

Investigating the Organisation of the Platypus Sex Chromosome Chain During Meiotic Prophase I

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Discipline of Genetics

School of Molecular and Biomedical Science

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Declaration

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Aaron Casey

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Abbreviations

°C: Degrees Celsius

μL: Microlitre

BAC: Bacterial Artificial Chromosome

CC: Cohesin Complex

cDNA: Complementary DNA

DAPI: 4'-6-Diamidino-2-phenylindole

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic Acid

FISH: Fluorescence *in situ* Hybridisation

GFP: Green Fluorescence Protein

HRR: Homolog Recognition Region

NE: Nuclear Envelope

NOR: Nucleolar Organising Region

PAR: Pseudoautosomal Region

PBS: Phosphate Buffered Saline

PFA: Paraformaldehyde

PI: Protease Inhibitor

SC: Synaptonemal Complex

SCC: Sister Chromatid Cohesion

SLX: SYCP3-like, X-linked

SLX2: SYCP3-like, X-linked 2

SLY: SYCP3-like, Y-linked

SMC1 α : Structural Maintenance of Chromosomes Alpha

SMC1 β : Structural Maintenance of Chromosomes Beta

SSC: Standard Saline Citrate

SYCP1: Synaptonemal Complex Protein One (a.k.a SCP1)

SYCP2: Synaptonemal Complex Protein Two

SYCP3: Synaptonemal Complex Protein Three

SYCE1: Synaptonemal Complex Element One

SYCE2: Synaptonemal Complex Element Two

SYCE3: Synaptonemal Complex Element Three

TEX12: Testis Expressed Twelve

TRF1/2: Telomeric Binding Factor 1/2

Abstract

Meiosis is a specialised form of cell division that occurs specifically in the gonads of sexually reproducing species. It comprises a round of DNA replication followed by two successive rounds of cell division to produce haploid gametes. Each stage is divided into four substages of prophase, metaphase, anaphase and telophase. Prophase I is the longest and most complex stage of meiosis during which homologous chromosomes pair and recombine. The evolution of heteromorphic sex chromosomes has led to a number of changes in meiotic organisation. This includes the non-pairing of sex specific parts of the heteromorphic sex chromosomes and their inactivation in many species.

The platypus has a unique set of 10 sex chromosomes with homology to bird sex chromosomes that exist as a chain during meiotic metaphase I. Questions of mode and extent of pairing and the existence of meiotic silencing remained unknown but can inform our understanding of the evolution and mechanisms of meiotic prophase I. Work presented in this thesis provides novel insights into evolution and meiotic organisation of the monotreme sex chromosome complex.

The platypus sex chromosome chain forms during zygotene in stepwise manner, with remarkable consistency beginning at the Y5 end of the chain and ending with the X1 (Chapter 1). Synapsis generally relies on 3 main proteins; SYCP1, SYCP2 and SYCP3. Surprisingly platypuses express three different copies of *SYCP3* (including a multicopy version on a Y chromosome), genes that generally exist as single isoforms in most other species. Particularly given the SYCP3Y isoform is male specific, this raises the possibility that

SYCP3 paralogs may have evolved in relation to the sex chromosome chain during prophase I (Chapter 2).

During pachytene, the asynaptic regions of the sex chromosomes adopt a state of folding, similar to that of the avian Z and W chromosomes during synaptic adjustment, albeit without the formation of a central element. During this time the cohesin complex is heavily loaded onto the axial elements of the asynaptic regions of the X and Y regions of the chain. Furthermore at mid-pachytene the asynaptic regions of the chain are pulled to a giant nucleolus at which time the cohesin appears to spread onto the chromosome loops of the asynaptic regions of the chain that are also coincident with DNA condensation (Chapter 3).

During platypus pachytene there is global transcriptional downregulation. We observe no localised phosphorylation of the histone H2AX, a hallmark of MSCI but we do observe localised patterns of H2AFY, H3K27me3 and H3K9me3 at a paranucleolar location, however the H2AFY and H3K27me3 showed some colocalisation with sex chromosomes, there was not consistent pattern and H3K9me3 was always associated with a section of chromosome 6 (Chapter 4).

Together these results provide novel insights into the meiotic organisation of the monotreme sex chromosome complex and the evolution of MSCI in mammals.

Chapter 1: Introduction

1.0 Overview of Meiosis in the Platypus

In recent years, study of the platypus has gained significant momentum. As one of the only five extant species of prototheria, it resides in a unique position as an earlier descendent than any other extant mammalian species. Coupled with the fact it is the only prototherian species whose genome has been sequenced makes the platypus an exciting model for mammalian genome evolution (Warren et al., 2008).

The most remarkable aspect of monotreme genomes is an extraordinarily complex sex chromosome system that shares homology with bird sex chromosomes (Grutzner et al., 2004b; Rens et al., 2007; Veyrunes et al., 2008b). The platypus has 10 sex chromosomes (Murtagh, 1977), comprising 5X and 5Y chromosomes, that exist as an alternating chain at metaphase I in the form $X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5$ (Grutzner et al., 2004b) (Figure 1). Such a large sex chromosome chain raises questions regarding the mechanism of fundamental meiotic processes like, recombination, chromosome pairing, segregation and meiotic silencing.

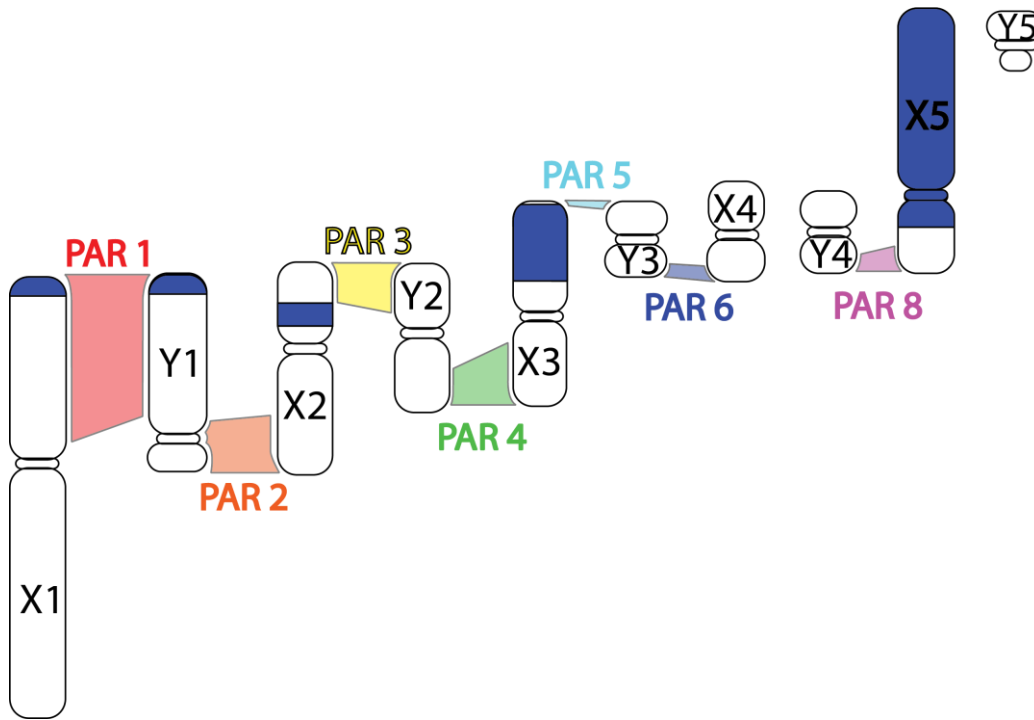


Figure 1. The platypus sex chromosome chain. Pseudoautosomal regions (PARs) are highlighted. Regions homologous to the chicken Z are highlighted in blue. Figure adapted from (Veyrunes et al., 2008b).

1.1 Introduction to Prophase I

Sexual reproduction is a highly successful form of reproduction that relies on meiosis, a specialised form of cell division, for its success. Prophase I is the longest and most complicated stage of meiosis, taking up to 90% of the time it takes for meiosis to complete (Alberts, 2002). It is initiated with the creation of double strand breaks across all of the chromosomes by SPO11 (Bergerat et al., 1997; Mahadevaiah et al., 2001), a protein that is highly conserved in eukaryotes. Repair occurs via numerous mechanisms, including homologous repair that results in exchange of sequence between homologues, ultimately leading to chromosomes with unique combinations of alleles (Bellani et al., 2005). For this repair to occur, homologous chromosomes must synapse, a process where the axial core of homologous chromosomes becomes physically joined by a proteinaceous complex called the synaptonemal complex. Based on the formation of the synaptonemal complex, prophase I

can be split into a number of substages, most notably leptotene, zygotene, pachytene and diplotene.

Heteromorphic sex chromosomes provide to be a unique case within the meiotic cell, particularly during prophase I and metaphase I where there exists checkpoints that, when triggered by asynapsed chromosomes, leads to apoptosis (Manterola et al., 2009; Reinholdt et al., 2009). Thus far two mechanisms to manage unpaired chromosomes have been discovered, the first is meiotic sex chromosome inactivation (MSCI), a process favoured in mammals (Reviewed by Hoyer-Fender, 2003b; and Turner, 2007) and the second is synaptic adjustment, a process observed in birds ZW chromosomes (Solari, 1977; Solari, 1992b; Schoenmakers et al., 2009b; Guioli et al., 2012b).

1.2 Chromosome Synapsis and the Consequences of Synaptic Failure

1.2.1 Chromosome Homology Search

Synapsis is preceded by a homology search, a process that relies heavily on telomeric function. At leptotene the telomeres bind to the nuclear envelope where they begin to move to a single pole. By zygotene, the telomeres are all located at a single pole (Scherthan et al., 1996), thus enabling a homology search by the chromosomes where they find their homologous partner, the DSBs then interact with their matching sequence on the homologous chromosome via interaxis bridges, bringing the homologues into close proximity (Reviewed by Page and Hawley, 2004). Over the duration of leptotene a proportion of the bridges mature into axial associations (Rockmill et al., 1995) that become initiation sites for SC formation. This gives the chromosomes a bouquet like appearance and is therefore referred to as the bouquet stage. At this time in many species, homologous

chromosomes begin to pair at many sites along the chromosome core (Weiner and Kleckner; Scherthan et al., 1994), while in others, pairing is initiated at a single site for each chromosome, known as the homolog recognition region (HRR) (Reviewed by Zetka and Rose, 1995). By pachytene, the homologous chromosomes have found their homologous partner, paired their entire length and the telomeres spread back out around the nuclear envelope, this time in pairs (Scherthan et al., 1996).

1.2.2 The Synaptonemal Complex

The synaptonemal complex is a tripartite structure that physically joins homologous chromosomes during prophase I. The synaptonemal complex appears as a three-dimensional, ladder like structure (Solari, 1970b; Holm and Rasmussen, 1977) (Figure 2). Running the length of the axial core of every chromosome is the axial element. The axial element comprises the two proteins; synaptonemal complex protein 2 (SYCP2) (Offenberg et al., 1991) and; synaptonemal complex protein 3 (SYCP3) (Heyting et al., 1988). The axial elements of homologous chromosomes are joined by transverse filaments each with a central element. The transverse filaments comprise a single protein: synaptonemal complex protein 1 (SYCP1) (Offenberg et al., 1991), while the central element has been shown to comprise many proteins: synaptonemal complex element 1 (SYCE1) (Costa et al., 2005), synaptonemal complex element 2 (SYCE2) (Costa et al., 2005), synaptonemal complex element 3 (SYCE3) (Schramm et al., 2011) and testis expressed 12 (Tex12) (Hamer et al., 2006). SYCP2 has been specifically shown to bind to SYCP1 providing a link between the axial elements and transverse filaments (Winkel et al., 2009).

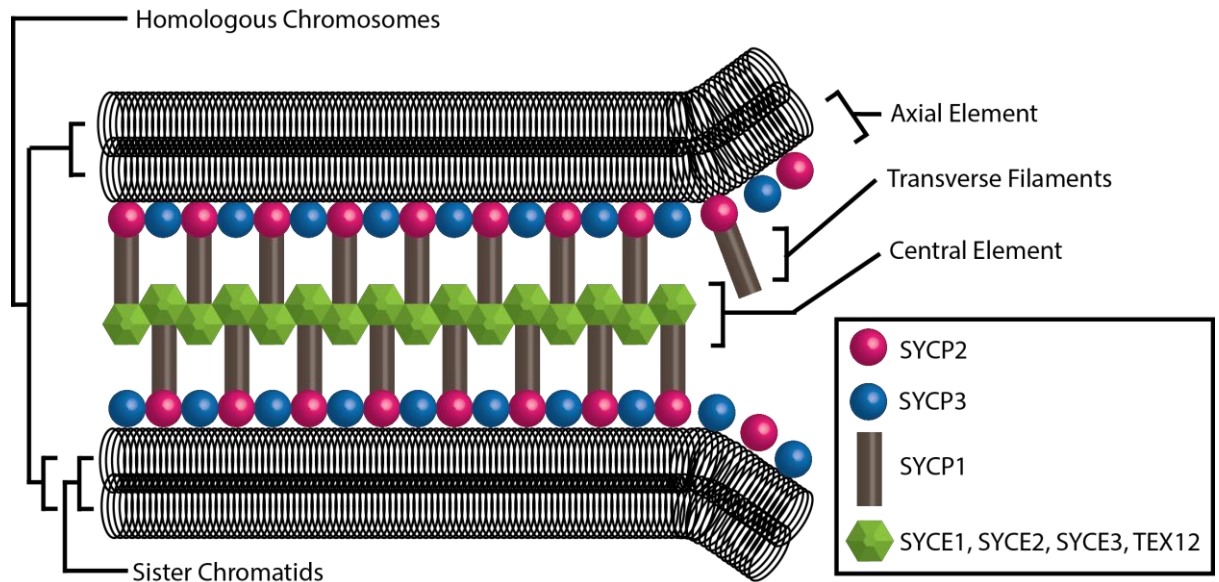


Figure 2. The synaptonemal complex. Sister chromatids are held together by the cohesin complex (not shown). Axial elements are comprised of SYCP2 and SYCP3. Transverse filaments are comprised of the sole protein SYCP1 and central elements are comprised SYCE1, SYCE2, SYCE3 and TEX 12.

The synaptonemal complex forms and dissolves during prophase I. At the beginning of leptotene, the proteins SYCP2 and SYCP3 begin to bind the chromosome axial core of every chromosome (Heyting et al., 1988; Offenberget al., 1998). As the cell enters zygotene, homologous chromosomes are pulled into close proximity, particularly at the telomeric ends which have all moved to a single pole (Scherthan et al., 1996). Thus begins the homology search, and when homologous chromosomes find each other, the transverse filaments and central element begin to form between them (Heyting et al., 1988; Offenberget al., 1991). By the beginning of pachytene the synaptonemal complex is fully formed between homologues. Meanwhile the sex chromosomes form a full synaptonemal complex only at their pseudoautosomal region (PAR), yet axial elements still form along the entire chromosomal core length (Solari, 1970b). As the cell moves into diplotene, the central element and transverse filaments dissolve and the homologues remain attached at chiasmata formed by homologous repair of DNA DSBs (Solari, 1970a). At late diplotene, the axial elements also dissolve, completing the role of the synaptonemal complex in meiosis of

most species (Solari, 1970a). As with most rules there is exceptions, and in this case, the role of the synaptonemal complex does not end at diplotene in metatherian mammals, instead the axial element protein SYCP3 and transverse filament protein SYCP1 are essential in keeping the sex chromosomes (that have no PAR) together in the form of a dense plate (Solari and Bianchi, 1975; Page et al., 2003a; Page et al., 2006; Namekawa et al., 2007b). In metatherians the dense plate ensures the sex chromosomes are tethered together up until anaphase, a time when the sex chromosomes are pulled toward opposite poles, after which time the dense plate dissolves, ending the role of the synaptonemal complex in meiosis.

1.2.3 The Cohesin Complex

Cohesins are central to a number of fundamental mechanisms in chromosome biology (Reviewed by Mehta et al., 2013), including DSB repair (Kim et al., 2002; Watrin and Peters, 2009), transcriptional regulation (Lara-Pezzi et al., 2004; Wendt et al., 2008), chromosome condensation (Ding et al., 2006; Heidinger-Pauli et al., 2010), homologue pairing and recombination (Xu et al., 2005; Herran et al., 2011; Lee and Hirano, 2011), DNA replication (Terret et al., 2009), and synaptonemal complex formation (Novak et al., 2008). Recently cohesin have been implicated in promoting nucleolar structure and function (Harris et al., 2013). Mitotic Cohesin itself is a multiprotein complex comprising four subunits: SMC3, SMC1 α , a α -kleisin subunit (Rad21), and a sister chromatid cohesin component (Stromal Antigen 1 or Stromal Antigen 2) (Reviewed by Peters et al., 2008). There are also meiosis specific isoforms of some components of the synaptonemal complex including the SMC1 isoform: SMC1 β , the α -kleisin subunit isoforms: Rad21L and Rec8, and the sister chromatid cohesin subunit isoform: Stromal Antigen 3 (SA3 or STAG3) (Reviewed by Bardhan, 2010; Lee and Hirano, 2011). The SMCs have a hinge domain that enables the two proteins to dimerise. At the other end of these proteins, the N and C terminals interact with ATPase

activity in conjunction with the kleisin protein family member and a stromal antigen protein family member, thus forming a ring like structure.

The cohesin complex has pivotal roles during prophase I, where at least 5 different forms of cohesin are known to exist (Lee and Hirano, 2011). Prior to leptotene and before the DNA is replicated, sister chromatids are held together by cohesin complexes containing SMC3, SMC1 α , RAD21 and SA1/SA2. As DNA is replicated, still prior to leptotene, three other cohesin complexes replace the original, these are (SMC3, SMC1 α , RAD21L and phosphorylated STAG3), (SMC3, SMC1 β , RAD21L and phosphorylated STAG3) and (SMC3, SMC1 β , REC8 and STAG3) (Lee and Hirano, 2011) (Figure 3). As prophase I progresses and enters zygotene, the (SMC3, SMC1 α , RAD21L and phosphorylated STAG3) and (SMC3, SMC1 β , RAD21L and phosphorylated STAG3) complexes are involved in the cohesion of non-sister chromatids. This association continues until mid pachytene (when early recombination intermediates change into late recombination intermediates), at which time the complexes are removed from the chromosomes, indicating a likely role in homologous repair (Lee and Hirano, 2011). From mid pachytene, when these complexes are removed, (SMC3, SMC1 β , RAD21 and STAG3) complexes replace the removed complexes, but instead of binding non sister chromatids, they bind sister chromatids. Meanwhile the (SMC3, SMC1 β , REC8 and STAG3) complex remains bound to the axial core into late diplotene (Lee and Hirano, 2011) (Figure 4).

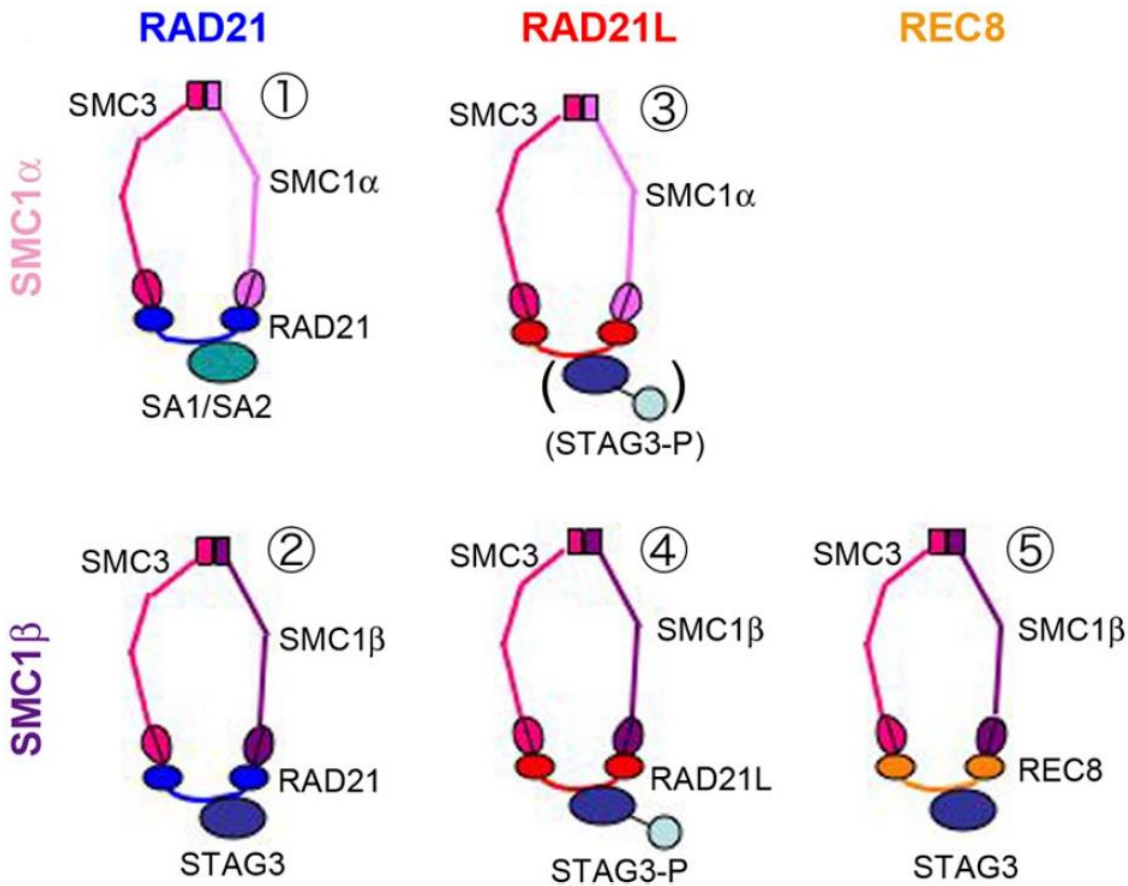


Figure 3. Putative cohesin complexes used in meiosis as revealed by immunoprecipitation assays as performed by (Lee and Hirano, 2011). Note that while most subunits are interchanged in each of the complexes, SMC3 is a part of every complex. The numbers assigned to each complex are the same assigned in figure 4. Figure from (Lee and Hirano, 2011).

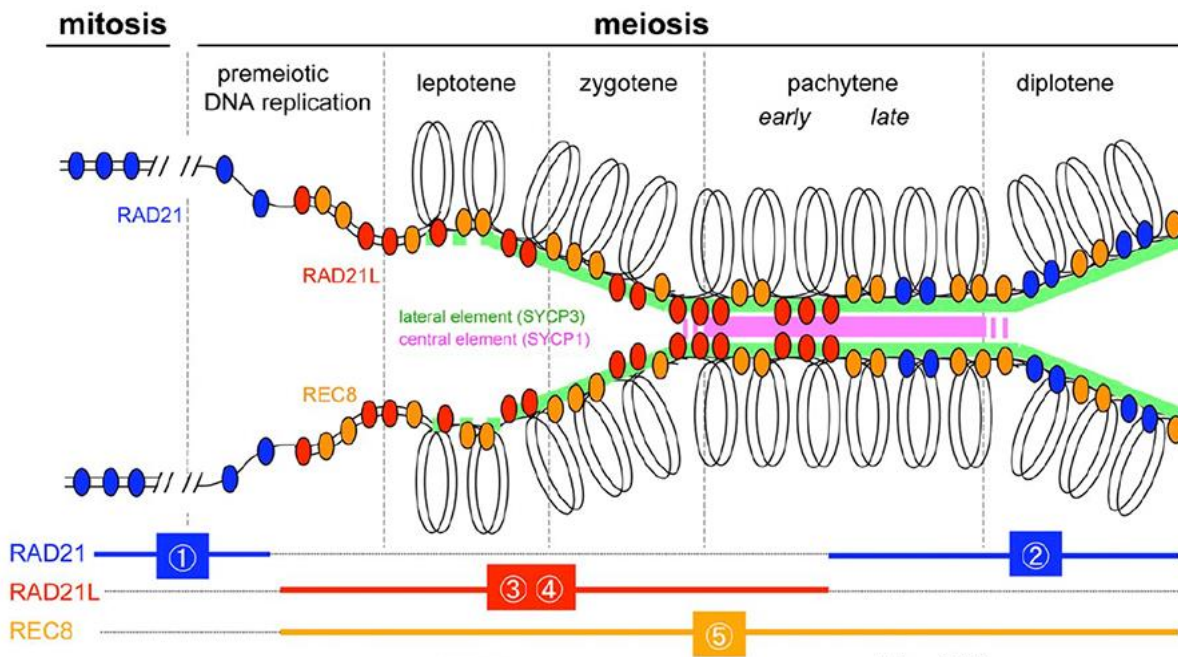


Figure 4. Distribution of the cohesin complexes (outlined in figure 3) during the processes of synapsis and recombination that occurs during prophase I. RAD21 complexes are in blue, RAD21L in red and REC8 complexes are coloured orange. Figure from (Lee and Hirano, 2011).

1.2.4 Unpaired DNA Checkpoint Avoidance Mechanisms

During meiosis checkpoint mechanisms ensure normal meiotic progression of genetically intact cells. A key checkpoint at pachytene is triggered by unpaired DNA, this checkpoint ensures that aneuploidy is avoided in the cell. If triggered this checkpoint leads to meiotic pathway arrest at pachytene and ultimately sterility in the organism (Manterola et al., 2009; Reinholdt et al., 2009).

1.2.4.1 Synaptic Adjustment (Pseudosynapsis)

Birds have a ZW sex chromosome system where females are the heterogametic sex. Birds have developed a unique mechanism by which they can avoid the pachytene checkpoint, in the form of synaptic adjustment, a form of pseudosynapsis. During pachytene, the larger Z chromosome wraps around the smaller W chromosome, and despite a lack of homology, a full synaptonemal complex is formed between the chromosomes (Solari, 1977; Schoenmakers et al., 2009b). This synaptic adjustment is likely to provide a mechanism by which the pachytene checkpoint is avoided, whereby the bird sex chromosomes appear to be completely paired, despite a general lack of homology. However whether this checkpoint exists at pachytene is a point of contention, with evidence that synaptic readjustment is error prone and during pachytene there exists a small portion of cells in which the pseudosynapsis between the Z and W does not occur (Guioli et al., 2012b). These cells do not arrest at pachytene, however whether there is another mechanism that helps the sex chromosomes avoid the pachytene checkpoint is currently unknown. It is also possible that these cells trigger a checkpoint at a later stage, specifically one that may be active during metaphase I (Manterola et al., 2009).

1.2.4.2 Meiotic Sex Chromosome Inactivation

Meiotic sex chromosome inactivation (MSCI) is a process whereby the sex chromosomes are made transcriptionally inactive during the prophase I stage of meiosis. While most often MSCI relies on synaptic progression to determine when and which chromosomes to target, in some species MSCI appears to be independent of synapsis and can even occur before synapsis begins (Cabrero et al., 2007).

In species that have MSCI, during the zygotene to pachytene transition, the sex chromosomes become transcriptionally silenced as evidenced by the differential incorporation of uridine by the sex chromosomes at this stage, which is lacking when compared with autosomes at this stage (Monesi, 1965a), furthermore there is also exclusion of RNA Polymerase II staining on the sex chromosomes (Khalil et al., 2004b) and increased DAPI staining when compared with the autosomes. Such silencing is mediated by histone variants/modifications. The silencing begins when the histone variant H2AX (histone 2A family member X), which is a major component of the meiotic nucleosome, is phosphorylated at serine 139 to form γ H2AX (Mahadevaiah et al., 2001). The sex chromosomes, now known as the sex body, become heterochromatic and are moved to the nuclear periphery. Subsequently the sex body is loaded with other histone modifications and variants that are associated with silencing in a wide variety of contexts. Such histone modifications and variants include H2AY (histone 2A family member Y - previously known as macroH2A1.2) (Hoyer-Fender et al., 2000b), uH2A (ubiquitinated Histone 2A) (Baarends et al., 1999), hyper- and hypo- acetylation of various histones (Khalil et al., 2004b) and H3K9me2 (Histone 3 Lysine 9 dimethylation) (de Vries et al., 2012).

Generally when meiotic chains are formed through translocation events, sterility ensues due to meiotic failure by the metaphase I stage most notably via the failure of MSCI (Manterola et al., 2009; Reinholdt et al., 2009).

Post meiotic sex chromatin (PMSC) was first reported in eutherian mammals (Namekawa et al., 2006) and later in metatherian mammals (Namekawa et al., 2007b). As the name suggests PMSC is an extension of MSCI whereby inactivation of the sex chromosomes continue into spermiogenesis. There are two theories as to the purpose of PMSC, the first theory suggests that its purpose is to ensure the paternal copy is identifiable after fertilisation, such that imprinted X-inactivation can occur in metatherians and in the placenta of eutherian mammals (Takagi and Sasaki, 1975; Xue et al., 2002). The second theory on the purpose of PMSC is that it ensures genes deleterious to spermiogenesis are not expressed throughout the stage.

PMSC is associated with a lack of Cot-1 signal, increase DAPI intensity, increased HP1 β , increased H3K9me₂, a lack of H3K27me₃, some H2AFY and a lack of H3K9ac (Namekawa et al., 2006; Namekawa et al., 2007b).

It is predicted that MSCI and PSMC are tolerated due to the presence of retrogene copies of X-linked genes on some autosomes (Reviewed by Jeremy Wang, 2004). Indeed when autosomal retrogene copies such as *Utp14b* are mutated, spermatogenic failure ensues (Baltus et al., 2004). In the platypus no retrogene copies of X-linked genes have been detected, a finding that has led to the prediction that MSCI or PMSC could not be tolerated in the platypus (Potrzebowski et al., 2008b). This theory however does not take into account that the genes contained on the platypus sex chromosomes are different to those on other mammalian sex chromosomes and in fact there may be no genes on the platypus sex chromosomes that are essential for spermatogenesis, and therefore the lack of autosomal

retrogenes of X-linked genes may be irrelevant to whether or not MSCI can occur in the monotremes.

1.2.5 Nucleolar Association of Sex Chromosomes During Prophase

During prophase I the nucleolus has a close association with the sex chromosomes and have therefore been implicated in having a role in sex chromosome organisation at this time (Kierszenbaum and Tres, 1974). In the mouse, nucleoli form on nucleolar organising regions (NORs) during zygotene, at pachytene these nucleoli disassociate with the NORs, coalesce and move into close association with the sex body, where it remains until diplotene (Tres, 2005). Similar nucleolar-sex body dynamics have been reported in other eutherians (Ohno et al., 1957; Holm and Rasmussen, 1977; Dresser and Moses, 1980), metatherians (Sharp, 1982) and even invertebrates (Viera et al., 2009). While the purpose of this association remains unclear, it has been observed that proteins that localise to the sex chromosomes during pachytene also accumulate in nucleoli (Escalier and Garchon, 2005b; Tsutsumi et al., 2011a). Thus it has been suggested that the functions may not only be related to chromosome synapsis, but also to chromatin condensation, recombination and even cell cycle progression (Tres, 2005)

1.3 Aims of this Study

Previous work on the platypus sex chromosomes, that started over 35 years ago, has concentrated on the meiotic chain at metaphase I. Thus to begin this project the initial aim was to determine if there was a specific order or timing of when the platypus sex chromosomes pair in prophase I (Chapter 1).

Next in order to determine how the chain forms this study analysed and visualised the proteins of the platypus synaptonemal complex (Chapter 2).

In recent times the role of the cohesin complexes in prophase I have become well established with some complexes required for normal axial element formation, while others are required to aid in keeping non sister homologues together at sites of crossovers. Thus the third aim of this study is to analyse the role of the sex chromosomes during platypus prophase I (Chapter 3).

Based on the lack of autosomal retrogenes copies of X/Y-linked genes, it has been predicted that MSCI does not occur in platypus, thus the final aim of this study is to determine the methods by which the platypus sex chromosome chain avoids triggering the prophase I checkpoint, whether it be MSCI, synaptic adjustment or a unique method (Chapter 4).

Chapter 2: Materials and Methods

2.1 Materials

All materials used were as described in each of the following manuscripts.

2.2 Methods

All methods were carried out as detailed in each of the following manuscripts.

Chapter 3: Formation of the Platypus Sex Chromosome Chain in Early Prophase I (Paper I)

Daish, T., Casey, A., Grutzner, F. (2009). Platypus chain reaction: directional and ordered meiotic pairing of the multiple sex chromosome chain in *Ornithorhynchus anatinus*. *Reprod Fertil Dev* 21: 976-984.

Statement of Authorship

Title of paper	Platypus chain reaction: directional and ordered meiotic pairing of the multiple sex chromosome chain in <i>Ornithorhynchus anatinus</i>
Publication Status	Published
Publication details	Daish T, Casey A, Grutzner F (2009) Platypus chain reaction: directional and ordered meiotic pairing of the multiple sex chromosome chain in <i>Ornithorhynchus anatinus</i> . <i>Reprod Fertil Dev</i> 21: 976-984.

Author Contributions

Name of Candidate	Aaron E. Casey	
Contribution to paper	Co-designer of experiment, responsible for carrying out fluorescence in situ hybridisation as well as collecting results and carrying out appropriate statistical analysis. Significant input on writing results and methods for the paper including figures 1, 2, 4, 5 and 7.	
Signature		Date 20.12.14

Name of Co-Author	Tasman J. Daish	
Contribution to paper	Co-designer of experiments. Carried out SYCP1 immunostaining and associated fluorescence in situ hybridisation experiments used in figures 3 and 6. Primary author of paper.	
Signature		Date 12/12/14

Name of Co-Author	Frank Grutzner	
Contribution to paper	Co-designer of experiments. Input on writing manuscript.	
Signature		Date 13/12/14

Daish, T., Casey, A. & Grutzner, F. (2009) Platypus chain reaction: directional and ordered meiotic pairing of the multiple sex chromosome chain in *Ornithorhynchus anatinus*.
Reproduction, Fertility and Development, v. 21 (8), pp. 976-984.

NOTE:

This publication is included on pages 17-25 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://dx.doi.org/10.1071/RD09085>

Chapter 4: Analysis of the Platypus Synaptonemal Complex Proteins. (Paper II)

Casey, A.E., Daish, T. J. and Grutzner, F. (2014). Identification and Characterisation of Synaptonemal Complex Genes in Monotremes. Submitted to "Gene"

Statement of Authorship

Title of paper	Identification and Characterisation of Synaptonemal Complex Genes in Monotremes
Publication Status	Submitted to "Gene"
Publication details	Casey, A. E., Daish, T. J. and Grutzner, F. (2014) Identification and Characterisation of Synaptonemal Complex Genes in Monotremes. Submitted (to Gene)

Author Contributions

Name of Candidate	Aaron E. Casey	
Contribution to paper	Primary designer of experiments. Carried out all experiments. Primary author of manuscript. Created all figures.	
Signature		Date 20.12.14

Name of Co-Author	Tasman J. Daish	
Contribution to paper	Input on experimental design and input on writing manuscript.	
Signature		Date 12/12/14

Name of Co-Author	Frank Grutzner	
Contribution to paper	Input on experimental design and input on writing manuscript.	
Signature		Date 13/12/14

Title

Identification and Characterisation of Synaptonemal Complex Genes in Monotremes

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Abstract

The platypus and echidna are the only extant species belonging to the clade of monotremata, the most basal mammalian lineage. The platypus is particularly well known for its mix of mammalian and reptilian characteristics and work in recent years has revealed that this also extends to the genetic level. Amongst the monotreme specific features is the unique multiple sex chromosome system (5X4Y in the echidna and 5X5Y in the platypus), which forms a chain in meiosis. This raises questions about sex chromosome organisation at meiosis and if this has led to changes in genes coding for synaptonemal complex proteins which aids pairing of homologous sequences. Here we investigate the key structural components of the synaptonemal complex in platypus and echidna, the synaptonemal complex proteins 1, 2 and 3 (*SYCP1*, *SYCP2* and *SYCP3*). *SYCP1* and *SYCP2* orthologues are present, conserved and expressed in platypus testis. *SYCP3* in contrast is highly diverged, but key residues required for self-association are conserved, while key residues required for tetramer stabilisation and DNA binding are missing. We also discovered a second *SYCP3*-like gene (*SYCP3-like*) in the same region. Comparison with the recently published Y-borne *SYCP3* amino acid sequences revealed that *SYCP3Y* is more similar to *SYCP3* in other

mammals than the autosomal monotreme SYCP3. It is currently unclear if the changes in the SYCP3 gene repertoire are related to meiotic organisation of the extraordinary sex chromosomes system.

