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**Localization of spindle assembly checkpoint proteins
in mouse spermatocytes**

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Lokalizacija proteina kontrolne točke diobenog vretena u mišjim spermatocitama

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Sažetak: Tijekom mejoze, od velike je važnosti pravilna segregacija kromosoma, koja se ostvaruje zahvaljujući bipolarnom povezivanju niti diobenog vretena s kinetohorama kromosoma. Ukoliko bipolarno povezivanje nije ostvareno, kontrolna točka diobenog vretena (engl. Spindle Assembly Checkpoint, SAC) odgađa anafazu djelovanjem specifičnog kompleksa SAC, kojeg čine i proteini MAD1, MAD2, BUB1 i 3F3/2 fosfoepitop. Ako ova kontrolna točka nije funkcionalna, neostvarivanje bipolarnosti orijentacije diobenog vretena rezultira nepravilnom segregacijom kromosoma i promjenom broja kromosoma u stanicama kćeri. U ovom radu proteini MAD2 i 3F3/2 fosfoepitop, koji su dio kompleksa SAC, imunološki su obilježeni u svrhu određivanja njihove lokalizacije. Korišteni su transgeni *Spo11β*-only miševi, u čijim mejotičkim stanicama češće dolazi do nebipolarnog povezivanja kromosoma i mikrotubula nego kod miševa divljeg tipa. Utvrđeno je da proteini MAD2 i 3F3/2 fosfoepitop kolokaliziraju s proteinom SYCP3 u metafazi I.

Ključne riječi: kontrolna točka diobenog vretena, bipolarno povezivanje, *Spo11β*-only miševi

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Localization of spindle assembly checkpoint proteins in mouse spermatocytes

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Abstract: During meiosis, appropriate segregation of chromosomes is of crucial importance, and it is achieved via bipolar orientation of kinetochore-microtubule attachment. Spindle assembly checkpoint (SAC), through a specific SAC complex consisting of MAD1, MAD2, BUB1 and 3F3/2 phosphoepitope and several other proteins, delays anaphase onset until all chromosomes are properly attached to the spindle. In case of malfunctioning SAC, cell will enter anaphase without proper kinetochore-microtubule orientation, and improper chromosomes segregation will occur, resulting in abnormal number of chromosomes in daughter cell. In this work proteins MAD2 and 3F3/2 phosphoepitope of the SAC complex were immunostained in order to determine their localization. Due to more frequent improper mitotic kinetochore-microtubule attachment, compared to the wild type, transgenic *Spo11 β* -only mice were used. It was determined that both MAD2 and 3F3/2 phosphoepitope colocalize with SYCP3 in metaphase I.

Keywords: spindle assembly checkpoint, bipolar attachment, *Spo11 β* -only mice

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1 Introduction

Sexually reproducing organisms produce haploid sex cells called gametes via the process of meiosis. This is one of the importances of meiosis, because it allows maintaining the same chromosome number in zygote, which is a diploid cell, through fusion of haploid gametes. Proper segregation of homologous chromosomes during meiosis I and sister chromatids during meiosis II is therefore essential. This is possible only with proper attachment of chromosomes to spindle. Surveillance mechanism that monitors if the attachment is correct is called the spindle assembly checkpoint and it acts by inhibiting chromosome or sister chromatid segregation until all chromosomes are not correctly attached to microtubules. Missegregation results in chromosome abnormalities, atypical number of chromosomes. There are two kinds of chromosome number defects: aneuploidy, which is abnormal number of chromosomes in a cell, and polyploidy, which is cell containing more than two complete sets of chromosomes. Polyploid human zygotes usually do not survive inside the uterus, resulting in spontaneous abortion, while aneuploidy can result in serious mental and physical disabilities. Such disorders are, for example, Down syndrome (trisomy 21), Edwards syndrome (trisomy 18), Patau syndrome (trisomy 13), or aneuploidy of sex chromosome, such as Turner syndrome and Klinefelter syndrome and other (Bond and Chandley, 1983; Vogt et al., 2008). Considering the severity of disorders caused by chromosomal abnormalities, it is important to elucidate mechanism preventing chromosomal missegregation and reasons of their failure.

