The role of STAG3 in mammalian meiosis

Dissertation

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“Claims that cannot be tested, assertions immune to disproof are veridically worthless, whatever value they may have in inspiring us or exciting our sense of wonder.”

– Carl Sagan
# Table of Contents

List of Figures ................................................................................................................. I
List of Tables ...................................................................................................................... II
Abbreviations ................................................................................................................... III
Acknowledgements .......................................................................................................... IV

1. Introduction .................................................................................................................... 1
   1.1. Gametogenesis ........................................................................................................ 1
       1.1.1. Spermatogenesis .......................................................................................... 2
       1.1.2. Oogenesis .................................................................................................... 4
   1.2. Meiotic prophase I ................................................................................................ 5
       1.2.1. Leptotene .................................................................................................... 6
       1.2.2. Zygotene .................................................................................................... 8
       1.2.3. Pachytene .................................................................................................. 9
       1.2.4. Diplotene .................................................................................................. 11
   1.3. Cohesin complex .................................................................................................. 11
       1.3.1. Structure of the cohesin complex and its role in sister chromatid cohesion ........................................ 11
       1.3.2. Cohesin dynamics during mitosis ................................................................ 13
       1.3.3. Other roles of cohesin complexes in eukaryotic cells .................................... 15
       1.3.4. Role of cohesin complex in meiosis .............................................................. 17
       1.3.5. Stromal antigen (STAG) cohesin subunit ..................................................... 20
   1.4. Aim of the project .................................................................................................. 22

2. Materials and Methods ............................................................................................... 23
   2.1. Materials ............................................................................................................... 23
       2.1.1. Mice and mouse cell lines .......................................................................... 23
       2.1.2. Bacteria ...................................................................................................... 24
       2.1.3. Primers and other oligonucleotides ............................................................. 25
2.1.4. Plasmids ...........................................................................................................26
2.1.5. Antibodies ........................................................................................................27

2.2. Methods ..................................................................................................................29
2.2.1. RNA extraction and RT-PCR .............................................................................29
2.2.2. Cryosectioning of testes and ovaries .................................................................30
2.2.3. Chromosome spreads ..........................................................................................31
2.2.4. Preparation of samples for FACS analysis ..........................................................31
2.2.5. Immunofluorescence analysis ............................................................................31
2.2.6. Telo-FISH ...........................................................................................................33
2.2.7. ImageJ analysis ...................................................................................................33
2.2.8. Cloning and production of recombinant STAG3 fragments ...............................33
2.2.9. Preparation of cell "pearls" from insect cell extracts ...........................................37
2.2.10. Gel filtration chromatography ..........................................................................37
2.2.11. Crosslinking of antibodies to the paramagnetic beads using AminoLink™ Coupling Resin ..................................................................................37
2.2.12. Protein extraction and immunoblotting ............................................................38
2.2.13. Differential salt extraction ................................................................................39
2.2.14. Protein immunoprecipitation and pulldowns ....................................................39
2.2.15. Coomassie Brilliant Blue and silver staining of gels .......................................41
2.2.16. Electrophoretic mobility shift assay (EMSA) ....................................................41

3. Results .........................................................................................................................42
3.1. STAG3-deficient mice are infertile .........................................................................42
3.2. Meiotic arrest in STAG3-deficient spermatocytes ..................................................43
3.3. Chromosome axis and synaptonemal complex formation are deficient in Stag3ko/ko spermatocytes .......................................................................................46
   3.3.1. STAG3 is necessary for the formation of the synaptonemal complex ..........47
   3.3.2. STAG3 is dispensable for the formation of DNA double-stranded breaks but is, at least indirectly, necessary for their processing ........................................49
3.4. Centromere and telomere cohesion is impaired in Stag3ko/ko spermatocytes ......51
3.5. Cohesin proteins in Stag3ko/ko spermatocytes ......................................................54
3.6. Deficiencies in Stag3\textsuperscript{ko/ko} oocytes largely parallel those in spermatocytes.

3.6.1. Chromosome axis and synaptonemal complex related proteins in Stag3\textsuperscript{ko/ko} oocytes.

3.6.2. Centromere and telomere cohesion is impaired in Stag3\textsuperscript{ko/ko} spermatocytes.

3.6.3. Other cohesins in Stag3\textsuperscript{ko/ko} oocytes.

3.7. The expression and purification of recombinant STAG3 in insect cells.

3.8. Immunoprecipitation of recombinant STAG3.

3.8.1. Pulldown of STAG3 interactors from mouse testis nuclear extracts.

3.8.2. Pulldowns from mouse nuclear extracts using recombinant STAG3 covalently crosslinked to an agarose resin.

3.9. Recombinant STAG3 binds DNA.

3.10. Introducing STAG3-GFP fusion protein into a mouse line.

3.11. Immunoprecipitation of STAG3-GFP containing cohesin complexes from STAG3-GFP mouse spermatocyte nuclear extracts.

4. Discussion.

5. Summary.


7. References.
List of figures

Introduction

Figure 1.1: Gametogenesis in mammals. .......................................................... 1
Figure 1.2: Testis organization and spermatogenesis ........................................ 3
Figure 1.3: Ovary organization and folliculogenesis .......................................... 5
Figure 1.4: Meiotic prophase I ....................................................................... 6
Figure 1.5: Assembly and organisation of the synaptonemal complex .............. 9
Figure 1.6: Structure of cohesin complex ........................................................ 12
Figure 1.7: Cohesin dynamics in mitosis .......................................................... 15
Figure 1.8: Cohesin complexes ....................................................................... 18
Figure 1.9: Overview of cohesins in meiosis and meiotic cohesin mutant phenotypes .... 19

Materials and Methods

Figure 2.1: STAG3 “knockout-first” allele ..................................................... 23
Figure 2.2: RT-PCR primer map of STAG3<sup>ko/ko</sup> allele .......................... 30
Figure 2.3: Recombinant STAG3 and its fragments ......................................... 34
Figure 2.4: In-Fusion Cloning Method ........................................................... 36
Figure 2.5: The principle of AminoLink™ coupling assay ............................... 38

Results

Figure 3.1: Characterization of spermatogenesis in Stag3<sup>ko/ko</sup> mice .......... 43
Figure 3.2: Axial element (AE) formation requires STAG3 ............................ 44
Figure 3.3: Staging of individual Stag3<sup>ko/ko</sup> tubules .................................. 45
Figure 3.4: Stag3<sup>ko/ko</sup> and synapsis-related proteins ............................... 47
Figure 3.5: IF staining for SC related proteins; Single colour channel and merged images ........................................................................................................ 49
Figure 3.6: IF staining for DMC1, SYCP3 and nucleic acid in juvenile mice ...... 50
Figure 3.7: Centromeres and telomeres in Stag3<sup>ko/ko</sup> spermatocytes .......... 51
Figure 3.8: Inverted colour images of Stag3<sup>ko/ko</sup> spermatocyte staining for centromeres and telomeres ................................................................. 52
Figure 3.9: Telo-FISH analysis of wt and Stag3<sup>ko/ko</sup> testis chromosome spreads. 54
Figure 3.10: Cohesins in wt and Stag3^{ko/ko} .................................................................55
Figure 3.11: Cohesin proteins in wt and in Stag3^{ko/ko} testis ........................................57
Figure 3.12: Synaptonemal complex and axis related markers in day 15 oocyte
chromosomes of wt and Stag3^{ko/ko} mice ....................................................................58
Figure 3.13: Centromeres and telomeres in day 15 oocyte chromosomes of wt and
Stag3^{ko/ko} mice ...........................................................................................................59
Figure 3.14: Inverted colour images of Stag3^{ko/ko} oocyte staining for centromeres and
telomeres ..........................................................................................................................60
Figure 3.15: Cohesin proteins in wt and Stag3^{ko/ko} oocytes ...........................................63
Figure 3.16: Purification of recombinant STAG3 ...............................................................65
Figure 3.17: Gel filtration chromatography of recombinant STAG3 .................................67
Figure 3.18: Immunoprecipitation of recombinant STAG3 with anti-GFP antibody ....70
Figure 3.19: SDS-PAGE analysis of pulldowns from mouse testis nuclear extracts using
recombinant STAG3 ............................................................................................................72
Figure 3.20: Pulldowns from mouse nuclear extract using immobilised recombinant
STAG3 ..................................................................................................................................74
Figure 3.21: Pulldowns from mouse nuclear extract using recombinant STAG3 in
different stringency conditions .......................................................................................75
Figure 3.22: Pulldowns from mouse nuclear extracts using recombinant STAG3
covalently crosslinked to the antibody-bead conjugate .................................................76
Figure 3.23: Electrophoretic Mobility Shift Assay of recombinant STAG3 and DNA ....78
Figure 3.24: FACS analysis of STAG3-GFP testis cells .......................................................79
Figure 3.25: Localization of STAG3-GFP on the chromosomes of mouse spermatocytes .................................................................81
Figure 3.26: Immunoprecipitation of STAG3-GFP containing cohesin complexes from
STAG3-GFP mouse testis nuclear extracts using anti-GFP antibody ..........................83
Figure 3.27: Silver staining analysis of anti-GFP precipitates from STAG3-GFP nuclear
extracts under increasing stringency conditions .............................................................86

Discussion

Figure 4.1: Excised ("ex") version of the deleterious STAG3 allele ..................................95
List of Tables

Table 2.1: Primary antibodies ................................................................. 27
Table 2.2: Secondary antibodies ............................................................ 28
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
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<tr>
<td>AA</td>
<td>axial associations</td>
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<tr>
<td>AE</td>
<td>axial element</td>
</tr>
<tr>
<td>APC</td>
<td>anaphase promoting complex</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>ataxia telangiectasia and Rad3 related</td>
</tr>
<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BRCA</td>
<td>breast cancer (protein)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CdLS</td>
<td>Cornelia de Lange syndrome</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CE</td>
<td>central element</td>
</tr>
<tr>
<td>DAPI</td>
<td>4', 6-diamino-2-phenylindole</td>
</tr>
<tr>
<td>DI</td>
<td>damage induced</td>
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<tr>
<td>DMC</td>
<td>disrupted meiotic cDNA</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpc</td>
<td>days post-coitum</td>
</tr>
<tr>
<td>dpp</td>
<td>days post-partum</td>
</tr>
<tr>
<td>DSB</td>
<td>double stranded break</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia, for example</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ESCO</td>
<td>establishment of sister chromatid cohesion protein</td>
</tr>
<tr>
<td>et al.</td>
<td>et alii</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>FISH</td>
<td>fluorescence in-situ hybridization</td>
</tr>
<tr>
<td>FSC</td>
<td>forward scatter</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>H2AX</td>
<td>histone protein H2A histone family, member X</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's buffered salt solution</td>
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<tr>
<td>His</td>
<td>histidine</td>
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<tr>
<td>HORMAD</td>
<td>HORMA domain-containing protein</td>
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<tr>
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<td>immunofluorescence</td>
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1. Introduction

1.1. Gametogenesis

Gametogenesis is a biological process by which diploid or haploid precursor cells undergo division and differentiation to form mature male (sperm) or female (egg) haploid gametes. Male and female mouse gametogenesis differ significantly, both morphologically and biochemically. In males, a single precursor (spermatogonia) divides and differentiates into 4 sperm cells (spermatogenesis), while in females, a single precursor (oogonia) develops into only one mature egg (oogenesis). Both of these processes occur in organs called gonads which facilitate the appropriate niche necessary for such dimorphism. Male gonads are called testes and female gonads are called ovaries. A diagram of both processes is shown in Figure 1.1.

**Figure 1.1 Gametogenesis in mammals.**

Primordial germ cells (spermatogonia and oogonia) either self-renew or differentiate and enter meiosis. The exchange of genetic material occurs in prophase I and cells divide 2 consecutive times. The first division is reductional, halving the number of chromosomes, while the second division is similar to mitosis. In case of spermatogenesis, the result is 4 spermatozoa, while in case of oogenesis the result is only one egg since the other 3 cells die via apoptosis (image adopted and modified from Kerkis et al. 2011).
1.1.1. Spermatogenesis

In higher vertebrates like mice, the male reproductive organ produces sperm from puberty to old age. It is encased in a tough membranous shell called tunica albuginea which contains a system of fine coiled tubes called seminiferous tubules which are immersed in the interstitial tissue. A thin layer of cells, containing germ stem cells lines the inner wall of every tubule and these cells differentiate radially inward. The stem cells, spermatocytes (cells undergoing meiosis) and spermatids (post-meiotic cells) are supported by Sertoli cells, which are stimulated by testosterone produced by Leidig cells located between seminiferous tubules. These Sertoli cells also form the blood-testis barrier by attaching to the inner extracellular matrix of the basal lamina, effectively making the layers and lumen of the tubules immunologically privileged sites. Leidig cells are embedded in the interstitial space along with immune and blood cells, nerves and lymph tissue and they mediate the endocrine signals received from the pituitary gland (for more information see Alberts, 2009; Mecklenburg and Hermann, 2016).

Spermatogenesis can be divided into 3 phases: stem cell proliferation, meiosis and spermiogenesis. Primary spermatocytes that enter meiosis are formed by a complex series of events involving spermatogonial stem cells. The fate of a germ stem cell depends on a ratio between renewal and differentiation factors. Spermatogonia type A either renew via mitosis, forming a chain of cells with intracellular bridges between each other, or differentiate. Since the chain is oriented such that only the first cell of the chain receives full renewal signalling from the stem cell niche, the signal is diluted through the chain to a level sufficient for breakage of the chain and further differentiation. The final product of this stem cell differentiation is spermatogonia type B, which enters meiosis.

Meiosis is a specialised cell cycle unique to germ cells. Similar to mitosis, the cells replicate DNA once, and then unlike mitosis, which is followed by one, meiosis is followed by 2 consecutive cell divisions (meiosis I and II) and results in 4 cells with a haploid instead of 2 cells with a diploid genome. This reductional cell division and the exchange of genetic material occurring between parental homologous chromosomes are crucial for sexual reproduction. Mouse interphase genome is divided into 40 DNA molecules (2n; representing 20 homologous, parental pairs of chromosomes), which upon entering the cell cycle replicate in the S-phase (4n; representing the replicated genome) and condensate into 40 sister chromatid pairs during prophase (in the cell cycle, a chromatid is a condensed DNA molecule). Unlike mitosis, upon entering meiosis, spermatocytes form specialized structures called chromosomal axes which are comprised of axial elements (AEs) and serve as proteinaceous scaffolds for each of the 40 sister chromatid pairs (also referred to as meiotic chromosomes). The cell then enzymatically introduces double stranded DNA breaks (DSB) throughout the genome which
then each chromosome repairs by using its parental homologue as template, effectively exchanging the genomic information. These structures comprised of 2 physically paired homologous chromosomes (altogether 4 chromatids) are called bivalents or tetrades. The architecture of these chromosomal formations is facilitated by the formation of a zipper-like structure called the synaptonemal complex (SC), which will be described in more detail later. During anaphase I, each chromosome of a bivalent is then segregated into a daughter cell, resulting in 2 cells with a reduced number of chromosomes (2n; representing 20 chromosomes containing 40 chromatids of mixed genetic material). In anaphase II, chromatids of each remaining chromosome are segregated into another 2 daughter cells, resulting all together in 4 haploid gametes (n; representing 20 recombinant DNA molecules). These gametes then undergo metamorphosis in the process called spermiogenesis.

Spermiogenesis is a process by which spermatids alter their morphology and cell content in the post-meiotic stage of gametogenesis. A large round shape is replaced by a smaller, spindle-like form. Acrosomes are formed for the purpose of penetrating oocytes during fertilization and a flagellum develops for the necessary motility. The midsection of the spermatid loses most of the cytoplasm but accumulates tightly packed mitochondria that are paramount for the high-energy demand supporting high mobility. The resulting spermatozoa have all necessary requirements to reach and fertilize the egg in the female oviduct. The gametogenesis in mice lasts 35 days starting from 4 days post-partum (dpp) and mice become fertile at age of 6 weeks. A diagram of testis morphology is shown in Figure 1.2.

**Figure 1.2 Testis organization and spermatogenesis**

Cross-section diagram of testis showing the seminiferous tubules, epididymis and vas deferens. Also, a cross-section of a seminiferous tubule showing spermatogonia, meiotic and post-meiotic cells as well as Sertoli cells. The immature sperm cells created in the tubules move to the epididymis where they finish metamorphosis and are ready to be ejaculated through vas deferens.
1.1.2. Oogenesis

Similar to development of sperm, primary oocytes entering meiosis also form the chromosomal axis, exchange genetic material and go through meiosis I and II. However, unlike spermatogenesis, which starts shortly after birth, oogenesis starts during fetal development at 13.5 days post coitum (dpc). Oogonia stem cells (primordial oocytes) travel to the developing ovary and mitotically divide to create primary oocytes, which are located in a primordial ovarian follicle. Also, meiosis starts before birth and proceeds to the end of diplotene where it is stopped in dictyate arrest until puberty. This pool of arrested primary oocytes is finite for the rest of the animal's life and once it is depleted the unit becomes sterile. Upon reaching puberty, a hormonal wave starts the first menstrual cycle, where a single primary oocyte is released from the dictyate arrest and finishes meiosis I, becoming a secondary oocyte and creating the first polar body. The follicle develops into a primary, secondary and tertiary follicle. Unlike spermatogenesis, only one oocyte is formed during oogenesis because during anaphase I and II, when the genetic material is being segregated, half of the chromatids are each time allocated by asymmetric division into a polar body that barely contains any cytoplasm, which then undergoes apoptosis. During ovulation, secondary oocyte is released into the oviduct, however, in order to complete meiosis II and create the second polar body, the secondary oocyte needs to be fertilized. The resulting mature ovum is at that point, after fertilization, called zygote. Further embryonic development continues by mitotic divisions of the zygote, which subsequently implants into the uterus wall at the stage called blastocyst. The follicle that released the secondary oocyte remains as corpus luteum, providing hormonal signalling which governs uteral preparation for implantation of the embryo. In humans, 14 days after ovulation and with the lack of embryo replacing the corpus luteum signalling, the corpus luteum becomes corpus albicans, which shuts down the signalling leading to the restart of the menstrual cycle. A diagram of ovary morphology is shown in Figure 1.3 (for more information see Alberts, 2009; Sen and Caiazza, 2013).
Meiosis I occurs in the primary follicles of embryonic ovaries and enter dictyotene arrest until puberty. Due to hormone induction during the first menstrual cycle, a primary oocyte is released from the dyctiotene and matures into a secondary oocyte, forming the first polar body in the process. Upon ovulation, secondary oocytes are released from the ovary into the oviduct, leaving behind corpus luteum, where meiosis II finishes only if fertilization occurs. Upon fertilization, a mature ovum with the second polar body is formed. https://biology-forums.com

1.2. Meiotic prophase I

For mitotic divisions to be possible, chromosomes (each comprised of a pair of sister chromatids) need to align at the equatorial plane of a dividing cell and bi-orient toward the spindle poles, which during anaphase allows the pulling forces of the mitotic spindle to segregate one of each sister chromatids into a different daughter cell. Unlike mitosis, bivalents formed in prophase I (comprised of 2 pairs of sister chromatids from the 2 homologues destined to undergo recombination) also need to bi-orient at the equatorial plane so that during anaphase I, the spindle can segregate each chromosome into a different daughter cell. Each of those cells then undergoes meiosis II, which is essentially the same as mitosis, but with half the number of chromosomes. However, to accomplish such a complex feat, the homologous chromosomes first need to be able to recognise each other, physically interact and exchange genetic material, which happens during meiotic prophase I. This requires a complex spatio-
temporal singling network and surveillance system which can be divided into 4 stages based on chromosome morphology: leptotene, zygotene, pachytene and diplotene. An overview of meiotic prophase I is shown in Figure 1.4, which will be explained step by step in the following sections.

**Figure 1.4 Meiotic prophase I**

An overview of events leading to axis and synaptonemal complex formation and their subsequent dissolution. The first four stages of prophase I are depicted (leptotene, zygonema, pachynema, and diplonema), together with the progression of synapsis events: localization of synaptonemal complex protein 3 (SYCP3; red) and 1 (SYCP1; green) and the appearance of major markers for recombination (colored circles). Homologous chromosomes, consisting of paired sister chromatids, are depicted as single looping lines radiating from the synaptonemal complex core. The timing of prophase I stages for male and female meiosis is shown above the cartoon image, representing the temporal differences in prophase I progression between male and female germ cells.

### 1.2.1. Leptotene

Changes in chromosomal structure, including chromatin condensation and recruitment of scaffold proteins, take place during early prophase I. The first stage of meiotic prophase I is leptotene where chromatin compaction already started and accumulation of SYCP3 and
SYCP2 forms the lateral elements (LEs) of the chromosomal axis. LEs together with cohesin complexes form the axial elements (AEs) (Kouznetsova et al. 2005, Fukuda et al. 2014).

Another important event during the formation of AEs is the recruitment of proteins containing HORMA domain, which is a highly conserved multifunctional module that acts as signal-responsive adaptor mediating protein-protein interactions (Aravin and Koonin, 1998; Rosenberg and Corbett, 2015). HORMAD1 and HORMAD2 interact with unsynapsed chromosomal axes and are removed with the formation of the synaptonemal complex (SC; forms between synapsed homologous chromosomes), but again reappear during dissolution of SC in diplotene (Wojtasz et al. 2009; Fukuda et al. 2010; Daniel et al. 2011). At the same time, DSBs are introduced genome-wide by an enzyme similar to the subunit A of DNA topoisomerase VI, named SPO11, in a non-random manner (Romanienko and Camerini-Otero 2000; Metzler-Guillemain and de Massy, 2000; Keeny 2008, Vrielynck et al. 2016). Two isoforms of SPO11 have been described before: SPO11α which was implicated in the sex body formation and maintenance and SPO11β which was shown to be the isoform responsible for the formation of DSB (Romanienko and Camerini-Otero 1990; Bellani et al. 2010; Kauppi et al. 2011). It was also recently shown that SPO11β requires TOPOVIBL, a protein similar to the subunit B of topoisomerase VI, for the formation of DSB (Robert et al. 2016).

Most of these DSBs are introduced at specific sites in the genome called hotspots (Gerton et al, 2000; Hwang and Hunter, 2011). Recent genome-wide mapping identified 15 000 – 20 000 hotspots in the mouse genome, each approximately 1 – 2 kilobases long, roughly 50 – 100 kb apart, which are positioned away from the regulatory sequences (Paigen and Petkov, 2010; Kumar and de Massy, 2010). The further processing of DSBs requires re-sectioning by a DNA damage sensor, the MRE complex, and subsequent activation of ATM and ATR kinases which are responsible for the phosphorylation of the histone H2AX (Rogakou et al. 1998; Mahadevaiah et al. 2001; Redon et al. 2002; Royo et al. 2013). The resulting γH2AX is a chromatin marker for recruitment of early recombinon protein RAD51 and its meiosis specific homologue DMC1 (Rockmill et al. 1995; Pittman et al. 1998). Chromatin modified in such manner is transcriptionally silenced, a phenomenon termed meiotic silencing of unsynapsed chromosomes (MSUC). During early to mid-leptotene stage, the interaction of meiotically induced DNA DSBs with matching sequences on the homologous chromosome brings the AEs of homologous chromosomes into alignment (Page and Hawley, 2004; Baudat and de Massy, 2012). The sites of these interactions are visualized as ~400-nm interaxis bridges (Albini and Jones, 1987; Tesse et al. 2003) of which a subgroup matures into a structure termed axial associations (AAs) that later serve as nucleation sites for SC formation (Wiltshire et al. 1998). Although some organisms like *Drosophila melanogaster* (McKim and Hayasi-Hagihara,1998) and *Caenorhabditis elegans* (Dernburg et al, 1998) do not require DSBs in order to form SCs, they are indispensable for SC formation in mammals. Mice lacking Spo11, and thus DSBs,
show aberrant SCs which results in promiscuous interactions between non-homologous chromosomes which eventually activates a checkpoint mechanism that kills the cell (Baudat et al. 2000, Romanienko and Camerini-Otero, 2000).

