Evolution of the Short Form of DNMT3A, DNMT3A2, Occurred in the Common Ancestor of Mammals

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Accepted: 10 June 2022

Abstract

Genomic imprinting is found in marsupial and eutherian mammals, but not in monotremes. While the primary regulator of genomic imprinting in eutherians is differential DNA methylation between parental alleles, conserved imprinted genes in marsupials tend to lack DNA methylation at their promoters. DNA methylation at eutherian imprinted genes is mainly catalyzed by a DNA methyltransferase (DNMT) enzyme, DNMT3A. There are two isoforms of eutherian DNMT3A: DNMT3A and DNMT3A2. DNMT3A2 is the primary isoform for establishing DNA methylation at eutherian imprinted genes and is essential for eutherian genomic imprinting. In this study, we investigated whether DNMT3A2 is also present in the two other mammalian lineages, marsupials and monotremes. We identified DNMT3A2 in both marsupials and monotremes, although imprinting has not been identified in monotremes. By analyzing genomic sequences and transcriptome data across vertebrates, we concluded that the evolution of DNMT3A2 occurred in the common ancestor of mammals. In addition, DNMT3A/3A2 gene and protein expression during gametogenesis showed distinct sexual dimorphisms in a marsupial, the tammar wallaby, and this pattern coincided with the sex-specific DNA methylation reprogramming in this species as it does in mice. Our results show that DNMT3A2 is present in all mammalian groups and suggests that the basic DNMT3A/3A2-based DNA methylation mechanism is conserved at least in therian mammals.

Key words: marsupials, eutherians, monotremes, DNA methylation, genomic imprinting.

Significance

While eutherian genomic imprinting is mainly regulated by DNA methylation, marsupial imprinted genes tend to lack promoter DNA methylation. In this study, we asked whether this difference is caused by the absence of a primary enzyme, DNMT3A2. We confirmed that DNMT3A2 is present in all mammalian lineages. As the primary enzyme must have been evolved in the common ancestor of mammals, our result suggests that the difference in DNA methylation levels between marsupial and eutherian imprinting is caused by the associated proteins rather than the enzyme itself.

Introduction

Genomic imprinting is an epigenetic process that causes differential expression of a subset of genes depending on their parental origin. Among vertebrates, genomic imprinting has been found in therian mammals, namely eutherians and marsupials, but there is no evidence for imprinting in monotremes or nonmammalian vertebrates (Pask et al. 2009; Renfree et al. 2009; Frésard et al. 2014; Griffith et al. 2016). Mammalian genomic imprinting is therefore thought to have evolved after the therian-monotreme divergence ∼184 million years ago (Cúneo et al. 2013). Genomic imprinting is known to regulate mammalian development and maternal behavior (Lefebvre et al. 1998;
Kaneda et al. 2004), indicating that the DMR of the conserved paternally expressed gene MEST is reprogrammed in developing germ cells in both marsupials and eutherians. As this gene is established by the action of DNMT3A in eutherians. As MEST in marsupials is imprinted but does not have a DMR, comparisons of DNMT3A-based mechanisms between marsupials and eutherians could elucidate the differences in DMR-based imprinting between the two mammalian lineages.

There is a novel short isoform of DNMT3A, DNMT3A2, identified in eutherians (Chen et al. 2002; Sakai et al. 2004). Further characterization of DNMT3A2 demonstrated that it is a more effective isoform of DNMT3A at establishing DNA methylation on imprinted genes (Sakai et al. 2004; Nimura et al. 2006; Suetake et al. 2006; Ma et al. 2015; Thakur et al. 2016). Marsupials also have DNMT3A, DNMT3B and DNMT3L (Yokomine et al. 2006; Ishihara et al. 2019), but the presence of DNMT3A2 is unknown. It is therefore possible that the evolution of DNMT3A2 occurred in the ancestor of eutherian mammals and caused the differences observed in the DNA methylation status of imprinted genes between eutherians and marsupials.

In this study, the evolution DNMT3A2 was investigated through the identification of isoforms of DNMT3A across vertebrates focusing on the two other mammalian lineages, marsupials and monotremes. By analyzing transcriptome data of a marsupial (the koala: Phascolarctos cinereus), a monotreme (platypus: Ornithorhynchus anatinus), and non-mammals (chicken: Gallus gallus; the bearded dragon: Pogona vitticeps), we evaluated the presence of DNMT3A2 across vertebrates. We further compared the gene and protein expression patterns of DNMT3As in the developing gonads of the marsupial tammar wallaby (Macropus eugenii) with the existing DNA methylation data for this species to determine conserved function. Here we report that the DNMT3A2 occurred in all three mammalian groups so it must have evolved in the common ancestor of mammals and that the basic DNMT3A3A2-based mechanism appears to be conserved at least in therian mammals.