The aim of this research was to determine localization of SAC proteins, MAD2 and 3F3/2 phosphoepitope on misaligned chromosome through immunostaining protocol on mouse spermatocytes in metaphase I. In this research spermatocytes of transgenic *Spo11β*-only mice were used. During meiosis I, in 70 % of cases their X and Y sex chromosomes fail to pair, resulting in improper attachment to microtubules and missegregation (Kauppi et al., 2011), which makes it an interesting material for studying the spindle assembly checkpoint.

Work was done based on previous researches done in Chromosome Stability research group, Genome Scale Biology Research Program, Biomedicum Helsinki, University of Helsinki.

2 Theoretical Background

2.1 Cell life cycle

Cell life cycle, or cell cycle, implies series of processes in eukaryotic cell that lead to cell division and self-replication. It consists of the two gap phases, G1 and G2, a S phase and M phase. G1, G2 and S phase together are referred to as interphase. Events specific for each phase are shown in Table 1.

Table 1. Cell cycle phases and characteristic events of each phase.

G1	Cell growth and G1 checkpoint, in order to make sure everything is ready for DNA replication.
S	Cell continues to grow, DNA condensed into chromosomes replicated.
G2	Cell growth continues, G2 checkpoint controls duplicated chromosomes, makes sure if everything is ready for entering M phase.
M	Cell stops to grow, and nuclear and cytoplasmic division starts.

Cell cycle is regulated through three main cell cycle checkpoints, the G1/S checkpoint, the G2/M checkpoint and the metaphase checkpoint, all previously mentioned in Table 1. Proteins specialized for each checkpoint determine if the existing conditions are favourable for the cell to enter the next phase of cell cycle. The main checkpoint proteins are cyclins and cyclin-dependent kinases, shortened Cdk. In the case of DNA damage, G1/S AND G2/M checkpoints halt the cell cycle progression, which allows the repairment of DNA damage before entering the next phase. Protein responsible for this halt is p53. During G1 phase, in conditions of DNA damage or degradation, cell can exit G1 phase and enter resting state, G₀ phase. This phase is common for terminally differentiated cells.

Another checkpoint, the spindle checkpoint, senses improper microtubule-kinetochore attachments or failure of microtubule to kinetochore attachment. Mutations of checkpoint proteins can have severe consequences, such as cell death or uncontrolled cell division that results in cancer (Cooper, 2000; Elledge, 1996).

2.1.1 Meiosis

Meiosis is a specific type of eukaryotic cell division, in which from diploid mother cell haploid daughter cells are being produced. Before meiosis, DNA was replicated during S phase, and each chromosome contains two identical sister chromatids that are attached at the centromere

and bound together by cohesion (Ishiguro and Watanabe, 2007). After one DNA replication, in meiosis II cell divisions occur, and by that meiosis is divided into meiosis I and meiosis II. Meiosis I is the reduction division, where homologous chromosomes are separated, while sister chromatids remain attached together in centromere, and this results in production of two daughter cells with haploid chromosome set. Meiosis II is mitosis-like division of cells produced in meiosis I, where sister chromatids separate and equally distribute into daughter cells. Both, Meiosis I and Meiosis II can be divided in four stages. Meiosis I in Prophase I, Metaphase I, Anaphase I and Telophase I, and Meiosis II in Prophase II, Metaphase II, Anaphase II and Telophase II. Major events of each meiotic phase can be seen in Figure 1.

