X chromosome inactivation in a female carrier of a 1.28 Mb deletion encompassing the human X inactivation centre

B. de Hoon1, Erik Splinter3, B. Eussen2, J. C. W. Douben2, E. Rentmeester1, M. van de Heijning4, J. S. E. Laven2, J. E. M. M. de Klein2, J. Liebelt5 and J. Gribnau1

1 Department of Developmental Biology, 2 Department of Clinical Genetics, and 3 Department of Obstetrics and Gynaecology, Erasmus MC, Rotterdam, The Netherlands
4 Cergentis B.V., Utrecht, The Netherlands
5 Division of Genetics and Molecular Pathology, Women’s and Children’s Hospital, North Adelaide, South Australia, Australia

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Authors for correspondence:
J. Liebelt
e-mail: jan.liebelt@sa.gov.au
J. Gribnau
e-mail: j.gribnau@erasmusmc.nl

X chromosome inactivation (XCI) is a mechanism specifically initiated in female cells to silence one X chromosome, thereby equalizing the dose of X-linked gene products between male and female cells. XCI is regulated by a locus on the X chromosome termed the X-inactivation centre (XIC). Located within the XIC is XIST, which acts as a master regulator of XCI. During XCI, XIST is upregulated on the inactive X chromosome and chromosome-wide cis spreading of XIST leads to inactivation. In mouse, the Xic comprises Xist and all cis-regulatory elements and genes involved in Xist regulation. The activity of the XIC is regulated by trans-acting factors located elsewhere in the genome: X-encoded XCI activators positively regulating XCI, and autosomally encoded XCI inhibitors providing the threshold for XCI initiation. Whether human XCI is regulated through a similar mechanism, involving trans-regulatory factors acting on the XIC has remained elusive so far. Here, we describe a female individual with ovarian dysgenesis and a small X chromosomal deletion of the XIC. SNP-array and targeted locus amplification (TLA) analysis defined the deletion to a 1.28 megabase region, including XIST and all elements and genes that perform cis-regulatory functions in mouse XCI. Cells carrying this deletion still initiate XCI on the unaffected X chromosome, indicating that XCI can be initiated in the presence of only one XIC. Our results indicate that the trans-acting factors required for XCI initiation are located outside the deletion, providing evidence that the regulatory mechanisms of XCI are conserved between mouse and human.

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1. Introduction
X chromosome inactivation (XCI) is a process that takes place in all female somatic cells, and results in almost complete transcriptional silencing of one of the X chromosomes. This process ensures that female XX cells have an equal dosage of X-chromosomal gene products compared to male XY cells [1]. XCI is initiated early during embryogenesis, where all cells of the female embryo proper randomly initiate inactivation of either the paternal or the maternal X chromosome. After the inactive state is established, the inactive X (Xi) is clonally passed on to all daughter cells. As a result, all females are a

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mosaic of cells with an active paternal X chromosome and cells with an active maternal X chromosome. The X inactivation ratio (XIR) between cells carrying an active paternal X chromosome and cells carrying an active maternal X chromosome differs between female individuals. In a female population the average XIR is 50 : 50, but ranges up to 100 : 0, as a result of chance, genetic predisposition or cell selection [2]. Within a female individual the XIR correlates between tissues of different origin [3].

The genetic elements involved in regulating XCI have been a subject of study for several decades. A region on the X chromosome was identified to be required for XCI. In human, this X inactivation centre (XIC) has been mapped to a 680–1200 kb region at chromosome Xq13 [4–6], deduced from several inversions and truncations involving the X chromosome in different individuals.

The genes that constitute the XIC are conserved in eutherians, and have been mostly studied in mouse. In contrast to the human XIC, the mouse Xic is less well defined and has been mapped to a 10–20 Mb region on the X chromosome [7,8]. Centrally located in the XIC/Xic is the long non-coding RNA (IncRNA) \( \text{XIST} \), which is essential for XCI [9,10]. Initiation of XCI is characterized by \( \text{XIST} \) upregulation on the future Xi, resulting in the \( \text{cis} \) spreading of \( \text{XIST} \) RNA, which recruits chromatin remodelling complexes that silence the X chromosome [11]. In mouse, several other IncRNA genes located within the Xic are involved in regulating XCI, through co-activation and -repression mechanisms acting to regulate \( \text{Xist} \), predominantly \( \text{in-cis} \) [12–16]. Several \( \text{trans} \)-acting X-encoded XCI activators ensure that XCI is only initiated in the presence of two X chromosomes [17]. In mouse the RING finger protein 12 (\( \text{Rnf12} \), also known as \( \text{Rlim} \)) represents the most prominent XCI activator [18,19]. \( \text{Rnf12} \) encodes an E3-ubiquitin ligase that targets \( \text{REX1} \), a repressor of \( \text{Xist} \), for proteasomal degradation [20]. Only in cells with more than one X chromosome present in the nucleus is the concentration of RNF12 and other XCI activators high enough to initiate XCI.