**Results**

**Tammar Wallaby has the Short Form of DNMT3A, DNMT3A2**

The 2,865 bp of tammar DNMT3A candidate sequence was identified by a blast search of the tammar genome database (Wallabase: https://wallabase.org). The comparison of the putative tammar DNMT3A with mouse Dnmt3a/Dnm3a2 identified highly conserved protein coding exons (fig. 1A and supplementary fig. S1). 5′-rapid amplification of cDNA end (RACE) reactions using a specific primer against the highly homologous region (fig. 1A) were then performed with adult tammar testis to characterize the marsupial DNM3A isoforms. The 5′-RACE products showed two distinct bands (fig. 1B), indicating that there were two isoforms. After cloning and sequencing of each
Two transcript variants of DNMT3A were identified. The first exon of the shorter isoform was located between exon 6 and exon 7 of the longer isoform. Based on the exon structure, we renamed the shorter isoform as DNMT3A2 (fig. 1). Combining specific primers against the DNMT3A2 and 3′-RACE reactions confirmed that the tammar DNMT3A2 is also a protein coding transcript (fig. 1C).

Platypus also has DNMT3A2 and a Unique Isoform of DNMT3A, DNMT3AOa

The 2,865 bp of platypus DNMT3A candidate sequence was identified by a blast search of the new platypus genome (mOrmA1.p.v4). 5′-RACE reactions were then performed using adult platypus testis to identify monotreme DNMT3A isoforms (fig. 2A). The 5′-RACE products showed four amplions, indicating that there were four isoforms. After sequencing each RACE product, four transcript variants of DNMT3A were identified. Isoform 4 encoded the shortest form of DNMT3A and its first exon was located between exon 6 and exon 7 of the longest form. Based on the exon structure, we renamed the shortest isoform as DNMT3A2 (fig. 2B). Using specific primers against the DNMT3A2 and isoform3, 3′-RACE reactions confirmed that the platypus DNMT3A2 and isoform3 were protein coding transcripts (fig. 2C). Isoform 3 contained a unique exon and encoded a unique protein. A BLAST search using NCBI BLAST did not reveal any similar sequences with this unique exon. Thus, we named isoform 3 as a novel isoform, DNMT3AO. anatinus specific isoform, DNMT3AOa (fig. 2C).

The Transcription Start Site of DNMT3A2 is Conserved Across Mammals

To identify conserved cis-regulatory elements at the transcription start site (TSS) of DNMT3A2 in mammals, genomic alignment was performed and visualized with AliTV program (Ankenbrand et al. 2017). The TSS of DNMT3A2 was highly conserved across mammals (fig. 3). Note that the TSS of platypus DNMT3A2 also has high homology with a region in the chicken DNMT3A locus. However, the range of the region is <62 bp and the sequence does...
not encode the first exon of mammalian DNMT3A2. The TSS of mammalian DNMT3A2 showed no homology with the DNMT3A locus of the bearded dragon lizard (P. vitticeps).

To identify common cis motifs around the TSS of DNMT3A2, the highly homologous region identified across mammals was subjected to multiple alignments by clustalW. As a result, highly conserved sequences around the TSS of DNMT3A2 across mammals were identified (fig. 4A; asterisks). Regions containing five or more highly homologous sequences were compared with known transcription factor binding motifs using the TOMTOM program in the MEME suite. From this analysis, two common regions were found that had significant matches with E-values <0.05 and q-values <0.05 (fig. 4A; dotted boxes). Region 1 contained upstream transcription factor 2 (USF2) and USF1 binding motifs (fig. 4B), and region 2 contained a T-box transcription factors (TBX15, TBX1, TBX4, TBX5) binding site and a Max dimerization protein (MGA) binding site (fig. 4C). Region 2 was downstream of the TSS and within the first exon of mammalian DNMT3A2.

