23). Work with parthenogenetic embryos has provided evidence for both a maternal and a paternal imprint (24–27). For example, X^MX^M gynogenetic embryos bearing two maternal genomes and (therefore two maternal X chromosomes [X^M]) block *Xist* expression despite possessing two X chromosomes, thereby arguing that the oocyte imprints the *Xist* gene to resist expression from the X^M in the early embryo. On the other hand, X^PX^P androgenetic embryos bearing two paternal genomes and (therefore two X^P) display two foci of *Xist* expression, indicating that the male germ line imprints the *Xist* gene for expression from the X^P in the early embryo. Little is known about the maternal imprint. Although de novo DNA methylation is dispensable (28), *Xist*'s antisense partner, *Tsix*, plays a critical role in protecting the X^M from silencing (29–31), and the H3K9me3 mark correlates with *Xist* silencing (32).

The nature of the paternal germline imprint and the extent of its influence in the early embryo have been under some debate. In one school of thought, the paternal germ line marks the *Xist* gene for expression in the female embryo, but the mark is not read until later in preimplantation development (17). Another model suggests that X^P silencing mechanistically follows from meiotic sex chromosome inactivation in the male germ line (16, 33–37) and is a direct extension of the postmeiotic sex

Significance

Mammals with imprinted X inactivation demonstrate exclusive silencing of the paternal X chromosome. X-inactivation-specific transcript (*Xist*) RNA directs this silencing and is imprinted to be expressed only from the father's X chromosome. Here we investigate how *Xist* is imprinted. Using a transgenesis system, we find that *Xist* imprinting depends on its unpaired state in the father's germ line. When *Xist* is present in two copies (one each on homologous chromosomes) and therefore is paired during meiosis, the gene does not demonstrate paternal-specific expression in the female embryo. However, when *Xist* is present in only one copy and therefore lacks a pairing partner during meiosis, the gene shows strong imprinted expression. Thus, the unpaired state plays a critical role in epigenetic transmission between generations.

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observed previously with YAC Tgs (39–41), intriguing phenotypes were observed in five of the six hemizygous transgenic lines, here named Tg2080, Tg2082, Tg2086, Tg2087, Tg2091, and Tg2092 (Fig. 1B). Two of the lines, Tg2080 and Tg2092, were unstable and were lost after a few generations.

Tg2080/+ had a male founder that carried two to seven Tg copies at a single integration site. The male founder was fertile. Crossing to WT females, however, resulted in F1 progeny that showed a female-specific Tg lethality. Tg2080/+ females died during the neonatal period. On the other hand, Tg2080/+ males were found at reduced frequency. These viability differences were not observed in prenatal embryos, which were recovered at expected Mendelian frequencies regardless of sex (Fig. 1C and Fig. S1). Tg2082/+ also had a male founder that showed a single integration site with four to seven Tg copies. Although both male and female F1 hemizygotes were viable, homozygosity of the Tg resulted in zygotic lethality of both sexes. By contrast, Tg2086 had a female founder with a single integration site and a Tg copy number of 2. Tg2086/+ mice had no gross phenotype. Both female and male offspring produced litters of expected size and at normal Mendelian frequencies (Fig. 1C).

On the other hand, for several hemizygous Tg lines (Tg2087, Tg2091, and Tg2092), sterility was a major issue. Intriguingly, in each case, the sterility was observed only in the male. Notably, all three lines had female founders. In the case of Tg2087/+, male sterility occurred when the autosomal Tg was homozygosed. In the case of Tg2091/Y and Tg2092/+, male sterility was observed in the hemizygous state. Tg2091 is an X-linked integration with 7–16 copies of the Tg. Tg2092 had two integration sites for a total of 30 Tg copies.

