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# *Ftx* is dispensable for imprinted X-chromosome inactivation in preimplantation mouse embryos

SUBJECT AREAS:

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X-chromosome inactivation (XCI) equalizes gene expression between the sexes by inactivating one of the two X chromosomes in female mammals. *Xist* has been considered as a major *cis*-acting factor that inactivates the paternally derived X chromosome (Xp) in preimplantation mouse embryos (imprinted XCI). *Ftx* has been proposed as a positive regulator of *Xist*. However, the physiological role of *Ftx* in female animals has never been studied. We recently reported that *Ftx* is located in the *cis*-acting regulatory region of the imprinted XCI and expressed from the inactive Xp, suggesting a role in the imprinted XCI mechanism. Here we examined the effects on imprinted XCI using targeted deletion of *Ftx*. Disruption of *Ftx* did not affect the survival of female embryos or expression of *Xist* and other X-linked genes in the preimplantation female embryos. Our results indicate that *Ftx* is dispensable for imprinted XCI in preimplantation embryos.

Female mammals have a unique mechanism of gene dosage compensation called X-chromosome inactivation (XCI), which is an essential epigenetic process for development. In mice, inactivation of the paternally derived X chromosome (Xp), but not of the maternally derived X chromosome (Xm), is initiated in the preimplantation embryos (imprinted XCI)<sup>1–3</sup>. This imprinted inactivation of the Xp is maintained in post-implantation extra-embryonic tissues. At the blastocyst stage, reactivation of the Xp occurs in the inner cell mass that will later form the embryo. Thus, the imprinted XCI is erased once, subsequently, random XCI of either the Xp or Xm chromosomes is initiated in the post-implantation embryo by a *cis*-acting region on the X chromosome termed the X-inactivation centre (Xic), which is essential as a genomic element for XCI<sup>4,5</sup>. Xic encodes a long non-coding (lnc)RNA *Xist*, which is expressed from the inactive X chromosome and represents the trigger for chromosome-wide silencing after implantation. Female mice with disrupted *Xist* alleles inherited from the father show severe growth retardation and die during post-implantation embryogenesis because of the failure of the imprinted XCI in extra-embryonic tissues<sup>6</sup>. Although *Xist* was also reported to play a major role in imprinted XCI before implantation<sup>7</sup>, the precise mechanism of imprinted XCI and how *Xist* expression is controlled remain unknown.

We recently identified a number of imprinted genes expressed predominantly from the inactive Xp at the preimplantation stage<sup>8</sup>, including three lncRNAs: *Xist*, *Ftx*, *Jpx*; two micro (mi) RNAs, miR-374-5p and miR-421-3p; and a protein-coding gene, *Zcchc13*. Combined with previous reports showing that *Tsix* was imprinted and expressed from the Xm<sup>9,10</sup>, these newly identified imprinted genes form a large cluster named the “*Tsix-Zcchc13* imprinted gene cluster”. Furthermore Okamoto *et al.* indicated that part of this cluster, from *Tsix* to *Ftx* excluding *Zcchc13*, is included in the 210 kb region that contains sufficient factors to trigger imprinted XCI<sup>11,12</sup>. They reported that a 210 kb single-copy transgene (Tg80) region containing *Tsix* to *Ftx* induced imprinted *cis*-inactivation in autosomes in preimplantation embryos when it was derived paternally. Among the four imprinted genes located in this transgene, namely *Tsix*, *Xist*, *Jpx*, and *Ftx*, *Ftx* is the most abundant transcript at the preimplantation embryo stage except for *Xist*. Furthermore, Chureau *et al.* proposed that *Ftx* is a positive regulator of *Xist*, as deletion of the *Ftx* promoter leads to decreased *Xist* expression in male embryonic stem (ES) cells<sup>13</sup>. However, its role in XCI remains to be elucidated for female embryos.

Taking these reports into consideration, we focused on the function of imprinted *Ftx* at the preimplantation stage. Using *Ftx*-deficient female mice, we analysed the effects of deletion of *Ftx* on female viability and imprinted XCI during preimplantation development.



