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Conserved H3K27me3-associated chromatin remodelling allows *STRA8* but not *MEIOSIN* expression in mammalian germ cells

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Abstract

In brief: Apart from mice, meiosis initiation factors and their transcriptional regulation mechanisms are largely unknown in mammals. This study suggests that STRA8 and MEIOSIN are both meiosis initiation factors in mammals, but their transcription is epigenetically regulated differently from each other.

Abstract: In the mouse, the timing of meiosis onset differs between sexes due to the sex-specific regulation of the meiosis initiation factors, STRA8 and MEIOSIN. Before the initiation of meiotic prophase I, the *Stra8* promoter loses suppressive histone-3-lysine-27 trimethylation (H3K27me3) in both sexes, suggesting that H3K27me3-associated chromatin remodelling may be responsible for activating *STRA8* and its co-factor *MEIOSIN*. Here we examined MEIOSIN and STRA8 expression in a eutherian (the mouse), two marsupials (the grey short-tailed opossum and the tammar wallaby) and two monotremes (the platypus and the short-beaked echidna) to ask whether this pathway is conserved between all mammals. The conserved expression of both genes in all three mammalian groups and of MEIOSIN and STRA8 protein in therian mammals suggests that they are the meiosis initiation factors in all mammals. Analyses of published DNase-seq and chromatin-immunoprecipitation sequencing (ChIP-seq) data sets confirmed that H3K27me3-associated chromatin remodelling occurred at the *STRA8,* but not the *MEIOSIN,* promoter in therian mammals. Furthermore, culturing tammar ovaries with an inhibitor of H3K27me3 demethylation before meiotic prophase I affected *STRA8* but not *MEIOSIN* transcriptional levels. Our data suggest that H3K27me3-associated chromatin remodelling is an ancestral mechanism that allows *STRA8* expression in mammalian pre-meiotic germ cells.

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Introduction

In mammals, germline sexual differentiation drives the establishment of sex-specific imprinting marks in the gametes that are important for the development of the next generation. The first sign of mammalian germline sexual differentiation is the onset of meiotic arrest in females and the onset of mitotic arrest in males (Alcorn & Robinson 1983, Spiller *et al.* 2017, Ishihara *et al.* 2021). In congenic mice with the C57BL/6 background, stimulated by retinoic acid gene 8 (*Stra8*) and the meiosis initiator (*Meiosin*) act together as transcriptional factors in preleptotene cells and activate meiosis-related genes to initiate meiotic prophase I (Anderson *et al.* 2008, Zhou *et al.* 2008, Kojima *et al.* 2019, Ishiguro *et al.* 2020).

Upregulation of these two meiosis initiation factors in a sex-specific manner is critical for the progression of germline sexual differentiation. This sex-specific protein expression at the onset of meiotic prophase I has been shown to be balanced by multi-level pre- and post-transcriptional regulation, such as the regulation of *Stra8* RNA levels by *Nanos2*-associated RNA degradation in males (Suzuki *et al.* 2010, Spiller *et al.* 2017, Saga 2022). In particular, pre-transcriptional changes in chromatin levels of these two factors may be important for understanding the regulatory mechanisms of meiotic initiation.

Coinciding with the first sign of germline sexual differentiation, epigenetic modifications such as DNA methylation and histone modifications are removed on a genome-wide scale (Matsui & Mochizuki 2014,

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Eguizabal *et al.* 2016), and then re-established in a sex-specific way in therian mammals (Monk 2015, Ishihara *et al.* 2019). The global wave of DNA demethylation affects non-imprinted genes including *Stra8* in mouse (Yamaguchi *et al.* 2012, Hargan-Calvopina *et al.* 2016) and the *Stra8* promoter loses DNA methylation in both sexes (Seisenberger *et al.* 2012). After losing DNA methylation, histone modifications appear to be important for the epigenetic regulation of *Stra8* expression in both sexes (Wang & Tilly 2010, Lesch *et al.* 2013, Yokobayashi *et al.* 2013, Hammoud *et al.* 2014). Before the onset of meiotic prophase I, the promoter of *Stra8* gains a bivalent chromatin status marked by the active histone mark, histone-3-lysine-4 trimethylation (H3K4me3) and the suppressive histone modification, histone-3 lysine-27 trimethylation (H3K27me3) in both sexes (Yokobayashi *et al.* 2013, Hammoud *et al.* 2014). This bivalent chromatin is known to have an inactive or poised transcriptional status (Blanco *et al.* 2020). Importantly, at the time germ cells initiate *Stra8* gene expression, the *Stra8* promoter gains active transcriptional status by losing the H3K27me3 marks in both sexes (Yokobayashi *et al.* 2013, Hammoud *et al.* 2014, Brind'Amour *et al.* 2015).

H3K27me3-associated chromatin remodelling may regulate *STRA8* and its cofactor *MEIOSIN* in mouse germ cells, but it is unclear whether epigenetic regulation of *STRA8* and *MEIOSIN* at the onset of meiotic prophase I is conserved in mammals, nor is it known whether STRA8 and MEIOSIN act as transcription factors for the onset of meiotic prophase I in all mammals. Little is known about the gene expression pattern and role of MEIOSIN in other vertebrates, but the STRA8 gene has been investigated in fish (Dong *et al.* 2013, Pasquier *et al.* 2016, Skaftnesmo *et al.* 2021). The *stra8* gene does not appear to be required for meiosis in Atlantic salmon (Skaftnesmo *et al.* 2021), and the *stra8* gene itself is not present in some of the fish species such as medaka (Pasquier *et al.* 2016). Amongst vertebrates, the role that STRA8 plays with MEIOSIN in meiotic initiation may have evolved in mammalian groups, but the extent to which STRA8 is functionally conserved in mammalian groups remains unclear. Therefore, it is important to examine mammals phylogenetically distant from mice, such as marsupials and monotremes, for the emergence of the STRA8-MEIOSIN interrelationship and their conserved role in the onset of meiotic prophase I in mammals.

In a marsupial, the tammar wallaby, *Macropus eugenii*, germline development, including the timing of meiotic entry in both sexes, has been extensively examined (Alcorn & Robinson 1983, Williamson *et al.* 1990, Ullmann *et al.* 1997, Hickford *et al.* 2011, 2017, Ishihara *et al.* 2021). In males, there is high expression of *STRA8* mRNA in adult testes (Hickford *et al.* 2017) when male germ cells capable of initiating meiosis are present (Williamson *et al.* 1990). In contrast to males, in females, *STRA8* upregulation begins around day 30 postpartum (pp) and there is high mRNA *STRA8* expression in around day 50 pp female ovaries in which approximately 50% of germ cells are capable of initiation of meiotic prophase I (Alcorn & Robinson 1983, Hickford *et al.* 2017). *STRA8* mRNA expression, therefore, coincides with the time when germ cells initiate meiotic prophase in both sexes. However, marsupial STRA8 protein localisation is unknown, monotreme *STRA8* mRNA expression has not been examined, and little is known about the STRA8 co-factor MEIOSIN in any mammal other than the mouse.

In this study, we investigated whether the epigenetic regulation of STRA8 and MEIOSIN is correlated with the timing of their RNA expression and the timing of the initiation of meiotic prophase I in all three mammalian groups. We examined the expression of both STRA8 and MEIOSIN in a marsupial, the tammar wallaby, two monotremes, the platypus (*Ornithorhynchus anatinus*) and the short-beaked echidna (*Tachyglossus aculeatus*) to determine whether they are present at the time of initiation of meiotic prophase I, similar to the mouse. To examine whether their expression is coordinated and regulated by the same sex-specific chromatin remodelling, we profiled changes in chromatin accessibility of both *Stra8* and *Meiosin* promoter regions in mouse fetal germ cells. Furthermore, we asked whether the bivalent chromatin status defined by H3K27me3 and H3K4me3 at the *Stra8* and *Meiosin* promoters in mice is a common feature in therian germ cells by comparing published chromatin-immunoprecipitation sequencing (ChIP-seq) data sets from the mouse and that of a widely divergent marsupial, the South American grey short-tailed opossum (*Monodelphis domestica*). Finally, using female tammar pouch young, we cultured ovaries with an inhibitor of H3K27me3 demethylation to evaluate the effect of H3K27me3 maintenance on *STRA8* and *MEIOSIN* expression before and after the onset of meiotic arrest. Here, we report that H3K27me3-associated chromatin remodelling at the promoter of *STRA8,* but not of *MEIOSIN*, is a conserved mechanism that allows *STRA8* expression in pre-meiotic germ cells of therian mammals.

