Landscape of DNA Methylation on the Marsupial X

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Abstract

DNA methylation plays a key role in maintaining transcriptional silence on the inactive X chromosome of eutherian mammals. Beyond eutherians, there are limited genome wide data on DNA methylation from other vertebrates. Previous studies of X borne genes in various marsupial models revealed no differential DNA methylation of promoters between the sexes, leading to the conclusion that CpG methylation plays no role in marsupial X-inactivation. Using reduced representation bisulfite sequencing, we generated male and female CpG methylation profiles in four representative vertebrates (mouse, gray short-tailed opossum, platypus, and chicken). A variety of DNA methylation patterns were observed. Platypus and chicken displayed no large-scale differential DNA methylation between the sexes on the autosomes or the sex chromosomes. As expected, a metagene analysis revealed hypermethylation at transcription start sites (TSS) of genes subject to X-inactivation in female mice. This contrasted with the opossum, in which metagene analysis did not detect differential DNA methylation between the sexes at TSSs of genes subject to X-inactivation. However, regions flanking TSSs of these genes were hypomethylated. Our data are the first to demonstrate that, for genes subject to X-inactivation in both eutherian and marsupial mammals, there is a consistent difference between DNA methylation levels at TSSs and immediate flanking regions, which we propose has a silencing effect in both groups.

Key words: **X chromosome inactivation, mammal, differential methylation, sex chromosomes, evolution, genomics.**

Article

Introduction

In therian (eutherian and marsupial) mammals, females have two copies of the X chromosome, one of which is transcriptionally silenced in somatic cells by an epigenetic process called X chromosome inactivation (XCI). Methylation of cytosine (5-methylcytosine—5mC) is an epigenetic modification associated with gene silencing on the X chromosome in eutherian mammals, where it is established relatively late on the inactive X chromosome (Xi) (Lock et al. 1987) and is essential for long-term maintenance of the XCI state (Mohandas et al. 1981; Graves 1982).

Eutherian XCI is facilitated by a noncoding RNA (ncRNA), called *XIST*, which is expressed from and spreads in *cis* along the nascent Xi (reviewed in Augui et al. 2011). *Xist* RNA interacts with a host of proteins, including epigenetic modifiers (Chu et al. 2015; McHugh et al. 2015; Minajigi et al. 2015) that mediate a cascade of epigenetic changes on the Xi including histone modifications, incorporation of variant histones, and ultimately DNA methylation of promoter-associated CpG islands (Lock et al. 1986; Norris et al. 1991; Tribioli et al. 1992; Escamilla-Del-Arenal et al. 2011).

XCI also occurs in marsupials, but in contrast to eutherians there is no *Xist* gene (Duret et al. 2006; Hore et al. 2007). Instead *Rsx* (RNA on the silent X), a noncoding RNA with *Xist*-like properties, is expressed exclusively from the nascent Xi to mediate its silencing (Grant et al. 2012). The marsupial Xi is characterized by a suite of histone modifications and variants that is similar, but not identical, to those observed on the eutherian Xi (Mahadevaiah et al. 2009; Rens et al. 2010; Chaumeil et al. 2011; Wang et al. 2014). The relevance of DNA methylation in marsupial XCI is poorly understood, a consequence of the limited number of genes analyzed and disparate species examined (Kaslow and Migeon 1987; Loebel and Johnston 1996; Wang et al. 2014).

Immunofluorescence studies labeling for 5mC demonstrated hypomethylation of the Xi compared with the active X (Xa) in marsupials (Rens et al. 2010; Ingles and Deakin 2015),

a counterintuitive result considering the correlation between DNA hypermethylation and gene silencing (reviewed in Jones 2012). CpGs associated with intragenic regions of *Hprt* in the common wallaroo (*Macropus robustus*), and *G6pd* in *Didelphis virginiana*, were shown to be hypermethylated in vivo on the Xa, but no correlation with expression was observed (Piper et al. 1993). With the exeption of CpG dinucleotides associated with the *Rsx* promoter, which are differentially methylated on the maternally and paternally derived X chromosomes (Wang et al. 2014), CpG islands associated with promoters of 27 genes show no differential DNA methylation between Xi and Xa (Kaslow and Migeon 1987; Loebel and Johnston 1996; Hornecker et al. 2007; Wang et al. 2014). These observations, which contrast Xi methylation in eutherians, led to the prevailing view that DNA methylation at promoters is not involved in marsupial XCI.

Platypus (a monotreme mammal) has a complex sex chromosome system in which males have five Xs and five Ys, and females have five pairs of Xs. This system shares no homology with therian sex chromosomes (Veyrunes et al. 2008); instead it shares considerable homology (especially X_5) with the bird ZW sex chromosome system (Grützner et al. 2004; Rens et al. 2007). Partial inactivation of X/Z-linked loci was observed in the homogametic sex of both platypus and chicken (Deakin et al. 2008; Livernois et al. 2013), analogous to the partial inactivation of genes on therian X chromosomes (Al Nadaf et al. 2010, 2012). Characterization of DNA methylation in monotremes is limited to one study using immunofluorescence (Rens et al. 2010), where no differential methylation was observed between X chromosomes at metaphase in female platypus. In chicken, there has been little focus on global Z chromosome DNA methylation, instead attention has centered on the male hypermethylated region (Teranishi et al. 2001; Itoh et al. 2011; Yang et al. 2016), or the genome more broadly (Li et al. 2011, 2015).

