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A whole-organism landscape of X-inactivation in humans

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Björn Gylemo, Maike Bensberg, Colm E Nestor

Crown Princess Victoria Children's Hospital, Department of Biomedical and Clinical Sciences (BKV), Linköping University, Linköping, Sweden

eLife Assessment

The study provides a **valuable** analysis of escape from X-inactivation based on three rare female GTEX-donors with non-mosaic X-inactivation. The methods and analyses are **solid** and broadly support the authors' claims. Their data are more comprehensive than those presented previously and add significant weight to evidence for which genes are inactivated or escape from X inactivation in humans.

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Abstract

As females are mosaic for X-inactivation, direct determination of X-linked allelic expression in bulk tissues is typically unfeasible. Using females that are non-mosaic (completely skewed) for X-inactivation (nmXCI) has proven a powerful and natural genetic system for profiling X-inactivation in humans. By combining allele-resolution data for one previously reported and two newly identified nmXCI females, we directly determined X-inactivation status of 380 X-linked genes across 30 normal tissues, including 198 genes for which XCI status is directly determined for the first time. Our findings represent a substantial advance in our understanding of human X-inactivation and will serve as a reference for dissecting the genetic origin of sex-bias in human traits. In addition, our study reveals nmXCI as a common feature of the human female population, with profound consequences for the penetrance and expressivity of X-linked traits in humans.

Introduction

During embryonic development in humans, female cells (46, XX) inactivate a single X-chromosome to balance dosage of X-linked gene expression between females (XX) and males (XY). The process of X-chromosome inactivation (XCI) begins during the peri-implantation stage in humans and is initiated by expression of the long non-coding RNA (lncRNA) *XIST* from the X inactivation center (XIC) of one X-chromosome in each female cell. *XIST RNA* coats the X-chromosome which in turn recruits protein complexes including chromatin modifiers which establish a facultative heterochromatin state. Silencing histone modifications and the deposition of DNA methylation

ultimately result in the transcriptionally silent inactive X-chromosome (Xi) (Supp Fig. 1A). The selective expression of *XIST* from only one X-chromosome needs to be tightly regulated. Whereas the exact mechanism by which a given X-chromosome in a female cell is selected for inactivation is unknown, the XIC in humans includes several lncRNAs that have been implicated in the regulation of *XIST* expression and X-inactivation (Supp Fig. 1A) (1). Although the initial choice of X-chromosome for inactivation is random, the inactivated X-chromosome is stably inherited in a clonal fashion throughout all subsequent cell divisions (2). Interestingly, the process of X-inactivation (XCI) is incomplete; approximately 15% of genes escape XCI and remain expressed from both X-chromosomes (3). These 'escape' genes are consequently more highly expressed in females than in males, although the degree of sex-biased expression of escape genes can vary and may be greater for those genes that lack a functional homologue on the Y-chromosome (4). The sex-biased expression of escape genes has been suggested to mediate sex differences in both the frequency and severity of several diseases (5, 6). In addition, genes in the pseudoautosomal regions (PAR1 and PAR2) of the X-chromosome also remain expressed from the Xi and are thus always bi-allelically expressed. PAR1 and PAR2 are short regions of homology between the X- and Y-chromosomes that are essential for pairing of the sex chromosomes during meiosis in males (7). In contrast to escape genes, which tend to be more highly expressed in female cells, genes in the PARs have been suggested to show male-biased expression (8). Consequently, the terms PAR and nonPAR are used to distinguish between the biallelic expression (escape) of genes within the PARs and those located elsewhere on the X-chromosome.

Human females are typically 'mosaic' for X-inactivation; female tissues contain a mixture of cells that have inactivated either the maternal or paternal X-chromosome (**Fig. 1A**). Mosaicism renders analysis of XCI in primary human tissues highly complex or unfeasible. Consequently, our knowledge of XCI and escape across human tissues is surprisingly sparse and typically based on indirect measures of XCI, such as DNA methylation, sex-biased expression or from observations in cell lines and animal models (4, 9). In a seminal study, Brown and colleagues reported XCI status for 639 genes by integrating data from three published studies that each used different indirect approaches and model systems to infer XCI status (3). This resource continues to serve as a valuable reference for inferred XCI status in humans.

Interestingly, rare cases of humans in which the same parental X-chromosome has been inactivated in all cells (non-mosaic XCI, nmXCI) have been reported (8, 10–12). Organism-wide nmXCI can arise from a constitutional genetic variant that directly interferes with inactivation of a specific parental X-chromosome, such as mutations in the promoter region of the *XIST* gene (10) and X-autosome translocations (13, 14). Disruption of the XIC locus or autosomal regions involved in the choice of X-chromosome to be inactivated have similarly been suggested to result in direct nmXCI, so called 'primary' skewing (15). Alternatively, selection against any deleterious constitutive genetic variant would result in clonal expansion of cells and indirectly result in nmXCI; 'secondary' skewing (**Fig. 1A**). As nmXCI females lack the confounding effect of mosaicism, they represent a powerful system to study human XCI by enabling direct determination of XCI status from bulk tissue samples. Indeed, using a single nmXCI female identified in the Genotype-Tissue Expression (GTEx) database (16), Tukiainen and colleagues generated the first comprehensive reference map of XCI status across human tissues based on direct determination of allele-specific expression of X-linked genes (8). However, as the ability to determine allele-specific expression is limited to the presence of an informative (heterozygous) genetic variant in the gene of interest, the XCI status of just 186 genes was determined across tissues in this one nmXCI female.

In this study, we identify two additional unrelated nmXCI females in the GTEx database. By combining allele-resolution data for the one previously reported nmXCI female and two newly identified nmXCI females, we directly determined X-inactivation status of 380 X-linked genes

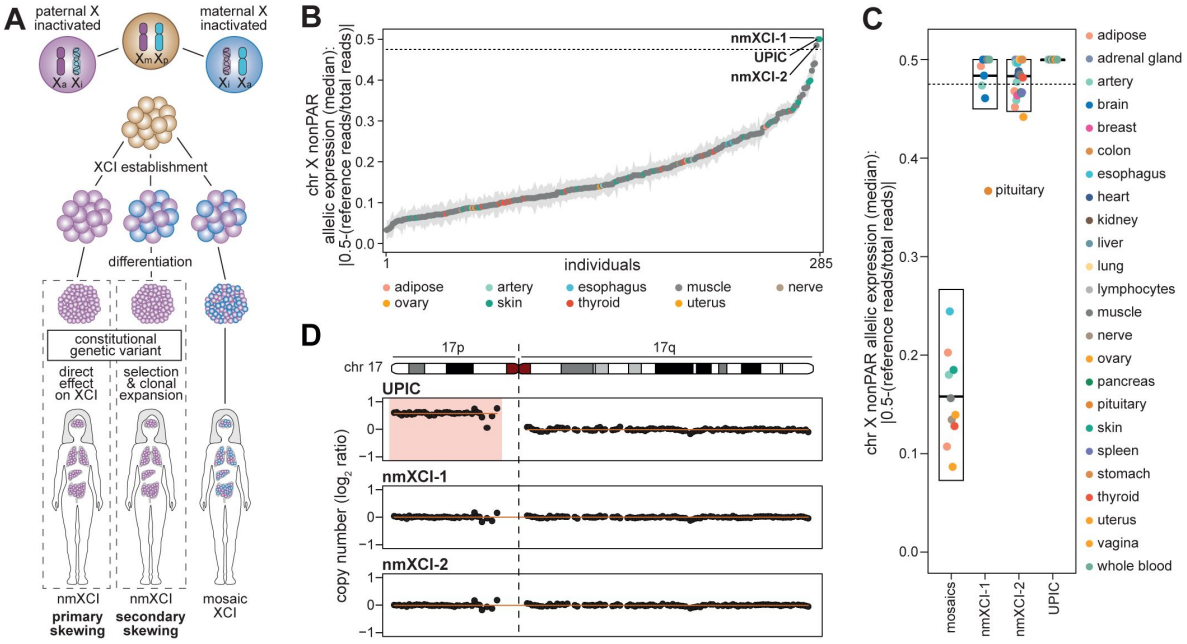

Fig. 1.