1.2.2. Zygotene

During the transition from leptotene to zygotene, telomeres of prophase I chromosomes cluster together and acquire a bouquet formation during which they attach to the nuclear envelope (NE) and move along the inner nuclear membrane (Alsheimer, 2009). To obtain such mobility, the telomers connect to the cytoskeleton and perinuclear motor proteins through meiosis-specific telomere protein complexes containing SUN/KASH-domain nuclear transmembrane proteins (Chikashige et al. 2006; Conrad et al. 2007; Penkner et al. 2009). The resulting chromosome “bouquet” is thought to promote homologous chromosome pairing, meiotic recombination and possibly metaphase plate alignment (Scherthan 2006; Tomita and Cooper, 2007). In mammals, there are two such proteins, SUN1 and SUN2. Deletion of SUN1 prevents telomere attachment to the NE and impairs homologue pairing and synapsis (Ding et al, 2007). Mammalian telomeres contain the shelterin complex which protects telomeres from the deoxyribonucleic acid (DNA) damage response and regulates telomere maintenance by telomerase (Palm and de Lange, 2008). Shelterin contains two double-stranded TTAGGG repeat binding proteins, TRF1 and TRF2, as well as TRF1 binding partner Rap1. Heterozygosity for the shelterin component TRF1 and disruption of the ATM kinase, the SMC1β cohesin, or the ATM kinase target H2AX alter telomere dynamics but not their attachment (Scherthan, 2007). Upon entering zygotene, the transient homologue interactions, mediated by RAD51/DMC1, stabilize by binding mismatch repair (MMR) proteins MSH4/MSH5 (Kneitz, 2000; Novak et al. 2001; Reynolds et al, 2013; Manhart and Alani, 2016). Msh4/Msh5 is a member of the ZMM family of meiotic proteins needed to form crossovers. zmm mutants are defective in synaptonemal complex formation and show less crossovers, suggesting a role in their formation (Surtees et al, 2004; Storlazzi et al, 2010). In budding yeast, Msh4/Msh5 has been shown to stabilize strand invasion intermediates in the later zygotene stage (Börner et al, 2004). It has recently been shown that sumoylation of MMR proteins stabilizes the invasion of DNA strands by forming D-loops which favours the crossover pathway and the reciprocal exchange between homologues (Reynolds et al, 2013). Non-sumoylated MMR proteins dissociate from the DSBs, which are subsequently repaired by a non-crossover pathway, resulting in non-reciprocal exchanges. The stable interaction of sumoylated MMR proteins is thought to nucleate the initiation of synaptonemal complex formation. The SC is a zipper-like structure composed of two lateral elements (LEs) that are joined together by transverse filaments (Page and Hawley, 2004; Costa et al, 2005). Transverse filaments are formed via dimerization of coiled-coil domain containing SYCP1 proteins (Hamer et al, 2006; Fraune et
al. 2011; Baudat et al. 2013), which bridge the gap between one LE and the central element (CE) comprised of SYCE proteins and TEX12 (Meuwissen et al, 1992; Bolcun-Filas et al. 2007; Davies et al. 2012). A diagram of SC formation and its structure is shown in Figure 1.5.

Figure 1.5 Assembly and organisation of the synaptonemal complex

A Assembly of synaptonemal complex (SC) components. In early prophase I, SYCP2 and SYCP3 accumulate on the chromosomes, forming the axial elements which later become part of the synaptonemal complex as lateral elements. The assembly of SC starts with the association of transverse filament protein SYCP1 with lateral elements, marking the beginning of central region formation. The final result is a structure extending along the whole length of the homologous chromosomes in a zipper-like manner. Adopted from Fraune et al. 2012.

B Schematic diagram of the synaptonemal complex organization. Lateral elements (LE), transverse filaments and central elements (CE) of the central region are depicted. Interaction of transverse elements connecting the homologues. Adopted from Page and Hawley, 2004.

Synaptonemal complex facilitates synapsis and subsequent formation of crossovers between the juxtaposed homologous chromosomes. By the end of zygotene, most of the DSBs have been repaired and autosomes synapsed, with an exception of X and Y chromosomes in males, which have only a short homology sequence.

1.2.3. Pachytene

Once all homologues are synapsed and every bivalent has a full SC established, the cell enters pachytene stage. Axes of autosomes in the bivalents displace HORMAD1 for SYCP1, and since all DSBs are repaired, γH2AX is removed from all the autosomal hot spots. In this stage
of meiosis, global transcription resumes at a significantly reduced rate. This transcriptional activation is accomplished by removal of silencing factors associated with γH2AX (Baarends et al. 2005; Ichijima et al. 2012). An exception to these genome wide changes are the sex chromosomes in males, X and Y. These chromosomes have only a short homology sequence called the pseudosomal region (PAR; Kauppi et al. 2012). Unlike on the autosomes, the DSBs on sex chromosomes remain unrepaired, which activates the checkpoint response involving ATR (Turner et al. 2005; Greaves et al. 2006; Royo et al. 2013) and BRCA1 (Turner et al. 2004). Phosphorylation of H2AX leads to transcriptional silencing of XY chromatin, a phenomenon termed meiotic sex chromosome inactivation (MSCI; McKee and Handel 1993; Yan and McCarrey, 2009). This silenced chromatin, along with all the associated proteins, is called the sex body (Solari, 1974; Turner et al. 2005; Bolcun-Filas and Schimenti 2012). Meiotic silencing involves two sets of proteins: “sensors”, which localize to AEs and sense asynapsis, and “effectors”, which localize to the chromatin loops associated with unsynapsed AEs, causing gene silencing over a considerable distance. SYCP3, HORMAD1, HORMAD2 and BRCA1 have been identified as sensors (Turner et al. 2004; Kouznetsova et al. 2009; Daniel et al. 2011; Wojtasz et al. 2012;) and the mediator of DNA damage checkpoint 1 (MDC1) and histone variant H2AFX as effectors (Fernandez-Capetillo et al. 2003; Ichijima et al. 2011). Failure to properly establish MSCI initiates the activation of meiotic prophase I checkpoint, which initiates apoptosis (Royo et al. 2010). This is accomplished when the finite amount of pachytene silencing factors, usually restricted only to the sex body, is diluted to the unrepaired DSBs on the autosomes, effectively re-establishing MSUC during pachytene. Insufficient silencing of the sex chromosomes results in the activation of Y chromosome Zfy genes that produce regulatory and cytotoxic proteins, which initially promote MSCI, then monitor its progress (since if MSCI is achieved, Zfy genes will be silenced), and finally execute cells with MSCI failure (Royo et al, 2013; Vernet et al. 2016).

During mid-pachytene, at least one DSB per autosome and one on the PAR will mature into a crossover. The resident MSH4/MSH5 complex on these DSBs serves as a platform for Mlh1/Mlh3 recruitment (Santucci-Darmanin et al. 2000). These breaks will give rise to future chiasmata, which are the only physical connection between homologues and necessary for proper segregation in metaphase I. The regulation of these maturation events is paramount and several mechanisms are involved to ensure an even distribution of crossovers that are not too close to each other. This process called crossover interference is poorly understood, but recent studies are trying to shed light on to the mechanisms involved in it (Zickler and Kleckner, 2016). Moreover, recently the SUMO ligase RNF212, a putative ubiquitin E3 ligase HEI10, and CNTD1 were identified as factors involved in selective stabilization of pre-CO sites and required for crossover formation (Hunter, 2015).
1.2.4. Diplotene

At the onset of diplotene, the synaptonemal complex starts to dissociate and homologs separate, however, they remain physically connected at the crossover sites, which are cytologically visible as chiasmata. The sister chromatids of the same chromosome are still held together by cohesion complexes. SYCP3 is still on the axes, but disappears after diplotene, remaining only on the centromeric region until anaphase II. Control of the redistribution of axial proteins still remains to be described. The removal of cohesins happens gradually with the dissolution of the synaptonemal complex and a subpopulation of cohesin complexes remains bound to centromeres which is important for proper segregation in both meiotic divisions.

1.3. Cohesin complex

1.3.1. Structure of the cohesin complex and its role in sister chromatid cohesion

In the cell cycle, replication of DNA produces two daughter chains, which need to remain closely associated throughout the process in order for the proper segregation to take place. In both mitosis and meiosis, this sister chromatid cohesion is established with a multiunit protein structure called the cohesin complex. Cohesins are evolutionarily conserved and play a role in many biological processes: cohesin ensures faithful chromosome segregation, regulates gene expression and preserves genome stability (Merkenschlager, 2010; Haering and Jessberger, 2012). The cohesin complex was first discovered in genetic screens that aimed to identify proteins involved in sister chromatid cohesion (SCC; Michaelis et al. 1997; Guacci et al. 1997).

In mammals, the mitotic cohesin complex is formed from 4 core cohesin subunits: SMC (structural maintenance of chromosome) proteins SMC1α and SMC3, a kleisin RAD21 and a stromal antigen protein (either STAG1 or STAG2). There is ample evidence that the first 3 cohesins form a ring-like structure which physically entraps the 2 sister chromatids and holds them together (Figure 1.6; Anderson et al. 2002; Haering et al. 2002; Huis in ‘t Veld et al. 2014). Each of the SMC proteins has a long α-helix domain, which is interrupted in the middle by a hinge domain. This enables a specific folding where two parts of the α-helix interact along their length forming a coiled-coil, bringing the C- and N-terminus together and forming a globular head domain containing an ATP nucleotide binding domain (NBD) of the ABC family. Similar structure with a hole in the middle can be found in other complexes able to manipulate DNA, such as the MCM licensing factors (replicative helicases), Rho transcription terminator (RNA helicase), PCNA (DNA replication processivity factor) or MSH2 and MSH6 sliding clamps (mismatch repair proteins), but all are of a full level of magnitude smaller in diameter. The hinge of the SMC proteins is a dimerization domain necessary for the interaction between SMC1 and SMC3, which results in a SMC1/SMC3 heterodimer containing 2 coiled-coil arms.
with an NBD on each end. Upon ATP binding and interaction with the kleisin molecule, the formation of the 30-nm wide ring is completed (Uhlmann, 2016). The kleisin RAD21 also binds the stromal antigen molecule, which contains armadillo repeats, structurally similar to HEAT repeats. Additionally, there are meiosis specific isoforms of cohesin proteins: SMC1β, kleisins REC8 and RAD21L and stromal antigen STAG3, which will be discussed later.

**Figure 1.6 Structure of the cohesin complex**

In mammals, the mitotic cohesin complex is formed from 4 core cohesin subunits: SMC1, SMC3, RAD21 and STAG. The complex is ring shaped and has a role in maintaining sister chromatin cohesion, regulates gene expression and preserves genome stability.

**A** SMC molecule folds in a way that 2 of its α-helices interact creating a coiled coil domain which also brings the C-and N-terminus in close proximity, forming a head domain containing an ATP nucleotide binding domain (NBD). The hinge domain, positioned between the 2 α-helices, is also a dimerization domain, allowing SMC1 and SMC3 to create a heterodimer with 2 coil-coiled arms with an NBD at each end. Upon ATP binding and kleisin interaction, the ring closes. Similar structure that entraps nucleic acids can be found in other protein complexes like PCNA, MCM and MSH2/6. The picture was adopted from Haering and Jessberger, 2012.

**B** The structure of cohesin complex entrapping 2 sister DNA molecules. Model of the cohesin ring encircling two chromatids. SMC3 (gray) is present in all cohesin complexes. There are two SMC1 genes and proteins: SMC1α (dark blue) and a meiosis-specific SMC1β (light blue). The tripartite ring closes via the association of an α-kleisin subunit, of which three variants exist: the ubiquitous RAD21 (dark turquoise), and two meiosis-specific forms, REC8 and RAD21L (light turquoise). A third component, of which there are three variants, associates with the complex via binding to the α-kleisin: canonical STAG1 or STAG2 (dark orange) or the meiosis-specific STAG3 (light orange) The picture was adopted from McNicoll and Stevense, 2013.
1.3.2. Cohesin dynamics during mitosis

During mitosis, cohesin complex is loaded on to the DNA in telophase, just after the reassembly of nuclear envelope. Loading of the cohesin complex onto mitotic chromosomes is performed by a kollerin complex comprised of SCC2 and SCC4 (Ciosk et al. 2000; Weitzer et al. 2003). SCC2 C-terminus contains HEAT repeats and is necessary for recruitment of cohesin on to the DNA, while the N terminus is required for binding to SCC4. Also, the interaction between SMC proteins of the cohesin complex and DNA is facilitated by their conformational change upon ATP binding and hydrolysis in their NBD domains. Upon entry into mitosis, SCC2/SCC4 dissociates from the DNA, however, recent studies showed that cohesin is reloaded on to the centromeres of mitotic chromosomes, suggesting that another mechanism of cohesin loading exists besides the kollerin complex. Regardless, once loaded after the S-phase, most of the cohesin complexes remain stably bound to the DNA until the activation of the prophase pathway and entrance into mitosis. For stable cohesion to be established, at least one of two conserved, consecutive lysine residues in the SMC3 subunit of the complex must be acetylated by acetyl transferases ESCO1 and ESCO2 (Unal et al. 2008; Whelan et al. 2012; Kouznetsova et al. 2016; Rivera-Colón et al. 2016). The main function of the acetylation event is to stabilize cohesin and prevent any precocious dissociation from DNA until the binding of factors necessary for disrupting the cohesion establishment upon entry into mitosis. Depletion of ESCO1 in mammalian cells causes cohesion defects but they remain viable, which suggests redundancy in the cohesion establishment function. These cohesin–DNA interactions can be reversed by the binding of WAPL to PDS5 (Gandhi et al. 2006; Kueng et al. 2006; Tedeschi et al. 2013), which directly interacts with the stromal antigen subunit of the cohesin complex. WAPL releases cohesin from DNA by opening a DNA “gate” between SMC3 and kleisin subunits (Chan et al. 2012; Buheitel and Stemmann, 2013; Eichinger et al. 2013; Huis in ’t Veld et al. 2014). If WAPL is depleted, the removal of cohesin is prevented and the bulk of cohesin remains on the chromosomes until metaphase (Peters et al. 2008). The maintenance of cohesion by the acetylation event is mediated by the recruitment of sororin, which displaces WAPL from PDS5, effectively stabilizing cohesion. However, SMC3 acetylation promotes sororin binding only when it occurs during DNA replication (Lafont et al, 2010; Nishiyama et al, 2010; Song et al, 2012). Sororin is also required for stabilization of cohesin on chromatin during replication (Gerlich et al, 2006; Schmitz et al, 2007).

Upon entry into mitosis, the bulk of cohesins in mammalian cells is removed from chromosome arms via prophase pathway, except of the cohesin associated with centromeres, which is removed during the metaphase to anaphase transition. One of the first events during the prophase pathway is the phosphorylation of the SMC1 and stromal antigen subunit of the
cohesin complex by polo-like kinase 1 (PLK1; Losada et al. 2002; Sumara et al. 2002; Lenart et al. 2007). Phosphorylation of other prophase and prometaphase proteins by AURORA B also contributes to cohesin dissociation from chromosomes arms (Sumara et al. 2004). The release of cohesin complex by WAPL, as described above, might be caused by a conformational change in the cohesin ring as a result of the interaction of WAPL/PDS5 with now phosphorylated SA2, which has a lower affinity for the PDS5/sororin complex.

During the prophase pathway, cohesin removal must be inhibited at the centromeres in order for the anaphase spindle to properly bi-orient the sister chromatids and afterwards segregate each into a different daughter cell. This is accomplished by the binding of shugosin (SGO1/2; McGuinness, 2005; Ishiguro et al. 2010; Tanno et al. 2015), which recruits the protein phosphatase 2A (PP2A) complex, which dephosphorylates SA2 and inhibits WAPL activity (Kitajima et al. 2006; Shintomi and Hirano 2009; Hara et al. 2014). This protection lasts until anaphase when activation of the anaphase promoting complex (APC/C) promotes degradation of securin (Nasmyth, 2004). Securin is an inhibitor of separase, which when released cleaves the kleisin subunit of the cohesin complex, releasing bi-oriented sister chromatids to the forces of the spindle. An overview of cohesin dynamics in mitosis is shown in Figure 1.7.
Cohesin dynamics in mitosis

Cohesin is loaded onto chromatin in early G1 phase, and this loading is assisted by the nipped-B-like protein. This heterodimer makes contact with all four cohesin subunits and may function as a molecular ‘shaft’ to convey energy from the ATP hydrolysis that takes place in the head domains SMC1α and SMC3 to the hinge region, which is thought to transiently dissociate to allow entry of the DNA. Subsequent binding of PDS5 and WAPL to cohesin, through RAD21 and stromal antigen (SA), promotes its unloading. The DNA fibre exits the complex through the interface created by the SMC3 head domain and the amino-terminal region of RAD21. During DNA replication, the cohesin acetyltransferases (CoATs) ESCO1 and ESCO2 acetylate (Ac) K105 and K106 in the N-terminal domain of SMC3, and sororin is recruited to PDS5, which displaces WAPL, although it remains bound to cohesin. Sororin-bound acetylated cohesin complexes encircling the two sister chromatids are stably bound to chromatin. In prophase, most cohesin dissociates from chromatin when polo-like kinase 1 (PLK1) phosphorylates (P) the SA subunit, and sororin is released from cohesin after being phosphorylated by aurora kinase B (AURKB) and cyclin-dependent kinase 1 (CDK1). Concomitantly, shugoshin 1 (SGO1) and its partner protein phosphatase 2A (PP2A) accumulate at centromeres to counteract the above-mentioned phosphorylation events and prevent cohesin dissociation. Centromeric cohesin remains on chromatin until anaphase, when cleavage of RAD21 by separase destroys the integrity of the cohesin ring. The cohesin complexes that are released during mitosis can be reused in the ensuing G1 phase after a cohesin deacetylase (histone deacetylase 8 (HDAC8) in human cells) removes acetyl groups from SMC3.

Adapted from Remeseiro and Losada, 2013.

1.3.3. Other roles of cohesin complexes in eukaryotic cells

The main role of cohesin is to maintain sister chromatin cohesion and to ensure the proper chromosome segregation during mitosis. Cohesins have also been shown to mediate DNA repair and regulate transcription by keeping distant DNA chains in close proximity, whether to bring a sister chromatid for homologous repair or an enhancer to the transcription machinery. DSB repair during the cell cycle can be accomplished by two different mechanisms. In G1
phase of the cell cycle, DSBs are repaired by non-homologous end joining (NHEJ), which easily results in loss of genetic information. In G2, the DSBs are repaired by homologous recombination (HR), where a sister strand is used as a template to replace the lost nucleotides, leaving the genetic information intact. Since close proximity of sister chromatids is necessary for HR, it can easily be concluded that sister chromatid cohesion might have a role in DSB repair. Biochemical approaches identified cohesins as components of the DNA damage response pathway (Jessberger et al. 1996), as well as screening studies trying to identify DNA damage hypersensitive mutants (Sjogren and Nasmyth 2001). Also, it has been shown that cohesin is recruited to the sites in close proximity to DSBs (Kim et al. 2002; Sjogren et al. 2007; Ito et al. 2014; Gelot et al. 2016). This damage induced (DI) cohesin can be reloaded onto the chromatin during G2 phase of the cell cycle and depends on checkpoint kinase 1 (Chk1), which phosphorylates SMC3 upon DNA damage and effectively counteracts WAPL, establishing a post-replication sister chromatid cohesion (Unal et al. 2008). Cohesin was also implicated in an intra-S DNA damage checkpoint, involving phosphorylation of two cohesin subunits, SMC1 and SMC3, in an ATM (ataxia-telangiectasia mutated) and NBS1 (Nijmegen Breakage Syndrome protein 1) dependent manner (Kim et al. 2002; Yazdi et al. 2002; Kitagawa et al. 2004; Jessberger, 2009). Additional complexes containing the SMC1/SMC3 heterodimer, similar to cohesin, have been suggested to be involved in DNA repair and/or in intra-S checkpoint function, such as the BRCA1 supercomplex (BASC; Yazdi et al. 2002). Cohesin was also reported to have a role in the G2-M checkpoint, independent of cohesin (Watrin and Peters, 2009). In these experiments, depletion of sororin from cells in culture had no effect on the G2-M checkpoint, which was consistent with its role in sister chromatin cohesion establishment and maintenance but not in chromatin association of cohesin.

The role of cohesin in gene expression was first suggested after studies that showed evidence of cohesin association with chromatin in post-replicative and non-dividing cells (Dix et al. 1997; Wendt et al. 2008). In general, transcription is often mediated by sequences located several kilobases away from a gene by the combined actions of transcriptional activators, silencing proteins and factors that modify chromatin. Studies in Drosophila reveal that chromosomal proteins required for sister chromatid cohesion also play critical roles in control of gene expression during development (Misulovin et al. 2008). Nipped-B protein, a Drosophila orthologue of a mammalian cohesin loader NIPBL, was discovered by screening experiments trying to identify genes involved in regulation of cut homeobox gene, necessary for fly wing development (Rollins et al. 1999). The regulation is mediated by a distant transcriptional enhancer located more than 80 kb upstream of the transcription start site. RNA interference (RNAi) experiments provided evidence of Nipped-B involvement in sister chromatid cohesion (Rollins et al. 2004; Seitan et al. 2006). However, reducing cohesin dosage increases cut expression in the developing wing, while reducing Nipped-B decreases expression. Moreover,
the binding of cohesin and Scc2, an ortholog of Nipped-B, have been mapped genome-wide in the yeast *Saccharomyces cerevisiae* (Donze et al. 1999; Glynn et al. 2004; Lengronne et al. 2004), where the binding of yeast cohesin associates with silent loci, including the telomeres and the HMR locus. In humans, mutations in NIPBL or the cohesin subunits SMC1α and SMC3 lead to a constellation of severe developmental defects known as Cornelia de Lange syndrome (CdLS), which appear to be independent of cohesins canonical role in chromatid cohesion (Krantz et al. 2004; Borck et al. 2007; Liu et al. 2009). In mammalian cells, cohesin is preferentially enriched at DNase I hypersensitive sites, occupied by CCCTC binding transcriptional insulator protein (CTCF; Parelho et al. 2008; Wendt et al. 2008). The insulating function of CTCF prevents the spread of heterochromatin between domains and largely depends on cohesin function around these sites (Splinter et al. 2006; Rubio et al. 2008; Wendt et al. 2008). The binding pattern of SMC1 cohesin is effected by CTCF, but not the other way around. CTCF is thought to target cohesin loading to the chromatin where genes need to be regulated by cohesins long-range interaction capability by forming chromatin loops. A mechanistic insight into cohesin role at these sites was given by a study proposing that the complex forms the topological basis for cell-type-specific intrachromosomal interactions at the developmentally regulated cytokine *IFNG* locus (Hadjur et al. 2009).

### 1.3.4. Role of cohesin complex in meiosis

During the meiotic prophase I, chromosomes are organized into an axis-loop form, and the meiotic cohesin complex is a major component of the chromosome axis, which later forms the lateral element of the SC (Page and Hawley; 2004). Meiotic cohesin complexes contain several isoforms of the conventional subunits: SMC1α can be replaced by SMC1β, kleisin RAD21 with REC8 or RAD21L (RAD21-like) and stromal antigens STAG1 and STAG2 with STAG3 (Revenkova et al. 2001; Prieto et al, 2002; Eijpe et al, 2003; Gutierrez-Caballero et al, 2011; Ding et al. 2016). The existence of these isoforms implies multiple combinations, resulting in potentially 18 different cohesin complexes. However, only 6 cohesin complexes have so far shown to exist in vivo (Figure 1.8).
Figure 1.8 Cohesin complexes

Shown are cohesin variants for which evidence exists. The canonical cohesin complex is present in all somatic cells and likely in early meiocytes (“Mit & Mei”). All other complexes are meiosis-specific (“Mei”). There may be other cohesin variants as well. Figure was adopted from Jessberger, 2011.