DNMT3A2 is a Mammal Specific Isoform of DNMT3A

Published transcriptome data sets of gonads derived from chicken and the bearded dragon were analyzed to identify potential isoforms by visualizing splicing junctions. Koala and platypus transcriptome data sets were also analyzed.
The transcription start sites of tammar, and platypus), chicken, and the central-bearded dragon were conserved across mammals. Genomic sequences of mammals (mouse, human, tammar, and platypus), chicken, and the central-bearded dragon were aligned, and their sequence homologies were visualized with AllTv program. The transcription start sites of DNMT3A2 were conserved across mammals. The first exon of platypus DNMT3A2 had a homology with chicken genome, although it was <62 bp. Exon structures of DNMT3A and DNMT3A2 are shown as black box connected with black lines in the aqua boxes.

Expression Patterns of DNMT3A2 During Gametogenesis are Conserved Across Therian Mammals

Figure 3. TSSs of DNMT3A2 are conserved across mammals. Multiple alignments confirmed presence of highly conserved region at the TSS of DNMT3A2 in mammals. Genomic sequences of mammals (mouse, human, tammar, and platypus), chicken, and the central-bearded dragon were aligned, and their sequence homologies were visualized with AllTv program. The transcription start sites of DNMT3A2 were conserved across mammals. The first exon of platypus DNMT3A2 had a homology with chicken genome, although it was <62 bp. Exon structures of DNMT3A and DNMT3A2 are shown as black box connected with black lines in the aqua boxes.

To further confirm the correlation between DNA methylation reprogramming and DNMT3A/3A2 expression, immunohistochemistry (IHC) was performed. Based on our western blotting analysis, the antibody used in this study could detect both DNMT3A and DNMT3A2 (supplementary fig. S2). In developing gonads, tammar DNMT3A/3A2 protein localization was always nuclear (fig. 7). Within ovaries, at day 16 pp, DNMT3A/3A2 was restricted to somatic cells (fig. 7A). By day 60 pp, when early folliculogenesis has commenced, several oocytes within primordial follicles adjacent to the cortex were DNMT3A/3A2-positive (fig. 7B). At day 120 pp, when DNA remethylation begins (Ishihara et al. 2019), some oocytes contained nuclear DNMT3A/3A2 but others were negative, seemingly with no respect to oocyte size or location (fig. 7C). At day 230 pp, some smaller oocytes within primary follicles had nuclear DNMT3A, as did most larger oocytes within secondary and tertiary follicles (fig. 7D).

At day 26 pp, the majority of germ cells were DNMT3A/3A2 negative (fig. 7E). At day 60 pp when DNA remethylation has commenced (Ishihara et al. 2019), it appeared there were more DNMT3A/3A2-positive cells compared with day 26 pp (fig. 7F). Day 110 pp testes contained similar numbers of DNMT3A/3A2-positive cells to day 60 pp (fig. 7G). However, in day 300 pp testes, DNMT3A/3A2-positive germ cells were not evident compared with day 110 pp (fig. 7H).

Discussion

The short form of DNMT3A, DNMT3A2 is critical in establishing DMR-based imprinting in eutherians. As most marsupial imprinted genes so far identified do not have DMRs, we predicted that the evolution of DNMT3A2 occurred after the eutherian-marsupial split 166 million years ago. However, DNMT3A2 was present not only in marsupials but also in monotremes, in which genomic imprinting has not been identified. No DNMT3A2 was identified in the chicken or the bearded dragon, but the TSS of DNMT3A2 was conserved across all mammals, suggesting that the acquisition of the new promoter for DNMT3A2 expression occurred in the common ancestor of mammals before the monotreme-therian split over 184 million years ago (Cúneo et al. 2013; fig. 8).
FIG. 4.—Common cis-acting elements around the TSS of DNMT3A2 across mammals. Multiple alignments of the TSS of mammalian DNMT3A2 identified common cis-acting elements. (A) Multiple alignments of the highly conserved region around the TSS of DNMT3A2 in mammals. The first exon of DNMT3A2 is shown in red-colored letters. There are two regions (regions 1 and 2) which had significant matches (E-value < 0.05 and q-value < 0.05) based on TOMTOM program in MEME suite. (B) Motif structure of the region 1. The region contained upstream transcription factor 2 (USF2) and USF1 binding motifs. (C) Motif structure of the region 2. The region contained T-box transcription factors (TBX15, TBX1, TBX4, TBX5) binding site and Max dimerization protein (MGA) binding site.