Identification of females with non-mosaic X-inactivation.

(**A**) The patterns of X-chromosome inactivation (XCI) in women resulting in mosaic (right female) or non-mosaic XCI (nmXCI). The presence of genetic variants can result in nmXCI females by (i) directly determining which X-chromosome can be inactivated (primary skewing, left female) or by (ii) imparting a selective advantage to a small number of cells (secondary skewing, middle female). Xa, active chr X; Xi, inactive chr X, Xm, maternal chr X, Xp, paternal chr X. (**B**) Single-tissue median allelic expression (AE) and standard error of all nonPAR genes on chromosome X (chr X) not previously classified as variable in all 285 women in GTEx. (**C**) Allelic expression per tissue of nonPAR chr X genes not previously classified as variable in mosaic females (median allelic expression < 0.475) and three females identified as non-mosaic, nmXCI-1, nmXCI-2 and UPIC (median allelic expression > 0.475). Boxplot indicating median, 25th and 75th percentile. (**D**) Copy number as log2 ratio of chromosome 17 (chr 17) for nmXCI females, UPIC, nmXCI-1 and nmXCI-2. Trisomy 17p in UPIC is highlighted.

across 30 normal tissues, including 15 tissues in which XCI has not previously been characterized. This unique dataset allowed investigation of tissue-specific escape from XCI in humans and generation of the most extensive multi-tissue map of human X-inactivation to date.

Results

Non-mosaic X-inactivation is common in human females

Whereas the frequency of nmXCI females in the general population was originally thought to be less than 1:500 (11), more recent work has suggested a frequency of as high as 1:50 (12). To identify nmXCI females we screened a single tissue of all 285 female donors (Table S1, Table S2) in the GTEx database (v8 release) (16).Calculating the allelic expression (AE, how much the ratio of Xa/Xi reads deviates from the expected 0.5) of all X-linked genes outside of the PARs (i.e. the nonPAR AE) allows assessment of skewing in female tissues. Briefly, allelic expression was determined by calling heterozygous SNPs on the X-chromosome using genomic data (whole exome sequencing (WES) or whole genome sequencing (WGS)) and subsequently counting the number of each base at every heterozygous SNP (hetSNP) position in RNA-seq data. The allelic expression can then be calculated as, abs(0.5 - (reference reads / total reads)), where a value of zero indicates complete biallelic expression and a value of 0.5 indicates complete monoallelic expression. We specifically exclude PAR genes from this calculation as they are not subject to XCI, and consequently, their Xa/Xi read count ratio is not significantly affected by XCI skewing. Three female samples showed an exceedingly high degree of skewing (median chr X nonPAR allelic expression > 0.475 i.e. less than 2.5% of reads coming from the 'inactive' allele), consistent with expression from a single parental chromosome, nmXCI (**Fig. 1B**). Extending allelic expression analysis to all available tissues across the three candidate females (**Table S3**) confirmed their complete nmXCI status (**Fig. 1C**). These three nmXCI individuals included the single previously identified nmXCI female, UPIC (8), confirming the accuracy of our screening approach (**Fig. 1B**, **Fig. 1C**).

nmXCI may result from deleterious genetic mutations causing primary (10) or secondary (acquired) skewing (15) or from stochastic non-random XCI (12). Whereas no small-scale mutations in the XIC were identified in any of the nmXCI females, trisomy 17p was observed in UPIC (**Fig. 1D**, **Supp Fig. 1B**). How trisomy 17p could result in non-random X-inactivation is unclear, but an unbalanced 17p:X translocation would result in strong selection for silencing of the abnormal X and consequential nmXCI (17). However, expression of genes on 17p was consistently higher than those on 17q across all tissues in UPIC, suggesting the duplicated 17p was not silenced (Supp Fig. 1C). Indeed, further investigation of donor metadata in GTEx revealed that UPIC had been diagnosed as mosaic for trisomy 17p by traditional karyotyping but did not provide a detailed karyotype. Interestingly, whereas all three females were nmXCI, females nmXCI-1 (GTEx ID: 13PLJ) and nmXCI-2 (GTEx ID: ZZPU) showed greater variation in allelic expression across tissues than UPIC (**Fig. 1C**), suggesting that their nmXCI may have resulted from clonal selection driven by constitutive genetic variants (secondary skewing).

A landscape of X-inactivation across normal human tissues

The ability to detect allele-specific expression is dependent on the presence of a heterozygous genetic variant (SNP) in the gene of interest. Using WES and WGS for both newly discovered nmXCI donors we identified 389 heterozygous SNPs across 316 X-linked genes (count and overlap between nmXCI-1 and nmXCI-2 of heterozygous SNPs and genes in **Fig. 2A**, distribution of genes covered along the X-chromosome in **Fig. 2B**, **Table S4**). Allelic expression ratios were highly consistent between nmXCI females and allowed us to identify known escape (PAR and nonPAR), inactive and variable (8) XCI genes (**Fig. 2C**). Next, we applied our analytical workflow to the previously identified nmXCI sample, UPIC, observing that the allelic expression levels seen using our analysis pipeline largely matched the findings of Tukiainen *et al* (**Fig. 2D**, **Supp Fig. 2A**)

(8). Notably, our inclusion of additional WGS data of UPIC and identification of 76 further SNPs allowed determination of allelic expression of 36 further genes in this individual (**Fig. 2E**). The use of updated gene-models, correction of reference alignment bias and more stringent quality filtering resulted in exclusion of 35 genes for which allelic expression was erroneously reported (**Fig. 2E**, **Supp Fig. 2B**).