The study of meiotic cohesins in mammals mostly involves gene knockouts/knockdowns and has a vast advantage over their mitotic counterparts because of tissue specific expression. For studies of their role in meiosis, mitotic cohesin genes must be conditionally inactivated in gonads only, either by timed excision with recombinases like cre or injections of expression inhibitors like RNAi. Failure to do so can lead to abnormal organism development, cancer or death. Unlike canonical cohesins, meiotic cohesin genes can be inactivated organism-wide, because they have no function in somatic tissue or that function is highly redundant, resulting in organism’s infertility in the worst-case scenario. An example of such redundant function is SMC1β general knockout which exhibited minor DNA repair defects in mouse fibroblasts (Mannini et al. 2015). So far, several knockout mice have been produced for in vivo functional analysis of meiotic cohesin subunits (Table 1.1).
### Table: Cohesion Mutants and Phenotypes

<table>
<thead>
<tr>
<th>Mutant</th>
<th>AEs</th>
<th>SCs</th>
<th>Arrest*</th>
<th>Sex Body</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smc1β&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>shorter (50 %)</td>
<td>shorter partial synapsis</td>
<td>T: IV</td>
<td>absent</td>
<td>MSCI failure, MSUC response, telomere defects, partial loss of SCC; DSB delay</td>
</tr>
</tbody>
</table>
| Revenkova et al., 2004
Adelfalk et al., 2009
Biswas et al., 2013 |             |                          |         |          |                                                                      |
| Rec8<sup>−/−</sup> | shorter      | shorter partial synapsis | T: ?    | C: late zygo | SYCP1 betw. sister chr.; premature loss of SCC; DSB impaired        |
| Bannister et al., 2004
Xu et al., 2005 |             |                          |         |          |                                                                      |
| Rad21L<sup>−/−</sup> | shorter      | shorter partial synapsis | T: ?    | C: mid zygo | non-homologous synapsis; DSB impaired                                 |
| Herran et al., 2011 |             |                          |         |          |                                                                      |
| Rec8<sup>−/−</sup> Rad21L<sup>−/−</sup> | absent      | absent                   | T: ?    | C: mid lepto | little cohesin loading; AEs similar to full STAG3 deficiency               |
| Llano et al., 2012
Ward et al., 2016 |             |                          |         |          |                                                                      |
| Stag3<sup>−/−</sup> ** | absent/ drastically shortened | absent/ drastically shortened | T: up to IV | C: lepto or mid zygo | partial centromere cohesion defect; SYCP1 betw. sister chr. |
| Fukuda et al., 2014
Hopkins et al., 2014
Llano et al., 2014
Winters et al., 2014 |             |                          |         |          |                                                                      |
| Stag3<sup>−/−</sup> Rad21L<sup>−/−</sup> | drastically shortened | absent/ drastically shortened | T: ?    | C: mid zygo | enhanced centromere cohesion defect; SYCP1 betw. sister chr.           |
| Ward et al., 2016 |             |                          |         |          |                                                                      |
| Stag3<sup>−/−</sup> Rec8<sup>−/−</sup> | drastically shortened | absent/ drastically shortened | T: ?    | C: mid zygo | enhanced centromere cohesion defect; AEs similar to Rec8<sup>−/−</sup> Rad21L<sup>−/−</sup> or full STAG3 deficiency; SYCP1 betw. sister chr. |
| Ward et al., 2016 |             |                          |         |          |                                                                      |

**Figure 1.9 Overview of cohesins in meiosis and meiotic cohesin mutant phenotypes.**

Some of the key phenotypes observed in individual mouse strains deficient for one or two cohesin proteins. The respective references are indicated. T: arrest stage based on most advanced stage of tubular development; C: arrest stage based on appearance of chromosomes and associated proteins; in several instances, the named stages are approximations due to difficulties determining the corresponding normal stage. The Stag3 mutants used by Hopkins et al, Ward et al., Fukuda et al., and Llano et al., were of distinct origin and express low levels of STAG3 and thus display a hypomorphic phenotype. Table was adopted from Biswas et al, 2016.

In general, depletion of meiosis-specific cohesin subunits, such as SMC1β, REC8 and RAD21L, causes meiotic blockage prior to the pachytene stage due to errors in pairing or the synaptic process in male mice. In SMC1β−depleted spermatocytes, formation of AEs is partially defective, with AEs markedly shortened and chromatin loops more extended (Revenkova et al. 2004; Novak et al. 2008). SMC1α complexes provide sufficient cohesion to keep sister chromatids together during prophase I, but still a high level of asynapsis is observed in Smc1β−/− meiocytes. Overexpression of SMC1α under Smc1β promoter in Smc1β−/− meiocytes managed to compensate for the majority of deficiencies (Biswas dissertation, 2014).
SMC1β-depleted oocytes are highly-error prone but proceed to metaphase II (Revenkova et al. 2004). In REC8-depleted spermatocytes, AEs are formed and partially synapsed, but the synopsis occurs between sister chromatids rather than homologous chromosomes (Bannister et al. 2004; Xu et al. 2005). REC8-null neonatal ovaries are devoid of oocytes and ovarian follicles, indicating that REC8-depleted oocytes never proceed beyond prophase I (Xu et al. 2005). In RAD21L-depleted spermatocytes, AEs are fragmented and poorly aligned, and synopsis occurs between non-homologous chromosomes (Herrán et al. 2011). Surprisingly, it has been reported that RAD21L-deficient females are fertile but develop an age-dependent sterility. Furthermore, it has been shown recently that mice lacking both REC8 and RAD21L fail to assemble their AEs, revealing that these two meiosis-specific kleisins are essential for the assembly of AEs. The pairing and recombination of homologous chromosomes during the meiotic prophase is necessary for the accurate segregation of chromosomes in meiosis. However, the mechanism by which homologous chromosomes achieve this pairing has remained an open question. Meiotic cohesins have been shown to affect chromatin compaction; however, the impact of meiotic cohesins on homologous pairing and the fine structures of cohesion-based chromatin remain to be determined. A recent report using live-cell imaging and super-resolution microscopy in fission yeast, demonstrated that the lack of meiotic cohesins alters the chromosome axis structures and impairs the pairing of homologous chromosomes (Ding et al. 2015). These results suggest that meiotic cohesin-based chromosome axis structures are crucial for the pairing of homologous chromosomes.

1.3.5. Stromal antigen (STAG) cohesin subunit

STAG or SA (stromalin) in vertebrates, was initially identified in human and Xenopus laevis cohesin complexes (Losada et al, 2000; Sumara et al, 2000), and named Scc3p in Saccharomyces cerevisiae (Michaelis et al, 1997). Despite very important recent progress, the functions of STAG proteins are still the least understood of any of the cohesin proteins. In vertebrates, there are three STAG variants called SA1-3, and the cohesin complex associates with one of them. SA1 and SA2 are ubiquitously expressed and appear to serve as interaction platforms of cohesin with other factors. For example, the insulator protein CTCF, which functionally interacts with and depends on cohesin, associates with SA2 (Xiao et al, 2011), which was also reported to interact with trans-activator proteins (Lara-Pezzi et al, 2004). SA1 also regulates transcription and cohesin binding to genomic sites also bound by CTCF (Remeseiro et al, 2012a). In addition, SA1 is required for sister chromatid cohesion at telomeres, while SA2 is necessary for centromeric cohesion (Canudas and Smith, 2009). In mice, heterozygous SA1 deficiency increases tumorigenesis, and embryonic fibroblasts derived from homozygous Stag1−/− mice show telomere-associated chromosome segregation defects and increased aneuploidy (Remeseiro et al, 2012b). SA1 is enriched at telomeres in
HeLa cells and directly binds telomeric DNA through a characteristic motif, an AT hook (Bisht et al, 2013). Phosphorylation of STAG proteins was reported in meiotic cells (Fukuda et al, 2012), and in mitotic cells, SA2 phosphorylation is required for dissolution of sister chromatid arm cohesion in prophase and prometaphase (Hauf et al, 2005). Potential roles of cohesins, including STAG proteins in human cancer, whether tumor-promoting or tumor-suppressing, depending on the circumstances such as overexpression, mutation, or protein loss, are currently debated (Solomon et al, 2011; Balbas-Martinez et al, 2013).

STAG3 was initially shown to be specific to spermatocytes where it associates with the synaptonemal complex (Pezzi et al, 2000). Moreover, STAG3 was found to be associated with axial elements and lateral elements and accumulates at the inter-sister chromatid domain, consistent with its potential role in sister chromatid cohesion (Prieto et al, 2001). STAG3 also associates with the paired and unpaired regions of the X and Y sex chromosomes and partially co-localizes with the inner centromeres and is also found at telomeres (Liebe et al, 2004). With progression of meiosis beyond pachynema, that is, with dissolution of the SC, STAG3 dissociates from the chromosome axes. At the metaphase to anaphase I transition, STAG3 disappears from chromosome arms and remains chromosome-associated at the centromeres. In anaphase I, STAG3 vanishes entirely and is not observed at later stages of meiosis. In other vertebrates, such as marsupials, a similar localization of STAG3 in spermatocytes was reported (Page et al, 2006) and expression patterns in human testis, ovary, spermatocytes, and oocytes are consistent with the observations in mice and a role in sister chromatid cohesion (Houmard et al, 2009; Nogues et al, 2009; Garcia-Cruz et al, 2010). In oocytes, a similar pattern of chromosome associations was observed where STAG3 is found along chromosome axes from leptonema to diplonema and dissociates during dictyate arrest (Prieto et al, 2004). In aged oocytes from senescence-accelerated mice, STAG3 levels, like those of other cohesin proteins, are significantly reduced (Liu and Keefe, 2008), consistent with the hypothesis of the loss of cohesin as a major contributor to increased age-dependent aneuploidy (reviewed in Jessberger, 2012). Recently, a 1-bp deletion in the Stag3 gene, that causes a frameshift, was found in patients of a family affected by premature ovarian failure. This gives rise to a small truncated protein of 194 amino acids of 1,225 amino acids (Caburet et al, 2014). Besides STAG3, the SA1 and SA2 (Prieto et al, 2002) proteins are also present in early prophase I, but their biological functions in meiocytes are unclear.
1.4. Aim of the project

Spermatogenesis is a crucial biological process in sexual reproduction and it is necessary for the creation of haploid gametes from diploid precursor cells. During meiosis, the chromosome architecture is rearranged into a structure called the chromosome axis which is necessary for the processes leading to crossover and proper chromosome segregation in anaphase I and II. These processes include DNA double strand break formation, homologous chromosome search and pairing, synaptonemal complex formation and the loading of cohesin complexes containing meiosis specific cohesin subunits. The specific meiotic roles of individual cohesin proteins are incompletely understood. The aim of this study is to describe the role of the only meiotic stromal antigen cohesin, STAG3, in mammalian meiosis.

The key aspects of describing this role are:

1. **Characterisation of STAG3-deficient mouse phenotype**

Since STAG3 was previously shown to interact with the cohesin ring only through association with meiotic kleisins RAD21L or REC8, STAG3-deficient mouse phenotype should be similar to that of RAD21L−/− REC8−/− double knockout, which was described recently (Llano et al, 2012). Immunofluorescence analysis of spermatocyte chromosome spreads from STAG3-deficient testis should provide insight into the key aspects of meiosis that depend on STAG3 function. This is achieved by probing for proteins crucial in above mentioned meiotic processes. Immunoprecipitation of meiotic cohesin complexes from STAG3-deficient testis should describe which complexes are responsible for whatever cohesion remains intact without the function of STAG3 containing complexes.

2. **Discovery of unknown STAG3 interactors using in vitro assays**

Immunoprecipitation of STAG3 containing cohesin complexes from wild type testis should provide more information about its interactors. Tagging the protein and overexpressing it in a protein expression system should provide ample material for in vitro protein-protein interaction assays like immunoprecipitation and pulldowns.

3. **Discovery of unknown STAG3 interactors by in vivo assays**

More reliable data can be collected from *in vivo* than *in vitro* studies. Tagging STAG3 and transfecting mouse zygotes should give rise to a new mouse line which already has the STAG3-containing cohesin complexes assembled under the same spatio-temporal conditions as in wt mouse.
2. Materials and Methods

2.1. Materials

2.1.1. Mice and mouse cell lines

To study the function of STAG3, a mouse line lacking functional STAG3 was created. Stag3\(^{ko/ko}\) embryonic stem (ES) cells were obtained from Knockout Mouse Project (KOMP) Repository, San Diego, USA (clone name EPD050_4_G09), which were derived from the parental ES line JM8A3.N1. The allele name is Stag3\(^{tm1a(KOMP)Wtsi}\) and is named ‘’ko’’ in this thesis to indicate that this is a knockout-first construct (Figure 2.1) and not a deletion allele.

![Figure 2.1 STAG3 “knockout-first” allele](image)

Insertion of the promoter-driven reporter cassette in the STAG3 gene also introduces an alternative splice acceptor site that bypasses whole exon 5 in this variant, making it a “knockout-first” allele. LoxP sites can be found inside the cassette and downstream of it, surrounding exon 5. Introducing cre recombinase into the cell, the promoter-driven reporter is excised along with exon 5 while the inducible reporter remains (this can be used to compare phenotypes between the “knockout-first” version and the actual removal of exon 5). The cassette is also surrounded by FRT sites which are the target of flp recombinase. When introduced in the cell, flp recombinase excises the cassette out of the gene rendering it active again. The remaining flox sites can still be used to excise exon 5 using cre recombinase which is useful for the creation of conditional knockouts. The image was taken from KOMP at www.komp.org

This construct uses a promoter-driven targeting cassette for the generation of a 'Knockout-first allele' (Skarnes et al., 2011) in C57BL/6N embryonic stem cells (Pettitt et al., 2009). This strategy relies on the identification of a 'critical' exon common to all transcript variants that, when deleted, creates a frame-shift mutation. The KO-first allele is flexible and can produce reporter knockouts, conditional knockouts, and null alleles following exposure to site-specific
recombinases Cre and Flp. ES cells were injected into C57BL/6 blastocysts and the resulting mice bred to homozygosity for this locus. Genotyping was performed using the following PCR primers: STAG3 Geno1, STAG3 Geno2, STAG3 Geno3 (see section Materials and Methods 2.1.3) and yielded the following products: Stag3 ko: primers 1 and 2 (product: 1412 bp); Stag3 wt: primers 1 and 3 (product: 1077 bp).

A STAG3-GFP transgenic mouse line was used for immunoprecipitation and other protein analysis experiments involving STAG3 in vivo. Bacterial Artificial Chromosome (BAC), containing full sequence STAG3 gene with an eGFP sequence at the end of the last exon, was obtained from the Transgenic Facility at Max Planck Institute of Molecular Cell Biology and Genetics (MPI-CBG), Dresden, Germany and injected into fertilized eggs of C57BL/6 mice. The zygotes were then transferred to pseudopregnant foster mothers and 3 weeks after birth, the mice genotyped for GFP and STAG3. The resulting mouse line had the exogenous gene inserted in an unknown position in the genome while the endogenous STAG3 gene was still active.

In an attempt to create a mouse line with a deficient endogenous STAG3 but with an active exogenous STAG3-GFP transgene, the newly acquired STAG3-GFP transgenic mouse was bred with a STAG3 heterozygote, STAG3\(^{ko/+}\) mouse (Figure 2.1). However, a full endogenous knockout is indistinguishable from the heterozygote if one attempts to genotype them because the wt gene is always present in the transgene. This problem will be addressed later in the thesis. SMC1β-LAP mouse line from the Jessberger lab was used as positive control for FACS analysis of STAG3-GFP cells. This mouse line was previously described (Murdoch et al, 2013). REC8\(^{-}\) mice, deficient for kleisin REC8 function, were used in some pulldown experiments as negative control. This mouse line has previously been described (Kudo et al, 2009; Biswas et al. 2016).

Animals were bred and maintained under pathogen-free conditions at the Experimental Center of the Medizinisch-Theoretisches Zentrum of the Medical Faculty at the Dresden University of Technology according to approved animal welfare guidelines, permission number 24-9168.24-1/2010-25 granted by the State of Saxony.

2.1.2. Bacteria

Stellar™ Competent Cells (Clontech) were used for all cloning and subcloning procedures. This includes the full length STAG3 and 5 of its fragments, as well as the viral baculovector. Chemically competent NEB™ 10-beta Competent E. coli (High Efficiency) were used for plasmid propagation.
2.1.3. Primers and other oligonucleotides

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**STAG3 Exon-exon junction primers:**

E3-F1  
TAGTCCCTCAACTAACGAAGACAG  
E3-F2  
CCCTCAACTAACGAAGACAC  
E3-F3  
AAGACAGCGACTTGGGAAGACAC  
E4-F1  
CGGAAGAAGCAGTCCCGGAAAG  
E4-F2  
TCTAGTGGAATGGCAAGAAATG  
E4-F3  
GTGGGAATGGCAAGAATGACAG  
E4-R1  
AAGTAATGGTGATGGCAGAGTC  
E4-R2  
CTGATTTCTTGGCCATTTCAC  
E4-R3  
GGTTGATGGCAGCTTCTTACCTC  
E5-F1  
TCTTTGTTGGATGAGTGGCAG  
E5-F2  
TTGTTGGATGAGTGGCAGATACAC  
E5-F3  
GGTGGATGAGTGGCAGATACAC  
E5-R1  
AGTTATCTGACATCCATCAAGAC  
E5-R2  
ATCTAGGCCTCGATCCACAAAGAC  
E5-R3  
GCCACTCATTCCACAAAGAC  
E6-R1  
TTCCGGTAAGTTGCGAGTATCCTC  
E6-R2  
CCGTTAGTGTTGAGTATCTC  
E6-R3  
GGTGTTGAGTATCTTGAATTGG

**Fragment insert primers:**

STAG3_F1  
AATAAAAAATCAGCGCCGCTCCCTACTTCTTTCGTGCACCTTC  
STAG3_F2  
AATAAAACATCGCCTCGCCGCTCCCTACTTCTTTCGTGCACCTTC  
STAG3_F3  
AATAAAACATCGCCTCGCCGCTCCCTACTTCTTTCGTGCACCTTC  
STAG3_R1  
AATAAAACATCGCCTCGCCGCTCCCTACTTCTTTCGTGCACCTTC  
STAG3_R2  
AATAAAACATCGCCTCGCCGCTCCCTACTTCTTTCGTGCACCTTC  
STAG3_R3  
AATAAAACATCGCCTCGCCGCTCCCTACTTCTTTCGTGCACCTTC  
Nested_F1  
TTCTAAGGCTTAAACCGGCTCCGGC  
Nested_F2  
GAAAGGACCTCTTGGGAGTGTGGG  
Nested_R1  
TGGCTAGGACCAAAAAGATGACC  
Nested_R2  
AGTGCTAGAGTAAGACCGCCCTCC  
STAG3_FW-NS  
CCTAAGGCTTAAACCGGCTCCGGC  
STAG3_REV-NS  
GAAATGCTCCATGTTCCACCTTCTTACCTTACCTTACC

**pOCC119 Stag3-GFP sequencing primers:**

Inf_pOCC119_F1  
GGGGCGCGCAGCGCCGCTCCCTACTTCTTTCGTGCACCTTC  
Inf_pOCC119_F2  
GGGGCGCGCAGCGCCGCTCCCTACTTCTTTCGTGCACCTTC  
Inf_pOCC119_F3  
GGGGCGCGCAGCGCCGCTCCCTACTTCTTTCGTGCACCTTC  
Inf_pOCC119_R1  
GGATCCCGGCGCGCGGGCGCGCGCTCCCTACTTCTTTCGTGCACCTTC  
Inf_pOCC119_R2  
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Inf_pOCC119_R3  
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Seq_pOCC119_F  
TCGAGCTCGAAACAAACAAAC  
Seq_pOCC119_R  
AACTTGTGGCGGGTCGTTACGTCG  
pOCC119_amp_5'  
GCCGCAGCGCCGCGGGATCCAGCACAGACG  
pOCC119_amp_3'  
GCCGCAGCGCCGCGGGATCCAGCACAGACG  
m13_F  
GTAAAACGACGGCCAG
m13_R CAGGAAACAGCTATGAC
His6_sq-F CCGCGAATTAATACGACTCAC
His6_sq-R CTGGTTAGCAGCCTGAATC

**Stag3 sequencing primers:**
Stag3sq-F1 TCTATGATGGCTTTCTATGGATG
Stag3sq-F2 AAGCAGTGAGTGAAGAGAGC
Stag3sq-F3 GAACCATGTTCAGACTCCTC
Stag3sq-F4 CATTGTCTGAGCTTCTCTCTG
Stag3_sq-R1 CTTATCTCCCTGCTCATTGGC
Stag3_sq-R2 CTGGCCTTTTTATGGAGAT
Stag3_sq-R3 CCTTGACTCTGTATATTTTCAC
Stag3_sq-R4 CCAATCTTGCATTCCTGGAG

**Gateway cloning primers**
GW1_F GGGGACAAGTTTGAAAGCAGGCTTGATGCCTACTCTGT
GW1_R GACCTTGAACTCTCTAAAGACTTGGGTCGAAAGAACATGTTTCACC
GW2_F CACCATGCCTACTCTGTGGTCACC
GW2_R TCAGAAAATCCTCCATGTTCAGCTCTGTG

**EMSA oligonucleotides**
Y1+cy5 Cy5-TGGATCCCGCATGACATTCGACGCTCTTCCGTG
Y1_compl ACCTAGGCGTACTGTAAGCGGCATTCGCTGAGAAGGCAC

2.1.4. Plasmids

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<tr>
<td>pDONR</td>
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<td>David Dreschel, MPI-CBG</td>
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2.1.5. Antibodies

Table 2.1 List of primary antibodies used.

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2.2. Methods

2.2.1. RNA extraction and RT-PCR

A single cell suspension was obtained from whole testes using Dounce homogenization, followed by centrifugation at 600 rpm for 10 min at 4°C. One ml of TRIzol was added to the cell pellet and incubated at room temperature (RT) for 5 min, followed by 200 µl of chloroform and incubated at RT for 3 min. The mixture was centrifuged at 1,770 g for 15 min and the aqueous phase used for RNA precipitation with 500 µl of isopropanol followed by centrifugation at 1,770 g for 10 min at 4°C. The pellet was washed with 70% ethanol, dried, and resuspended in 50 µl of RNAase-free water. One µg of total RNA was used per 20 µl of reverse transcription reaction (SuperScript II, Invitrogen). The mixture was incubated for 5 min at 65°C before adding the 5× first-strand buffer and DTT (10 mM final concentration). The mixture was chilled and heated to 42°C before adding the SuperScript reverse transcription enzyme (10 u/µl final concentration). The reaction was incubated for 50 min at 42°C, followed by inactivation at 70°C for 15 min. 2 µl of the RT reaction was used as template in a standard 50 µl PCR (denaturation at 95°C for 30 s, annealing at 60.5°C for 30 s, and elongation at 72°C for 1 min). The primers used were: E3 fwd, E4 rev, E4 fwd, E5 rev (see section Materials and Methods 2.1.3) and yielded the following products: exon 3 – exon 4 junction: primers E3 fwd and E4 rev: (product: 108 bp in both wt and ko); exon 4 – exon 5: primers E4 fwd and E5 rev (product: 158 bp only in wt; Figure 2.2).
Knockout-first (promoter)

![RT-PCR primer map of STAG3 allele](image)

**Figure 2.2 RT-PCR primer map of STAG3 allele**

Presence of the insertion cassette in STAG3 gene results in a cDNA without exon 5. This combination of primers can detect exon 3 – exon 4 junction in both wt and ko, while exon 4 – exon 5 junction can only be found in wt.

### 2.2.2. Cryosectioning of testes and ovaries

Whole testes or ovaries were fixed in 4% formaldehyde in PBS pH 7.4 at room temperature for 30 min. Following fixation, samples were incubated in 30% sucrose for 16 h at 4°C, and immersed in O.C.T Compound (Tissue-Tek 4583) in specimen molds (Tissue-Tek 4566 Cyromold 15 × 15 × 5 mm) and frozen at -80°C. 7 μm sections were cut using a Leica CM1900 cryostat microtome and placed onto microscope slides (StarFrost K078; 76 × 26 mm). Sections were immersed in cold methanol for 10 min, then in cold acetone for 1 min, and dried for 10 min. The slides were washed in 1 × PBST (PBS plus 0.1% Tween-20), then subsequently blocked in 2% BSA in 1× PBS for 30 min, and incubated with primary antibodies at 4°C for 16 h. Primary antibodies used in Figure 3.2 and Figure 3.3 were: anti-SYCP3 (mouse monoclonal, hybridoma cell line supernatant) and anti-γH2AX phospho-Ser139 (1:500, mouse monoclonal IgG1, Millipore 05-636). Slides were washed three times in 1× PBS and incubated with secondary antibodies for 1–2 h at 22°C. Slides were washed three times in 1× PBS and mounted using VectaShield mounting media (Vecta Laboratories, H-1000) containing 1 μg/ml DAPI and 24 × 50 mm coverslips (Engelbrecht, K12450, depth 0.13–0.17 mm). Testis sections were imaged using a Zeiss Axiopt microscope at 20× or 40× magnification with oil of refractive index 1.518 (Zeiss, Immersol 518 F).
Ovaries were sectioned (7 µm thick sections) to compare the number of oocytes between adult wt and ko mice and every 10th section was put on a microscope slide (StarFrost K078; 76 × 26 mm). The slides were probed with anti-NOBOX (oocyte marker; Suzumori et al. 2002) and NOBOX-positive foci counted.