In human, the regulation of XCI is less clear. Although the XIC has been localized, the requirement for \( \text{trans} \)-regulatory mechanisms has not been studied. To examine whether XCI in human is also regulated by \( \text{trans} \)-regulatory mechanisms acting on the XIC, which contains all the \( \text{cis} \)-regulatory elements to direct \( \text{XIST} \) expression, we have identified and studied a female carrier with a deletion of the XIC.

2. Results
A 26 year old, intellectually normal, non-dysmorphic woman presented with primary amenorrhoea, and was diagnosed with hypergonadotrophic ovarian failure due to ovarian dysgenesis, also known as premature ovarian insufficiency (POI). Blood examination showed increased FSH levels (more than 40 IU l\(^{-1}\)), with decreased oestrogen (less than 60 pmol l\(^{-1}\)). Height was between the 75th–90th centile and no stigmata of Turner syndrome were evident on examination. External genitalia were unambiguously female. Breast development, pubic and axillary hair distribution were normal female at the time of examination following several years of oral contraceptive hormone treatment. On transvaginal ultrasound examination a small uterus was visible, measuring 1.9 × 0.9 × 1.4 cm\(^3\), with hypoplastic endometrium of 0.1 mm. No clear ovarian tissue was identified; however, an area of thickening without antral follicles 0.6 cm in length was seen in the left ovarian fossa and a similar region 1 cm in length, in the right ovarian fossa. Karyotype analysis of blood cells showed a normal 46XX karyotype. DNA was isolated from peripheral blood and was used for molecular SNP karyotyping, using the Human OmniExpress-24 SNP-array from Illumina. Several regions showing loss of heterozygosity were identified, which might be explained by a history of parental consanguinity. However, only one single significant copy number change was identified corresponding with a region showing loss of heterozygosity, which was located on chromosome X and included a deletion of the XIC (figure 1). No other copy number changes were identified (electronic supplementary material, figure S1).

The centromeric breakpoint of the identified deletion was located in a 148 kb long segment between SNPs rs650032 and rs650032. This region is characterized by a large inverted segmental duplication, including \( \text{DMRTC1} \) and \( \text{DMRTC1b} \), complicating the precise mapping of this breakpoint (figure 2a). The location of this breakpoint determines if the duplication is intact and present as two copies of \( \text{DMRTC1} \) and \( \text{DMRTC1b} \), or partially deleted and consequently present as a single copy of \( \text{DMRTC1} \) on the affected X chromosome. The telomeric breakpoint of this region was located in a 17 kb region, delineated by SNPs rs12668161 and rs5981589. A deletion encompassing the XIC and as small as this, has not been reported before, which prompted us to study this deletion in detail. To characterize the breakpoint regions more precisely we performed qPCR to determine the copy number at consecutive positions along the breakpoint regions. As a control, DNA from a healthy female and a healthy male was used. qPCR analysis along the centromeric breakpoint region, detected two copies centromeric of the inverted duplication in this region, and one copy telomeric. Three qPCR amplicons within the duplication all indicated the presence of 1.5 copies, suggesting the breakpoint is located in the middle of the inverted duplication (figure 2b). To map the deletion at the single nucleotide level, targeted locus amplification (TLA) was performed on frozen lymphoblastoid cells. TLA technology entails sequencing of all sequences that occur in physical proximity to a primer pair used for targeted amplification. Using this technology, with primer sets located on the telomeric side of the deletion, we found an increase in coverage in the centre of the inverted duplication, indicating the position of the centromeric breakpoint (figure 3a,b). Among the obtained reads the break-spanning read was also identified (figure 3c). The flanking sequence of the centromeric breakpoint maps to both duplions of the inverted duplication, but the orientation of the sequence corresponds to the centromeric duplication. This maps the deletion to ChrX: 72.080.568–73.367.054 (hg19), a region of 1.28 Mb, and among the genes involved in the deletion are \( \text{XIST} \), \( \text{TSIX} \) and other \( \text{cis} \)-regulatory genes and elements in XCI (electronic supplementary material, table S1).