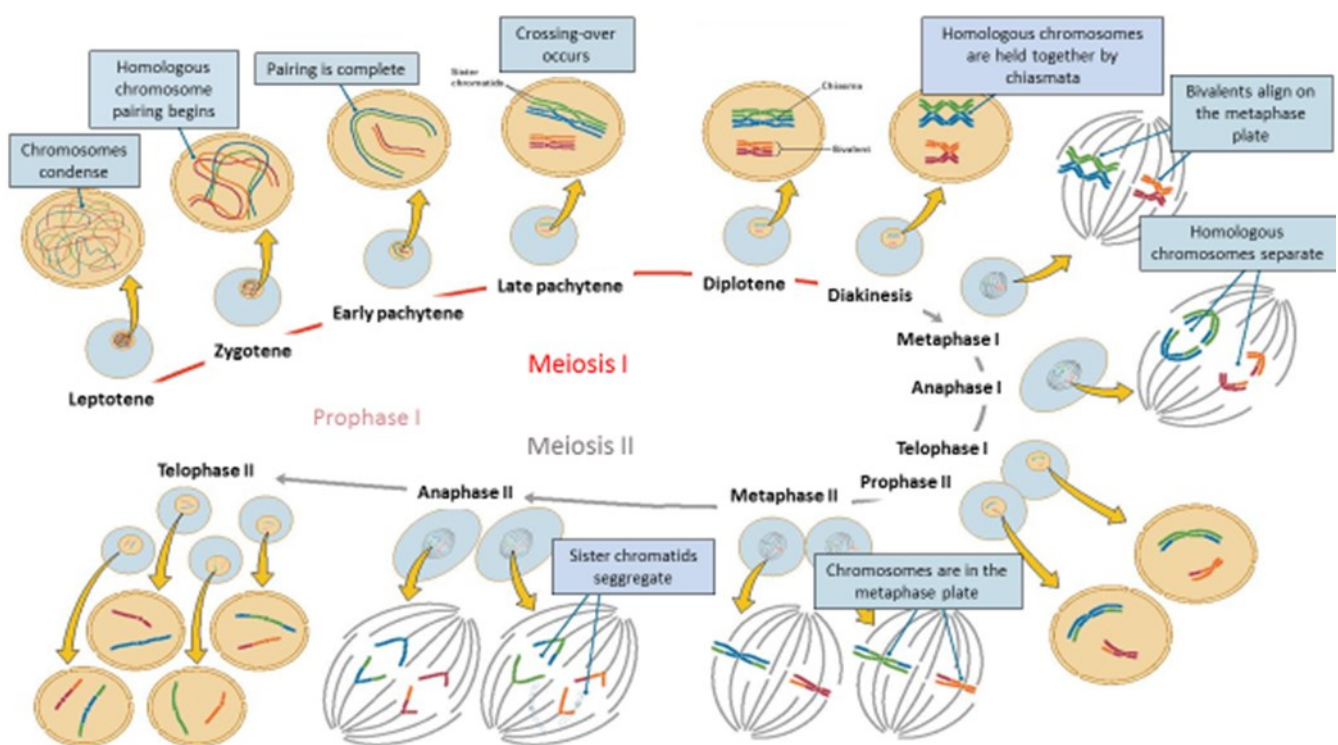


Figure 1. Meiosis phases and specific events for each phase. (Slightly modified from Ussery, 1998)

Although meiosis and mitosis are similar, meiosis differs from mitosis in two very important aspects, number of chromosomes in the end of process and genetic recombination. While in the end of mitosis from one diploid mother cell two diploid daughter cells are produced, meiosis of one diploid mother cell results in production of 4 haploid daughter cells. Prophase I is the longest stage of meiosis and of crucial importance for sexual reproduction of eukaryotes. Its duration is significantly longer than duration of mitotic prophase and it is divided into 5 stages: leptotene, zygotene, pachytene, diplotene and diakinesis. During leptotene, individual chromosomes condense. In zygotene pairing of homologous chromosomes starts and it is

known as synapsis. At that point, meiosis-specific proteinaceous structure forms between two homologous, the synaptonemal complex (SC) (Schulz-Schaeffer, 1980). The structure of the SC consists of three major proteinaceous structures, two axial (AE) or lateral elements (LE), a central element which is located between the lateral elements, and the transverse filaments that are the link between the lateral elements (Heyting, 1996). The meiosis specific proteins SYC2 and SYCP3 and cohesion complex make the most of LEs (Dobson et al., 1994; Lammers et al., 1994; Offenberg et al., 1998; Winkel et al., 2009). In the pachytene, as pairing of homologous is completed, bivalents are formed.