2.2.3. Chromosome spreads

The tunica albuginea was removed from the testes, and testis tubules were incubated in 500 µl of 1 mg/ml collagenase for 10 min at 32°C. A single tubule suspension was obtained by pipetting and then centrifuged for 5 min at 380 g at 22°C. The pellet was resuspended in 0.05% trypsin and incubated for 5 min at 32°C with agitation of 350 rpm. Trypsin activity was neutralized by adding 200 µl of DMEM containing 10% FCS. The single cell suspension was filtered through a 40-µm strainer by centrifugation at 380 g for 10 s and then centrifuged at 320 g for 5 min at 22°C. The pellet was resuspended in 500 µl of PBS. For embryonic ovaries, a single ovary was incubated in PBS + 5 mM EDTA (pH 7.2) for 2 min at 4°C, incubated in a droplet of 1 mg/ml collagenase in 1× PBS for 2 min at 4°C, washed in 1× PBS, and macerated in 15 µl of 1× PBS. The single cell suspension was obtained by diluting the macerate 10× in PBS. 1-2 µl of single cell suspension was added to each well of a 10-well slide (Thermo Scientific, ER-308B-CE24, 10 well, 6.7 mm) containing 0.25% NP-40; cells were lysed for 2 min and fixed with 25 µl of 1% PFA, 5 mM sodium borate pH 8.5, 0.15% Triton-X 100. The slides were incubated in a wet chamber for 1 h, dried for 30 min to 1 h, washed two times with 0.5% Photo-Flo (KODAK, 146 4510), washed two times with PBS, dried, and stored at -20 °C.

2.2.4. Preparation of samples for FACS analysis

The single cell suspension was obtained as in section Materials and Methods 2.2.3. The cells were centrifuged at 380 g for 5 minutes at 4°C and PBS replaced with FACS basic buffer (20 mM HEPES, pH 7.2, 1.2 MgSO4·7H2O, 1.3 mM CaCl2·2H2O, 6.6 mM sodium pyruvate, 4 µg/mL DNase). Hoechst dye was added to final concentration of 5 µg/mL and the cells were incubated for 1 hour at 32°C in complete dark with agitation of 350 rpm. The cells were again centrifuged at 380 g for 5 minutes at 4°C and the buffer replaced with fresh, Hoechst-free basic FACS buffer to avoid background signal and diluted 1/10 for FACS analysis.

2.2.5. Immunofluorescence analysis

Chromosome spreads were either blocked in a buffer containing 2% BSA, 0.1% Tween-20 in PBS or 10% goat serum for at least 1 hour at room temperature before the primary antibody treatment. Slides were incubated with the primary antibody for at least 2 h at 37 °C, washed 3× with 2% BSA, 0.1% Tween-20 in PBS and incubated with the secondary antibody for 1 hour at 37 °C. The slides were again washed 3× with 2% BSA, 0.1% Tween-20 in PBS and
allowed to dry for 10 min and afterwards mounted with VectaShield mounting media (Vecta Laboratories, H-1000) containing 1 µg/ml DAPI and 24 × 50 mm coverslips (Engelbrecht, K12450, depth 0.13–0.17 mm).

Nuclear spreads were staged based on axis development, which was assessed mainly by detection of SYCP3. Dotty SYCP3 signal all over the nucleus corresponded to preleptotene stage while short stretches of axis to leptotene stage. Preleptotene stage cells have active DNA replication, so EdU labelling can be used in combination with SYCP3 staining to identify preleptotene spermatocytes and to confirm the axis morphology that characterizes them. Long and incomplete axis are formed in mid-zygotene where SYCP1 staining shows partial synopsis of the chromosomes. Late zygotene is characterised by whole axes and almost complete synopsis. To identify substages of wt pachytene spermatocytes, a combination of SYCP3 and H1t staining was used (Inselman et al, 2013). Early pachytene: thickness of synapsed axis pairs is uniform along the entire bivalent, the sex body is crescent shaped with a very short PAR region and H1T staining shows low intensity or no signal. Mid-pachytene autosomal axis has same characteristic as early pachytene but increased H1t signal. To distinguish early from mid-pachytene, H1t signal intensity was measured in all pachytene cells that do not have late pachytene characteristics and the cut-off was set in the median. These data were used in experiments where SYCP3 was excluded from the staining and yet pachytene staging was necessary. Late pachytene spermatocytes autosomes show thickening of SYCP3 signal at their end, the H1t staining intensity is above any of the previously measured stages and the sex body shows a specific "&" shape, with short, hair-like protrusion. Early diplotene cell autosomes show "bubbles" of de-synapsis which looks similar to late zygotene, except the shape of these non-synapsed regions differs. The autosomes in the synaptonemal complex formation process close much smaller angles between the 2 axes and the area between them is elongated while the dissolving of the synaptonemal complex shows bigger angles at the juncture between the 2 axes and the area between them is more round-shaped. Also, the whole process of prophase I progression starts with high levels of double-stranded DNA breaks (DSBs) in leptotene which can be detected by γH2AX staining. As the breaks are repaired and the cells progress, γH2AX staining gets concentrated on the unsynapsed axes, and finally, only to the sex chromosomes in pachytene because X and Y chromosomes remain unsynapsed all the way to the first division. However, the process of de-synapsis does not recruit any more γH2AX which allows an easy way to distinguish late zygotene from early diplotene. Late diplotene has the process of de-synapsis completed and the chromosomes are held together only by chiasmata. Fluorescence was visualized with Zeiss Axiophot fluorescence microscope and images were analysed in ImageJ version 1.51h.
2.2.6. Telo-FISH

For telomere detection, Telomere PNA FISH Kit/FITC (DakoCytomation K5325) was used. Chromosome spreads prepared as described in section Materials and Methods 2.2.3 were pre-treated with TBS1 solution for 2 min, then 3.7% formaldehyde for 2 min and washed in TBS 1 and TBS2 two times for 5 min. Pre-treatment Solution was diluted 1:20000 in TBS and added to the wells. The slides were washed with TBS3 and TBS4 two times for 5 min. The slides were immersed in cold ethanol series 70%, 85% and 95% 3 times, 2 min each and air dried in a vertical position. 10 µl of Telomere PNA Probe/FITC was added to the wells and covered with an 18x18 cover slip. The cover slip edges were covered with plastic glue to prevent evaporation of the sample and the slides placed in a 80 °C, preheated incubator for 5 min and then at RT in a dark room for 30 min. The slides were briefly washed in 1:50 diluted Rinse and 5 min in the Wash Solution at 65 °C. Imersion in the ethanol series was repeated and the slides air dried. The slides were mounted with VectaShield mounting media (Vecta Laboratories, H-1000) containing 1 µg/ml DAPI and 24 × 50 mm coverslips (Engelbrecht, K12450, depth 0.13– 0.17 mm).

2.2.7. ImageJ analysis

To perform ACA, RAP1 and DMC1 foci quantifications and other colocalization experiments, images were analysed in ImageJ. Multi-channel RGB color immunofluorescence images were separated into grayscale images and in the cases of ACA signal, where the foci were easily distinguishable from the background, a background threshold was set and the foci were automatically counted by the program. In other cases, the foci were counted by hand with an attempt to identify similar type foci and sort them in the same manner.

To confirm the co-localization signals as genuine, the 2 main channels were always turned 90 degrees clockwise relative to each other. If there was a significant difference between the number of foci in these two conditions, the co-localizations were considered genuine. To make sure that the shape of the cell does not influence the results, only the spreads with similar shaped, circular nuclei were used. In this case, ImageJ analysis was necessary for unbiased quantification. For the comparison of these samples, student T-test with p<0.05 was used.

2.2.8. Cloning and production of recombinant STAG3 fragments

To create the recombinant STAG3 construct and its fragments, pOCC119 vector obtained from MPI-CBG Protein Expression Facility was used (Figure 2.3A).
**Figure 2.3 Recombinant STAG3 and its fragments**

A  Polyhistidine (His6) and Maltose Binding Protein (MBP) are upstream of STAG3 cDNA sequence, separated by a 3C protease cleavage site, and Green Fluorescent Protein (GFP) is downstream, separated by a TEV cleavage site.

B  Map of the fragments of STAG3 relative to the full sequence of cDNA.

The selection property of the vector is the ampicillin resistance gene. Upstream of the cloning site, the plasmid has a polyhistidine (His6) sequence fused to a maltose binding protein sequence (MBP) which is separated from the cloning site by a 3C protease recognition site, while downstream it has a green fluorescent protein (GFP) sequence, separated from the cloning site by a TEV protease recognition site. Both full length STAG3 and fragments of STAG3 cDNA (Figure 2.3B) were created by PCR amplification from pCR-XL-TOPO STAG3 plasmid (IMAGE clone 8862033) using primers with overhangs homologous to the vector cloning site (see section Materials and Methods 2.1.3) and cloned into pOCC119 by In-Fusion Cloning procedure (Figure 2.4; CloneTech 121416). The amplification of STAG3 cDNA and gene fragments was performed with Q5® High-Fidelity DNA Polymerase, using this setup:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q5 Buffer 5x</td>
<td>10 µl</td>
</tr>
<tr>
<td>dNTPs Mix (10 mM)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Fw primer (10 mM)</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>Rev primer (10 mM)</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>DNA template (500 pg/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Q5 Polymerase</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Water</td>
<td>32.5 µl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>50 µl</strong></td>
</tr>
</tbody>
</table>
Thermic protocol

1. 95 °C, 2 min
2. 95 °C, 10 sec
3. 62 °C, 45 sec
4. 72 °C, 2 min (1 Kb / min)
5. Go to step 2 and repeat 30 times
   72 °C, 10 min
   4 °C
A vector can be linearized with restriction enzymes that leave ends with overhangs or blunt ends, however, 3’ overhangs should be avoided because the In-Fusion Enzyme truncates them (since they cannot be replicated) resulting in loss of information. The 2 primers used in PCR amplification of a gene of interest should contain overhangs on their 5’ end that are homologous to a minimum of 15 base pairs at both ends of the linearized vector. Orientation of the insert is determined by which primer contains which overhang. Once both vector and insert are purified, they are mixed in 1:2 ratio, respectively, and incubated with the In-Fusion Enzyme at 50 °C for 15 min. The enzyme truncates any 3’ overhangs creating 3’ indents, revealing 5’ overhangs in both vector and insert which are complementary. After alignment, the enzyme polymerases any non-aligned strands but no ligation occurs. Once the bacteria are transformed, their own repair mechanisms repair nicked DNA. Image obtained from Clontech at www.clontech.com
The sequences of dsDNA fragments ready for cloning contained 15 base pairs on both ends that were homologous to the linearized pOCC119 vector ends (linearization was achieved using restriction enzyme digestion by EcoRV and NotI at 37 °C for 16 hours). Both linearized vector and fragments were purified, using a PCR DNA and Gel Band Purification Kit (GE Healthcare, 28903470), and 5X In-Fusion HD Enzyme Premix was added. The reaction setup is as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X In-Fusion HD Enzyme Premix</td>
<td>2 µl</td>
</tr>
<tr>
<td>Linearized vector</td>
<td>2 µl (50 ng)</td>
</tr>
<tr>
<td>Purified PCR fragment</td>
<td>5 µl (100 ng)</td>
</tr>
<tr>
<td>dH2O</td>
<td>1 µl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>10 µl</td>
</tr>
</tbody>
</table>

2.2.9. Preparation of cell “pearls” from insect cell extracts

Insect cells infected with the baculovirus were grown in a growth medium for 72 hours and collected by centrifugation at 300 g for 10 minutes. The visibly green pellet was resuspended in the thin layer of leftover medium and collected by 25 ml pipet. Drop by drop of this green, dense suspension was dropped into a small tank filled with liquid nitrogen, resulting in so-called “pearls” which were then stored at –80 °C. 150 µg of pearls was resuspended in the lysis buffer (2 x PBS, 1% NP – 40, protease inhibitors, 5 µg/ml benzonase SIGMA) and the nuclear membranes were broken by 20 passages through the rough homogenizer using a tight pestle. To remove the cell debris, the extract was clarified by centrifugation through a 40 µm filter at 6 000 g for 20 min.

2.2.10. Gel filtration chromatography

The filtration curve was first standardized in reference to the void volume which was determined by loading Blue Dextran on to the gel filtration column. The curve speed was set to 0.5 mL per centimetre and fractions collected at every 0.5 mL. 500 µg of purified recombinant STAG3 was loaded on to a gel filtration column after 30 min incubation in 5 different conditions: PBS, 0.5% SDS, 20 mM spermidine, 20 mM EDTA or 1.5 M NaCl.

2.2.11. Crosslinking of antibodies to the paramagnetic beads using AminoLink™ Coupling Resin

To covalently crosslink the recombinant STAG3 to an agarose resin, AminoLink™ Coupling Resin protocol was applied (Figure 2.5). 2 mL of resin was transferred into a column and
allowed to settle by gravity flow. The column was equilibrated with PBS, the purified recombinant STAG3 was added and the resin bed re-suspended. In a fume hood, 40 µl of Cyanoborohydride Solution was added to the reaction slurry (final concentration was 50 mM NaCNBH₃) and the column was rotated for 16 hours at 4°C. A sample from the column supernatant was collected before and after the incubation to assess the efficiency of the coupling reaction and measured on nanodrop. After 16 hours, the column was washed with PBS followed by 4 ml of quenching buffer (1M Tris•HCl, pH 7.4). The coupling process was repeated with the quenching buffer instead of recombinant STAG3 and for 30 min instead of 16 hours to block all other available active sites (crosslinking of remaining active sites with primary amines of Tris in excess concentrations). The resin was washed in 10 ml of 1 M sodium chloride and afterwards with PBS containing 0.05% sodium azide and stored at 4°C.

Figure 2.5 The principle of AminoLink™ coupling assay

The activated support resin contains aldehyde functional groups that spontaneously react with primary amines on proteins or other molecules. The Schiff base bonds that form is reduced to stable secondary amine bonds in the presence of the mild reducing agent, sodium cyanoborohydride. Coupling efficiency by this reductive amination mechanism is typically greater than 80%, regardless of the ligand’s molecular weight or pI. Once the ligand is immobilized, the prepared resin can be used for multiple rounds of affinity purification in simple gravity-flow. Image adopted from ThermoFisher Scientific at www.thermofisher.com

2.2.12. Protein extraction and immuno blotting

For protein extraction and immunoblotting, nuclear extracts were prepared essentially as described in Jessberger et al, 1993. Briefly, the tunica albuginea was removed from the testes and a single cell suspension created using Dounce homogenization by grinding the tissue 25 times through a smooth surface homogenizer with a loose pestle in buffer B (5 mM KCl, 2 mM DTT, 40 mM Tris (pH 7.5), 2 mM EDTA, and protease inhibitors). The nuclear membranes were broken by 40 passages through the rough homogenizer using a tight pestle pestle. The nuclear suspension was centrifuged at 1,180 g for 3 min, the nuclear pellets resuspended in buffer C (5 mM KCl, 1 mM DTT, 15 mM Tris (pH 7.5), 0.5 mM EDTA, and protease inhibitors), and nuclear proteins were extracted by adding ammonium sulphate (AS; pH 7.4) to a concentration of 250 mM and incubating on ice for 30 min. Samples were centrifuged at 234,000 g for 30 min at 4°C. Supernatant was collected and protein content measured by Bradford before being stored at -20°C in Laemmli buffer for Western analysis. 5 μg of protein was run on an SDS–PAGE gel, transferred to a nitrocellulose membrane, and blocked in 5%
milk in PBST for 1 h at 22°C. Primary antibodies were added at 1 µg/ml in PBST for 16 h at 4°C. A biotinylated protein ladder (Cell Signaling Technology 7727) was loaded onto the gels and detected using an anti-biotin HRP (Cell Signaling Technology 7075). Also, PageRuler™ Plus Prestained Protein Ladder which was detectable by itself in visible light was used. Blots were washed three times in PBST and developed using chemiluminescent HRP substrate (Millipore, WBKLS) and imaged on a Kodak ImageStation 2000MM or Amersham Imager 600.

All extracts were prepared using a solution mix comprised of protease inhibitors, which included 1 mM Phenylmethylsulphonyl fluoride (PMSF) and Complete EDTA free protease inhibitor cocktail tablets (Roche, 11873580001), and phosphatase inhibitor 0.5 Sodium orthovanadate.

2.2.13. Differential salt extraction

For stepwise salt extraction of nuclei, the nuclei were first incubated for 30 min, on ice in a hypotonic buffer to allow nucleoplasmic proteins to diffuse out of the nuclei. The nuclei were then centrifuged (800 rpm, 5 min, 4°C) and the supernatant taken. The nuclei were washed once with the same buffer and same low speed centrifugation. The nuclei were then resuspended in the same buffer with 25 mM ammonium sulphate added to extract proteins loosely bound to chromatin, and incubated for 30 min on ice. Again, the nuclei were centrifuged, the supernatant taken (25 mM fraction), washed once, and resuspended and incubated for 30 min on ice in the same buffer containing 250 mM ammonium sulphate to extract proteins tightly chromatin-associated. After centrifugation at 1,770 g for 10 min at 4°C to pellet the remnant nuclei, the supernatant was taken (250 mM fraction).

2.2.14. Protein immunoprecipitation and pulldowns

Protein extracts for immunoprecipitation and pulldown experiments were obtained as described above (see section Materials and Methods 2.2.10). The protein concentration was measured either by Bradford Assay or nanodrop. The latter was preferable since in these experiments the relative amount of proteins to input was sufficient for the results to be comparable. Immunoprecipitation conditions used in individual experiments were as follows:

Figure 3.11: 150 µg of total nuclear protein extract was diluted in PBS to a volume of 400 µl and 200 µl of anti-SMC1β hybridoma supernatant was added to a final volume of 600 µl. 300 µg of protein G-coupled paramagnetic beads (Dynabeads® Protein G, Thermofisher Scientfic) were added to the solution and incubated for 16 h with rotation of 8 rpm at 4°C. Controls were made under same conditions but with 20 µg of general mouse IgG instead of hybridoma. The beads were washed 5 times in PBS and eluted by boiling at 95 °C and 5% inputs were loaded on to the 8% SDS gel next to the IP reactions.
Figure 3.18: 300 µg of paramagnetic protein G Dynabeads were incubated with 4 µg of goat anti-GFP antibody for 30 min at 22 °C. The beads were washed with PBS 2 times and incubated with 150 µg of purified, protease 3C cleaved recombinant STAG3 for 16 h at 4°C. The beads were washed with PBS 3 times and eluted by boiling at 95 °C.

Figure 3.19: 300 µg of paramagnetic protein G Dynabeads were incubated with 4 µg of goat anti-GFP antibody for 30 min at 22 °C. The beads were washed with PBS 2 times and incubated with 150 µg of purified, protease 3C cleaved recombinant STAG3 for 2 h at 4°C. The beads were washed with PBS 3 times and added to 150 µg of mouse testis nuclear extract (extraction described in section Materials and Methods 2.2.9). The final ammonium sulphate concentration was set to either 65 mM or 185 mM. The beads were washed with PBS 3 times and eluted by boiling at 95 °C.

Figure 3.21: First, 150 µg of mouse nuclear extract was diluted in 0.8 x PBS to set the final ionic strength to that of PBS. Secondly, 300 µg of paramagnetic protein G Dynabeads were incubated with 4 µg of goat anti-GFP antibody for 30 min at 22 °C. The beads were washed with PBS 2 times and incubated with 150 µg of purified, protease 3C cleaved recombinant STAG3 for 2 h at 4°C. The beads were washed with PBS 3 times and added to the adjusted ionic strength mouse nuclear extracts. The final IP conditions were set to 0.1% TritonX-100, 0.1% Brij 58 and 0.01% SDS in PBS and the pulldown incubated for 16 hours at 4°C. Beads of each condition were washed with their respective IP buffer 3 times and eluted by boiling on 95°C.

Figure 3.22: Covalently crosslinked anti-GFP-beads were incubated with 150 µg of mouse nuclear extract, which was diluted to final concentration of 65 mM ammonium sulphate, for 16 hours at 4 °C. The beads were washed 3 times with PBS and eluted by boiling at 95 °C.

Figure 3.26: 300 µg of paramagnetic protein G Dynabeads were incubated with 2 µg of goat anti-GFP antibody for 30 min at 22 °C. The beads were washed 2 times with PBS and added to 150 µg of STAG3-GFP mouse line nuclear extract, which was adjusted to a final concentration of 65 mM ammonium sulphate, and incubated for 2 hours at 4°C. The beads were washed with PBS 3 times and eluted by boiling at 95 °C.

Figure 3.27: First, 150 µg of STAG3-GFP mouse nuclear extract was diluted in 0.8 x PBS to set the final ionic strength to that of PBS. Secondly, 300 µg of paramagnetic protein G Dynabeads were incubated with 2 µg of goat anti-GFP antibody for 30
min at 22 °C. The beads were washed with PBS 2 times and added to the adjusted ionic strength mouse nuclear extracts. The final IP conditions were set to 0.1% Tween-20, 0.1% TritonX-100, 0.1% Brij 58. 500 mM NaCl, 0.01% and 0.1% SDS in PBS and the immunoprecipitation incubated for 2 hours at 4°C. The beads were eluted by boiling at 95 °C.

2.2.15. Coomassie Brilliant Blue and silver staining of gels

For silver staining, gels were first incubated in a fixing solution (methanol : acetic acid : water = 45 : 5 : 45) for 30 min and washed in water 4 times during 1 hour to remove the acid completely. The gel was sensitized with 0.02% sodium thiosulphate for 2 minutes and washed with water 2 times, 1 minute each and then incubated in the silver staining solution (0.1 % silver nitrate) for 20 minutes in the dark. The gel was washed 2 times with water, 1 minute each and developed in 0.04% formalin, 2% sodium carbonate solution. The developer was quenched by low pH by adding 100 ml of 1% acetic acid which was also used for storage.

The Coomassie staining was performed by boiling the gel in 1% Coomassie Brilliant Blue solution followed by an overnight incubation in de-staining solution (methanol : acetic acid : water = 45 : 2 : 45). The gel was washed in water the next day to return it to its original size and stored in water.

2.2.16. Electrophoretic mobility shift assay (EMSA)

The dsDNA used in this experiment was prepared by incubating two 46 bp complementary strands of oligonucleotides (see section Materials and Methods 2.1.3) in a decreasing temperature gradient (70 °C - 25 °C) for 55 minutes, out of which one strand had a CY5 chromophore at the 5’ end. A series of 5 decimal dilutions was made from 160 nM purified recombinant STAG3 (for preparation see section Materials and Methods 2.2.7) and 100nM dsDNA added to each in a total volume of 20 µl. The reaction was incubated for 30 min at 4°C and loaded on to a 4% agarose gel. The gel was run for 2.5 hours at 70 V at 4°C. Infrared camera was used to detect the CY5 chromophore.
3. Results

3.1. STAG3-deficient mice are infertile

To assess the role of STAG3 in mammalian meiosis, a mouse model lacking STAG3 protein function was used. The embryonic stem cell Stag3<sup>tm1a(KOMP)Wtsi</sup> was obtained from KOMP and injected into blastocysts to produce the respective Stag3 mutant strain. This strain carries a knockout-first cassette in intron 4, which disables gene expression by providing a splice acceptor located in the lacZ part of the insert, after which further splicing is not possible (see section Materials and Methods 2.1.1). The mice were bred to obtain homozygosity and the strain named Stag3<sup>ko/ko</sup> to indicate its ‘knockout-first’ design. The resulting mice were healthy but infertile. The testes of Stag3<sup>ko/ko</sup> males were 30% size and weight of wild-type (wt) mice (Figure 3.1A). To determine the presence of Stag3 mRNA, RT-PCR was performed, which confirmed the disruption of transcription and termination of splicing. Primers were designed such that the PCR product spans wt spliced and alternative, insert cassette mediated spliced exon-exon junctions of exons 3 – 4 and 4 – 5, respectively. The 108 base pair (bp) band representing the exon 4 – exon 5 junction was missing in Stag3<sup>ko/ko</sup> which confirmed the disruption of Stag3 gene expression (Figure 3.1B). To confirm the absence of STAG3 protein, Stag3<sup>ko/ko</sup> nuclear extracts were analysed by immunoblotting (IB; Figure 3.1C), which revealed no STAG3 band in the Stag3<sup>ko/ko</sup> extracts, except for a non-specific band just above the STAG3 expected size which is also present in wt. This is also confirmed by anti-SMC1β immunoprecipitation, which is described later in this chapter, section 3.5. The lack of STAG3 was also shown by anti-STAG3 immunostaining of wt and Stag3<sup>ko/ko</sup> spermatocyte chromosome spreads (Figure 3.1D).
Figure 3.1: Characterization of spermatogenesis in Stag3<sup>ko/ko</sup> mice.