A comparative analysis of the DNA methylation landscape on X or Z chromosomes in phylogenetically informative species is key to our broader understanding of dosage compensation. Here, we use reduced representation bisulfite sequencing (RRBS) to examine the patterns of CpG methylation on sex chromosomes in representative species from extant mammalian linages (eutherians, marsupials, and monotremes) and a bird. In platypus and chicken, we find no differential DNA methylation on sex chromosomes between the sexes, indicating that 5mC plays no global role in sex chromosome gene silencing in these species. As expected, transcription start sites (TSSs) of genes subject to XCI are hypermethyated in female mouse, and include the regions ~2.5 kb either side of TSSs. In female opossum, TSSs of X-borne genes subject to inactivation are not hypermethylated; rather, regions flanking TSSs are hypomethylated. Therefore, although absolute levels of DNA methylation of genes subject to XCI differ between eutherian and marsupial mammals, both produce uniform DNA methylation profiles across TSSs. Our data provide the first evidence of nonrandom differential DNA methylation of the X chromosome between a male and female representative marsupial.

Results

Reduced representation bisulfite sequencing (RRBS) was used to measure male and female DNA methylation in mouse, opossum, platypus, and chicken liver samples. Profiles of absolute DNA methylation values were recorded, and male:female ratios calculated, with particular focus on the on the X (mammals) or Z (bird) chromosomes. We looked for regions of differential DNA methylation between the sexes, on the X or Z, which would indicate a role in sex chromosome gene silencing.

Large Scale Differential DNA Methylation between the Sexes

Only CpG dinucleotides sampled in both sexes were retained for analysis, which were classified as mapping to an autosome or sex chromosome (supplementary table S1, Supplementary Material online). Large regions of differential methylation between the sexes were not evident on the autosomes in any species (fig. 1 and supplementary figs. S1 and S2, Supplementary Material online). In platypus, no net differential methylation was detected on any X chromosome (supplementary fig. S1, Supplementary Material online). This observation was mirrored in chicken, in which there was no net differential methylation observed on the Z chromosome between the sexes (supplementary fig. S1, Supplementary Material online).

Globally, the X chromosome in mouse had similar methylation levels between the sexes, although there were regions with a bias toward females (fig. 1). In opossum the X chromosome was globally hypermethylated in males compared with females (fig. 1), which contrasts with eutherians, and results from hypomethylation of the X in females (supplementary fig. S2, Supplementary Material online), known to be the Xi from immunofluorescence studies (Rens et al. 2010; Ingles and Deakin 2015).

Differential DNA Methylation within Genomic Features

In both sexes, within an annotated genomic feature, reads at all sampled CpGs were scored as either bisulfite converted (nonmethylated), or not converted (methylated). The absolute level of methylation within a feature was calculated (across all sampled CpGs) as a proportion of methylated reads out of total read depth. Features were considered differentially methylated if there was a difference in absolute DNA methylation between the sexes of $>15\%$ and q-value <0.05.

The R package MethyKit (Akalin et al. 2012) was used to estimate DNA methylation within annotated genomic features, including: promoters (1 kb 5′ of annotated TSSs), gene bodies, CpG islands, LINE1, and 300-kb tiles. These features were binned according to their location on an autosome or sex chromosome (an X in mammals or Z in chicken). In each species, and for all genomic features examined on autosomes, differential methylation was not biased toward either sex (fig. 2 and supplementary fig. S3, table S2, and data S1–S4, Supplementary Material online).