To understand the basis of these phenotypes, we first examined *Xist* expression in somatic cells of the progeny. We isolated tail-tip fibroblasts from male and female progeny of each transgenic line and performed *Xist* RNA FISH with accompanying DNA FISH to distinguish the transgenic locus from the endogenous *Xic*. Robust expression of *Xist* RNA was observed from the hemizygous transgenic locus in both male and female cells from all transgenic lines (Fig. 1D). The *Xist* “cloud” produced by the Tg was qualitatively similar to the endogenous *Xist* cloud in the female cell. In the case of hemizygous Tg2087/+, for example, an *Xist* cloud was observed over the transgenic locus in 74.6% of male cells ($n = 114$). Among female cells, 59% showed two *Xist* clouds, one over the Xi and one over the autosomal insertion site;

Fig. 2. Male germline defects in *Xist* transgenic mice. (A) Male hemizygous Tg2092 mice are sterile and have small testes. Shown are representative testes from a transgenic male and a WT littermate control male. (B) Pachytene arrest in the male germ line of hemizygous Tg2092 and Tg2091 mice. PS, pachytene spermatocytes; RS, round spermatids; SC, Sertoli cells; Sp, spermatozoa. (C) γ H2AX and SCP1 immunostaining in pachytene spermatocytes reveal the XY body in WT mice and aberrant foci formation in Tg2092 mice. (D) Normal XY-body formation in the pachytene spermatocytes of hemizygous Tg2091 mice. Immunostaining fluorescence for γ H2AX and DNA FISH for Tg BAC8 integration are shown. (E) Normal XY-body formation in the pachytene spermatocytes of homozygous (Tg/Tg) Tg2087 males. Immunostaining fluorescence for γ H2AX and DNA FISH for Tg BAC8 are integration shown. (F) Defective spermatozoa of hemizygous Tg2091 male: Morphological abnormality in sperm heads of Tg2091 is visualized with DAPI in the spermatocytes spread. (G) Defective sperm cells of homozygous (Tg/Tg) Tg2087 males. Mature sperm isolated from epididymis of the homozygous Tg/Tg male (sterile) and the hemizygous +/Tg male (fertile). Sperm morphology was visualized by differential interference contrast.

several hundred kilobases without being detectable at the level of immunostaining. This asynapsis and any resulting recruitment of the double-strand break repair mechanism could lead to localized silencing (43, 44). Interestingly, 2091 Tg/Y and 2087 Tg/Tg males showed morphological anomalies in the spermatozoa. In the case of Tg2091, dysmorphic head shapes and failed separation of meiotic tetrads were readily apparent (Fig. 2F). There was an absence of mature spermatozoa in the epididymis. In the case of Tg2087, the homozygous males carried mature sperm in the epididymis but failed to sire offspring. Examination of mature spermatozoa revealed heterogeneous morphological abnormalities ranging from a kinked tail to a missing head (Fig. 2G). These anomalies were present only in the homozygous state and provided logical explanations for the male-specific sterility. The female counterparts were fully fertile.

Xist Imprinting: Epigenetic Effects of Transgene Hemizygosity. Unlike the high-copy-number Tg2092/+ line, transgenic lines with low-copy *Xist* integrations generally showed normal XY bodies in pachytene spermatocytes (Figs. 2D and E and 3A). In the male germ line, large regions of asynapsed chromatin are known to be subject to meiotic silencing, as observed in cases of chromosomal translocations (43, 45), and can interfere with meiotic sex chromosome inactivation (46). Our *Xic* Tg was 200 kb, and its insertion at low copy numbers (two to seven times) did not obviously interfere with the meiotic synapsis. Intriguingly, however, the ectopic *Xic* chromatin was changed by the unpaired state of the Tg, most evidently during the pachytene stage of meiosis (Fig. 3A). DNA FISH using BAC8 as a probe showed the ectopic *Xic*, when hemizygous (+/Tg) in the male germ line, to be compact chromatin visualized as a small and tight pinpoint signal (Tg2086 is shown as an example in Fig. 3A). This compact signal is typical of observations of BAC8 Tgs in somatic cells (e.g., in mouse embryonic fibroblasts) (Fig. 1D) and therefore was not surprising.