A Testis samples from wt (Stag3<sup>+/+</sup>) and Stag3<sup>ko/ko</sup> mice, 40 days of age.

B RT-PCR analysis of testis mRNA from wt (Stag3<sup>+/+</sup>) and Stag3<sup>ko/ko</sup> mice. The primer pairs are shown indicating the respective exons (E3, E4, E5); the expected size (bp) of the PCR products is provided.

C Immunoblot of testis nuclear extracts of the indicated mice, probed with anti-STAG3 or anti-SMC3 antibody as indicated. The anti-STAG3 antibody recognizes a specific band corresponding to the predicted molecular weight (139 kDa) of STAG3, which was present in wt but absent in Stag3<sup>ko/ko</sup> extracts. An unspecific band is marked by an asterisk. A gel was loaded in parallel using the same extracts, and the corresponding membrane was probed with an antibody directed against SMC3, which has the same predicted molecular weight (139 kDa). The pictures are representative of three independent experiments. M = biotinylated protein marker.

D Immunofluorescence staining of spermatocyte chromosome spreads of wt and Stag3<sup>ko/ko</sup> mice, probed with anti-SYCP3 antibody for AEs and SCs and anti-STAG3; nucleic acids were stained with DAPI. The stages of wt prophase I spermatocytes are indicated, and two examples of Stag3<sup>ko/ko</sup> chromosome spreads are provided. Cells that show the most developed albeit very small SYCP3-positive axis-like structures and the least γH2AX staining to be the most advanced. These leptotene-like cells typically show up to 40 separate SYCP3-positive dots or very short axial structures (n = 65). Size bars indicate 10 μm.

3.2. Meiotic arrest in STAG3-deficient spermatocytes

Infertility of STAG3-deficient mice indicates disruption of meiotic process, so analysis of testis sections from Stag3<sup>ko/ko</sup> and wt mice was performed. The sections were stained for: SYCP3, which localizes to AE and SC; for the phosphorylated form of histon H2AX (γH2AX), which marks unsynapsed regions of chromosomes; and with DAPI to visualise the chromatin (Figure 3.2). The sections revealed the tubules to be less than half in diameter. Tubules of seminiferous epithelium cycle Stage I and IV, obtained from Stag3<sup>ko/ko</sup> testis, contained cells
Figure 3.2: Axial element (AE) formation requires STAG3.

A Immunofluorescence staining of testis sections of wt and Stag3\textsuperscript{ko/ko} mice, probed with anti-SYCP3 and anti-\textgamma H2AX; nucleic acids are stained with DAPI.

B Immunofluorescence staining of spermatocyte chromosome spreads of wt and Stag3\textsuperscript{ko/ko} mice, probed with anti-SYCP3 antibody for AEs and synaptonemal complexes; nucleic acids were stained with DAPI. The stages of wt prophase I spermatocytes are indicated, and two examples of Stag3\textsuperscript{ko/ko} chromosome spreads showing different SYCP3 staining patterns are provided. Size bars indicate 10 μm.

C Immunofluorescence staining of spermatocyte chromosome spreads of wt and Stag3\textsuperscript{ko/ko} mice, probed with anti-SYCP3 and anti-\textgamma H2AX; nucleic acids were stained with DAPI. The stages of wt prophase I spermatocytes are indicated, and six examples of Stag3\textsuperscript{ko/ko} chromosome spreads showing different staining patterns are provided. In agreement with the analysis of the testicular sections, those cells that show the most developed albeit very small SYCP3-positive axis-like structures and the least \textgamma H2AX staining are assumed to be the most advanced. These leptotene-like cells typically show up to 40 separate SYCP3-positive dots or very short axial structures (n = 65).

Size bars indicate 10 μm.
positive for SYCP3 and γH2AX. In wt testis, cells progressing from stage I to IV show an incremental compaction of γH2AX as more and more DSBs are repaired. Stag3\(^{ko/ko}\) cells with less and more compact γH2AX signal were considered more advanced. Only some axial structures marked with SYCP3 were detected and they were very short (Figure 3.1D). The fact that there was any SYCP3 in these cells defines them as at least leptonema stage of meiotic prophase I, however, the general absence of AEs prevents precise staging by observing chromosome architecture. Another way to determine their stage is by their cell associations within the tubule (Figure 3.3).

Figure 3.3: Staging of individual Stag3\(^{ko/ko}\) tubules

Stag3\(^{ko/ko}\) testis tubules. The tubules were stained with anti SYCP3, anti γH2AX and DAPI. Single channel DAPI images are provided in inverted colour to highlight chromatin features. The stages of the seminiferous tubules are indicated with roman numbers. Some cells are annotated as examples. Inm = intermediate spermatogonia; Bm = B spermatogonia; PL = preleptonema; L/Z = leptonema/zygonema; P =pachynema. No cells beyond early pachynema are observed at any stage.

Progression up to tubular stage IV was observed in a manner similar to wt testis, at least as far as the tubule content is concerned. This was also shown by analysing juvenile male mice at days 11, 13, and 15 post-partum (pp), whose cells reach late zygonema just before juvenile stage IV. There was only one cell layer with no pachytene cells observed in Stag3\(^{ko/ko}\) tubules beyond stage V. Stage VI tubules showed only spermatogonia B type cells, however, wt tubules also harbour pachytene cells. Stage VIII tubules of Stag3\(^{ko/ko}\) testis showed only cells
in stages earlier than zygotene, indicated by low SYCP3 signal, while wt testis tubules already have pachytene and diplontene cells in this stage. This is also true for all subsequent tubule stages. Early pachytene cells of Stag3\(^{\text{ko/ko}}\) testis were observed only in stages I to IV, indicated by SYCP3- and γH2AX-positive signal, along with immature spermatogonia. These data indicate that Stag3\(^{\text{ko/ko}}\) testis has spermatogenesis arrested at tubular stage IV.

In summary, the most advanced chromosomal stage should be considered as leptotene-like and the most advanced tubular stage as stage IV, since no pachytene cells were observed beyond it. Because of this, the term ‘leptotene-like’ will be used to describe the Stag3\(^{\text{ko/ko}}\) cell stages from now on, since no or only very short AEs can be detected. However, it is important to note that wt leptonema significantly differs from this leptotene-like state.

### 3.3. Chromosome axis and synaptonemal complex formation are deficient in Stag3\(^{\text{ko/ko}}\) spermatocytes

To take a closer look at the chromosome architecture of Stag3\(^{\text{ko/ko}}\) spermatocytes, chromosome spreads were prepared and immunostained with anti-SYCP3 and anti-γH2AX antibodies. Only dot-like patches and/or stretches of SYCP3 signal was observed, confirming the cell’s inability to form AEs. These stretches might resemble very short AEs or they might as well be aggregates of SYCP3 or a combination of both (Figure 3.2B). An example of such amorphic SYCP3 aggregation was observed in some cells which were deemed less advanced, while in other such cells the pattern was diffused along the chromatin. In cells considered more advanced, the dot-like pattern of SYCP3 staining sometimes reached 40 dots (equivalent to the number of mouse chromosomes), of which some were slightly elongated. However, most cells displayed between 10 and 40 round spots. This is in line with the Stag3\(^{\text{ko/ko}}\) testis section analysis, where stage VIII tubules and beyond showed weak SYCP3 signal in leptoneme and zygotene spermatocytes compared to a much stronger signal in stages I to IV, which harbour pachytene cells in wt testis. As the cells progress, more DSBs are repaired, the homologous chromosomes start to pair and the damage response and unsynapsed chromosome marker γH2AX is displaced from paired AEs in wt spermatocytes. This is manifest in a more compact γH2AX signal which finally in pachytene only exists on the chromatin containing X and Y chromosomes. This chromatin is termed the sex body and the X and Y chromosomes comprising it pair only in a short segment, the pseudoautosomal region or PAR (Figure 3.2C). Considering that there are no AEs in Stag3\(^{\text{ko/ko}}\) spermatocytes and thus no chromosome pairing and pairing-associated removal of γH2AX from autosomes, the γH2AX signal staining pattern does become generally more compact. (Figure 3.2C and Figure 3.3). Some cells were observed to display a single cloud of γH2AX, which indicates development up to stage IV.
3.3.1. **STAG3 is necessary for the formation of the synaptonemal complex**

To determine whether there is indeed no formation of synapsis between chromosomes, chromosome spreads of Stag3\(^{ko/ko}\) and wt spermatocytes were stained for SYCP3 and SYCP1, which is a marker for the SC. The SYCP3-positive only axes mark for the absence of synapsis, while axes positive for both SYCP1 and SYCP3 reveal the synapsed regions. Wt spermatocytes showed the expected pattern of SYCP1 signals at appropriate stages, while SYCP1 signals in Stag3\(^{ko/ko}\) leptotene-like spermatocytes were detected in at least half of the cells. Examples of cells with and without SYCP1 signals are shown in Figures 3.4A and 3.5A. In cells that show abundance of SYCP1 signal, many but not all foci co-localize with SYCP3 signal.

![Immunofluorescence staining of spermatocyte chromosome spreads of wt and Stag3\(^{ko/ko}\) mice, probed with anti-SYCP3 for axial elements (AEs) and anti-SYCP1 for synapsed axes; nucleic acids were stained with DAPI. Four examples of Stag3\(^{ko/ko}\) nuclei are shown, representing different levels of SYCP1 staining. Five SYCP3/SYCP1 structures are shown magnified at the bottom of the figure and are numbered.](image)

**Figure 3.4:** **Stag3\(^{ko/ko}\) and synapsis-related proteins**

A Immunofluorescence staining of spermatocyte chromosome spreads of wt and Stag3\(^{ko/ko}\) mice, probed with anti-SYCP3 for axial elements (AEs) and anti-SYCP1 for synapsed axes; nucleic acids were stained with DAPI. Four examples of Stag3\(^{ko/ko}\) nuclei are shown, representing different levels of SYCP1 staining. Five SYCP3/SYCP1 structures are shown magnified at the bottom of the figure and are numbered.

B Immunofluorescence staining of spermatocyte chromosome spreads of wt and Stag3\(^{ko/ko}\) mice, probed with anti-SYCP3 for AEs and anti-HORMAD1 for unsynapsed axes; nucleic acids were stained with DAPI. Three examples of Stag3\(^{ko/ko}\) spreads are shown, displaying different levels of HORMAD1 staining, including occasional aggregates and local accumulation.

C Immunofluorescence staining of spermatocyte chromosome spreads of wt and Stag3\(^{ko/ko}\) mice, probed with anti-SYCP3 for AEs and anti-DMC1 for double-strand break repair foci.

Size bars indicate 10 μm.
This co-localization never fitted completely but was found in close proximity, overlapping in the majority of their respective surfaces. The magnified examples are shown in Figure 3.4A, bottom right.

However, the majority of SYCP3 signal did not co-localize with SYCP1, indicating prevalent lack of synapsis. SYCP1 signal that did not co-localize with SYCP3 may represent non-specific deposits of SYCP1 that does not associate with any AEs. The foci positive for both SYCP1 and SYCP3 were either partially or fully co-localizing as dingle dots or a row of dots, but never as full stretches. This may indicate either non-specific deposits of these proteins or a failed attempt in generating initiation sites for SC formation using the very short stretches of AEs. These data confirm the absence of SCs in Stag3<sup>ko/ko</sup> spermatocytes.

Another way to observe the absence of SCs is to observe the presence of another marker which is present on the AEs before synapsis and removed from AEs as the SC forms between homologous chromosome axes. HORMAD1 associates with AEs in leptotene and remains bound as long as chromosomes stay unsynapsed (Wojtasz et al. 2009; Fukuda et al, 2010, Daniel et al. 2011). Immunostaining of wt and Stag3<sup>ko/ko</sup> spermatocyte chromosome spreads with anti-HORMAD1 antibody revealed the expected staining pattern in wt spreads, while the STAG3-deficient cells showed that HORMAD1 indeed associates with chromatin in the absence of STAG3 (Figure 3.4B and Figure 3.5B). Most of the HORMAD1 signal co-localized with SYCP3 and the most intense signal was associated with the SYCP3 dots and short stretches, but not larger aggregates. It is important to note that since HORMAD1 signal is diffusely distributed around the chromatin, some of the signal co-localization might have happened by chance. In summary, HROMAD1 signal mostly associates with SYCP3 dots and short stretches in the absence of STAG3. This brings more evidence in favour of the assumption that the large SYCP3 signal clusters are SYCP3 aggregates and do not represent AEs.
3.3.2. STAG3 is dispensable for the formation of DNA double-stranded breaks but is, at least indirectly, necessary for their processing

The processing of DSBs requires the chromosomal axis, and since the formation of AEs and SCs is clearly impaired in Stag$^{3\text{ko/ko}}$, one might expect the formation of DSBs and their processing to be impaired as well. To further assess this, immunostaining of wt and Stag$^{3\text{ko/ko}}$ spermatocyte chromosome spreads with anti-DMC1, a meiosis-specific recombinase that is involved in meiotic homologous recombination-mediated DSB repair (Habu et al. 1996; Pittman et al. 1998; Yoshida et al. 1998), was performed. The Stag$^{3\text{ko/ko}}$ spermatocytes showed an abundant amount of DMC1 foci distributed along the chromatin (Figure 3.4C and Figure 3.5C). This shows that STAG3 is not necessary for their formation. However, meiotic processing of
DSBs is not possible since synapsis is necessary to bring the homologous chromosomes in a close enough proximity for the use of homologous DNA chains as repair templates.

The difficulty in assessing the exact number of DMC1 foci rises from the lack of axis and thus SYCP3-DMC1 co-localization, however, an estimation was made between 100 and 200 foci in Stag3<sup>ko/ko</sup> cells. This is consistent with the inability of these cells to progress through pachytene since wt pachytene cells have less than 40 DMC1 foci localizing to the SC. These data indicate a deficiency in DSB processing in the absence of STAG3, since DSBs are introduced in the chromatin loops without an established axis necessary for their repair. To observe DMC1 foci of both wt and Stag3<sup>ko/ko</sup> spermatocytes in only leptotene and zygotene cells, 11- and 13- day-old mouse testes were analysed, respectively (Figure 3.6). A similar number of DMC1 foci was observed in Stag3<sup>ko/ko</sup> spermatocytes at both time points, while the number of foci in wt zygtonema was reduced compared to wt leptotena. In some rare Stag3<sup>ko/ko</sup> cells which displayed some axis-like structures, some DMC1 foci were co-localizing with these SYCP3 signals, which might suggest that STAG3 is not required for axis association of DMC1. However, given the sheer number of DMC1 foci located on the loops, these rare cases of signal co-localization might happen by chance.

![Figure 3.6: IF staining for DMC1, SYCP3 and nucleic acid in juvenile mice.](image-url)

Staining of wt and Stag3<sup>ko/ko</sup> testis nuclei from juvenile mice of day 11 and 13 pp for DMC1, SYCP3 and nucleic acid. An additional, rare example of Stag3<sup>ko/ko</sup> nuclei showing some axes-like structures is shown for day 13 pp. Size bar indicates 10 μm.
3.4. Centromere and telomere cohesion is impaired in Stag3ko/ko spermatocytes

SYCP3 foci and stretches were most often found in DAPI-intense pericentromeric regions of heterochromatin, so it was interesting to determine their relationship with the centromeres. The chromosome spreads of wt and Stag3ko/ko spermatocytes were stained with ACA (anti-centromere antibodies) and anti-SYCP3 to see if any of the approximately 40 SYCP3 foci are in the vicinity of or even co-localizing with centromeres. An average of 71% (n=18) of SYCP3 signals in Stag3ko/ko cells were indeed close to or partially overlapped with the centromeric signal (Figure 3.7A).

Figure 3.7: Centromeres and telomeres in Stag3ko/ko spermatocytes.

A Immunofluorescence staining of spermatocyte chromosome spreads of wt and Stag3ko/ko mice, probed with anti-SYCP3 and anti-centromere antibody (ACA); nucleic acids were stained with DAPI. Three examples of Stag3ko/ko spreads are shown to indicate centromere cluster formation and to highlight partial co-localization of SYCP3 with centromeres and the structures of these regions. Three areas are provided as magnified excerpts. Size bars indicate 10 μm.

B Immunofluorescence staining of spermatocyte chromosome spreads of wt and Stag3ko/ko mice, probed with anti-SYCP3 and anti-RAP1 to stain telomeres; nucleic acids were stained with DAPI. Three examples of Stag3ko/ko spreads are shown to represent different stages and to show an example of telomere cluster formation, which is shown in a magnified excerpt as well. Size bars indicate 10 μm.
However, complete overlap of ACA and SYCP3 signals was never observed, even though the SYCP3 signal is found in the pericentromeric region most of the times. Different cells showed different ratio of co-localization, which might indicate different stages of development between them. Many of the ACA foci appear in pairs, indicating possible loss of sister chromatin cohesion. To determine whether this is true, the number of ACA foci was quantified. 20 synapsed chromosomes in pachynema of wt spermatocytes bring their 4 centromeres in such close proximity that only 20 ACA foci are observable. Since Stag3\(^{ko/ko}\) spermatocytes have no SC formed, full centromeric cohesion would show 40 ACA signals, while complete loss of centromeric cohesion would count 80 foci. The average number of clearly separated centromere signals obtained from the quantification of Stag3\(^{ko/ko}\) spermatocytes was 55 ± 8 (n = 36). This clearly shows a partial loss of centromeric cohesion (Figures 3.7A and 3.8A).

Figure 3.8 Inverted colour images of Stag3\(^{ko/ko}\) spermatocyte staining for centromeres and telomeres.

A  SYCP3 and ACA; a magnified picture of a pair of centromere signals, indicating loss of sister cohesion, is showed.
B  SYCP3 and RAP1; Three examples of telomere counting, and their magnified images indicating loss of sister telomere cohesion, are showed
The minimum numbers of spots counted and a telomere cluster are indicated. Size bars indicate 10 μm.
In case of these clearly separated pairs of ACA foci, the SYCP3 signal often appears as small extensions between or to the side of them. In some cases, SYCP3 connects the 2 ACA foci or appears as 2 separate lines originating between the ACA foci and extending to both sides. This is clearly visible in examples provided in Figure 3.7A. There are also cells where centromere signals cluster in groups of up to 10, and most but not all of them co-localize with the SYCP3 signal. Unlike the small round foci and stretches of SYCP3, large aggregates of SYCP3 signal typically do not co-localize with the ACA foci, providing further evidence that these are un-specific deposits of SYCP3. Together, these data suggest that SYCP3 forms structures specifically at centromeres, possibly indicating vain attempts to initiate the formation of AEs.

Mouse centromeres are telocentric so the cohesion state of telomeric chromatin is also of great interest. Association of STAG3 with SMC1β, a cohesion subunit shown to protect telomeres (Priteo et al. 2001; Adelfalk et al. 2009) possibly implicates STAG3 into telomere maintenance as well. In order to determine whether STAG3 affects telomere cohesion, wt and Stag3ko/ko spermatocyte chromosome spreads were stained with anti-RAP1 and anti-SYCP3 (Figure 3.7B). Since mouse primary spermatocytes have 40 chromosomes and each of the 2 chromatids in a chromosome contributes 2 telomeres, the telomere count in these cells goes up to 160 foci, depending on how many of them are in cohesion. Pachytene wt cells with complete synapsis show 40 RAP1 foci. When cells are unable to form SCs, like in the case of Stag3ko/ko spermatocytes, there should be 80 observable telomeres if all sister chromatids are in cohesion and 160 observable telomeres in the case of complete loss of telomeric cohesion. There were more than 40 signals observed in Stag3ko/ko spermatocytes, which confirms the failure of synapsis. However, it was not possible to precisely count the number of foci because some cells display clusters of RAP1 foci that usually contain more than 10 tightly overlapping foci. Other cells show co-localization of several RAP1 foci with SYCP3 dots or stretches and some even show foci of different intensities next to each other. This could indicate a loss of telomeric cohesion, but it could also be telomeres from different chromosomes. Nevertheless, quantifying the foci in cells that do not show large clusters of foci showed up to 114 signals per cell (Figure 3.8B; n = 14), which is probably a significant underestimation. These data suggest that STAG3 is necessary for telomeric cohesion in primary spermatocytes and that there is at least partial loss of telomeric sister chromatid cohesion in these cells lacking STAG3.

Since not all individual RAP1 foci co-localize with SYCP3 signals, it is logical to assume that these telomeres maintain cohesion even without the formation of structures containing SYCP3. Many of the cells displayed clusters of RAP1 foci co-localizing with SYCP3 signal which is reminiscent of telomere bouquet formation, an event in wt cells which brings chromosome ends in proximity next to each other during leptotene/early zygotene (Scherthan. 2001; Siderakis, Tarsounas. 2007). These data suggest that telomere bouquet formation still occurs in the
absence of STAG3 and that these cells reach at least late leptotene. These results were confirmed by telomere-FISH, which also displayed up to 117 foci and telomere clusters (Figure 3.9). The average number of FISH telomere signals was 74 (n=23) in cells not displaying any clusters. When compared to SMC1β-deficient spermatocytes (Adelfalk et al. 2009), no extended stretches or telomere bridges were observed in Stag3^ko/ko spermatocytes. However, these cells also advance further than STAG3-deficient cells, even forming almost complete synopsis with many pachytene-like chromosome structures.

![Stag3^+/+] Late Leptonema  Zygonema  Pachynema

![Stag3^ko/ko]

Figure 3.9 Telo-FISH analysis of wt and Stag3^ko/ko testis chromosome spreads. Three prophase I stages of wt and three examples of Stag3^ko/ko nuclei, including one showing a telomere cluster, are showed. Size bar indicates 10 μm.

3.5. **Cohesin proteins in Stag3^ko/ko spermatocytes**

The presence of any sister chromatid cohesion in STAG3-deficient spermatocytes implies that at least some cohesin complexes are still present on the chromosomes. To confirm this, wt and Stag3^ko/ko spermatocyte chromosome spreads were stained for SMC3, a universal cohesin subunit present in all cohesin complexes (Figure 3.10A). Unlike wt cells where SMC3 localizes along the entire axis of the chromosomes, Stag3^ko/ko spermatocytes showed dotty, diffused
pattern of SMC3 foci along the entire chromatin. There was no specific pattern of localized SMC3 signal in any nuclear region, which suggests that the cohesin complexes are evenly distributed on the chromatin, still providing some sister chromatin cohesion. SMC1α and SMC1β proteins, of which either can heterodimerize with SMC3, were also present on the chromatin in the same diffused pattern with no specific localization pattern (Figure 3.10B, C).

Figure 3.10: Cohesins in wt and Stag3ko/ko.
Immunofluorescence staining of spermatocyte chromosome spreads of wt and Stag3ko/ko mice, probed for SYCP3 and:
A  SMC3 as a universal cohesin;
B  SMC1α as a universal cohesin;
C  SMC1β as a meiosis specific cohesin;
D  RAD21 or RAD21L as universal and meiosis specific kleisins, respectively.
Nucleic acids were stained with DAPI. Size bars indicate 10 lm.

The kleisins RAD21 and meiosis-specific RAD21L were both detected on the chromatin of Stag3ko/ko spermatocytes, although the RAD21L signals were weak.