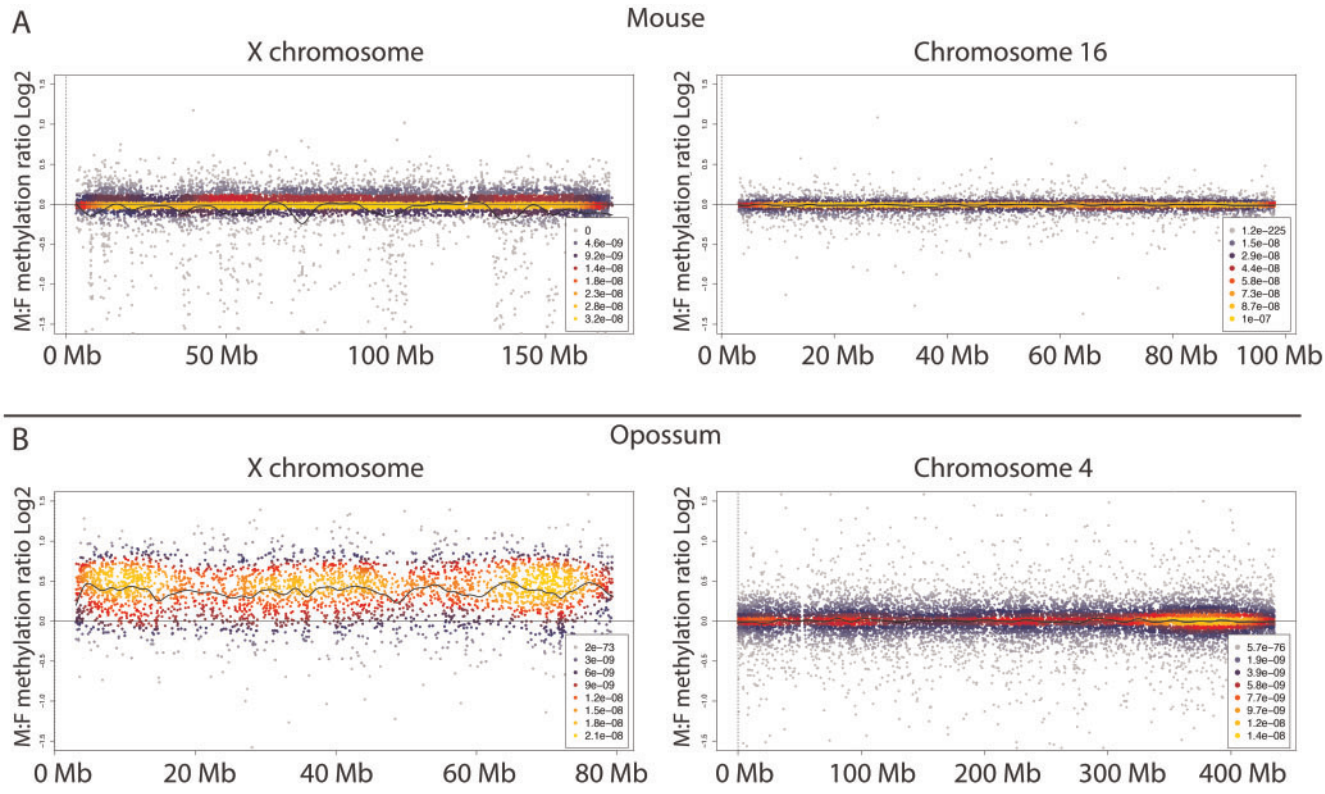

Fig. 1. Heatscatter plots of male to female DNA methylation ratios along the length of the X and a representative autosome for (*A*) mouse and (*B*) opossum. Each dot represents the M:F ratio within a 10-kb window on a log2 scale. Above zero is higher methylation in males, below zero is higher methylation in females. Gray through to yellow represent increased local data density. Smoothed lines of best fit are black (see Materials and Methods).

There was no evidence that differential methylation of genomic features was biased toward either sex on the Xs in platypus, nor the Z chromosome in chicken (supplementary fig. S2, Supplementary Material online and table 1). However, as expected, in mouse, all features on the X chromosome were more often hypermethylated in females than in males. This was particularly evident for X-borne CpG islands that associate with promoters (i.e., ±1 kb of annotated TSSs), where 270 of 299 (90.3%) were hypermethylated in females relative to males (fig. 2, table 1, and supplementary data S1, Supplementary Material online), supporting previous observations that CpG island hypermethylation is important for eutherian XCI.

Genomic features on the opossum X chromosome were hypomethylated in females compared with males (fig. 2 and table 1), mirroring the global DNA methylation pattern (fig. 1). In females, 112 of the 255 (43.9%) 300-kb windows on the X were hypomethylated. This contrasted greatly with the putative regulatory CpG islands that associate with promoters, of which only 17 of 206 (8.3%) were hypomethylated in females (fig. 2, table 1, and supplementary data S2, Supplementary Material online). These observations are the first to demonstrate that the Xi is not uniformly demethylated in marsupials.

DNA Methylation at TSSs

A metagene analysis was employed to examine the pattern of DNA methylation of different bins of promoters. For CpGs ±10 kb of annotated TSSs, with a read depth of >8 in both sexes, the percent methylation level was calculated. CpGs were binned according to location (X/Z chromosome or an autosome), expression (expressed or not expressed—data from Brawand et al. 2011), and XCI status (inactivated or escapee). For each bin DNA methylation levels were plotted for both sexes across TSSs with ggplot2 (Wickham 2009) using a generalized additive model regression line with 95% confidence intervals (see Materials and Methods).

Comparison of methylation at autosomal TSSs between the sexes was used to control for systemic biases between samples. For each species, DNA methylation at autosomal TSSs was equivalent between the sexes for both expressed (fig. 3*A* and *B*; supplementary fig. S4*A*, Supplementary Material online) and nonexpressed genes (supplementary figs. S5*A* and *B* and S4*B*, Supplementary Material online). In platypus and chicken, there was also no differential DNA methylation detected between males and females at TSSs on the sex chromosomes (supplementary fig. S4*C* and *D*, Supplementary Material online), suggesting against a role in gene silencing on the Xs in platypus or the Z in chicken.