## Results

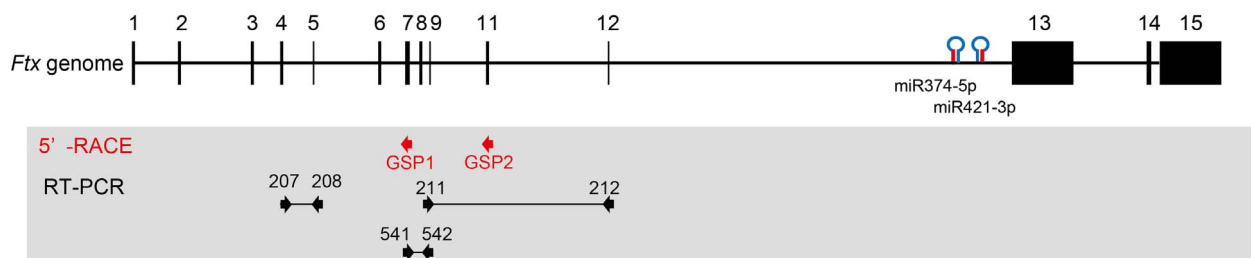
**Generation of *Ftx*-deficient mice.** To generate *Ftx*-deficient mice, we determined the transcription start site of *Ftx* using 5'-rapid amplification of cDNA ends (RACE). We designed two gene-specific primers (GSPs) at exon 7 (GSP1) and exon 11 (GSP2) of *Ftx* based on information from mouse genomic sequences (UCSC mm9; <http://genome.ucsc.edu/>) (Figure 1a). We recently reported that *Ftx* is imprinted and exhibits predominant expression in female blastocysts<sup>8</sup>. Consistent with this, we detected two major bands in female blastocysts using both primers (Figure 1b), indicating that these transcription start sites were indeed imprinted. Sequencing of these two major bands revealed that the transcripts were transcribed from exons 1 and 3 (24 and 17 clones, respectively, Figure 1c). In addition to these two major start sites, a few clones were also identified from exon 4 as start sites (two clones, Figure 1c). Therefore, we disrupted *Ftx* by homologous recombination (Figure 2a). The targeting vector replaced exons 1–4 with a neomycin (Neo) cassette in the mutated allele, resulting in complete disruption of imprinted *Ftx* lncRNA expression. Correct targeting of the *Ftx* allele in ES cell clones by homologous recombination was confirmed by polymerase chain reaction (PCR) amplification (Figure 2b). Two independent *Ftx*-deficient ES cell lines, #64 and #77, were injected into blastocysts to create germline chimeras. The mutated allele derived from recombinant ES cells was transmitted to female offspring from chimeric males, and the Neo cassette was then removed by mating with CAG-FLPe transgenic mice (Figure 2c). We confirmed complete loss of *Ftx* expression in *Ftx*-deficient blastocysts by reverse transcription (RT)–PCR analysis (Figure 2d, RT-PCR primers (211/212) amplified exon 9–12, and primers (541/542) amplified exon 7–9 as shown in Figure 1a).

**Expression of miRNAs in *Ftx*-deficient blastocysts.** Two miRNAs, miR-374-5p and miR-421-3p, are located within the *Ftx* intron in the same orientation as the *Ftx* transcript (Figure 1a). We confirmed that these miRNAs were not expressed in female blastocysts (+<sub>m</sub>/–<sub>p</sub>) when the *Ftx*-targeted allele was derived from the father, whereas these miRNAs were predominantly expressed in wild-type female blastocysts (+<sub>m</sub>/+<sub>p</sub>) (Figure 2e). This finding indicated that the *Ftx*-deficient mice failed to express miR-374-5p and miR-421-3p simultaneously, and suggested that both miRNAs were transcribed from the same promoter as *Ftx*.