In order to determine which meiotic cohesin complexes are present on the chromatin of STAG3-deficient spermatocytes, immunoprecipitation experiments with anti-SMC1β antibody from wt and Stag3ko/ko testis nuclear protein extracts was performed. SMC1β is a meiosis-specific SMC cohesin subunit, so precipitates obtained by these experiments should be from
spermatocytes only and contain the majority of meiosis-derived cohesin complexes. SMC1β was present in both wt and Stag3<sup>ko/ko</sup> extracts, as shown in Figure 3.11A, as well as the universal cohesin subunit SMC3, although a small fraction of it bound non-specifically to the beads as visible from the IgG control. Kleisin, RAD21 was not detected in the precipitates, which is in line with other studies showing no interaction between STAG3 and RAD21 (Lee and Hirano, 2011). However, a meiosis-specific kleisin REC8 was detected in precipitates from both wt and Stag3<sup>ko/ko</sup> extracts, as was expected. Interestingly, there was significantly less REC8 in both extracts and precipitates from the mutant cells, which might indicate decreased stability of this particular kleisin in the absence of STAG3. Another meiosis-specific kleisin, RAD21L was detected at wt-like signal levels. STAG3 band was present only in the wt precipitates and clearly missing from the Stag3<sup>ko/ko</sup> precipitates, which is another proof that Stag3<sup>ko/ko</sup> spermatocytes do not contain STAG3 and that the band present in both inputs and previous IB experiments (Figure 3.1C) is indeed non-specific background. Both of the other STAG variants, SA1 and SA2 were co-precipitated with SMC1β. Interestingly, probing for these proteins showed much stronger signals in the IPs from Stag3<sup>ko/ko</sup> spermatocytes, which may imply that the absence of the meiosis-specific stromal antigen might elicit a compensation attempt by other SAs.

Immunoprecipitation experiments showed the presence of cohesin subunits in the extracts and that they interact as expected, but whether these complexes are bound to chromatin and to what extent cannot be derived from these data. To assess this, testis nuclear extracts from wt and Stag3<sup>ko/ko</sup> mice were obtained using a differential salt extraction method with increasing salt concentrations of 0, 25 and 250 mM ammonium sulphate (AS; Figure 3.11B). Two cohesins present in all testis cells, SMC3 and SMC1α, as well as meiosis-specific cohesins, SMC1β and REC8, dissociated from chromatin at high salt concentration in wt spermatocytes, which implies that they are tightly bound to chromatin. REC8 was managed to be extracted with 25 mM salt concentration and a fraction if it even leaked out of the nucleus before salt was added. These conditions are usually used to extract the nucleoplasmic fraction of proteins, which is still not bound to chromatin, which may indicate that REC8 accumulates in the nucleoplasm at even larger quantities in Stag3<sup>ko/ko</sup> spermatocytes compared to wt. Together, these data show that cohesin complexes are present on the chromosomes of Stag3<sup>ko/ko</sup> spermatocytes.
Figure 3.11 Cohesin proteins in wt and in Stag3\textsuperscript{ko/ko} testis

A Immunoprecipitation of cohesin complexes from wt and Stag3\textsuperscript{ko/ko} testis nuclear extract using anti-SMC1\(\beta\) antibody (1\(\beta\)) or control IgG as indicated. Precipitates and 5% of input extracts are probed with the indicated antibodies for specific cohesin proteins.

B Stepwise extraction of proteins from wt and in Stag3\textsuperscript{ko/ko} testes nuclei using 0, 25, and 250 mM ammonium sulphate (AS) as indicated. Extracts were immunoprobed using the antibodies indicated on the right. *Unspecific band; M = marker; kDa numbers refer to marker signals.
3.6. Deficiencies in Stag3^{ko/ko} oocytes largely parallel those in spermatocytes.

Most of the adult Stag3^{ko/ko} female mice lacked ovaries altogether, while others had significantly reduced ovary size with no oocytes present. To observe meiotic prophase I and get the data comparable to male deficiencies described above, chromosome spreads from 15 day old embryonic ovaries were analysed by immunofluorescence (Figure 3.12). At this time point, leptotene to very late zygotene staged oocytes can be found in wt ovaries. Staining with anti-SYCP3 revealed extremely short axis-like structures and dots, as well as larger aggregates, which is similar to Stag3^{ko/ko} spermatocytes.

![Image](image_url)

**Figure 3.12** Synaptonemal complex and axis related markers in day 15 oocyte chromosomes of wt and Stag3^{ko/ko} mice

Immunofluorescence staining of chromosome spreads with anti-SYCP3 and:

A SYCP1 as a synopsis marker;

B HORMAD1 as a marker for unsynapsed regions;

C γH2AX to show progression through the initial stages of prophase I;

Size bars indicate 10 μm.

58

About 80% of the cells displayed small dots of SYCP1 which overlapped with SYCP3 about 40% of the time (Figure 3.12A). As in Stag3^ko/ko spermatocytes, HORMAD1 localized diffusely throughout the entire chromatin and also overlapped with SYCP3, which is to be expected due to its widespread presence (Figure 3.12B). No specific structure or localization pattern was detected. Consistent with the spermatocyte data, γH2AX signal was found to be ranging from a diffused pattern and large quantities to more concentrated clouds in those cells that show abundancy of short SYCP3 stretches and numerous foci (Figure 3.12C). Cells that have many defined SYCP3 spots and little γH2AX were considered the most advanced.

3.6.2. Centromere and telomere cohesion is impaired in Stag3^ko/ko spermatocytes

Stag3^ko/ko oocytes also displayed pairs of ACA signal which was counted above 40 foci, usually between 41 and 68 (n = 12), and many of these overlapped with SYCP3 signals. Centromere clusters were also observed in some oocytes (Figure 3.13A), most likely belonging to a more advanced stage of early zygotene.

Figure 3.13  Centromeres and telomeres in day 15 oocyte chromosomes of wt and Stag3^ko/ko mice

Immunofluorescence staining of chromosome spreads with anti-SYCP3 and:
A  ACA as a centromere marker;
B  RAP1 as a telomere marker;
Size bars indicate 10 μm.
Smaller clusters could also be found in many oocytes, rendering the quantification of ACA foci an underestimation since only clearly separated foci were counted. Examples of images used for counting are provided in Figure 3.14A. In comparison, wt oocytes showed 38-40 centromere signals, which is also indicative of how difficult it is to count all foci.

Similar to Stag3\textsuperscript{ko/ko} spermatocytes, RAP1 foci were analysed in embryonic oocytes. The telomere signals also greatly varied in quantity. Figure 3.13B shows three examples of Stag3\textsuperscript{ko/ko} oocytes of different stages (Figure 3.14B shows RAP1 inverted colour spots used for counting).

![Figure 3.14](image)

**Figure 3.14 Inverted colour images of Stag3\textsuperscript{ko/ko} oocyte staining for centromeres and telomeres.**

Examples of immunofluorescence, inverted color images obtained by staining for SYCP3 and:

A. ACA as a centromere marker;
B. RAP1 as a telomere marker.

The minimum numbers of centromere or telomere spots counted and an example of centromere clusters are provided. Wt cells in leptonema show 40 centromeres since the axes are not yet synapsed, and show between 40 and 80 telomeres in zygonema depending on the status of synapsis. In the example shown, 44 telomere spots are detected, consistent with the advanced zygotene stage. Size bars indicate 10 μm.
The number of RAP1 foci that were clearly separate from others was always above 80, between 82 and 122, which is also probably a gross underestimate due to the presence of telomere clusters and signal strength variety. These foci were often observed in pairs, which suggests a loss of sister chromatid cohesion, at least in the telomeric region. 80 telomeres are expected to be observable if no synapsis between homologous chromosomes is present and all sister chromatids are in cohesion, which is expected in Stag3<sup>ko/ko</sup> oocytes due to the lack of AEs, and 160 are expected if there is a complete loss of sister chromatid cohesion. Thus, any number of foci above 80 indicates at least partial loss of telomeric cohesion. No telomere extensions, bridges or any other observable defects were detected, which is consistent with the spermatocyte data. Together, these data show that Stag3<sup>ko/ko</sup> oocytes display at least partial loss of centromeric and telomeric sister chromatid cohesion.

### 3.6.3. Other cohesins in Stag3<sup>ko/ko</sup> oocytes

Like in Stag3<sup>ko/ko</sup> spermatocytes, cohesin proteins such as SMC3, SMC1α, SMC1β, RAD21, and low levels of RAD21L were detected in Stag3<sup>ko/ko</sup> oocytes spreads and localized diffusely in dotty patterns throughout the chromatin (Figure 3.15).
Figure 3.15 Cohesin proteins in wt and Stag3<sup>ko/ko</sup> oocytes

Immunofluorescence images of wt and Stag3<sup>ko/ko</sup> oocyte chromosome spreads, probed for SYCP3 and:

A  SMC3, a universal cohesin;
B  RAD21L, a meiosis specific kleisin;
C  RAD21, a universal kleisin;
D  SMC1β, a meiosis specific cohesin;
E  SMC1α, a universal cohesion;
F  STAG3, a meiosis specific strimalin antigen.

Nucleic acids were stained by DAPI. Size bars indicate 10 μm.
3.7. The expression and purification of recombinant STAG3 in insect cells

In order to understand the biological functions of STAG3 it is necessary to identify the parts of the meiotic machinery that it interacts with. This includes both meiosis-specific and mitotic proteins. As already shown by the IP data above, STAG3 interacts, directly or indirectly, with the cohesin ring components like SMC3, SMC1β and RAD21L or REC8. Out of the two kleisins, REC8 seems to be the more important component of the SMC1β/STAG3 containing cohesin rings in axis formation given its decreased levels in the Stag3\(^{ko/ko}\) mice SMC1β pulldowns, compared to RAD21L (Figure 3.11A). Furthermore, no other single cohesin mutant showed such extreme phenotype as Stag3\(^{ko/ko}\), leading to the consideration that STAG3 might function as a platform for the interaction between the majority of meiosis specific cohesin rings and the rest of the meiotic prophase I machinery. To identify the proteins that directly or indirectly interact specifically with STAG3 containing cohesin rings, immunoprecipitation experiments from wt mouse testis nuclear protein extracts, using anti-STAG3 antibody, were performed. Unfortunately, the available antibodies, which successfully recognised the denatured form of the protein in immunoblotting and immunofluorescence experiments, had poor binding affinity for the STAG3 native structure. A different approach, using genetic engineering to create a recombinant STAG3, was taken to address this issue. Fusing protein tags to STAG3 and expressing it in cell culture would allow quick and abundant isolation and purification of the recombinant protein which can be further used in a plethora of in vitro experiments such as immunoprecipitation and protein-protein interaction assays. In theory, mixing purified STAG3 recombinant protein with a mouse testis nuclear extract would result in the formation of cohesin complexes in vitro which can be immunoprecipitated with an antibody targeting one of the tags and then analysed by immunoblotting and/or mass spectrometry. The ability of cohesin complexes to form in vitro was already previously shown in our lab. Detecting novel STAG3 interactors would open doors to a cornucopia of potential future studies of the meiotic machinery, while studying the binding properties of STAG3 to some of the key interactors might further shed light onto the biological function of STAG3 itself. Ideally, using full STAG3 sequence in these in vitro experiments should reveal most of the STAG3 interactors, while using carefully designed fragments of STAG3 should roughly map the domains involved in the interaction process. However, to more precisely map the key amino acid residues necessary for proper interaction, compensation studies in which the candidate residues were mutated and then introduced back into the Stag3\(^{ko/ko}\) mouse in an attempt to rescue the mutant, i.e. using CRISPR/CAS9, would be necessary.

cDNA sequences for protein tags were added upstream and downstream of the full STAG3 cDNA and its 5 different fragments, which were then each cloned into a baculovirus-derived plasmid vector (see section Materials and Methods 2.2.7). The tags used were: green
fluorescence protein (GFP) at the C-terminus and maltose-binding protein (MBP) and polyhistidine (His-Tag) at the N-terminus. To determine proper integration of these constructs and their sequence, primers for polymerase chain reaction (PCR) were constructed (See section Materials and Methods 2.1.3). The virus containing this vector was created and used to infect insect cell cultures which then overexpressed the recombinant STAG3. One of the advantages of having a GFP tag fused to a protein being overexpressed in such a system is the visible green fluorescence of the cells, making the transfection process efficiency easier to monitor by naked eye, and of the concentrated protein solution making the purification column and fractions containing the protein green. The protein was purified from the insect cell extract using Ni-NTA Agarose column (Invitrogen R901-15), containing a nickel-charged affinity resin, which binds the polyhistidine tag of the protein (see section Materials and Methods 2.2.8). An example of this purification process is provided in Figure 3.16.

![Figure 3.16: Purification of recombinant STAG3.](image)

Insect cell lysate containing recombinant STAG3 was passed through a Ni-NTA Agarose column. The column was washed with high salt and eluted with 250 mM imidazole after which the purified protein was desalted/concentrated and incubated with 3C protease for 2 hours. Individual fractions were collected for SDS-PAGE analysis using Coomassie Brilliant Blue for protein visualisation. Flow-through 1 was collected during high salt wash and flow-through 2 during desalting/concentration. 3 different concentrations of bovine serum albumin (BSA) was used as a loading control. Pre-stained protein marker bands (M) are shown in kiloDaltons (kDa). The recombinant STAG3 size is 214 kDa and the cleavage products are 167 kDa and 47 kDa.
The column was not able to bind all of the recombinant protein and the flow-through was visibly green. At first, one might think that there is simply too much protein for the column capacity, but increasing the amount of resin did not affect the amount of protein in the flow-through. Moreover, filter of the desalting/concentration column was visibly green and the final protein yield was lower. Finally, in some instances during the site-specific protease cleavage reaction, which removes the His-MBP-tag and lasts 2-16 hours, the protein precipitated either partially or completely which was measurable by the size of the green pellet, nanodrop or Bradford protein assay and SDS-PAGE analysis. One possible reason for this precipitation effect might be the loss of the MBP tag which is empirically known to improve protein solubility. However, leaving the purified protein for 2-16 hours without the protease in some instances also resulted in precipitation to various degrees. Except high ion strength, no additional conditions that cause precipitation were identified.

Since precipitation occurs gradually over time, it is easy to assume the recombinant protein’s ability to aggregate. To discern whether this is true and to what degree, gel filtration chromatography experiments were performed with the purified recombinant STAG3. Blue Dextran was loaded onto the column to indicate the void volume (Figure 3.17A). The major fraction containing the recombinant STAG3 was eluted from the void volume, indicating at least 4 proteins aggregating together (Figure 3.17B). In an attempt to dissolve the aggregates, the experiment was repeated under several different conditions. Interestingly, strong ionic detergent conditions, such as 0.5% SDS, failed to break apart the aggregates of recombinant STAG3 (Figure 3.17C), but in the presence of a polycation, 20 mM spermidine, the profile showed only a small fraction of the protein in the void volume while forming additional peaks at 60 ml and 65 ml, which is roughly between dimeric and monomeric range (Figure 3.17D). Under the conditions of 20 mM EDTA, which is in lower concentrations used as a metal ion sequester, the profile showed a single saturated peak at 107 ml (Figure 3.17E), that is, the peak was not measurable by the detector. The signal peak under high ionic strength conditions, i.e. 1.5 M NaCl, was in the same position as under PBS conditions but had drastically lower amplitude (Figure 3.17F) indicating loss of protein. This is consistent with the previous observation that the purified protein instantly precipitates when mixed with the mouse nuclear extract which contains 250 mM ammonium sulphate (AS) or 500mM NaCl. Besides analysing the aggregate state by gel filtration, it is worth mentioning that the green protein pellet formed by precipitation of the purified protein could readily be dissolved in solutions standardly used for preventing protein aggregation like 0.5 M arginine buffer (pH 8.0).
Figure 3.17 Gel filtration chromatography of recombinant STAG3
Effect of 5 different conditions on recombinant STAG3 was measured by gel filtration chromatography.

A  Blue Dextran; control for identifying the void volume fraction
B  PBS
Figure 3.17 Gel filtration chromatography of recombinant STAG3

C  SDS, 0.5%
D  Spermidine, 20 mM
Figure 3.17 Gel filtration chromatography of recombinant STAG3

E  EDTA, 20 mM
F  NaCl, 1.5 M
3.8. Immunoprecipitation of recombinant STAG3

In order to mix the insect cell expressed, purified recombinant STAG3 with the mouse testis nuclear protein extract, it first needed to be immobilized on a resin. Previous attempts to mix the two solutions resulted in immediate precipitation of the fluorescent protein. The mouse protein extract contained 250 mM ammonium sulphate, which when diluted decreased the amount of precipitate, but never completely. This could either be a consequence of the change in salt concentration or the change in concentration of the protein itself. Desalting the mouse extract was possible but undesirable due to protein loss. In any case, preparing the bait separately and then adding the mouse extract was preferable because the beads-bait complex can easily be transferred from buffer to buffer and because of the possibility to make controls between steps. An example of immunoprecipitation of recombinant STAG3 with anti-GFP is shown in Figure 3.18. To confirm that the visible band is recombinant STAG3, the purification product was cleaved by 3C protease that targets a junction site between STAG3 sequence and His6–MBP–tag (from now on referred to as MBP-tag) and immunoprecipitated using anti-GFP.

![Figure 3.18 Immunoprecipitation of recombinant STAG3 with anti-GFP antibody.](image)

Purified STAG3 was incubated with 3C protease for 16 hours (cleaved) after which G-protein-coupled paramagnetic beads, preincubated with anti-GFP antibody, were added. Beads were separated from the solution by magnet, the protein-antibody conjugate was eluted by boiling for SDS-Page analysis and Coomassie Brilliant Blue was used for protein visualisation. Lanes marked for Input and Eluate show parts of the purification process. The recombinant STAG3 size is 214 kDa and the cleavage products are 167 kDa and 47 kDa (MBP indicated).
Cleaving the 214 kDa recombinant STAG3 yielded a 167 kDa STAG3 containing fragment and 47 kDa MBP-tag. The immunoprecipitation lane showed enrichment in STAG3-GFP fragment but no MBP-tag. The other two lower bands are heavy and light immunoglobulin chains.

Another way to immobilize the recombinant STAG3 focused on its MBP component. Amylose coupled paramagnetic beads were used to pulldown the protein directly from the crude cell lysate in high enough quantity for subsequent pulldown experiments from the mouse testis nuclear extracts. In this case, once the mouse proteins are added, the bait-target complex could be eluted by 3C protease cleavage. However, this wasn’t possible because 3C was unable to cleave the protein once it was bound to the beads. Usually, 1-2 h is enough for the complete cleavage of 250 µg of recombinant STAG3, however, an over-night incubation with bead-bound recombinant STAG3 did not yield any cleavage products once the beads were eluted by boiling. For this reason, skipping the 3C cleavage altogether, pulling down the full-length protein and eluting by boiling was preferable.

### 3.8.1. Pulldown of STAG3 interactors from mouse testis nuclear extracts

To obtain more information about the interactors of STAG3, pulldowns from mouse testis nuclear extracts were performed using the full length or cleaved product of recombinant STAG3 bound to either amylose or anti-GFP antibody–protein G bead conjugates. One example for such pulldown is shown in Figure 3.19. Full length recombinant STAG3 was immunoprecipitated with anti-GFP antibody bound to paramagnetic beads which was then used to pulldown STAG3 interactors from the mouse testis nuclear extract. The beads were eluted by boiling and loaded on to a gel for SDS-PAGE analysis. When comparing the lane containing only bait to the lane with bait and mouse nuclear proteins, no differences between band profiles were observed. Same results were obtained when using the STAG3-GFP fragment or full length STAG3 bound to amylose beads as bait (data not shown).
Recombinant STAG3 was immunoprecipitated from insect cell extracts using anti-GFP antibody bound to protein G-coupled paramagnetic beads. Using this conjugate as bait, pulldowns (PD) from mouse testis nuclear extracts were performed under 2 different salt concentrations, 65 and 185 ammonium sulphate (AS). (M) pre-stained protein marker. Recombinant STAG3 size is 214 kDa. The gel was stained with Coomassie.

To detect specific direct and indirect interactors like REC8 and SMC3, respectively, immunoblot analyses of these pulldowns from mouse nuclear extracts were performed. One example is shown in Figure 3.20, where the pulldown was done under 2 different salt concentration conditions, 85 and 170 mM ammonium sulphate, and the membrane probed with anti-GFP and anti-REC8 antibodies. Pulldowns were also done with testis nuclear extract from a REC8-deficient mouse line (REC8<sup>−/−</sup>; see section Materials and Methods 2.1.1) as control. The anti-GFP probed membrane (Figure 3.20A) confirmed that the bait contained recombinant STAG3 at expected band size 214 kDa, although some degradation product was observed. However, REC8 was not detected in either of the conditions (Figure 3.20B). One
reason for this might be the inaccessibility of STAG3 interactors to their respective binding domains of the recombinant STAG3 which could be relieved by introducing more stringent conditions under which the pulldowns are performed. Another reason might be that REC8 is unstable in cells devoid of STAG3 or it needs to associate with the SMC cohesin subunits before it can bind STAG3.

The experiment was repeated using the same stock of previously prepared bait (Figure 3.18, eluate lane), the ionic strength of the mouse nuclear extracts adjusted to that of PBS and PBS containing TritonX-100, Brij58 or SDS was added to achieve their final concentration of 0.1%, 0.1% and 0.01%, respectively. Probing with polyclonal anti-SMC3 revealed weak bands under all 3 conditions at the expected size of 139 kDa (Figure 3.21A). However, SMC3 antibody used in some of the previously described experiments also sometimes revealed weak signals even in IgG control experiments (i.e. Figure 3.11A). However, probing the same membrane with anti-SMC1β antibody didn’t reveal any expected bands in this pulldown (Figure 3.21B).
Figure 3.20 Pulldown of STAG3 interactors using immobilised recombinant STAG3

Pulldowns of STAG3 interactors from wt and REC8⁻/⁻ testis nuclear extract using immobilized recombinant STAG3 or just anti-GFP antibody. Immunoprecipitation was performed under 2 different ionic strength conditions, 185 and 65 mM ammonium sulphate (AS). Precipitates and 5% of input extracts were probed with:

A  anti-GFP antibody
B  anti-REC8 antibody
Figure 3.21 Pulldowns from mouse nuclear extract using recombinant STAG3 in different stringency conditions

Pulldowns from mouse wt nuclear extracts using immobilized recombinant STAG3 in increasing stringency conditions. Extracts were adjusted to PBS ionic strength and PBS containing Triton-X 100, Brij 58 or SDS were added to their final concentration of 0.1%, 0.1% and 0.01%, respectively. Precipitates and 5% of input extracts were probed with

A  anti-SMC3
B  anti-SMC1β
3.8.2. Pulldowns from mouse nuclear extracts using recombinant STAG3 covalently crosslinked to an agarose resin

In some instances, covalently crosslinking the bait to a resin can improve the yield. Recombinant STAG3 was crosslinked to agarose beads using AminoLink™ Coupling Resin (see section Materials and Methods 2.2.10) overnight and the amount of protein measured at 0 and 16 hours of incubation. The efficiency of crosslinking was 85% and the resin was visibly green. Pulldown experiments from mouse nuclear extracts were performed using crosslinked recombinant STAG3 (Figure 3.22) and the blot probed with anti-SMC3 antibody. Only one non-specific band could be observed in the pulldown compared to the control crosslinking reaction which did not contain any bait and increasing the amount of the beads revealed a few more non-specific signals. The blot showed below was overexposed to insure detection of even the weakest signal.