The inactivation status of X genes has been reported for mouse brain, spleen, and ovary (Berletch et al. 2015), and that catalogue of mouse X-borne genes is adopted here. The TSSs of expressed genes subject to XCI were hypermethylated in females compared with males (fig. 3*C*). This contrasted with the subset of eight genes sampled in our data that escape XCI

Table 1. Proportion of X/Z Chromosome Features with >15% More Methylation in Either Male or Female and *q*-Value <0.05.

	Mouse		Opossum		Platypus		Chicken	
Feature	Male	Female	Male	Female	Male	Female	Male	Female
300-kb Tiles	0/559	19/559	112/255	0/255	2/284	4/284	11/275	6/275
LINE1	178/9268	292/9268	107/1118	0/1118	2/3492	4/3492	10/517	4/517
Gene bodies	7/763	213/763	148/412	0/412	4/400	16/400	25/594	39/594
Promoters	6/382	224/382	36/228	0/228	1/165	3/165	3/354	8/354
CGIs	2/411	329/411	52/416	1/416	15/896	12/896	8/646	15/646
Orphan CGIs	2/133	80/133	35/219	0/219	14/773	12/773	6/283	11/283
Promoter-associated CGIs	0/299	270/299	17/206	2/206	1/124	0/124	2/419	5/419

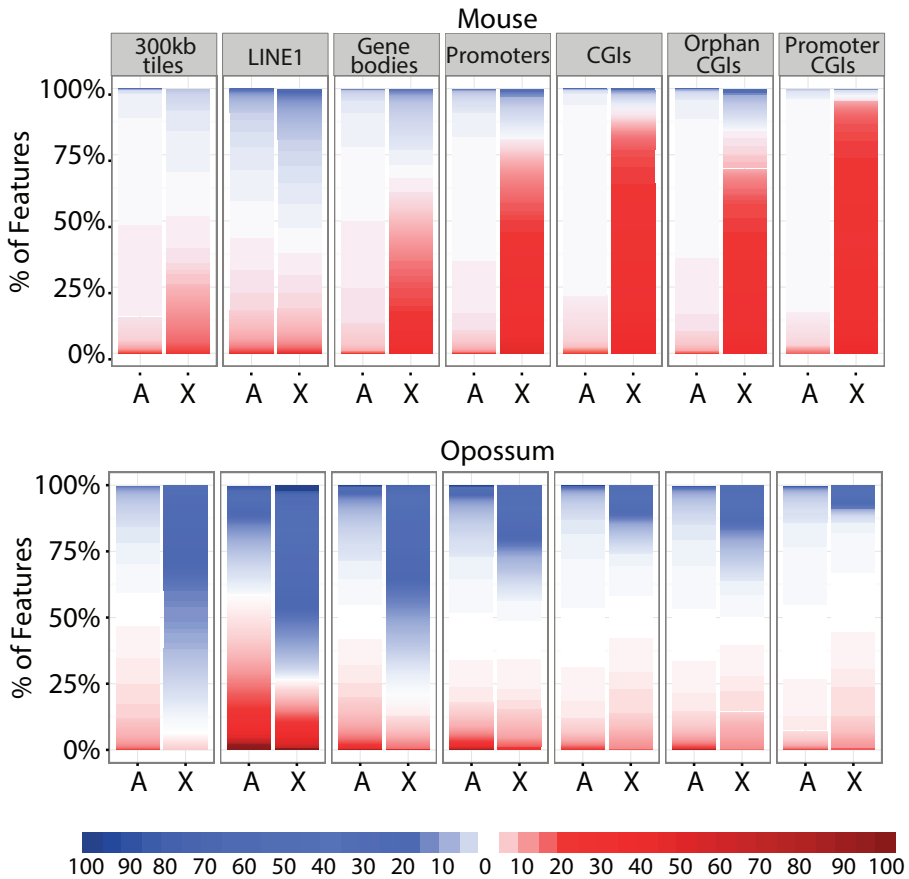

Fig. 2. Differential DNA methylation between the sexes of annotated genomic features in mouse and opossum. For each feature, the left bar chart (*A*) represents features annotated on autosomes; the right bar chart (X) represents features annotated on the X. White represents equal methylation between the sexes. Darker shades of red indicate increasing DNA methylation in females. Darker shades of blue indicate increased DNA methylation in males. The scale is percentage DNA methylation difference between the sexes. Numbers of features sampled for each species are provided in supplementary table S4, Supplementary Material online. Plotted from data in supplementary data S1 and S2, Supplementary Material online.

in at least two of brain, spleen, and ovary (so likely escaping in liver too, from which our data were generated), where the DNA methylation difference between the sexes was <5% at the TSSs (supplementary fig. S6, Supplementary Material online). Using CpG DNA methylation levels in male as a measure for their methylation on the Xa in females, Xi DNA methylation level was inferred (see Materials and Methods). At TSSs of genes subject to inactivation, DNA methylation was 40% higher on Xi (fig. 3*E*—purple line) than on Xa (fig. 3*C*—blue line).