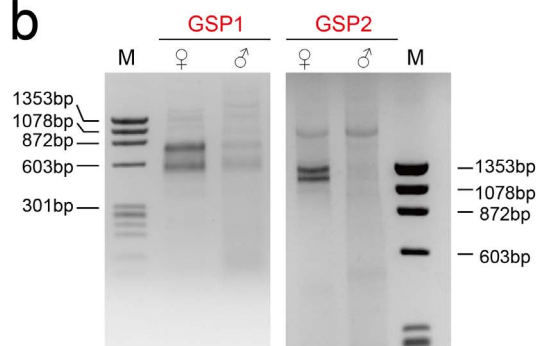
**Mating of *Ftx*-deficient mice.** Paternal transmission of the *Xist*-deficient allele produces female-specific embryonic death because of failure of imprinted XCI in extra-embryonic cell lineages<sup>6</sup>. We examined whether paternal transmission of *Ftx* would affect female development, as with *Xist*. Mating *Ftx*-deficient mice (–/Y) with wild-type *Mus mus domesticus* (C57BL/6N) mice (+/+) generally produced offspring at the expected Mendelian ratios (Table 1, chi-square = 0.52). A similar result was observed in another mouse line from recombinant ES cell line #77 (Supplementary Table S1, chi-square = 0.52). Moreover, other crosses, namely +/– × +/Y, +/– × –/Y, and –/– × –/Y, also produced offspring at the expected Mendelian ratios (chi-square >0.1). All offspring of *Ftx*-deficient mice were viable and fertile which indicated that deleting *Ftx* had no serious effects on female viability.

**Expression of *Xist* in *Ftx*-deficient blastocysts.** *Xist* was reported to be upregulated by *Ftx* in male ES cells<sup>13</sup>. Based on this report, we examined whether disruption of *Ftx* would affect *Xist* expression in preimplantation embryos. First, we carried out quantitative (q)-PCR, and found that the expression level of *Xist* in the *Ftx*-deficient