![Figure 3.22](image.jpg)

**Figure 3.22** Pulldowns from mouse nuclear extracts using recombinant STAG3 covalently crosslinked to the antibody-bead conjugate

Pulldowns from mouse nuclear extracts using recombinant STAG3 crosslinked to agarose beads. Input and precipitates were probed with anti-SMC3. Pre-stained protein marker sizes are shown in kilodaltons (kDa).
3.9. Recombinant STAG3 binds DNA

Because the recombinant STAG3 cannot bind STAG3 interactors in any of the tested conditions \textit{in vitro}, perhaps other factors, that are only present in the mouse testis, are necessary. Previous studies of yeast protein rec11, a homologue of mammalian STAG3, showed that rec11 must first bind DNA before it can interact with the cohesion complex components (Sakuno and Watanabe, 2015). Another study showed that timely phosphorylation of rec11 is necessary for loading of SC associated elements (Phandis et al, 2015). Conditions in meiotic cells or testis may contain yet unknown factors/events that are not present in insect cells. To recombinant STAG3 binds DNA, Electrophoretic Mobility Shift Assay (EMSA) was performed (see section Materials and Methods 2.2.15) with decimal dilutions of purified recombinant STAG3 and 100 mM, 46 base pair double stranded DNA (dsDNA) fragment in which one DNA chain was labelled with a CY5 chromophore (Figure 3.23). Protein concentrations above 1.6 nM could easily retard the movement of the DNA through the gel, while lower than 0.16 nM failed to do so. However, to further investigate the DNA binding properties of recombinant STAG3, experiments with different length nucleic acid, single-stranded DNA, DNAs with secondary structures and other variations should be performed. In conclusion, STAG3 seems to have the ability to bind DNA, at least \textit{in vitro} and at protein concentrations above 0.16 nM.
Decimal dilutions of purified recombinant STAG3 were incubated for 15 minutes at 4 °C with 100 nM, 60 base pair dsDNA in which one chain was labelled with CY5 chromophore. The 4% gel was run on 70 V for 2 hours, 4 °C.

3.10. Introducing STAG3-GFP fusion protein into a mouse line

It has become clear that under the conditions tested so far, the insect cell expressed recombinant STAG3 is not able to bind STAG3 interactors from the mouse nuclear extract in vitro. So in order to introduce a labelled STAG3 into a mouse line, a Bacterial Artificial Chromosome (BAC) containing STAG3-GFP under STAG3 promoter was used (see section Materials and Methods 2.1.1). The BAC was injected into 168 mice zygotes which were then transferred into surrogate females and the progeny was genotyped for GFP. Only one female
was genotyped as GFP-positive and bred with wt males. The resulting mouse line had both the STAG3 and the exogenous GFP-tagged transgene integrated at an unknown locus in the genome. FACS analysis was performed on STAG3-GFP testis single cell suspension and compared to GFP negative littermates (negative control) and SMC1β-LAP (positive control) mice cells (n=2, Figure 3.24).

**Figure 3.24 FACS analysis of STAG3-GFP testis cells**

Single cell suspension of STAG3-GFP testis cells was analysed by fluorescence-activated cell sorting (FACS), along with the testes from wt littermates (negative control) and SMC1β-LAP mice (positive control). The data was gated for GFP positive events according to the negative control. STAG3-GFP cells showed significantly more GFP positive events compared to GFP negative littermates and a fairly similar profile pattern to SMC1β-LAP cells, although there were 3 times more positive events. Hoechst staining analysis of GFP positive events revealed that a subpopulation of these cells corresponds to primary spermatocytes (4n).

The data was gated following the analysis of GFP negative littermate cells, which was then used in the analysis of STAG3-GFP and SMC1β-LAP mice cells. Hoechst dye was added to distinguish different cell cycle stage cells according to the amount of DNA they contain and 100 000 events were analysed per sample. STAG3-GFP sample showed significantly more GFP positive events and the profile looked similar in pattern to SMC1β-LAP, although there were 3 times more GFP positive events. The Hoechst analysis of GFP positive events revealed a subpopulation of cells corresponding to primary spermatocytes (4n).

In order to verify expression and localization of the transgene expressed STAG3-GFP, immunostaining of spermatocyte chromosome spreads with anti-GFP or anti-STAG3 antibody
was performed. The background of anti-GFP staining (Figure 3.25B) was high and the signal much lower in intensity compared to the anti-STAG3 staining (Figure 3.25A), which might be an indication of lower expression when compared to the endogenous STAG3 or the specificity of the two antibodies is not equitable. Only pachytene staged cells showed correct localisation of the GFP signal along the chromosome axes, while other stages showed no such pattern. The experiment was controlled for the possibility of signal “bleed through” from the red channel to the green channel by repeating the experiment without adding the anti-GFP primary antibody, which resulted in no signal in the green channel. Also, two different blocking procedures, either with 10% goat serum or 2% BSA, were attempted, yielding no difference in signal pattern or intensity. Moreover, to see if STAG3-GFP, along with its expression levels, retains the full biological function of endogenous STAG3, these mice were bred with the STAG3<sup>ko/+</sup> mice in hope to knockout the endogenous gene while the transgene remains as the only source of STAG3 expression. However, the genotyping for such a knockout presents a challenge which will be discussed later.
Figure 3.25 Localization of STAG3-GFP on the chromosomes of mouse spermatocytes

Zygotene and pachytene staged cells in STAG3-GFP transgenic mouse spermatocytes. The chromosome spreads were stained for SYCP3 and:

A  STAG3; stains for both the STAG3-GFP fusion protein and endogenous STAG3.
B  GFP; stains for STAG3-GFP fusion protein.
Size bars indicate 10 μm.
3.11. Immunoprecipitation of STAG3-GFP containing cohesin complexes from STAG3-GFP mouse spermatocyte nuclear extracts

STAG3 in vitro experiments suggested the possibility that the protein might require certain parts of the spatio-temporal regulation provided by the meiotic machinery to bind its interactors. This may involve some modification of STAG3 before or upon loading to the chromosomes, such as timely phosphorylation like shown in yeast (Phandis et al, 2015), which would be necessary for further recruitment of downstream factors necessary for AE and SC formation. The advantage of expressing STAG3-GFP in mouse spermatocytes is a sure way to circumvent these possibilities during IP experiments. Immunoprecipitation from both wt and transgenic mouse spermatocyte nuclear extracts, using anti-GFP antibody to precipitate STAG3-GFP along with its interactors, and immunoblot analysis were performed. The predicted size of STAG3-GFP is 165 kDa and its expression is clear from the input in the blot probed with anti-GFP antibody (Figure 3.26A). One clear band at the same position was observed in STAG3-GFP, but not wt precipitates, although the signal intensity was similar to the 10% input signal. To compare the relative expression of the transgene expressed STAG3 and the endogenous STAG3, the blot was probed with anti-STAG3 antibody (3.26B). Both STAG3 and STAG3-GFP protein bands were detected in the input of transgenic nuclear extracts at 140 and 165 kDa, respectively, while only the former was detected in the wt input. The relative intensity between the bands indicates a significantly higher expression of the endogenous STAG3 compared to the transgene expressed protein. Surprisingly, both were also detected in the precipitates of the transgenic extracts, with the STAG3-GFP signal dominating in intensity, while none were present in the wt. The presence of both proteins might suggest a possible multimerization event that might even be native to endogenous STAG3. This possibility will be discussed more later.

The same blot was analysed for the direct and indirect known interactors, REC8 and SMC1β, respectively. REC8 was detected at the predicted size just above 70 kDa in both inputs and the precipitates from the transgenic extract, but not in wt precipitates (3.26C). It is important to mention that the overall signal of the blot is lower because the exposure level of the membrane during detection was limited by the saturated non-specific band signal just below 250 kDa, which might explain why REC8 signal is so weak in the precipitate and both inputs. SMC1β was detected at the predicted size of 150 kDa in both inputs and precipitates from transgenic extracts, but not in wt precipitates (3.26D). However, precipitate signal intensity from the transgenic extract was significantly lower than in 10% input.
Figure 3.26 Immunoprecipitation of STAG3-GFP containing cohesin complexes from STAG3-GFP mouse testis nuclear extracts using anti-GFP antibody

The precipitates were analysed by immunoblotting and the same membrane probed against:

A  GFP: the STAG3-GFP signal band was detected at expected size of 165 kDa in both input and IP, while wt control showed no bands at the designated size.

B  STAG3: both endogenous STAG3 and STAG3-GFP bands were detected in the input of STAG3-GFP extracts, at 139 and 165 kDa, respectively. The STAG3 band was visibly stronger than STAG3-GFP band. Surprisingly, both bands were detected in the IP of STAG3-GFP extracts, with STAG3 band being very faint. Only STAG3 band was detected in the wt input, while no bands were detected in the wt control IP.
Figure 3.26 Immunoprecipitation of STAG3-GFP containing cohesin complexes from STAG3- GFP mouse testis nuclear extracts using anti-GFP antibody

C  REC8: Both the STAG3-GFP and wt inputs contained the REC8 band at 80 kDa, although the signal was weak. The band was also present in the STAG3-GFP IP and absent from the wt IP.

D  SMC1β: the SMC1β band was detected in both STAG3-GFP and wt inputs at the expected size of 141 kDa. The band was present in the STAG3-GFP IP, as expected, but not in the wt control IP.
Now that the known interactors were detected, it is safe to assume that the cohesin complexes incorporate STAG3-GFP which then might recruit other components of the meiotic machinery. To detect these proteins, future experiments should involve Mass Spectrometry (MS) analysis of these precipitates. In the meantime, optimal IP conditions to increase the yield of specific proteins and reduce the background, should be identified. The current problem with previous experiments was the yield of the bait. Immunoblot analysis can detect very small amount of protein, but for MS analysis, after analysing the sample by SDS-PAGE and Coomassie Brilliant Blue staining, it is also recommended for the bait band to be predominant. However, neither Coomassie nor silver staining analysis showed such a predominant band. To address this issue, silver staining analysis of precipitates obtained under increasing stringency conditions was performed (Figure 3.27). Increasing the amount of protein input under optimal conditions might give a stronger signal with significantly lower background. Lower stringency conditions showed minor improvement (3.27A) while higher stringency conditions, specifically 0.01 % SDS, had a significantly lower background (3.27B), but the prominent bands were still not visible by eye. Since immunoblot analysis of IPs in some of these conditions revealed the presence of SMC1β, SMC3 and REC8, one would expect that these bands should be among the last to disappear with higher stringency. No bands were detected in 0.1 % SDS treated precipitates. Further immunoprecipitation experiments with increased amount of nuclear extract in 0.01 % SDS should be performed, however, using larger IP volumes of the same protein concentration as used in these experiments is recommended rather than concentrating the nuclear extracts.
Figure 3.27 Silver staining analysis of anti-GFP precipitates from STAG3-GFP nuclear extracts under increasing stringency conditions.

The precipitates were obtained under increasing stringency conditions:

A PBS, Tween-20 (0.1%), Triton-X 100 (0.1%), Brij 58 (0.1%). A pipetting error can be visible in the wt sample of Triton-X 100 condition. 5% of wt and transgene (tg) inputs and pre-stained protein ladder lanes (M) are marked.

B NaCl (500 mM), SDS (0.01% and 0.1%). The gel also contains an IP sample with 10 times more protein input in PBS. 5% of wt and transgene (tg) inputs and pre-stained protein ladder lanes (M) are marked.
4. Discussion

Meiosis is a crucial biological function for creation of haploid gametes necessary for sexual reproduction. The process itself includes formation of the chromosomal axis, synapsis of homologous chromosomes and recombination of their genetic material. Failing to properly execute any of these processes leads to detection and elimination of these cells by a meiotic surveillance system via apoptosis. An exception of this can be found in mammalian female gametogenesis where cells can progress to the end of oogenesis, resulting in aneuploidies manifesting as a plethora of genetic diseases if fertilization occurs. The cohesin complex is a proteinaceous ring-like structure with the main function of establishing cohesion between newly replicated sister DNA molecules, but is also involved in meiotic chromosomal axis formation and maintenance, synaptonemal complex formation, genetic recombination and gene expression regulation (Parelho et al. 2008; Merkenschlager, 2010; Haering and Jessberger, 2012). Any disruption in function of cohesin complexes results in a loss of sister chromatid cohesion, disruption of chromosomal axis architecture or defective synapsis, which leads to activation of the meiotic surveillance system that kills the cell. STAG3 is a meiosis specific stromal antigen (SA) component of the cohesin complex. Previous studies identified STAG3-containing complexes to associate with the chromosomal axis, synaptonemal complex and the sex body in males. However, no studies were made involving a completely STAG3-deficient mouse, which was addressed in this doctoral thesis. The aim of this study was to describe the STAG3 mutant in detail and try to shed some light onto the biological role of STAG3 in mammalian meiosis.

STAG3\(^{ko/ko}\) spermatocytes show the most severe phenotype of all single cohesin mutants

Stag3\(^{ko/ko}\) spermatocytes lack AEs and their spermatogenesis is aborted at a leptotene-like stage, which is the earliest stage of arrest among all the single cohesin mutants. For the same reason, staging of these cells is not possible by standard analysis of chromosome architecture. However, observing cell interactions in testis seminiferous tubules allows staging by tubular development. Together with this analysis and the fact that SYCP3 was found in dot-like structures and short stretches, SYCP1 in spotty deposits, γH2AX in reduced amounts and telomeres in clusters, it is easy to assume that the most advanced cells reach late zygotene. This assumption is tentative because, other than tubular cell associations, zygonema as such is defined by clearly visible AEs that are undergoing synapsis and by other chromosome features which are heavily perturbed in Stag3\(^{ko/ko}\) spermatocytes and embryonic oocytes.
Therefore, these cells will from now on be referred to as leptotene-like, since the terminology in this case is not clearly defined. Both male and female meiocytes lacking the STAG3 protein showed a drastic phenotype with virtually no AEs and thus no SCs. Many of these cells display dotty foci of SYCP3, as well as aggregates, but only a small amount have short stretches and filaments that resemble short axis-like structures. These structures represent either AE initiator units or non-specific aggregates of SYCP3, which tends to form filamentous structures in vitro by itself (Yuan et al, 1996). Also, SYCP3 signal in these cells tends to orbit the centromeres and telomeres, sometimes even protruding into the ACA or RAP1 foci and partially overlapping, especially in those cells where these foci cluster. The SYCP3 signal never overlaps completely with ACA foci, but extends to the side or between pairs of ACA foci instead, sometimes forming two filaments starting between centromere pairs and extending to the sides perpendicular to the direction these foci pairs face. SYCP1, which marks the synapse, is present in very small amount of foci, which sometimes overlap with SYCP3 in small dot like structures, but not in all cells. In comparison, mice spermatocytes lacking cohesin REC8 exhibit shortened AEs and show evidence of synapsis between sister chromatids (Xu et al, 2005), which might explain these SYCP1/SYCP3 positive foci, if indeed these are legitimate co-localization events. High resolution microscopy images might provide evidence to support this. Formation of DSBs does not seem to depend on STAG3, since DMC1 foci can still be detected in Stag3\(^{ko/ko}\) spermatocytes. This is consistent with other cohesin deficiencies such as Smc1\(^{β−/−}\) mouse (Revenkova et al, 2004; Biswas et al, 2013), which also showed the presence of DMC1, as well as RAD51 foci. Presence of relatively large amounts of DMC1 foci in most of the cells suggests that these DSBs do not repair efficiently. By the end of zygotene, wt meiocytes contain less than 50 DMC1 foci, which are the result of DSB processing of the initial pool of 120 - 200 foci in early zygotene. Most of the Stag3\(^{ko/ko}\) spermatocytes display more than 100 foci, but more precise counting was proven to be difficult, so deriving precise stages from these numbers was not possible. These data suggest that DSBs are not efficiently processed in the absence of STAG3, which is similar to the delayed repair exhibited by Smc1\(^{β−/−}\) spermatocytes (Biswa et al, 2013), as well as Rec8\(^{−/−}\) Rad21L\(^{−/−}\) spermatocytes (Llano et al, 2012), which exhibit a similar phenotype as Stag3\(^{ko/ko}\) spermatocytes. This may also be the consequence of cells failing to develop enough for these DSBs to undergo repair. Another indication of delayed DSB repair is the persistence of γH2AX signal in the majority of Stag3\(^{ko/ko}\) spermatocytes, as well as the failure of their chromosomes to synapse. As wt cells progress, γH2AX signal is displaced from synapsed AEs, except from X and Y chromosomes which remain unsynapsed for the most of their sequence. Even though Stag3\(^{ko/ko}\) spermatocytes lack AEs and SCs, the diminishing of γH2AX signal and its concentration into cloud formations within cells that progress the furthest according to tubular stage development, suggests that the ATM-mediated phosphorylation of H2AX cannot be maintained despite the failure to synapse. Initial
assumption was that ATM activity requires the formation of AEs and that the maintenance of H2AX phosphorylation requires AE-associated proteins such as HORMAD1 (Fukuda et al, 2010; Daniel et al, 2011). However, HROMAD1 is still present in Stag3\(^{ko/ko}\) spermatocytes, although diffusely distributed along the chromatin, which is still interesting because HORMAD1 binds AEs of unsynapsed chromosomes and these cells have no AEs as such. There was no data on HORMAD1 binding pattern in the analysis of the Rec8\(^{ko/ko}\) Rad21L\(^{ko/ko}\) spermatocytes (Llano et al, 2012).

Sister chromatid cohesion is only partially impaired in STAG3-deficient meiocytes, indicating involvement of other cohesin complexes in their maintenance. Quantification of ACA foci revealed an average of 55 foci per mutant cell, which indicates that there is no synapsis between homologous chromosomes and that the sister chromatid cohesion is partially dissolved. A full dissolution of sister chromatid cohesion would count 80 clearly separated ACA foci. Similar data concerning sister chromatid cohesion was provided in studies of REC8\(^{-/-}\), RAD21L\(^{-/-}\) and SMC1β\(^{-/-}\) mice, although their levels were reduced (Bannister et al, 2004; Revenkova et al, 2004; Xu et al, 2005; Herran et al, 2011; Biswas et al, 2013). This is also in line with a recent study of a STAG3 hypomorph mouse generated by insertional mutagenesis (Caubert et al, 2014). Another recent study showed that the SMC1α-based complexes present in prophase I provide a substantial fraction of centromeric sister chromatid cohesion (Biswas et al, 2013). In the absence of STAG3, SMC1β and/or SMC1α complexes containing other STAGs, SA1 and/or SA2, should be the ones responsible for the remaining sister chromatid cohesion. It was shown before that only a small fraction of SMC1β complexes should interact with either SA1 or SA2 (Lee and Hirano, 2011), however, this fraction appears to be increased in Stag3\(^{ko/ko}\) spermatocytes as visible from Figure 3.11A. Since it is clear from these data that SA1- and/or SA2-containing cohesin complexes provide the remaining cohesion in STAG3-deficient spermatocytes, it is safe to assume that they carry this function in wt spermatocytes as well, albeit, probably in smaller amount.

Just as centromeric sister chromatid cohesion, telomeric sister chromatid cohesion also depends on STAG3. Quantification of both RAP1 and telomere-FISH signal in Stag3\(^{ko/ko}\) spermatocytes revealed more than 80, but never all 160 foci. It is not possible to guarantee that all foci were counted due to a weak signal strength of some of the individual foci, however, it is safe to conclude that STAG3 has at least a partial roll in telomere sister chromatid maintenance. Unlike SMC1β, which was shown to have a role in protecting telomeres from damage such as breaks, large extensions and inter-chromosomal telomere bridges (Adelfalk et al, 2009), STAG3 was not shown to carry a similar role, as no such telomere perturbations were revealed by RAP1 staining or FISH experiments in Stag3\(^{ko/ko}\) meiocytes. This may suggest that a different type of SMC1β-containing complex is responsible for telomere protection, such as SMC1β/SA1 or SMC1β/SA2 perhaps. However, it is also possible that
Stag3\textsuperscript{ko/ko} spermatocytes never develop far enough to exhibit telomere perturbations that are present in SMC1\textsuperscript{β}\textsuperscript{−/−} cells, which make to as far as early pachytene stage. Moreover, it was shown in mitotic cells that SA1 binds directly to DNA through its unique AT-hook to promote sister chromatid cohesion at telomeres (Bisht et al, 2013). This notion is intriguing, since it reveals a possibility that STAG3 might also work alone to promote telomeric sister chromatid cohesion, without the need for the whole cohesin complex.

STAG3-deficient meiocytes do not form AEs and SCs, unlike other meiosis-specific cohesin mutants, which contain AEs that are very short and SCs that are not complete on every chromosome pair (Bannister et al, 2004; Revenkova et al, 2004; Xu et al, 2005; Herran et al, 2011). This highlights STAG3 as a crucial component of the meiotic fraction of cohesin complexes necessary for axis formation and sister chromatid cohesion. In comparison, REC8\textsuperscript{−/−} RAD21L\textsuperscript{−/−} mutant (Llano et al, 2012) also exhibits an almost complete lack of axis, which is not surprising since RAD21L and REC8 are the only kleisins that have been shown to interact with STAG3, connecting it to the rest of the cohesin complex. This is supported by the data retrieved from immunoprecipitation experiments in this study, where RAD21 was not detected in wt SMC1\textsuperscript{β} precipitates (Figure 3.11A) and was observable only as weak signal on pre-pachytene chromosomal axes. Depletion of STAG3 most likely affects RAD21L- and REC8-based complexes, displaying a similar phenotype to the “double knockout” of these kleisins. These data, together with the fact that out of the two kleisins only REC8 exhibits sister chromatid cohesion impairment, suggest that other combinations of cohesin complex subunits is possible. SMC cohesin subunits SMC3, SMC1\textalpha and SMC1\textbeta are present in STAG3-deficient spermatocytes and localize throughout the chromatin, which suggests that other cohesin complexes are indeed present. REC8 and RAD21L are also present, however the stability of REC8 is somewhat reduced, suggesting a decrease in its stability in the absence of STAG3. More evidence of this was offered by the differential salt extraction experiments, where almost all of the REC8 protein was detected in the low-salt fraction, indicating loose chromatin association of this particular meiotic kleisin in Stag3\textsuperscript{ko/ko} spermatocytes. Interestingly, previous studies showed little or no association of meiotic cohesin subunits with SA1 or SA2 (Ishiguro et al, 2011; Lee and Hirano, 2011), suggesting that cohesin complexes, almost entirely comprised of mitotic cohesins, are responsible for what is left of sister chromatin cohesion in Stag3\textsuperscript{ko/ko} meiocytes. This study also showed that precipitation of SMC1\textbeta containing cohesin complexes co-precipitates both SA1 and SA2 in very small amounts, as indicated by very weak signals (Figure 3.11A), which was even more prominent in spermatocytes devoid of STAG3. It is possible that the absence of STAG3 elicits an upregulation in the levels of SA1 and SA2 in these mutants (not visible from total testis extracts). Another possibility is that the stability of SA1 and SA2 is increased by association with cohesin complexes usually interacting with
STAG3 now that STAG3 is out of the competition. Also, with its high affinity partner STAG3 gone, SMC1β might bind SA1 and SA2 much more stably than in wt conditions.

SMC1α-based complexes were found to be responsible for a substantial amount of cohesion in early meiotic prophase I, after which SMC1β type complexes take over (Eijpe et al, 2000). Consistently, studies on SMC1β−/− meiocytes observed sustainable sister chromatid cohesion at centromeres and along chromosome arms (Revenkova et al, 2004; Biswas et al, 2013), which can only be provided by SMC1α-based complexes in this mutant. Several groups have reported IP data obtained from testis nuclear extracts or somatic cell overexpression systems, providing evidence of SMC1α association with RAD21 or RAD21L (Gutierrez-Caballero et al, 2011; Ishiguro et al, 2011; Lee and Hirano, 2011). Conversely, Ishiguro et al, 2011, provided evidence against the interaction of SMC1α with RAD21L. Together, this study on STAG3-deficient mice and the study on the meiotic kleisin double knockout Rec8−/− Rad21L−/− mice confirm that cohesin is required for the formation of AEs and SCs, which when applied to the data obtained from SMC1β-deficient mouse leads to the conclusion that SMC1α-based complexes significantly contribute to the formation of AEs and SCs. The existence of SMC1α/RAD21 complex is in line with IP and pulldown experiments (Gutierrez-Caballero et al, 2011; Ishiguro et al, 2011), which also showed the interaction of STAG3 with these complexes, although the levels detected were very low. The evidence of SMC1α interacting with RAD21L and REC8 is controversial, since only some studies could provide it (Revenkova et al, 2004; Ishiguro et al, 2011; Lee and Hirano, 2011). However, data from several groups support that SMC1β forms complexes with each of the three kleisins (Revenkova et al, 2004; Gutierrez-Caballero et al, 2011; Ishiguro et al, 2011; Lee and Hirano, 2011), and according to the data in this study, considering such strong phenotype of Stag3ko/ko meiocytes, STAG3 probably associates with each of these complexes. If we consider the dosage phenotype of STAG3, described by Fukuda et al, 2014, reduced levels of STAG3 can still provide cohesion for the formation of AEs, even though they are much shorter than in wt, and for detectable, but aberrant synopsis. RAD21 and RAD21L were found to localize on those AEs, but not REC8, suggesting that STAG3 is paramount for REC8-containing cohesin complexes. Another group also reported a STAG3 mutant, obtained by a lentiviral insertion in exon 8 of the STAG3 gene (Llano et al, 2014). It was not clear if this was a complete knockout, since continuous stretches of the SC marker SYCP1 was detected colocalizing with short axes marked by SYCP3 and no immunoblot or mRNA RT-PCR data was presented. This suggest that at least partial synopsis still occurs, which is strikingly similar to that of STAG3 hypomorph phenotype described by Fukuda et al, 2014. In comparison to these mutants, STAG3 mutants described in this study bear the most severe phenotype. However, the similarities between these mutants are obvious, like partial loss of centromeric cohesion and the presence of other cohesins observed on chromosome spreads.
All together, these data suggest that meiotic chromosome AEs and SC formation depend on SMC1α- and SMC1β-based cohesin complexes containing STAG3. SMC1α complexes contribute in the initial phases of meiosis I, after which SMC1β complexes almost completely take over. However, in either case, the presence of STAG3 in these complexes is paramount for the entirety of the meiotic prophase I. None of the other individual cohesin mutants exhibited such a drastic phenotype, which highlights STAG3 as the most important meiotic cohesin.