Next, we integrated data from all three nmXCI individuals (nmXCI-1, nmXCI-2 and UPIC), allowing direct allelic expression detection of 380 X-linked genes across 30 tissues, including 15 tissues for which XCI status has not been directly assessed previously. While 195 genes and 17 tissues could only be examined in a single individual, 185 genes and 13 tissues were examined across multiple nmXCI females, allowing for selected inter-tissue and inter-individual comparisons (**Fig. 3A**, **Fig. 3B**). Classification of allelic imbalance (mono-allelic | bi-allelic) of individual alleles was initially determined by the binomial test ($P_{ADJ} < 0.01$), according to best practice (18). However, upon visual inspection we identified several well characterised escape and inactive genes that may have been mis-classified as variable escape (**Fig. 3C**). Inappropriate classification of allelic imbalance results from the well documented sensitivity of the binomial test to data over-dispersion, which frequently occurs with read count data (18, 19). Consequently, we further manually curated the allelic expression status of all 380 genes using the empirical guideline of allelic ratio > 0.4 as indicating mono-allelic expression and the potential consequences of high and low read counts. Our three criteria for manual re-classification were (i) *low power*, indicating genes with a low statistical power but consistent escape pattern, (ii) *low read count*, indicating genes with a consistent escape pattern but with non-significant escape in a single tissue, and (iii) *over-estimation*, genes in which a high read counts inflated the binomial p-value. (**Fig. 3C**, **Supp Fig. 3, Table S4, Table S5**). This manual curation resulted in the re-classification of allelic expression status of 32 genes (**Table S5, Supp Fig. 3**). Our classifications of XCI status based on direct determination of allele-specific expression represent one of the most extensive and high-confidence maps of X-inactivation to date (**Fig. 3D**, **Fig. 3E**, **Supp Fig. 4**). Allelic expression for all genes independent of XCI status was highly correlated between individuals (**Supp Fig. 5A**), and by including three nmXCI individuals in our analysis we were able to determine cross-tissue XCI status for 13 genes which were only covered in one tissue in the previous assessment based on UPIC alone (**Fig. 4A**). Interestingly, whereas we reveal *EGFL6*, *TSPAN6* and *CXorf38* as variable escape genes, these were previously classified (Balaton *et al*) as inactive, variable and escape, respectively. Finally, we compared our classification with the classification of X-linked escape status from donor UPIC as determined by Tukiainen and colleagues (4, 9, 12). As expected, our classification was largely consistent with that of Tukiainen *et al*, but also revealed the possible misclassification of XCI status of several genes and further added the XCI status for 198 genes for which XCI status had not previously been directly assessed (**Fig. 4B**). Our direct assessment of allelic expression further allowed for validation of XCI status previously determined by indirect measures (3, 8). We confirmed XCI status for many previously classified inactive and escape genes whereas most reported variable escape genes are re-classified as inactive based on our analysis (Supp Fig. 5B, Supp Fig 5C). However, as the XCI classifications reported here are largely based on inter-tissue comparisons within individuals, most previous classifications were based on inter-individual comparisons of XCI, confounding direct comparisons between studies.

Discussion

Here we describe an extensive multi-tissue map of human X-inactivation directly determined from allele-specific expression of X-linked genes. This data represents a doubling of the number of X-linked genes for which XCI status has been directly determined across multiple normal tissues and individuals. Our results suggest that XCI is stable both within and across individuals and that tissue-specific escape from X-inactivation is rare (11.6% of total genes (44 variable genes / 380 total covered X-linked genes)) and often challenging to characterize. Variability in X-linked gene

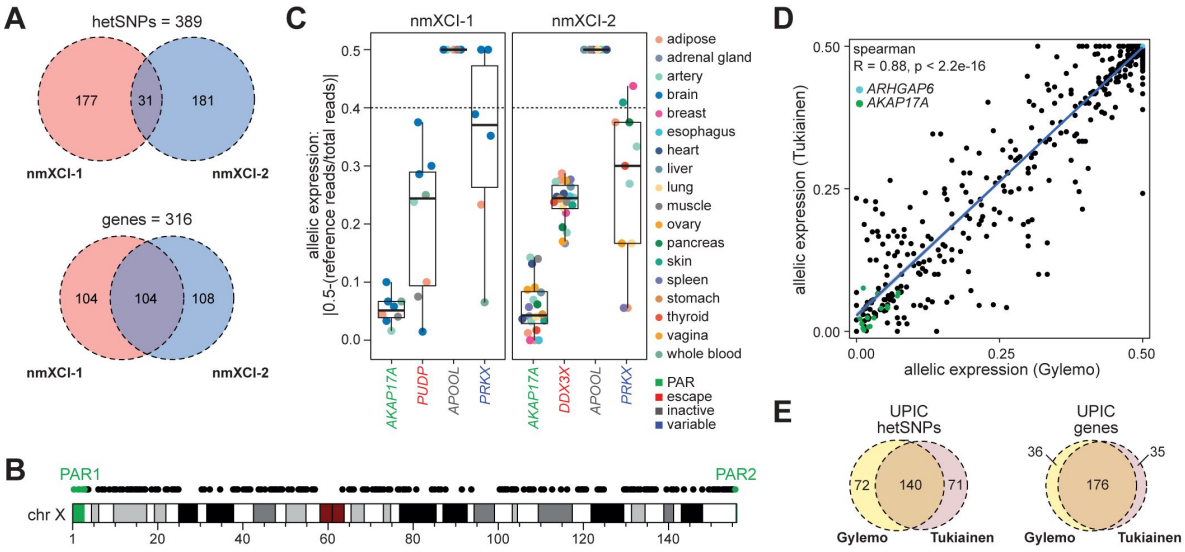

Fig. 2.

Characterization of two novel human nmXCI females.

(A) Overlap of genic heterozygous SNPs (hetSNPs) (upper) and genes with hetSNP (lower) across the two novel nmXCI females (nmXCI-1 and nmXCI-2). Genic hetSNPs were identified using both WES and WGS for each individual. (**B)** Distribution of assessed genes across the X-chromosome. Genes located in the pseudoautosomal region 1 (PAR1) and PAR2 are highlighted in green. **(C)** Allelic expression per tissue for well-characterised PAR, (*AKAP17A*), escape (*PUDP, DDX3X*), inactive (*APOOL*) and variable (*PRKX*) genes (8). Boxplot indicating median, 25th and 75th percentile. **(D)** Spearman correlation of allelic expression values using our analysis approach (Gylemo) and the Tukiainen *et al* analysis pipelines for female UPIC. **(E)** Overlap of genic hetSNPs (left) and genes (right) identified by our analysis (Gylemo) and the Tukiainen *et al* analysis pipeline in female UPIC.