Highlights

- Monotremes express orthologues of SYCP1, SYCP2 and SYCP3 in testis.
- In contrast to SYCP1 and SYCP2, SYCP3 is highly diverged and duplicated in monotremes.
- In monotremes the autosomal SYCP3 is more diverged than a recently identified Y copy of the SYCP3 gene.
- Changes in monotreme SYCP3 may be related to the evolution of the monotreme sex chromosome complex.

Keywords

Synaptonemal complex, monotremes, meiosis, SYCP1, SYCP2, SYCP3.

Introduction

During prophase I homologous chromosomes align and pair with one another. This pairing is triggered by the homologous repair of DNA double strand breaks (DSBs) and aided by formation of a tripartite structure called the synaptonemal complex (SC) (Reviewed by Page and Hawley, 2004). The SC assembles as a three-dimensional ladder-like structure comprising two axial elements (called lateral elements in the fully formed SC) joined by transverse filaments and a central element (Solari, 1970b; Holm and Rasmussen, 1977). The axial element comprises two proteins; synaptonemal complex protein 2 (SYCP2) (Offenberg et al., 1991; Offenberg et al., 1998) and synaptonemal complex protein 3 (SYCP3) (Heyting et al., 1988). A single protein comprise the transverse filaments, synaptonemal complex

protein 1 (SYCP1) (Offenberg et al., 1991), while the central element has numerous components: synaptonemal complex element 1 (SYCE1) (Costa et al., 2005), synaptonemal complex element 2 (SYCE2) (Costa et al., 2005), synaptonemal complex element 3 (SYCE3) (Schramm et al., 2011) and testis expressed 12 (Tex12) (Hamer et al., 2006). The lateral element SYCP2 protein has been shown to bind SYCP1 providing the only demonstrated structural link between the axial elements and transverse filaments (Winkel et al., 2009).

The SC forms and disassembles during prophase I to mediate association of chromosome homologues. At leptotene, the chromosomes are organised along the axial elements (Heyting et al., 1988; Offenberg et al., 1998) and at this stage DNA DSBs interact with their matching sequence on the homologous chromosome via interaxis bridges, bringing the homologues into close proximity (Reviewed by Page and Hawley, 2004). Over the duration of leptotene a proportion of the bridges mature into axial associations (Rockmill et al., 1995) that become initiation sites for SC formation. As the cell enters zygotene, homologous chromosomes continue to be pulled into close proximity, particularly at telomeric ends which focus to a single pole (Scherthan et al., 1996). As homologous sequences associate, the transverse filaments and corresponding central elements begin to assemble (Heyting et al., 1988; Offenberg et al., 1991). At pachytene the SC is fully formed between autosomal pairs while the sex chromosome SC is restricted to pseudoautosomal regions (PARs) (Solari, 1970b), with only axial elements being present along the entire length of the chromosomes (Solari, 1970b). At diplotene, the central elements and transverse filaments disassemble with chromosomal association maintained by chiasmata, the sites of recombination (Solari, 1970a). By late diplotene, the axial elements are also lost thus completing the role of the SC in meiosis of most species (Solari, 1970a). In metatherian mammals which lack a sex chromosome PAR, the axial element proteins SYCP3 and SYCP1 are essential in maintaining sex chromosome association through diplotene via formation of the 'dense plate' (Solari and

Bianchi, 1975; Page et al., 2003a; Page et al., 2006). This structure tethers the sex chromosomes until anaphase I after which time the dense plate dissolves (Page et al., 2006).

The scale and mode of SC assembly is conserved from yeast to humans (von Wettstein et al., 1984), however it was originally proposed to have arisen through convergent evolution due to a lack of detectable sequence homology of components across taxa. Recent findings reject this hypothesis, showing a common link between all studied Metazoa, particularly for SYCP1 and SYCP3 (Fraune et al., 2012). Further study of central element proteins revealed SYCE2 and Tex12 arose as early as Eumetazoa; SYCE1 in Bilateria and SYCE3 much later in Teleostomi (Fraune et al., 2013). Of all the component SC proteins, SYCP3 is the most highly conserved at the amino acid (aa) level and can be split into several functional domains. Two such domains are involved in self association; an N-terminal 6aa (Nt6) and a C-terminal 6aa (Ct6) domain with two other domains being responsible for DNA binding; basic patch 1 (BP1) and basic patch 2 (BP2) (Tarsounas et al., 1997; Baier et al., 2007; Syrjänen et al., 2014). The first conserved self-association motif (Nt6) reveals that 5 of 6 amino acids are conserved between zebrafish and human, with the middle amino acid substituted for a similar polar neutral amino acid. The second conserved self-association motif (Ct6) has 100% similarity at the amino acid level between zebrafish and human. Heterologous expression of mutated SYCP3 (either at the Nt6 or Ct6) results in failure of the proteins ability to polymerise and form 5-10 nm fibres (that resemble SYCP3 ultrastructures observed in meiotic cells) and thus such mutations are predicted to affect the role of SYCP3 in the meiotic cell (Yuan et al., 1998; Baier et al., 2007; Syrjänen et al., 2014). The first DNA binding motif (BP1, KXXKKR) is conserved from zebrafish to human. The second DNA binding motif (BP2, KRKR) shows 100% conservation at the amino acid level between zebrafish and human. SYCP3 deletion results in impaired axial element assembly leading to defects in synapsis, chiasmata formation, chromosome condensation (Yuan et al., 2000; Liebe et al., 2004) and ultimately meiotic

arrest and sterility in males, while in females aneuploidy is observed with a resulting increase in embryonic death (Yuan et al., 2002). Recently SYCP3 has been shown to exist as a tetramer consisting of four-helix bundles and coiled coil motifs with DNA binding N-terminal sequences that protrude from each end of the core (Syrjänen et al., 2014). In conjunction with the ability of these tetramers to interact with one another, SYCP3 is proposed to provide the structural framework for the axial element structure.

The platypus has 21 autosomal pairs and an extraordinary sex chromosome system comprising 5X and 5Y chromosomes that form an alternating chain (X1Y1X2Y2X3Y3X4Y4X5Y5) during meiosis (Grutzner et al., 2004b). Previously we have shown this complex forms in a highly ordered stepwise manner during zygotene (Daish et al., 2009). This pairing is mediated by multiple PARs of varying size, with some yet to be identified, specifically between the X5Y5 and X4Y4 chromosome pairs. This has interesting implications for SYCP3 function given that marsupials, who also lack a detectable PAR, form a dense plate enriched with SYCP3 (Page et al., 2003a) and SYCP1 (Page et al., 2006) to maintain X and Y chromosome proximity. Here we identify and characterise key SC component genes and report the expression of three distinct copies of SYCP3 in platypus testis.

Materials and Methods

Genome Database Search

All genome database searches were carried out on the ensembl asia database website (<http://asia.ensembl.org/index.html>).

Domain prediction

To obtain domain predictions, amino acid sequences were entered into interpro (<http://www.ebi.ac.uk/interpro/>) using default settings.

Sequence alignments

Sequence alignments were carried out using Geneious (<http://www.geneious.com/>) and MAFFT (Kato et al., 2002). For protein alignments the algorithm was set to “Auto”, the scoring matrix set to “BLOSUM62”, the gap open penalty set to “1.53” and offset value set to “0.123”. For nucleotide alignments the algorithm was set to “Auto”, the scoring matrix to “200PAM/k=2”, gap open penalty to “1.53” and offset value to “0.123”.

Phylogenetic tree generation

Trees were created using Geneious (<http://www.geneious.com/>) using the MrBayes plugin (Huelsenbeck and Ronquist, 2001) with the rate matrix (fixed): poisson, rate variation: gamma, outgroup: zebrafish, gamma categories 4, chain length: 1,100,000, subsampling frequency: 200, heated chains: 4, burn-in length: 100,000, heated chain temp: 0.2, random seed: random, branch lengths ~ exponential: 10, shape parameter ~ exponential: 10

Immunocytochemistry

Meiotic spreads were performed using a dry down protocol (Peters et al., 1997) with a paraformaldehyde pH of 10 for platypus material. Immunostaining was carried out as previously described (Schoenmakers et al., 2009b) with the following antibodies: rabbit anti-mouse SYCP1 (Novus, NB3000-229), mouse anti-human SYCP2 (abcam, ab67694), mouse anti-hamster SYCP3 (Novus, NB100-2065), rabbit anti-mouse SYCP3 (abcam, ab15092), guinea pig anti-SYCP3 (serum, a kind gift from Christa Heyting). All antibodies were used at a 1:200 dilution.

RNA extraction

RNA was extracted using TRIzol Reagent (Life Technologies) according to manufacturer's instructions. Briefly, tissue approximately 0.5cm³ was homogenised in a mortar and pestle using liquid nitrogen in 1mL of TRIzol. The resulting ground material was scraped into a micro centrifuge tube and once defrosted was allowed to incubate for 5 minutes at room temperature. 0.2mL of chloroform was added to the tube, the tube was shaken vigorously for 15 seconds and allowed to incubate for another 2 minutes. The tube was then centrifuged at 12,000 g for 15 minutes. The upper aqueous phase was transferred to a fresh tube and 0.5mL of isopropyl alcohol was added to the aqueous phase in the new tube. The sample was once again incubated for 10 minutes at room temperature and subsequently centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was aspirated and the pellet was washed with 1mL of 75% ethanol. The sample was vortexed and then centrifuged at 7,500 g for 5 minutes at 4°C. The ethanol was aspirated and the pellet was dried at room temperature. The RNA pellet was then dissolved using sterile milli-Q water heated to 60°C

cDNA synthesis

An Invitrogen SuperScript™ III Reverse Transcriptase kit was used to generate the cDNA libraries from both platypus and echidna testis using 5µg total RNA. Reactions were carried out as per the First-Strand cDNA synthesis instructions with no alterations. Briefly, 1 µl of oligo(dT)₂₀, 5µg RNA, 1µL of 10mM dNTP mix and distilled water were added to a microcentrifuge tube to a total of 13µL. The tube was heated to 65°C for 5 minutes, incubated on ice for 1 minute, centrifuged and the following was added to the tube: 4µL of 5X First-Strand buffer, 1µL of 0.1 M DTT, 1µL of RNaseOUT and 1µL of SuperScript III RT. The tube was incubated for 60 minutes at 50°C and the reaction inactivated by heating 70°C for 15 minutes.

Primer design

Primers were designed for SYCP2, SYCP2-like, SYCP3, SYCP3-like and SYCP3Y (Supplementary Table 1) using the program Geneious. The primer sets were used to confirm expression in platypus and echidna testis cDNA samples, platypus beta actin primers were used as a control.

DNA binding site prediction

Amino acid sequences were entered into the online tool, BindN, located at <http://bioinfo.ggc.org/bindn/> with options left as standard. To confirm BindN was effective at determining DNA binding sites, human SYCP3 sequence was entered into the program and it identified both BP1 and BP2 as DNA binding sites.

Protein homology

Protein homology was predicted using the protein homology/analogy recognition version 2.0 (Phyre2) server, located at <http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>.

Results

An ensembl database search yielded a likely orthologue of *SYCP1* (ENSOANG00000004520), however we could not identify platypus *SYCP1* exon 1 and 2 due to a sequence gap in this region in the platypus genome assembly. A BLAT search using the identified platypus *SYCP1* sequence against the mouse database revealed 5 of the top 8 hits aligned to mouse *SYCP1*. Furthermore interpro domain prediction identified platypus *SYCP1* protein as having a *SYCP1* detailed signature match. To further confirm that this is the *SYCP1* orthologue, synteny analysis was performed which revealed that the predicted *SYCP1* resided between the genes *NRLH5* and *TSHB*, the same genes flanking *SYCP1* since the divergence of the reptilian lineage (Supplementary Figure 1). Finally we conducted a multi-species orthologue

alignment with the platypus SYCP1 amino acid sequence (data not shown). A Bayesian tree was generated from the resulting alignment and platypus SYCP1 located as expected between the therian mammals and reptilian lineage which included birds (Figure 1a). An antibody raised against SYCP1 was used in immunocytochemistry experiments on platypus meiotic spreads (Figure 2) to confirm its location within the SC. Interestingly there was a large number of SCs which appeared to have one or more breaks in the otherwise linear staining pattern. (Figure 2, white arrows).

The platypus genome database contains two *SYCP2* genes: *SYCP2* (ENSOANG00000009282) and another unique *SYCP2-like* (ENSOANG00000007192) sequence. Synteny analysis of *SYCP2* places it between *PPP1R3D* and *PHACTR3*, a position conserved in all vertebrates examined (Supplementary Figure 2). *SYCP2-like* resides on a different contig to *SYCP2*, however domain analysis reveals they both contain a SYCP2 detailed signature match. Platypus *SYCP2* and *SYCP2-like* amino acid sequences were aligned with the same species set applied to SYCP1 for tree generation (Figure 1b). Platypus *SYCP2* was located in the expected position between marsupials and reptiles, while *SYCP2-like* had a more basal position within the *SYCP2* clade. A commercially available antibody raised against *SYCP2* was unsuccessfully tested in immunocytochemistry experiments on platypus meiotic spreads (data not shown). To confirm *SYCP2* and *SYCP2-like* expression in testis, we performed RT-PCR on cDNA from several monotreme tissues using the primer pairs outlined in Supplementary Table 1. Only platypus *SYCP2* expression was detected in testis (Supplementary Figure 4) while *SYCP2-like* expression was not detected in any tissue tested (Supplementary Figure 5) (for *Beta actin* controls, see Supplementary Figure 9). Sequencing of the *SYCP2* band revealed that exons 38-40 of the predicted *SYCP2* gene are spliced out of the platypus transcript (data not shown).

The platypus genome contains three different *SYCP3* genes: we identified *SYCP3* (ENSOANG00000032000) and *SYCP3-like* (ENSOANG00000029439) and an additional Y-linked copy (*SYCP3Y*) was recently reported (Cortez et al., 2014). Synteny analysis reveals *SYCP3* is located between *GNPTAB* and *CHPT1*, a position that is conserved in vertebrates since the divergence of the reptilian lineage. Unlike other species, we identified a second *SYCP3* (*SYCP3-like*) copy between *SYCP3* and *GNPTAB* (Supplementary Figure 3). Interpro domain analysis of the predicted proteins revealed that *SYCP3* and *SYCP3Y* contain a Cor1/Xlr/Xmr domain, which was not in the predicted *SYCP3-like* sequence (data not shown). For tree generation amino acid sequence of platypus *SYCP3*, *SYCP3-like* and *SYCP3Y* were aligned with *SYCP3* from the same species used in the *SYCP1* alignment where they clustered together in their expected position between therian mammals and the reptilian lineage (Figure 1c). Platypus *SYCP3* has a poorly conserved BP1 with only 1 of 6 amino acids conserved and only 1 other amino acid is replaced with a similar amino acid when compared with human. Similarly, BP2 has only 2 of 4 amino acids conserved with no similarity at the 2 remaining residues. BindN also failed to predict a DNA binding site at either BP1 or BP2. At the Nt6, 3 of the 6 amino acids are conserved with the other 3 residues being replaced with similar amino acids and therefore is predicted to be functionally conserved. At the Ct6, all 6 of the amino acids are conserved between human and platypus *SYCP3* (Figure 3). Platypus *SYCP3-like* also has a poorly conserved BP1 with only 1 amino acid conserved and a single residue replaced with a similar amino acid when compared with human sequence. At BP2, 1 of the 4 amino acid residues are conserved with a further residue replaced with a similar amino acid. BindN failed to predict DNA binding sites in the same region as BP1 or BP2 in platypus *SYCP3-like*. At the Nt6, 3 of the 6 amino acid residues are conserved with a further 2 replaced with similar amino acids. At the Ct6 4 of the 6 residues are conserved with the remaining 2 being replaced with similar amino acids (Figure 4). For platypus *SYCP3Y*, at BP1,

4 of the 6 amino acids are conserved with BindN predicting a DNA binding site in the same region. For BP2 the sequence is 100% conserved with human SYCP3 and thus predicted to retain its DNA binding ability. At the SYCP3Y Nt6, 3 of the 6 amino acids are conserved with the other 3 being replaced with similar amino acids (Figure 5). The Ct6 is located at the end of the last exon of SYCP3 and platypus SYCP3Y reported by Cortez et al. (2014) is missing the last exon. This sequence was obtained via RNA-seq data and confirmed by PCR and therefore the sequence may be truncated or the analysis may have missed the final exon due to technical reasons.

Each of the SYCP3 sequences were analysed using the Phyre2 server. Platypus SYCP3 returned a hit of human SYCP3 with 98.8% confidence and 63% coverage (145 residues). Platypus SYCP3-like returned a hit of human SYCP3 with 100% confidence and 64% coverage (150 residues). Platypus SYCP3Y returned a hit of human SYCP3 with 100% confidence and 68% coverage (152 residues).

Several SYCP3-like proteins have been described in mouse, each being located on sex chromosomes. Sly (SYCP3-like, Y-linked) is an SYCP3-like protein essential for spermiogenesis in mouse (Cocquet et al., 2009). Slx (SYCP3-like, X-linked) is a spermatid expressed cytoplasmic protein (Reynard et al., 2007) and Slx2 (SYCP3-like, X-linked 2, previously Xlr6) localises to the sex body during pachytene (Shi et al., 2013). Sly has a conserved Nt6 and BP2, but lacks a conserved BP1 and is completely missing the Ct6. Slx is moderately conserved at the Nt6, however it lacks conservation at the Ct6 and both of the basic patches. Slx2 is conserved at the Nt6 and BP2, but lacks conservation at BP1 and the Ct6. In this light we aligned platypus and echidna SYCP3 sequences with Sly, Slx and Slx2 to determine if any of the monotreme proteins may undertake similar function to the mouse SYCP3-like proteins. However after individual alignments with the mouse protein

sequences, the monotreme protein isoforms showed better conservation with murine SYCP3 than any of the sex-linked SYCP3-like proteins (data not shown).

To determine the localisation of platypus SYCP3 in meiotic nuclei, we tested a range of SYCP3 antibodies on meiotic spreads, including commercially available antibodies (see methods) and several antibodies we had generated against the platypus SYCP3 sequence. However could not detect any positive staining using either the commercially available or commercially generated SYCP3 antibodies. To confirm that all three *SYCP3* transcripts were specifically expressed in the testis we used RT-PCR with primers specific to each transcript on cDNA from platypus testis and several somatic tissues. Bands of expected size were observed for *SYCP3* (Supplementary Figure 6), *SYCP3L* (Supplementary Figure 7) and *SYCP3Y* (Supplementary Figure 8) (for *Beta actin* controls, see Supplementary Figure 9). This expression was specific to testis and sequencing of the PCR products confirmed them as *SYCP3*, *SYCP3L* and *SYCP3Y* transcripts.

To obtain echidna *SYCP3* sequence we designed primers based on the platypus *SYCP3* open reading frame. RT-PCR for echidna *SYCP3* showed testis specific expression of a single product, but surprisingly the echidna transcript was only ~600bp in length, 100bp shorter than platypus (Supplementary Figure 10) (for *Beta actin* controls, see Supplementary Figure 12). Sequencing confirmed deletion of the entire exon 6 from the echidna transcript. Similar to platypus, echidna Ct6 is 100% conserved while the Nt6 showed only 2 of 6 residues conserved with the remaining 4 replaced with similar amino acids. Furthermore neither of the basic patches are conserved with only 1 residue conserved in BP1 with 1 other residue replaced with a similar amino acid. Bp2 has only 2 conserved amino acids and no others replaced with similar amino acids (Figure 6). Furthermore BindN failed to locate DNA binding sites at either of these locations. In addition, interpro was unable to predict a

Cor1/Xmr/Xlr domain within the echidna sequence (data not shown). Echidna SYCP3Y sequence data was acquired from RNA-seq confirming its testis expression (Cortez et al., 2014). We then designed primers based on this sequence and performed RT-PCR on several tissues to confirm that echidna SYCP3Y expression was restricted to testis (Supplementary Figure 11) (for *Beta actin* controls, see Supplementary Figure 12). The echidna SYCP3Y sequence was aligned with murine SYCP3 and again showed conservation at the Nt6 with 3 of the 6 residues conserved and a further 2 replaced with similar amino acids, while Ct6 is missing. Similar to platypus SYCP3Y, BP1 and 2 are conserved with 4 of 6 residues conserved at BP1 and 3 of 4 at BP2 with the remaining residue replaced with a similar amino acid (Figure 7). Using interpro we were unable to predict a Cor1/Xmr/Xlr domain within the echidna SYCP3 sequence (data not shown). Phyre2 analysis showed Echidna SYCP3 returning a match with human SYCP3 with 100% confidence and 56% coverage (112 residues) and echidna SYCP3Y matching with human SYCP3 with 100% confidence and 80% coverage (140 residues).

30 key residues have been reported as important for SYCP3 tetramer formation in human through hydrophobic interaction, hydrogen bonding, cation interaction or salt link formation (Syrjänen et al., 2014). We first compared these residues in chicken, which diverged from the common ancestor before monotremes, and found 25 of these residues are conserved with a further 4 replaced with similar amino acids. Platypus SYCP3 shows as few as 6 residues conserved with human and only a further 3 being replaced with similar amino acids. Platypus SYCP3-like has 9 residues conserved with human with a further 4 replaced with similar amino acids. Platypus SYCP3Y has 17 residues conserved with human and a further 5 replaced with similar amino acids. Echidna SYCP3 has as few as 10 critical residues conserved with a further 3 replaced by similar amino acids. Echidna SYCP3Y has 14 critical residues conserved with a further 4 replaced by similar amino acids.

In order to determine what selective pressures were acting on the *SYCP3* genes, we next compared the number of non-synonymous substitutions per non-synonymous site (K_a) to the number of synonymous substitutions per synonymous site (K_s) to obtain K_a/K_s ratios. To run the analysis we used the “ K_a/K_s calculation tool” available at <http://services.cbu.uib.no/tools/kaks> with default settings. The results revealed there to be positive selection pressure on the monotreme *SYCP3* sequences after their divergence from the common mammalian ancestor and before their divergence from one another (Supplementary Table 2 and Supplementary Figure 13). Furthermore, since the divergence of platypus and echidna *SYCP3* from the common monotreme ancestor, there has been positive selection on the echidna sequence (Supplementary Table 2 and Supplementary Figure 13). All the other *SYCP3* genes appear to be undergoing purifying selection including the Y-borne (*SYCP3Y*) sequences (Supplementary Table 2 and Supplementary Figure 13).

Discussion

Monotremes have maintained a remarkably complex sex chromosome system which forms a chain at meiosis. Our difficulties applying *SYCP3* antibodies to investigate pairing of sex chromosomes led us to investigate whether the key SC genes are conserved in monotremes. We identified and characterised *SYCP1*, *SYCP2*, *SYCP2*-like, *SYCP3*, *SYCP3*-like and *SYCP3Y* in platypus along with *SYCP3* and *SYCP3Y* in echidna using a combination of database searches, protein alignments, synteny analysis, protein analysis (InterPro and Phyre2) RT-PCR and sequencing.

SYCP1 has a 51.8% pairwise identity between platypus and mouse while *SYCP2* has 32.8% pairwise identity between the same species. The low pairwise alignment score of platypus and murine *SYCP2* is somewhat surprising given the functional relationship between *SYCP2* and *SYCP1*. Interestingly the region of *SYCP2* shown to be required for interaction with

SYCP1 (aa 1376-1505) (Winkel et al., 2009) is even less conserved with 23.3% pairwise similarity, suggesting that this functional relationship may have changed in monotremes consistent with our inability to detect SYCP2 using commercially available antibodies on meiotic spreads.

Both conserved basic patches (BP1 and BP2) are required for SYCP3 to bind DNA (Syrjänen et al., 2014). For platypus and echidna SYCP3 and platypus SYCP3-like, these patches are poorly conserved, indicating that their function may not be conserved. Furthermore BindN DNA binding analyses failed to predict potential DNA binding domains anywhere near these sites, further supporting the idea that DNA binding capability is not conserved in these platypus sequences. However it cannot be ruled out that new unique DNA binding domains have evolved in these proteins, that cannot be predicted by BindN. Interestingly, for platypus and echidna SYCP3Y, both basic patches show significant conservation, furthermore BindN predicts DNA binding domains at these same sites.

For normal self-assembly, SYCP3 orthologues require conservation of the Nt6 and Ct6. For platypus and echidna SYCP3, platypus SYCP3-like and platypus and echidna SYCP3Y, Nt6 is well conserved. However Ct6 is only conserved in platypus and echidna SYCP3 and platypus SYCP3-like, with the region containing Ct6 missing from both platypus and echidna SYCP3Y. Given the lack of conservation at the Ct6, SYCP3Y may not be able to self-associate and form polymers in the same way as other SYCP3 homologues. However, only protein expression in a heterologous system will definitively resolve this question. While at least one of the SYCP3 isoforms (SYCP3 or SYCP3-like) likely play an orthodox role in SC assembly, it is also possible that the platypus SYCP3 isoforms perform roles unique to monotremes (such as Slx in mouse).

In mouse, SYCP3 and SYCP3-like proteins function in a broad range of roles from the synaptonemal complex to roles essential for spermiogenesis in mouse (Cocquet et al., 2009), there is also a spermatid expressed cytoplasmic protein (Reynard et al., 2007) and a protein that localises to the sex body during pachytene (Shi et al., 2013). While we have confirmed our SYCP3 isoforms have homology to mammalian SYCP3, we have found no great similarity to any of the murine SYCP3-like proteins. It is therefore uncertain whether any of our isoforms undertake similar roles to those in mouse, whether they all function in the SC or whether some have unique functions. To shed more light on the distribution and function of the monotreme SYCP3 isoforms during meiosis, antibodies specific to the various platypus and echidna SYCP3 isoforms need to be generated for use in immunostaining of meiotic spreads. Also the expression of these isoforms in a heterologous system, both individually and in combination with one another will provide valuable data on which of these proteins are able to form higher order structures, either individually or in combination with one another.

Our analysis of platypus meiotic nuclei stained with SYCP1 indicated that there were discontinuous SCs along chromosomes, a finding not reported in other species. We speculate such breaks occur at centromeric sequences or sites of recombination however investigation of this is hampered by the lack of reagents, such as centromere proteins. In a previous study such antibodies failed to produce signals in platypus, most likely due to divergence of such sequences in monotremes (Alkan et al., 2010)

Tetramer formation/stabilisation is dependent on key SYCP3 residues (Syrjänen et al., 2014). It is possible platypus SYCP3Y is able to form/stabilise tetramers in the same manner as human, however for platypus SYCP3, SYCP3-like, or echidna SYCP3 and SYCP3Y, they may

have evolved new functional interactions based on our analyses showing that the key residues are poorly conserved in these platypus and echidna sequences.

Together this first analysis of SC genes in monotremes shows that while some regions of these genes remain conserved, there are other functionally important domains that have undergone significant change through sequence divergence, duplication, and transposition onto Y chromosomes. Future functional studies are required to answer questions on how these changes affect SC assembly and function and whether this relates to the evolution of the complex monotreme sex chromosome system.

Acknowledgements

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Artwork and Tables with Captions.

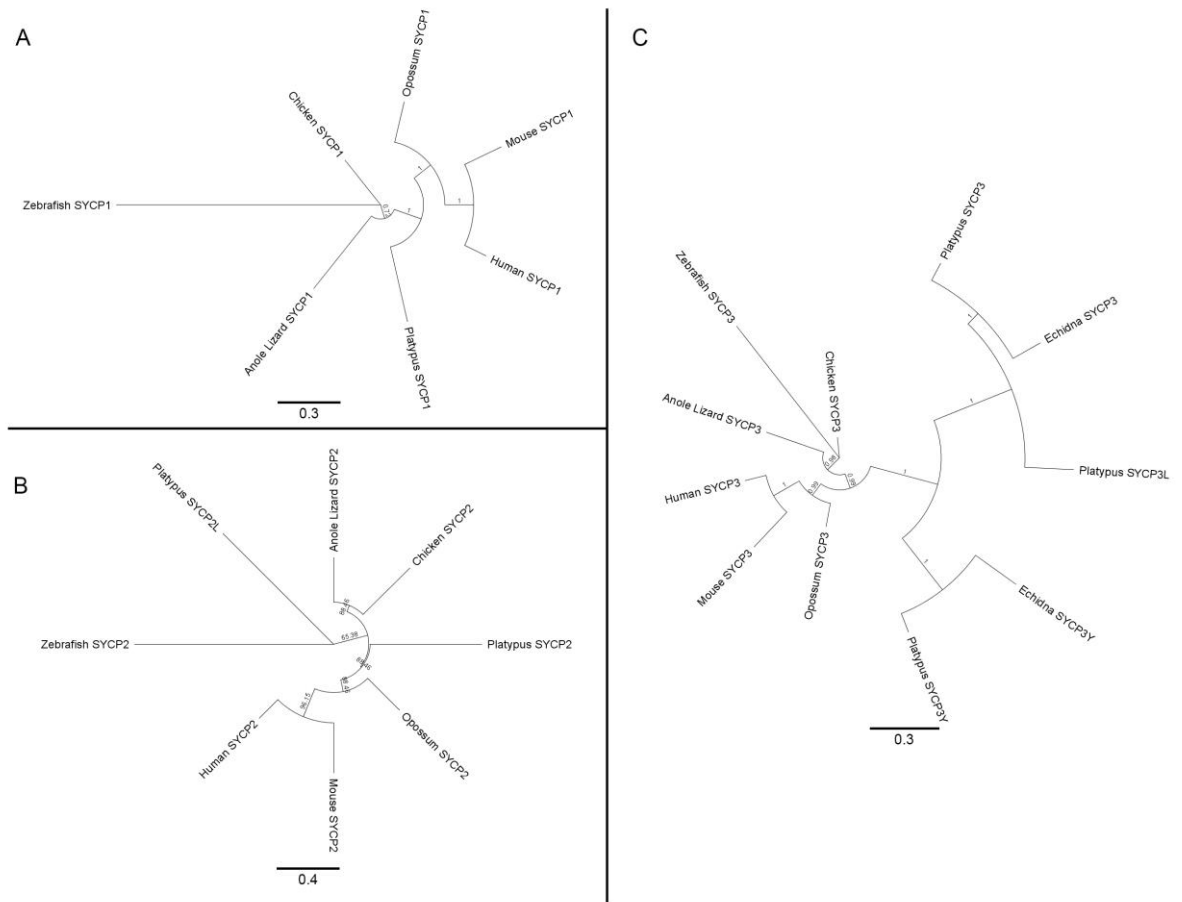


Figure 1. Clades generated using Mr Bayes from amino acid sequence for SYCP1 (a), SYCP2 (b) and SYCP3 (c). Branch labels represent posterior probability and scale bar represents substitutions per site.

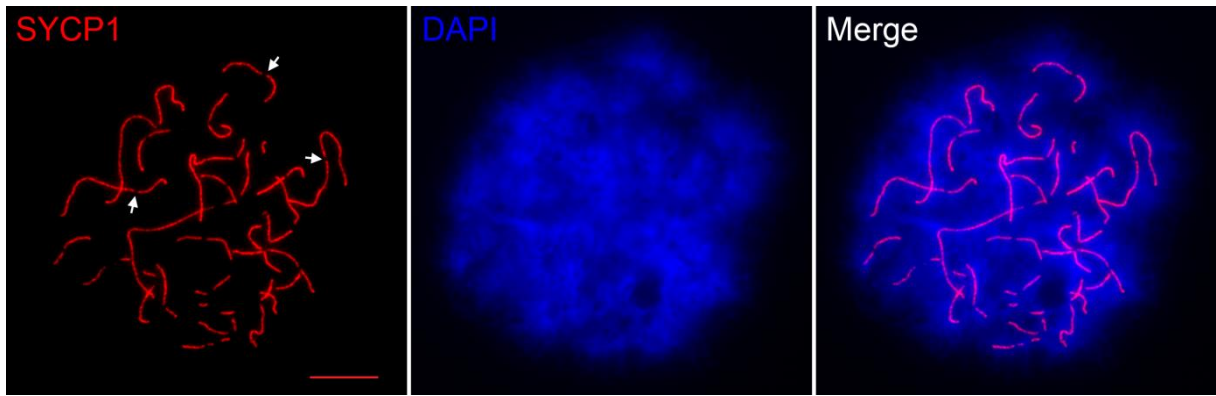


Figure 2. Immunolocalisation of SYCP1 (red) on platypus meiotic spreads. Breaks in the synaptonemal complex of larger chromosomes are indicated (white arrows). DAPI counterstain (blue).

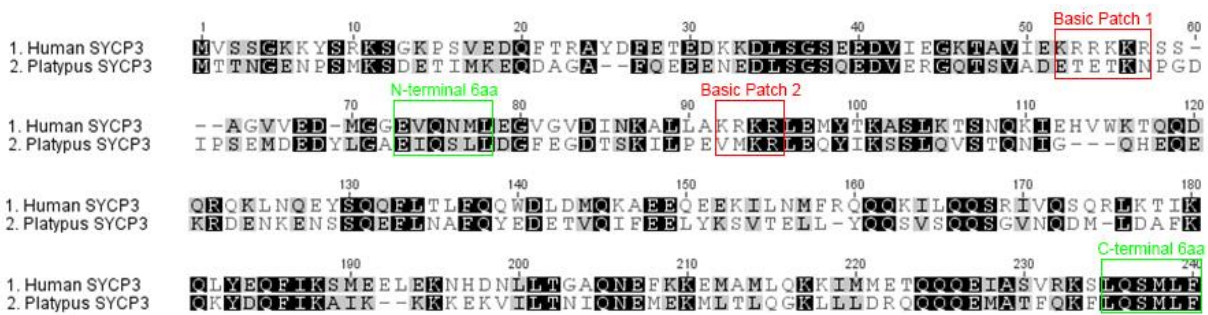


Figure 3. Alignment of platypus SYCP3 with human SYCP3. Conserved motifs are coloured black, similar residues are coloured grey and dissimilar residues are coloured white. Both basic patches are poorly conserved while the N- and C- terminal 6aa regions are conserved.

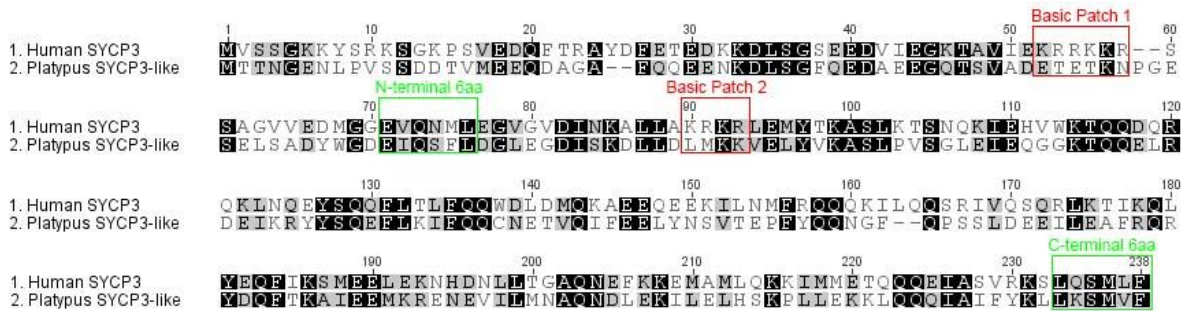


Figure 4. Alignment of platypus SYCP3-like with human SYCP3. Conserved motifs are coloured black, similar residues are coloured grey and dissimilar residues are coloured white. Both basic patches are poorly conserved while the N- and C- terminal 6aa regions are conserved.