Materials and methods

Animals and tissue collection

Tammar wallabies of Kangaroo Island origin were from our breeding colony at the University of Melbourne. Post-natal wallabies were aged and killed humanely as previously described (Poole *et al.* 1991, Renfree 2002, Hickford *et al.* 2011). All tammar experiments were approved by the University of Melbourne Animal Experimental Ethics Committees and followed the Australian National Health and Medical Research Council (2013) guidelines (National Health and Medical Research Council (Australia) 2013). Gonads were dissected immediately after death and one gonad was

snap-frozen in liquid nitrogen for expression analysis and the other was cryo-fixed with OCT compound. Platypus testis was collected from wild-caught animals under permits from NSW Parks and Wildlife and ethically approved by the University of Melbourne Animal Ethics committees. Due to the strict ethical regulation and the difficulty to obtain wildlife permits, we could not collect any new samples of male or female platypus. Echidna gonads were collected opportunistically from injured animals brought into the Currumbin Wildlife Hospital (SE Queensland) that required euthanasia for animal welfare reasons under permits from the Queensland Department of Environment and Science. All other tissues were immediately snap-frozen in liquid nitrogen.

Unlike mice, tammar germ cells continue to proliferate after birth, reaching a peak at about day 50 pp. The onset of meiotic prophase I in female germ cells from day 25 and leptotene and zygotene stages appear by day 30 (Alcorn & Robinson 1983). In males, spermatogenesis does not begin until 19 months of age and is complete by 25 months (Williamson *et al.* 1990).

RNA extraction and cDNA synthesis

Snap-frozen tissues were used for RNA extraction using the GenElute Mammalian total RNA Miniprep Kit (Sigma-Aldrich) following the manufacturer's instructions. To remove residual genomic DNA, the extracted RNA was treated with the DNA-free DNase treatment and removal kit (Thermo Fisher Scientific). Samples were used as a template for cDNA synthesis using SuperScript IV First strand Synthesis System (Invitrogen).

Identification and expression analysis MEIOSIN in the tammar wallaby

A BLAST search of the tammar genome database (Wallabase; https://wallabase.science.unimelb.edu.au) was performed using the mouse MEIOSIN protein sequence to identify a putative *MEIOSIN* gene. Tammar *MEIOSIN* specific primers were made based on the putative sequence using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Supplementary Table 1, see section on supplementary materials given at the end of this article). PCR reactions were performed using GoTaq Green master mix (Promega Corporation). After confirmation of tammar *MEIOSIN* by sequencing, tissue-specific expression analysis of tammar *MEIOSIN* was performed by PCR using brain, heart, lung, liver, kidney, adult ovary and adult testis with *MEIOSIN* primers described in Supplementary Table 1.

Identification and expression analysis of MEIOSIN and STRA8 in monotremes

BLAST searches of the new platypus genome (mOrnAna1.pri.v4 and echidna genome (mTacAcu1.pri) (Zhou *et al.* 2021) were performed using the tammar MEIOSIN and STRA8 protein sequence to identify putative *MEIOSIN* and *STRA8* genes. As fresh platypus tissues were unavailable, published platypus RNA-seq data sets using several tissues were analysed to estimate tissue-specific expression patterns of monotreme *MEIOSIN*. Publicly available raw RNA-seq data sets (GEO accession: GSE97367) (Marin *et al.* 2017) 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. The trimmed reads were aligned to the platypus reference genome (mOrnAna1.p.v1) using HISAT2 with a parameter --dta (Kim *et al.* 2019). After alignment with HISAT2, mapped reads were used as inputs of Stringtie (Pertea *et al.* 2015) to calculate TPM (transcript per million) values of *MEIOSIN* gene. To estimate tissue-specific expression pattern of echidna *STRA8* and *MEIOSIN*, RT-PCR using several adult tissues, testis, ovary, liver, lung and kidney was performed with specific primers described in Supplementary Table 1.

5′ and 3′ rapid amplification of cDNA ends

To determine the full-length *MEIOSIN* gene in the tammar and platypus, RACE (rapid amplification of cDNA ends) experiments were performed with a SMARTer RACE 5′/3′ kit (Clontech). The first round RACE reaction was performed with adult testis cDNA using SeqAmp DNA Polymerase (Clontech) with gene-specific primers (Supplementary Table 1). The nested RACE was performed by GoTaq DNA polymerase (Promega), and the RACE products were cloned using pGEM-T Easy Vector (Promega) and Stellar competent cells (Clontech). Plasmids were extracted using Wizard Plus SV Minipreps DNA Purification System (Promega) and sequenced.

Reverse-transcription quantitative PCR

Reverse-transcription quantitative PCR (RT-qPCR) primers (Supplementary Table 1) were designed from the known tammar *MEIOSIN* and amplified a 117 base pair product. T-qPCR was performed on a Quantstudio 5 (Thermo Fisher Scientific) on gonadal cDNA of both sexes from day 10 pp to adult and various adult tissues using the SYBR Green PCR kit (Qiagen). TATA-box binding protein (TBP) and hydroxymethylbilane synthase (HMBS) were selected as the housekeeping genes. Expression levels of the tammar *MEIOSIN* were normalised to the geometric mean of the expression levels of housekeeping genes. Differences in the gene expression levels between stages were analysed by Tukey–Kramer's multiple comparison test in R.

Immunofluorescence

The following antibodies were optimised on tammar gonads: rat anti-mouse Stra8 antibody (Ishiguro *et al.* 2020), rat anti-mouse MEIOSIN antibody (Ishiguro *et al.* 2020) and guinea pig anti-mouse Sycp3 (Ishiguro *et al.* 2020). Embedded frozen samples were serially sectioned at 8 µm. Sections were treated with 4% (w/v) paraformaldehyde (PFA) for 1 min to fix fresh tissues, washed by PBS and incubated in 0.1% (v/v) Triton X-20 in PBS for 5 min at room temperature to permeabilise the tissue. The sections were incubated for 1 h with 10% (w/v) goat serum for blocking and then incubated with primary antibody solution (1:500) at RT for 1 h. The sections were washed three times with PBS and incubated with fluorescent secondary

antibodies (1:500) for 1 h. Sections were washed three times with PBS again and incubated for 10 min with 4', 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). DAPI-treated sections were mounted with a fluorescence mounting solution. The control for all treatments was no primary antibody. These slides were treated in the same manner as described above except primary antibody treatment was omitted. Images were collected on a Nikon A1R Confocal Laser Microscope System (Nikon).

DNase-seq analysis

To characterise the chromatin status of the promoter of mouse *Stra8* and *Meiosin* before and after the onset of meiosis in female germ cells, DNase-seq data sets derived from fetal germ cells in both sexes were analysed. Published raw ChIP-seq data (GEO accession: GSE109770) (Li *et al.* 2018) was downloaded from NCBI SRA. Using an Oct4-EGFP transgene, primordial germ cells were isolated from embryonic day (E) 9.5, E10.5, E12.5, E13.5, E14.5 and E16.5 embryos by fluorescence-activated cell sorting (Li *et al.* 2018). Samples from E12.5 onwards were collected separately by sex (Li *et al.* 2018). Details of the experimental procedures can be found in the original paper (Li *et al.* 2018). All DNase-seq reads were trimmed using TrimGalore! (v0.6.5) with default settings to remove adaptor sequences, low-quality reads and less than 20 bp reads. The trimmed reads were aligned to the mouse reference genome (mm10) using Bowtie2 (v2.3.5.1) (Langmead & Salzberg 2012). The mapped reads were used for peak-calling using MACS2 (v2.1.3.3) (Zhang *et al.* 2008) with a q-value threshold of 0.01. Bedgraph coverage tracks were generated from the mapped reads using Deeptools2 (v3.3.1) (Ramírez *et al.* 2016).

The coverage was calculated as the number of reads per 10-bp bins across the genome and normalised using reads per kilobase per million mapped reads (RPKM). The bedgraph coverages tracks were visualised using Spark (v2.6.2) (Kurtenbach & Harbour 2019).