X-inactivation status in opossum has been determined for 174 of the 490 annotated protein coding X genes in brain, and 134 X genes in extra-embryonic membranes (Wang et al. 2014). Of these, the same 24 genes escaped XCI in both tissue types, with the remaining subject to silencing. Because the set of escaper genes is consistent between tissues, the inactivation status of X genes in brain was used to assign inactivation status of genes in liver for this study. We sampled 120 genes that were classified as subject to XCI (Wang et al. 2014). In male, 100 were expressed, and 20 were not expressed; whereas

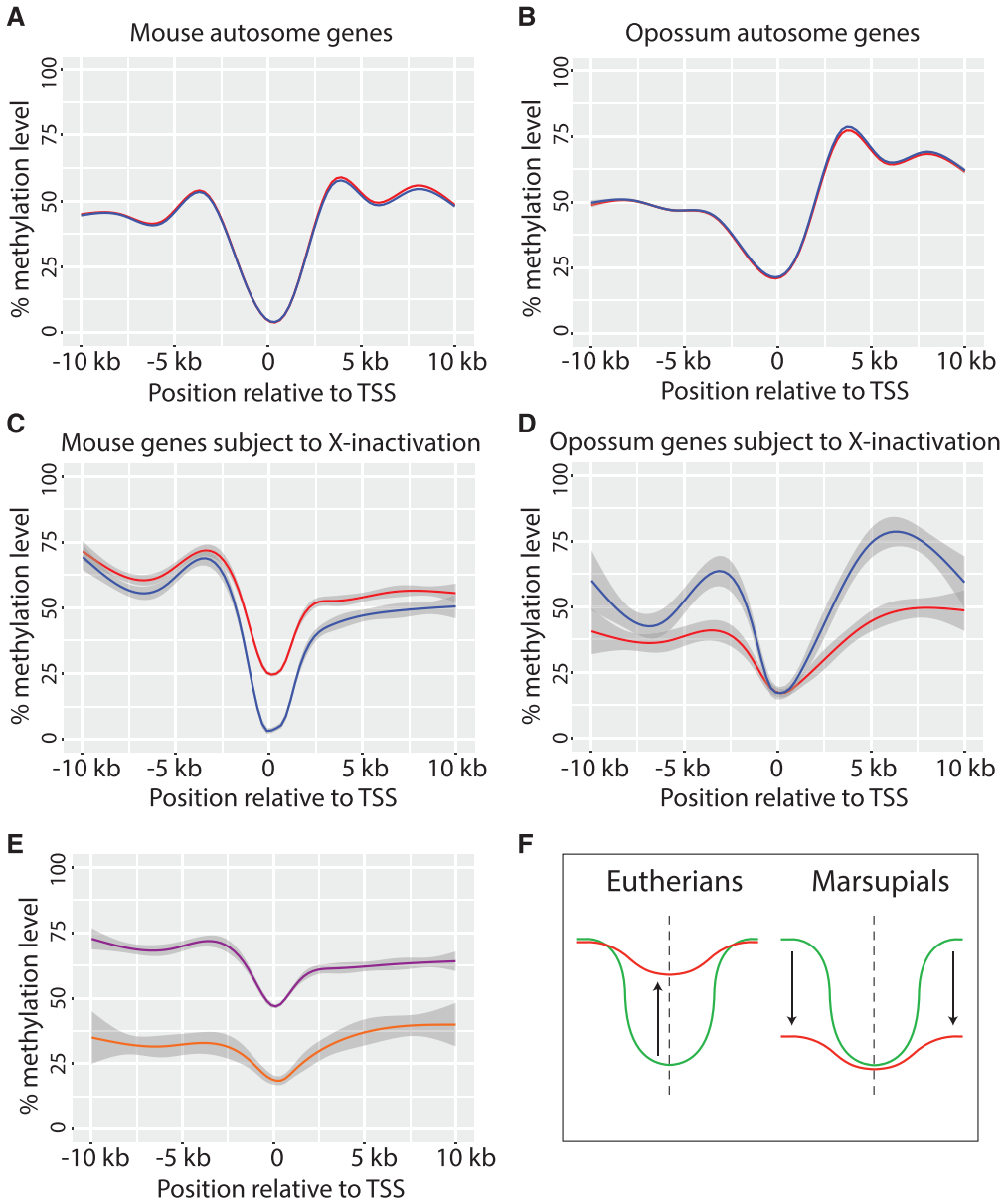

Fig. 3. DNA methylation levels of CpGs, sampled at a read depth >8, ±10 kb from annotated transcription start sites in mouse and opossum of all expressed genes on autosomes (*A* and *B*) and gene subject to XCI (*C* and *D*). Smoothed regression lines for best fit of CpG methylations levels are plotted for male (blue) and female (red) with 95% confidence intervals (gray shadowing). All genes are expressed in liver (expression data from Brawand et al. 2011). Individual methylation levels at each CpG are not plotted due to high density of data. The total number of CpGs sampled for the total number of TSSs (genes) are available in supplementary table S3, Supplementary Material online. (*E*) An estimation of DNA methylation levels (see Materials and Methods), across transcription start sites of expressed genes subject to XCI, on the Xi in mouse (purple) and opossum (orange). The methylation profiles on Xi are uniform compared with the Xa (represented by male methylation levels for X-borne TSSs—fig. 3*C* and *D*). (*F*) Model to depict a uniform DNA methylation profile across transcription start sites (TSSs) of gene subject to X inactivation in eutherians and marsupials. On the active X (green lines) the level of DNA methylation is relatively low at TSSs (dashed lines), which is flanked by high DNA methylation. On the inactive X (red lines) the difference in DNA methylation level between TSSs and flanking regions is reduced. In eutherians TSSs are hypermethylated to effect this, whereas in marsupials the flaking regions are hypomethylated.

in female 107 were expressed and 13 were not expressed (supplementary table S3, Supplementary Material online).

For expressed genes subject to XCI, we observed differential DNA methylation profiles between the sexes (fig. 3*D*). DNA methylation ±1 kb from TSSs was the same in both sexes, but in females, regions flanking the TSS were hypomethylated. Approximately 3-kb upstream from TSSs of genes subject to XCI, DNA methylation was 20% lower in females. Approximately 6-kb downstream TSSs of genes subject to XCI, DNA methylation was 30% lower in females. Across TSSs of genes that are subject to XCI, but which are not expressed, male and female DNA methylation patterns were overlapping (supplementary fig. S5*D*, Supplementary Material online). For the 21 genes we sampled that escape XCI, DNA methylation across TSSs was the same in both sexes (supplementary fig. S6, Supplementary Material online).

Importantly, observations of equal DNA methylation between the sexes at the TSS, and reduced flanking methylation in females were reflected in analyses of genes subject to XCI in an Australian marsupial (tammar wallaby) (supplementary fig. S7, Supplementary Material online).