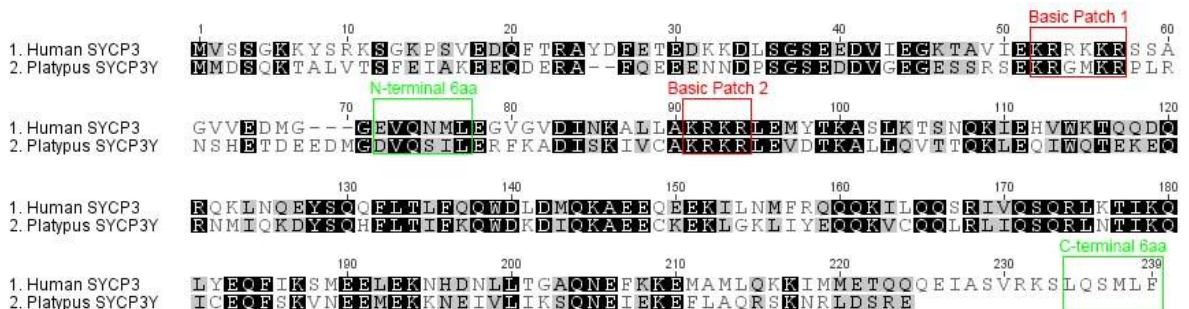


Figure 5. Alignment of platypus SYCP3Y with human SYCP3. Conserved motifs are coloured black, similar residues are coloured grey and dissimilar residues are coloured white. Both basic patches are conserved as is the N-terminal 6aa regions, however the C-terminal 6aa is missing completely.

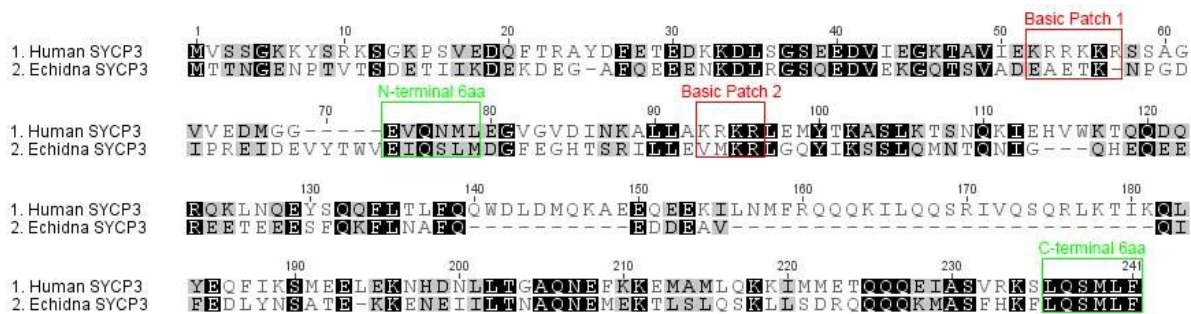
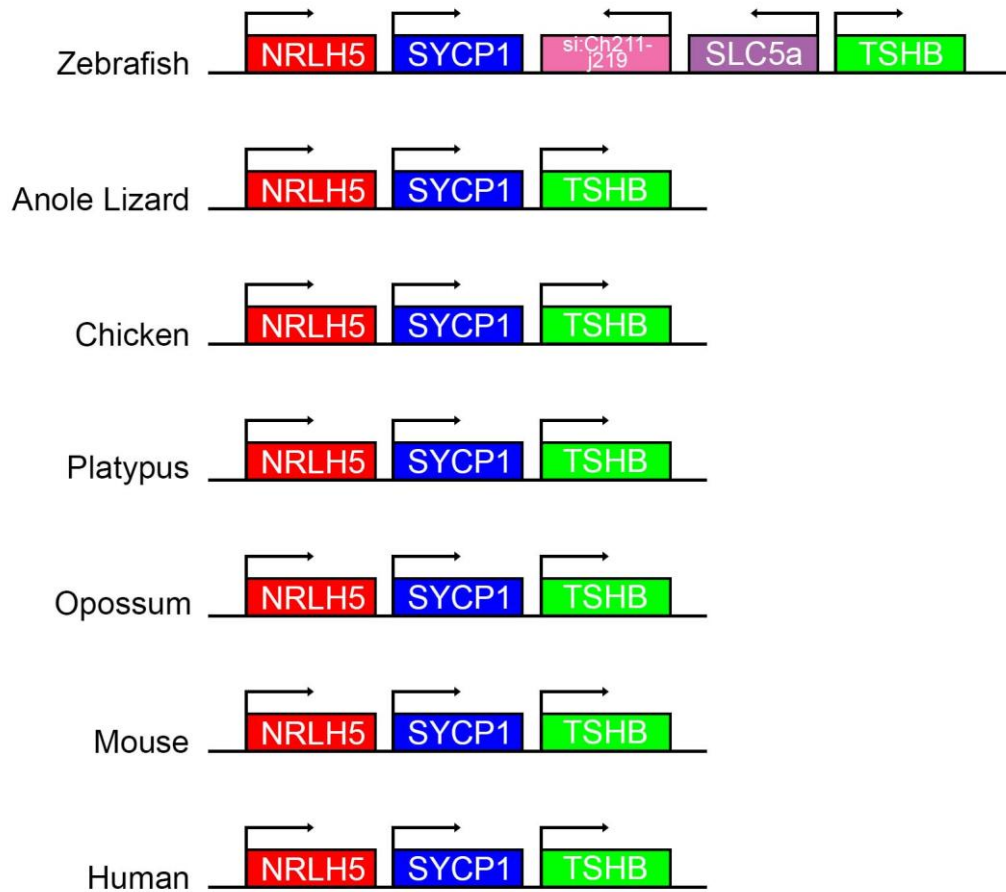


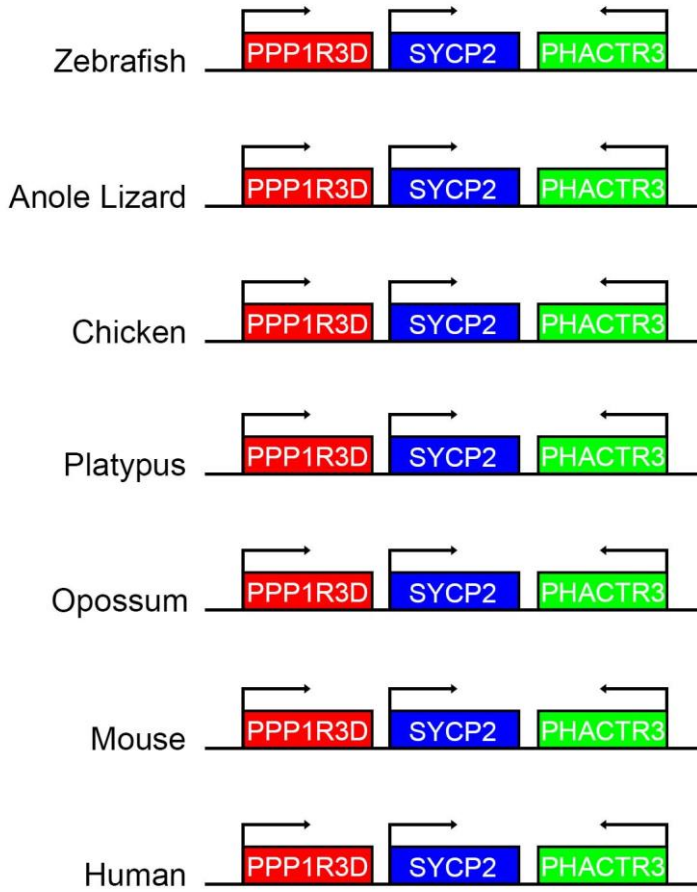
Figure 6. Alignment of echidna SYCP3 with human SYCP3. Conserved motifs are coloured black, similar residues are coloured grey and dissimilar residues are coloured white. Both basic patches are poorly conserved while the N- and C- terminal 6aa regions are conserved.



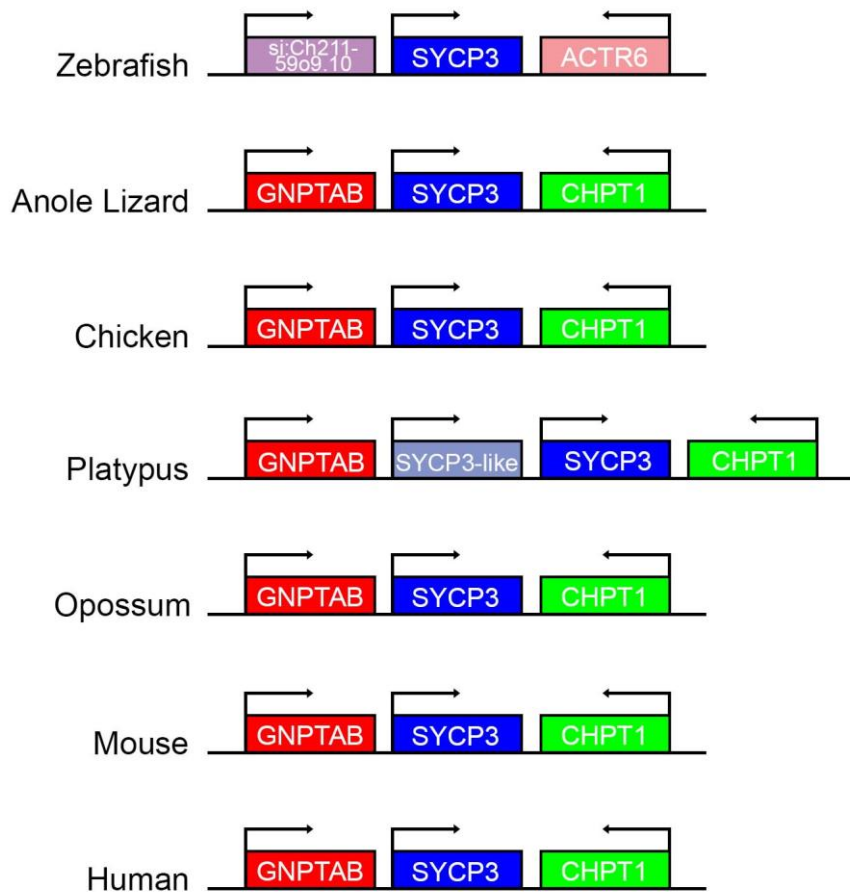
Figure 7. Alignment of echidna SYCP3Y with human SYCP3. Conserved motifs are coloured black, similar residues are coloured grey and dissimilar residues are coloured white. Both basic patches and the N-terminal 6aa are conserved, however the C-terminal 6aa is missing completely.



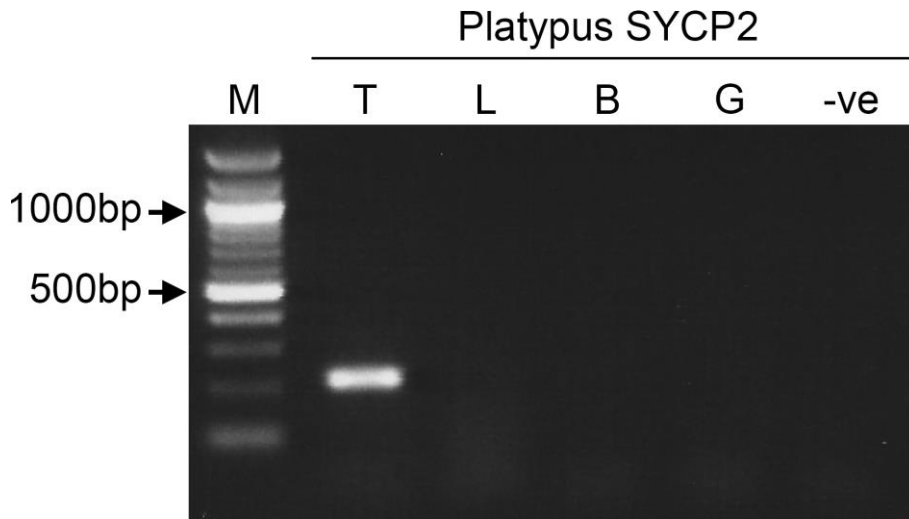
Supplementary Figure 1. Synteny analysis of *SYCP1*. In all species, *SYCP1* resides downstream of *NRLH5* and upstream of *TSHB*. In zebrafish there has been an insertion of two genes downstream of *SYCP1* and upstream of *TSHB*.



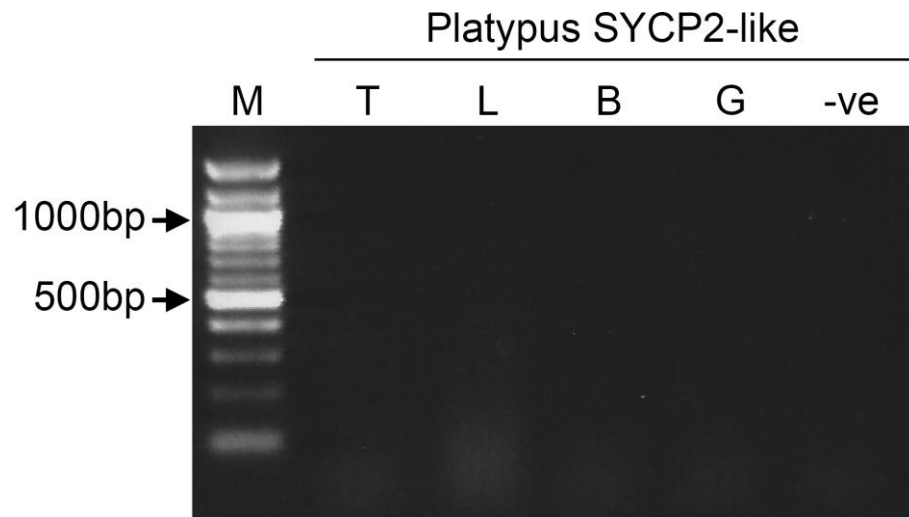
Supplementary Figure 2. Synteny analysis of *SYCP2* and its surrounding genes. In all species *SYCP2* resides downstream of *PPP1R3D* and upstream of *PHACTR3*.



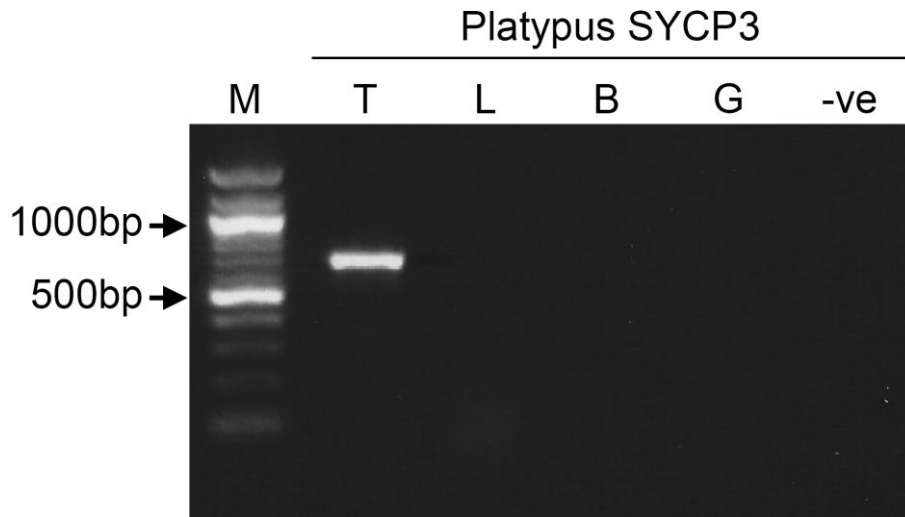
Supplementary Figure 3. Synteny analysis of *SYCP3* and its surrounding genes. In all species except for zebrafish, *SYCP3* resides upstream of *GNPTAB* and downstream of *CHPT1*. In the platypus an *SYCP3-like* gene was found to reside upstream of *GNPTAB* and downstream of *SYCP3*.



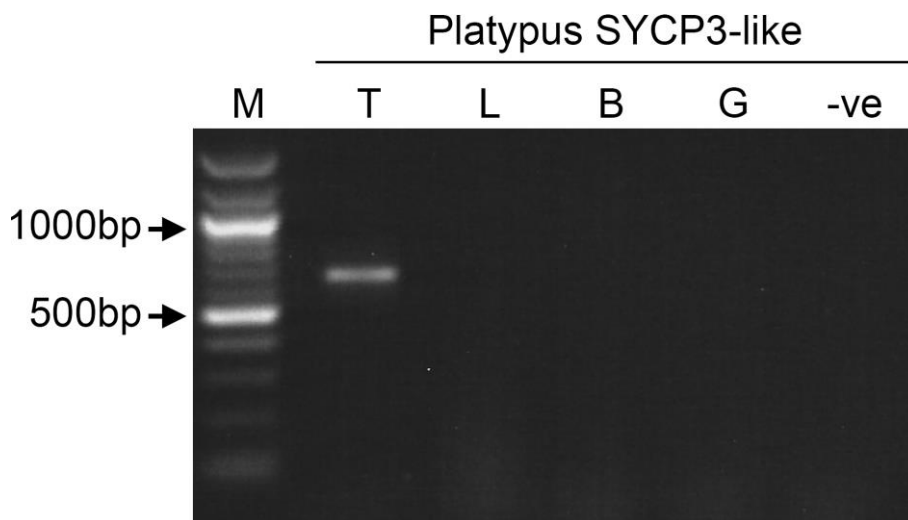
Supplementary Figure 4. RT-PCR using platypus *SYCP2* specific primers on testis (T), liver (L) and brain (B). A band of expected size is observed only in the testis lane. In the genomic control (G) the primers span a distance too great to amplify and produce a band, however this confirms that the testis band is produced from cDNA, not genomic.



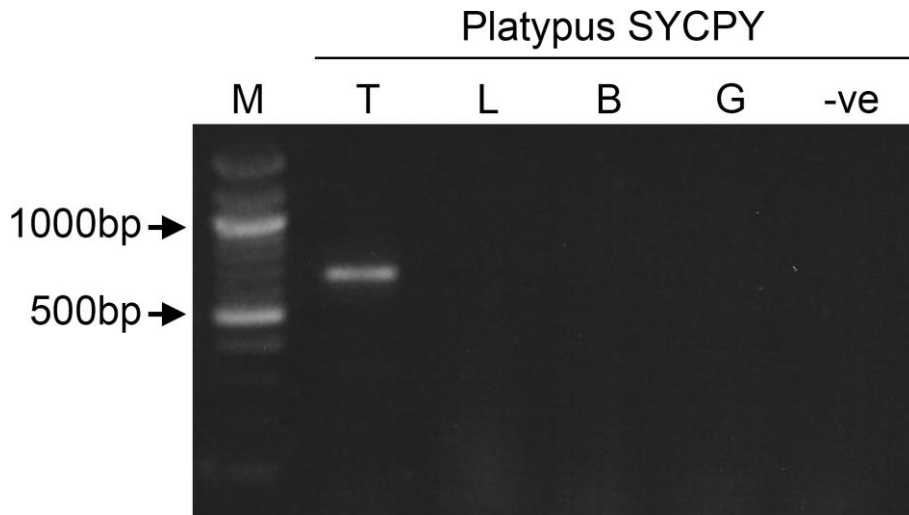
Supplementary Figure 5. RT-PCR using platypus *SYCP2L* specific primers on testis (T), liver (L) and brain (B). No band of expected size is observed in any lane, confirming that *SYCP2L* is not expressed in testis or any other tissue tested.



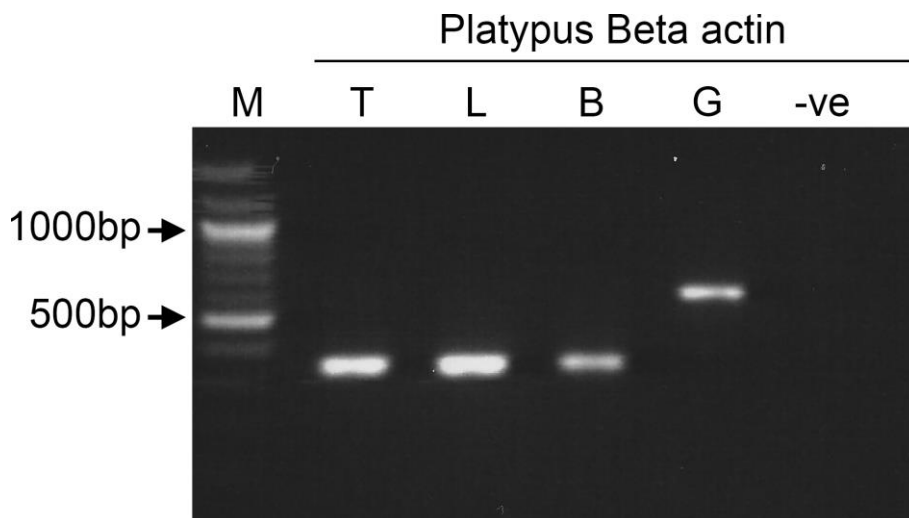
Supplementary Figure 6. RT-PCR using platypus *SYCP3* specific primers on testis (T), liver (L) and brain (B). A band of expected size is observed only in the testis lane. In the genomic control (G) the primers span a distance too great to amplify and produce a band, however this confirms that the testis band is produced from cDNA, not genomic.



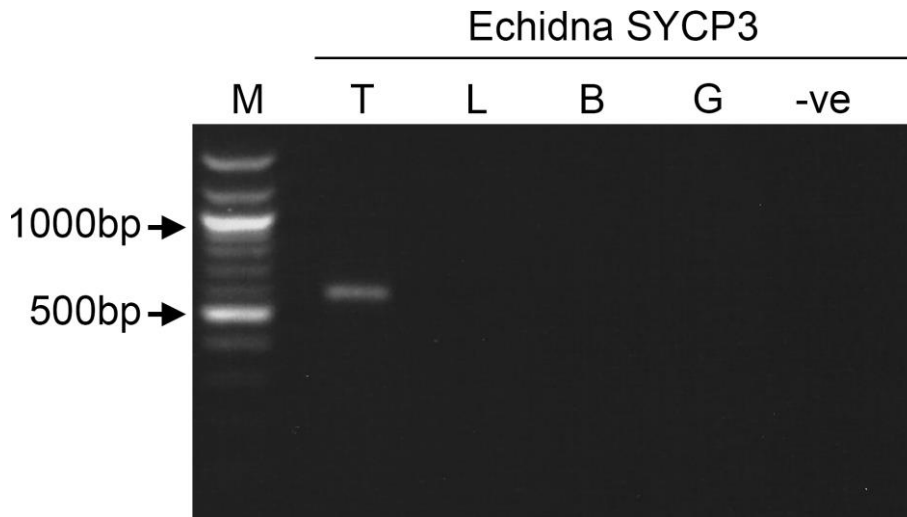
Supplementary Figure 7. RT-PCR using platypus *SYCP3L* specific primers on testis (T), liver (L) and brain (B). A band of expected size is observed only in the testis lane. In the genomic control (G) the primers span a distance too great to amplify and produce a band, however this confirms that the testis band is produced from cDNA, not genomic.



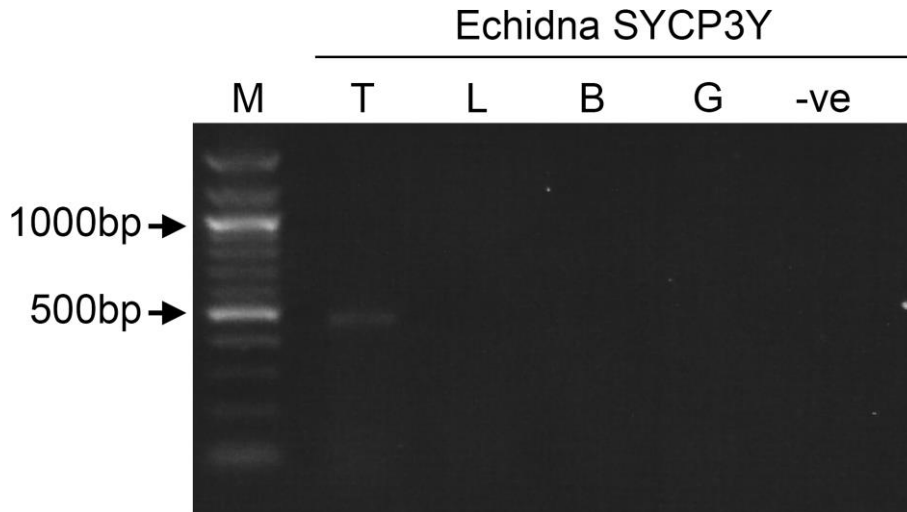
Supplementary Figure 8. RT-PCR using platypus *SYCP3Y* specific primers on testis (T), liver (L) and brain (B). A band of expected size is observed only in the testis lane. In the genomic control (G) the primers span a distance too great to amplify and produce a band, however this confirms that the testis band is produced from cDNA, not genomic.



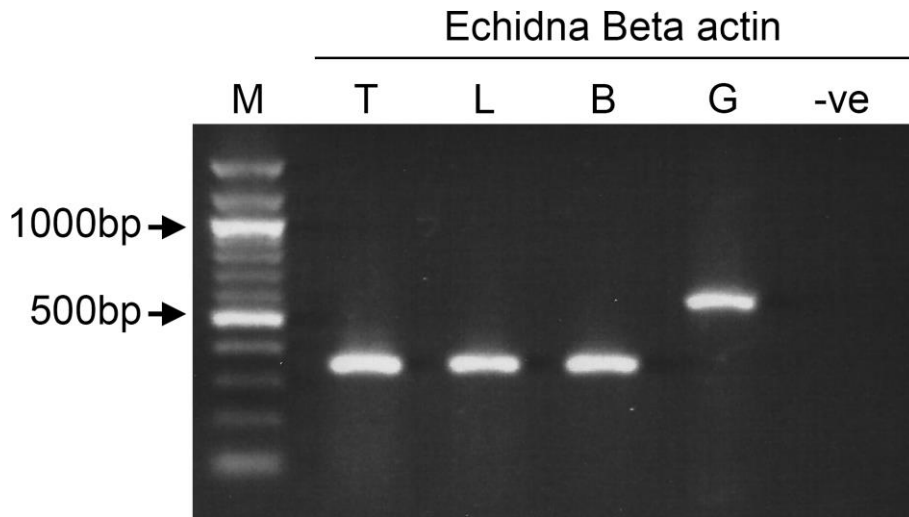
Supplementary Figure 9. RT-PCR using platypus *Beta actin* specific primers on testis (T), liver (L) and brain (B). A band of expected size is observed all three cDNA lanes and the genomic control (G) confirming that appropriate concentrations of cDNA/DNA were used in platypus RT-PCR experiments (Supplementary Figures 4-8).



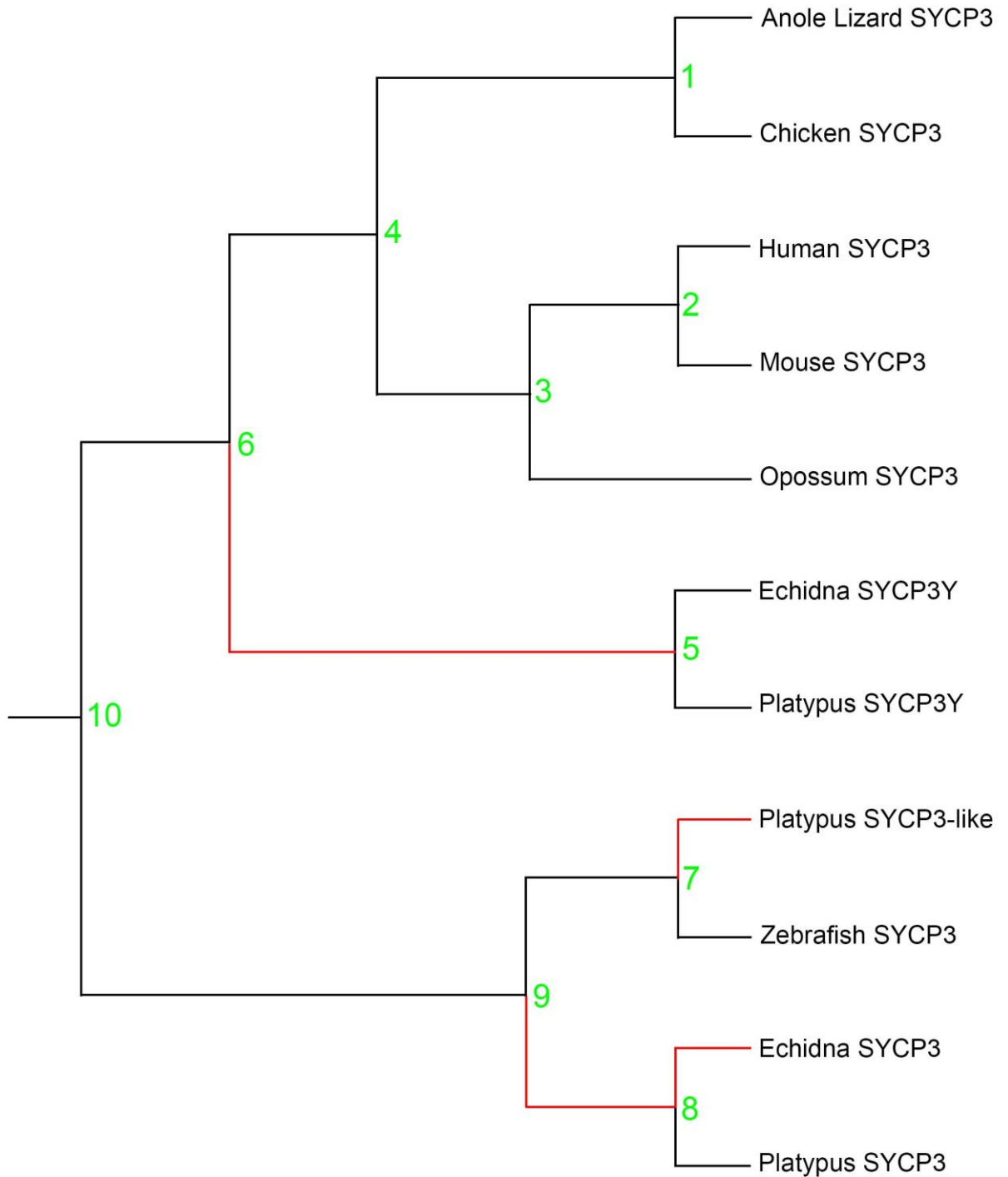
Supplementary Figure 10. RT-PCR using echidna *SYCP3* specific primers on testis (T), liver (L) and brain (B). A band of expected size is observed only in the testis lane. In the genomic control (G) the primers span a distance too great to amplify and produce a band, however this confirms that the testis band is produced from cDNA, not genomic.



Supplementary Figure 11. RT-PCR using echidna *SYCP3Y* specific primers on testis (T), liver (L) and brain (B). A band of expected size is observed only in the testis lane. In the genomic control (G) the primers span a distance too great to amplify and produce a band, however this confirms that the testis band is produced from cDNA, not genomic.



Supplementary Figure 12. RT-PCR using echidna *Beta actin* specific primers on testis (T), liver (L) and brain (B). A band of expected size is observed all three cDNA lanes and the genomic control (G) confirming that appropriate concentrations of cDNA/DNA were used in echidna RT-PCR experiments (Supplementary Figures 10 and 11).



Supplementary Figure 13. Tree generated and used in calculation of Ka/Ks ratios. Tree was generated using an online Ka/Ks calculation tool (see methods for detail). Red lines indicate positive selection while black lines indicate negative selection. Green numbers refer to nodes used in supplementary table 2 that contains the Ka and Ks values for each individual branch.

Supplementary Table 1. Primers used in RT-PCR in this study.

	Forward	Reverse	Expected product size
SYCP2	5' GGCTGCAAGTCCATCATCTATAGA 3'	5' GGAAGTACCTCAGTGTTCATT 3'	315 bp
SYCP2-like	5' GGGGAAAACCTTCCAGTTTC 3'	5' CAGAAATGGAGTTTAGGGTCCTCAA 3'	393 bp
SYCP3	5' ATGACAACCAACGGGGAAAAC 3'	5' TCAAATAACATGGACTGAAGAAAAC 3'	696 bp
SYCP3-like	5' GGGGAAAACCTTCCAGTTTC 3'	5' TTGTTGCTGCAGCTTCTTT 3'	651 bp
SYCP3Y	5' GATGGACTCCCAGAAAACAG 3'	5' CCAATCTATTCTTGCTTCGTTG 3'	653 bp
eSYCP3Y	5' CCTGAAAAGCGAGGGATGAA 3'	5' CGTGCTTCTGTTGAAAGGAT 3'	464 bp
bAct	5' ATCCGTAAGGACCTGTATGC 3'	5' CCGGACTCATCGTACTCTTG 3'	~350 bp

Supplementary Table 2. Ka/Ks ratios as used in Supplementary Figure 13. Red text highlights figures indicating positive selection.

Node#	Ka/Ks Branch1	Ka Branch1	Ks Branch1	Ka/Ks Branch2	Ka Branch2	Ks Branch2
1	0.2612	0.08142	0.3117	0.1105	0.02982	0.2698
2	0.3877	0.04672	0.1205	0.6918	0.09191	0.1329
3	0.1899	0.04229	0.2227	0.1657	0.023	0.1388
4	0.1459	0.03168	0.2171	0.1175	0.02505	0.2131
5	0.6613	0.102	0.1542	0.8392	0.06318	0.07528
6	0.6559	0.1092	0.1665	1.0851	0.1684	0.1552
7	1.5489	0.1913	0.1235	0.3955	0.3339	0.8442
8	1.071	0.08723	0.08144	0.8228	0.05877	0.07143
9	0.5054	0.04673	0.09246	2.6939	0.1651	0.06128
10	0.5911	0.08226	0.1392	0.7614	0.1001	0.1314

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Chapter 5: Dynamics of the Platypus Sex Chromosome Chain Throughout Prophase I (Paper III)

Casey, A.E., Daish, T. J. and Grutzner, F. (2014). Temporal and spatial cohesin recruitment on platypus sex chromosomes during meiotic prophase I. Manuscript in preparation

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Contribution to paper	Input on experimental design and input on writing of manuscript.	
Signature		Date 19/12/14

Title

Temporal and spatial cohesin recruitment on platypus sex chromosomes during meiotic prophase I.

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ABSTRACT

The cohesin complex is vital for chromosome organisation meiosis and mitosis. In addition to the important function in sister chromatid cohesion, these complexes are important for recombination, DSB repair, chromosome pairing and segregation. Egg-laying mammals (monotremes) feature an unusually complex sex chromosome system comprising 5 X and 5 Y chromosomes in platypus that form a chain at meiotic prophase. This system raises fundamental questions regarding meiotic organisation and segregation of this large sex chromosome complement during meiosis. Our previous work revealed the 10 sex chromosomes pair via 9 pseudoautosomal regions in a specific order during prophase however nothing is known about non-histone proteins associated with the formation of the meiotic chain. Here we show that platypus sex chromosomes associate with a large nucleolar body and accumulate cohesin during prophase I. Detailed analysis revealed a highly dynamic loading profile of SMC3 on the sex chromosomes which culminates in a differential loading on the chromosomal axis of XY shared regions compared with the chromatin of asynapsed X and Y specific regions during prophase progression. At late prophase I, cohesin accumulation is largely lost from the sex chromatin and resolves into

subnuclear compartments. This is the first report of a protein accumulation coinciding with formation of the sex chromosome chain in monotremes. Our work suggests a role for cohesin in monotreme sex chromosome organisation during meiotic prophase I.