ChIP-seq analysis

To characterise the epigenetic status of the promoter of mouse *Stra8* before and after the onset of meiosis, ChIP-seq data sets derived from adult germline stem cells (AGSCs), spermatocytes and spermatids were analysed. Published raw ChIP-seq data (GEO accession: GSE49624 (mouse) (Hammoud *et al.* 2014)) was downloaded from NCBI SRA. Mouse AGSCs were isolated by magnetic cell sorting separator using anti-CD117 antibody (Hammoud *et al.* 2014). Spermatocytes and spermatids were collected from male C57Bl/6 mice by centrifugal elutriation (Hammoud *et al.* 2014). Details of the experimental procedure can be found in the original paper (Hammoud *et al.* 2014). As well as mouse ChIP-seq data sets, ChIP-seq data obtained from pachytene spermatocytes and round spermatids of the grey short-tailed opossum were analysed to estimate a conserved epigenetic status of the promoter of marsupial *STRA8* after the onset of meiosis. Published raw ChIP-seq data (GEO accession: GSE68507 (opossum) (Lesch *et al.* 2016)) were downloaded from NCBI SRA. Opossum pachytene spermatocytes and round spermatids were collected using a STAPUT gradient (Lesch *et al.* 2016). Details of the experimental procedure can be found in the original paper (Hammoud *et al.* 2014). All ChIP-seq reads were trimmed using TrimGalore! (v0.6.5) with default settings to remove adaptor sequences, low-quality reads and less than 20 bp reads. The trimmed reads were aligned to either the mouse reference genome (mm10) or the opossum genome (MonDom5) using Bowtie2 (v2.3.5.1) Low mapping quality reads (MAPQ < 30) including non-mapped reads were removed by Samtools (v1.9), and PCR duplicates were filtered by Picard tools MarkDuplicates (v2.25.0). After the filtration, the mapped reads were used for peak-calling using MACS2 (v2.1.3.3) with the parameters --broad --broad-cutoff 0.01 --nomodel by comparing with Input controls. Bedgraph coverage tracks were generated from the mapped reads using Deeptools2 (v3.3.1). The coverage was calculated as the number of reads per 10 bp bins across the genome and normalised using RPKM. The bedgraph coverage tracks were visualised using Spark (v2.6.2).

In vitro assay for H3K27me3 on STRA8 and MEIOSIN transcriptional levels in the presence of demethylation inhibitor

Collagen dental sponges (Dental Solutions Israel, Ashdod, Israel) were immersed in DMEM plus 10% (v/v) fetal bovine serum (Thermo Fisher Scientific) until sponges were saturated with the media. Ovaries from either day 23 pp or day 33 pp females were cultured on top of the sponge for 20 h at 37°C for tissue acclimatisation. Media was replaced with DMEM plus 10% (v/v) fetal bovine serum with the addition of either 0.25 μM dimethyl sulfoxide (DMSO) or 100 μM GSK-J1 (Jumonji H3K27 demethylase inhibitor, Sigma-Aldrich) in 0.25 μM DMSO and cultured for 40 h. The solutions were then replaced with fresh media supplemented with either DMSO or GSK-J1 in DMSO for an additional 40 h. After a total of 5 days (100 h) culturing, total RNA was extracted from the explants by GenElute Mammalian Total RNA Miniprep Kit following the manufacturer's instructions. To remove residual genomic DNA, the extracted RNA was treated with DNA-free DNase treatment and removal kit, and 150 ng of the sample RNAs was used as a template for cDNA synthesis using the SuperScript IV First-Strand Synthesis System. RT-qPCR was performed on a Quantstudio 5 using the SYBR Green PCR kit. *HMBS* was selected as the housekeeping gene. Expression levels of the tammar *STRA8* and *MEIOSIN* were normalised to the expression levels of the housekeeping gene. Differences in the gene expression levels between control and GSK-J1 treatment were analysed by paired *t*-test after confirming the normality of data by the Shapiro–Wilk test in R.

Results

Putative meiosis initiators are highly expressed in the adult testis of the tammar and both monotremes

BLAST searches of the tammar genome using the mouse *Meiosin* sequence identified a 1665 bp sequence of the putative tammar orthologue of *MEIOSIN*. To identify

monotreme *MEIOSIN* and *STRA8*, BLAST searches of the echidna genome (mTacAcu1.pri) and the new platypus genome (mOrnAna1.pri.v4) (Zhou *et al.* 2021) were also performed using the putative tammar MEIOSIN and STRA8 sequences. As a result, one candidate each for *MEIOSIN* and *STRA8* in echidnas, two candidates for *MEIOSIN* and one candidate for *STRA8* were identified in platypus. Annotations of the neighbouring genes of the putative *MEIOSIN* orthologue in the tammar and echidna genomes showed synteny to corresponding genes in mouse genome (Fig. 1A), suggesting that the *MEIOSIN* gene is conserved between the tammar and the echidna genomes. While mouse MEIOSIN and echidna putative MEIOSIN contain two major functional domains (a basic helix-loop-helix (bHLH) domain and a high mobility group (HMG) box (Supplementary Fig. 1)), the putative tammar MEIOSIN and platypus MEIOSIN candidates had only the HMG box. To confirm the presence of a bHLH domain in the tammar MEIOSIN and the platypus MEIOSIN, 5′ and 3′ rapid amplification of cDNA ends (RACE) reactions were performed. Sequencing of the 5′ and 3′ RACE products confirmed the full length of the tammar *MEIOSIN* transcript and the platypus *MEIOSIN* gene (Fig. 1B and C). The tammar *MEIOSIN* gene and the platypus *MEIOSIN* gene encode 639 amino acids and 495 amino acids, respectively. The putative proteins contain the two functional domains, bHLH and HMG box (Fig. 1B, C and Supplementary Fig. 1). The putative STRA8 proteins contain conserved bHLH domain in mammals (Supplementary Fig. 2). Consistent with its potential role as a meiosis initiation factor, RT-PCR analysis demonstrated that the tammar *MEIOSIN* transcript was abundant in adult testis as was tammar *STRA8* (Fig. 1D). In the adult ovary, tammar *STRA8* transcript was not found, while *MEIOSIN* transcript was barely detectable. (Fig. 1D). MEIOSIN and STRA8 were undetectable in the other adult tissues examined in this study (Fig. 1D). To estimate the tissue-specific expression of monotreme *MEIOSIN* and *STRA8*, RT-PCR experiments using several adult echidna tissues and analysis of published platypus transcriptome data sets were performed. Echidna *STRA8* and *MEIOSIN* were abundant in adult testes (Fig. 1E). In the adult echidna ovary, *STRA8* transcript was not found, while *MEIOSIN* transcript was barely detectable. (Fig. 1E). *MEIOSIN* and *STRA8* were undetectable in the other adult echidna tissues examined in this study (Fig. 1E). The platypus *MEIOSIN* and *STRA8* mRNA were also highly expressed in adult testes but not in the other adult tissues examined (Fig. 1F).

Changes in tammar MEIOSIN transcript levels during gametogenesis coincide with the onset of meiotic prophase I in both sexes

Male and female wallabies differ in the timing of entry of their germ cells into meiotic prophase I (Ullmann *et al.* 1997, Ishihara *et al.* 2021). Since tammar *STRA8* gene expression pattern is correlated with the timing of the initiation of meiotic prophase I in both sexes (Hickford *et al.* 2017), its potential partner MEIOSIN may have a similar expression pattern during gametogenesis. The correlation of *MEIOSIN* transcription levels with germ cell development in both sexes was investigated to examine its possible function in marsupial meiosis. In males, a statistically significant upregulation of *MEIOSIN* transcriptional level was only observed in adult testes in which germ cells have the potential to initiate meiotic prophase I ($P < 0.05$, Tukey–Kramer's multiple comparison test) (Fig. 2A). In females, *MEIOSIN* transcript was abundant in day 60 pp ovaries when approximately 50% of germ cells have entered meiotic arrest (Alcorn & Robinson 1983, Ullmann *et al.* 1997) (Fig. 2B).