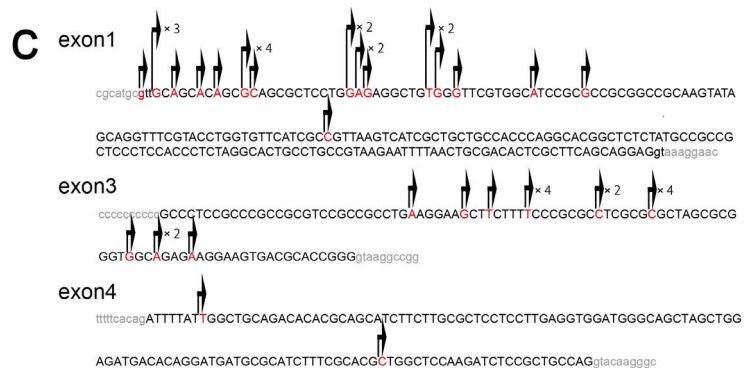
**a**



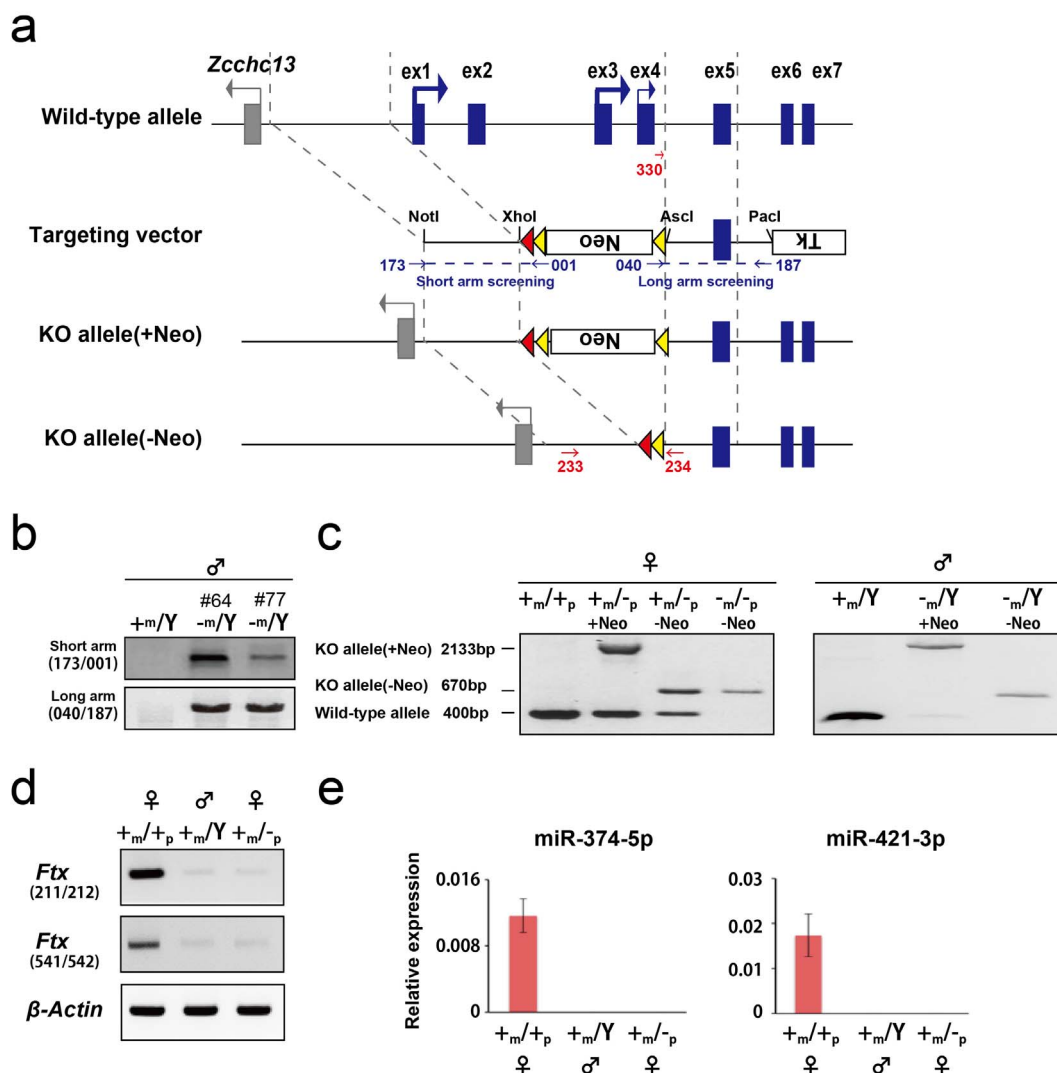
**b**



**c**



**Figure 1 | Identification of the 5' ends of *Ftx* by 5'-RACE.** (a) *Ftx* genomic structure in the mouse genome is shown. Exons are indicated by rectangles (black). The two miRNAs, miR-374-5p and miR-421-3p, are clustered in the intron of *Ftx*. The locations of the gene-specific primers (GSP1 and GSP2) used for 5'-RACE analysis are shown as red arrows. The RT-PCR primer positions used to assess the *Ftx* expression levels are shown as black arrows. (b) Ethidium bromide-stained gel of 5'-RACE products: ♀, female blastocysts; ♂, male blastocysts; M, molecular-weight markers (PhaiX174 HaeIII digest). (c) Summary of 5'-RACE sequences. Transcriptional start sites of each clone are indicated in red and with arrows. The numbers of clones of the same sequence are shown to the right of the arrows. The starts sites tended to distribute within each exon. Reported exon sequences are indicated by capital letters.



**Figure 2 | Targeted disruption of the *Ftx* gene.** (a) Scheme for generating the *Ftx* knockout (KO) allele. *Ftx* exons are shown as blue boxes. Two major start sites and one minor one are indicated by heavy and light arrows, respectively. FRT sites, denoted as yellow triangles, flank the neomycin-resistance gene (*Neo*) as a positive gene selection marker, and *loxP* sites are shown as red triangles. Primers used for validating gene recombination in ES cells are shown as blue arrows. Primers used for genotyping are shown as red arrows. (b) Validation of recombination on the chromosomal long and short arm target sequences of ES cells (cell lines #64 and #77). (c) Genotyping results for wild-type females (+<sub>m</sub>/+<sub>p</sub>, where m is the maternally derived X chromosome and p is the paternally derived X chromosome), heterozygous females (+<sub>m</sub>/-<sub>p</sub>), homozygous females (-<sub>m</sub>/-<sub>p</sub>), wild-type males (+<sub>m</sub>/Y, where Y is the Y chromosome) and hemizygous males (-<sub>m</sub>/Y). The PCR primers (233, 234, and 330) used for genotyping are depicted in Fig2-a. (d) Expression of *Ftx* in *Ftx*-deficient blastocysts. PCR primers used for RT-PCR are shown in parentheses. Uncropped images of the full-length gels are presented in Supplementary Figure S1. (e) The expression levels of miR-374-5p and miR-421-3p were quantified by q-PCR in wild-type female (+<sub>m</sub>/+<sub>p</sub>), male (+<sub>m</sub>/Y) and heterozygous female (+<sub>m</sub>/-<sub>p</sub>) blastocysts. The ratios of the number of cDNA copies for each miRNA to that of cDNA copies for miR-295 are indicated as expression levels. The bar graphs and lines show the mean ± standard deviation (SD) (*n* = 3).