Ishihara et al. Genomé Biol. Evol. 14(7) https://doi.org/10.1093/geb/evac094 Advance Access publication 23 June 2022
Fig. 5.—Visualizations of splicing junctions of *DNMT3A* confirmed *DNMT3A2* in koala and platypus but not in other vertebrates. Visualizations of splicing junctions of vertebrate *DNMT3A* identified *DNMT3A2* in koala and platypus but not chicken and bearded dragon. Published gonadal transcriptome data sets derived from koala, platypus, chicken, and a dragon lizard were mapped against their genomic sequences and splicing junctions of *DNMT3A* were visualized by sashimi plot. Splicing junctions were shown as lines connecting two exons and numbers of junction reads were shown on the junction lines. Exon structures of *DNMT3A* were shown as black boxes under the sashimi plot. (A) Koala. In testis, koala transcriptome data analysis successfully detected the short form, *DNMT3A2* (orange circle). (B) Platypus. In both sexes, platypus transcriptome data analysis successfully detected the short form, *DNMT3A2* (orange circle). (C) Chicken. Chicken transcriptome data analysis could not identify *DNMT3A2* in gonads. (D) The central-bearded dragon, *Pogona vitticeps*. Transcriptome data analysis did not identify *DNMT3A2* in gonads.
The highly homologous region around the TSS of DNMT3A2 across mammals contained a number of consensus binding motifs. The putative USF1/2 binding sites were located in the TSS of the tammar DNMT3A2 and upstream of the TSS of DNMT3A2 in the other mammals examined in this study. USFs generally bind to genomic regions close to TSS of target genes and regulate their expression (Rada-Iglesias et al. 2008). For example, USF1 binds to the first exon of the N4bp2l1 gene and regulate its expression in mice (Watanabe et al. 2019). This suggests that USF1/2 binding may contribute to the expression of DNMT3A2 in mammals. Moreover, we found a putative binding site of T-box transcription factors such as TBX15 and TBX5. The putative TBX factor binding site was downstream of the TSS of DNMT3A2. However, Tbx5 binds to a motif located downstream of the TSS of Fgf10 and controls its expression in developing mouse limbs (Delgado et al. 2021). Thus, T-box transcription factors regulate target gene expression even when they bind to binding sites downstream of the TSS. These data suggest that these T-box transcription factors may regulate DNMT3A2 expression in mammals as seen in mouse Fgf10. TBX15 is known to be required for
skeletal development of the limb, vertebral column, and head (Singh et al. 2005). In mice, Dnmt3a/3a2 deficiency shows defects in osteoclast differentiation (Nishikawa et al. 2015). The other T-box transcription factor, TBX5, is involved in cardiovascular development (Steimle and Moskowitz 2017), and inactivating DNMT3A/3A2 contributes to cardiovascular disease (Sano et al. 2018). Therefore, the short isoform, DNMT3A2, in addition to establishing DNA methylation-based imprinting in germ cells, may have a functional role in mammalian bone and cardiovascular development. Further characterization of the molecular mechanisms that regulate DNMT3A2 expression could elucidate possible links between DNMT3A2, T-box transcription factors and mammalian development.

A cofactor of DNMT3A, DNMT3L is essential for adding DNA methylation on selected gene loci, including imprinted genes in eutherians (Bourc’his et al. 2001; Hata et al. 2002; la Salle et al. 2007; Glass et al. 2009). Across mammals, DNMT3L is conserved in both marsupials and eutherians but not in monotremes (Yokomine et al. 2006; Renfree et al. 2009). These data suggest that monotremes lack an ability to recruit DNA methylation on selected gene loci to

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**Fig. 7.**—DNMT3A/3A2 protein localization during tammar gametogenesis. Immunohistochemistry of DNMT3A/3A2 during oogenesis and spermatogenesis revealed sex-specific DNMT3A/3A2 upregulation in developing germ cells. (A) Ovary at day 16 pp. (B) Ovary at day 60 pp. (C) Ovary at day 110 pp. (D) Ovary at day 192 pp. (E) Testis at day 26 pp. (F) Testis at day 60 pp. (G) Testis at day 120 pp. (H) Testis at day 300 pp. Black and red arrows represent germ cell with DNMT3A/3A2 staining and DNMT3A/3A2 negative germ cells, respectively. Brown color: positive staining. Scale bars: 20 μm.
Evolution of DNMT3A2

![Diagram showing the evolution of DNMT3A2 across species.](image-url)