Author Summary

During meiosis, homologous chromosomes pair up and exchange genetic material between the maternal and paternal homologues. For normal chromosome pairing to occur, a complex multi-protein structure is assembled between the homologues. XY sex chromosomes are unusual in that they remain unpaired over most of their length. Platypus feature an extraordinarily complex sex chromosome system consisting of 5 X and 5 Y chromosomes. During meiosis all ten sex chromosome line up and form a chain in preparation for the first meiotic division. For the first time we show that a protein complex called cohesin could play an important part in sex chromosome organisation in platypus. Importantly we find that cohesin loading differs between the paired and unpaired parts of the chain. Tight association of the sex chromosomes with a large nucleolar structure called the nucleolus and differential cohesin accumulation suggest a role in the formation and organisation of this remarkable sex chromosome complex.

INTRODUCTION

Cohesins are key non-histone proteins of the structural maintenance of chromosome (SMC) group. Traditionally cohesins are described as the molecular glue that keeps sister chromatids physically attached after DNA replication during both mitosis and meiosis. More recently it has been discovered that cohesins are also central to a number of other fundamental mechanisms in chromosome biology (Reviewed by Mehta *et al* (Mehta et al., 2013)) including transcriptional regulation (Lara-Pezzi et al., 2004; Wendt et al., 2008), DNA double strand break (DSB) repair (Kim et al., 2002; Watrin and Peters, 2009), chromosome

condensation (Ding et al., 2006; Heidinger-Pauli et al., 2010), DNA replication (Terret et al., 2009), homologue pairing and recombination (Xu et al., 2005; Herran et al., 2011; Lee and Hirano, 2011) and synaptonemal complex (SC) formation (Novak et al., 2008; Fukuda et al., 2014; Hopkins et al., 2014; Winters et al., 2014). Cohesins have also been implicated in promoting nucleolar structure and function (Harris et al., 2013).

Mitotic cohesin is a multiprotein complex comprising four subunits: SMC3, SMC1 α , an α -kleisin subunit (Rad21), and a sister chromatid cohesin component (Stromal Antigen 1 or Stromal Antigen 2). There are also meiosis specific isoforms of some components of the cohesin complex (CC) including SMC1 β (structural maintenance of chromosomes isoform), Rad21L and Rec8 (α -kleisin subunit isoforms), and the sister chromatid cohesin subunit isoform Stromal Antigen 3 (SA3/STAG3).

Meiotic cohesin subunits appear to act in functionally specific combinations (Lee and Hirano, 2011). While most cohesins are involved in sister chromatid cohesion, some, such as the RAD21L containing complexes, function in non-sister chromatid cohesion between homologous chromosomes (Lee and Hirano, 2011). One significant common property between the different cohesin complexes is their utilisation of the SMC3 subunit.

In meiotic cells, immunostaining of cohesin complexes show the same localisation as the SC to the point that some investigations now report the CC to be part of the SC (Hopkins et al., 2014). Prior to leptotene, RAD21L and REC8 accumulate along the axial core of all chromosomes and at this stage, both have predicted roles in sister chromatid cohesion. During leptotene and zygotene RAD21L physically associates with non-sister chromatids of homologous chromosomes likely aiding DSB repair. Following DSB resolution, RAD21L containing complexes are replaced with RAD21/SMC1 β complexes which bind sister chromatids once again (Lee and Hirano, 2011).

The nucleolus has long been implicated in sex chromosome organisation during prophase I due to their close association at pachytene (Kierszenbaum and Tres, 1974). During mouse zygotene, nucleoli form on nucleolar organising regions (NORs) however at pachytene these nucleoli disassociate with the NORs, coalesce and move into close association with the sex body until diplotene (Tres, 2005). Similar nucleolar-sex body organisation has been reported in other eutherians (Ohno et al., 1957; Holm and Rasmussen, 1977; Dresser and Moses, 1980), metatherians (Sharp, 1982) and even invertebrates (Viera et al., 2009). The purpose of this association remains unclear, however, several proteins that localise to the sex chromosomes during pachytene are also observed to accumulate in nucleoli (Escalier and Garchon, 2005b; Tsutsumi et al., 2011a).

Cohesins have been shown to localise to the nucleolus (Valdeolmillos et al., 2007) implicating a role in nucleolar structure and/or function and have differential sex chromosome association patterns in different species. In mouse RAD21L is recruited to the chromatin loops of the X and Y sex body, a pattern similar to γ H2AX but appearing in late pachytene until mid diplotene (Herran et al., 2011; Ishiguro et al., 2011) suggesting a possible role of cohesin in sex chromosome pairing and sex body formation. In the horse the axial element of the sex chromosomes have significantly more of the cohesin subunit SMC3 loaded when compared with autosomes (Baumann et al., 2011) further suggesting a role in sex chromosome organisation during prophase I.

The platypus carries a highly unusual sex chromosome system comprising 5X and 5Y chromosomes (Murtagh, 1977; Grutzner et al., 2004b) with seven known PARs (X1Y1, Y1X2, X2Y2, Y2X3, X3Y3, Y3X4 and Y4X5) that mediate pairing to form an alternating XY 10 sex chromosome chain during meiosis I (Grutzner et al., 2004b; Rens et al., 2004; Daish et al., 2009). The complexity and homology of monotreme and avian sex chromosomes raise

fundamental questions about meiotic pairing, recombination, meiotic sex chromosome inactivation and sex chromosome segregation. While we have previously shown that the chain forms in a specific order and that this order is not dependent on the extent of XY homology (Daish et al., 2009), there is no information about synaptonemal complex formation and structural maintenance of chromosome protein association during chain assembly.

In the present study we examine the dynamics of cohesin/synaptonemal complex formation during meiotic prophase I in platypus, paying particular attention to the sex chromosome chain. Our finding of tight nucleolar association and differential cohesin accumulation on the chromatin loops of the asynapsed regions of the sex chromosomes implicate a possible role in sex chromosome pairing.

RESULTS

At meiotic prophase I platypus sex chromosomes accumulate SMC3 while in close proximity to a large nucleolar body

In order to investigate the dynamics of SC formation in monotreme prophase I, we used antibodies raised against the central element protein SYCP1 and the cohesin subunit SMC3 to visualise chromosome axial cores. SMC3 is a component of all meiotic cohesin complexes discovered to date and platypus SMC3 is highly conserved with over 99% pairwise identity with human SMC3 at the amino acid level (Figure S1). Western blotting using whole cell lysates from platypus testis identified a single protein species of the expected size (Figure S2).

SYCP1 immunostaining showed the expected SC staining pattern in platypus prophase cells. However the SMC3 antibody detected unusually strong accumulations not observed in the

mouse (Figure 1). We also noted that the focus of accumulation was consistently associated with a round, large DAPI negative structure (Figure 1). Immunostaining for STAG3 (a kind gift from Jose Barbero) showed an identical staining pattern to SMC3 (Figure S3). In mouse, a similar association exists between the sex body and nucleolus in late prophase I (Kierszenbaum and Tres, 1974; Tres, 2005). We therefore expected this DAPI-poor region in platypus cells to be the nucleolus. Silver staining confirmed that this region is rich in protein indicative of a single large nucleolus (Figure 2, red arrow) and the same size as the circular DAPI void seen in meiotic spreads. Platypus chromosome 6 bears a single large nucleolar organising region (Murtagh, 1977). We used immunostaining and BAC DNA FISH to determine the position of the chromosome 6 nucleolar organising region (NOR) relative to the nucleolus during meiotic prophase. Using a platypus chromosome 6 paint in combination with anti-SYCP1 and anti-SMC3 antibodies, we observed the DAPI poor region first forms near one end of chromosome 6 during late zygotene (Figure 3, zygotene panel). This association is maintained through pachytene and continues into diplotene (Figure 3, pachytene and diplotene panels) but ceases prior to prometaphase where no nucleolar structure is observed (Figure 3, prometaphase panel).

Next we investigated SMC3 accumulation dynamics specifically on the platypus sex chromosomes. We carried out immuno-FISH on meiotic spreads using anti-SMC3 and anti-SYCP1 antibodies followed by two Y chromosome specific BAC probes targeting Y5 (Oa_Bb – 152P15) and Y2 (CH236-145P9). Y5 and Y2 reside consistently within the marked SMC3 accumulation regions demonstrating that the platypus sex chromosomes undergo cohesin loading in prophase I (Figure 4).

Cohesin accumulation on sex chromosomes is highly dynamic through prophase I

In order to characterise the sex chromosome loading of SMC3 in detail we used anti-SYCP1 to identify the different prophase stages followed by SMC3 and DNA FISH with sex chromosome specific BAC probes.

Leptotene; Both mouse and platypus early prophase I stage nuclei lack SYCP1 staining as it precedes commencement of synapsis. During this stage we observed a homogeneous speckling of SMC3 signal distributed throughout the nucleus (Figure 5, leptotene panel).

Zygotene; In platypus we observed very few axial elements (SMC3) which also lacked a corresponding central element (SYCP1). In contrast, mouse axial element formation preceded central element assembly as previously reported (Yang et al., 2006) (Figure 5, zygotene panel).

Pachytene; We were able to identify eight substages of pachytene in mouse based on previous work (Quack and Noel, 1977; Turner et al., 2004b) (Figure 6). Platypus meiotic cells are refractory to standard surface spreading techniques, resulting in the SC's appearing less dispersed compared to mouse, however, based on SC length we were able to identify eight comparable pachytene substages from stage a (early pachytene) to stage h (late pachytene), with early and late stages having long and short SC's respectively (Figure 6).

Stage a/b - in early pachytene the sex chromosome chain is evident as a long thin element which at first (stage a) is only discernible via double immunostaining with SYCP1 and SMC3 and appearing as the only chromosomal elements not containing central elements. The sex chromosome chain then maintains a consistent conformation however with a marked increase in the amount of SMC3 localising to the chain, such that it can be identified with SMC3 immunostaining alone (stage b).

Stage c – folding of the sex chromosome chain is apparent at multiple points reminiscent of the synaptic adjustment observed between the Z and W chicken sex chromosomes (Solari, 1977).

Stage d-f - the majority of the chain appears to further coalesce and by this stage several elements of the chain associate with the nucleolar body.

Stage g - the entire condensed chain is located adjacent to one hemisphere of the large nucleolar body and the cohesin loading now appears to homogenously cover the area occupied by the contracted sex chromosome chain.

Within stage g pachytene cells, the unpaired regions of the sex chromosome chain coalesce and locate to a paranucleolar position. This region also colocalises with an intense DAPI signal further highlighting the condensation of the sex chromosome complex (Figure 6 and Figure 9, also Daish et al. submitted). This condensed body of X and Y specific DNA is also coincident with a more homogenous SMC3 signal. The X and Y specific FISH signals, including the Y4 chromosome paint, resided entirely within this region of SMC3 paranucleolar accumulation (Figure 8, Figure 9 and 10). Thus it appears that at this stage SMC3 accumulates over all of the X and Y specific chromatin, as no FISH signals were observed outside of this region. This association of intense SMC3 staining with a DAPI intense region remains until early diplotene after which time the intense SMC3 staining colocalises with DAPI poor foci that correspond to the protein bodies described above (Figure 6 and Figure 7).

Stage h - From late pachytene we observed formation of small round DAPI negative SMC3 positive spheres (Figure 7). These protein accumulations are also observed in the silver staining (Figure 2, pink arrows/arrowheads). Initially the protein bodies increase in number with up to five large (Figures 2 and 7, pink arrowheads) and numerous small foci visible

during early diplotene (Figures 2 and 7, pink arrows). This suggests that SMC3 is rapidly removed from the sex chromosome chromatin at late pachytene and subsequently concentrated into DAPI negative proteinaceous foci.

During diplotene platypus SCs rapidly contract and the axial cores and central elements concurrently disassemble

During diplotene the murine central element begins to dissolve while the axial elements are still clearly visible resulting in completely desynapsed chromosomes being joined only by chiasmata (Solari, 1970a). In contrast to this, we never observed desynapsing SCs in platypus. Instead the axial cores appear to rapidly compress to form a zig-zag pattern while more SMC3 protein bodies form and as the central elements and axial cores dissolve (Figure 7, late diplotene panel). Furthermore, by diplotene there is no longer any association of SMC3 with the sex chromosomes. This is in stark contrast to mouse where by prometaphase, only the X and Y chromosomal axial elements have SMC3 (Figure 7, prometaphase inset) while the autosomes show only a few scattered SMC3 foci. In platypus late prometaphase, we consistently observed two different sized spherical SMC3 positive regions, both of which were DAPI negative (Figure 7, red and yellow arrows). In both cases there was also a small region of SYCP1 staining directly adjacent to the SMC3 foci (Figure 7, blue arrows), reminiscent of the organisation of the dense plate observed in marsupials.

Differential SMC3 loading on paired and unpaired regions of platypus sex chromosomes at prophase I

In order to investigate the distribution of SMC3 on the sex chromosome chain in more detail we immunostained for SMC3 in combination with DNA FISH with a selection of BACs and

chromosome painting probes targeting XY specific and shared regions of the 10 sex chromosomes in platypus. After SMC3 immunostaining, imaged cell coordinates were recorded prior to a series of DNA FISH experiments. Firstly we used probes targeting each end of the chain, including four X1 and a Y5 specific BAC (Table 1). From early zygotene to mid pachytene both probe sets hybridised to chromosomal DNA which lacked a central element (SYCP1) but with axial element associated SMC3 staining. Subsequent DNA FISH with a Y4 specific probe also showed colocalisation with regions lacking a central element. By contrast, from zygotene through to diplotene, probes targeting XY shared regions (PARs) localised to elements with cohesin loading and a fully formed central element (Figure 8).

We next determined if there is a difference in SMC3 loading between XY shared and XY specific regions on the platypus sex chromosome chain during pachytene. In a separate immuno-FISH experiment, we carried out BAC DNA FISH in five consecutive experiments on the same cells to determine the location of almost the entire chain at mid pachytene and SMC3 loading in the same set of nuclei. The experiments were carried out using BACs in the following order: 1; five X5 BACs and four X1 BACs, 2; X1Y1 (PAR1) and Y4X5 (PAR 8), 3; X3 and Y3, 4; Y3X4 (PAR6) and X2Y2 (PAR3), 5; Y2 and Y5. This showed that the X and Y specific sequences (X1, Y2, X3, Y3, X5 and Y5) consistently reside within SMC3 accumulations lacking a central element (Figures 9a and 9b). In contrast, the PARs (X1Y1, X2Y2, Y3X4 and Y4X5) were outside or on the periphery of the SMC3 staining and in all cases had an associated central element.

From late pachytene, some X and Y specific probes lost association with SMC3 concomitant with the formation of protein bodies, while the PAR probes maintained localisation within SMC3 positive chromosomal regions with central elements (Figure 8). By late diplotene,

there were no observed associations of the X and Y specific regions with SMC3 which had been rapidly removed from the sex chromosome chromatin by this stage (Figure 8).

DISCUSSION

This is the first detailed analysis of the organisation of the most complex mammalian sex chromosome system at male meiotic prophase. We found a remarkably complex and dynamic sex chromosome organisation which includes anchoring to a nucleolar structure, backfolding, progressive condensation and importantly differential recruitment of SMC3 to paired and unpaired parts of the sex chromosome chain.

The consistent association of the sex chromosome chain with a large singular nucleolus, beginning in zygotene and continuing throughout pachytene and into diplotene contrasts to what is seen in other species, such as mouse, where this association begins later, during pachytene, and ceases earlier, by diplotene (Tres, 2005). Relocation of proteins has also been reported during meiotic association of the XY body and nucleolus in pachytene (Escalier and Garchon, 2005b; Tsutsumi et al., 2011a).

In mouse it is well documented that in early prophase, axial elements form before assembly of the central element in prophase (Dobson et al., 1994). In platypus, we observed simultaneous formation of axial and central elements. In mouse during central element disassembly the axial elements persist in diplotene, while in platypus both parts of the SC dissolved at the same time. In platypus the unpaired regions of the sex chromosome chain lose most of their SMC3 loading by early diplotene, whereas in mouse, not only does this association continue into late diplotene, but by pro-metaphase only the sex chromosomes still exhibit SMC3 staining at the axial core. Whether this change in SC formation is related to the sex chromosome complex is currently unknown and requires further investigation. Furthermore, in SMC3 staining and silver staining experiments we observed small round

spheres of signal, these round spheres are DAPI poor and are reminiscent of the protein bodies that form at the centromeres in avian lampbrush chromosomes where they are implicated in having a role in structural maintenance of these chromosomes (Krasikova et al., 2005).

We observed a remarkably different recruitment of cohesin to the X and Y specific regions in the complex sex chromosome system in platypus. In the majority of species investigated thus far, cohesin loading on the sex chromosomes appears weaker compared with the autosomes, likely due to the fact there is generally only one chromosomal axis on the sex chromosomes, whereas the signal observed between autosomal homologues is created from the close proximity of two axial cores (Eijpe et al., 2000; Valdeolmillos et al., 2007; del Priore and Pigozzi, 2012). Such a phenomenon has also been observed for grasshopper B chromosomes (Viera et al., 2004). Interestingly, in grasshopper preleptotene cells the X chromosome lacks SMC3, however by leptotene the levels on the X are similar to the autosomes and by pachytene the loading appears weaker than that observed on the autosomes (Valdeolmillos et al., 2007), indicating that the distribution of cohesin on sex chromosomes can be stage specific and dynamic. In contrast to this, other mammals such as the armadillo, have SMC3 signals on the unpaired sex chromosome axial cores indistinguishable in intensity from autosomal SMC3 despite the signal being produced from a single axial core (Sciurano et al., 2012). This indicates there is more cohesin loading onto sex chromosome axial cores than on the autosomes. In horse this is even more pronounced, where the X shows even greater SMC3 recruitment at its axial core than that seen on the autosomes (Baumann et al., 2011). In the rat it has been observed that cohesins form foci scattered along the axial core length, similar to a 'beads on a string' pattern (Eijpe et al., 2000). Overall this seems to indicate a trend towards altered cohesin density on sex chromosomes.

In platypus in early pachytene, the cohesin subunit SMC3 is present on autosomal SCs as in other species, however it undergoes increased recruitment onto the unpaired sex chromosome axial cores. These results are somewhat similar with those previously reported in horse where SMC3 is enriched on the axial core of the sex chromosomes (Baumann et al., 2011). However, in platypus, we only see this enrichment on the unpaired regions of the sex chromosome chain and not the PARs. We also find enrichment of SMC3 on monotreme sex chromosomes is fundamentally different in that SMC3 is also recruited to the chromatin loops on unpaired regions. This enrichment is transient and disappears by late diplotene with complete loss from the unpaired regions of the sex chromosome chain (Figure 7 and 10). SMC3 enrichment also colocalises with an intense DAPI signal that disappears by late diplotene. This is in contrast to what happens in mouse, where by pro-metaphase, only the sex chromosomes have axial core SMC3. These observations raise the question of whether cohesins have a different function when on the unpaired proportion of sex chromosomes such as a role in gene silencing, a property of cohesin that has recently emerged (Seitan et al., 2013) including the insulation of promoters (Wendt et al., 2008). This is supported by our observation of a highly organised and hierarchical pairing of individual sex chromosome pairs (Daish et al., 2009) and higher order organisation of the chain during prophase including specific association with the large nucleolus which also shows SMC3 enrichment. Such enrichment has also been observed in plants where it may affect gene expression (Yuan et al., 2012). Cohesin has also been reported to be present at specific heterochromatic regions such as centromeric heterochromatin where it is recruited by Suv4-20h2 (Hahn et al., 2013) and at the mating-type heterochromatic region of fission yeast where it is recruited by Swi6(HP1) (Nonaka et al., 2002). Together with the above described observations of sex chromosome condensation, cohesin recruitment to the chromatin of the platypus sex chromosomes may be a signature of heterochromatin formation. In

monotremes so far there is no reported evidence for transcriptional silencing of their sex chromosomes but the absence of retrogenes has led to predictions that MSCI does not occur in platypus (Potrzebowski et al., 2008b), however the DAPI positive staining which weakens coincident with SMC3 removal may suggest a state of transient chromatin silencing occurs on platypus sex chromosomes during pachytene. A similar transient sex chromatin silencing has been observed in birds during late prophase I (Solari, 1977).

Our results show that reorganisation of SMC3 distribution coincides with sex chromosome condensation during pachytene. It is interesting that in birds the ZW pair undergo synaptic adjustment and equalisation (Solari, 1977; Schoenmakers et al., 2009b). Possibly this method of sex chromosome organisation is a signature of a common ancestral mechanism of managing heteromorphic sex chromosomes in the case of monotremes and birds that share extensive homology (Grutzner et al., 2004b; Veyrunes et al., 2008b). Synaptonemal complex proteins are known to play a role in the association of sex chromosomes that lack homology. In marsupials, SC protein accumulation forms the dense plate at the nuclear periphery which is proposed to mediate and maintain sex chromosome association in the absence of any PAR region (Page et al., 2003a). Cohesin is essential for normal SC function, and accumulation on the axial elements of sex chromosomes has been observed in several mammalian species. Our results show that this accumulation is also conserved in monotreme mammals. To our knowledge this is the first report showing an association of SMC3 on XY specific chromatin and not just on the axial core. The coating of unpaired chromatin with SMC3 may aid self-association of the large sex chromosome complex independent of homology.

In summary we have shown that organisation of the platypus sex chromosome complex during prophase shows distinct and dynamic cohesin loading that is differential between paired and unpaired parts of the sex chromosome chain (Figure 11). Consistent association

of the NOR, sequential folding of the chain and increased DAPI intensity which is quickly reversed at the pachytene to diplotene transition suggests that cohesin accumulation may play a vital role in meiotic organisation and potentially segregation of the platypus sex chromosome complex.

METHODS

Specimen Collection

Platypus specimens were captured by netting in 2002 (AEEC permit R.CG.07.03 (F.G.), Environment ACT permit LI 2002 270 (J.A.M.G.), NPWS permit A193 (R.C.J.)) and 2008 (AEC permit no. S-49-2006 (F.G.)) at the Upper Barnard River (New South Wales, Australia) during the breeding season. Mouse testis were obtained from three week old animals (Swiss white).

Storage Preparation

The method of meiotic sample preparation and storage was the same as previously described by Daish et al (2009) (Daish et al., 2009). Briefly the testis were placed in a petri dish containing 1 x PBS with 1 x Protease Inhibitor (Roche). The surrounding tunica albuginea was punctured and the testis material was teased apart using needles resulting in the release of meiotic cells and sperm from the ruptured tubules. A pipette was used to break up and flush the remaining material to ensure maximal release of cells. The resulting cell suspension was mixed with DMSO (to a final concentration of 10%), slow cooled and stored in liquid nitrogen for future use.

Meiotic Spreads

Meiotic spreads of testis material were prepared by a method based on that previously described by Peters et al (1997)(Peters et al., 1997). Briefly, a 75 μ L aliquot of the testis 10% DMSO suspension was thawed. Either 75 μ L 0.3M sucrose or 75 μ L of 75 μ M KCl was added to

the testis material and left to sit at room temperature for 5 minutes. Next 50µL of 1% PFA/0.15% Triton X-100 pH 9.5 was dropped onto slides and spread with a pipette tip. 20µL of the testis material/hypotonic solution was then dropped onto the PFA coated slides. Slides were kept in a moist chamber for 2 hours and then washed in 50 mL of 1 X PBS. Slides were air dried and used immediately for immunostaining or stored at -20°C.

Immunostaining

Immunostaining was carried out as per Schoenmakers et al (2009) (Schoenmakers et al., 2009b). Briefly, slides were blocked in 1 X PBS with 0.5% w/v BSA and 0.5% w/v milk powder three times for five minutes and incubated in a humid chamber with a primary antibody (1:200 dilution) in 1 X PBS/10% w/v BSA for either 2 hours at 37°C or overnight at room temperature. Slides were then washed in 1 X PBS three times for five minutes each and blocked in 10% v/v goat serum in blocking buffer for three times for five minutes each and incubated at 37 °C for 2 hours in a humid chamber with a secondary antibody (1:400 dilution) in 10% v/v goat serum in blocking buffer. Blocking buffer: 5% w/v milk powder in 1 X PBS centrifuged at 13,200 for 10 minutes. Finally slides were washed three times for five minutes in 1 X PBS, dipped in DAPI (1:5000 dilution) for one minute, washed twice with MQ water and mounted with Vectashield (Vecta Laboratories).

Antibodies

To observe the central element we used an antibody (Novus, NB300-229) raised against the central element protein; Synaptonemal Complex Protein 1 (SYCP1). We employed the use of an SMC3 antibody (Abcam, Ab9263) to visualise the axial core in platypus since it has been previously shown that SMC3 recruitment follows synaptic progression (Valdeolmillos et al., 2007)

DNA FISH

A method of Fluorescence In Situ Hybridisation (FISH) based on the original protocol described by McDougall et al (1972) was optimised for PFA fixed meiotic material. Briefly, BAC DNA probes were directly labelled using Klenow and a random 9mer primer overnight with either SpectrumOrange or SpectrumGreen 2'-deoxyuridine-5'-triphosphate (Abbott Molecular). To reduce the background signal on slides, labelled probes were subsequently co-precipitated with salmon sperm and sonicated platypus male genomic DNA. Pellets were dissolved in deionised formamide and 2 X hybridisation buffer containing dextran sulfate. Slides containing the PFA fixed meiotic material were dehydrated in an alcohol series (70-100% ethanol) for 5 minutes each, treated with RNase A (100µg/mL) for 30 minutes at 37 °C, pepsin (to a final concentration of 0.005%) for 10 minutes at 37°C, fixed in formaldehyde (final concentration of 1% formaldehyde) in PBS/50mM MgCl₂, dehydrated in an alcohol series (70-100% ethanol) for 5 minutes each, denatured in 70% formamide /2 X SSC at 70°C for 7 minutes for the first FISH and 3 minutes for each subsequent FISH on the same slide, dehydrated (70-100% ethanol) for 5 minutes each and air dried ready for hybridisation with the labelled probe. The probes were applied to the dry slide and covered with a coverslip, sealed with rubber cement and incubated overnight at 37°C in a moist chamber. The coverslip was removed and slides were washed three times in 50% formamide/2 x SSC followed by 2 x SSC. Slides were washed once in 2 X SSC at 42 °C for 5 minutes, once at 60 °C in 0.1 X SSC for 5 minutes and finally once at 42 °C in 2 X SSC for 5 minutes. Slides were then stained with DAPI for one minute, washed twice with MQ water and mounted with Vectashield (Vecta Laboratories).

DNA FISH Probes

Table 1. Probes used in DNA FISH experiments*.

Probe Name	Chromosome	X/Y specific or PAR	Paper first cited
CH236-804 O01	X1	Specific	-
CH236-378 F21	X1	Specific	Veyrunes et al. (2008b)
CH236-200 G3	X1	Specific	Veyrunes et al. (2008b)
CH236-341 C3	X1	Specific	Veyrunes et al. (2008b)
CH236-286 H10	X1Y1	PAR	Veyrunes et al. (2008b)
CH236-78 K11	X2Y2	PAR	Veyrunes et al. (2008b)
Oa_Bb-145 P9	Y2	Specific	-
CH236-158 M16	X3	Specific	Veyrunes et al. (2008b)
Oa_Bb-462 C1	X3Y3	PAR	Tsend-Ayush et al. (2009)
Oa_Bb-397 I21	Y3	Specific	-
CH236-639 O23	Y3X4	PAR	Veyrunes et al. (2008b)
Y4 paint	Y4	Specific	Grutzner et al. (2004b)
Oa_Bb-466 A15	Y4X5	PAR	-
CH236-820 A16	X5	Specific	Veyrunes et al. (2008b)
CH23-634 B19	X5	Specific	-
CH236-830 M18	X5	Specific	-
CH236-236 A5	X5	Specific	Veyrunes et al. (2008b)
CH236-752 F12	X5	Specific	Veyrunes et al. (2008b)
Oa_Bb-152 P15	Y5	Specific	(Tsend-Ayush et al. (2009))

*Probes were purchased from two libraries, the female platypus library held at the Children's Hospital Oakland Research Institute (CH236) and the male platypus library from the CUGI BAC/EST Resource Centre, Clemson, South Carolina USA (Oa_Bb)

Image Acquisition and Processing

Slides were visualised using a Zeiss AxioImager 2.1 microscope equipped with a 10x ocular and 10x, 20x, 63x and 100x objective lenses. Fluorescent tags were visualised using 3 filters: DAPI, for

DAPI stained DNA; GFP, for SpectrumGreen and Alexa 488 and; DS red, for SpectrumOrange and CY3. Images were recorded via an Axiocam CCD-camera and Zeiss Axiovision software. Unless otherwise stated, images were captured using the 100x objective lens, appearing as 1000x with the 10x ocular. Images were processed using Axiovision (Zeiss), GIMP 2.8 (<http://www.gimp.org/>) and ImageJ (National Institutes of Health, United States; <http://rsb.info.nih.gov/ij>).

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Figures

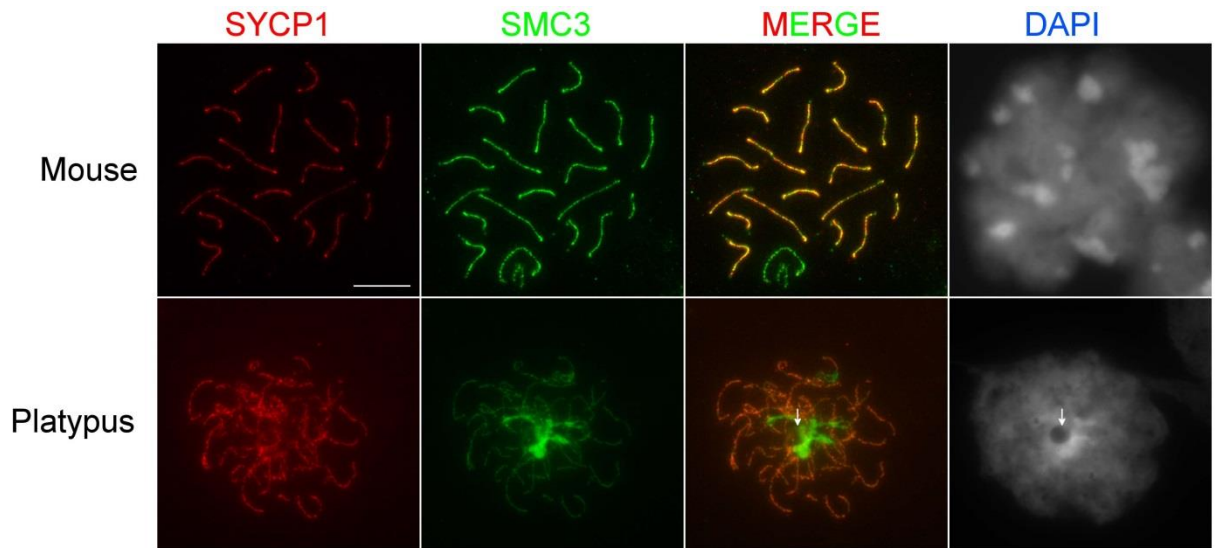
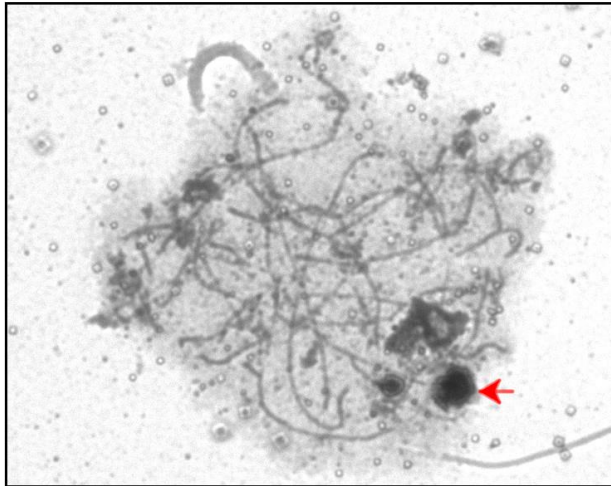
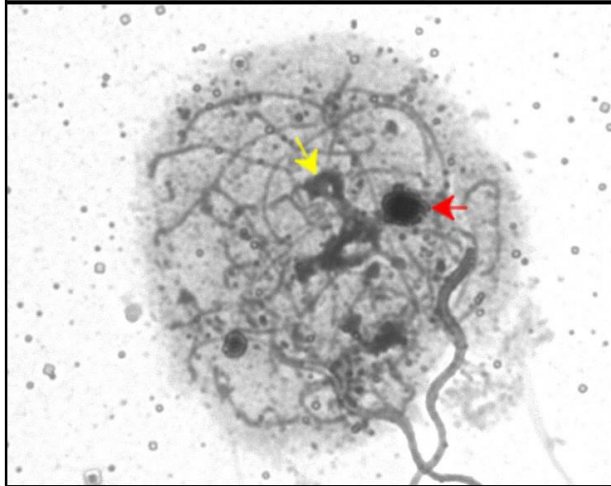


Figure 1. SMC3 and SYCP1 localisation in mouse (upper panel) and platypus (lower panel) pachytene cells. SMC3 localises to the axial core of all chromosomes while SYCP1 localises only to synapsed chromosomes, thus, the sex chromosomes appear as the only chromosomes exhibiting axial core signals without a central element signal. The signal in the platypus showed a significantly higher level of SMC3 recruitment on regions without a corresponding axial signal. This region also consistently associated with a large, spherical, DAPI poor region of the cell (white arrow). Scale bar = 10 μ m.

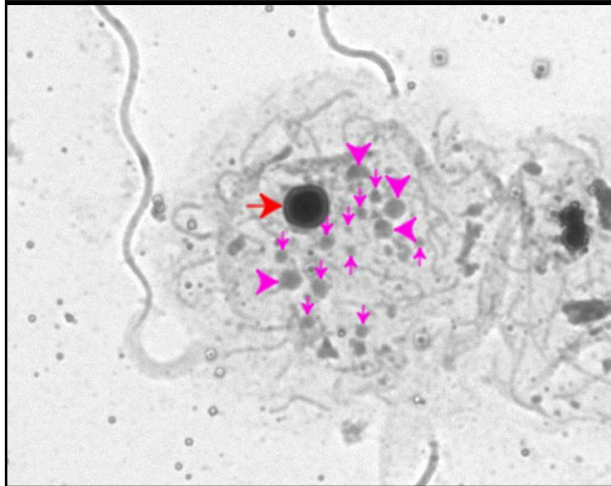
Zygotene



Pachytene



Diplotene



Pro-
Metaphase

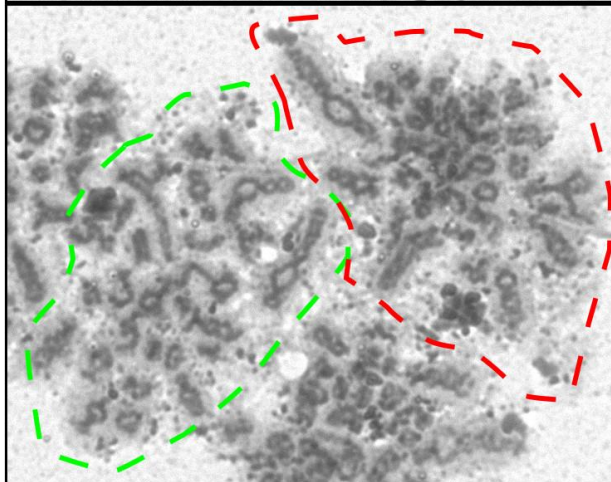


Figure 2. Silver staining of platypus pachytene cells. From zygotene to diplotene there is a large spherical structure that is heavily stained that represents the platypus single giant nucleolus (red arrow). During pachytene there is a second darker stained region (yellow arrow), that is less intense than the nucleolus to which it is attached. This region most likely correlates to the region of SMC3 accumulation (ie the sex chromosomes). In diplotene many smaller spherical bodies are visible, usually four larger bodies (large pink arrowheads) and numerous smaller bodies (small pink arrowheads). By pro-metaphase (two cells are outlined by red and green dashes), there is no obvious nucleolus and the axial core clearly has significant protein recruitment, having a darker core than seen in any of the other stages. Scale bar = 10 μ m.