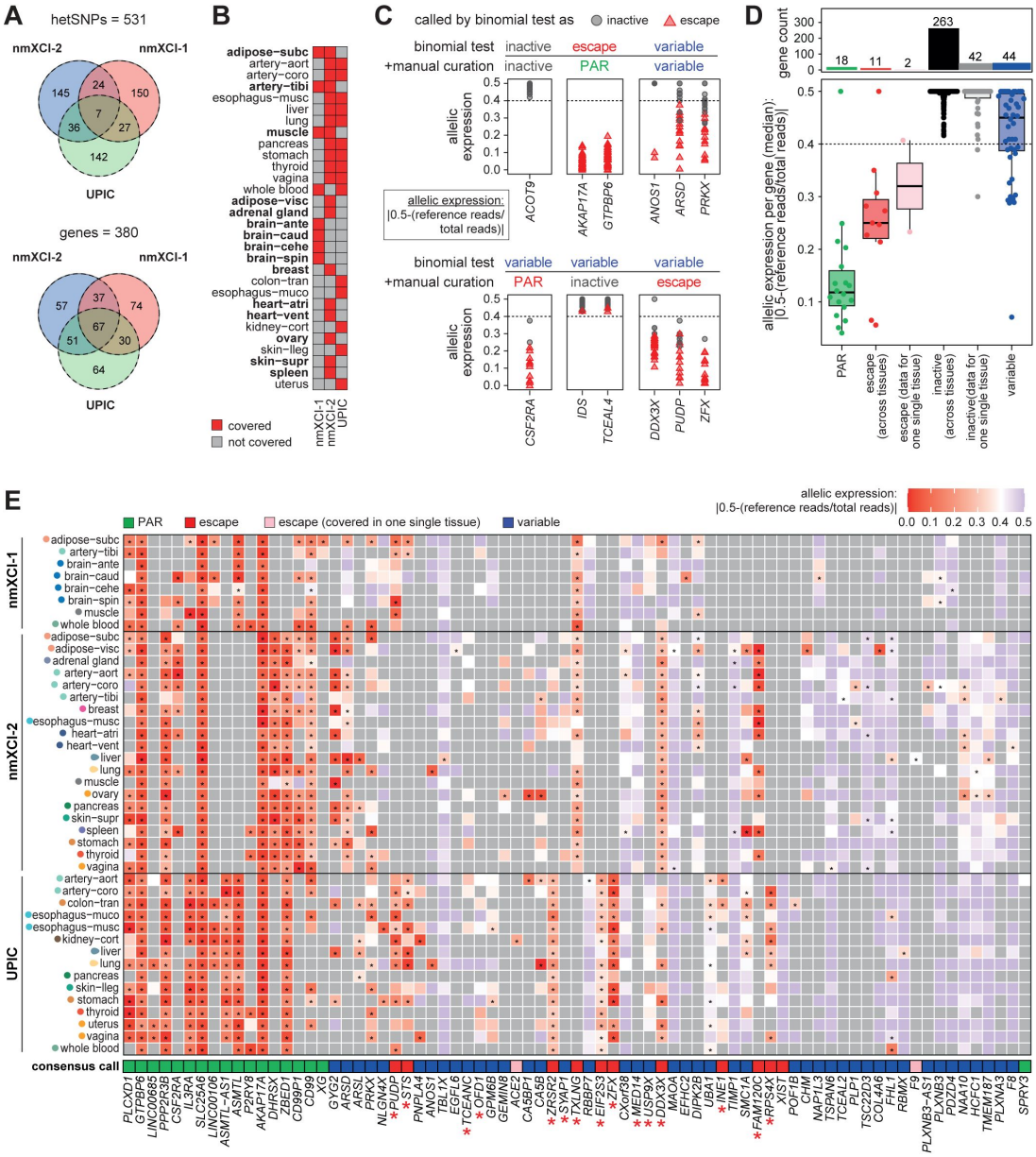

Fig. 3.

An extended landscape of X-inactivation in humans.

(A) Overlap of genic heterozygous SNPs (hetSNPs) (upper) and genes with hetSNP (lower) across the three females (nmXCI-1, nmXCI-2 and UPIC) with non-mosaic X-inactivation (nmXCI). **(B)** Tissues covered in each nmXCI female. Tissues not covered in UPIC are indicated in bold. **(C)** Examples of genes classified by the binomial test alone and after manual curation. Allelic expression across tissues is shown with XCI status based on the binomial test indicated as inactive (grey circle) or escape (red triangle). **(D)** Allelic expression of X-linked gene categories (lower) and number of genes included in each category (upper). Genes classified as escape and inactive are separated based on whether allelic expression was determined across multiple tissues or in a single sample (data for one single tissue). Boxplot indicating median, 25th and 75th percentile. **(E)** Heatmap showing the allelic expression of all genes that show constitutive or variable escape from XCI. Black asterisks within the tiles indicate a significant expression from the inactive X-chromosome (i.e. escape, FDR-corrected binomial q-value < 0.01). The 'consensus call' tile is the assigned XCI status across tissues and individuals for each gene with genes classified as variable including both intra- and interindividual variation. Red asterisks indicate genes in which manual curation of XCI status was performed. Grey tiles indicate missing data. **(B, E)** Tissue abbreviations can be found in Table S7.

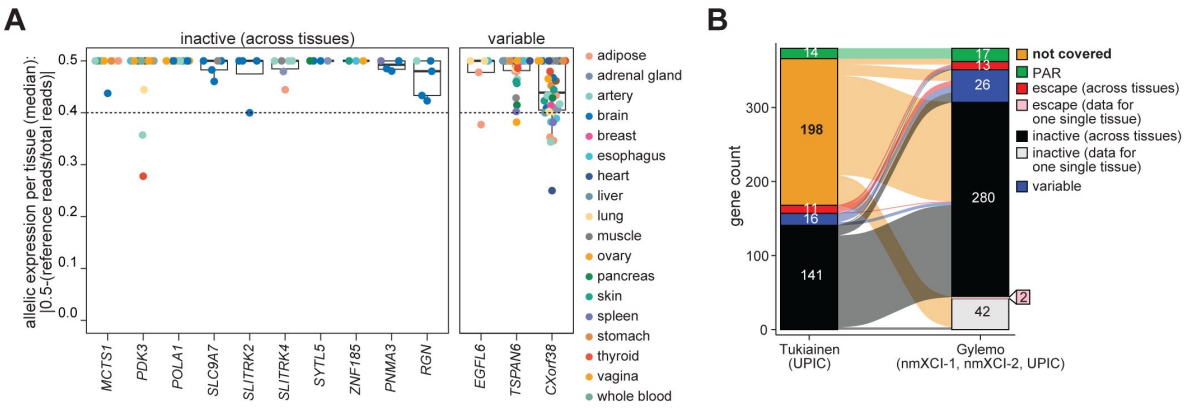

Fig. 4.

Classification and novel XCI assessment of X-linked genes.

(A) Allelic expression across all available tissues in all three nmXCI females for genes which were only covered in one tissue in the previous assessment based on UPIC alone (8) **(B)** Alluvial plot showing classification of escape status of X-linked genes based on our analysis (Gylemo) compared to a previous assessment based on UPIC alone (Tukiainen) (8). Genes classified as escape and inactive are separated based on whether allelic expression was determined across multiple tissues or in only one sample (data for one single tissue).

expression is important to characterize, as XCI variability can directly modify expressivity of X-linked pathogenic variants. This provides a mechanism for sex-biased expression differences in certain biological contexts and tissues, which in turn can modify disease-risk.

nmXCI females allow for direct determination of allele-specific expression of X-linked genes, even in bulk tissue samples. Our study further highlights the power of such natural genetic systems to shed light on XCI in humans. Whereas the origin of nmXCI in females is typically unknown, nmXCI females likely harbor one or more mutations that (i) directly affect the choice of X-chromosome to be inactivated (primary skewing) or (ii) result in selection against cells carrying an active mutated X-chromosome (secondary/acquired skewing). As such, the X-linked genetics of nmXCI females is clearly atypical and could result in aberrant establishment and maintenance of XCI in these females. Whereas the highly similar patterns of XCI observed across the three genetically independent nmXCI females analyzed here suggests that such affects are subtle, additional multi-tissue studies of XCI in mosaic females is required to confirm the wider applicability of our findings. Finally, our identification of two additional nmXCI females among the 285 females in the GTEx database further substantiates recent claims of extreme skewing of XCI as a frequent and major modifier of trait penetrance and expressivity in the human population (12, 20).