The deletion was confirmed by DNA-FISH, using several BACs mapping to different regions of the X chromosome. DNA-FISH using BACs containing \( \text{PHKA2} \) and \( \text{RNF12} \) confirmed the presence of two X chromosomes in all nuclei, while DNA-FISH using BACS covering \( \text{XIST} \) or \( \text{IPX} \) indicated...
these regions to be present on only one X chromosome in all cells (figure 4b). XCI was analysed by XIST RNA-FISH on cells from a lymphoblastoid cell line of the patient. All examined cells contained a XIST RNA cloud, indicating XCI is initiated in cells with the 1.28 Mb deletion (figure 4b). To assess the XIR, allele specific methylation analysis of fragile X mental retardation 1 (FMR1), on DNA isolated from peripheral blood, showed predominant inactivation of one allele (93 : 7%), indicating that XCI is severely skewed in the patient (data not shown). To determine which X chromosome was inactivated, sequential DNA-RNA FISH was performed for XIST RNA and the JPX locus, included in the deletion. XIST RNA domains co-localized with the JPX locus of the unaffected X chromosome, indicating that all trans-acting factors are still present. These results and the human deletion presented here indicate that JPX, and other sequences including TSIX, previously implicated in trans-regulation of XCI, through X-pairing and RNA mediated recruitment mechanisms, are not required to properly initiate the human XCI process in-trans [13,21].

Whether the deletion described here represents the entire cis-acting XIC remains elusive. This human cis-XIC is most likely delineated by the topologically associating domain(s) (TAD) in which XIST resides. TADs were identified by studies examining the higher order chromatin structure, indicating limitation of spatial interactions to cis domains of approximately 1 Mb in size [15,22]. Analysis of available data on TADs in human H1 male ES cells and IMR90 female fibroblasts, indicates that the human XIC of H1 ES cells is included in a single large TAD, ranging from DMRTC1 to RNF12, not including RNF12 [22]. In human IMR90 fibroblasts containing an Xi, this region is divided into two smaller TADs with the boundary at the XIST/TSIX locus which now includes RNF12, similar to mouse (figure 5).

This change in higher order topology might be instructive in or the consequence of XCI and considering these data, the human XIC might extend beyond the studied deletion from DMRTC1 to FTX, and might include RNF12.

3. Discussion

Here we identified a patient with a deletion encompassing the human XIC. Our results demonstrate that in the presence of the identified 1.28 Mb deletion, XCI can still occur on the unaffected X chromosome. Therefore, the region involved in the deletion, extending from FTX to the first duplicon of DMRTC1, does not affect trans-regulation of XCI, but most likely covers most elements and genes involved in cis-regulation of human XCI. The identified deletion, and the absence of an X-inactivation phenotype resemble the ΔXTX and Δ(Xite-Dxmit171) deletions that were studied in mouse embryonic stem (ES) cells [16,17]. The ΔXTX deletion involves only Xist, Tsix and Xite, whereas the Δ(Xite-Dxmit171) deletion also includes the neighbouring regulators Jpx and Ftx. ES cells with heterozygous ΔXTX and Δ(Xite-Dxmit171) deletions, inactivate the wild-type X chromosome, indicating that all trans-acting factors are still present. These results and the human deletion presented here indicate that JPX, and other sequences including TSIX, previously implicated in trans-regulation of XCI, through X-pairing and RNA mediated recruitment mechanisms, are not required to properly initiate the human XCI process in-trans [13,21].
Our results indicate that the region involved in the deletion does not include important trans-acting factors. Although the identity of these factors remains elusive so far, our results are compatible with a role for human RNF12 in trans-activation of human XCI, as RNF12 is not included in the deletion and still present on both X chromosomes of the female individual described here (figure 3a). A role for RNF12 in human XCI is further supported by a recent finding that female carriers of a mutation in RNF12 show exclusive XCI of the mutated X [23]. Skewed XCI could be the consequence of cell selection processes, but male carriers with the same mutation are born only displaying X-linked intellectual disability, which might implicate a direct involvement of RNF12 in XCI. This finding is also concordant with mouse studies which show that one functional copy of Rnf12 is required for establishment of the inactive X chromosome [16]. The XIC deletion identified here is comparable to the region described to represent the human XIC, which was defined by genetic studies involving large X chromosomal abnormalities resulting from truncations and translocations. At first the XIC was delineated by an X;14 translocation in an XXY male and the breakpoint of an