As with mouse, the CpG methylation levels on Xi were inferred from Xa methylation in male (see Materials and Methods). Approximately 3-kb upstream from TSSs of genes subject to XCI, DNA methylation was 30% lower on Xi (fig. 3*E*—orange line) than on Xa (fig. 3*D*—blue line); whereas ~6-kb downstream, DNA methylation was 40% lower on Xi than on Xa. As such, absolute DNA methylation levels on the Xi at TSSs of genes subject to XCI differed markedly between mouse and opossum, ranging from ~45% to 70% and ~20% to 40%, respectively (fig. 3*E*). However, in both species DNA methylation across the promoters of these genes was more uniform on Xi compared with the Xa, which we propose acts as a part of the epigenetic silencing signal on the X in both eutherian and marsupial mammals.

Discussion

Here, we present RRBS data generated from liver DNA derived from male and female mouse, opossum, platypus, and chicken. In opossum there was no differential DNA methylation between males and females at TSSs of genes subject to XCI, whereas flanking regions were hypomethylated in females. Our data are the first to identify nonrandom differential DNA methylation between the sexes of genes subject to XCI in marsupials.

No differential DNA methylation was detected between the sexes on the chicken Z, or any of the X chromosomes in platypus (fig. 1). Frustratingly, only 437 Mb of the 1.84 Gb of assembled platypus sequence is anchored to chromosomes (Warren et al. 2008), which greatly reduced the number of genes that could be analyzed. Improved assemblies will enable tabulation of genomic features and promoters located on specific X chromosomes, rather than examining all Xs as one. For instance, our current analysis is limited because we cannot partition loci to X_5 (the oldest X) and X_1 (the youngest X), which may differ in DNA methylation profile. Notwithstanding these limitations, we conclude that there is no differential DNA methylation between the sexes on the Z chromosome in chicken, or across all the X chromosomes in platypus, precluding an obvious role in sex chromosome gene silencing.

As expected, in female mice there was hypermethylation of the X at CpG islands associated with promoters (fig. 2 and table 1). Hypermethylation was also observed at TSSs of expressed genes subject to XCI (fig. 3*C*), whereas genes that escape XCI displayed no differential DNA methylation between the sexes near TSSs (supplementary fig. S6, Supplementary Material online). In addition to validating our pipeline, this observation accords with previous findings of hypermethylated promoters, which negatively regulate gene expression and contributes to the maintenance of eutherian XCI (Escamilla-Del-Arenal et al. 2011).

The pattern of DNA methylation on the marsupial X chromosome and its role in XCI has been investigated for many years. Differential DNA methylation between the sexes of X-borne gene promoters has not been observed in female marsupials (Kaslow and Migeon 1987; Loebel and Johnston 1996; Hornecker et al. 2007; Wang et al. 2014), which contrasts immunofluorescence evidence that demonstrates the Xi is globally hypomethylated in marsupials (Rens et al. 2010; Ingles and Deakin 2015); an unexpected observation considering that hypermethylation correlates with transcriptional silencing. General consensus in the literature has been that DNA methylation does not play an obvious role in marsupial XCI (Cooper et al. 1993). Differential DNA methylation was previously detected in the *Rsx* promoter (Wang et al. 2014); however, we did not sample the *Rsx* promoter in our RRBS data.