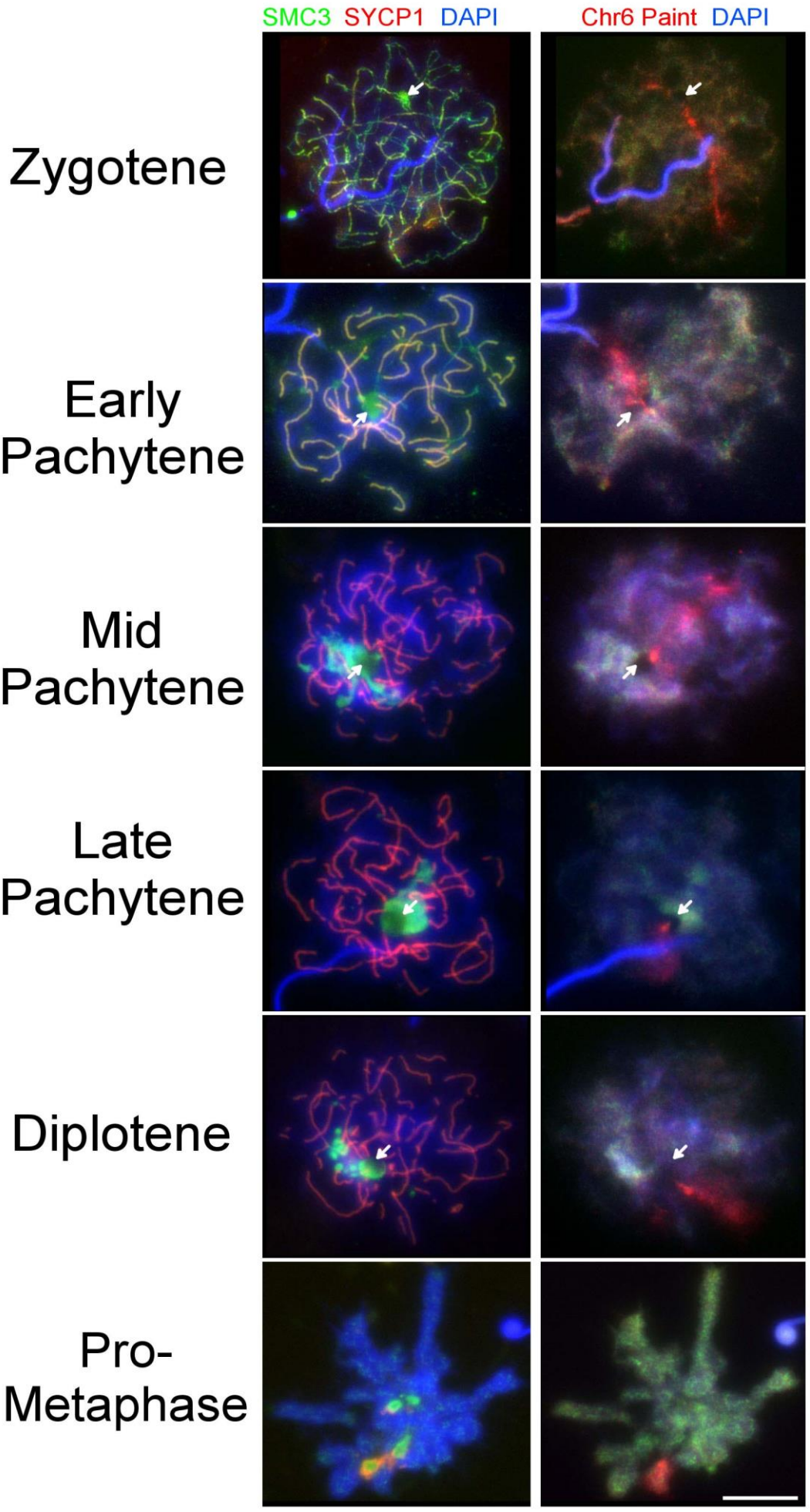


Figure 3. Chromosome 6 localisation within platypus prophase I cells. In zygotene, chromosome 6 intersects the DAPI poor region, there is also SMC3 recruitment at this region. At this stage the DAPI poor region is located closer to the nuclear periphery. In early, mid and late pachytene cells, as well as diplotene cells, the DAPI poor region is located closer to the centre of the cell. In all cases, the chromosome 6 paint intersects this region on the opposite pole to where the sex chromosomes are located. By pro-metaphase, we observe chromosome 6 at the nuclear periphery, but adjacent to the most intense SMC3 stained region that also has some SYCP1 association. Scale bar = 10 μ m.

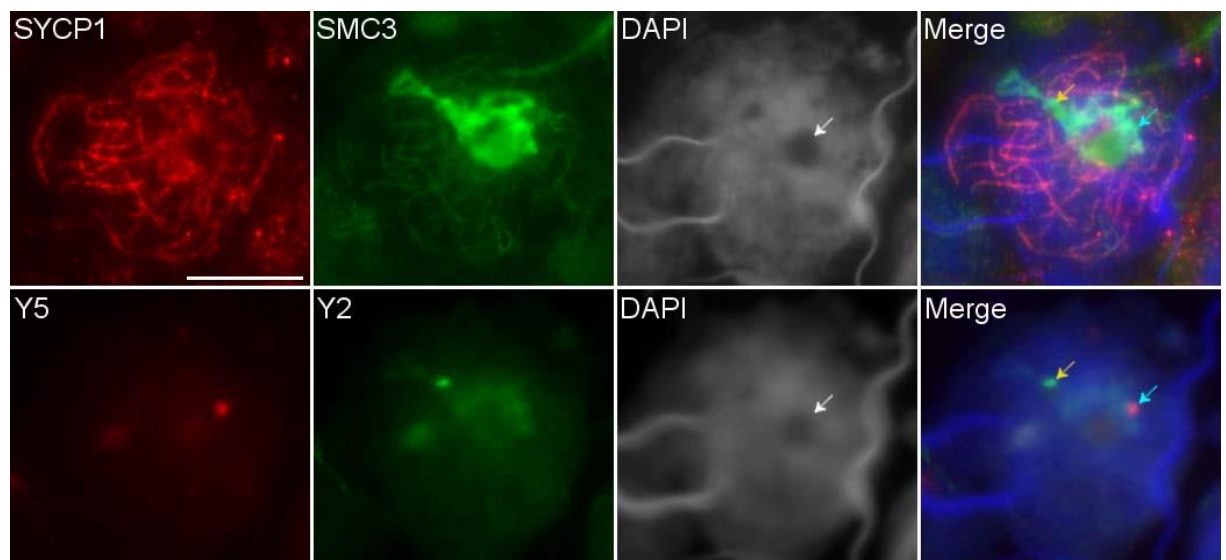


Figure 4. Colocalisation of sex chromosomes with SMC3 accumulation in platypus pachytene cell nuclei. Y specific probes; Y2 (yellow arrow) and Y5 (green arrow). The region of SMC3 accumulation is also associated with a large DAPI poor region (white arrow). Scale bar = 10 μ m.

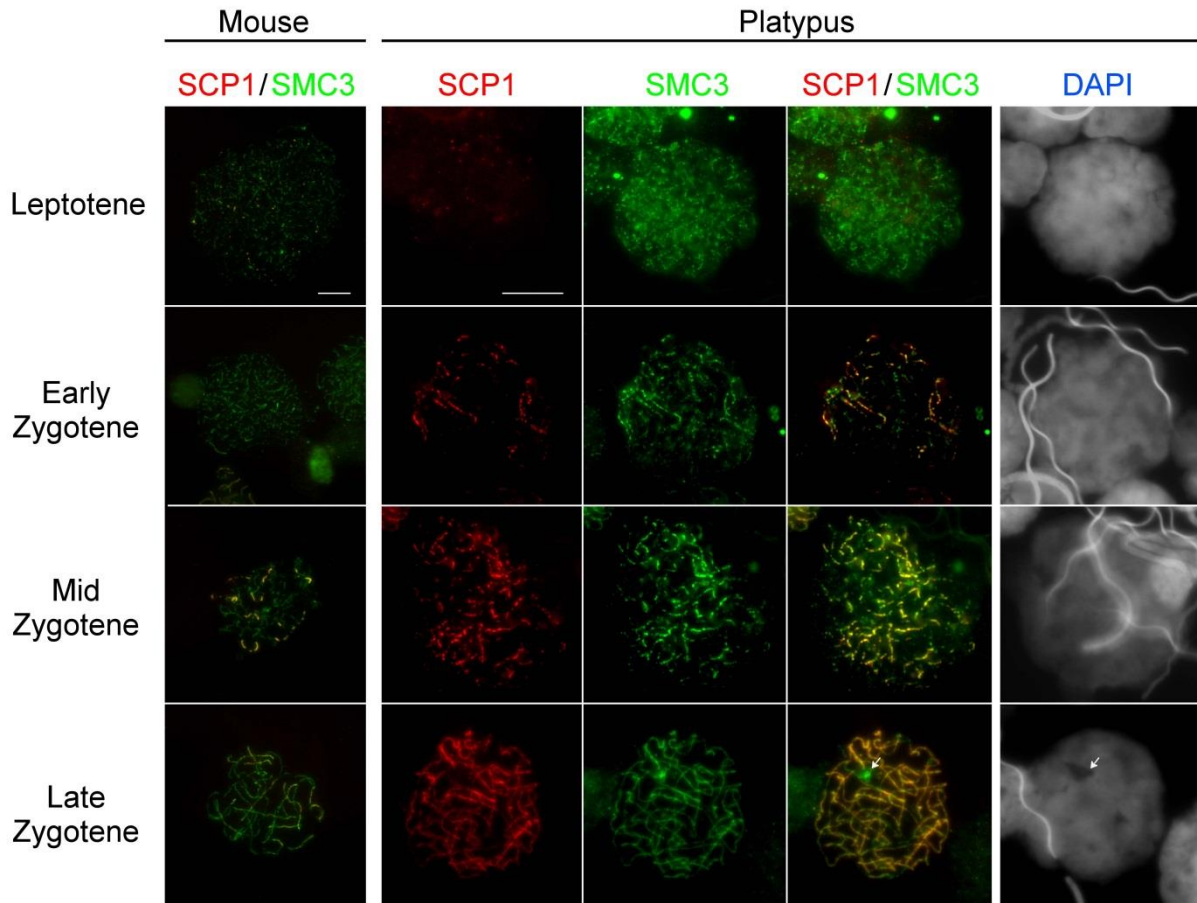


Figure 5. Immunolocalisation of SMC3 and SYCP1 in mouse and platypus early prophase I cells. In leptotene, both platypus and mouse cells exhibit a homogenous speckling of cohesin throughout the nucleus. By mid zygotene in mouse, many axial elements are visible with only a few with a partial central element. In contrast to this, in platypus we observe that most formed axial elements also have a central element. In late zygotene the giant nucleolus begins to form (white arrow). Scale bar = 10µm.

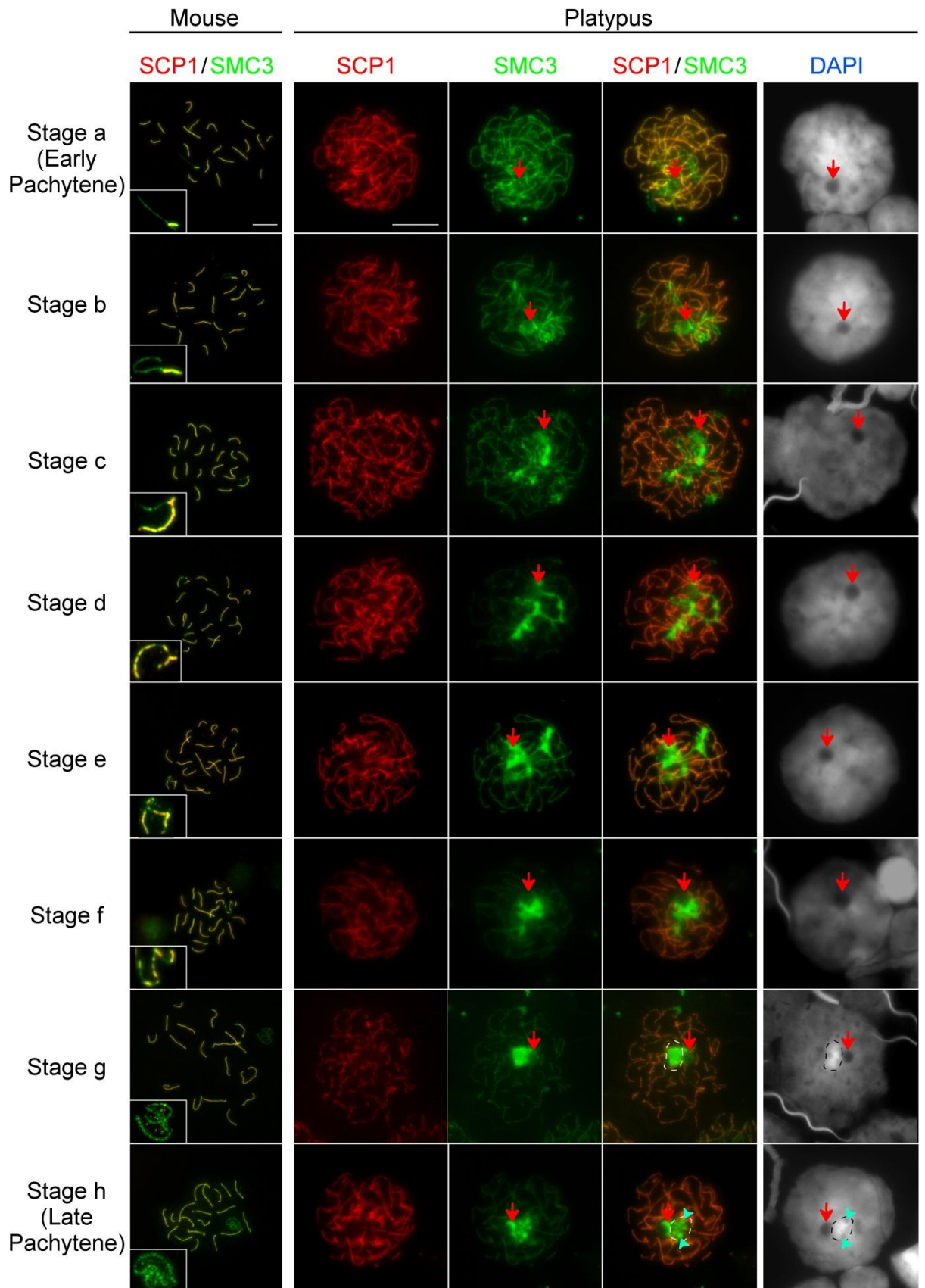


Figure 6. Substages of mouse and pachytene cells based on their sex chromosome configuration and synaptonemal complex length. There are eight distinct mouse sex

chromosome patterns for each stage (insets). In all stages the platypus sex chromosome chain is attached to a giant nucleolus (red arrow). In platypus stage g and h nuclei, the region of significant cohesin accumulation colocalises with a DAPI intense region (black/white dashed surround). In platypus late pachytene, protein bodies are observed (light blue arrowheads). Scale bar = 10µm.

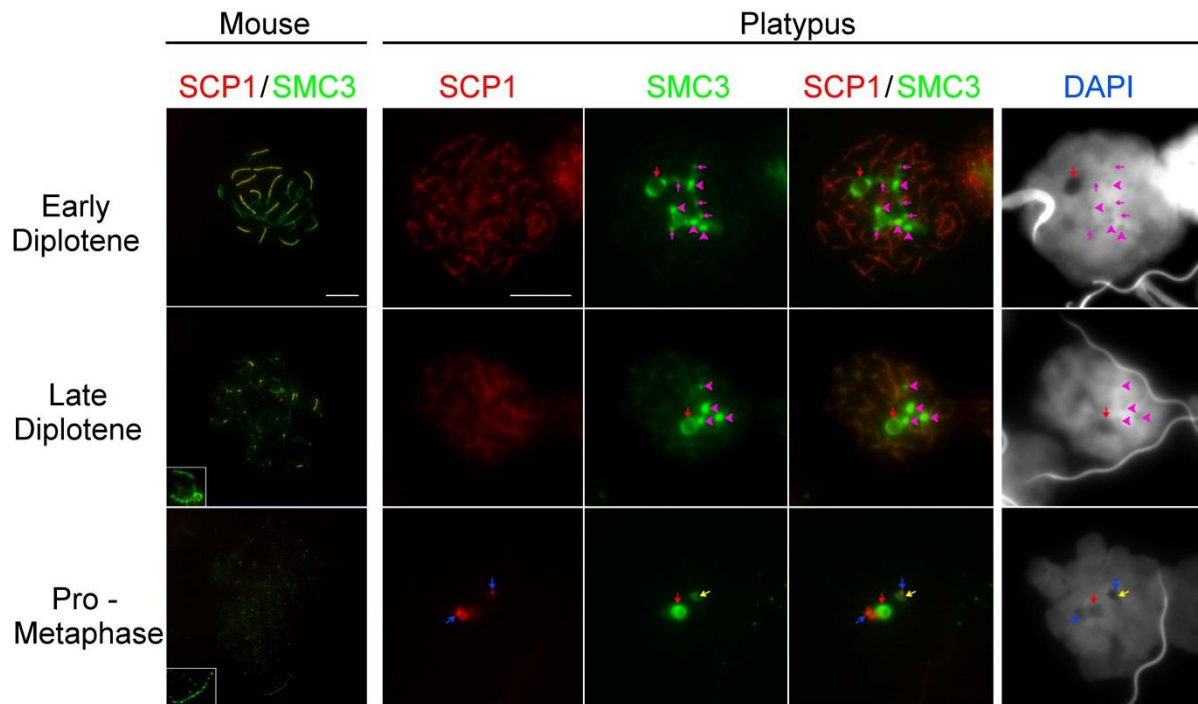


Figure 7. Diplotene to pro-metaphase in mouse and platypus prophase I cells. In platypus, from early diplotene four large protein bodies (large pink arrow heads) and many small protein bodies (small pink arrowheads) are observed and the large nucleolus persists (red arrow). In mouse pro-metaphase cells, only the sex chromosomes have SMC3 staining at the axial core, while all such staining is lost from the autosomes. In platypus there is only weak SMC3 staining scattered on the chromosomes in pro-metaphase. Also in platypus pro-metaphase cells, there is a larger region that contains SMC3 (red arrow) and a smaller region that contains SMC3 (yellow arrow). Both regions also have a smaller associated region containing SYCP1 (blue arrows). Scale bar = 10µm.

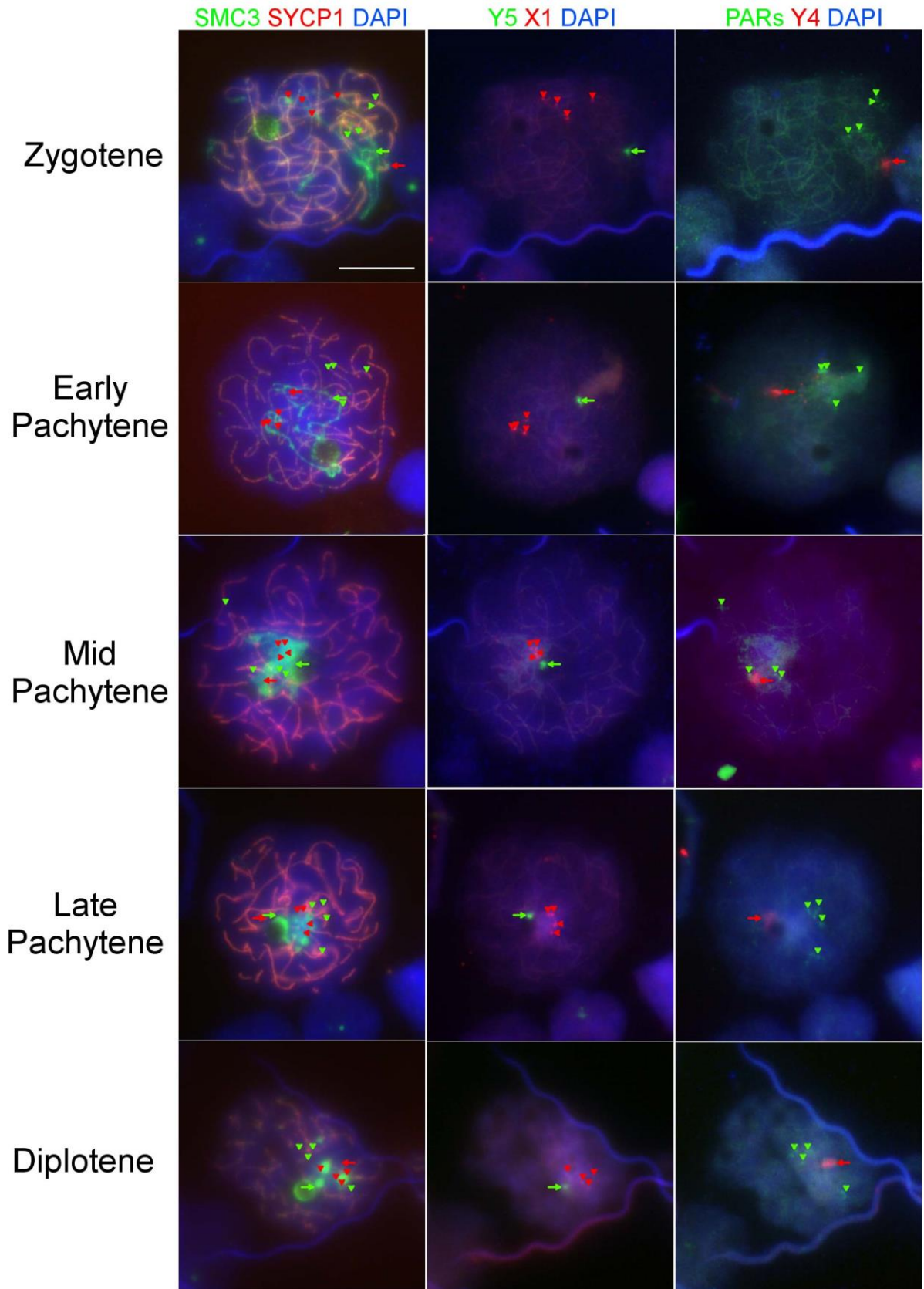


Figure 8. Sex chromosome localisation during prophase I. Four X1 probes (red arrowheads), a Y5 probe (green arrow), a Y4 paint (red arrow) and four PAR probes (green arrowheads) are visible in each of the stages. From zygotene to pachytene, the X and Y specific probes localise to regions that have only SMC3 labelling, but no central element staining.

Importantly in mid pachytene, no X or Y specific signal extends to a region outside of that which has increased SMC3 accumulation. In contrast, the PAR probes consistently localise outside of this region and localise over, or adjacent to, central elements. In diplotene, the X and Y specific probes localise to regions adjacent to, but not within, protein bodies containing SMC3, while the PAR probes localise to regions over, or adjacent to, central elements. Scale bar = 10 μ m.

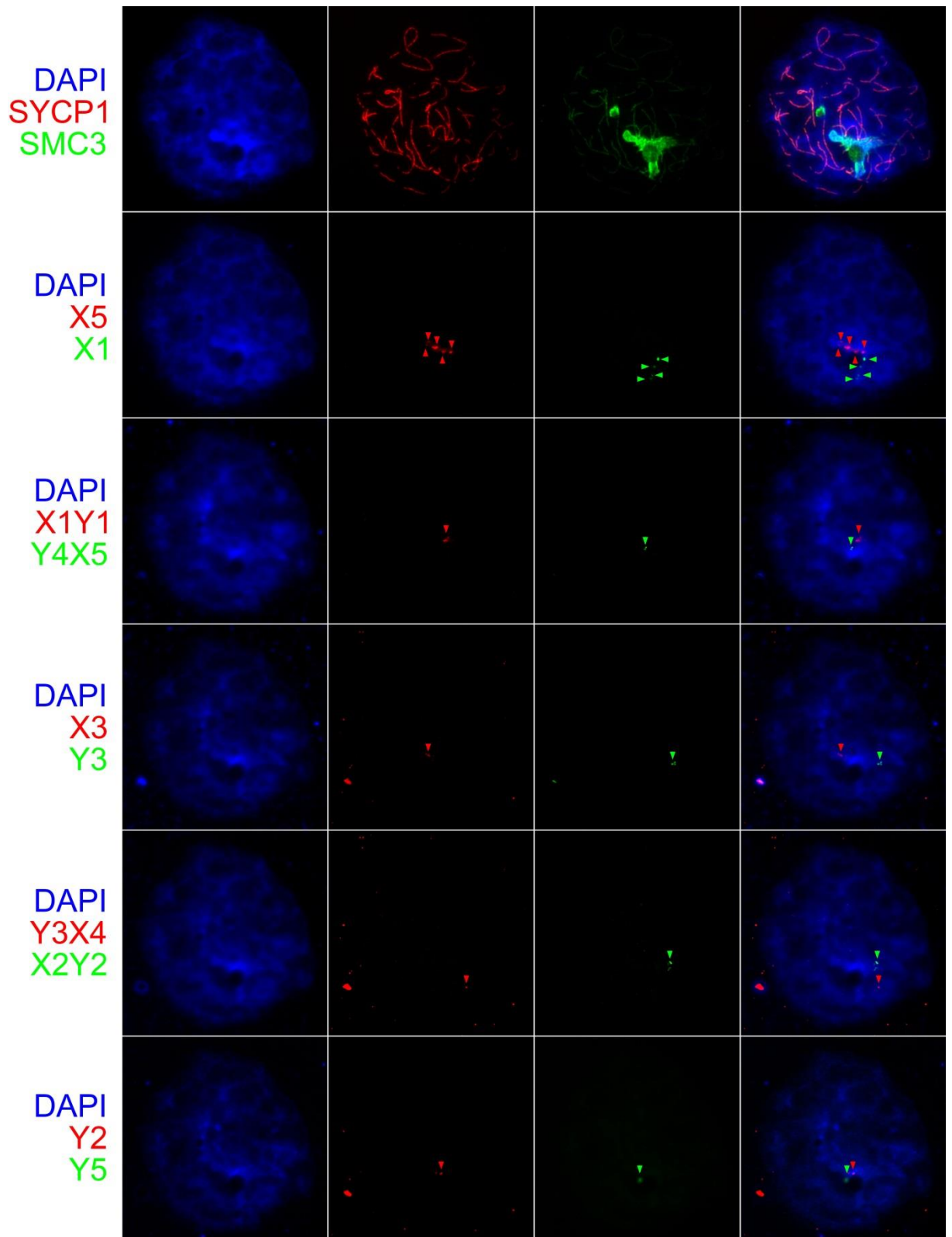


Figure 9. Sequential sex chromosome specific immuno-FISH on a platypus stage g pachytene cell. For each sequential experiment red probes are marked with red arrows and green probes with green arrows.

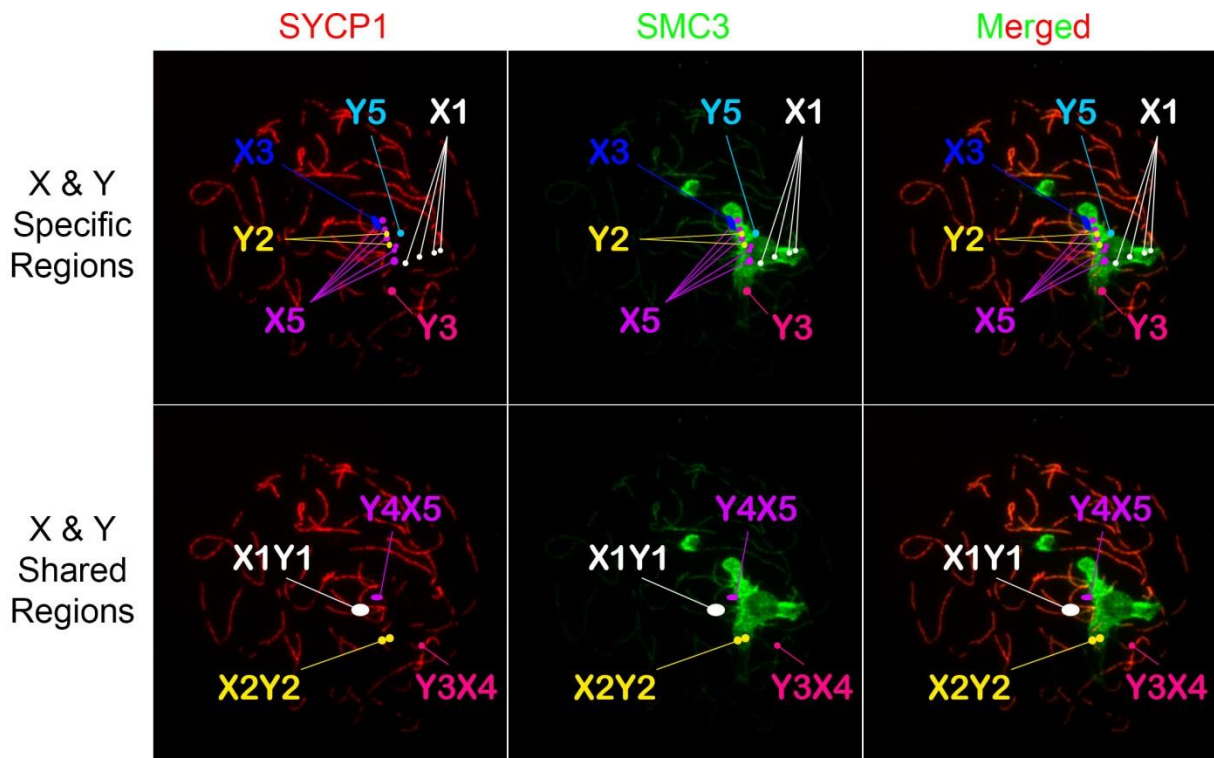


Figure 10. Overlay of the various probes onto the original immunostaining image from figure 9. It is clear that for all cases of X and Y specific probes they reside within the region with significant cohesin recruitment, while the PAR probes reside outside the region with significant cohesin recruitment. Scale bar = 10µm.

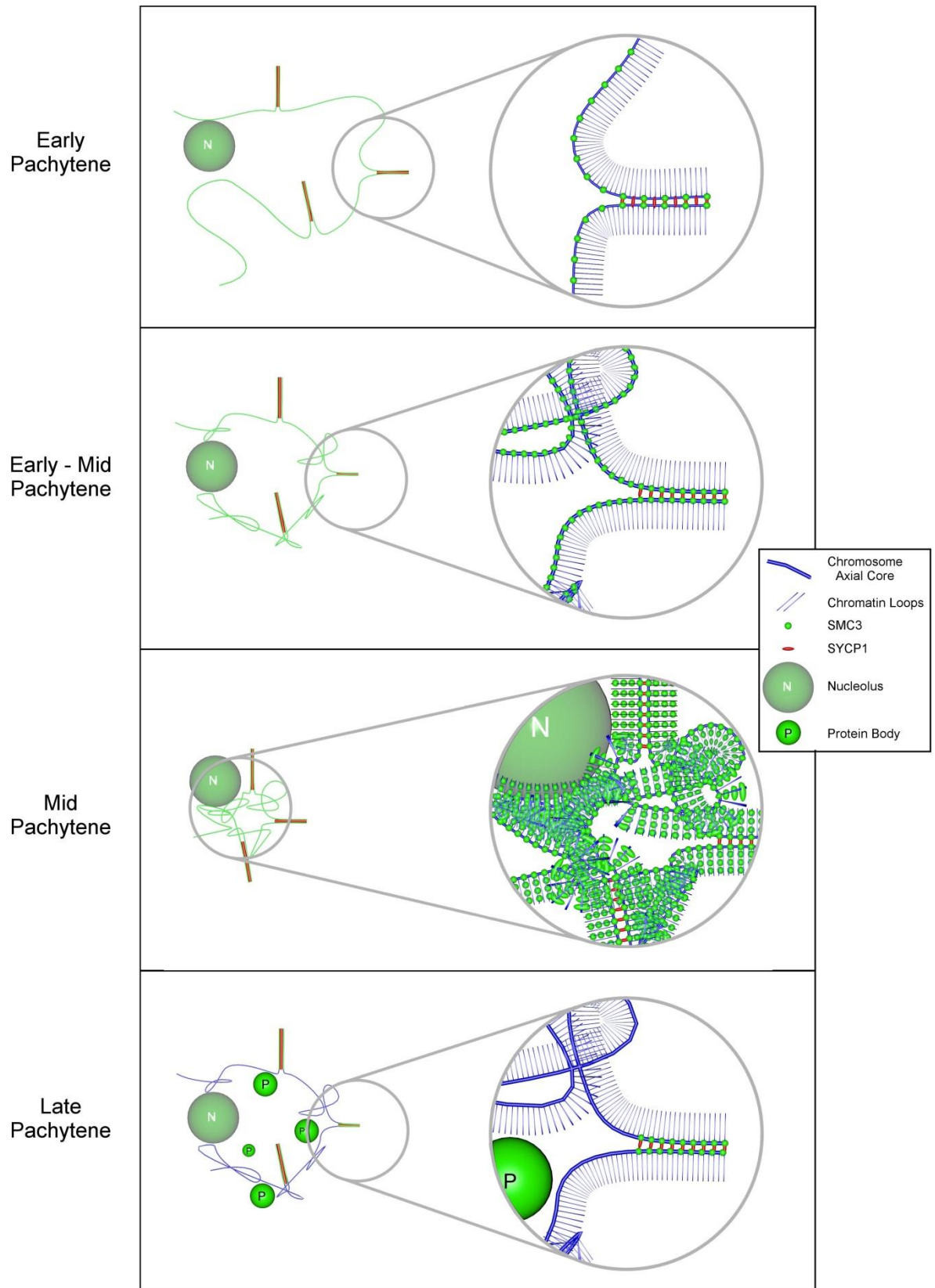


Figure 11. Schematic of platypus sex chromosome and cohesin dynamics during pachytene.