blastocysts was not affected significantly; it was the same as in wild-type blastocysts (Figure 3a). Next, we analysed *Xist* expression by RNA-fluorescence *in situ* hybridisation (FISH) in the *Ftx*-deficient blastocysts. A single *Xist* RNA accumulation signal was detected in a *Ftx*-deficient as well as a wild-type blastomere at the blastocyst stage, indicating that the localisation of *Xist* had not been disturbed (Figure 3b). Figure 3c shows summary of *Xist* RNA FISH analysis. There was no significant difference of the number of the cells with *Xist* RNA signal between *Ftx*-deficient and wild-type embryos. These data indicate that deleting *Ftx* had no apparent effect on *Xist* expression at the preimplantation stage.

#### Effects of *Ftx* disruption on imprinted XCI in preimplantation embryos.

Our results with crossing experiments among *Ftx*-deficient

mice and normal *Xist* expression in the *Ftx*-deficient blastocysts indicated that disruption of *Ftx* has no major role on imprinted XCI. However, the effect of *Ftx*-disruption on each X-linked gene was still uncertain. We next performed restriction enzyme fragment polymorphism (RFLP) analysis to examine the allelic expression patterns of *Xist* and four X-linked genes, *Fmr1*, *Rnf12*, *Fgd1*, and *Padh1*, which are all subject to imprinted XCI. We took advantage of a single nucleotide polymorphism found in each gene between C57BL/6N and the Japanese wild mouse *M. m. molossinus* (JF1), and examined the expression patterns of all five genes in *Ftx*-deficient blastocysts. The expression patterns of all genes were similar to those of wild-type embryos (Figure 3d). Taken together, the results indicate that disruption of *Ftx* expression produced no obvious effects on imprinted XCI during preimplantation development in mice.

Table 1 | Mating of *Ftx*-deficient mice (line #64)

Cross	Male pups		Female pups		Total pups	Mean litter size
(♀ × ♂) +/+ × -/Y	+/Y 40		+/- 45	+/O* 1		
	40		46		86	7.8
Cross	Male pups		Female pups		Total pups	Mean litter size
(♀ × ♂) +/- × +/Y	+/Y 25	-/Y 23	+/+ 32	-/+ 21		
	48		53		101	7.2
Cross	Male pups		Female pups		Total pups	Mean litter size
(♀ × ♂) +/- × -/Y	+/Y 28	-/Y 26	+/- 23	-/- 22		
	54		45		99	7.0
Cross	Male pups		Female pups		Total pups	Mean litter size
(♀ × ♂) -/- × -/Y	-/Y 36		-/- 46			
					82	6.8

\*: XO female.

## Discussion

In this study, we examined whether *Ftx* might be involved in the mechanisms of imprinted XCI at the preimplantation stage. Although *Xist* deletion results in female-specific embryonic death when it is derived from the father because of the lack of XCI in extra-embryonic tissues<sup>6</sup>, disruption of *Ftx* did not affect survival rates or litter sizes in both sexes, demonstrating that this ncRNA is not necessary for the survival of female mice. Further studies indicated that *Ftx* disruption did not cause major deficiency in the expression of *Xist* or the expression of X-linked genes in preimplantation embryos. These results are inconsistent with the hypothesis that *Ftx* is a positive regulator of *Xist* in XCI, as proposed by Chureau *et al.*<sup>13</sup>. Because they analysed the expression of *Xist* using *Ftx*-deficient XY male ES cells that had one active X chromosome and lacked XCI, their results might not reflect the situation for female cells *in vivo*. Chureau also reported that, in addition to *Xist*, *Ftx* deletion decreased the expression levels of the adjacent imprinted genes, *Jpx* and, *Zcchc13*<sup>13</sup>. It would be of interest to examine whether these adjacent genes are also affected in female cells. We were also interested in whether *Ftx* is involved in the onset of the imprinted *Xist* upregulation during early preimplantation stages because previous studies have shown that *Xist* RNA upregulation is detected from the two- to four-cell stage onward<sup>14,15</sup>. We used q-PCR to examine the *Ftx* expression at preimplantation stages and found that the expression begins at the blastocyst stage (Figure S1). This suggests that *Ftx* is not involved in the onset of this *Xist* upregulation. As far as we examined, *Ftx* disruption had no apparent effects on imprinted XCI at the preimplantation stage. This indicates that *Ftx* is dispensable for imprinted XCI, and that the remaining genes included in the 210 kb regulatory region of imprinted XCI—*Tsix* and/or *Jpx*—might play essential roles in the regulation of imprinted *Xist*.