Differential DNA methylation between the sexes is only observed near TSSs of expressed genes that are subject XCI, so correlates perfectly with XCI. Close inspection of 20-kb windows at TSSs of expressed genes subject to XCI established no differential DNA methylation between the sexes at TSSs. However, differential DNA methylation between the sexes was detected in regions that flank TSSs. In contrast, genes that escape XCI and genes subject to XCI, but not expressed, had no differential methylation between the sexes across their TSSs.

The patterns observed in opossum were consistent to those we observed for a limited number of genes in tammar wallaby brain, liver, spleen, and cultured fibroblasts. Additionally, in common wallaroo (*Macropus robustus*) blood, CpGs in the gene body of *Hprt* were hypomethylated on the Xi (Piper et al. 1993). And in *Didelphis virginiana* liver, heart, and brain, hypomethylation on the Xi was observed in the gene body of *G6pd* (Kaslow and Migeon 1987). These observations suggest that hypomethylation flanking TSSs on the inactive X is consistent across different marsupial species and tissues.

Mounting evidence suggests that the spatial distribution of methylated cytosines across promoters correlates with gene expression levels, rather than average methylation across entire promoters. Low DNA methylation at TSSs, flanked by higher DNA methylation (a U-shaped profile), correlates with increased gene expression (Vanderkraats et al. 2013; Edgar et al. 2014; Kapourani and Sanguinetti 2016). A profile of uniform DNA methylation across TSSs, irrespective of absolute level, correlates with reduced gene expression (Kapourani and Sanguinetti 2016). Unique to our analysis is the observation of differential "flattening" (between the sexes) of the DNA methylation profile at TSSs for genes subject to XCI, in both mouse and opossum (fig. 3*C* and *D*). We propose that the flattening acts as part of the epigenetic machinery to maintain silence of genes on the X chromosome in all therian mammals.

Although genes subject to XCI in eutherian and marsupial mammals share a common distribution of DNA methylation across promoters, there is a stark difference between the strategies by which this is achieved. In eutherians the level of DNA methylation is rendered more uniform across the

promoter by hypermethylation at TSSs, whereas in marsupials, hypomethylation of regions flanking TSSs achieves the same outcome. In both groups, the difference in DNA methylation between males and females (whether it be at TSSs in mouse or flanking TSSs in opossum) remains consistent at ~20% (fig. 3C and D).

Superficially, our observations contrast those reported in the literature; but, our data do remain consistent with previous observations. We demonstrated global hypomethylation of the X chromosome in females, as previously observed (Rens et al. 2010; Ingles and Deakin 2015). However, we also observe an equal level of DNA methylation (~20%) in both sexes at TSSs of genes subject to XCI (fig. 3D). Previous work (Kaslow and Migeon 1987; Loebel and Johnston 1996; Hornecker et al. 2007; Wang et al. 2014) examining promoter DNA methylation on the X in marsupials did not detect differential methylation between sexes because regions immediately upstream of TSSs were examined. We demonstrate that no differential methylation is detected until >1-kb upstream of TSSs.

This study provides a comprehensive between sex analysis of DNA methylation patterns on the sex chromosomes in representative amniote vertebrates. Our data suggest that a uniform distribution of DNA methylation across TSSs of genes subject to XCI is common in both eutherian and marsupial mammals. Surprisingly, in marsupials DNA methylation is low across these TSSs, whereas in eutherians DNA methylation is high.

Materials and Methods

Ethics and Samples

All animal experiments were approved by the Australian National University Animal Experimentation Ethics Committee (approval number R.CG.14.08) and the Garvan/St Vincents Animal Ethics Committee (#13/35).

Liver samples of 8-day-old male and female chicks (*Gallus gallus*), adult platypus (*Ornithorhynchus anatinus*), adult opossum (*Monodelphis domestica*), and adult mouse (*Mus musculus*) were collected fresh and snap frozen. Male and female tammar wallaby (*Macropus eugenii*) brain, liver, and spleen were also collected and snap frozen, and primary fibroblast cell cultures were established from ear clips of the same individuals. One male and one female was available for chick, platypus, opossum, and tammar wallaby. Six males and six females were available for mouse. Genomic DNA was isolated with the DNeasy Blood & Tissue Kit (QIAGEN). DNA quality was evaluated by agarose gel electrophoresis and spectrophotometer.

Sequenom MassARRAY EpiTYPER Analysis

Tammar wallaby DNA methylation was examined for four X-borne genes using the Sequenom MassArray EpiTYPER (Sequenom, San Diego, CA) as previously described (Novakovic et al. 2011). Primers (supplementary table S5, Supplementary Material online) were designed using Agena Bioscience's EpiDesigner software (http://www.epidesigner.com; last accessed November 24, 2017). PCR amplification was performed after bisulfite conversion of genomic DNA with the Qiagen EpiTect Bisulfite kit (Qiagen, Australia) according to the manufacturer's instructions. All PCR amplifications, and downstream processing, were carried out in triplicate.