In early pachytene, the sex chromosome chain appears as a long thin element with unremarkable SMC3 accumulation. By early-mid pachytene, the sex chromosome axial core

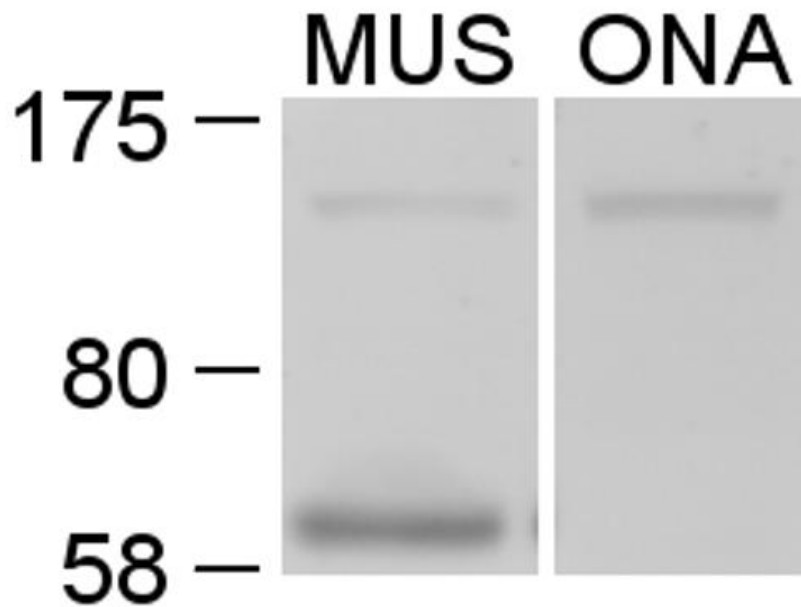
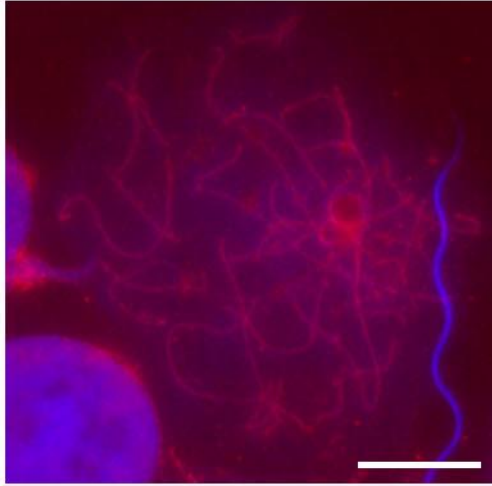


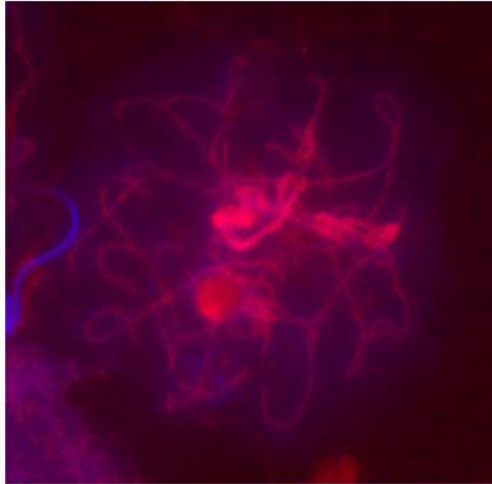
Figure S2. Western analysis of primary antibody. Nuclear extracts from testis were obtained from mouse (MUS) and platypus (ONA) which were subjected to immunoblot analysis using the polyclonal SMC3 antibody. In platypus and the mouse control, a band is observed at the expected size of 140kDa. The mouse control also shows a second band at roughly 60kDa, while in the platypus, only the band of expected size is observed.

STAG3 DAPI

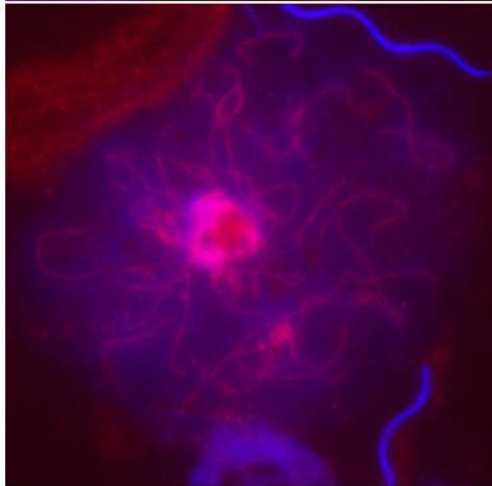
Early
Pachytene



Early-Mid
Pachytene



Mid-Late
Pachytene



Diplotene

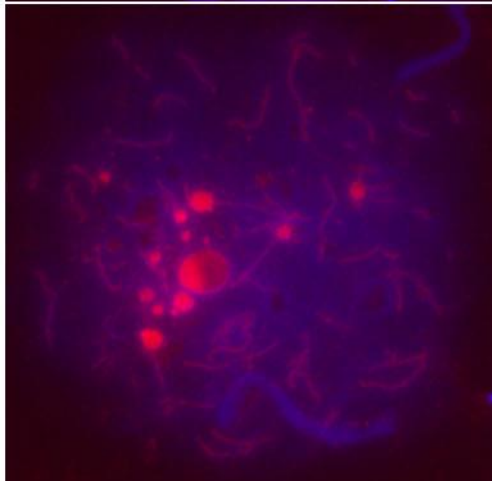


Figure S3. Immunostaining patterns of STAG3 in platypus prophase I cells. Patterns from early pachytene through to diplotene are represented. The patterns observed match those observed in SMC3 immunostained platypus prophase I cells.

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Chapter 6: Sex Chromosome Inactivation in the Platypus During

Prophase I (Paper IV)

Casey, A.E., Daish, T.J. and Grutzner, F. (2014). Monotreme sex chromosome and repressive histone mark nucleolar association in meiosis may be the first step in mammalian MSCI evolution. Submitted to "PLOS Genetics"

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Author Contributions

Name of Candidate	Aaron E. Casey	
Contribution to paper	Co-designer of experiments. Carried out immunostaining and FISH for H3K9me2, H3K9me3, RNApolIII and γ H2AX in γ -irradiated pachytene cells. Had input on writing of manuscript and significant input on generation of figures.	
Signature		Date 20.12.14

Name of Co-Author	Tasman J. Daish	
Contribution to paper	Co-designer of experiments. Methanol acetic acid fixed meiotic material with DNA FISH. Fibroblast and meiotic irradiation. γ H2AX, H2AFY, RPA32, Fibrillarlin immunostaining. Cot-1 RNA FISH. Sedimentation fractionation of meiotic samples. RT-PCR. Primary manuscript author.	
Signature		Date 12/12/14

Name of Co-Author	Frank Grutzner	
Contribution to paper	Co-designer of experiments and input on writing of manuscript.	
Signature		Date 19/12/14

Monotreme sex chromosome and repressive histone mark nucleolar association in meiosis may be the first step in mammalian MSCI evolution

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Abstract

Heteromorphic sex chromosomes present many challenges to the complex programme of synapsis, recombination and segregation in meiosis I. Monotreme mammals have an elaborate sex chromosome arrangement with the platypus having 5X and 5Y highly differentiated sex chromosomes and therefore present additional and unique complexity throughout meiosis in terms of unpaired DNA checkpoints, recombination and segregation. We have assessed the state of meiotic silencing in platypus prophase I using a combination of molecular approaches to detect whether platypus sex chromosomes have a functional meiotic silencing pathway. We found no evidence for a therian like programme of meiotic silencing in platypus instead observing an avian like generalised transcriptional suppression in pachytene and an absence of hallmark epigenetic marks on the sex chromosomes.

Interestingly we observed nucleolar tethering of the sex chromosome complex and association with a perinucleolar region of silenced chromatin. This may represent the first steps of the evolution of a sex chromosome specific silencing program in mammals.

Author Summary

Meiosis involves a highly regulated program of gene regulation and the critically important process of silencing gene expression on the X and Y sex chromosomes. Disruptions to this process result in sterility and cell death and therefore is an absolute requirement for successful production of viable sperm and fertility. Platypus are the most ancient mammalian lineage called monotremes yet surprisingly their sex chromosomes do not have the same set of genes as other mammals and currently it is not known whether sex chromosome gene silencing is required as in other mammals. Intriguingly, platypus have 10 sex chromosomes which form an alternating XY chain during meiosis which raises fascinating questions about how mammals evolved from this system to the current human XY system. Here we have studied gene expression and chromosome dynamics in platypus meiosis and discovered that the common mammalian form of meiotic silencing evolved after the split from monotremes and provide insight into the nature of how this process may have evolved by observing the behaviour of the future mammalian sex chromosomes in monotreme meiosis.

Introduction

The evolution of heteromorphic sex chromosomes resulted in substantial changes in the mammalian meiotic cell in regard to the homologue pairing and recombination programme and in terms of checkpoints which regulate meiotic progression following unpaired DNA detection. Homologue pairing, or synapsis, is aided by assembly of the synaptonemal complex (SC) and is required for the resolution of Spo11-induced DNA double stranded breaks (DSBs) and chiasmata formation, the points at which the paternal and maternal genetic material undergo recombination (Zickler and Kleckner, 1999; Page and Hawley, 2003; Cole et al., 2010). Therian sex chromosome homology, pairing and recombination is restricted to the pseudoautosomal region (PAR), consequently this leaves the majority of the sex chromosome DNA unpaired through meiotic prophase (Burgoyne, 1982). Numerous strategies have independently emerged through evolution to deal with the presence of heterologous sequences during the period of synapsis and recombination revealing the dynamic and adaptive nature of regulatory mechanisms in the germline in response to sex chromosome divergence.

The hallmark feature of pachytene meiotic cells in male mammals is the transcriptional silencing of genes residing on unpaired sex chromosome DNA, termed Meiotic Sex Chromosome Inactivation (MSCI) (Monesi, 1965b; Kierszenbaum and Tres, 1974; McCarrey et al., 1992; Turner, 2007). This response is thought to have been borne from a more general mechanism which detects and silences all unpaired sequences (Meiotic Silencing of Unpaired Chromatin; MSUC) via utilisation of DNA damage repair (DDR) pathway components and recruitment of epigenetic remodelling effectors (Schimenti, 2005; Zamudio et al., 2008). While MSUC is thought to prevent transmission of cells with aberrant pairing arrangements by enforcing a checkpoint and triggering a programmed cell death response (Turner et al., 2006), therian MSCI is postulated to prevent the exchange of genetic material between

heterologous sequences or the escape from MSUC checkpoint detection (McKee and Handel, 1993; Inagaki et al., 2010).

Variations on the MSCI theme are present in diverse species spanning large evolutionary timeframes such as fungi, nematode, insects, birds and various mammals such as opossum, mouse and human however there are fundamental differences in the mode by which the sex linked genes are repressed as well as recent conflicting conclusions on the presence of a functioning silencing mechanism in specific species. For example, mammals and Nematode repress meiotic expression by direct epigenetic modification while *N. crassa* achieves meiotic silencing post-transcriptionally via an Argonate/RISC pathway response termed Meiotic Silencing of Unpaired DNA (MSUD) (Shiu et al., 2001) and recent reports have disputed the presence of MSCI in *Drosophila* and chicken (Mikhaylova and Nurminsky, 2011; Guioli et al., 2012a). Clear distinctions are also present in the manner by which sex chromosomes associate through meiosis; while the mouse and human XY mediate pairing initially by PAR synapsis, the marsupial XY which lack a PAR are tethered separately to a dense body structure (Solari and Bianchi, 1975; Page et al., 2003b) and in female chicken the ZW undergo complete pseudosynapsis (synaptic adjustment) between heterologous sequences (Solari, 1992a).

Monotremes present a fascinating opportunity to further our understanding of meiotic silencing in an evolutionary context. The majority of sex chromosome DNA has homology to the chicken Z however the heterogametic sex, unlike chicken, is male (Rens et al., 2007; Veyrunes et al., 2008a). Platypus have a complex 5X and 5Y sex chromosome system which via multiple PAR pairing forms a chain during prophase I in preparation for alternate XY segregation (Grutzner et al., 2004a; Rens et al., 2004; Daish et al., 2009). Thus determining the status of monotreme MSCI may not only reveal potentially novel meiotic silencing

mechanisms but also refine our understanding of how therian-like MSCI programmes evolved.

In this study we have taken a first look at the status of MSCI in platypus using DNA FISH, immunohistochemistry and expression analyses to characterise the epigenetic and sex chromosome linked gene activity through prophase I. Surprisingly, unlike other mammals, we did not detect a sex chromosome specific silencing mechanism but observed a more chicken-like schedule of general transcriptional repression with an absence of the hallmark epigenetic MSCI modification, phosphorylation of the H2AFX histone variant. In addition, we saw similarities with chicken regarding the nature of heterologous sex chromosome self-association but also more therian-like associations with repressive histone variants/modifications and the nucleolus. This study reveals avian and mammalian aspects of sex chromosome meiotic dynamics in platypus potentially supporting the idea of a transition from global to sex chromosome specific silencing in mammals.

Results

The platypus sex chromosome chain forms a condensed body at pachytene

We performed serial DNA FISH on methanol:acetic acid fixed meiotic cells using sex chromosome specific BAC probes to visualise the distribution of sex chromosomes and assess chromatin compaction status. All cells observed with obvious condensed chromatin fibres indicative of advanced prophase I and autosomal synapsis (evident by single PAR foci indicating synapsis confirming late pachytene stage according to previous work (Daish et al.,

2009) had a large DAPI intense body present (Figure 1). All probes targeting PARs, X5 and Y2 along the sex chromosome chain showed co-localisation with the DAPI intense mass indicating its primary composition is sex chromatin.

We next wanted to determine the distribution of individual chain elements relative to a large nucleolar-like body (NLB) whose presence is restricted to the pachytene stage in platypus (Casey et al (a) submitted). In order to investigate the association of the sex chromosome chain with this structure we used multiple BAC probes targeting regions along the meiotic sex chromosome chain (X1q, PAR1, Y2, PAR5, PAR8, X5, Y5) to characterise the proximity of individual sex chromosomes in pachytene nuclei following surface spreading and PFA fixation. DNA FISH signals were collated and relative positions overlaid on a representative DAPI image of a pachytene stage spermatocyte (Figure 2). We observed there to be a specific NLB proximity hierarchy for chain elements with the Y5 end maintaining tight association at pachytene with the sex chromosomes becoming more spatially distributed toward the X1 end of the chain. From Y2 the tested sex chromosomes show many signals in close association with this structure similar to the Y5 which appears tethered suggesting there occurs a transient close association of the majority of the chain during pachytene.

To assess the status of nucleolar activity during the period of sex chromosome association we used an antibody to detect Fibrillarin, an important nucleolar protein component, the presence of which is indicative of active ribosomal gene transcription (Kressler et al., 2010). In combination with anti-SYCP1 to identify pachytene spermatocytes we observed Fibrillarin-positive nucleoli in cells prior to synapsis and a loss of Fibrillarin in cells with advanced stages of synapsis indicating active ribosomal gene transcription had ceased prior to its association with the sex chromosomes (Figure 3).

Platypus pachytene spermatocytes have perinucleolar repressive histone accumulations frequently not associated with sex chromatin

There is currently no direct evidence to suggest MSCI occurs in monotremes. However due to the lack of retrogenes in monotremes (Potrzebowski et al., 2008a) and chicken (International Chicken Genome Sequencing, 2004; Wang et al., 2005; Toups et al., 2011), otherwise required as meiosis specific backup genes in mammals during the period of sex chromosome silencing, it has been suggested that MSCI evolved after their divergence and prior to the metatherian split (Potrzebowski et al., 2008a). Our observation that the sex chromosomes coalesce into a dense mass during pachytene led us to ask whether signature histone modifications or variants are recruited to effect facultative heterochromatin formation on platypus sex chromatin.

Using immunostaining on surface spreads we detected strong enrichments for H3K27me₃, H2AFY and H3HK9me₃ adjacent to the large NLB in pachytene spermatocyte nuclei (Figure 4a, 4b panels E and B-D, 4c left panel). The most marked H3K27me₃ and H2AFY accumulations were restricted to SYCP1 positive pachytene spermatocytes and maintained a tight association with the NLB. We observed a number of H2AFY configurations through pachytene ranging from fibrillar NLB-tethered radiations (Figure 4b panel B) to larger para-NLB positive areas (Figure 4b panels B and C). In order to investigate whether this H2AFY accumulation is specific to the sex chromosome complex we conducted DNA FISH experiments. From our observation that the Y5 end of the meiotic chain was tethered to the NLB through pachytene we expected the H2AFY foci to be coincident with the para-NBL sex chromatin. However, while specific chain elements such as X5 and Y5 were sometimes

observed within the H2AFY foci (Figure 4b panels F, G and H), these and additional regions of the chain such as PAR5, Y2 and X1 (Figure 4b panel H) were consistently either outside or in close proximity to the H2AFY positive domain. Similarly the H3K27me3 domain was consistently outside or opposite the para-NLB DAPI intense region where the sex chromosomes are located.

We next asked whether the single rDNA-containing NOR, located on chromosome 6, had lost association with the nucleolus during this period by using a DNA paint probe to chromosome 6. We observed a region of chromosome 6 to maintain close association with the NLB in pachytene cells (Figure 4c right panel) when rDNA transcription had ceased and interestingly has partial colocalisation with the repressive histone mark H3K9me3 (Figure 4c).

Absence of prophase I phosphorylation of histone H2AFX on autosomes or sex chromosomes

The hallmark of meiotic sex chromosome inactivation is phosphorylation of the histone variant H2AFX (γ H2AFX) on pachytene sex chromatin. We could not detect this histone modification at any stage of prophase on surface spreads or western blotting using total testis lysates or extracted histones (data not shown). Neither a first or second wave of H2AFX modification which normally occurs with Spo11-mediated DNA DSB induction commencing during leptotene or sex chromosome specific enrichment during pachytene respectively was observed. In order to exclude the possibility that the absence of γ H2AFX was due to technical reasons or failure of the antibody to bind the target epitope we next asked whether we could detect this response to DNA DSBs in platypus somatic cells under the same conditions following DSB induction by gamma irradiation. Following a 5 (data not

shown) or 10 Grey irradiation we were able to detect H2AFX phosphorylation in both mouse (Figure 5a B and F) and platypus fibroblasts (Figure 5a D and H) at 1 and 4 hours post treatment showing that our antibody was able to detect the epitope using our immunostaining protocol. While we observed a marked and dose dependent increase in γ H2AFX in response to DSB induction in platypus fibroblasts this response appeared reduced and delayed relative to mouse. In addition we irradiated cultured mouse and platypus meiotic cells in order to detect a γ H2AFX response however, while mouse pachytene cells displayed a marked increase following exposure (Figure 5b B and F) we failed to detect any equivalent response in platypus pachytene nuclei (Figure 5b D and H). Furthermore we detected the presence of the repair pathway sensor kinase ATM at induced γ H2AFX foci in fibroblasts indicating a conserved damage response pathway is functional in platypus (Figure S1). Thus we demonstrate the ability of our antibody to detect induced γ H2AFX and also observe the phosphorylated epitope sequence in platypus (Figure S2) and therefore consider the possibility that an alternative histone variant or modification may be functioning to mark and stabilise chromatin following meiotic DNA DSBs.

The DDR machinery component RPA is present in early prophase spermatocytes and lacks preferential sex chromosome association in pachytene

To establish the nature of meiotic DSB dynamics in platypus prophase I in light of the lack of detectable γ H2AFX we used an antibody against the replication protein A subunit (RPA32) to visualise the temporal and spatial distribution of DNA damage repair (DDR) foci. The heterotrimeric RPA complex functions in replication, DNA repair and recombination to stabilise the ssDNA ends at sites of SPO11-mediated DSBs during meiotic prophase and thus

is a required component of transition recombination nodules (Moens et al., 2007). Consistent with observations in mouse and human (Moens et al., 2002; Moens et al., 2007; Oliver-Bonet et al., 2007) platypus prophase I cells follow a similar temporal pattern of DSB formation and resolution with RPA32 foci peaking in early zygotene (Figure 6A) and reducing in number through to the mid pachytene stage of full synapsis evidenced by SYCP1 and SMC3 immunostaining (Figure 6B, C and D). RPA foci are seen initially to evenly decorate the asynaptic chromosomal axes (Figure 6, row A) prior to resolution into more restricted and limited domains as SC formation progresses into pachytene (Figure 6, rows B and C). We did not observe RPA foci distributions indicative of sex chromosome specific accumulation in early-mid stage pachytene spermatocyte nuclei staged by the presence of the large NLB, an adjacent DAPI intense mass and SYCP1/SMC3 staining (Figure 6, rows D and E). Previous reports describe persistence of RPA foci on the unsynapsed X and Z of mouse and chicken respectively at late pachytene (Plug et al., 1997; Moens et al., 2002; Guioli et al., 2012a) therefore we asked whether such restriction of foci to unpaired sex chromosome DNA also occurs in platypus by assessing their frequency and distribution in mid-late pachytene nuclei in more detail. Using characteristic DAPI staining as criteria for identifying pachytene stage spermatocytes, RPA foci numbers were counted in each nuclei and scored for NLB proximity where the sex chromosome chain has been shown to coalesce at this time (Casey et al (a) submitted). Of the 138 pachytene nuclei observed, 31% lacked an RPA signal, 50% had 1-3 foci and 8% had 6 or more foci, (Table 1). We reasoned that recombination nodules which resulted in crossover events, chiasmata formation and occurring on sex chromosome PARs, would lose RPA in concert with the autosomes if MSCI is not functioning in platypus. Therefore late pachytene stage RPA foci would represent either lagging autosomal and sex chromosome PAR DSB resolution events or foci persisting due to presence of unpaired sex chromosome DNA. We did not find convincing evidence for sex chromosome chain specific

RPA foci accumulation due to the low frequency of 'lagging' foci being positioned in close proximity to the NLB (eg 2/35 and 6/35 near NLB foci for nuclei with 5 and 4 RPA foci respectively). Furthermore we did not observe nuclei with 6 or more foci having any paranucleolar grouping or linear foci decorations consistent with sex chromosome chain specific accumulation (Figure 6 rows B, C and D). In addition, the use of an anti-SMC3 antibody, shown by us to heavily accumulate and therefore mark sex chromosomes in pachytene (Casey et al (a) submitted) failed to identify sex chromosome specific RPA32 localisations (Figure 6 SMC3 panels).

Platypus meiotic cells undergo a transient period of global transcriptional repression through mid-late prophase I

We next asked whether the platypus sex chromosomes undergo transcriptional silencing at pachytene (i.e. MSCI) by assessing the distribution of active gene transcription using Cot-1 RNA FISH and RNA PolIII immunostaining on testis surface spreads. The consequence of MSCI on sex chromosome gene activity is made obvious through the visualisation of Cot-1 and RNA PolIII holes over the sex chromosomes in pachytene spermatocytes in numerous mammals including marsupials, mouse and human (Turner et al., 2004a; Namekawa et al., 2007a; de Vries et al., 2012). Surprisingly we observed significant global reductions in Cot-1 probe hybridisation specifically at the pachytene stage (Figure 7, panels B and E) indicating a general suppression of gene transcription transiently occurs in platypus during the meiotic stage when MSCI is otherwise established in marsupials and eutherians. Furthermore we could not detect any obvious differential Cot-1 RNA staining between the peri-NLB region and the remaining nucleus (Figure 7 panel E). Prior to the drop in Cot-1 we observed a wave of global H3K9me2 staining further supporting the presence of a state of general

transcriptional repression (Figure 8). This repressive epigenetic modification is evident throughout the nucleus from early pachytene and is absent in zygotene and late pachytene/diplotene nuclei and importantly no enrichment could be detected specifically around the NLB consistent with the homogeneous Cot-1 profile. RNA PolIII immunostaining provided further support to the Cot-1 profile with a generalised loss through pachytene (Figure 9a Early, Mid and Late Pachytene panels) however by diplotene there was an increase in the RNA PolIII signal indicating general transcriptional reactivation occurs following the period of pachytene specific suppression (Figure 9a, diplotene panel). SMC3 staining was employed to determine pachytene stages and sex chromosome location. Similar to the Cot-1 distribution we could not detect any sex chromosome specific changes in RNA PolIII staining over the sex chromatin using signal intensity mapping in either mid or late pachytene nuclei (Figure 9b).

Gene specific repression of autosomal and sex linked loci in *Platypus* pachytene cells

In order to directly investigate gene activity in meiotic cells we isolated pachytene enriched cell populations by gravity sedimentation followed by RT-PCR of sex chromosome specific and autosomal genes. To this end cell suspensions from total platypus testis were applied to a BSA gradient to isolate different cell types based on size. We successfully harvested pachytene cell populations with at least 70% purity based on characteristic DAPI appearance (Figure 10a) and confirmed by immunostaining for SYCP1 (Figure 10b). RNA was extracted from equivalent cell numbers from each fraction and gene specific semi-quantitative RT-PCR performed on cDNA from 3 fractions with different pachytene complements and total testis. The three gradient fractions used for expression analysis contained 42% (n=194 nuclei), 70% (n=352 nuclei) and 2.5% (n=161 nuclei) pachytene cells for fractions 4(F4), 6(F6) and 9(F9)

respectively (Figure 10a). Consistent with the loss of Cot-1 RNA FISH and RNA PolII signal in SYCP1 positive cells we saw an obvious drop in PCR product abundance in fractions with high pachytene cell complements (Figure 11). Transcript abundance increased in fractions with higher non-pachytene cell content with F6, F4, F9 and total testis having lowest to highest product abundance and highest to lowest pachytene complements respectively. The suppression in transcriptional output was not observed to be biased regarding gene location with autosomal, PAR and sex chromosome linked loci showing reductions in transcript abundance in pachytene enriched fractions compared to fractions with reduced pachytene content and total testis (Figure 11).

Discussion

Transcriptional silencing of unpaired DNA during prophase I of meiosis is a conserved mechanism by which the consequence of aberrant synapsis is prevented or heterologous sex chromosomes avoid checkpoint activation (Turner, 2007; Inagaki et al., 2010). Therian sex chromosomes undergo a programme of transcriptional silencing via accumulation of DNA damage repair machinery components and repressive epigenetic modification (MSCI). This is one of only two known chromosome wide silencing mechanisms (the other being X chromosome inactivation). MSCI has been extensively studied in eutherian and metatherian mammals and recently two major papers have also investigated this in the female chicken heterogametic ZW system (Schoenmakers et al., 2009a; Guioli et al., 2012a). Monotremes are the third major group of mammals and exhibit a complex sex chromosome system which shares homology to bird sex chromosomes (Grutzner et al., 2004a; Rens et al., 2007; Veyrunes et al., 2008a). There has been no direct evidence on whether MSCI is present in these animals. Our data provide first insight into aspects of meiotic silencing in monotremes.

Platypus sex chromosomes coalesce into a dense mass in pachytene spermatocytes

Using a combination of surface spreading and serial DNA FISH we were able to visualise the majority of platypus sex chromosome elements within a large heterochromatic mass showing dynamic folding within the sex chromosome complex (described in detail in Casey et al. (a) submitted), reminiscent of the state of synaptic adjustment observed between the chicken ZW. Formation of a DAPI intense sex body represents an established state of meiotic silencing in therians while in chicken a state of equalisation between heterologous sequences also involving SC assembly. However, its relevance to MSCI, while established in mouse remains unclear in chicken where this 'equalised' configuration may circumvent the requirement for DDR machinery persistence on the otherwise unpaired sex chromatin. Given the absence of detectable DDR components from mid-pachytene specifically on the sex chromosomes we take this observation and the coalesced sex chromatin in platypus pachytene nuclei to represent a more chicken-like state of sex chromosome self association. The fact we do not observe an accompanying SYCP1 positive region within the sex chromatin mass at this time suggests the mode of sex chromosome self-association differs from that occurring in chicken where SC components appear to mediate ZW interactions at least during the equalisation stage.

Our observation that condensed and self-associated platypus sex chromosomes are tethered to a large nucleolar body raises obvious questions regarding its requirement and contribution to sex chromatin formation. It has long been known that in mouse spermatocytes during prophase I nucleoli detach from the autosomes in early pachytene, coalesce and migrate to the synapsed and silenced sex chromosomes however the reason for this interaction remains unclear (Kierszenbaum and Tres, 1974; Knibiehler et al., 1981).

This process precludes involvement of ribosomal gene transcription evidenced by lack of proximity to the autosomal NORs. Nucleoli contain an abundance of proteins not directly involved in rRNA production and the loss of rRNA transcription during the time of XY association implicates a chromatin modifying function. Similarly we show loss of orthodox nucleolar activity during the period of physical attachment to the condensed sex chromosome chain in late pachytene and speculate an involvement in facilitating a yet to be defined sex chromosome specific chromatin modification. Few proteins have been characterised which show accumulation to both sex chromatin and nucleoli regions at pachytene (Escalier and Garchon, 2005a; Tsutsumi et al., 2011b) however *Xlr* gene family members are proposed to have some involvement in coordinating meiotic silencing and we predict a similar relationship in monotremes. Interestingly the *Xlr* family member which has nucleolar and sex chromosome enrichment, *Xlr6*, has sequence similarity with the SC component *SYCP3* through the conserved *Xlr* family Cor1 motif (Tsutsumi et al., 2011b). We also report here that platypus chromosome 6, which contains the only NOR, maintains nucleolar association at late pachytene and notably accumulates the repressive histone mark H3K9me2. As yet the significance of such observations are yet to be determined in the context of sex chromatin dynamics, meiotic silencing or the homology of monotreme chromosome 6 to the therian X (Veyrunes et al., 2008a).

A transient period of global transcriptional repression occurs through pachytene

We have assessed the transcriptional status of meiotic prophase cells in platypus males using cell enrichment, immunohistochemistry, Cot-1 RNA FISH and RT-PCR to determine the status of sex chromosome specific transcriptional silencing. Unlike observations in mouse

describing global pre-pachytene transcriptional repression followed by autosomal transcriptional reactivation (Monesi, 1965b; McCarrey et al., 1992; Ellis et al., 2005; Turner et al., 2005; Burgoyne et al., 2009), we observe a global suppression of expression to occur and be maintained through pachytene similar to recent findings in chicken (Guioli et al., 2012a). Dramatic reductions in Cot-1 RNA FISH signals in pachytene cells was preceded by detection of a repressive histone mark (H3K9me2) and further supported by the observed drop in detectable transcripts of both sex chromosome and autosomal genes. Our data provide no evidence for differential transcriptional activity between the synapsed autosomes and the sex chromosome chain in pachytene spermatocytes using our methods. Current evidence suggests a generalised reactivation occurs in diplotene based on our RNA PolII staining. Our work using an anti-SMC3 antibody allowed more accurate staging and identified pan nuclear H3K9me2 enrichment in early pachytene followed by a global loss at diplotene which is similar to observations in mouse but not in terms of the late pachytene sex chromosome specific enrichment (Khalil et al., 2004a; Greaves et al., 2006). The observed perdurance of the genome wide transcriptional drop through pachytene may be linked to the pseudo-pairing status of the sex chromosomes which transiently coalesce and maintain a para-NLB association.

Platypus sex chromosomes are not consistently associated with a large paranucleolar accumulation of the repressive histone variant H2AFY

The large H2A variant H2AFY has long been associated both with XIST-dependent silencing of the inactive X chromosome in female somatic cells and the meiotic XY body (Costanzi and Pehrson, 1998; Mermoud et al., 1999; Hoyer-Fender et al., 2000a; Richler et al., 2000). In

addition to the direct repressive transcriptional environment imparted by H2AFY recruitment, more specific roles such as strengthening sex chromosome PAR associations and sister chromatid cohesion to aid correct segregation have also been put forward (Turner et al., 2001; Hoyer-Fender, 2003a). Our observation that a large para-NLB H2AFY foci accumulates specifically at pachytene and only transiently associates with a few sex chromosomes is intriguing as it departs from the orthodox mammalian H2AFY-sex chromosome paradigm. Is this an early utilisation of this variant which is then more specifically recruited and integrated into the therian MSCI and XCI pathways following monotreme divergence? The presence of the considerable amount of unpaired sex chromosome DNA is not sufficient for H2AFY recruitment as it has been shown to be excluded from the XY chromatin in XY^{Tdyml} female mouse oocytes demonstrating that incorporation is independent of the presence of asynaptic chromosomes (Hoyer-Fender et al., 2004). Interestingly our observed persistent nucleolar association with chromosome 6 (which has the only NOR in platypus) may also implicate a role for H2AFY in negatively regulating rDNA transcriptional activity as recently described (Cong et al., 2014). Our observation of para-NLB H3K27me3 and H3K9me3 accumulations are intriguing as they also don't appear to be associated directly with the sex chromosomes similar to H2AFY therefore we find no evidence to implicate these repressive marks in a sex chromosome specific silencing role. In addition the amount of H2AFY on total testis is greater in platypus compared to mouse (Supplementary Figure 3). It would be interesting to determine whether these marks occupy the same region around the NLB at pachytene and colocalise with regions of chromosome 6.

The platypus sex chromosome chain lacks a therian-like meiotic silencing program

The presence of unsynapsed autosomes or unpaired XY DNA at pachytene triggers the accumulation of many DDR machinery components which are proposed to protect the cell from meiotic progression until synaptic completion or escape unpaired DNA checkpoint surveillance respectively (Turner et al., 2005; Turner et al., 2006). We used an antibody targeting the RPA32 subunit to determine whether the considerable unpaired sex chromosome DNA of platypus accumulates and maintains repair pathway components indicative of meiotic silencing. We hypothesised two possible scenarios; Firstly our above observations that spermatocytes undergo a generalised phase of transcriptional repression through pachytene during the period when the sex chromosomes have extensive self-association reflects recent observations in chicken (Schoenmakers et al., 2009a; Guioli et al., 2012a). The lack of observed silencing marks such as γ H2AFX on the pseudosynapsed ZW implied a therian-like MSCI pathway did not function or was not essential in chicken. Secondly the presence of unpaired DNA would trigger a conserved MSCI pathway and we would detect DDR component accumulation on heterologous sequences at pachytene similar to generalised observations in mammals and the asynaptic ZW (Guioli et al., 2012a). The observed RPA32 foci dynamics demonstrated that the conserved genome-wide first wave of DNA DSBs in early prophase is present in platypus. Our inability to detect a coincident wave of phosphorylated H2AFX is difficult to reconcile as we also were able to detect early prophase Spo11 foci (data not shown) indicating functionality of a conserved DSB pathway and the fact we were able to detect the γ H2AFX epitope following DSB induction in cell culture. Our previous work (Casey et al (a) submitted) demonstrated massive accumulation of the cohesin subunits SMC3 and STAG3 specifically on the sex

chromatin through prophase I and most markedly at pachytene which together with recent observations demonstrating a regulatory function for cohesin in governing the spread of H2AFX serine 139 phosphorylation (Caron et al., 2012) may account for repression of this conserved DSB response in platypus. However, this obviously fails to account for the lack of γ H2AFX detection on the autosomes during the considerable early prophase wave of DSB induction.

If a therian-like response to unpaired DNA operated in platypus meiosis, we predict the persistence of RPA foci specifically on the sex chromosome chain beyond the stage at which autosomal foci were resolved. Using serial immunostaining for RPA32, SYCP1, SMC3 in combination with characteristic DAPI staining to identify mid to late pachytene spermatocytes we were unable to identify persistence of sex chromosome specific RPA32 foci. We reasoned the presence of the substantial unpaired XY DNA, demonstrated to be tethered to the large NLB at this stage, would be an obvious presence in nuclei even if restricted to a subpopulation of late pachytene cells as occurs in chicken which have inefficient ZW synaptic adjustment (Guioli et al., 2012a). The fact we do not observe any late pachytene nuclei with RPA32 foci restricted to the position of the sex chromosomes suggests platypus sex chromosome self-association is efficient and therefore precludes involvement of key meiotic checkpoint machinery. It also is feasible that a limited region of the sex chromosome chain undergoes detection due to partial pseudosynapsis however we find this unlikely in terms of sex chromosome specific detection as only 16.14% (36/223) of RPA32 foci were positioned adjacent the nucleolus in late pachytene nuclei with 5 or less RPA32 foci. We therefore contend the majority of latent RPA32 foci present in pachytene nuclei are autosomal and find no evidence to support the idea that sex chromosome DNA triggers discreet checkpoint pathway responses outside that observed to occur in concert with the autosomal DSB resolution programme. It remains possible that additional and as yet

untested silencing marks recognise and accumulate on the sex chromosomes however argue against our methods failing to detect a subpopulation of nuclei with RPA32 foci restricted to sex chromosomes with incomplete or aberrant heterologous synapsis.

A recent report on the presence of functional X-borne retrogenes in mammals postulated the emergence of therian-like MSCI occurring prior to the metatherian-eutherian split and after monotreme divergence based on their absence in platypus (Potrzebowski et al., 2008a). As chicken also lacks autosomal backup genes and therefore does not follow the 'out-of-X' trend observed in other lineages (International Chicken Genome Sequencing, 2004; Wang et al., 2005; Toups et al., 2011) and has a similar Z chromosome gene content to platypus X5, we support the idea that a therian-like MSCI checkpoint does not operate in platypus. However our results may offer insights about the evolutionary path at the origin of therian MSCI. We show accumulation of multiple repressive histone marks tightly linked to both the nucleolus and the future therian X chromosome. Maybe in platypus we see the beginning of a shift from transient genome wide meiotic transcriptional suppression to sex chromosome specific silencing (MSCI). This trajectory may have been triggered by the combination of paranucleolar association of a host of repressive histone variants and modifications originally established to regulate NOR activity through pachytene prior to the co-opting of this machinery into the MSCI mechanism observed in other mammals.