On the other hand, when present in the homozygous stage (Tg/Tg), the *Xic* chromatin became decompacted, with the DNA FISH signals appearing much more diffuse than typical for

somatic cells (Fig. 3A, Lower). To quantitate this difference, we performed DNA FISH and compared the sizes of the BAC8 transgenic signals in Tg/Tg versus +/Tg cells (Fig. 3B). We captured z-stack images at 0.2- μ m intervals and measured the size and spread of the DNA FISH signals, expressed in terms of a “condensation index,” as defined by the normalized compass (see the legend of Fig. 3B). A significant difference was observed between the sizes of the Tg loci in +/Tg versus Tg/Tg cells, with loci in Tg/Tg cells occupying a greater territory and, hence, being more open (Fig. 3B). On the other hand, when the 2086 Tg/Tg spermatocytes had not yet entered meiosis (spermatogonial cells), the BAC8 transgenic loci were well separated, and both loci appeared as small pinpoints (Fig. 3C). We conclude that pachytene chromatin is treated differently during pachytene and that the hemizygous, unpaired state of BAC8 sequences resulted in a greater degree of chromatin compaction.

The compaction state of chromatin has been well correlated with the gene activity state, with condensed chromatin associated with gene repression and decondensed chromatin associated with gene activation (47–49). Therefore one might predict that the Tg would be better expressed in the more open chromatin of the homozygous state. However, we were surprised to see increased *Xist* expression in the +/Tg state and lower expression in the Tg/Tg state (Fig. 3D; elevated *Xist* expression of the +/Tg cells was not associated with a large *Xist* cloud). This paradoxical finding is consistent with a previous report indicating that *Xist* expression is favored by the heterochromatic state of the *Xic* in somatic cells (50) and also is consistent with *Xist* being expressed only from the silent Xi (8). Thus, hemizygosity affects not only the chromatin compaction of the Tg but also its expression status during male pachytene. We conclude that *Xist* expression is favored, in general, by condensed chromatin.

Paternal Effects: Xist Imprinting Promoted by Hemizygosity. The effect of the hemizygous state on *Xist* expression was arguably small (twofold). However, because *Xist* normally is expressed only at very low levels in the male germ line (several RNA copies

Fig. 4. Imprinted *Xist* expression in the transgenic preimplantation embryo. (A) Transgenic *Xist* expression was observed in trophectoderm cells of blastocyst-stage (E3.5) embryos in which the Tg was inherited exclusively from the father (+/Tg) by crossing heterozygous fathers to WT mothers. *Xist* and *Tsix* RNA FISH were performed using strand- and locus-specific LNA or DNA oligonucleotide probes. Trophectodermal cells were sampled from the mural trophectoderm surrounding the blastocoel to avoid inner cell mass cells located at the polar region of the embryo. Arrowheads indicate Tg insertion sites. (B) No transgenic *Xist* expression was detected in trophectoderm cells of blastocyst-stage (E3.5) embryos when the Tg was inherited exclusively from the mother (Tg/+) by crossing heterozygous mothers to WT fathers. *Xist* RNA FISH was performed using locus-specific LNA probes. (C) Transgenic *Xist* expression in the blastocyst was affected by the Tg zygosity of the father but not of the mother. For each transgenic embryo (E3.5 blastocyst) with Tg derived from the father, the percentage of cells showing transgenic *Xist* expression was counted. At least five embryos were analyzed for each category. [†]TgXist RNAs in Tg/Tg cells were qualitatively different, ranging from small pinpoints to modest RNA clouds (Fig. 5). Averages $\pm$ SE are shown. * $P < 0.05$ as calculated by Student's *t* test comparing the result of Tg/Tg father with that of +/Tg fathers, both from Tg2086. By convention, the maternal genotype is noted first: Tg/+, maternal transmission; +/Tg, paternal transmission. (D) *Xist* and BAC5 DNA FISH was performed after the RNA FISH of cells shown in A, with the BAC5 probe (probe 2 in Fig. 1A) marking the endogenous *Xic*. The Tg (arrowheads) is indicated by *Xist* DNA signal (red) that is not associated with the BAC5 DNA signal (green).

dramatically in the embryos inheriting the Tgs from a homozygous father, and *Xist* was absent in most of the cells (example 1 in Fig. 5B). In resulting female embryos, the number of trophoblastic cells with two *Xist* RNA clouds (one transgenic, one endogenous) was reduced similarly, and the transgenic *Xist* cloud was mostly absent (example 3 in Fig. 5B). Additionally, among the minority of Tg/Tg cells that did express transgenic *Xist*, the *Xist* foci ranged from small pinpoints to modest RNA clouds and therefore were qualitatively different from normal *Xist* clouds (Fig. 5). Thus, although the Tg carries sufficient genetic information to imprint *Xist*, imprinting cannot be executed properly when the Tg is present in the homozygous state during male meiosis.