Materials and methods

Allele-specific expression analysis for initial identification of nmXCI females

For the initial screen to identify nmXCI females in GTEx pre-processed SNPs were called from WES data from GTEx (GTEx_Analysis_2017-06-05_v8_WholeExomeSeq_979Indiv_VEP_annot.vcf.gz, see *https://gtexportal.org/home/methods* for specifics on data processing). Downloaded data was lifted over from hg19 to hg38 with LiftoverVcf (GATK v.4.1.9.0) and bcftools (v1.10.2) (21) was utilized to include only hetSNPs on the X-chromosome. One RNA-seq sample per female was downloaded from AnVIL using the Gen3 client, as aligned BAM files (see *https://gtexportal.org/home/methods* for specifics on data processing). Tissues used for the screen were selected based on abundance in the entire data set (muscle, n=239; skin-lleg, n=21; thyroid, n=1; adipose-subc, n=2; artery-tibi, n=2; esophagus-muco, n=2; nerve, n=2; adipose-visc, n=1; ovary, n=1; uterus, n=1; see *Table S7* for full list of abbreviations). Following data download, allelic expression (AE) analysis of allele counts for hetSNPs was retrieved from RNA-seq data using GATK ASEReadCounter (v.4.1.9.0), requiring a minimum base quality of 20 in the RNA-seq data. To remove potentially spurious sites, conservative filters were applied to the data: variant call read depth in WES >= 20 per allele, minor allele read count > 10% of the total read count, and RNA-seq read depth > 10 reads. If a gene carried multiple hetSNPs, only the one with the highest RNA-seq read count was included. AE data from the screen can be found in *Table S2* and *Table S3*. XCI skew of each individual participant was assessed by calculating the single-tissue median AE of all nonPAR chr X genes not detected previously as variable (8) (Table S1) in all 285 women in GTEx. Median chr X nonPAR AE of higher than 0.475 (equates to < 2.5% of reads coming from the inactive X-chromosome) indicates expression from a single parental chromosome and was used as a cutoff to distinguish between mosaic and non-mosaic XCI (nmXCI) females. As a control, UPIC was included. UPIC is a female which has previously been shown to have nmXCI (8). As UPIC is not included in GTEx_Analysis_2017-06-05_v8_WholeExomeSeq_979Indiv_VEP_annot.vcf.gz, SNP calling was performed using WES data and AE analysis was performed, ultimately leveraging her AE data as a positive control.

Detailed analysis of allele-specific expression and XCI status in nmXCI females

For further assessing XCI status of the three females with nmXCI (high median chr X nonPAR AE), the remaining RNA-seq tissue samples available for all three donors were downloaded from GTEx and processed as described above.

We further downloaded WES and whole-genome sequencing (WGS) data available for the three nmXCI females, UPIC, 13PLJ (nmXCI-1) and ZZPU (nmXCI-2) and performed SNP calling. Briefly, WES BAM and WGS CRAM files for each donor were downloaded from AnVIL using the Gen3 client. The files were sorted and converted into fastq files using Samtools (v1.9) (21). Raw fastq reads were quality trimmed using FastP (v.0.20.0) (22) with default settings. BWA-MEM (23) was used for mapping reads to the human genome build 38 (hg38, GCA_000001405.15_GRCh38_no_alt_analysis_set.fna) using default settings. Sambamba v0.8.2 (24) was used to mark duplicate reads. The GATK best practice germline short variant discovery pipeline was used to process the aligned reads, using base quality score recalibration and local realignment at known insertions and deletions (indels) (GATK v.4.2.6.1). Indels and SNPs were called jointly across all samples, for both WGS and WES data. Default filters were applied to indel and SNP calls using the variant quality score recalibration (VQSR) approach of GATK. All RNA-seq samples available for each participant (Table S6) were downloaded from AnVIL as aligned BAM files using the Gen3 client. Samtools (v1.9) (21) was used to sort and convert the BAM files to fastq files and quality trimming was done with FastP (v.0.20.0) (22) with default settings. RNA-seq reads were aligned with STAR 2-pass mode (v2.7.10a) (25) with GCA_000001405.15_GRCh38_no_alt_analysis_set.fna and --sjdbGTFfile gencode.v40.annotation.gtf index. WASP filtering was performed to reduce reference bias (26). Briefly, GATK SelectVariants was used to extract hetSNPs (that passed the VQSR filtering) for each individual from either the WGS or WES VCFs, and subsequently passed to STAR via --varVCFfile and–waspOutputMode. Reads failing WASP filtering were removed. Following data pre-processing, AE analysis of the allele counts for hetSNPs was retrieved from RNA-seq data using GATK ASEReadCounter. Heterozygous variants that passed VQSR filtering were first extracted for each sample from WES and WGS VCFs using GATK SelectVariants. Following, sample-specific VCFs and RNA-seq BAMs were input to ASEReadCounter requiring minimum base quality of 20 in RNA-seq data. ASEReadCounter outputs from WGS and WES pipelines were joined. If overlapping sites were identified, the hetSNP read counts were merged and the AE counts from the assay with the highest total read count was kept. To remove potentially spurious sites, conservative filters were applied to the data (variant call read depth >= 10 per allele and minor allele read count > 10% of the total variant read count for WES/WGS data, and an RNA-seq read depth of more than 7 reads). If a gene carried multiple hetSNPs, the hetSNP detected in the highest number of tissues was kept. If two hetSNPs were detected in the same number of tissues the one with the highest RNA-seq read count was kept. The 'pituitary' sample from nmXCI-1 was excluded as it had a lower median chr X nonPAR AE than all other tissues from the same individual. Furthermore, the 'lymphoblasts' sample was excluded from donor UPIC since it is unclear how EBV-transformation of lymphocytes affects the X-chromosome. The final ASE table for all X-linked genes in the nmXCI females can be found in Table S4.

X-inactivation status categorization

Tissue-specific X-inactivation status categorization was performed as previously described (8). Briefly, XCI status of genes was assessed by comparing the allelic read count ratios at each filtered X-chromosomal hetSNP, in each tissue individually. Whether there was significant expression from the inactive X-chromosome (escape) at each hetSNP was tested with a one-sided binomial test where the reads from Xi compared to the total read count was expected to be significantly greater than 0.025 or 2.5% (hypothesized probability of success = 0.025). FDR correction was applied to p

values from the binomial test for each of tissues separately using the rstatix R package version 0.7.2 (Kassambara A. 2023). q values < 0.01 were considered significant and indicative of XCI escape for the hetSNP and tissue.

To assess across-tissue XCI status of genes, we leveraged the q values obtained above. If a given hetSNP showed significant Xi expression in all tissues in which the gene is expressed, the SNP was categorized as 'escape (across tissues)', a hetSNP with non-significant Xi expression across all tissues in which the gene is expressed was classified as 'inactive (across tissues)'. If a gene was significant in more than one but not in all tissues in which it is expressed the gene was classified as 'variable'. Genes for which we had AE data in only one tissue were either labeled "inactive (data for one single tissue)" or "escape (data for one single tissue)" based on the binomial test. Finally, genes residing in the pseudo-autosomal region (PAR) were labeled 'PAR'. Upon inspection of the classification results several well characterized escape genes and inactive genes were mis-classified as variable escape. Consequently, we manually curated allelic expression status of all 380 genes using the empirical guideline of allelic ratio >0.4 as indicating mono-allelic expression while also considering the consequences of high and low read counts. Our three criteria for manual re-classification were low read count, low power and over-estimation, indicating genes with a low statistical power but consistent escape pattern (low power), consistent escape pattern but with non-significant escape in single tissue (low read count) and over-estimation due to high read counts inflating the binomial p-value (over-estimation). The list of genes that were manually curated, their change in XCI status and the criteria for their manual classification can be found in Supp Fig. 3 and Table S5.