Materials and Methods

Sample Collection

Platypus were captured at the Upper Barnard River (New South Wales, Australia) under the following permits; AEEC permit R.CG.07.03 (F.G.), Environment ACT permit LI 2002 270 (J.A.M.G.), NPWS permit A193 (R.C.J.) and AEC permit no. S-49-2006 (F.G.). Animals were euthanized with an intraperitoneal injection of 0.1 mg/g pentobarbital (Lethabarb). Mouse testis were obtained from three week old animals (Swiss white).

Gravity sedimentation

Total testis cell lysates were stored in 90% PBS/10% DMSO at -80°C and thawed at room temperature prior to use. 500ul of thawed total testis suspension was diluted in 9.5ml ice cold PBS, filtered through a 70µm cell strainer (Falcon, #2530), pelleted at 700rpm for 5 minutes and resuspended in 10ml ice cold RPMI media 1640 (Gibco, #21870-076). A 2-4% continuous bovine serum albumin gradient was formed using a 100ml total volume dual chamber SG100 gradient maker (Amersham Biosciences) in a 250ml Lenz Bistabil separating funnel (Sigma -Aldrich). The 10ml cell suspension was manually loaded onto the gradient, sedimentation was for 3.5 hours at room temperature and 10X 10ml fractions then decanted from the funnel. Cell fractions were pelleted 5min at 700rpm, resuspended in 200ul PBS for surface spreading and RNA extraction.

Antibodies and BAC clones

The following antibodies were used for surface spreading and fibroblast immunohistochemistry at 1:300 dilution. Anti- H2AFY rabbit (Upstate), -SYCP1 rabbit (Novus Biologicals), -SMC3 rabbit (Abcam), -γH2AFX rabbit (Abcam), -H3K9me2 mouse (Abcam), -H3K9me3 mouse (Abcam), -Fibrillarin mouse (Abcam), -Spo11 mouse (Novus Biologicals) and -ATM phospho S1981 mouse (Abcam), -Histone H4 rabbit (Upstate), -RNA PolII mouse (Upstate) -RPA32 rabbit (Abcam). Secondary antibodies were used at 1:500 and 1:2000 for

immunohistochemistry and western blotting respectively; Alexa Fluor 488 goat anti-rabbit, 568 goat anti-mouse (Invitrogen), anti-mouse AP and –rabbit AP (Rockland).

BAC probes were obtained from Children's Hospital Oakland Research Institute (CHORI, Oakland, CA, USA) or CUGI BAC/EST Resource Centre, Clemson, South Carolina USA: X1q (CH236-378 F21), PAR1 (CH236-286 H10), PAR3 (CH236-78 K11), PAR4 (CH236-165 F5), PAR5 (CH236-462 C1), PAR6 (CH236-639 O23), PAR8 (OA_Bb-466 A15), Y2 (CH236-178 N20), Y5 (CH236-152 P15) and X5 (CH236-236 A5).

Semi-quantitative RT-PCR

Equivalent cell numbers from each gravity sedimentation elution fraction were determined using a haemocytometer followed by standard TriZol (Invitrogen) RNA extraction according to the manufacturers' instructions. 130ng of total RNA was used as template for cDNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen). All cDNA samples underwent 28 amplification cycles, all primer sets had 55°C annealing temperature.

Protocols for surface spreading, DNA FISH and immunohistochemistry were as described in Daish et al (2009).

Acknowledgements

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Figures

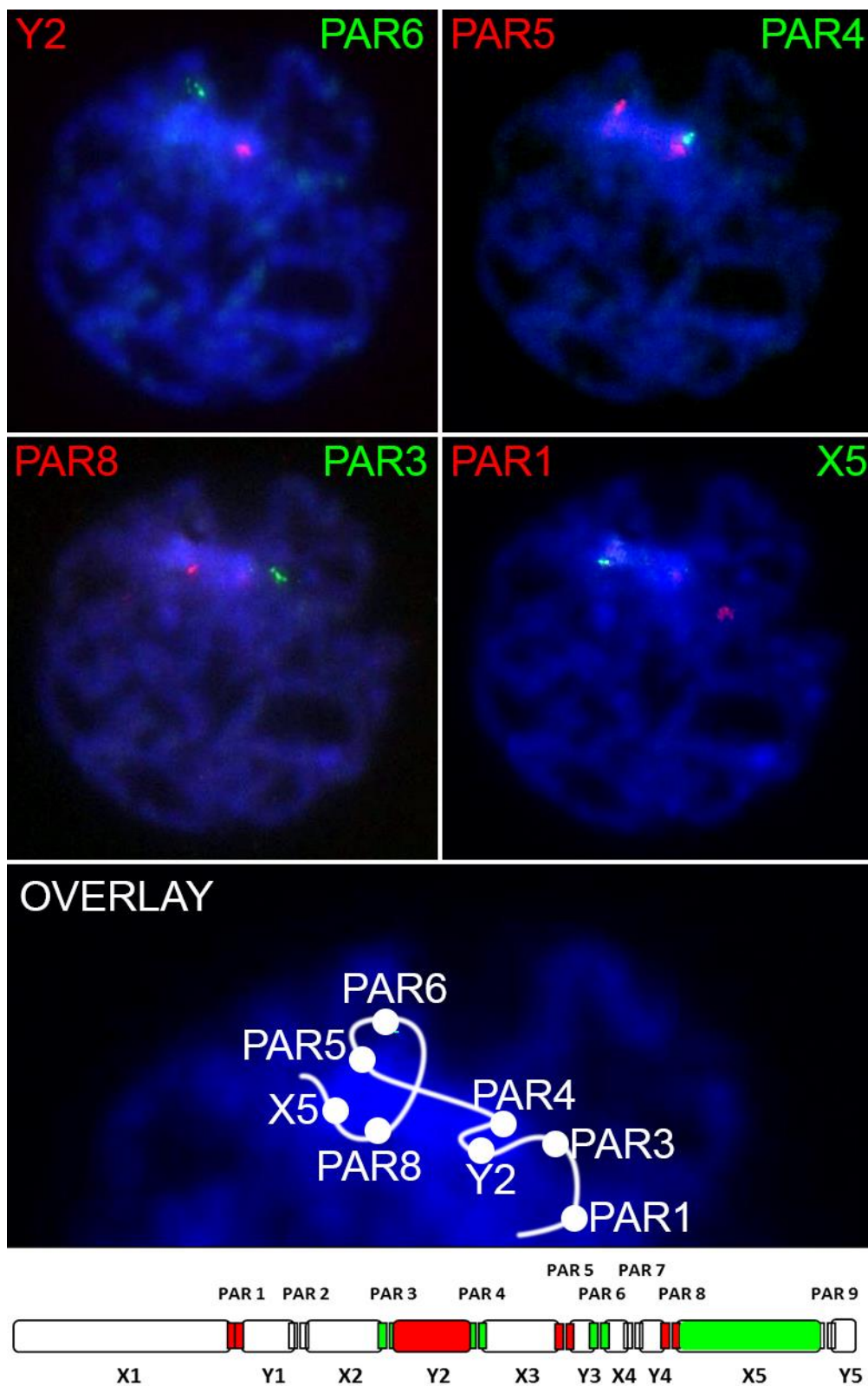


Figure 1. Sex chromosome chain conformation at pachytene. Methanol:acetic acid fixed spermatocytes underwent serial BAC probe DNA FISH hybridisations targeting indicated

chain elements. Cell coordinates and signals were recorded prior to additional dual colour labelling experiments. All signals are positioned within or immediately adjacent a DAPI intense heterochromatic mass (A arrow) indicating its primary composition is sex chromosome chromatin. Condensed chromosome configuration is indicative of pachytene stage and the DAPI intense mass was consistently observed in similarly staged nuclei (data not shown). Overlay shows representative sex chromosome chain conformation. Bottom figure shows BAC probe positions in sex chromosome chain with corresponding label colour.

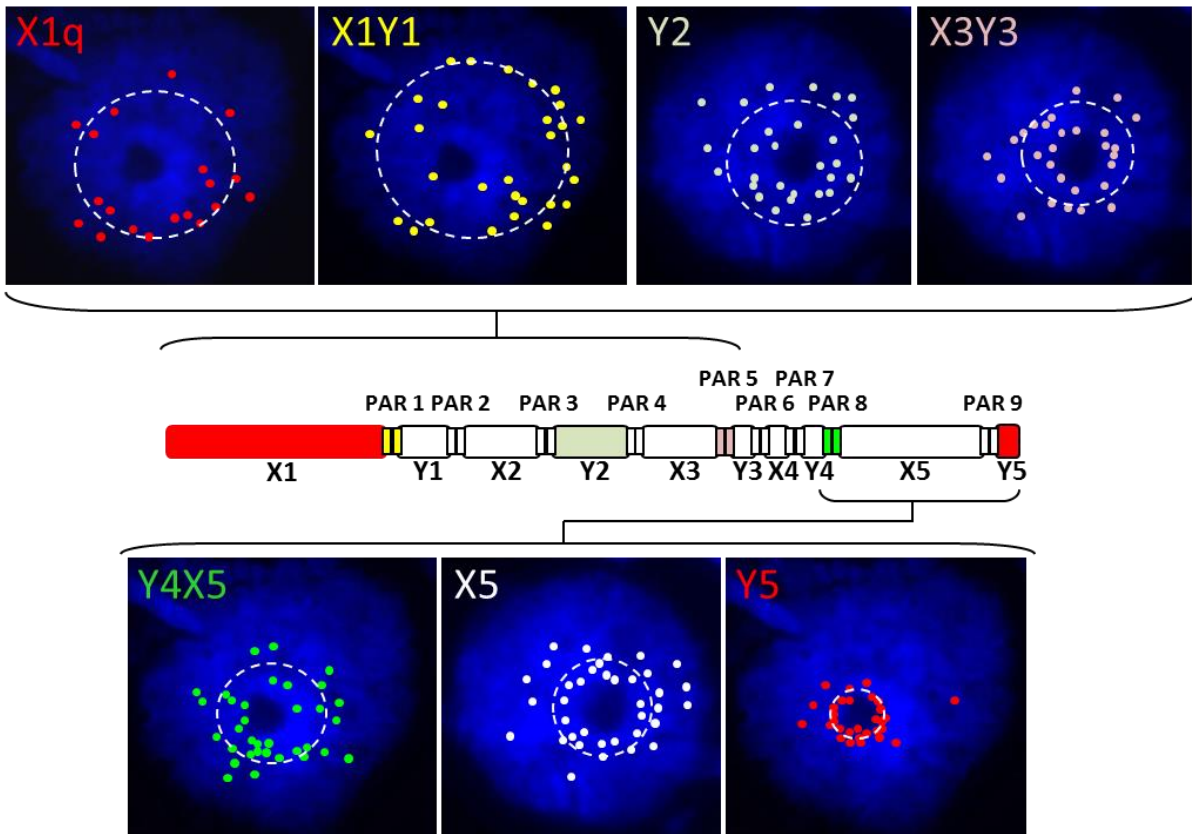


Figure 2. Sex chromosome distribution relative to the nucleolar-like-body in pachytene spermatocytes. DNA FISH was performed on PFA fixed meiotic spreads and individual signals recorded based on proximity to the large DAPI void in pachytene spermatocytes termed the nucleolar-like-body (NLB). Dual colour DNA FISH enabled two sex chromosome chain element positions to be assessed per nuclei and positions of each element were collated and overlaid on a representative DAPI stained pachytene nucleus. An ordered hierarchy of NBL proximity was observed with Y5 having consistent close association and the X1 end of the chain displaying more variable positions. Signal numbers for each sex chromosome element were as follows: X1q 19, X1Y1 (PAR1) 29, Y2 28, X3Y3 (PAR5) 27, Y4X5 (PAR8) 34, X5 37 and Y5 29. Note all 'internal' chain elements (Y2, PAR5, PAR8 and X5) except X1Y1 (PAR1) show a proportion of signals closely associated with the NLB. Middle panel shows a sex chromosome schematic with the 7 DNA BAC probe positions along the chain.

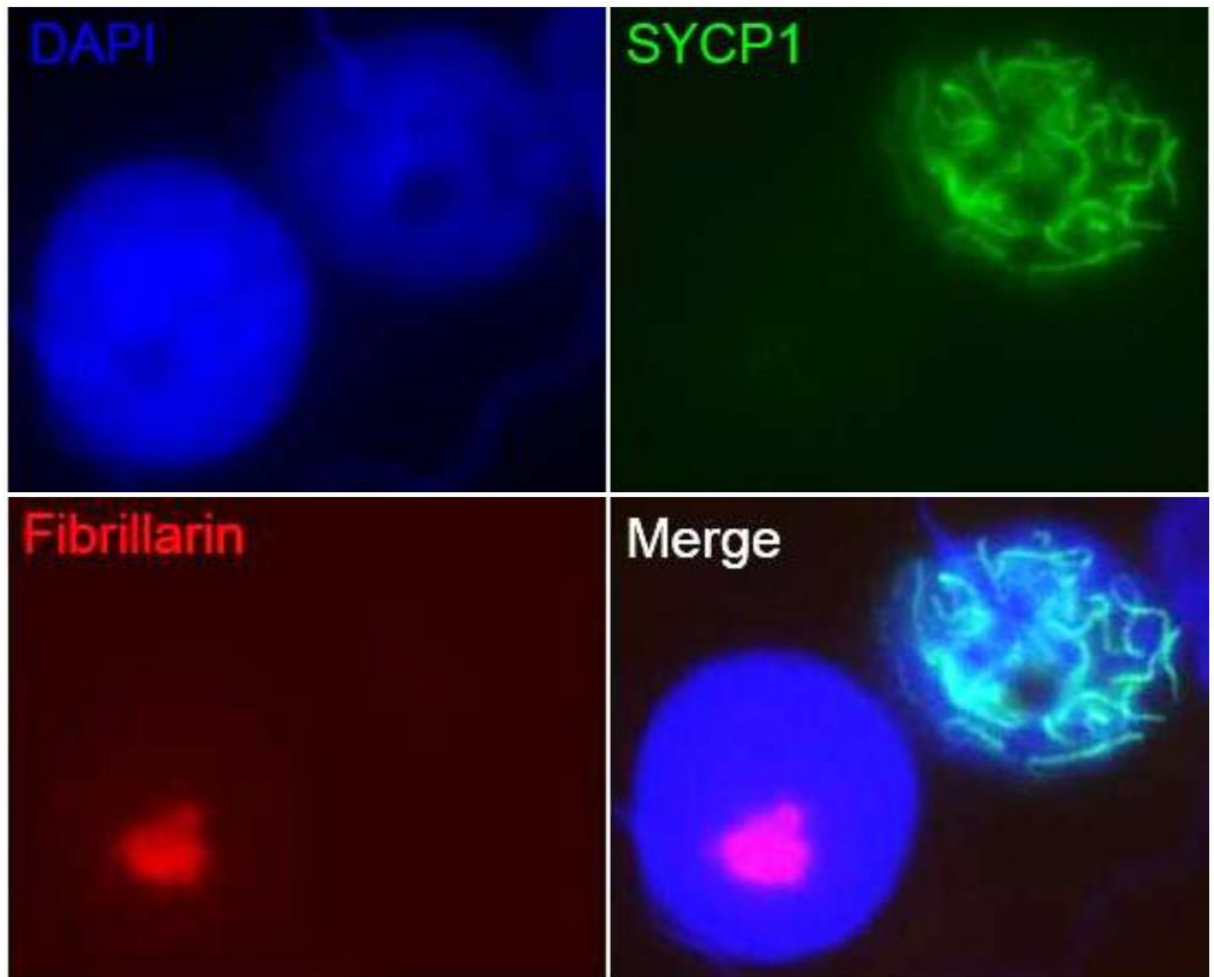


Figure 3. Nucleolar and chromosome 6 NOR function in platypus pachytene spermatocytes. To determine whether the nucleolus maintains function during the period of sex chromosome association surface spreads were immunostained for the nucleolar marker protein Fibrillarin and SYCP1. Fibrillarin is not present in the NLB during pachytene when sex chromosome association occurs indicating rRNA biogenesis is suspended during pachytene.

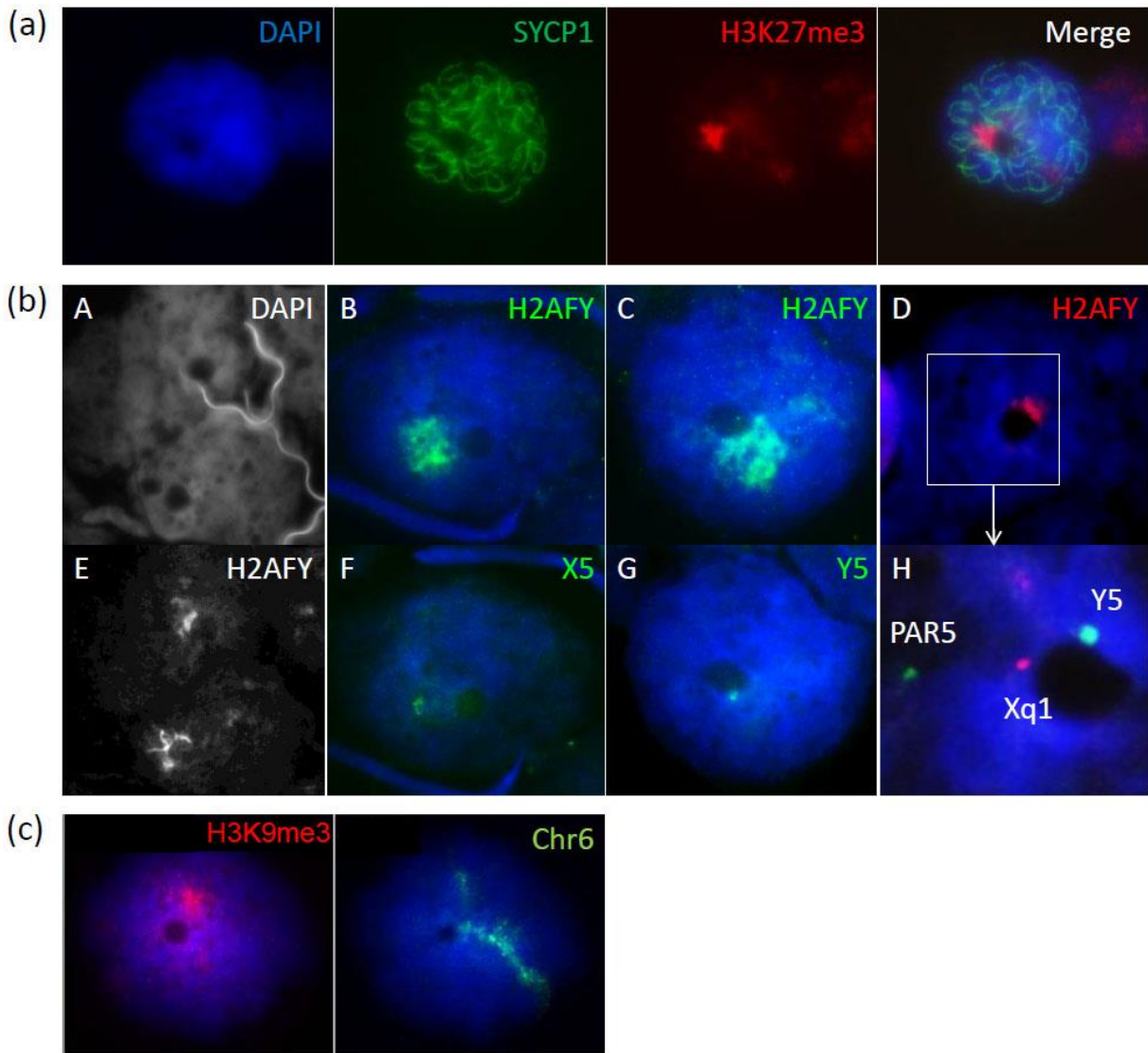


Figure 4. Distribution of H3K27me3, H2AFY and H3K9me3 sex chromosome co-localisations in platypus pachytene spermatocytes. Surface spread platypus testis cells were stained for H3K27me3 and SYCP1, H2AFY or H3K9me3 followed by BAC or chromosome 6 paint DNA FISH. (a) H3K27me3 staining occurs in SYCP1 positive nuclei. Note its location occurs outside or opposite the DAPI intense para-NLB region. (b) Pachytene nuclei show marked H2AFY accumulations adjacent a large nucleolar like body with differing conformations being observed from smaller restricted foci (A, E and D), more dispersed fibrillar arms (E) and larger paranucleolar blocks (B and C). Chain elements toward the Y5 end of the chain were observed to infrequently co-localise with the large H2AFY region (F and G) while other sex chromosomes were rarely observed to be associated with H2AFY (H). (c) The single NOR on

chromosome 6 maintains connection with the nucleolus during pachytene (right panel) with regions colocalising with H3K9me3 (left panel).

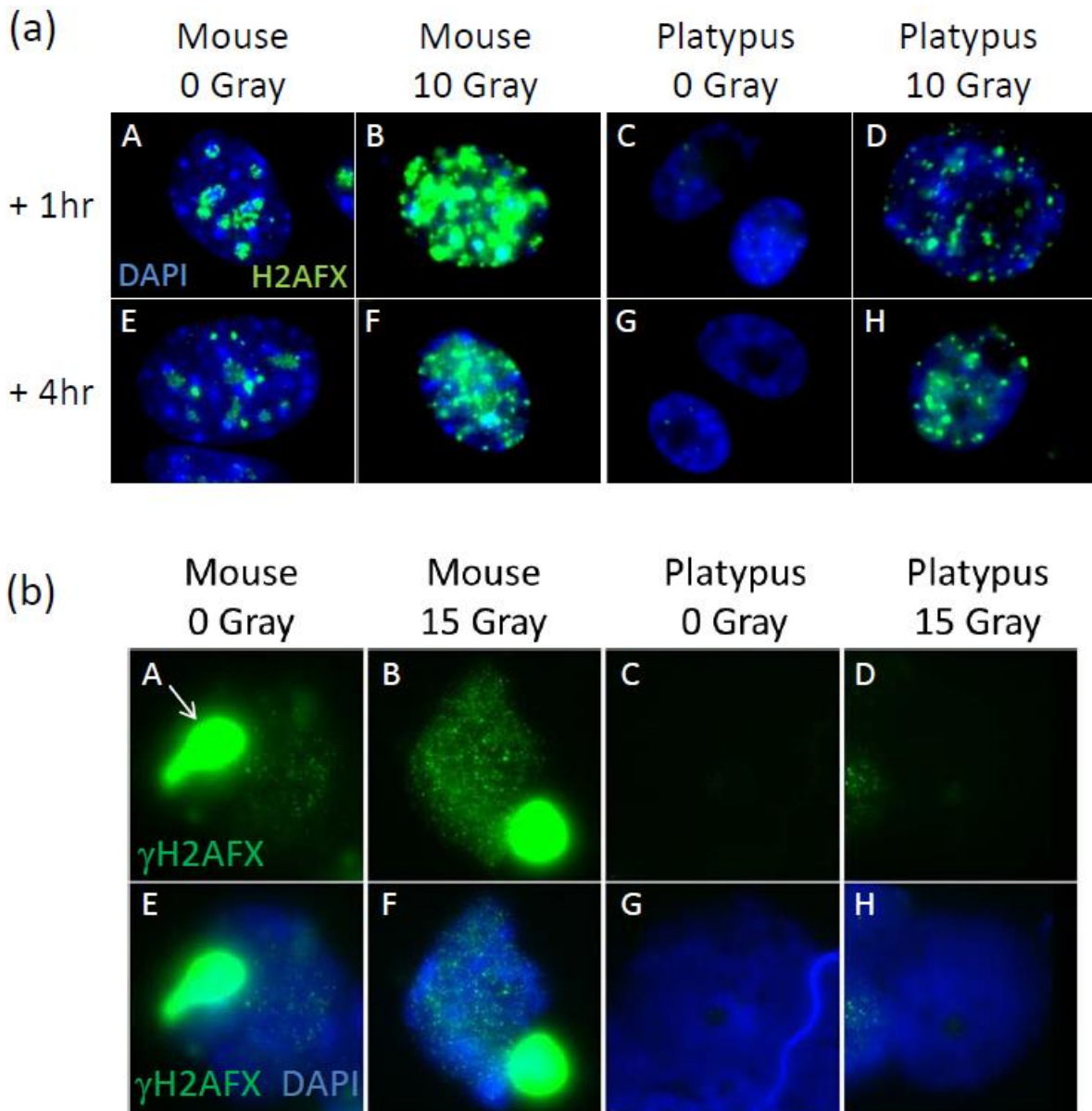


Figure 5. Irradiated fibroblasts and meiotic cells immunostained for phosphorylated H2AFX.

(a) Cultured mouse and platypus fibroblasts were treated with 10 Gray of ionising radiation then immunostained with anti- γ H2AFX at 1 and 4 hours post-treatment followed by DAPI staining. The treated mouse fibroblasts exhibit a rapid induction of detected phosphorylated H2AFX Serine 139 at 1 hr post treatment (B) compared to controls (A and E) which is reduced by 4 hrs post treatment (F). Platypus fibroblasts similarly showed an increase in γ H2AFX staining following treatment however this appeared significantly reduced (D and H) compared to mouse. Note untreated platypus cells (C and G) had negligible staining compared to mouse. (b) Mouse and platypus cryopreserved testis lysates were

cultured overnight and exposed to 15 Gray of ionising radiation and immunostained for γ H2AFX at 1hr post-treatment. A radiation-induced DNA damage response was detected in mouse pachytene nuclei (B and F) staged by the presence of the γ H2AFX-positive sex body (arrow A). No equivalent response indicated by increased γ H2AFX staining was detected in platypus meiotic cells following irradiation (D and H). Identification of platypus pachytene cells were based on characteristic DAPI appearance with DAPI void representing the NLB (G and H).

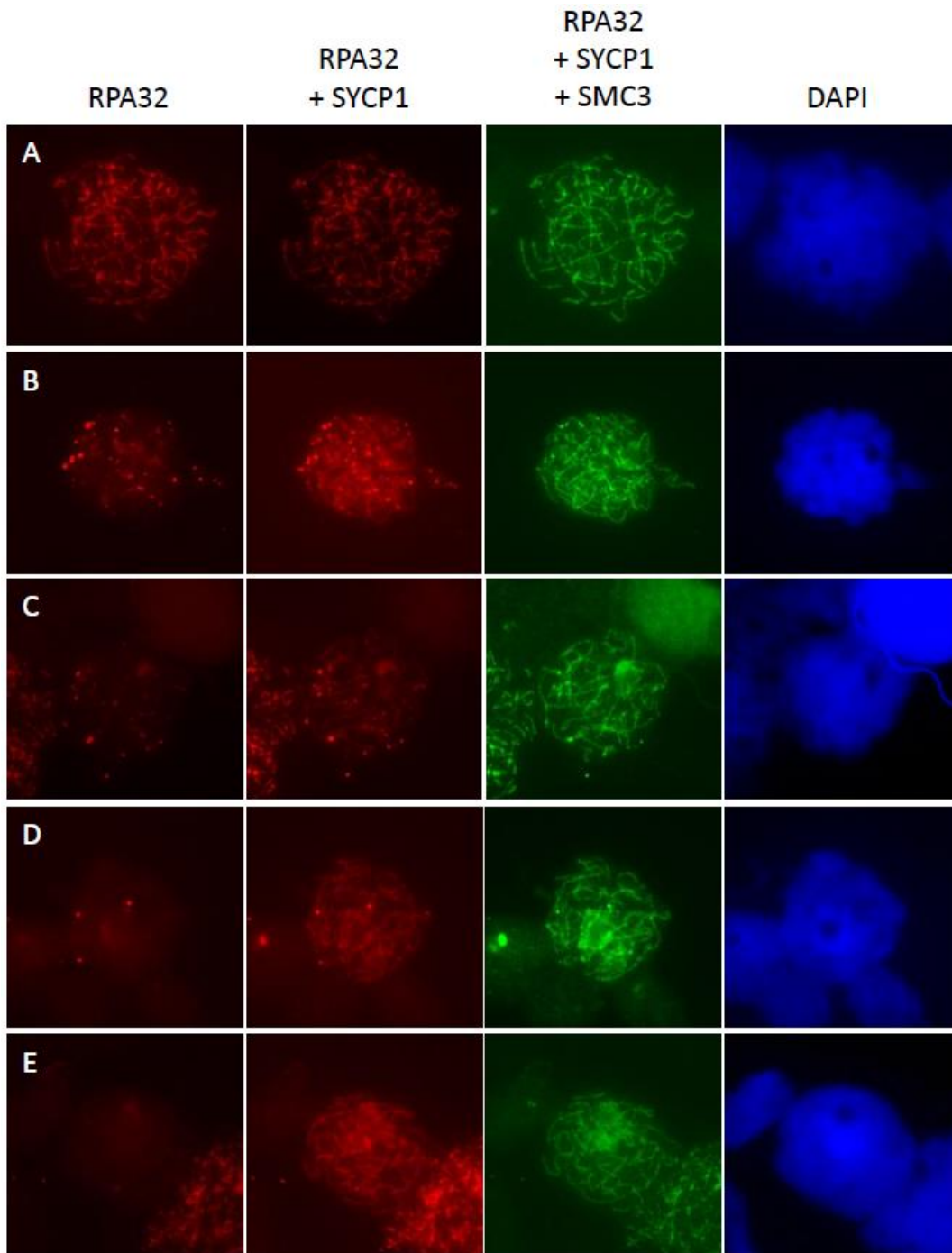


Figure 6. RPA32 foci dynamics in prophase I. Platypus testis surface spreads were immunostained with anti-RPA32, nuclei coordinates recorded followed by serial staining and image capture for SYCP1 and SMC3. Staging was assessed through a combination of SYCP1 and RPA32 foci characteristics and number respectively with SMC3 used to identify sex chromosome positions. Serial staining was required due to the three antibodies being raised in rabbit. RPA32 foci were seen to evenly decorate the condensing chromosomes prior to

the presence of advanced synaptonemal complex assembly (A RPA32 and RPA32 + SYCP1). As SYCP1 staining becomes less discontinuous RPA32 foci numbers are reduced (B, RPA32 and RPA32+SYCP1) prior to the heavy accumulation of SMC3 on the sex chromosomes (B RPA32+SYCP1+SMC3). As the sex chromosomes location becomes apparent due to SMC3 accumulation (C RPA32 + SYCP1+SMC3 and D RPA32 + SYCP1+SMC3) RPA32 foci are further reduced and lack associated with the sex chromosomes (C RPA32 and D RPA32). 31% of pachytene cells observed did not have RPA32 foci (E) indicating DNA DSBs are resolved through this period. No conformations indicative of sex chromosome specific RPA32 persistence were observed supported by only 5% (2/32) of RPA32 foci in nuclei with 5 RPA32 foci being in close proximity to the nucleolar-like-body where the sex chromosomes are positioned (see Table 1 and D RPA32 + SYCP1+SMC3 for RPA32 foci frequencies and NLB proximity and sex chromosome position respectively).

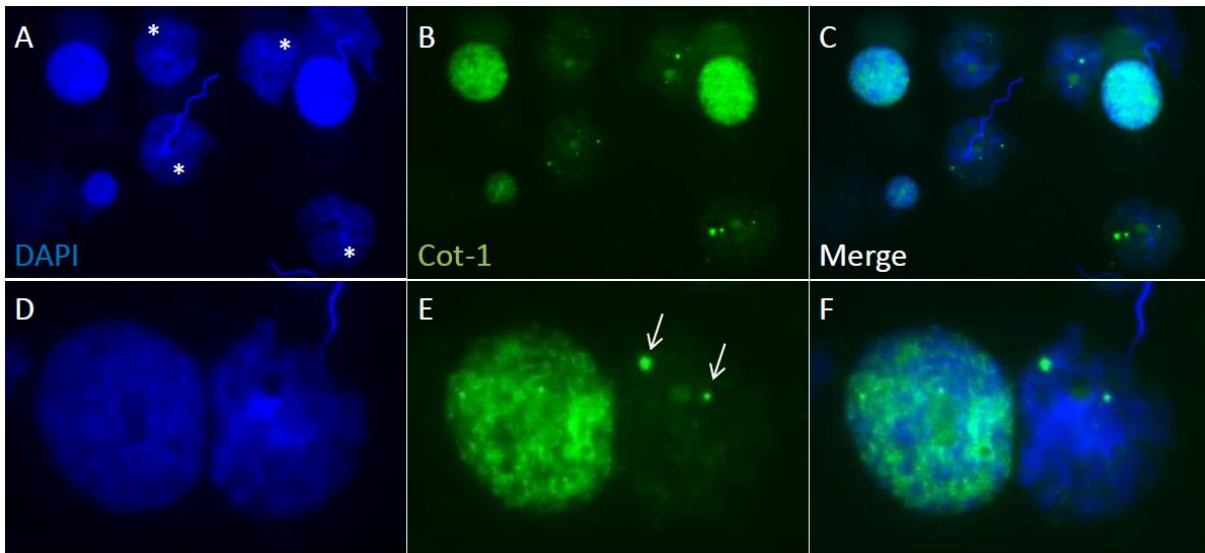


Figure 7. Cot-1 RNA FISH staining on platypus meiotic spreads. Platypus testis cell surface spreads were hybridised with a Cot-1 RNA FISH probe (green) and counterstained with DAPI. Nuclei with a characteristic DAPI appearance with large DAPI void (A asterisks and D) have a marked reduction in Cot-1 RNA FISH signal while other cell types are positive for Cot-1 hybridisation (B). Note 2-3 strong Cot-1 foci are present in pachytene cell nuclei (E arrows).

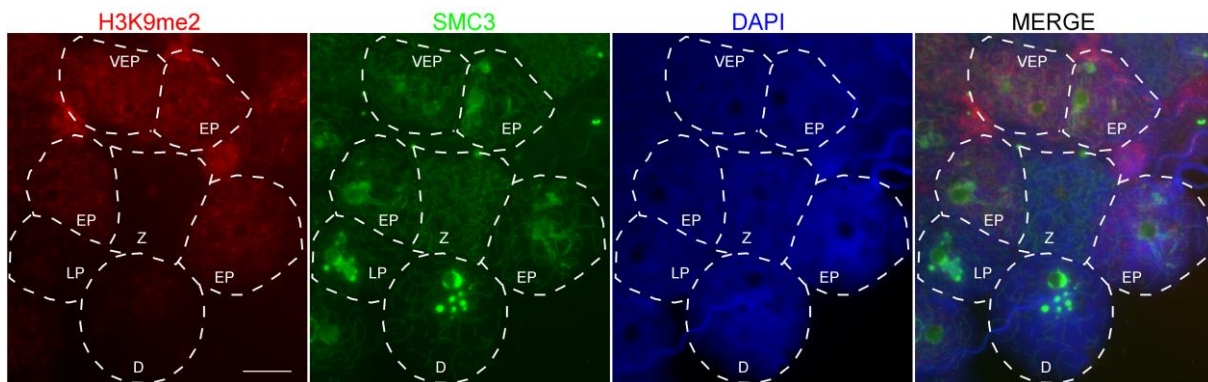


Figure 8. H3K9me2 dynamics through prophase I. Platypus meiotic surface spreads were immunostained for H3K9me2 and SMC3. Consistent with the loss of RNA FISH Cot-1 staining in pachytene nuclei (Figure 7) there was generalised increases in the H3K9me2 repressive histone mark through pachytene and reductions in both zygotene and diplotene which precede and follow pachytene respectively (H3K9me2 panel). H3K9me2 staining was also reduced in late pachytene (LP) nuclei. Staging and sex chromosome positions were

determined by SMC3 accumulation dynamics (SMC3 panel). Z=zygotene, VEP=very early pachytene, EP=early pachytene, LP=late pachytene and D=diplotene.