Figure 2. XIC deletion identified PCR. (a) The XIC deletion as identified by comparative genomic hybridization (CGH). A schematic representation of the X-chromosome is shown, with the deleted region marked in red. (b) Comparison of qPCR results to CGH. A magnification is shown of the regions containing the breakpoints. The top panel shows qPCR results along the breakpoint regions, with copy number plotted against chromosomal position. In the map of the chromosomal region below, the inverted duplication in the centromeric breakpoint region is marked in blue.

Figure 3. XIC deletion identified by TLA. (a) TLA coverage is plotted against chromosomal position. Arrow indicates the location of the primer sets. Star indicates the increase in coverage on the other side of the deleted region. A genetic map of the region is shown below, with the inverted duplication in blue. (b) Magnification of the centromeric breakpoint region. (c) The break-spanning read, with the sequence centromeric of the breakpoint marked in blue and the sequence telomeric of the breakpoint marked in green.
isodicentric X chromosome (idic(X)) [4,5,24]. Based on these rearrangements the XIC was estimated to measure at least 0.8 Mb in size with a maximum of 2.89 Mb (figure 5). Later the telomeric boundary was redefined by studies involving another rearranged X chromosome (rea(X)), reducing the estimated size of the XIC to 0.68–1.2 Mb [6]. In all these individuals XCI is still initiated, leading to inactivation of the X;14 translocation product, or the mutated idic(X) and rea(X) X chromosomes, demonstrating the presence of sufficient levels of XCI activators for initiation of XCI. Interestingly, the rea(X) does not contain an intact copy of RNF12. These results demonstrate that either RNF12 does not function as a trans-acting activator, or that other human trans-acting activators exist next to RNF12 and are located on the proportion of the X chromosome not deleted from rea(X). In support of this latter hypothesis, mouse studies also hinted at the presence of additional XCI activators besides Rnf12, as ES cells carrying a heterozygous Rnf12 deletion still initiate XCI, although at a reduced frequency [18,19,25]. Although our results suggest RNF12 is a trans-acting activator of human XCI and are concordant with mouse studies, further investigation is required.

![image of DNA-FISH results](https://example.com/dna-fish-results.jpg)

**Figure 4.** The X-chromosome carrying the deletion is preferentially active. (a) At the top, a map of the X-chromosome is shown with the location of the BAC probes that were used indicated in blue. The location of the deletion as identified by CGH and TLA is marked in red. Below, representative images of DNA-FISH for regions located inside or outside the deleted region are shown. BAC CTD-2183M22, containing PHKA2, and BAC CTD 2530H13, containing RNF12, are located outside the deleted region. BAC CTD-2183M22, containing XIST, and BAC RP13-36G14, containing JPX, are located within the deleted region. Results from analysing at least 30 metaphases are shown in the tables to the right. (b) Representative pictures of sequential RNA-DNA-FISH. Two representative nuclei are shown with RNA-FISH results for XIST RNA in green, and DNA-FISH results for BAC RP13-36G14 in yellow and BAC CTD-2180H21 in red. The table to the right shows the percentage of nuclei with overlapping signals of XIST RNA and BAC RP13-36G14 DNA.
The female individual presented here suffers from ovarian dysgenesis. As our results indicate complete skewing of XCI, with the affected X chromosome remaining active, the deletion itself might be involved in ovarian dysgenesis. Prior work has also implicated chromosome Xq13, harbouring the XIC, in amenorrhoea, as several cases have been described with a breakpoint in this region and primary amenorrhoea and various signs of Turner syndrome [26]. Hypothesizing that one of the genes in the identified deletion is responsible for the ovarian dysgenesis phenotype, we assessed all involved genes by gene ontology (GO) analysis using the AmiGO 2 browser. GO terms involved in gonadal differentiation, and included in the 1.28 Mb deletion studied here. XCI is 100% skewed and as a result cells carrying the XIC deletion will contain only one active copy of DMRTC1, leading to ovarian dysgenesis. Interestingly, functional loss of other protein coding genes, including CDX4 and CHIC1 appears well tolerated, which might be explained by redundant mechanisms, as reported for CDX4 in mice, or escape of XCI.