Copy number analysis

Copy number analysis was performed using the R-package, QDNASeq (27). Briefly, whole genome sequencing data for all three nmXCI females was mapped to the human reference genome (hg38) by BWA-MEM (23) using default parameters. Raw copy numbers were then estimated by counting the number of reads mapped to non-overlapping bins of 15 kb unless stated otherwise.

Investigating chromosome 17p and 17q arm expression

Transcriptome mapping was performed using Salmon v1.9.0 (--gcBias --seqBias -- numBootstraps 100) (28) using known Refseq transcripts (NM_* and NR_*) from the GRCh38.p12 assembly (GCF_000001405.38_GRCh38.p12_rna.fna.gz) as a reference. Library type options were IU. Raw fastq reads were quality trimmed using FastP (v.0.20.0) with default settings. Abundance estimates were converted to h5 format using Wasabi (*https://github.com/COMBINE-lab/wasabi*). Sleuth was used for data normalization and normalized gene abundances (29, 30). Expression matrix (TPM) can be found in Table S8.

Data and code availability

All data and analysis scripts are included in this manuscript or are available on GitHub *https://github.com/ColmNestorLab/tissue_XCI*.

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Additional information

Author contributions

C.E.N and B.G designed research; B.G. performed research; C.E.N., B.G. and M.B. wrote the paper; M.B. provided insights and discussions.

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Additional files

Supplemental Tables

Supplementary figures

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Author information

Björn Gylemo
Crown Princess Victoria Children's Hospital, Department of Biomedical and Clinical Sciences (BKV), Linköping University, Linköping, Sweden
ORCID iD: 0000-0001-5253-6737

Maike Bensberg
Crown Princess Victoria Children's Hospital, Department of Biomedical and Clinical Sciences (BKV), Linköping University, Linköping, Sweden

Colm E Nestor
Crown Princess Victoria Children's Hospital, Department of Biomedical and Clinical Sciences (BKV), Linköping University, Linköping, Sweden
ORCID iD: 0000-0001-5853-1769

For correspondence: colm.nestor@liu.se

Editors

Reviewer #1 (Public review):

Summary:

This manuscript investigates genes that escape X-Chromosome Inactivation (XCI) across human tissues, using females that exhibit skewed or non-random XCI. The authors identified 2 female individuals with skewed XCI in the GTex database, in addition to the 1 female skewed sample in this database that has been described in a previous publication (Ref.16). The authors also determined the genes which escape XCI for 380 X-linked genes across 30 different tissues.

Strengths:

The novelty of this manuscript is that the authors have identified the XCI expression status for a total of 380 genes across 30 different human tissues, and also discovered the XCI status (escape, variable escape, or silenced) for 198 X-linked genes, whose status was previously not determined. This report is a good resource for the field of XCI, and would benefit from additional analyses and clarification of their comparisons of XCI status.

https://doi.org/10.7554/eLife.102701.2.sa4

Reviewer #2 (Public review):

Summary:

Gylemo et al. present a manuscript focused on identifying the X-inactivation or X-inactivation escape status for 380 genes across 30 normal human tissues. X-inactivation status of X-linked

genes across tissues is important for understanding sex-specific differences in X-linked gene expression and therefore traits, and the likely effect of X-linked pathogenic variants in females. These new data are significant as they double the number of genes that have been classified in the human, and double the number of tissues studied previously.

Strengths:

The strengths of this work are that they analyse 3 individuals from the GTex dataset (2 newly identified, 1 previously identified and published) that have highly/ completely skewed X inactivation, which allows the study of escape from X inactivation in bulk RNA-sequencing. The number of individuals and breadth of tissues analysed adds significantly to both the number of genes that have been classified and the weight of evidence for their claims. The additional 198 genes that have been classified and the reclassification of genes that previously had only limited support for their status is useful for the field.

In analysing the data they find that tissue-specific escape from X inactivation appears relatively rare. Rather, if genes escape, even variably, it tends to occur across tissues. Similarly if a gene is inactivated, it is stable across tissues.

Comments on revised version:

The authors have answered all of my queries. While they have not been able to pinpoint the genetic cause of the highly skewed XCI cases in their cohort, I agree this is beyond the scope of this study. I have no further requests.

https://doi.org/10.7554/eLife.102701.2.sa3

Reviewer #3 (Public review):

Summary:

Nestor and colleagues identify genes escaping X chromosome inactivation (XCI) in rare individuals with non-mosaic XCI (nmXCI) whose tissue-specific RNA-seq datasets were obtained from the GTEX database. Because XCI is non-mosaic, read counts representing a second allele are tested for statistical significant escape, in this case > 2.5% of active X expression. Whereas a prior GTEX analysis found only one nmXCI female, this study finds two additional donors in GTEX, therefore expanding the number of assessed X-linked genes to 380. Although this is fewer than half of X-linked genes, the study demonstrates that although rare, nmXCI females are represented in RNA-seq databases such as GTEX. Therefore this analytical approach is worthwhile pursuing in other (larger) databases as well, to provide deeper insight into escape from XCI which is relevant to X-linked diseases and sex differences.

Strengths:

The analysis is well-documented, straight-forward and valuable. The supplementary tables are useful, and the claims in the main text well-supported.

Weaknesses:

There are very few, except that this escape catalogue is limited to 3 donors, based on a single (representative) tissue screen in 285 female donors, mostly using muscle samples. However, if only pituitary samples had been screened, nmXCI-1 would have been missed. Additional donors in the 285 representative samples cross a lower threshold of AE = 0.4. It would be worthwhile to query all tissues of the 285 donors to discover more nmXCI cases, as currently fewer than half of X-linked genes received a call using this very worthwhile approach.

Comments on revised version:

The authors incorporated some textual changes, but deferred any new analysis, or expansion from these two new skewed donors to include more individuals/tissues, or going more in depth for individual genes to future manuscripts. They appear to have that option at eLife.

https://doi.org/10.7554/eLife.102701.2.sa2

Reviewer #4 (Public review):

Summary:

This study by Gylemo et al. investigates genes that escape X-Chromosome Inactivation (XCI) by analyzing RNA-sequencing data from three female individuals with highly skewed XCI identified in the GTEx database-two newly reported and one previously described. Utilizing these rare non-mosaic XCI cases, the authors assess allelic expression patterns across 30 normal human tissues to classify the XCI status of 380 X-linked genes, including 198 not previously annotated. The study provides a broader and more comprehensive catalog of XCI escape, contributing valuable insights into sex-specific gene expression and the potential implications of X-linked variants in disease.