The major event occurring in pachytene is the crossing over, equal exchange of genetic material between chromatids of homologous chromosomes and each chromosome pair undergoes at least one crossover (Schulz-Schaeffer, 1980; Jones, 1984). In order for crossing over to be achieved, programmed-double strand breaks need to happen. Protein that catalyzes this double-strand breaks is the Spo11 (Keeney et al., 1997).

In the diplotene, synaptonemal complex is being degraded and the bivalents remain attached in the site of chiasmata (Schulz-Schaeffer, 1980). After the degradation of the SC, SYCP3 remains localized only at centromeres of chromosomes (Bisig et al., 2012). In diakinesis, stage of late prophase I, chromosomes condense more and spindle microtubules attach to kinetochores of homologous chromosomes, which stay in form of bivalents due to connection between them in chiasmata (Schulz-Schaeffer, 1980).

Great importance of meiosis lies in its production of genetically diverse cells. Diversity is a result of three events during meiosis: genetic recombination during prophase I, independent segregation of homologues during anaphase I and independent segregation of sister chromatids during anaphase II (Pittman and Schimenti, 1998). Independent segregation of homologous and sister chromatids was first proposed by Mendel in “Law of Segregation” and “Law of Independent Assortment”. Cells produced with meiosis undergo further differentiation processes and develop into gametes: sperm or ova.

2.2 Kinetochores structure and function

The kinetochore is a highly complex protein structure essential for proper chromosome segregation during mitosis and meiosis. Kinetochores of each chromosome are assembled on opposite sides of the centromere and they are responsible for interactions between the

chromosome and microtubules (MTs) during cell division. By electron microscopy, kinetochore appears as a trilaminar structure.

The deepest layer is the inner plate, localized next to centromere, containing in its structure centromeric chromatin, which is persistent all throughout the cell cycle, DNA-binding proteins, such as CENP-A AND CENP-B, and structural proteins (Maiato et al., 2004). Outside the inner plate, there is the outer plate, which together with the fibrous corona is considered the "outer domain". The outer domain consists mostly of known MT-interacting proteins and checkpoint proteins that are crucial for proper kinetochore-MT attachments. The outer plate contains microtubule binding sites (Fukagawa and Wulf, 2009). In the fibrous corona motor proteins CENP-E and cytoplasmic dynein are localized and are responsible for the movement of chromosomes during cell division.

For accurate chromosome segregation in mitosis bi-orientation of sister-kinetochores before anaphase onset is essential, which means that sister-kinetochores are attached to microtubules from opposite spindle poles. Bi-orientation is also known as amphitelic attachment. There are three other modes of kinetochore-microtubule attachments, monotelic, syntelic and merotelic attachment shown in Figure 2.