*Tsix*, an antisense transcript to *Xist*, is another lncRNA that functions as a repressor of *Xist* during the initiation of random XCI in post-implantation embryos<sup>9,10</sup>. It inhibits the expression of *Xist* on one female X chromosome to maintain the active state of the randomly chosen one. It is imprinted and expressed from maternally derived X chromosomes at the preimplantation stage<sup>10</sup>. However, whether *Tsix* is also required for the imprinted expression of *Xist* in preimplantation embryos remains unclear. *Tsix* expression was reported to start later than *Xist* expression from Xp alleles, implying that there were mechanisms other than *Tsix* that repress *Xist* expression from Xm alleles<sup>16</sup>. Further study is required to reveal the role of *Tsix* in imprinted XCI at the preimplantation stage.

*Jpx* is located just downstream of the *Ftx* locus. Its deletion in XX female ES cells results in failure of accumulation of *Xist* on the inactive X chromosome and XCI in differentiated ES cells, indicating that *Jpx* can upregulate *Xist* expression in random XCI<sup>17</sup>. However, how *Jpx* regulates *Xist* expression *in vivo* is not known. In the case of imprinted XCI, there are only three paternally expressed imprinted genes, *Xist*, *Ftx*, and *Jpx*, located in the 210 kb transgene. Apart from *Ftx*, *Jpx* is the only candidate gene that potentially upregulates *Xist*. Based on its proposed role in ES cells<sup>17</sup>, studies in *Jpx*-targeted mice will be of interest to delineate the necessity of this imprinted ncRNA in imprinted XCI.

In this study, we focused on imprinted XCI, but not on random XCI. Because the *Tsix-Zcchc13* cluster is included within the Xic region that plays a major role in random XCI, it is necessary to elucidate whether *Ftx* is involved in random XCI in post-implantation embryos. Further study is required to elucidate the mechanism of XCI, but the results presented in this paper will help clarify the answer.