Strengths:

The primary strength of this work lies in its expanded scope: it doubles the number of tissues and significantly increases the number of X-linked genes with known XCI status compared to previous studies. By focusing on rare individuals with non-random XCI, the authors provide a unique opportunity to observe allelic expression and classify escape status with more confidence. Their findings that escape from XCI is relatively consistent across tissues (rather than tissue-specific) enhance the understanding of XCI mechanisms. The methodology is robust, the data are well-documented, and the supplementary resources are comprehensive. This study thus represents a valuable resource for the XCI field and a promising basis for future investigations.

Weaknesses:

Despite its strengths, the study is limited by its reliance on only three individuals, which restricts statistical power and generalizability. Concerns were raised regarding the comparability of XCI status across tissue types and cell lines, particularly given that previous classifications may have used cancer or immortalized cells. Additionally, more could be done to explore the genetic basis behind the observed skewed XCI, which might affect the conclusions about escape patterns. Finally, the authors are encouraged to expand their approach to additional RNA-seq datasets or single-cell analyses to validate their findings and potentially discover more individuals with skewed XCI, which would deepen understanding of this important biological phenomenon.

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Author response:

The following is the authors' response to the original reviews

> ***Public Reviews:***

> ***Reviewer 1 (Public review):***
>
> *(1) The authors state that they have reclassified the allelic expression status of 32 genes (shown in Table S5, Supplementary Figure 3). The concern is the source of the tissue or cell line which was originally used to make the classification of XCI status, and whether the comparisons are equivalent. For example, if cell lines (and not tissues) were used to define the XCI status for EGFL6, TSPAN6, and CXorf38, then how can the authors be sure that the escape status in whole tissues would be the same? Also, along these lines, the authors should consider whether escape status in previous studies using immortalized/cancer cell lines (such as the meta-analyses done in Balaton publication) would be different compared to healthy tissues (seems like it should be). Therefore, making comparisons between healthy whole tissues and cancer cell lines doesn't make sense.*

Indeed, many previous classifications were based on clonal cell lines, which could result in atypical patterns of escape due to the profound and varied effects of adaptation to culture. However, one of the primary goals of our study was to directly determine allele-specific expression from the X-chromosome in healthy primary tissues, in part to exclude the potential confounding effects of cell culture.

Whereas we do perform comparisons with cell culture-based classifications, we also provide detailed comparisons with the previous classification of Tukiainen *et al*, which also uses primary human tissues. In addition, whereas the comparison with Balaton *et al* is not optimal, we hold that it is valuable as it reveals which genes may exhibit aberrant escape patterns in culture. Finally, despite the above reservations, our comparison revealed an overwhelming agreement with previous research which suggests that in the vast majority of cases, escape appears to be correctly maintained in culture.

> *(2) The authors note that skewed XCI is prevalent in the human population, and cite some publications (references 8, 10-12). If RNAseq data is available from these female individuals with skewed XCI (such as ref 12), the authors should consider using their allelic expression pipeline to identify XCI status of more X-linked genes.*

Indeed, we completely agree and are in the process of obtaining this data which has proven complex and time-consuming in the currently regulatory environment.

> *(3) It has been well established that the human inactive X has more XCI escape genes compared to the mouse inactive X. In light of the author's observations across human tissues, how does the XCI status compare with the same tissues in mice?*

This is a very interesting point, and a comparison we are currently working on. However, this is a major undertaking and one that is outside of the scope of this study. We do appreciate the differences in mice and humans on X-chromosome level and could only speculate on the overlap being relatively small as the number of escapees in mice has been shown the be far lower than in humans.

> ***Reviewer 2 (Public review):***
>
> *In my view there are only minor weaknesses in this work, that tend to come about due to the requirement to study individuals with highly skewed X inactivation. I wonder whether the cause of the highly skewed X inactivation may somehow influence the likelihood of observing tissue-specific escape from X inactivation. In this light, it would be interesting to further understand the genetic cause for the highly skewed X inactivation in each of these three cases in the whole exome sequencing data. Future additional studies may*

> *validate these findings using single-cell approaches in unrelated individuals across tissues, where there is normal X inactivation.*

We thank the reviewer for their positive assessment of our work. This is a point we have and continue to grapple with. We cannot rule out that the genetic cause of complete skewing may influence tissue-specific XCI. Moreover, the genetic cause for the non-mosaic XCI is currently unclear and is likely to vary between individuals, which could also result in inter-individual variation in tissue-specific escape. We are currently performing large prospective studies in the tissues of healthy females to specifically address this point.

> ***Reviewer 3 (Public review):***
>
> *There are very few, except that this escape catalogue is limited to 3 donors, based on a single(representative) tissue screen in 285 female donors, mostly using muscle samples. However, if only pituitary samples had been screened, nmXCI-1 would have been missed. Additional donors in the 285 representative samples cross a lower threshold of AE = 0.4. It would be worthwhile to query all tissues of the 285 donors to discover more nmXCI cases, as currently fewer than half of X-linked genes received a call using this very worthwhile approach.*

We thank the reviewer for their positive assessment of our work. Of course, we agree that a tissue-wide screen in all individuals would have been optimal and is a line of research we are currently pursuing. However, the analysis of allele-specific expression in all 5,000 RNA-seq samples is a massive undertaking and was simply not practicable within the time-scale of this study.

> ***Recommendations for the authors:***
>
> ***Reviewer #2 (Recommendations for the authors):***
>
> *Thanks to the authors for an interesting manuscript! I enjoyed reading it and the care that has gone into explaining the analyses and the findings. There are a few recommendations that I have for strengthening the work.*

We thank the reviewer for the nice feedback. Much appreciated.

> *(1) I would like to see a genetic analysis of the three individuals, to try and identify the genetic causes of the skewed X inactivation beyond just considering the XIC or translocations. The cause of the highly skewed X inactivation would be of interest to many.*

This is certainly a very interesting avenue of research and one that we are currently focusing on. However, in the current study we simply had too few skewed XCI females to assess this in an exhaustive manner. To tackle this issue, we have begun a prospective study of healthy females to identify additional non-mosaic females.

> *(2) I wonder whether the cause of the skewed XCI may somehow influence the assessment of tissue-specific escape? If there is a problem with X inactivation itself, perhaps escape would also be different, making it appear more constitutive than tissue-specific?*

This is a point we have and continue to grapple with. We cannot rule out that the genetic cause of complete skewing may influence tissue-specific XCI. Moreover, the genetic cause for the non-mosaic XCI is currently unclear and is likely to vary between individuals, which could result in inter-individual variation in tissue-specific escape.

> *I think the abstract is likely a bit inaccessible to those outside the field. I am in the X inactivation field, but don't use the term non-mosaic X inactivation, but rather would call it highly skewed, or non-random X inactivation. In my view, it would be simpler for the abstract to call non-mosaic XCI highly skewed XCI instead, or to use more words to ensure it is clear for the reader.*

We agree that the terminology of completely skewed/non-mosaic XCI could be more clearly defined in the abstract and have clarified this. “Using females that are non-mosaic (completely skewed) for X-inactivation (nmXCI) has proven a powerful and natural genetic system for profiling X-inactivation in humans.”

> *I would consider calling the always escape genes constitutive escapees, while the variable may be facultative.*

This is something we have also considered and have received differing feedback on. However, we will definitely keep this in mind for future publications.

> *Line 132, it would be useful to explain median >0.475 as less than 2.5% of reads coming from the inactive allele here, not just in the methods. Can you also explain why this cutoff was chosen?*

We thank the reviewer for this clarification. A clarification has been added to the main text as suggested.