Reduced Representation Bisulfite Sequencing

Reduced representation bisulfite sequencing (RRBS) was conducted by Zymo research. Briefly, libraries were prepared from 200 to 500 ng genomic DNA digested with 60 units of TaqI and 30 units of MspI (NEB) sequentially. Size-selected TaqI-MspI fragments (40–120 and 120–350 bp) were filled in and 3′-terminal-A extended, extracted with DNA Clean & Concentrator-5 kit (Zymo Research). Ligation to preannealed adapters containing 5′-methyl-cytosine instead of cytosine was performed using the Illumina DNA preparation kit and protocol. Purified, adaptor ligated fragments were bisulphite-treated using the EZ DNA Methylation-Direct Kit (Zymo Research). Preparative-scale PCR was performed and purified DNA PCR products were subjected to a final size selection on a 4% NuSieve 3:1 agarose gel. SYBR-green-stained gel slices containing adaptor-ligated fragments of 130–210 or 210–460 bp in size were excised. Library material was recovered from the gel (Zymoclean Gel DNA Recovery Kit) and sequenced on an Illumina HiSeq genome analyzer.

Mouse liver RRBS data have been deposited in the NCBI Gene Expression Omnibus under accession number GSE84573. Opossum, platypus, and chicken liver RRBS data are available in the NCBI short read archive under BioProject PRJNA356776.

RRBS Data Analysis

All fastq files were filtered with Trimmomatic-0.30 (Bolger et al. 2014) with the option SLIDINGWINDOW: 5:15. Paired forward and reverse reads were mapped to respective indexed genomes with bismark_v0.9.0 (http://www.bioinformatics.babraham.ac.uk/projects/bismark/; last accessed November 24, 2017) using the following options: –non_directional -bowtie2 -D 20 -R 3 -N 0 -L 20.

Sorted SAM files were read into the R package MethylKit (Akalin et al. 2012) with the read.bismark command. Read context was set to CpG, minimum coverage set to 1, and minimum quality set to 20. The command filterByCoverage was used to create an object of bases with coverage of at least one, and a second object of bases with coverage of at least nine. Both were normalized with the command normalizeCoverage. Normalized objects with coverage of at least one were used for estimations of methylation within genomic features (e.g., LINE1, gene bodies, and windowed analysis). Normalized objects with coverage of at least nine were used to estimate methylation of specific CpG dinucleotides in both sexes.

Male:female DNA methylation ratios were calculated within 10- and 300-kb sliding windows with the tileMethylCounts command in methyKit. These ratios were used for circos plots (300-kb windows) and linear plots (10-kb windows). The regionCounts command in methlyKit was used to calculate methylation values in each sex for

annotated LINE1, gene bodies, promoters (i.e., 1-kb upstream of annotated TSSs), and CpG islands. Male:female ratios were calculated from these values.

Circos plots were created with RCircos (Zhang et al. 2013). The RCircos.Histogram.Plot function was used to plot absolute DNA methylation levels within 300-kb windows (see above) for both male and female. The 10 kb windowed heatscatter plots were plotted with the R package LSD.

For metagene analysis of DNA methylation levels at TSSs, male and female methylation vales were calculated at CpG dinucleotids (±10 kb from TSSs) with a read depth of >8 (in both sexes). Genes on the X chromosome in mouse and opossum were binned into those that escaped XCI, and those subject to XCI (both expressed and not expressed). Bins were also generated for expressed and nonexpressed autosomal genes in each species. For each bin DNA methylation levels were plotted around TSSs with the R package ggplot2 (Wickham 2009). A generalized additive model with default smoothing (cubic smoothing spline) was used with the predict function in R to fit a regression line with 95% confidence intervals.

Analysis of TSSs in single mouse male and female pairs (supplementary fig. S8*A*–*D*, Supplementary Material online) resulted in almost identical curves to those observed for the six male and six female replicates combined, validating the use of single replicates in the other species. DNA methylation across TSSs was also analyzed using publically available mouse whole genome bisulfite sequencing (WGBS) data for male and female (Cole et al. 2017). DNA methylation at TTSs of autosomal genes were identical between the sexes (supplementary fig. S8*E*, Supplementary Material online), whereas DNA methylation at TSSs of genes subject to XCI was increased in female (supplementary fig. S8*F*, Supplementary Material online), validating the patterns observed in the RRBS data.

For each CpG associated with genes subject to XCI, the DNA methylation level in male (with a single active X) was used as a measure of DNA methylation level on Xa in female. In female, the total methylation level measures both the Xa and Xi. Therefore, the level of CpG DNA methylation on Xi was estimated by: 1) Calculating the difference of DNA methylation between sexes at each CpG by subtracting the male methylation value from the female. 2) Adding this value to the female methylation value. A generalized additive model regression line with 95% confidence intervals was plotted with ggplot2 as above.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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