STRA8 and MEIOSIN proteins are localised in pre-leptotene germ cells in the tammar adult testes and pouch young ovaries

To characterise where tammar STRA8 and MEIOSIN proteins were expressed in male germ cells at the onset of meiosis, immunofluorescence of adult testes was performed using synaptonemal complex 3 (SYCP3), a marker of the meiotic chromosome axis. In C57BL/6 mice, MEIOSIN and STRA8 proteins appear in two phases of male and female germ cells; the first 'early period' of preleptotene when Sycp3 staining is weakly diffuse and the second 'middle period' of preleptotene when there are patchy aggregates without forming the meiotic chromosome axis (Gaysinskaya & Bortvin 2015, Ishiguro *et al.* 2020). Therefore, we considered meiotic initiation as including these two phases. In adult testes, STRA8 was localised in both cytoplasm and nucleus of preleptotene spermatocytes that had a speckled pattern of SYCP3 staining along the periphery of seminiferous tubules as in mice (Anderson *et al.* 2008, Tedesco *et al.* 2009, Gaysinskaya & Bortvin 2015, Kojima *et al.* 2019, Ishiguro *et al.* 2020) In contrast, STRA8 was not observed in spermatocytes in the progress of meiotic prophase that showed thread-like SYCP3 staining (Fig. 3A). Similarly, MEIOSIN was also localised in preleptotene spermatocytes (Fig. 3B) but not in pachytene spermatocytes which had a 'dense-plate' of SYCP3 staining, a marsupial-specific structure observed during X–Y pairing (Page 2003, Page *et al.* 2006).

To characterise where tammar STRA8 and MEIOSIN were localised in female germ cells, the localisation of tammar STRA8 and MEIOSIN in female germ cells was examined in day 57 pp ovaries. STRA8 and MEIOSIN were detected only in preleptotene germ cells which had a slightly speckled pattern of SYCP3 staining (Fig. 4A and B). In contrast, both STRA8 and MEIOSIN were not observed in germ cells in the progress of the meiotic prophase that had the thread-like SYCP3 staining.

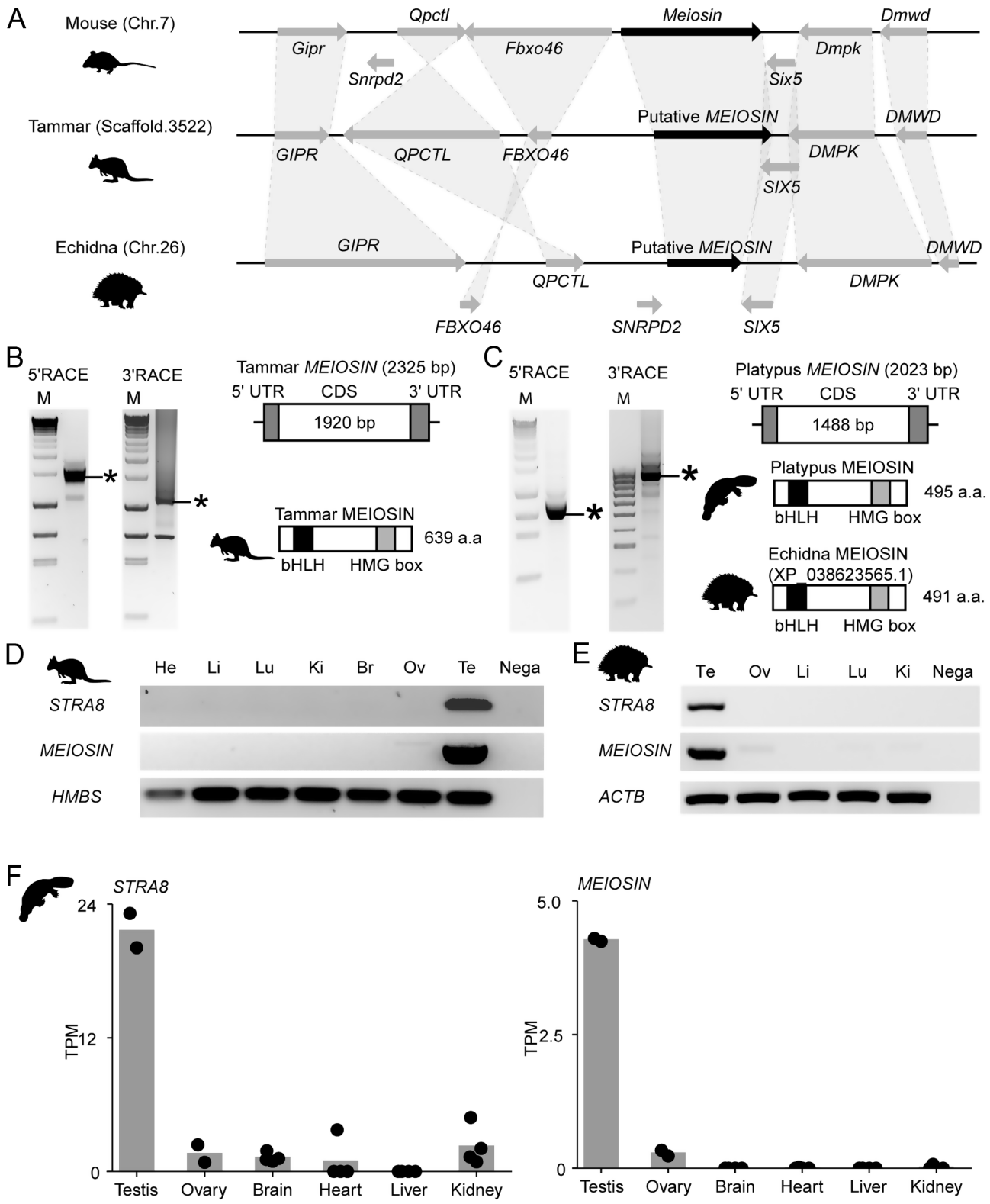

Figure 1 Identification and tissue-specific expression of the tammar and monotreme *MEIOSIN* and *STRA8* orthologue. (A) Conserved synteny of the mammalian *MEIOSIN* gene locus. The arrows show the orientation from 5′ to 3′. (B) Identification of full-length tammar MEIOSIN transcript by 5′ and 3′ RACE. Asterisks show RACE products containing partial *MEIOSIN* sequence. M, molecular-weight size marker. The identified full-length tammar *MEIOSIN* transcript and the encoded protein are represented by boxes. Two functional domains, basic helix-loop-helix (bHLH) and high mobility group (HMG), are represented by black and grey coloured boxes, respectively. (C) Identification of full-length platypus

Figure 1 Continued.

MEIOSIN transcript by 5′ and 3′ RACE. Asterisks show RACE products containing partial *MEIOSIN* sequence. M, molecular-weight size marker. The identified full-length platypus *MEIOSIN* transcript and the encoded protein are represented by boxes. The echidna MEIOSIN protein is also represented by boxes. Two functional domains, basic helix-loop-helix (bHLH) and high mobility group (HMG), are represented by black and grey coloured boxes, respectively. (D) The tissue-specific expression of the tammar *STRA8* and *MEIOSIN* transcripts was examined by RT-PCR using adult tissues. *Hydroxymethylbilane synthase* (*HMBS*) was selected as a reference gene. Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Nega, negative control (water); Ov, ovary; Te, testis. (E) The tissue-specific expression of the echidna *STRA8* and *MEIOSIN* transcripts was estimated by RT-PCR using several adult tissues. *Beta-actin* (*ACTB*) was selected as a reference gene. (F) Transcriptome analysis of platypus *STRA8* and *MEIOSIN*. Mean values of TPM (transcript per million) are represented by a bar graph and values of each biological replicate are represented by a dot-plot.

Sex-specific chromatin remodelling occurs at the Stra8 but not Meiosin promoter in mouse female germ cells

Since STRA8 and MEIOSIN were both present in therian preleptotene cells, it is possible that the sex-specific *MEIOSIN* mRNA expression which is coordinated with that of *STRA8* is controlled by the same epigenetic regulation as seen in mouse *Stra8* (Yokobayashi *et al.* 2013), but this remains to be confirmed. DNaseI hypersensitive sites (DHSs), which reflect chromatin accessibility, were examined in mouse fetal germ cells from published data (Li *et al.* 2018) to obtain insights into similarities and differences in sex-specific chromatin remodelling between *STRA8* and *MEIOSIN* during germline sexual differentiation. From embryonic day 9.5 (E9.5) to E10.5 when there is no *Stra8* expression (Fig. 5A), there were no significant DHSs at the promoter of *Stra8* (Fig. 5B). However, in females, significant DHS sites appeared at E12.5 and remained until E13.5 when many germ cells express *Stra8* (Fig. 5A and B). After E14.5, coinciding with the reduction of the *Stra8* transcript levels (Ishiguro *et al.* 2020, Niu & Spradling 2020), the significant peak disappeared from the promoter of *Stra8* (Fig. 5A and B). Although the loss of promoter DNA methylation is observed at E13.5 in both sexes (Seisenberger *et al.* 2012), the changes in chromatin accessibility at the promoter of *Stra8* were only observed in females (Fig. 5B). This change in chromatin status at the *Stra8* promoter coincided with the known changes in H3K27me3 status that occurs only in females (Yokobayashi *et al.* 2013). In contrast to *Stra8*, the *Meiosin* promoter had a statistically significant DHS, showing that the chromatin remains open ($q < 0.01$), regardless of the sexes (Fig. 5A and B).