**Fig. 8.** Evolution of DNMT3A2 occurred in the common ancestor of mammals. Phylogenetic tree and DNMT3 family. DNMT3A family member proteins of each animal are listed under the silhouette of each animal. The important isoform of DNMT3A, DNMT3A2 has evolved in the common ancestor of mammals (red letters). The platypus lacks a cofactor of DNMT3A, DNMT3L, but the platypus has the platypus-specific isoform of DNMT3A, DNMT3AOa (blue letters).

establish genomic imprinting. However, in this study, the novel platypus-specific isoform DNMT3AOa was identified. Although the exact function of DNMT3A2 is currently unknown, this novel isoform may increase the capacity for the platypus to add DNA methylation onto their genome. The capacity of DNMT3AOa to add DNA methylation on platypus genome needs to be determined.

In mice, Dnmt3a3a2 expression shows distinct sexual dimorphisms during gametogenesis and correlates with DNA methylation reprogramming in both sexes (la Salle et al. 2004; Lucifero et al. 2007). Our analysis showed that patterns of tammar DNMT3A and DNMT3A2 expression were also different between the sexes during gametogenesis and correlated with the known DNA methylation patterns across gametogenesis in the tammar. Although the previous mouse studies did not separate Dnmt3a2 expression from Dnmt3a expression in developing germ cells (la Salle et al. 2004; Lucifero et al. 2007), the combined expression of mouse Dnmt3a3a2 and tammar DNMT3A/DNMT3A2 have similar patterns during gametogenesis. Our IHC analysis further confirmed that DNMT3A/3A2 localization also showed sex-specific differences consistent with DNA methylation reprogramming in this species. These data suggest that the sex-specific global DNA methylation reprogramming in therian mammals is mediated by a conserved DNMT3A/DNMT3A2-based mechanism. Although we could not confirm DNMT3A/3A2 expression in developing platypus gonads due to limited access to samples, it would be interesting to determine if this pattern is conserved across all mammals.

As well as DNMT3A3A2 expression, the expression pattern of its co-factor DNMT3L in the tammar is also similar to mouse Dnmt3l (Ishihara et al. 2019). Therefore, based on this study and previous studies (Yokomine et al. 2006; Renfree et al. 2009; Ishihara et al. 2019), we confirmed that marsupials have all the known molecular machineries, DNMT3A3A2 and DNMT3L, required for catalyzing DNA methylation on imprinted genes. Despite this, some marsupial imprinted genes lack promoter DNA methylation. For example, in the tammar, the conserved imprinted gene MEST lacks a DMR on its promoter (Suzuki et al. 2005, 2018; Das et al. 2012). However, in mice, Mest DMR establishment in oocytes occurs in the later stages of oogenesis (Lucifero et al. 2004) and during tammar oogenesis, late upregulation of DNMT3A relative to DNMT3A2 was observed. As the combined expression of mouse Dnmt3a/Dnmt3a2 and tammar DNMT3A/DNMT3A2 has similar patterns during oogenesis, this suggests that the lack of DNA methylation on the promoter of marsupial MEST might be caused by differences in DNMT3A and/or its associated proteins between marsupials and eutherians. However, confirmation of this requires examination of the specific expression pattern of Dnmt3a relative to Dnmt3a2 in the mouse during oogenesis.

This study confirms that the overall mechanism and expression pattern of DNMT3A/DNMT3A2 is conserved among therian mammals. However, given this conservation, it remains unclear as to why some marsupial imprinted genes lack DMRs on their promoters. DNMT3A2 was also present in monotremes. In addition, a novel DNMT3A isoform was identified in monotremes which may contribute to establish the global DNA methylation levels. Further examination of monotremes could provide important insights into evolution of mammalian genomic imprinting.

**Materials and Methods**

**Animals**

Tammar wallaby (*M. eugenii*) samples of Kangaroo Island, South Australia origin, were collected from captive animals from our colony maintained by the University of Melbourne. Gonads were snap-frozen immediately after dissection. Platypus (*O. anatinus*) tissues were collected from wild-caught animals under permits from NSW Parks and Wildlife and ethically approved by the University of Melbourne Animal Ethics committees. All animal handling and husbandry were in accordance with the National Health and Medical Research Council of Australia (2013) guidelines.