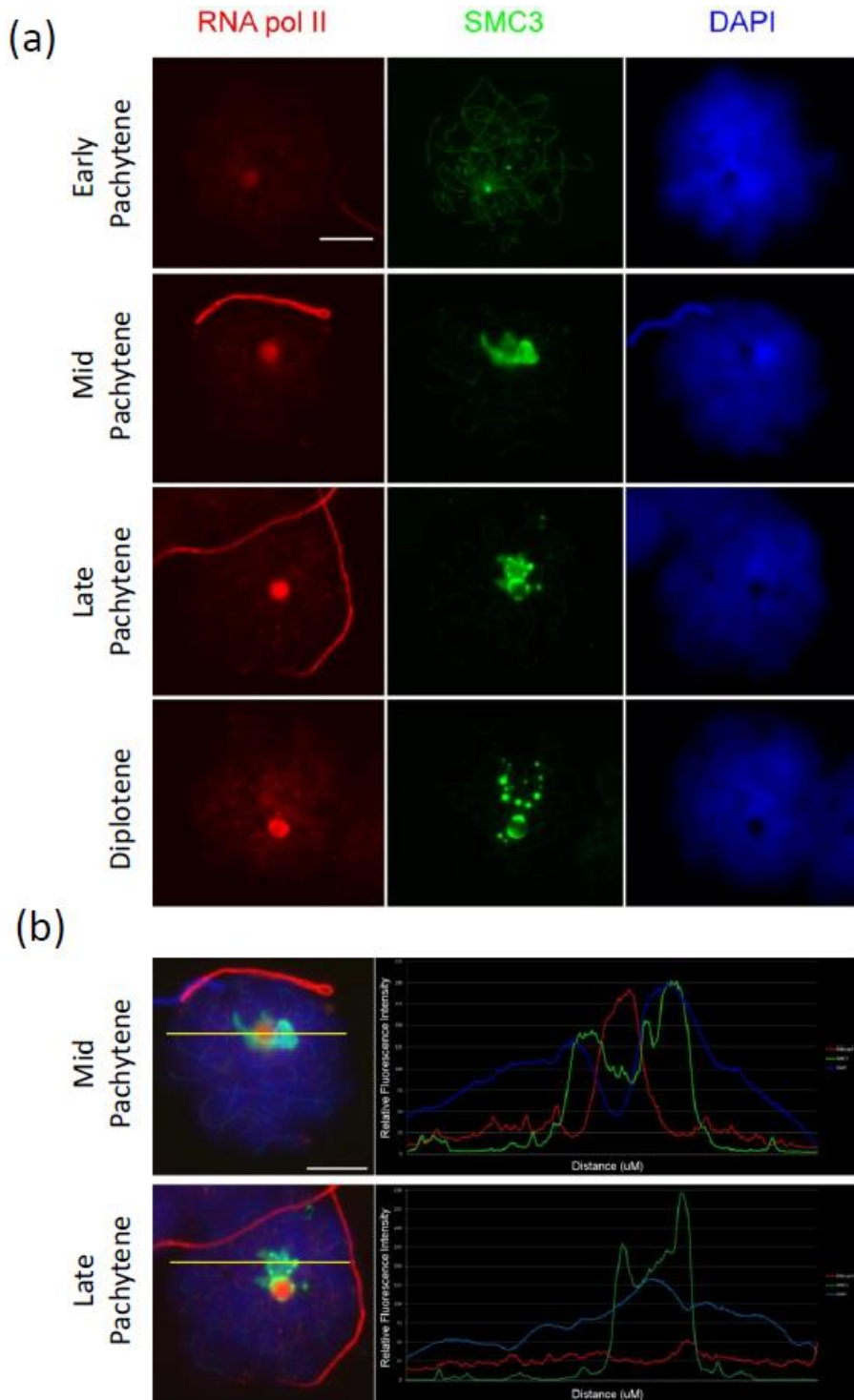


Figure 9. RNA PolII staining through prophase I. Platypus meiotic surface spreads were immunostained for RNA PolII and SMC3 (a) followed by colour intensity mapping (b). (a) RNA PolII staining is low throughout pachytene and increases in diplotene (bottom panel) indicating a generalised reactivation of transcription occurs following the pachytene specific transcriptional drop. Prophase stage and sex chromosome location was determined by SMC3 staining (middle column panels). (b) Signal intensity profiles were conducted in mid and Late

pachytene nuclei in (a) to determine whether relative RNA PolIII staining was reduced over the sex chromosomes. While general RNA PolIII signals were low at these stages there was no detectable reduction over the sex chromosomes relative to other nuclear regions. Horizontal yellow line indicates position of the intensity slice.

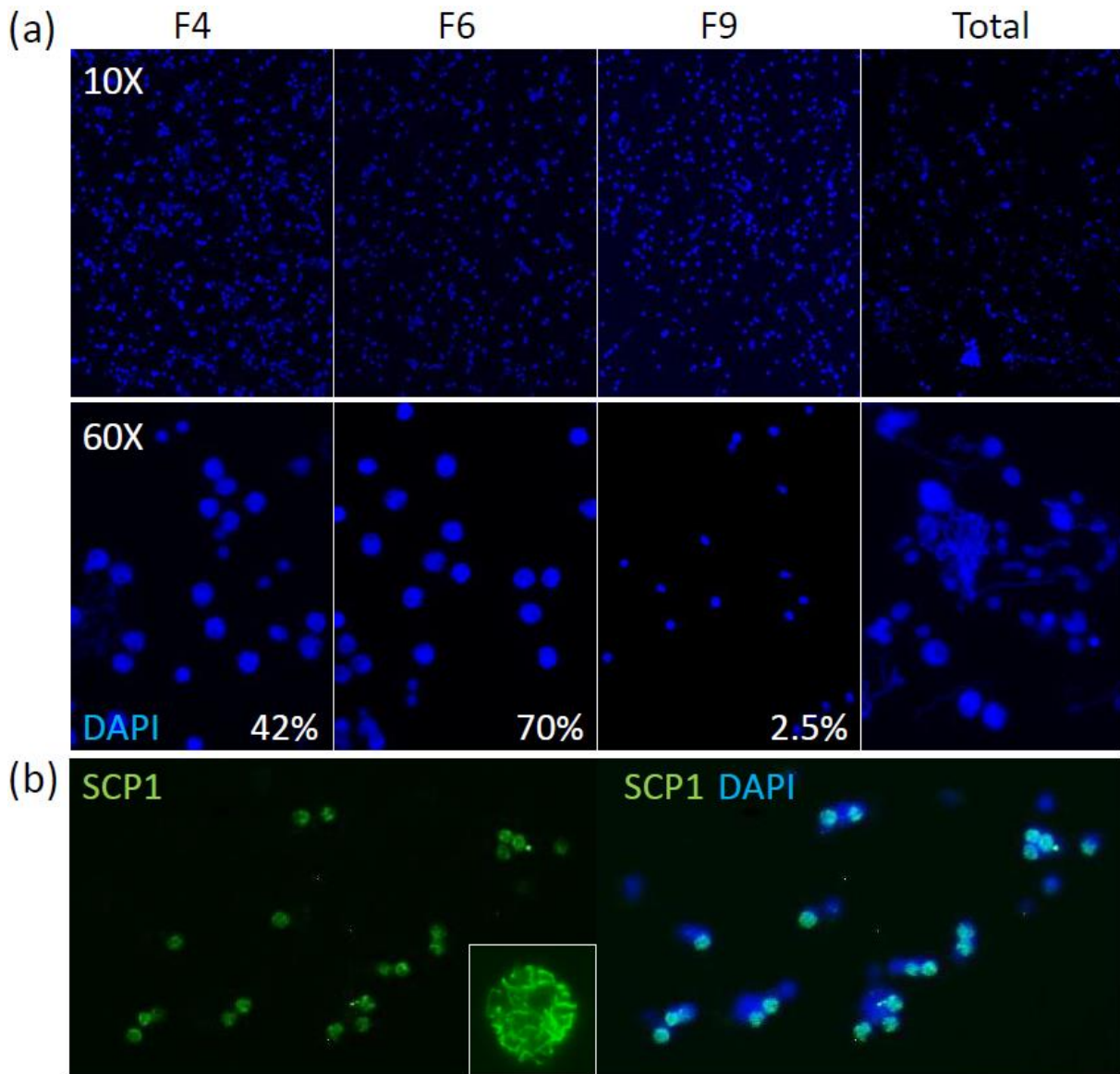


Figure 10. Platypus testis sedimentation gradient fractions. Total testis cell suspensions were applied to a continuous 2-4% BSA gradient and fractions eluted following 3 hours. Cells from each fraction were surface spread on slides and DAPI stained (a) or immunostained for SYCP1 (b). (a) Columns are gradient fractions numbered as they were eluted from the sedimentation chamber, rows are objective magnification. F6 has the highest pachytene enrichment (70%) followed by F4 (42%) and F9 (2.5%). Total = total testis not put through column. (b) Pachytene cell enrichments were confirmed using an anti-SYCP1 antibody to identify pachytene stage meiotic cells (inset left panel).

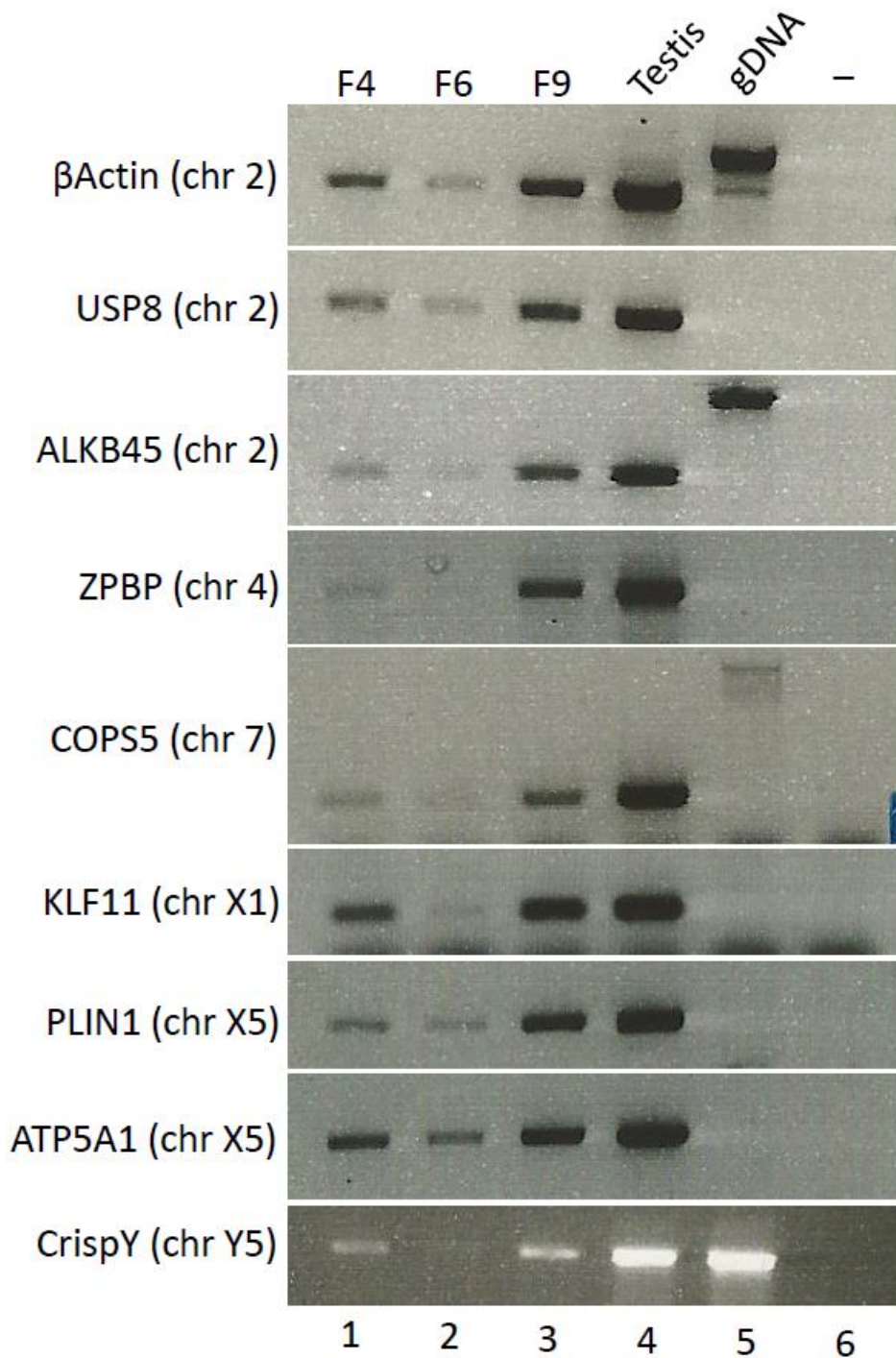
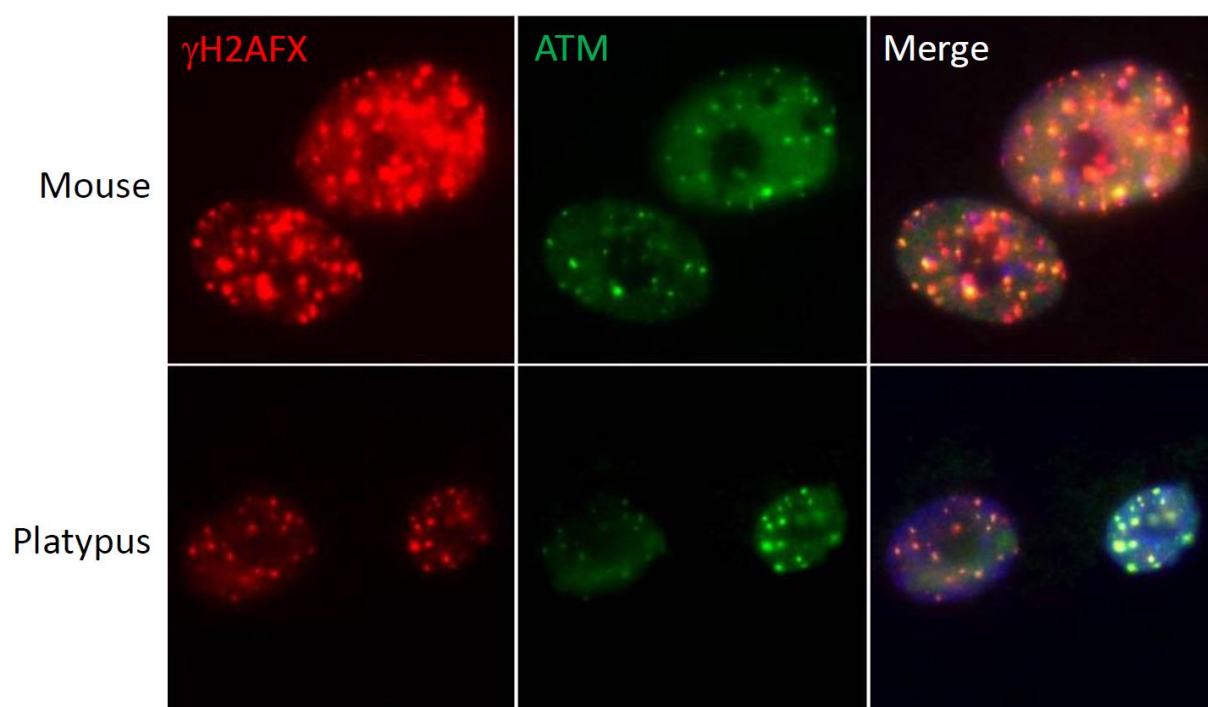


Figure 11. Autosomal and sex linked gene expression in gradient sedimentation separated testis cell populations. RNA from indicated gradient sedimentation fraction cells was extracted followed by cDNA synthesis and semi-quantitative RT-PCR. Platypus pachytene enriched cell populations have reduced expression of autosomal and sex linked genes. Lanes 1-3 have cDNA templates from gradient sedimentation fractions 4 (F4), 6 (F6) and 9 (F9) with respective pachytene cell enrichments of 42%, 70% and 2.5%. As pachytene content

increases the transcript abundance decreases independent of genomic location. Controls were total testis from the same animal as that used for fractions (Testis, lane 4), platypus male genomic DNA (gDNA, lane 5) and no template (-, lane 6). Note visual absence of some gDNA controls are due to high molecular weights or intron size. Chromosomal location for each amplified gene transcript given in parenthesis left of panels.

Supplementary Figures



S1. DNA DSB induction in mouse and platypus fibroblasts. Fibroblast cells were exposed to 10 Gray of ionising radiation and dual immunostained for phosphorylated H2AFX (γ H2AFX) and ATM after 1 hour to detect double strand break repair foci. Marked induction of DNA breaks was observed and both antibodies showed signal co-localisation demonstrating the ability of the γ H2AFX antibody to detect DSB in platypus.


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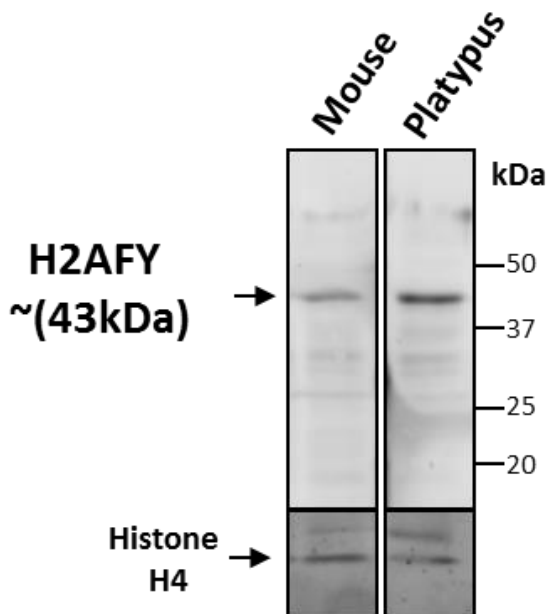
Mouse_γH2AFX      MSGRGKTGGKARAKAKSRSSRAGLQFPVGRVHRLLRKGGHYAERVGAGAPVYLAAVLEYLT 60
Platypus_γH2AFX  MSGRGKTGGKARAKAKSRSSRAGLQFPVGRVHRLLRKGGHYAERVGAGAPVYLAAVLEYLT 60
*****

Mouse_γH2AFX      AEILELAGNAARDNKKTRII PRHLQLAIRNDEELNKKLLGGVTIAQGGVLPNIQAVLLPKK 120
Platypus_γH2AFX  AEILELAGNAARDNKKTRII PRHLQLAIRNDEELNKKLLGGVTIAQGGVLPNIQAVLLPKK 120
*****

Mouse_γH2AFX      SSATVGPKAPAVC KKASQASQEY 143
Platypus_γH2AFX  SGAASSPKAPNA- KKGAQASQEY 142
*.*: .**** . **.:*****
                ↑
                SER 139

```

S2. Amino acid alignment of mouse and platypus H2AFX. The anti-γH2AFX detects the epitope boxed in red with the phosphorylated Serine 139 position indicated with an arrow.



S3. H2AFY protein detection in mouse and platypus testis. An anti-H2AFY antibody was used to detect H2AFY protein levels in total testis lysates from mouse and platypus. Bottom panels are histone H4 loading controls.

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Chapter 7: Discussion

7.1 Summary of Results

Almost 40 years after the discovery of the platypus meiotic chain (Murtagh, 1977) and ten years after the identification of the sex chromosomes (Grutzner et al., 2004b), this is the first thorough investigation of the organisation of the meiotic sex chromosome chain in platypus. While there are many examples of mammalian species with multiple sex chromosomes, the majority of these exist in the form X_1X_2Y , with the minority as XY_1Y_2 (Yoshida and Kitano, 2012), none of which come close in size to the platypus sex chromosome chain; $X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5$. Such a complex chain raises interesting questions as to how it is organised and even tolerated during meiosis. Thus the principle aims of this study were to investigate formation of the chain and to examine how it is assembled and organised at meiosis I. The work presented here has made significant inroads into our understanding these aims, specifically in regards to the organisation of the chain, including the timing of its formation, its tight association with the large nucleolus and its differential association with significant cohesin loading. Finally this work has added to the pool of knowledge on the evolution of the process of mammalian MSCI.

7.1.1 Formation of the Platypus Sex Chromosome Chain

Generally the sex chromosomes pair much later in zygotene than autosomes (Kauppi et al., 2012), however in platypus we observed that a number of the sex chromosomes were pairing before most of the autosomes. Furthermore we observed sex chromosome pairing was not random, where the chain forms beginning at the Y5 end with chromosomes added sequentially, finishing with the addition of X1. Surprisingly, this order has no correlation with the size of either the PARs or chromosomes. These findings reveal that there is a requirement for strict coordination of pairing sex chromosomes when such a large chain is

involved. In the future, it will also be of great interest to determine if there is any such coordination in a species with several translocations, particularly ones that include the sex chromosomes, such as those in various strains of mice.

We have yet to gain any data on the echidna chain. A study of the formation of the echidna sex chromosome chain will prove particularly interesting since the Y5 chromosome (the first to pair in the platypus sex chromosome chain) is fused onto the Y3, in the middle of the echidna sex chromosome chain (Rens et al., 2007). If the order of pairing of the chain is chromosome dependent then we would expect to observe the middle of the chain pairing first, else we may observe an ordered pairing from one end as we observe in the platypus, or we may possibly observe a random order of pairing.

7.1.2 The Platypus Large Nucleolus

From late zygotene and into diplotene, the platypus sex chromosome chain has a strict association with a large nucleolus. This association both precedes and succeeds a similar association in mouse that only occurs in pachytene (Tres, 2005). Another point of contrast is that in platypus the nucleolus never leaves the NOR as it does in mouse (Tres, 2005) and the site of attachment of the NOR on the large nucleolus is always on the opposite pole to the sex chromosomes, supporting previous findings of a bipolar nucleolus (Dresser and Moses, 1980). This emphasises the importance of the nucleolus in platypus prophase I and also supports a general trend of association between the nucleolus and sex chromosomes during prophase I that has been reported in an array of species, including other eutherians (Ohno et al., 1957; Kierszenbaum and Tres, 1974; Holm and Rasmussen, 1977; Dresser and Moses, 1980), metatherians (Sharp, 1982) and even invertebrates (Viera et al., 2009). The purpose of this association is not clear, however, several proteins that localise to the sex chromosomes during pachytene are also observed to accumulate in nucleoli (Escalier and

Garchon, 2005b; Tsutsumi et al., 2011a), suggesting that among other possible roles, the nucleolus may act as a store for specific proteins, such that they can be rapidly loaded onto the chromatin of the sex chromosomes.

In the echidna genome there is three NORs, including one on the X₅ (Rens et al., 2007). It will therefore be important to determine what happens in the echidna prophase I cell in regards to nucleolar activity. Of particular interest will be to determine whether the nucleoli coalesce as they do in mouse, whether any nucleoli, particularly the X₅, remain attached to the NOR, as occurs in platypus, and finally to observe the size of the nucleolus that attaches to the sex chromosome chain, assuming one does. It would also be of interest to attempt to determine whether any proteins that translocate from the nucleolus to the sex chromosomes in other species also translocate from the monotreme nucleolus to the sex chromosome chain using immunocytochemistry techniques.

7.1.3 The Platypus Synaptonemal Complex

Platypus has relatively well conserved SYCP1 and SYCP2 proteins, while the SYCP3 protein is much less conserved. This is at odds with other findings that regions of the SYCP3 protein are the most well conserved of the synaptonemal complex proteins (Fraune et al., 2012). Interestingly the Ka/Ks ratios suggest that before the split of platypus and echidna from their common ancestor there was positive selection acting on SYCP3, these values also suggest that since the split of platypus and echidna there has been positive selection on the echidna, but not the platypus, copy of SYCP3. Further to this, platypus expresses SYCP3-like and SYCP3Y proteins, which are unique to the platypus or possibly monotreme lineage. The conservation of various domains and residues in the different monotreme SYCP3 isoforms (see table 1) make it difficult to resolve the function of these isoforms and the implications for this are unknown.

Table 1. Conservation of domains and residues critical for SYCP3 formation and function.

	BP1	BP2	Nt6	Ct6	Other residues required for tetramer stabilisation
Platypus SYCP3	✘	✘	✓	✓	6/30 conserved + 3/30 similar residues (9/30)
Echidna SYCP3	✘	✘	✓	✓	9/30 conserved + 4/30 similar residues (13/30)
Platypus SYCP3-like	✘	✘	✓	✓	17/30 conserved + 5/30 similar residues (22/30)
Platypus SYCP3Y	✓	✓	✓	✘	10/30 conserved + 3/30 similar residues (13/30)
Echidna SYCP3Y	✓	✓	✓	✘	14/30 conserved + 4/30 similar residues (18/30)

Tick marks indicate a conserved domain and a cross indicates unconserved domains. Explanation of the domains is located in the manuscript titled "Identification and Characterisation of Synaptonemal Complex Genes in Monotremes".

While there is a possibility that all of the platypus SYCP3 protein isoforms may function in formation of the synaptonemal complex, it is also possible that the paralogues have gained new function, similar to the sex chromosome linked paralogues identified in mouse. In mouse it is reported that SYCP3-like, X-linked (slx) has a cytoplasmic role of unknown function (Mueller et al., 2008), SYCP3-like X-linked 2 (SLX2) localises to the sex body coincident to the phosphorylation of H2AX at the beginning of pachytene (Shi et al., 2013) and SYCP3-like, Y-linked (sly) coats the sex chromatin in spermatids (Cocquet et al., 2009). Therefore future work will concentrate on cloning these genes for expression such that they can be utilised to generate antibodies that can be used in immunocytochemistry experiments. They will also be expressed in heterologous systems, both individually and in combination, to determine whether fibril formation, and thus self-association, occurs for each SYCP3 isoform both individually and in combination with one another.

7.1.4 Accumulation of Cohesin on the Sex Chromosomes During Meiotic

Prophase I

Cohesins are essential for a number of fundamental mechanisms in chromosome biology, including keeping sister chromatids attached after replication (Mehta et al., 2013), transcriptional regulation (Lara-Pezzi et al., 2004; Wendt et al., 2008), DNA double strand break (DSB) repair (Kim et al., 2002; Watrin and Peters, 2009), chromosome condensation (Ding et al., 2006; Heidinger-Pauli et al., 2010), DNA replication (Terret et al., 2009), homologue pairing and recombination (Xu et al., 2005; Herran et al., 2011; Lee and Hirano, 2011) synaptonemal complex (SC) formation (Novak et al., 2008; Fukuda et al., 2014; Hopkins et al., 2014; Winters et al., 2014) and promoting nucleolar structure and function (Harris et al., 2013). In meiotic prophase I, cohesins have been shown to have multiple roles. Some cohesins function to keep sister chromatids together after the replication step that occurs prior to leptotene, while other cohesins have roles in ensuring non-sister chromatids, from homologous chromosomes, remain in close proximity (Lee and Hirano, 2011). In the platypus, cohesins show an unusual reorganisation in prophase I, and show increased loading on the asynapsed regions of the platypus sex chromosome chain. In late pachytene, it also appears that the cohesins spread onto the chromatin of the asynapsed regions of the sex chromosomes, a time when there is also a colocalisation with a DAPI intense region, suggesting a possible relationship between cohesin loading and condensation of X and Y specific DNA. While this appears to be a unique role of cohesin in meiosis, it is supported by recent work where cohesin enrichment has been predicted to affect gene expression in plants (Yuan et al., 2012). Specific heterochromatic regions, including centromeric heterochromatin, also present cohesin accumulation, where it is recruited by Suv4-20h2 (Hahn et al., 2013) the mating-type heterochromatic region of fission yeast exhibits the same type of cohesin accumulation, where it is recruited by Swi6(HP1) (Nonaka et al., 2002).

This study has shed light on the role of the cohesin subunits SMC3 and STAG3 in platypus meiosis, however we have yet to examine the roles of other cohesin subunits such as REC8, RAD21, RAD21L, SMC1 α and SMC1 β . Furthermore we are yet to report on the role of any of these subunits in echidna meiosis, studies that will prove of great interest given the echidna also contains a large chain that contains some conserved and some unique elements compared to the platypus chain composition. Therefore future work will concentrate on characterising the localisation of the other meiotic cohesin components in platypus prophase I cells and to determine the localisation of all of the meiotic cohesin proteins in echidna prophase I cells.

7.1.5 Meiotic Silencing in the Platypus

In platypus pachytene cells we observe a DAPI intense region that is not apparent in either zygotene or diplotene, this region is apparent in both PFA and methanol/acetic acid fixed cells and is thus not likely an artefact of experimental technique. DNA FISH using sex chromosome specific probes reveal that the DAPI intense region is coincident with the platypus sex chromosomes, indicative of a condensed state. When the DAPI intense region is visible at a paranucleolar region, it colocalises with significant cohesin loading on the asynapsed region of the sex chromosome chain. Condensation of the sex chromosomes is an almost universal process in mammals as part of MSCI, however the similarity between platypus and other mammals ends there. Our assessment of transcription within meiotic prophase cells using Cot-1 RNA FISH, RT-PCR and immunocytochemistry with H3K9me2 and RNAPolIII reveal that global transcriptional repression occurs for the duration of pachytene, which is similar to that which occurs in birds, and different to that which occurs in other mammals, where global transcriptional repression occurs in early pachytene, with the transcriptional reactivation of autosomes soon after.

It is also of interest that we failed to observe phosphorylation of the histone variant H2AX, in platypus pachytene cells, this applies to the first wave that occurs in zygotene that is a part of the DDR pathway and the second wave which localises to the chromatin loops of the sex chromosomes at the beginning of pachytene, a hallmark of the initiation of MSCI. The fact that the first wave of H2AX phosphorylation is not observed in platypus is indicative that another histone variant/modification may have evolved to be used in the platypus DDR pathway and it is possible that such a histone variant/modification may also be used to coat the chromatin of the sex chromosomes during pachytene, therefore a study into how DSBs are resolved in platypus without the use of γ H2AX is necessary in the future.

Consistent with observations in other species we also report H3k27me3 and H2AFY accumulation at a para-nucleolar location, however there was no stable association between these accumulations and the platypus sex chromosomes. Our results from RPA32 staining also suggests that there is no DDR machinery retained on the sex chromosomes in pachytene or later, indicative of a lack of classic MSCI. We also report that there is H3K9me3 accumulation on a portion of the platypus chromosome 6, a curious finding since the same chromosome becomes the single X chromosome in therian mammals.

These findings indicate that in platypus there appears to be a shift from the synaptic adjustment and longer global transcriptional downregulation observed in birds to the shorter global transcriptional downregulation and sex chromosome specific silencing of MSCI observed in therian mammals.

Indeed there are few histone modifications/variants that have been linked to the unpaired regions of the platypus sex chromosome chain and these show no consistent colocalisation with all of the unpaired regions of the chain. However, there are a number of histone modifications/variants that have yet to be tested including H2AZ and SUMOylation,

therefore a study that will expand on this knowledge is necessary to provide further understanding of the evolution of MSCI in mammals.

7.2 Limitations of the Platypus Model

The platypus as a model has many limitations, firstly material is a rare and precious commodity and thus all experiments must be planned in a way to minimise use of material. Secondly, compared with other mammals, platypus proteins are the most diverged and thus antibodies raised against specific proteins (usually mouse or human) often fail to recognise the platypus homologue. Finally, genetic manipulation of the species is out of the question.

References

The following references are only for the manuscripts cited in the introduction and discussion. All other references are located at the end of their respective manuscripts.

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Amendments

The following changes and additions have been made to this thesis

Page xi, Line 18 should read: "Chapter 3"

Page xii, Line 2 should read: "Chapter 4"

Page xii, Line 9 should read: "Chapter 5"

Page xii, Line 15 should read: "Chapter 6"

Page 3, Line 6 should read: "Thus far, two mechanisms...".

Page 4, Line 8 should read: "1.2.2 The Mammalian Synaptonemal Complex".

Page 6, Line 5 should read: "In metatherians the dense plate (a structure made of the synaptonemal complex proteins SYCP3 and SYCP1) ensures the sex chromosomes..."

Page 6, Line 9 should read: "1.2.3 The Mammalian Cohesin Complex".

Page 7, Lines 6-9 should read: "As DNA is replicated, still prior to leptotene, three other cohesin complexes replace the original, these are: 1) SMC3, SMC1 α , RAD21L and phosphorylated STAG3; 2) SMC3, SMC1 β , RAD21L and phosphorylated STAG3; and 3) SMC3, SMC1 β , REC8 and STAG3"

Page 11, Line 13 should read: "PMSC is associated with a lack of Cot-1 (DNA in the range of 50-300 bp that is enriched with repetitive sequence) FISH signal, increased DAPI intensity..."

Page 12, Line 3, the following should be inserted:

"1.2.5 Retrogenes in Meiosis"

“The X chromosome contains genes that are required for spermatogenesis. MSCI prevents the expression of these genes at the time during which they are required. As a result autosomal backup genes evolved by retroposition. These retrogenes are created by the reverse transcription of mRNA sequences and are therefore duplicates of a source gene, albeit lacking any introns. X chromosome genes required for spermatogenesis have been shown to have retrogene copies located on autosomes, which are specifically expressed during meiosis when their source genes are silenced via MSCI/PMSC. A lack of sex chromosome derived retrogenes in the autosomes of platypus has led to the suggestion that MSCI evolved in the therian lineage, after the split from monotremes (Potrzebowski et al., 2008).”

Page 12, Line 22 should read: “Next, in order to determine how the chain forms, this study...”

Page 13, Line 4 should read: “...to analyse the role of the cohesin complex during...”

Page 27, “Publication Status” should read: “Accepted for publication in the journal ‘Gene’.”

*Note that the accepted version has been significantly revised.

Page 37, Line 6 before “Interpro domain...” the following should be inserted: “SYCP3 proteins contain a Cor1/Xlr/Xmr domain, where Cor1 is a component of the chromosome core in meiotic prophase chromosomes, Xlr is a lymphoid cell specific protein and *Xlm* is specifically transcribed in the testis and localises to the nuclei of spermatocytes.”

Page 39, Line 6 should read: “However, we could not...”

Page 66, Line 9 should read: “...organisation during meiosis and mitosis.”

Page 73, Line 23 should read: "...staining (Figure 2, pink arrows/arrowheads) resembling protein bodies (spherical bodies rich in specific proteins including cohesins) previously described (Krasikova et al., 2005)."

Page 115, Line 1 should read: "...2009))"

Page 115, Line 14-16 should read: "However, despite the differential spatial distribution, each of the BACs tested had many signals in close proximity with this structure, suggesting there occurs a transient close association of the majority of the chain during pachytene."

Page 163, Line 3 should read: "7.2 Monotremes as a Model"

Page 163, Line 9 should read: "Despite the difficulties of working with platypus, their position as the most basal living mammal has provided important and fundamental insights into the evolution of the mammalian genome. Complexity and homology of platypus sex chromosomes has already revolutionised our understanding of sex chromosome evolution in mammals. Work presented in this thesis has provided important insights into how such a complex composition is organised in early meiosis and how sex chromosome silencing mechanisms at meiosis evolved in mammals. For future work it will be important to include the echidna in such comparisons. Finally, there are still many open questions regarding monotreme meiosis, for example the presence and frequency of recombination on sex chromosomes and autosomes, or, understanding the mechanism behind the highly accurate alternate segregation of the chain that generates either 5X or 5Y bearing sperm. Now the tools and techniques are available to tackle those questions which will provide insights into the molecular basis for the extraordinary biology of monotremes and the evolution of fundamental mechanisms of chromosome biology."

Page 175, Line 14-17 should be deleted.