Discussion

Both the maternal and paternal germ lines play critical roles in imprinting the X chromosome for dosage compensation (24–27, 29, 32). Although the maternal germ line lays down a repressive mark that precludes *Xist* expression from the X^M in the early embryo, the paternal germ line imprints a permissive mark that drives *Xist* expression from the X^P (Fig. 6A). Our present work has elucidated one aspect of how the imprint is applied to *Xist* on the X^P . The work demonstrates that (i) the information necessary for paternal *Xist* imprinting is contained with the 200-kb BAC8 Tg and (ii) the cross-generational imprinting effect results directly from the unpartnered or hemizygous state of the *Xist* locus in the male germ line.

In our transgenic model (Fig. 6B), the 200-kb BAC8 Tg includes essential noncoding regulatory elements flanking *Xist* and is sufficient for ectopic *Xist* expression and long-range chromatin silencing *in cis*. The unpartnered *Xist* Tgs account for several hundred kilobases to several megabases of nonhomologous DNA sequence on autosomes (Fig. 1B) and are chromatinized in a manner similar to the XY body (Fig. 2). The phenomenon we observe is likely related to the extent of nonhomology rather than to Tg copy number per se. Higher copy numbers of the Tg would promote extensive hemizygosity and, in our study, are correlated with phenotypic defects affecting viability and/or fertility. Judging by the highly visible accumulation of γ -H2AX at Tg sites in many lines (Fig. 2), we infer a behavior analogous to the meiotic sex chromosomes and propose that the hemizygous—and therefore unpartnered or unpaired—state of *Xist* in the male germ line partly drives the imprinting of this gene. One attractive mechanism might involve meiotic silencing by unpaired DNA (MSUD) or chromatin (37, 52), a genome-defense phenomenon first described in fungi meiosis where the absence of pairing or synapsis triggers a silencing mechanism limited to the unpaired chromatin segment (52). The phenomenon also occurs in mammals (37).

We postulate that chromatin modifications resulting from the unpaired state of the X-inactivation center may be directly responsible for imprinting *Xist* and account for the paternal-specific *Xist* expression in the next generation. Thus, *Xist* imprinting appears to depend on both (i) sequences specific to the *Xic* contained within the 200-kb BAC8 Tg that would render *Xist* receptive to imprinting, and (ii) passage of those sequences through the male germ line as a nonhomologous, unpaired chromosomal region to mark the *Xist* gene (Fig. 6). At the same time, the unpaired state of the X chromosome may result in imprinting of the X-linked repetitive elements (14). Together, the imprinting of *Xist* and repetitive elements may partially underlie imprinted XCI. In mice, genomic imprinting of autosomal loci occurs prenatally in prospermatogonia (53) and without obvious involvement of meiotic asynapsis. Thus, different mechanisms may be used by imprinted XCI and autosomal imprinting.

Materials and Methods

Generation of Transgenic Mice. Purified BAC8 DNA (clone 399K20) (54) was microinjected pronuclearly into fertilized embryos, which were transplanted into recipients for the creation of transgenic mice. Microinjection and the production of transgenic animals were carried out by the Transgenic and Gene Targeting Facility (Massachusetts General Hospital). The presence and

Fig. 6. *Xist* imprinting is promoted by the hemizygous state in the paternal germ line. (A) In the WT germ line, imprints are applied to both the X^M and the X^P . The X^M imprint (gray) is repressive for *Xist* expression in the early embryo, whereas the X^P imprint (green) is permissive for *Xist* expression. In the XY body, *Xist* lacks a pairing partner, and the unpaired state promotes its imprinting. (B) In the transgenic animal, imprinting of *Xist* is promoted by the hemizygous state (green) of the Tg in the male germ line, and Tg*Xist* is expressed in the early embryo. When the Tg is homozygous (paired; gray), *Xist* expression is repressed in the early embryo.