**Gonadal RNA Extraction and cDNA Synthesis**

Snap-frozen gonads were used for RNA extraction using the GenElute Mammalian total RNA Miniprep Kit (Sigma-Aldrich, St Louis, MO, USA) following the manufacturer’s instructions. The extracted RNA was treated
with the DNA-free DNase treatment and removal kit (Thermo Fisher Scientific, Waltham, MA, USA) to remove residual genomic DNA then 200 ng of RNA was used as a template for cDNA synthesis using SuperScript IV First strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Approximately 1 µg of RNA was used as a template for cDNA synthesis using a SMARTer RACE 5′/3′-kit (Clontech, Mountain View, CA, USA).

5′- and 3′-Rapid Amplification of cDNA Ends

To determine the TSSs of the marsupial and monotreme DNMT3A transcripts, 5′-RACE experiments were performed using the SMARTer RACE 5′/3′-kit (Clontech). Putative tammar and platypus DNMT3A sequence were searched by a blast search of the wallaby genome loaded from NCBI (https://www.ncbi.nlm.nih.gov) and the bearded dragon (Wallabase: https://wallabase.org). To locate conserved genome (Wallabase: https://wallabase.org) and the platypus genome (mOrnAna1.p.v4), respectively. After identifying the putative sequences, gene-specific primers were designed for the downstream RACE experiments. The first round RACE reaction was performed with adult tammar testis or adult platypus testis cDNA using SeqAmp DNA Polymerase (Clontech) with the gene-specific primers (supplementary table S1). The nested 5′- and 3′-RACE was performed by GoTaq DNA polymerase (Promega, Fitchburg, WI, USA) and the RACE products cloned using pGEM-T Easy Vector (Promega) and JM109 competent cells (Promega). Plasmids were extracted using Wizard Plus SV Minipreps DNA Purification System (Promega) and sequenced by Sanger Sequencing method with the vector specific primers (supplementary table S1).

Genomic Comparison of DNMT3A Locus Across Vertebrates

Genomic sequences of several vertebrate DNMT3As [mouse (Mus musculus), human (Homo sapiens), tammar (M. eugenii), platypus (Or. anatinus), chicken (G. gallus), and the bearded dragon (P. viticeps)] including up to 3,000 bases of upstream sequence of the TSS was downloaded from NCBI (https://www.ncbi.nlm.nih.gov) and Wallabase (https://wallabase.org). To locate conserved genomic region across vertebrates, genomic alignments and visualization were performed by AllTv (Ankenbrand et al. 2017). To identify conserved transcription factor binding sites, the identified conserved region across mammals was subjected to multiple alignment by clustalW. Regions containing five or more highly conserved sequences around the TSS of DNMT3A2 across mammals were identified and compared with known transcription factor binding motifs by motif searches with JASPR CORE database (2020) using TomTom function in the MEME Suite (https://meme-suite.org/meme/index.html; Bailey et al. 2015). Motifs with an E-value <0.05 and q-value <0.05 were considered as potential transcription factor binding motifs.

Transcriptome Analysis

Publicly available raw RNA-seq data sets of koala (GEO accession: GSE128122; Yu et al. 2019), platypus (GEO accession: GSE97367; Marin et al. 2017), chicken (GEO accession: GSE97367; Marin et al. 2017), and the bearded dragon (BioProject accession: PRJEB5206; Georges et al. 2015) were downloaded from NCBI SRA (https://www.ncbi.nlm.nih.gov/sra). All RNA-seq reads were trimmed using TrimGalore! (v0.6.5; https://github.com/FelixKrueger/TrimGalore) with default settings to eliminate adaptor sequences, poor quality reads, and very short (<20 bp) reads. The trimmed reads were aligned to each genome (koala: phaCin_unsw_v4.1; platypus: mOrnAna1.p.v1; chicken: GRCg6a; the bearded dragon: pv1.1) using HISAT2 (Kim et al. 2019). Biological replicates were merged to detect splicing variants by increasing sequence depth. The reads were used as inputs of ggsashimi program (Garrido-Martín et al. 2018) to visualize splicing junctions.

Reverse transcription Quantitative RT-PCR

Reverse transcription quantitative RT-PCR (RT-qPCR) primers (supplementary table S1) were designed from the known tammar DNMT3A and DNMT3A2 sequences and amplified 113 and 76 bp products, respectively. RT-qPCR was performed on a Quantstudio 5 (Thermo Fisher Scientific) on gonadal cDNA of both sexes from day 10 pp to adult using the SYBR Green PCR kit (QIAGEN, Hilden, Germany). TATA-box binding protein (TBP) and hydroxy-methylbilane synthase (HMBS) were selected as the housekeeping genes. Expression levels of the DNMT3As were normalized to the geometric mean of the expression levels of housekeeping genes. Differences in the gene expression levels between stages were analyzed by Tukey–Kramer’s honestly significant difference test in R.