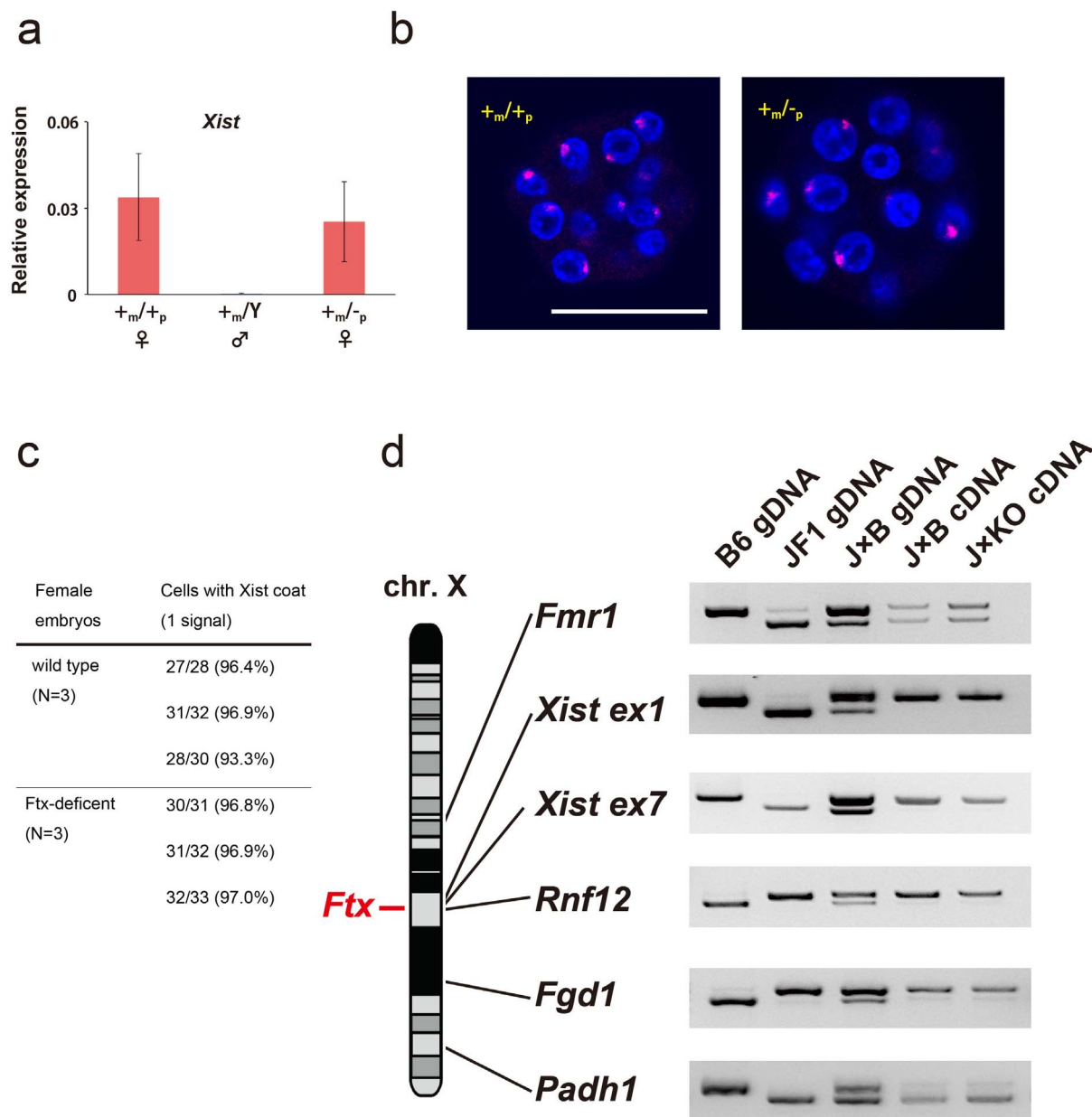
## Methods

**Animals.** All animal experiments were performed according to the guidelines of the Committee on the Use of Live Animals in Teaching and Research of Tokyo Medical and Dental University, and animal protocols were reviewed and approved by the animal welfare committee of the Tokyo Medical and Dental University (Permit Number: 0130228A).

**Embryo collection.** For blastocyst collection, 8-week-old C57BL/6N female mice (Crea Inc., Tokyo, Japan) were superovulated with an intraperitoneal (i.p.) injection of 5 IU of pregnant mare serum gonadotropin (Aska Pharmaceutical Co. Ltd., Tokyo, Japan) followed by an i.p. injection of 5 IU of human chorionic gonadotropin (hCG; Aska Pharmaceutical Co. Ltd.) 48 h later. These superovulated female mice were mated with C57BL/6N(+Y) or *Ftx*-deficient mice (-Y) and blastocysts were collected from the uterus on 3.5 dpc from around 92–100 h post-hCG.

**5'-RACE.** Total RNA was prepared from male and female wild-type blastocysts using TRIzol (Invitrogen, Carlsbad, CA, USA). 5'-RACE was performed on RNA prepared from blastocysts with GSP1 and GSP2 (Supplementary Table S2) using SMARTer RACE cDNA Amplification kits (Clontech, Mountain View, CA, USA). To separate male and female blastocysts, we used a non-invasive sexing method by tagging X chromosomes with an enhanced green fluorescent protein (EGFP) transgene<sup>18–20</sup>. With this method, the female (XX<sup>EGFP</sup>) blastocysts can be separated from the non-fluorescent male (XY) blastocysts by mating X<sup>EGFP</sup>Y male mice to wild-type female (XX) mice. The RACE products were subsequently cloned into pCR4-TOPO (Invitrogen) for sequencing. Sequencing was performed using BigDye Terminator v3 Cycle Sequencing kits (ABI, Life Technologies, Carlsbad, CA, USA).