The cutoff was applied to account for potential variations in skewing, given that we screened only a single tissue sample per individual. Although nmXCI females are theoretically expected to have 0% of reads originating from the 'inactive' allele, this is not always observed due to (a) technical errors such as PCR or sequencing inaccuracies, or (b) differences in skewing between tissue types.

> *Lines 156-160 describe how the heterozygous SNPs were identified in relation to Figure 2. I read these in the methods so that I could understand Figure 1, so I suggest moving this section up.*

We have moved the section as suggested by the reviewer.

> *Line 156, consider adding in a sentence to describe what is shown in Figures 2A and B i.e, the overlap of SNPs and spread along the X.*

We have added a sentence describing what is shown in Figures 2A and 2B as suggested by the reviewer.

> *Line 217, it would be useful to give the % of genes that show tissue-specific escape, to quantify rare.*

We have added a sentence quantifying ‘rare’ at the suggested line.

> *(4) Typos:*
>
> *Line 119, missing 'the most' before extensive (and remove an).*

We thank the reviewer for pointing this out. This error has been corrected.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *Some results in the supplementary figures were quite striking. What is going on with DDX3X and ZRSR2? How come total read counts are so different between individuals?*

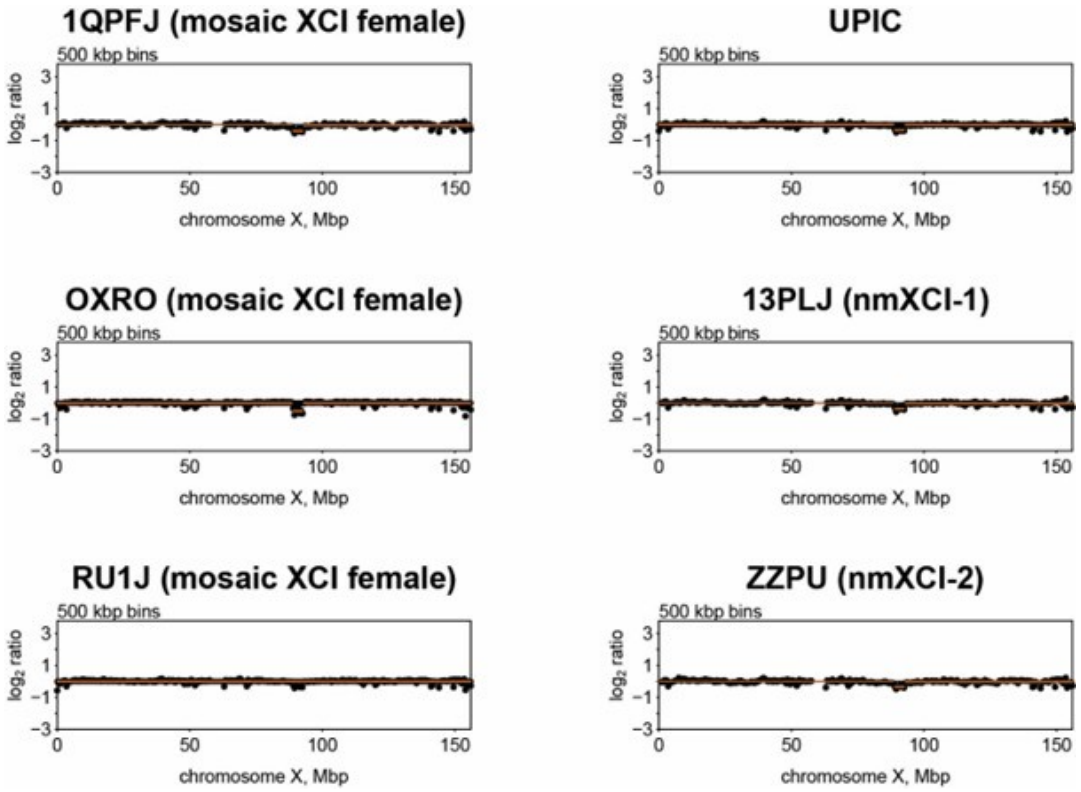
Indeed, this is a very intriguing observation and one that we have simply failed to understand thus far. We are currently performing a large prospective study to obtain greater number of non-mosaic females and tissues samples. Hopefully, additional observations across females will allow us to gain further insights into the inter-individual behaviour of DDX3X and ZRSR2.

> *One item I would like to see added is some analysis to address the cause of these extremely skewed XCI individuals. The copy number analysis suggests there are some segmental deletions on the X in all three nmXCI cases. Where are these deletions, and do any fall in the region of the X-inactivation centre? Have the authors performed any analysis of potentially deleterious X-linked variants in the WGS or WES data? Why are these donors so skewed? It's interesting that UPIC was still more skewed than the other two.*

The segmental deletions the reviewer points out are not segmental deletions, the same variation in coverage is found in all females we've looked at including females with a mosaic XCI (see Author response image 1 below where the same pattern of slightly lower read counts is observed at the same sites in all female samples). No deletions were identified in the XIC region. No analysis was performed of deleterious X-linked variants. Why the donors are so skewed is unknown and intriguing. Indeed, identifying the origin of extreme skewing (including the females in this study) is now the main focus of the group. Whereas UPIC had trisomy 17, which has likely resulted in the observed skewing, we have not yet found a genetic variant that could explain the skewing observed in 13PLJ or ZZPU.

Author response image 1.

Copy number as log2 ratio using 500kb bins across the X-chromosome for 3 mosaic XCI females (1QPFJ, OXRO, and RU1J) and 3 nmXCI females, UPIC, nmXCI-1 and nmXCI-2.



> *This is not necessary to address with new analyses, but as alluded to above, the authors could screen more than a single representative tissue. And to apply this analysis to larger databases (UK biobank), which the authors may be planning to do already.*

This an avenue of research we are currently investigating.

> *The code is well-documented and accessible. Additional information on the manual reclassification (to deal with inflated binomial P-values) would be helpful. Why not require a minimal threshold for escape (10% of active X allele) in addition to a significant binomial P (inactive X exp. > 2.5% of active)?*

We thank the reviewer for this positive assessment of the code.

Indeed, how to define 'escape' is a vexed issue, and one we feel has been given undue weight within the field. In reality, studies of escape are often dealing with sparse data (e.g. read depth), few observations (genes and individuals) and substantial amounts of missing data. Thus, it is unlikely that a standard statistical approach will be sensitive and specific across different studies and data types. Similarly, cut-offs, though useful would also need to be adjusted to the data type and quality in any given study.

Whereas we initially used a significant binomial P-value as our sole test (often quoted as 'best practice'), this resulted in wide-spread inflation of P-values. Thus, we switched to manually curating the allelic expression status of all 380 genes using the empirical guideline of allelic ratio >0.4 (also a commonly used cut-off) as indicating mono-allelic expression. We considered combining the binomial P-value with the cut-off but felt that this would result in an overly complex definition of escape and would unnecessarily exclude many genes from classification, due to the opposing effects of low/high read depth on the binomial and cut-off approaches respectively.

Indeed, due to the difficultly of both accurate and objective ‘classification’ of escape that we placed an emphasis on clearly displaying all data for each gene in each individual to allow readers to see all the data on which each classification was based.

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