Immunohistochemistry

Extensive testing of different antibody concentrations and pretreatments (enzyme digestion and heat treatment in different buffers) using various tammar tissues and organs was performed to optimize each antibody. PFA-fixed, paraffin-embedded wallaby gonads were sectioned at 6 μm and sections were mounted on Superfrost slides (Thermo Fisher Scientific, Victoria, Australia). Slides were deparaffinized, rehydrated, permeabilized in 0.1% (v/v) Triton X-100 in tris-buffered saline (TBS; 50 mM Tris HCl, 150 mM NaCl, pH 7.4) for 15 min and then blocked in 6% hydrogen peroxide in water for 10 min. Slides requiring heat treatment were heated in a water bath at 99 °C in the appropriate buffer (supplementary table S2) for 30 min and then left at room temperature for another 30 min to cool. Nonspecific staining was blocked using 10% serum from the appropriate species for 30 min at room temperature.

Genome Biol. Evol. 14(7) https://doi.org/10.1093/gbe/evac094 Advance Access publication 23 June 2022 11
Primary antibodies were diluted as described in supplementary table S2 in TBS/0.1% bovine-serum albumin/5% appropriate serum and then applied to the sections for 16 h at 4 °C. Negative control sections were incubated with an equivalent concentration of the appropriate immunoglobulin. Signal was amplified using biotinylated goat anti-rabbit or rabbit anti-mouse secondary antibodies and then an ABC/HRP kit (DAKO, New South Wales, Australia) and visualized with DAB (Sigma). Sections were lightly counterstained in hematoxylin and then coverslipped in DPX. At least three (usually four to five) gonads were stained for each stage for each antibody. Sections were viewed using an Olympus BX51 System microscope and photographed using an Olympus DP70 digital microscope camera.

Western Blot

Protein was extracted from day 64 pp tammar PY testis (n = 4 pooled), day 80 pp ovary (n = 5 pooled), and day 23 of gestation fetus (n = 2 pooled) by homogenization in RIPA buffer (50 mM Tris base, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate). Fifty micrograms of each sample were electrophoresed in a 7.5% SDS-PAGE gel and then transferred to a PVDF membrane (Amersham Hybond-P). The membrane was blocked in 5% skim milk dissolved in TBST (TBS, 50 mM Tris HCl, 150 mM NaCl, pH 7.5 with 0.1% Tween 20) at 4 °C overnight and then incubated at room temperature with the anti-DNMT3A antibody (ab13888, 2 µg/ml) for 2 h, followed by a 1 h incubation with a HRP-conjugated goat anti-mouse secondary antibody diluted in TBS with 5% skim milk and 0.1% Triton X-100. Chemiluminescence using the Amersham ECL Western Blotting Detection Reagents kit (GE Healthcare Life Sciences) was used to detect bound antibody.

Supplementary Material

Supplementary figure and supplementary table are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

Author Contributions

T.I., D.H., J.C.F., O.W.G., S.S., and M.B.R. discussed and designed the research; T.I. performed most of the research; D.H. performed IHC experiment; J.C.F. and M.B.R. collected platypus tissues; J.C.F. extracted RNA from platypus tissues; O.W.G., S.S., and M.B.R. provided supervision; T.I., J.C.F., O.W.G., S.S., and M.B.R. discussed the data and T.I., D.H., J.C.F., O.W.G., S.S., and M.B.R. wrote the paper.

Acknowledgments

The authors thank all members of the Wallaby research group for helpful discussions and assistance with sampling. They thank Professors Peter Temple-Smith, Geoff Shaw, and Dr Kath Handasyde for collecting platypus samples. This study was supported by a Melbourne Research Scholarship to Ti and grants from the Australian Research Council to MBR and Prof. Geoff Shaw.

Data Availability

Raw RNA-seq data sets of koala (GEO accession: GSE128122), platypus (GEO accession: GSE97367), chicken (GEO accession: GSE97367), and the bearded dragon (BioProject accession: PRJEB5206) were publicly available on NCBI SRA (https://www.ncbi.nlm.nih.gov/sra).

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Associate editor: Dr. Rachel O’Neill