STRA8 but not MEIOSIN promoter retains the bivalent chromatin in therian male germ cells

To confirm similarities and differences in the chromatin status of mouse *Stra8* and *Meiosin* before and after the onset of meiosis in males, ChIP-seq data sets (Hammoud *et al.* 2014) derived from differentiating Kit$^+$ AGSCs, spermatocytes and spermatids were analysed. Kit$^+$ AGSC (pre-meiotic germ cells) lacked the suppressive modification, H3K27me3, at the promoters of both *Stra8* and *Meiosin,* while the active modification, H3K4me3, was present at the promoters analysed by MACS2 ($q < 0.01$), coinciding with the active transcriptional status before the onset of meiosis (Fig. 6A). In both meiotic cells (spermatocytes) and post-meiotic cells (round spermatids), the promoter of *Stra8* gained statistically significant ($q < 0.01$, *MACS2*) H3K27me3 marks so that the promoter became bivalent (Fig. 6A) reflecting the inactive/poised chromatin status. In contrast to *Stra8*, neither spermatocytes nor spermatids had statistically significant H3K27me3 at the promoter of *Meiosin* (Fig. 6A).

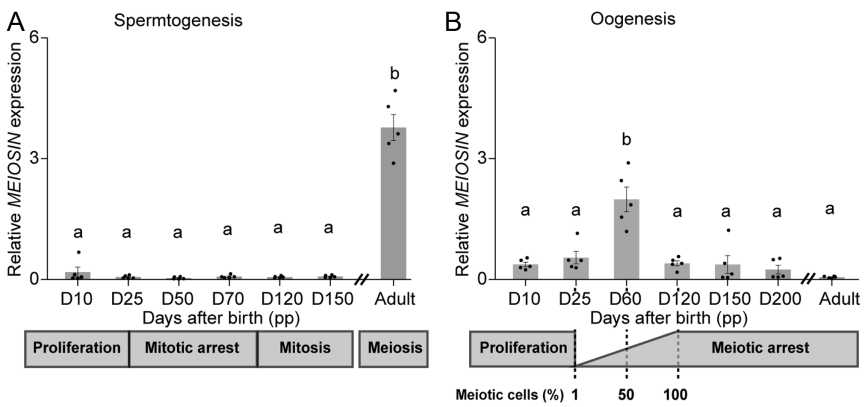

Figure 2 Expression of *MEIOSIN* during tammar gametogenesis. (A) *MEIOSIN* expression during tammar spermatogenesis. (B) *MEIOSIN* expression during tammar oogenesis. Relative gene expression against two housekeeping genes, *HMBS* and *TBP*, was shown as a bar graph. The error bar represents the standard error of the mean (S.E.M.). Each dot points represent expression levels of MEIOSIN in each sample at each developmental stage. Bars labelled with the same letters represent values that do not differ significantly by Tukey–Kramer's multiple comparison test at $P < 0.05$. The corresponding developmental stages of male and female tammar germ cells based on previous publications (Alcorn & Robinson 1983, Ullmann *et al.* 1997, Ishihara *et al.* 2021) are shown at the bottom.

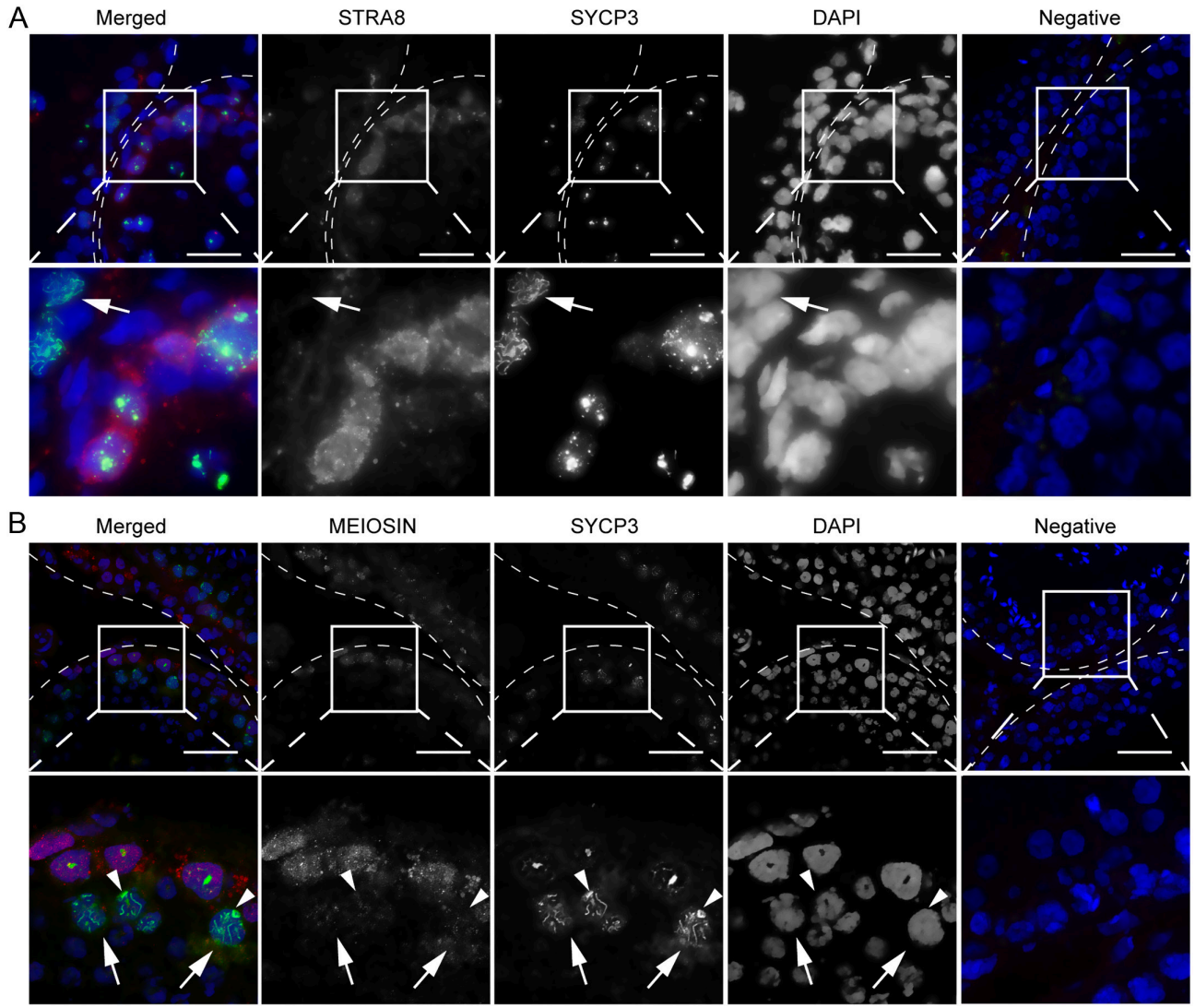

Figure 3 Protein localisation of tammar STRA8 and MEIOSIN in the adult testis. (A) STRA8 localisation in the adult tammar testis. The white dashed lines represent seminiferous tubules. Red, green and blue represent STRA8, SYCP3 and DAPI, respectively. (B) MEIOSIN localisation in the adult testis. The white dashed lines represent seminiferous tubules. Red, green and blue fluorescence represent MEIOSIN, SYCP3 and DAPI, respectively. The white arrows represent meiotic cells which had meiotic chromosomes visualised by SYCP3 staining. The marsupial-specific 'dense plate' structure is shown by white arrowhead. Negative controls represent the testis section incubated with secondary antibodies only (no primary antibodies). Scale bars: 50 μm.

To confirm whether the bivalent chromatin status at the *STRA8* but not *MEIOSIN* promoter after the onset of meiosis is conserved between marsupial and eutherian mammals, published ChIP-seq data sets (Lesch *et al.* 2016) derived from the grey short-tailed opossum were analysed. Opossum meiotic cells (pachytene spermatocytes) and post-meiotic cells (round spermatids) also had bivalent chromatin at the *STRA8* promoter (Fig. 6B) reflecting the inactivated/poised status of the *STRA8* promoter after the onset of meiosis. In contrast to *STRA8*, neither spermatocytes nor spermatids had statistically significant H3K27me3 at the promoter of *MEIOSIN* (Fig. 6B).