In summary, our studies show that XCI is normally initiated in a female carrier with a 1.28 Mb deletion of the XIC, and indicate that the trans-acting information required for female specific initiation of XCI is located outside the identified deletion. Our findings highlight the evolutionary conservation of pathways and mechanisms involved in regulation of XCI in eutherians.

4. Material and methods

(a) Targeted locus amplification

Cross-linked chromatin, was fragmented and re-ligated, and two sets of primer pairs were designed for the sequence telomeric of the deletion, and used in individual TLA amplifications according to de Vree et al. [28]. PCR products were purified and pooled, prepared for sequencing using the Illumina NexteraXT protocol, and sequenced on an Illumina Miseq sequencer. Reads were mapped using a BWA-SW algorithm allowing partial mapping. In case of a deletion, existing interactions with the deleted region are abolished and new interactions with the new neighbouring sequence are produced.

(b) DNA-FISH

Cells were arrested in metaphase using colcemid for 2 h, treated with 0.075 M KCl, and fixed with methanol/acetic acid. Slides were dehydrated, denatured and incubated with probe mixture as described in [18]. Probes, indicated in figure 4a, were detected with a FITC conjugated mouse-anti-digoxigenin antibody or
Alexa 594 conjugated streptavidin. Metaphase spreads were first identified in DAPI and subsequently the number of red and green foci were counted.

(c) DNA-RNA-FISH
Cytoplasmic preparations were prepared and cells were fixed with 4% PFA, and RNA-FISH was performed as described before [17]. Cells were analysed for presence of an XIST RNA cloud and photographs were taken. Subsequently slides were subjected to DNA-FISH. Cells were denatured and incubated with probe mixture and detected with a FITC conjugated mouse-anti-digoxigenin antibody or Alexa 594 conjugated streptavidin. Cells analysed for RNA-FISH were re-analysed for a DNA-FISH signal. Pictures from DNA-FISH and RNA-FISH were overlaid and scored for co-localization of signals.

(d) RT-PCR
Quantitative RT-PCR was performed using Platinum Taq DNA polymerase (Life Technologies), SYBR green (Sigma Aldrich) according to the manufacturer's instructions. Primer sets and their chromosomal locations are listed in electronic supplementary material, Methods 1.

(e) SNP and CNV detection
Molecular SNP karyotyping was performed on a Human OmniExpress-24 SNP-array (Illumina). Data analysis was performed with the BioDiscovery’s SNP-FASST2 segmentation algorithm, an extension of the FASST2 segmentation algorithm (a hidden Markov model [HMM] based approach). B-allele frequency probes were assigned to a range of possible states and a combination of the BAF and log-R states were used to make the final copy number and allelic event calls. The significance threshold for segmentation was set at $5 \times 10^{-9}$ also requiring a minimum of eight probes per segment and a maximum probe spacing of 1000 kb between adjacent probes before breaking a segment. The log ratio thresholds for single copy gain and single copy loss were set at 0.25 and −0.2, respectively. The homozygous frequency threshold was set to 0.85. The homozygous value threshold was set to 0.8. The heterozygous imbalance threshold was set to 0.4. The minimum loss of heterozygosity length was set at 300 kb.

Ethics. The participant was recruited through the Childhood Overgrowth (COG) Study, which began recruitment in 2005, approved by the London Multicenter Ethics Committee (05/MRE02/17). Informed consent was obtained from all participants.

Data accessibility. This article has no additional data.

Authors’ contribution. B.d.H., J.E.M.M.d.K. and J.G. designed the experiments. B.d.H., E.S., B.E., J.C.W.D., E.R., M.v.d.H. and J.S.E.L. performed the experiments. J.L. collected patient material and conducted the initial screen. B.d.H. and J.G. wrote the manuscript.

Competing interests. We do not have any competing interests.

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