**Insect cell expressed recombinant STAG3 showed no evidence of participating in de novo formation of cohesin complexes using cohesin subunits from mouse nuclear extracts**

The baculoviral infection of insect cells, using a vector containing the peptide of interest and the subsequent overexpression and purification of that peptide is a well-known method of studying protein-protein interactions (more information can be found in Fernandez and Hoeffler, 1999). An example of such use of baculoviral vector/insect cell system is the co-expression of SA2 and RAD21 (Zhang et al, 2013). This method was chosen due to several advantages: an eukaryotic expression system provides posttranslational modification to the peptide of interest, although one cannot guarantee that all necessary modifications of the transgene will be implemented; prokaryotic vectors are usually less reliable with large inserts and STAG3 cDNA is more than 4 kilobases long; availability of the well-established method at the MPI-CBG protein facility; a previous study demonstrated co-expression of mitotic stromal antigen SA2 and its kleisin interactor RAD21 in the same system (Zhang et al, 2013). Expressing the full length STAG3 and immobilizing it on a resin can be used as bait in a pulldown assay from a mouse nuclear protein extract. Once the binding interactors are detected by mass spectrometry (MS), the experiments would be repeated with fragments of STAG3, effectively mapping the binding regions for groups of these proteins. However, western blot analysis should first detect the known interactors like REC8, SCM1b and SMC3, which was not the case in this study using the recombinant STAG3.

The expression of recombinant STAG3 was abundant in insect cells, initially concluded from green fluorescence of cell culture and prepared “pearls” and later by gel electrophoresis and Coomassie staining. The purification of the recombinant protein indicated a problem with its His-tag binding capacity. The column was not able to bind all of the recombinant protein and the flow-through was visibly green. At first, one might think that there is simply too much protein for the column capacity, but increasing the amount of resin did not affect the amount of protein in the flow-through. This might be explained as a partial degradation of the protein, which could be shown by anti-GFP immunoprecipitation of the flow-through and immunoblot analysis probed for GFP and STAG3. Another explanation is that the polyhistidine tag might be
obscured during the aggregation of the protein. Recombinant STAG3 has the ability to aggregate, as was shown by gel filtration chromatography. Therefore, the purified protein might be the sub-population that still wasn’t aggregated at the time of cell lysis. The aggregates themselves are pretty stable, considering that 0.5% SDS solution wasn’t able to dissolve them. Interestingly, 20 mM EDTA or spermidine had a significant effect on aggregate stability. Why a polycation and a metal sequester would affect the aggregates and a strong ionic detergent would not, is not intuitively clear. The need for a metal ion might be implicated in either STAG3 biological function or its aggregation capability, but there is no data in the current literature supporting this. Other in vivo chelation experiments targeting Ca\(^{2+}\), Mg\(^{2+}\) or other metal ions might prove more insight into this problem.

Regardless of its ability to aggregate, immunoprecipitation of the purified recombinant STAG3 was successful, which was confirmed by SDS-PAGE of the sample subjected to proteolytic cleavage with the site-specific protease 3C. However, using this immunoprecipitated recombinant STAG3 as bait in pulldown experiments from mouse nuclear protein extracts showed no additional protein bands. Moreover, immunoblot analysis showed no known interactor bands present. This may be explained in the same manner as the abundance of the recombinant STAG3 in the flow-through during the purification process. The ability of the protein to aggregate might mask the binding sites for REC8, which in turn renders other cohesins not able to participate in the formation of STAG3 containing cohesin complexes. Another explanation for this involves the possible spatio-temporal regulation of the meiotic stromal antigen cohesin subunit. The recruitment of STAG3 onto the meiotic chromosomal axis might require other events in meiosis, like phosphorylation or DNA binding. It is known that STAG3 is phosphorylated during meiosis (Fukuda et al, 2012, 2014). Also, it was shown in yeast that the STAG3 homologue Rec11 needs to bind DNA first before it can interact with other components of the cohesin complex (Phadnis et al, 2015).

Analysis of the STAG3 primary protein structure reveals a domain rich in positive amino acid residues, which might be responsible for the protein’s affinity for the negatively charged DNA. Additionally, STAG3 is mostly comprised of armadillo type folds, structurally similar to HEAT repeats. Stacked armadillo repeats (ArmR) are each typically consisting of 42 amino acids arranged in three α-helices. These superhelical structures present an extensive solvent-accessible surface that is well suited for binding large substrates such as proteins and nucleic acids (Andrade et al, 2001), which might also be contributing to the STAG3 affinity for DNA, as well as many other proteins involved in meiosis. Altogether, these data provide evidence that the recombinant STAG3 interacts with a double stranded DNA (dsDNA) fragment. However, further experiments are necessary to implicate DNA binding as necessary for proper STAG3 cohesin complex formation and cohesion establishment. Such experiments would involve mapping the precise regions of the protein, or even amino acid residues necessary for
DNA interaction. Mouse mutants lacking these STAG3 domains should show impairment in cohesin complex establishment.

**STAG3-GFP fusion protein most likely compensates for the loss of endogenous STAG3 in mice.**

*In vitro* experiments using STAG3 overexpressed in insect cells raised concerns about the possible lack of either proper post-translational modification or presence of other signalling factors necessary for meiotic cohesin complex formation. To mitigate any unknown lack of meiotic spatio-temporal regulation and/or posttranslational modification of STAG3, *in vivo* established STAG3-GFP containing cohesin complexes would be very advantageous for its study. The tag is necessary for immunoprecipitation experiments due to the lack of functional anti-STAG3 antibodies that recognise the native form of STAG3 and attempts to generate monoclonal antibodies during the course of this thesis had failed. STAG3-GFP containing BAC was injected into mouse zygotes and the resulting mice bred with the STAG3 heterozygote (STAG3<sup>ko/+</sup>). As explained in section Materials and Methods 2.1.1, the STAG3<sup>ko/ko</sup> mice are completely deficient for STAG3 protein and this deficiency is the result of an alternative splicing site provided by an insertional cassette with flox sites inside and outside of the cassette. Genotyping relies on PCR detection of the insertional cassette for the STAG3 floxed allele and of a DNA segment for the wt allele which is otherwise split and separated by the cassette. Using these genotyping primers, STAG3<sup>+/-</sup> mouse will show only the wt band, STAG3<sup>ko/+</sup> will show both the floxed band and the wt band and STAG3<sup>ko/ko</sup> will show only the floxed band. However, introducing STAG3-GFP transgene into the genome of this mouse line makes genotyping difficult but still crucial to discern if the transgene is fully functional. If the transgene is present in the STAG3 knockout background and the phenotype of these mice is identical to the wt mice, then it is safe to assume that the transgene fully compensates for the loss of endogenous STAG3. The difficulty of this genotyping method lies in the fact that STAG3-GFP sequence will always be detected as wt positive, regardless if the endogenous STAG3 locus is STAG3<sup>ko/+</sup> or STAG3<sup>ko/ko</sup>, effectively making the heterozygote indistinguishable from the knockout. None of the possible primer combinations can make this genotyping viable without mapping the precise location of the transgene insert in the genome. However, the solution to this genotyping conundrum lies in the function of the insertion cassette itself. By crossing these mice with a Cre expressing mouse, the neomycin reporter gene of the insertional cassette is cut out, creating a different version of a still deleterious allele (Figure 4.1, tm1b).
The original floxed allele can be subjected to Cre recombinase which is produced in a mouse and controlled by meiotic vasa promoter, ensuring spatio-temporal specificity. The recombinase is expressed only in spermatogonia cells about to enter meiosis. Introducing this allele into the STAG3 floxed line allows detection of a STAG3 knockout without using the standard wt primers (see section Materials and Methods 2.1.1). Genotyping for GFP will detect the presence of the transgene, while using a primer pair, of which one binds 500 bp upstream of loxP 1 site and the other 500 bp downstream of loxP 3 site, gives a 1000bp product in case of the new allele (“ex” for “excised”) and a much bigger product in case of the original floxed allele (“fl” for “floxed”). It is important to note that the absence of the neo protein cannot be used as an allele distinguishing method since both excised and wt alleles lack the neo reporter. This method still cannot distinguish between all heterozygotes and knockouts but it can detect a subset of knockouts which have both versions of the knockout allele in the endogenous STAG3 locus (STAG3\textsuperscript{ex/fl} and STAG3-\textsuperscript{GFPex/fl}).

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While breeding of these mice is proceeding, preliminary analysis of GFP positive mice from the STAG3-GFP line was performed. FACS analysis of single cell suspension obtained from STAG3-GFP testis revealed significantly more events of high green fluorescence compared to the wt and a pattern fairly similar to that of SMC1b-LAP, although the expression in STAG3-GFP mouse was weaker. Other experiments, like immunoprecipitation and immunofluorescence, also showed a STAG3-GFP signal to be weaker than the wt signal. Several explanations are possible for this generally lower expression profile of STAG3-GFP compared to the endogenous STAG3. One possibility is that sufficient levels of STAG3 negatively regulate its own expression, and if the STAG3-GFP has a bit lower affinity for its partners, due to the GFP tag, the equilibrium of binding kinetics would selectively shift in favour
of the endogenous STAG3. Since the absence of STAG3 positively regulated the levels and possibly, indirectly the stability of SA1 and SA2 (Figure 3.11A), it is possible that STAG3 can regulate its own levels. This possibility will get more credence once the STAG3-GFP<sup>ex/fl</sup> mouse is analysed and comparably higher levels of STAG3-GFP are detected. Another possibility is that the transgene was integrated close to a heterochromatin area or a possible enhancer isn’t readily available to reach this site, resulting in lower expression. However, this possibility might only be confirmed if the STAG3-GFP<sup>ex/fl</sup> mouse exhibits a hypomorphic phenotype where the STAG3-GFP levels are reduced enough to be comparable to the mutants described by Fukuda et al, 2014, and Llano et al, 2014. Hoechst dye was added to distinguish GFP positive cells by their DNA count and the analysis indeed revealed a GFP positive primary spermatocyte population.

Immunofluorescence staining of STAG3-GFP chromosome spreads, with anti-GFP antibody, revealed a pachytene localization pattern similar to that of STAG3 in wt, although the signal was much weaker. Surprisingly, other prophase I stages showed no defined patterns of GFP signal and a lot of background. If this particular antibody batch exhibits generally higher background and lower affinity for GFP or STAG3-GFP is expressed in lower levels than the endogenous STAG3, it is possible that only synapsed chromosomes had strong enough signal to be visible. To clarify this, a well-established positive control which has a similar localization pattern and frequency is necessary. The closest to such a control would be an SMC1β-LAP mouse because the majority of SMC1β-containing cohesin complexes also contain STAG3. Immunofluorescence analysis of these chromosome spreads will be performed during the revision of this thesis, once more mice are available, and hopefully added afterwards. Moreover, staining with anti-STAG3 antibody showed a wt STAG3 localization pattern and signal strength in all stages. These data suggest that STAG3 positive foci are either solely comprised of STAG3-GFP in case of the knockout or a mix of endogenous STAG3 and STAG3-GFP proteins in case of the heterozygote, since both GFP and STAG3 signals were detected on these chromosomes. Also, in case of the later, the ratio might be in favour of the endogenous STAG3, which might reflect the same difference between STAG3-GFP and endogenous STAG3 levels, as observed by immunoblot analysis (Figure 3.26B). However, these assumptions are valid only if SMC1β-LAP positive control reveals a GFP signal similar in pattern and intensity as the STAG3 signal.

Immunoprecipitation experiments and immunoblot analysis of STAG3-GFP nuclear protein extracts, using anti-GFP antibody, revealed the presence of cohesin subunits expected to be a part of STAG3 containing cohesin complex. The REC8 signal was much weaker than SMC1β signal, which corresponds to the dual nature of STAG3 binding to meiotic kleisins. Further probing with anti-RAD21L antibody should reveal a signal similar or weaker than REC8, which together with REC8 should explain the SMC1β signal strength. However, this cannot be
quantified by immunoblot analysis since the signal strengths are not necessarily linear, and different antibodies yield different signal strength. Interestingly, although probing the blot with anti-STAG3 antibody revealed both the endogenous STAG3 band and the transgene band, the latter was much weaker in signal. Even though the band signal between different probing of the same membrane depends on antibody specificity and exposure duration and might not correlate linearly, a difference between the relative signal strength of specific bands of the same probing is significant. This difference can be explained if STAG3 forms oligomers as a functional cohesin subunit. *In vitro* experiments with recombinant STAG3 revealed its ability to aggregate. While this might have been a characteristic of that particular fusion protein, it is also possible that such feature was a symptom of an unregulated biological function of STAG3 to form dimers, or even multimers. However, it is important to note that GFP itself has a tendency to multimerize. If STAG3 had another tag except GFP or if a better anti-STAG3 antibody was available, a reverse IP would confirm if this is indeed an oligomerization event. Other than the negative control being clear in that blot, the evidence of STAG3 ability to oligomerize is rather weak. To strengthen the data showing that STAG3-GFP fully compensates for the loss of the endogenous STAG, it is crucial to analyse the testes with STAG3-GFP in STAG3 knockout background.

Interpretation of these data relies on the assumption that the transgene fully compensates for the endogenous STAG3 function. During these preliminary experiments, 21 GFP positive mice were sacrificed and testes taken for different analyses, out of which 16 were genotyped as STAG3 heterozygotes (STAG3<sup>h<sup>+/+</sup></sup>), which are indistinguishable from homozygote knockouts by the means of this genotyping protocol. A subpopulation of these erroneously genotyped heterozygotes are actually homozygous knockouts, which according to Mendelian law of inheritance contains 5 mice. Interestingly, even though only a few of these testes were taken for IF analysis and showed no phenotype, none of these 16 pairs of testes were different in size from wt. Statistically, assuming that the transgene cannot compensate for the loss of the endogenous STAG3, the p-value of selecting zero reduced-size testes from 16 heterozygous mice of this line is p<0.05 (= 0.015). This is strong statistical evidence that the transgene can fully compensate for the function of endogenous STAG3. Another way to get more data before the Cre mouse line is ready, is to sacrifice another 15 “homozygous” STAG3-GFP mice, use one testis to make nuclear extracts and one to make 1 chromosome spread slide for each of these mice. Immunoblot analysis of these extracts should reveal approximately 5 testes which have no endogenous STAG3 band and only the STAG3-GFP band. The corresponding slide can be used for a quick preliminary analysis of chromosome architecture and the presence of other cohesins.

Mass spectrometry analysis of anti-GFP immunoprecipitation experiments using nuclear extracts from STAG3-GFP mice is crucial for detection of novel STAG3 interactors. Once the
list of potential candidates is available, a few selected should be used in protein interaction assays to identify key binding domains. Subsequent compensation studies will be needed, where mutations are introduced into these domains and transfected into the STAG3 knockout background by following identical procedure used to obtain STAG-GFPex/fl mice. However, once both "excised" and "floxed" alleles are obtained and maintained in the colony, this whole procedure should take significantly less time.
5. Summary

The process of mammalian spermatogenesis is crucial for sexual reproduction because it results in formation of gametes. During meiosis, each chromosome assembles into a specialized structure called the chromosomal axis which is used as a platform for meiotic processes. Genome-wide double stranded DNA breaks (DSBs) are introduced and subsequently repaired via homologous recombination, which as a consequence has the pairing of homologous chromosomes and exchange of genetic material between them. Also, the resulting chiasmata remain as a physical link between homologous chromosomes, providing resistance to the tension created by the meiotic spindle until all bivalents are bidirectionally aligned on the metaphase plate. The cohesin complexes are tripartite ring-like structures that primarily maintain sister chromatid cohesion after the DNA replicates. Other functions of cohesin complexes involve DNA repair and gene expression regulation, as well as still poorly described roles in the intricate network of meiotic signalling pathways and interaction events. Meiosis-specific cohesin subunits are implicated in chromosomal axis formation, synaptonemal complex formation and DSB formation. These include SMC1β, kleisins RAD21L and REC8 and stromal antigen STAG3. The aim of this study was to describe the role of STAG3 in mammalian meiosis.

Newly generated STAG3-deficient mice of both sexes are sterile with meiotic arrest. In these mice, meiotic chromosome architecture is severely disrupted as no bona fide axial elements (AE) form and homologous chromosomes do not synapse. Axial element protein SYCP3 forms dot-like structures, many partially overlapping with centromeres. Asynapsis marker HORMAD1 is diffusely distributed throughout the chromatin, and SYCP1, which normally marks synapsed axes, is largely absent. Centromeric and telomeric sister chromatid cohesion are impaired. Centromere and telomere clustering occurs in the absence of STAG3, and telomere structure is not severely affected. Other cohesin proteins are present, localize throughout the STAG3-devoid chromatin, and form complexes with cohesin SMC1β. No other deficiency in a single meiosis specific cohesin causes a phenotype as drastic as STAG3 deficiency. STAG3 emerges as the key STAG cohesin involved in major functions of meiotic cohesin.

To further describe STAG3 function, a genetic construct coding for STAG3 tagged with green fluorescent protein (GFP) on C-terminus and polyhistidine-maltose binding protein (His6-MBP) on N-terminus, was created and incorporated into a baculoviral vector used to infect insect cell cultures. The overexpressed fusion protein was purified from these cells using a nickel purification column, also known as HisTrap. The purified protein was immobilised on paramagnetic beads as bait in pulldown assays from wild type mouse nuclear extracts for detection of STAG3 interactors. No known interactors were detected in these experiments.
filtration chromatography analysis showed that this fusion protein forms aggregates which are resistant to disruption by strong ionic detergents like SDS but easily disassembled by metal ion sequesters like EDTA, polycation spermidine and high ionic strength conditions like 500 mM NaCl. The fusion protein also demonstrated the ability to bind double stranded DNA, as shown by electrophoretic mobility shift assay (EMSA).

Since *in vitro* experiments demonstrated that above described recombinant STAG3 is not able to form *de novo* cohesin complexes from mouse nuclear extracts, *in vivo* experiments were the obvious next step. A Bacterial Artificial Chromosome (BAC), containing the STAG3 gene and a GFP sequence inserted directly before the open reading frame STOP codon, was injected into fertilized mouse eggs, which were then transferred into pseudo-pregnant female mice. The newly born GFP-positive mouse was used for breeding to create a colony that can eventually give rise to mice deficient in endogenous STAG3 function (STAG3 knockout) expressing the STAG3-GFP fusion protein under STAG3 promoter from a different locus in the genome. Preliminary experiments indicated that the cohesin complexes containing STAG-GFP compensated for the loss of endogenous STAG3. Using anti-GFP antibodies, immunoprecipitation experiments (IPs) directly from STAG3-GFP nuclear extracts revealed the expected cohesin subunit interactors. Future experiments should involve mass spectrometry analysis of these IPs once the optimal IP incubation conditions are determined. Hopefully, new STAG3 interactors will be discovered and subjected to further interaction studies. Moreover, the analysis of STAG3-deficient mice expressing the STAG3-GFP fusion protein is crucial in order to confirm that STAG3-GFP completely compensates for the loss for endogenous STAG3.
6. Zusammenfassung

Der Ablauf der Spermatogenese ist entscheidend bei der sexuellen Reproduktion, da er zur Bildung der Gameten führt. Während der Meiose formen die einzelnen Chromosomen spezialisierte Strukturen, die chromosomale Achse genannt werden und als Gerüst für meiotische Prozesse dienen. Genomweite DNA-Doppelstrang-Brüche (DSBs) werden erzeugt und anschließend durch homologe Rekombination repariert, welche zur Folge hat, dass sich die homologen Chromosomen aneinander lagern und genetisches Material untereinander austauschen. Die resultierenden Chiasmata verbleiben als physikalische Verbindung zwischen den homologen Chromosomen und liefern Widerstand gegenüber der durch die meiotische Spindel aufgebaute Spannung bis alle Bivalenten bidirektionell in der Metaphasenplatte ausgerichtet sind.


Um die Funktionen von STAG3 weiter zu beschreiben, wurde ein genetisches Konstrukt, das für STAG3 codiert, welches C-terminal dasgrün fluoreszierende Protein (GFP) sowie N-
terminal ein Polyhistidin-Maltose-Bindeprotein (His6-MBP) enthält, erzeugt und in einen baculoviralen Vektor eingebracht um Insektenzellkulturen zu infizieren. Das überexprimierte Fusionsprotein wurde mittels einer Nickel-Purifikationsäule, die auch als HisTrap bekannt ist, aus diesen Zellen aufbereitet. Das aufbereitete Protein wurde mittels paramagnetischen “beads” immobilisiert und als Köder in “pulldown”-Assays mit nukleären Wildtyp-Maus-Extrakten verwendet um Interaktionspartner des STAG3 zu finden. Dabei konnten keine bekannten Interaktionspartner gefunden werden. Die Analyse mittels Gelfiltrations-Chromatographie zeigte, dass dieses Fusionsprotein Aggregate bildet, die widerstandsfähig gegenüber Zerstörung mit starken ionischen Detergenzien wie SDS sind, sich aber leicht durch Komplexbildner von Metallionen wie EDTA, “polycation spermidine” und hoch ionische Bedingungen wie 500mM NaCl zerlegen lassen. Weiterhin zeigte das Fusionsprotein im “electrophoretic mobility shift assay” (EMSA) die Fähigkeit doppelsträngige DNA zu binden.

Da die in vitro Experimente zeigten, dass das oben beschriebene rekombinante STAG3 nicht in der Lage ist de novo Cohesinkomplexe von nukleären Maus-Extrakten zu bilden, waren in vivo Analysen der nächste offensichtliche Schritt. Ein Bacterial Artificial Chromosome (BAC) mit STAG3 und der GFP-Sequenz, welche direkt vor dem Stopcodon des offenen Leserasters eingeführt wurde, wurde in befruchtete Mausoozyten injiziert und diese in pseudoträchtige Mäuse transferiert. Die neu geborenen GFP-positiven Mäuse wurden in der Zucht genutzt, um eine Kolonie von Mäusen erzeugen zu können, die defizient bezüglich der endogenen STAG3 Funktion (STAG3-Knockout) ist und die das STAG3-GFP-Fusionsprotein unter dem STAG3-Promoter von einem anderen Locus exprimieren. Vorläufige Ergebnisse weisen darauf hin, dass die Cohesinkomplexe mit STAG3-GFP den Verlust des endogenen STAG3 kompensieren können. Immunopräzipitationsexperimente (IPs) mit anti-GFP Antikörpern und nukleären Extrakten aus STAG3-GFP-Mäusen zeigten die zu erwarteten interagierenden Cohesinunteneinheit. Künftige Experimente sollten, nach Bestimmung der optimalen Inkubationsbedingungen für die IP, massenspektroskopische Untersuchungen dieser IPs beinhalten. Hoffentlich können neue Interaktionspartner des STAG3 entdeckt werden, die dann für weitere Interaktionsstudien genutzt werden. Zudem ist die Analyse der STAG3-defizienten Mäuse mit Expression des STAG3-GFP Fusionsproteins entscheidend, um zu bestätigen, dass STAG3-GFP für den Verlust des endogenen STAG3 kompensieren kann.
7. References


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