Maintenance of H3K27me3 was sufficient to prevent an increase in STRA8 transcriptional levels before the onset of meiotic arrest in the female tammar

Although H3K27me3-associated chromatin remodelling was present at the *STRA8* but not *MEIOSIN* promoter in mice and male opossum, it is still not clear whether this remodelling affects transcription levels before the onset of meiotic prophase I in marsupials. To test the effect of removal of H3K27me3 on the levels of marsupial *STRA8* and *MEIOSIN* transcripts during the onset of meiotic prophase I, *in vitro* culture experiments with GSK-J1,

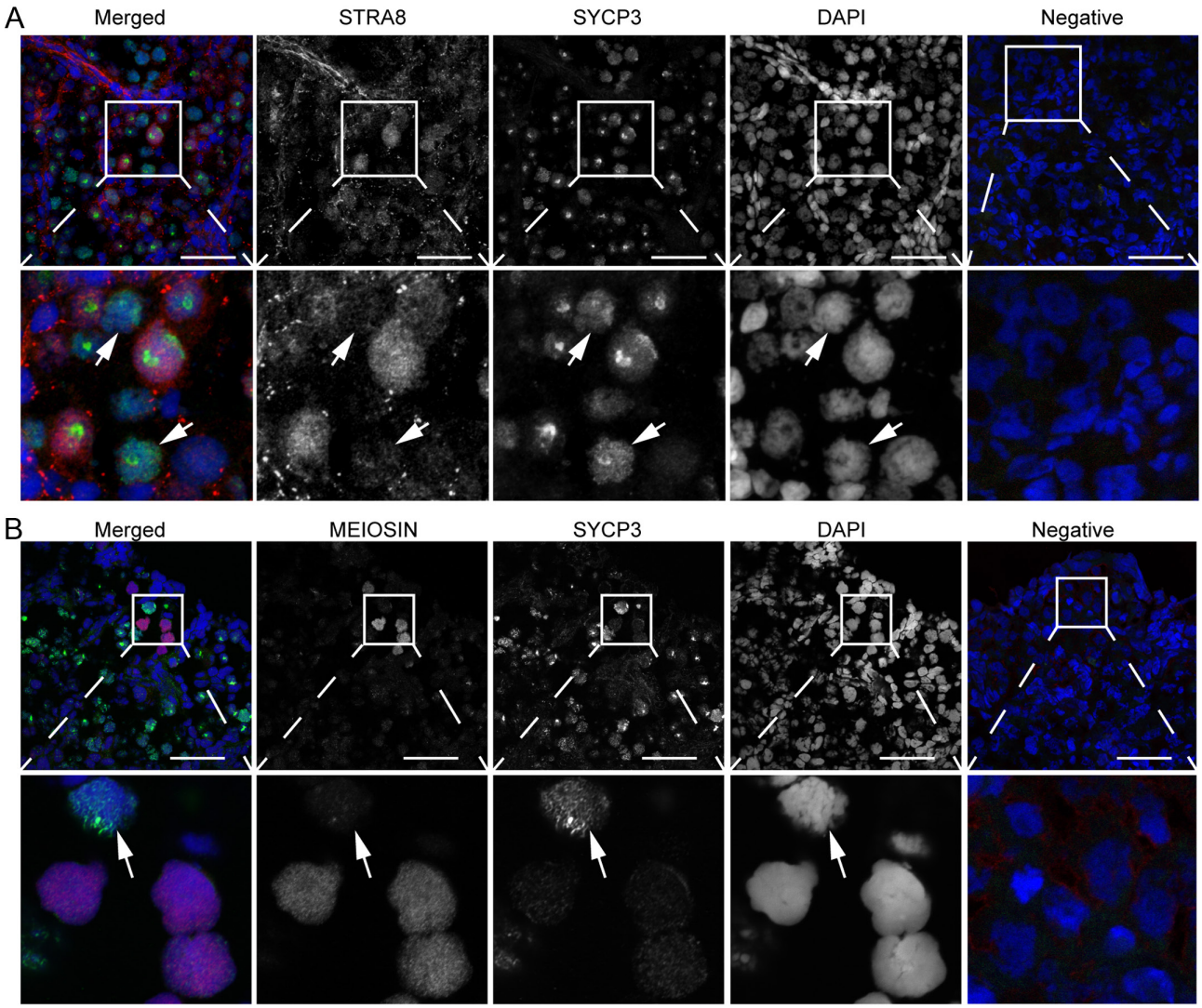

Figure 4 Protein localisation of the tammar STRA8 and MEIOSIN in the day 57 pp tammar ovary. (A) STRA8 localisation in the day 57 pp tammar ovary. Red, green and blue represent STRA8, SYCP3 and DAPI, respectively. (B) MEIOSIN localisation in the day 57 pp tammar ovary. Red, green and blue represent MEIOSIN, SYCP3 and DAPI, respectively. White arrows represent meiotic cells which had meiotic chromosomes visualised by SYCP3 staining. Negative controls represent the ovary section incubated with secondary antibodies only (no primary antibodies). Scale bars: 50 μm.

an inhibitor of JMJD3s (an H3K27me3 demethylator), were performed using female tammar pouch young ovaries (Fig. 7A). In females, *STRA8* expression is known to reach a plateau from day 30 pp while some germ cells begin meiotic prophase I (Hickford *et al.* 2017). To test the effect of the chemical on Stra8 and Meiosin expression, ovaries from day 23 pp (before the entry into meiotic prophase 1) and ovaries from day 33 pp after STRA8 expression reached a plateau were used. After 5 days of treatment with 100 mM GSK-J1, there was a statistically significant ($P < 0.05$, paired *t*-test) decrease of *STRA8* transcript levels in ovaries from pouch young (PY) aged day 23 pp (before the entry into meiotic prophase 1). There was no significant difference in *MEIOSIN* transcript levels between GSK-J1 treatment and control (Fig. 7B). However, there was no significant effect of the inhibitor on either *STRA8* or *MEIOSIN* transcript levels in ovaries from PYs aged day 33 pp (i.e. after the onset of meiotic arrest in a proportion of germ cells that occurs progressively between day 25 and 70 pp (Alcorn & Robinson 1983) (Fig. 7C).

Discussion

MEIOSIN and STRA8 are conserved meiosis initiation factors in mammals

Changes in tammar *STRA8* transcriptional levels coincide with the onset of meiosis in both sexes (Hickford *et al.* 2017). Examination of *MEIOSIN* in the tammar and the