**Construction of the *Ftx*-targeting vector.** The targeting vector was designed to replace exons 1–4 of *Ftx* with a pNT1.1 Neo cassette. A 5.3 kb *NotI*–*XhoI* fragment as



**Figure 3 | Expression of *Xist* in *Ftx*-deficient blastocysts.** (a) q-PCR analysis of *Xist* RNA in *Ftx*-deficient blastocysts. The ratios of the number of cDNA copies for *Xist* to that of cDNA copies for a housekeeping gene,  $\beta$ -actin, are indicated as expression levels. The bar graphs and lines show the mean  $\pm$  standard deviation (SD) ( $n = 3$ ). (b) RNA FISH analysis for *Xist* (red) expression in *Ftx*-deficient blastocysts. The nuclei of blastocysts were stained with DAPI (blue). Scale bar, 50  $\mu$ m. (c) Summary of *Xist* RNA FISH analysis. Three wild-type ( $+_m/+_p$ ) and Three *Ftx*-deficient ( $+_m/-_p$ ) blastocysts were examined. Cells with 2 *Xist* signals were not observed. (d) Analysis of X-linked gene expression levels in *Ftx*-deficient blastocysts. No differences in the allelic expression patterns of X-linked genes between JF1 and C57BL/6N embryos were detected using RFLP. Uncropped images of the full-length gels are presented in Supplementary Figure S1.

a short chromosomal arm and a 5.0 kb *AscI*–*PacI* fragment as a long arm were obtained by PCR using genomic DNA of a bacterial artificial chromosome clone (RP23, #239G16) as a template. Primers used for constructing the *Ftx* targeting vector are listed in Supplementary Table S2.

**Generation of *Ftx*-deficient mice.** The targeting vector was linearized with *NotI* digestion and electroporated into ES cells (EGR-G101) derived from C57BL/6N mice (Crea, Inc.). ES cell clones that were candidates for possessing the targeted allele were isolated by positive selection with G418. Correct targeting of the *Ftx* allele in ES cell clones by homologous recombination was confirmed by PCR. Positive ES cell clones were subsequently used to generate chimeras by injection into 8-cell stage ICR strain embryos. Injected embryos were transferred to ICR strain pseudopregnant foster mothers, resulting in the birth of chimeric mice. Male chimeric mice were crossed with C57BL/6N female mice to obtain F1 heterozygous female offspring, and then crossed with CAG-FLPe transgenic mice (B6.Cg-Tg (CAG-FLPe)) to delete the Neo

cassette. The *Ftx*-deficient mice used in the present study were from a C57BL/6N background. The primers used for confirming homologous recombination are listed in Supplementary Table S2.

**q-PCR analysis.** Total RNA of embryos was extracted using TRIzol (Invitrogen) and contaminating DNA was digested using RNase-free DNase (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized using reverse transcriptase SuperScript III (Invitrogen). For miRNA expression analysis, total RNA was reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (ABI). The TaqMan MicroRNA assays used (with identification numbers in parentheses), were as follows: mmu-miR-374-5p (001319), mmu-miR-421-3p (002700), and mmu-miR-295 (000189). Mature miRNAs were amplified with TaqMan Universal PCR Master Mix II (ABI) using a LightCycler 480 Instrument (Roche, Basel, Switzerland). The q-PCR reactions were performed using samples collected from three independent preparations.



**FISH.** *Xist*-specific probes were prepared by nick translation with Cy3-dCTP (GE Healthcare, Little Chalfont, UK) from p*Xist* 1986–9498. Blastocysts were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min on ice and incubated in 0.1% Triton X-100 in PBS for 10 min on ice. Hybridisation was carried out at 42 °C overnight. The nuclei of embryos were stained with DAPI. *Xist* signals were observed using a confocal scanning laser microscope (LSM 510; Carl Zeiss, Jena, Germany).

**Polymorphic analysis.** (J × B) or (J × KO) F1 embryos were obtained from mating 8- to 12-week-old wild-type JF1 (J) female mice with wild-type C57BL/6N (B) or *Ftx*-deficient male mice (KO). Polymorphisms of X-linked genes between JF1 and C57BL/6N were detected by RFLP analysis. Primers and restriction enzymes used for polymorphic analysis are summarized in Supplementary Table S2.

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## Author contributions

M.S. and S.K. performed most experiments, assisted by Y.F., M.O. and I.F.; S.K., Y.F. and M.O. generated mutant mice. M.S. and S.K. analysed the data. M.S. and S.K. wrote the manuscript and all authors discussed the results and commented on the manuscript.

## Additional information

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

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