copy number of BAC8 was assessed by PCR and quantitative PCR (qPCR) on tail or toe DNA using primers against the BAC8 vector sequence as well as five unique genomic regions in the 194.4-kb BAC8 insert sequence. *Hprt* and *Pgk* genomic primers were used for internal controls. Primers used were pB4 forward: AGTTGGAACCTCTTACGTGCCGAT and reverse: ATGTGGTGTGACCGGAA-CAGAGAA; Sp85 forward: GGGTCTCTACCTATCAACAAATAGAATC and reverse: TTAAGGAAGGAACCTTAGACAGGAATGTAG; *Xist*_E1 forward: CGGTT-CTCCGTGGTCTCTC and reverse: GGTAAGTCACCATACACAC; BAC8_3 forward: GGTTTAGGCTCCATTCTTAAGACCTCAT and reverse: GGCTACA-GAGCTAGTCCAAGACACCA; BAC8_4 forward: ATCCAGGACACCAGG-ATTCTCC and reverse: CATCTAGAACTAGCTTGAGAGG; BAC8_5 forward: TTCAATTGTTTAATGAAATATCCACA and reverse: GGGGTATCATAAAGGTG-CTG; *Hprt* forward: CTGCTACTTCAACTCCTGGTGTGC and reverse: AGGCGA-ATTGGGATGTAGCTCAG; and *Pgk1* forward: AAGGGCTCAGATTCTCAGGACTC and reverse: GTTTCGGATTCTGACCGATGGTC. Transgene integration was verified by DNA FISH on fibroblasts isolated from skin or tail. Mouse experiments were conducted under oversight of the Institutional Animal Care and Use Committees (IACUC) at Massachusetts General Hospital and University of California, Irvine.

RNA FISH, DNA FISH, and Immunostaining. FISH and immunostaining in cells were performed as described in refs. 55 and 56. FISH and immunostaining in meiotic spermatocytes and embryos followed the published protocol (57). For immunofluorescence, cells were blocked with PBS, 0.2% Tween20, 1% BSA. Anti-H3K27me3 (no. 39535; Active Motif) (1:100), anti- γ H2AX (JBW301; Upstate) (1:5,000), or anti-SCP1 biotin (Novus Biologicals) (1:300) were incubated at room temperature for 1–2 h, followed by three washings of 5 min each in PBS and 0.2% Tween20, were incubated with secondary antibodies at 1:500 for 1 h at room temperature followed by three more washings, and were mounted in VECTASHIELD with DAPI (Vector Laboratories). For *Xist* RNA-FISH, a fluorescein-12-dUTP-labeled DNA probe or Cy5-labeled locked nucleic acid (LNA) was used. Tsix RNA-FISH was performed using Cy3-labeled ssDNA oligo probes. For DNA FISH combined with RNA FISH

immunostaining, RNA FISH or immunostaining was performed first. Slides were postfixed in 4% (wt/vol) paraformaldehyde, treated with RNase A, and denatured. Images were collected using Nikon Eclipse 90i and Zeiss LSM780 microscopes and were analyzed with Volocity software (PerkinElmer).

Quantitative Measurement of the Chromatin Domain. Z-section images were captured with the Zeiss LSM780 confocal system. Image analysis and measurements were performed using Volocity software on projected 2D planes generated from stacked z-sections. The normalized compass is defined as a/A , where a measures the area of Tg chromatin spread by DNA FISH and A measures the corresponding nucleus size by DAPI.

qRT-PCR. Total RNA was isolated using TRIzol (Life Technology) and treated with TURBO DNase (Life Technology). One microgram of RNA was reverse-transcribed

using the SuperScript III first-strand reverse transcription kit (Life Technology). qPCR was performed using VeriQuest SYBR qPCR Master Mix (Affymetrix) on the Chromo 4 DNA engine (Bio-Rad). Ct values were normalized to *Gapdh* to calculate the relative expression levels. Primer pairs were as follows: Xist NS33, CAGAG-TAGCGAGGACTGAAGAG; NS66, GCTGGTTCGTCTATCTTGTTGGG (58); *Gapdh* forward, ATGAATACGGCTACAGCAACAGG; *Gapdh* reverse, GAGATGCTCAGT-GTTGGGGG (38).

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