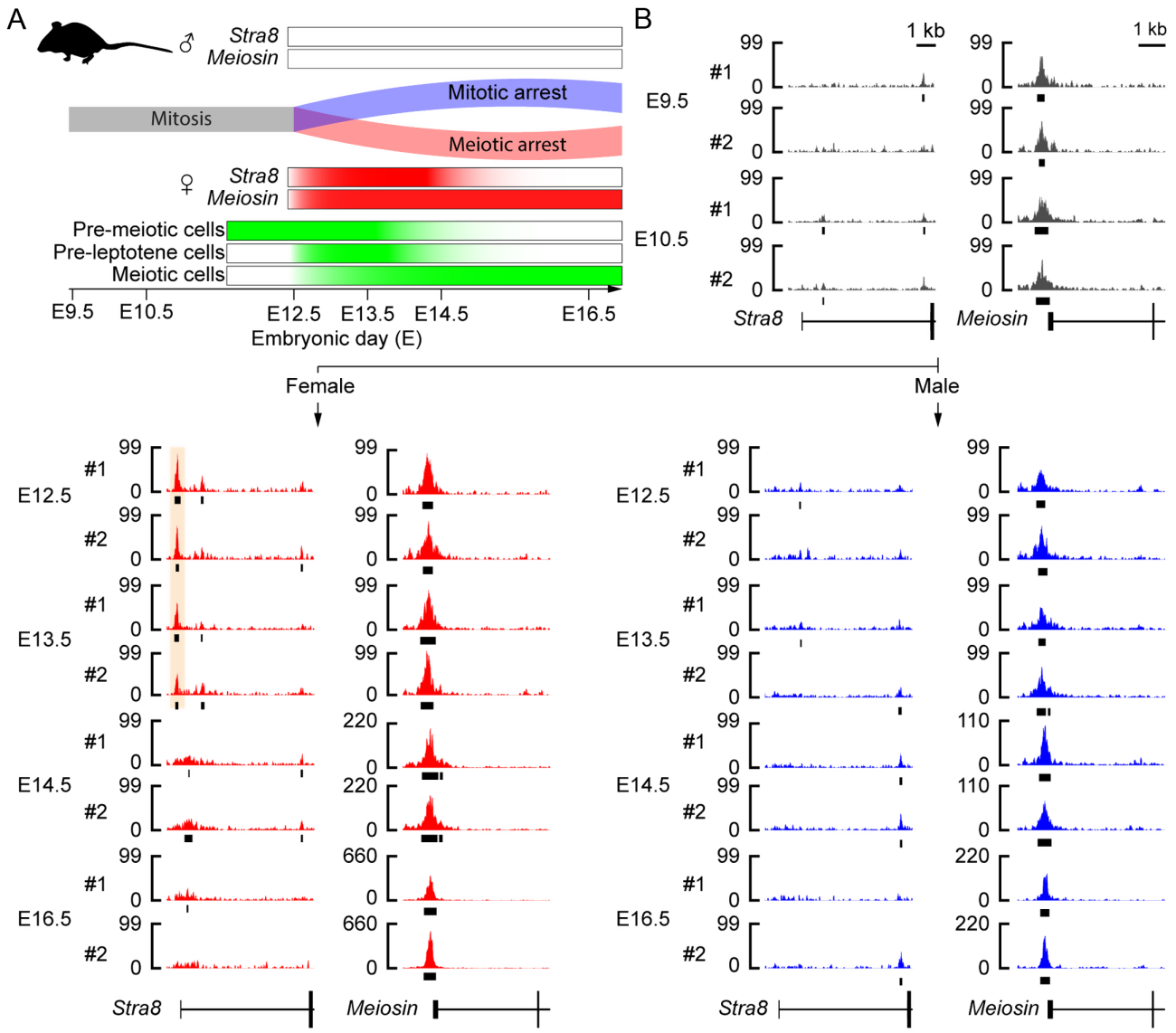

Figure 5 Chromatin remodelling occurs at the promoter of *Stra8* but not *Meiosin* in developing female germ cells in the mouse. (A) Schematic diagram of mouse fetal gametogenesis. Information about *Stra8* and *Meiosin* expression in both sexes is based on Ishiguro *et al.* (2020). Cell type information is based on Niu and Spradling (2020). (B) DNase-seq analysis of mouse fetal germ cells in both sexes. As the original study did not separate samples at E9.5 and 10.5 based on sexes, the coverage tracks of DNase-seq before germline sexual differentiation are represented by the grey-coloured tracks. After germline sexual differentiation, the coverage tracks of DNase-seq of each sex are represented by the red-coloured tracks (females) and the blue-coloured tracks (males), respectively. The Y-axis of the coverage tracks represents RPKM values. Black boxes under the coverage tracks represent statistically significant DNase hypersensitive sites based on MACS2 ($q < 0.01$). Orange-coloured box represents the region that is remodelled before and after the onset of meiosis. Two biological replicates are shown as #1 and #2, respectively.

two monotremes confirmed that the gene contains the two conserved domains, bHLH domain and HMG-box, in all three mammalian groups. Since the two domains are known to regulate the DNA-binding activity of transcription factors (Štros *et al.* 2007, Murre 2019), it suggests that both marsupial and monotreme MEIOSIN can act as a transcription factor, as in the mouse (Ishiguro *et al.* 2020). In the tammar, *MEIOSIN* was highly expressed in adult testes at the time germ cells initiated meiosis. *MEIOSIN* and *STRA8* were also highly expressed in adult monotreme testes. Up-regulation of tammar *MEIOSIN* expression in females occurred at the time when meiotic prophase I was initiated. Although we could not confirm the protein localisation of monotreme STRA8 and MEIOSIN due to the limited access to samples and lack of specific antibodies, we confirmed that both tammar STRA8 and MEIOSIN proteins were only present in preleptotene cells in both males and females. This strongly suggests that STRA8 and MEIOSIN meiosis initiation factors have been conserved for at least 180 million years of mammalian evolution (Luo *et al.* 2011, Cúneo *et al.* 2013).

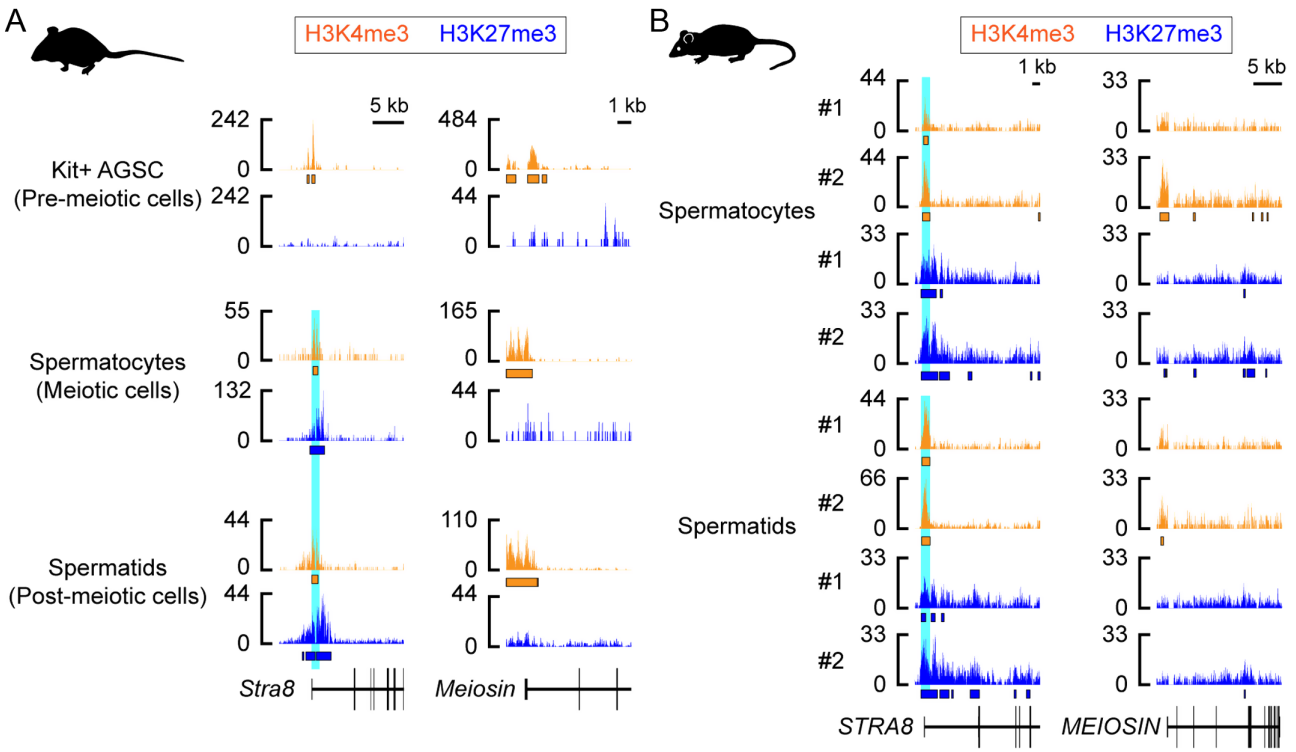

Figure 6 *STRA8* promoter has a conserved bivalent chromatin status in male germ cells after the onset of meiosis in therian mammals. (A) ChIP-seq analysis of male germ cells before and after the onset of meiosis in mouse. The coverage tracks of the active histone modification, H3K4me3, and the suppressive histone modification, H3K27me3, are represented by orange-coloured tracks and blue-coloured tracks, respectively. AGSCs, adult germline stem cells. (B) ChIP-seq analysis of male germ cells after the onset of meiosis in the grey short-tailed opossum. Two biological replicates are shown as #1 and #2, respectively. The Y-axis of the coverage tracks represents RPKM values. Boxes under the coverage tracks represent statistically significant peaks of each histone mark based on MACS2 ($q < 0.01$). Aqua-coloured boxes show bivalent chromatin regions where statistically significant H3K4me3 and H3K27me3 are present.

Conserved H3K27me3-associated chromatin remodelling and STRA8 gene expression

To initiate meiosis, *STRA8* and *MEIOSIN* need to be expressed in preleptotene cells. In mice, the removal of a suppressive histone mark, H3K27me3, occurs at the promoter of *Stra8* before the onset of meiosis in both sexes (Lesch *et al.* 2013, Yokobayashi *et al.* 2013, Hammoud *et al.* 2014). The data from the ChIP-seq analysis of mouse male germ cells before and after the onset of meiosis (Hammoud *et al.* 2014) showed that H3K27me3 re-establishment was accompanied by inactivation of the promoter of *Stra8* after the onset of meiosis in males. This is consistent with the loss of *Stra8* transcripts in male germ cells after the onset of meiosis (Hermann *et al.* 2018). As in mice, male germ cells in opossums had H3K27me3 at the promoter of *STRA8* after the onset of meiosis. This suggests that H3K27me3 is gained after entering meiotic prophase I and re-defines chromatin status as transcriptionally inactive 'bivalent' status to inactivate the *STRA8* transcription after the onset of meiotic prophase I in therian mammals. Mouse female germ cells also remodelled chromatin before and after the onset of meiosis. The pattern coincided with the loss of H3K27me3 and the temporal *Stra8* expression in germ cells. In the female tammar, preventing the removal of H3K27me3 with the demethylase inhibitor GSK-J1 before the onset of meiotic arrest significantly decreased *STRA8* expression. However, there was no effect of GSK-J1 on *STRA8* expression after the onset of meiotic arrest. Since *MEIOSIN* expression did not change, this reduction might not be due to the loss of germ cells. It is still possible that the maintenance of H3K27me3 in other genomic loci such as an unknown activator of *STRA8* affects its expression. However, after the onset of meiotic arrest after day 33 pp, there were no significant differences in *STRA8* and *MEIOSIN* expression between GSK-J1 treated samples and control samples. Therefore, the reduction of *STRA8* transcript levels with GSK-J1 treatment before meiotic arrest is more likely to be caused by the maintenance of H3K27me3 that occurs at the *STRA8* promoter in female tammar before the onset of meiosis. In male mice, *Stra8* transcripts are controlled by NANOS2 with CCR4-NOT deadenylase complex (Suzuki & Saga 2008, Suzuki *et al.* 2010, Saga 2022). Therefore, there is another layer of transcript regulation

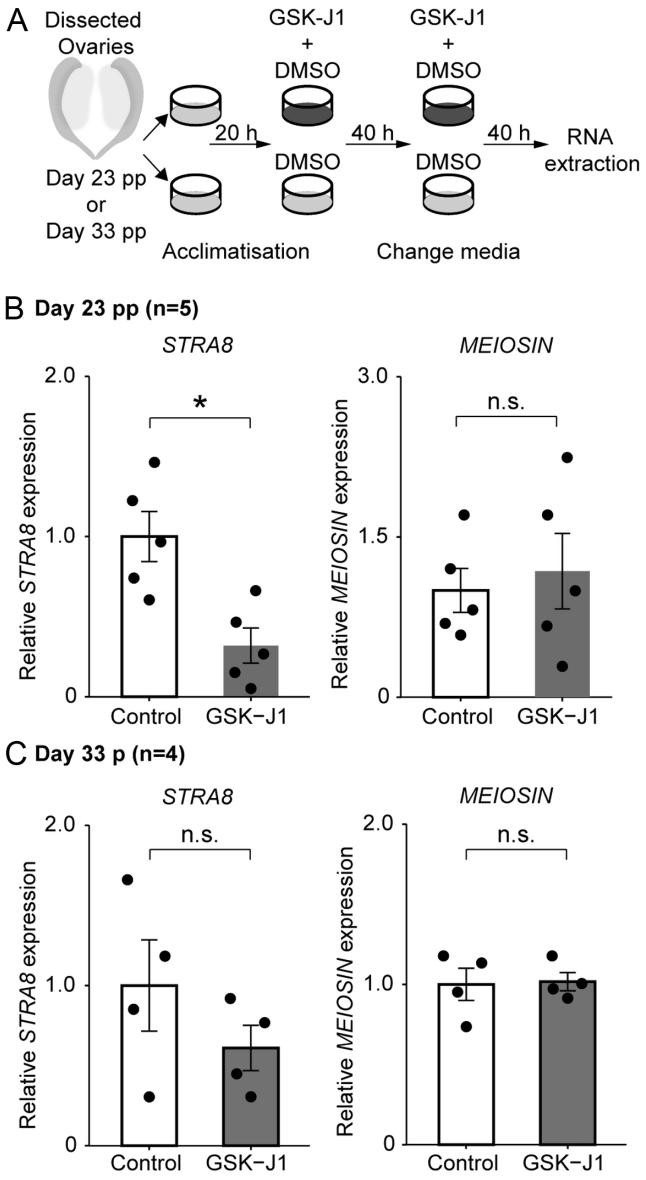

Figure 7 Removal of H3K27me3 before the onset of meiotic arrest is sufficient to regulate *STRA8* expression in cultured tammar ovaries. (A) Schematic diagram of the *in vitro* culture experiment. (B) Effects of 100 μM GSK-J1 on the expression of *STRA8* and *MEIOSIN* in day 23 postpartum (pp) ovaries ($n = 5$). The asterisk represents statistically significant difference based on paired *t*-test at $P < 0.05$. (C) Effects of 100 μM GSK-J1 on the expression of *STRA8* and *MEIOSIN* in day 33 pp ovaries ($n = 4$). All samples were normalised by a house keeping gene, *HMBS*. Relative expressions against control are shown as bar graphs. Error bars represent the standard error of the mean (S.E.M.).

for the *Stra8* gene in mice. Although almost nothing is known about NANOS2 in marsupials as yet, it is possible that NANOS2-associated mRNA degradation controls *STRA8* transcript amount in marsupials in concert with epigenetic changes in chromatin. The role of marsupial NANOS2 in *STRA8* and possibly *MEIOSIN* transcripts should be investigated in future studies.

MEIOSIN gene expression may be regulated by multilayered mechanisms

During mouse spermatogenesis, male germ cells respond to retinoic acid (RA) signalling twice; the first time in the transition from undifferentiated-to-differentiating spermatogonia and the second in the transition of differentiating spermatogonia to preleptotene cells (Oatley & Griswold 2020). However, only the second RA pulse induces meiosis in male mice by causing both MEIOSIN and STRA8 protein expression in preleptotene cells (Ishiguro *et al.* 2020, Oatley & Griswold 2020). Since the first RA pulse induces *Stra8* but not *Meiosin* transcripts, this suggests that *MEIOSIN* gene expression is regulated by other mechanisms. Although there is continuous *Meiosin* expression in fetal mouse ovaries, MEIOSIN protein is only present in pre-leptotene cells (Ishiguro *et al.* 2020). Similarly, there was very weak tammar *MEIOSIN* gene expression even in adult ovaries, but the protein was only present in preleptotene cells and there is no STRA8 expression, so temporal MEIOSIN protein expression may depend on post-transcriptional mechanisms rather than on pre-transcriptional regulation based on chromatin status. In contrast, even though there is continuously open chromatin at the promoter of *Meiosin* in fetal male germ cells, *Meiosin* expression is not observed in fetal testes (Ishiguro *et al.* 2020). The open chromatin may recruit yet unknown factors to regulate the sex-specific *Meiosin* expression. DMRT1 is a possible candidate because this protein is known to bind the *Meiosin* promoter in spermatogonia (Ishiguro *et al.* 2020). Since DMRT1 expression prevents premature meiotic entry in male germ cells (Matson *et al.* 2010), the open chromatin-based association with DMRT1 might be a possible pathway to prevent precocious *MEIOSIN* gene expression and to control the timing of the onset of meiotic prophase I in males. However, DMRT1 can also promote *Stra8* gene expression in the presence of RA in female germ cells (Krentz *et al.* 2011, Feng *et al.* 2021). The role of DMRT1 binding at the *MEIOSIN* promoter in the sex-specific *MEIOSIN* gene upregulation remains to be determined.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/REP-22-0286.

Declaration of interest

The authors declare no competing interest.

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Author contribution statement

TI, KI and MBR designed the research; TI performed experiments; TI, MBR, KI and JF analysed data; TI and MBR collected the marsupial tissue; MBR and JF collected the monotreme tissue; TI, JF, OWG, KI and MBR discussed the data. TI and MBR edited and all authors approved the paper

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