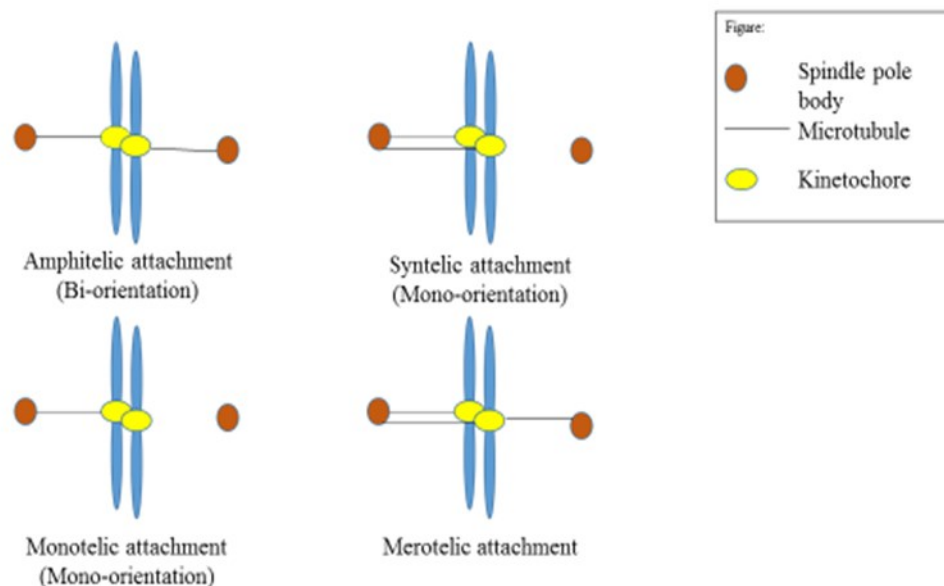


Figure 2. Types of kinetochore-microtubule attachments in mitosis (figure based on figure from Logarino et al., 2004).

When microtubule-kinetochore attachment is achieved, movement of microtubules guides chromosomes towards the spindle equator. If attachment was correct, this results in alignment of chromosomes into equatorial plate during metaphase (Maiato et al., 2004). In meiosis I, unlike in mitosis, homologous chromosomes separate but sister chromatids stay attached together in centromere. Meiotic recombination between bivalents is essential for accurate separation of homologous chromosomes. When meiotic recombination is absent, missegregation of homologous occurs, which results in aneuploidy of gametes.

After meiotic recombination, the bond between homologs in chiasmata is preserved due to cohesion complex in the chromosome arms. This enables their attachment to meiosis I spindle apparatus, in such manner that each homologue from a pair is attached to microtubules from an opposite spindle pole and the cohesion rings along non chromosome arms prevents too early segregation of homologs. When attached correctly, tension caused by pulling forces from microtubules from opposite spindle poles, is generated in chiasmata of homologues. Tension in chiasmata is a signal that homologous are bi-oriented and when it is applied, anaphase II onset in meiosis II occurs. Cleavage of cohesin at chromosome arms allows homologs to segregate (Buonomo et al., 2000).

2.3 Spindle assembly checkpoint (SAC)

During mitosis and meiosis equal partition of previously duplicated chromosomes into daughter cells is crucial. It can be achieved only in case of bi-orientation of homologous in meiosis I and bi-orientation of sister chromatids in meiosis II and mitosis. In case of improper attachment, special mechanism prevents anaphase onset until bi-orientation is not achieved, which allows cell to correct improper attachments. This mechanism is called spindle assembly checkpoint or mitotic checkpoint.

Genetic screens in *Saccharomyces cerevisiae* revealed main components of SAC, that include MAD (mitotic-arrest deficient) and BUB (budding uninhibited by benzimidazole) proteins. MAD genes are MAD1, MAD2 and MAD3 (BUBR1 in humans) and BUB gene BUB1 (Hoyt, 1991; Li and Murray 1991). Proteins encoded by these genes are conserved in all eukaryotes (Chen, 1996) and recruit to kinetochores in hierarchical manner (Funabiki et al., 2013).

However, the exact mechanisms of these proteins recruitment to kinetochores remains unknown, but it has been discovered that phosphorylation plays an essential role in checkpoint. From numerous kinases effecting the checkpoint, kinetochore kinase monopolar spindle 1

(MPS1) was shown to have the biggest effect on the checkpoint. Another kinase with an important role in the checkpoint, but also in the error correction, is the Aurora B kinase (Funabiki et al., 2013; Foley et al., 2012; Vader et al., 2008). The platform for SAC signalling is the KMN network, which is composed of kinetochore null protein (KNL1), miss-segregation 12 (MIS12) and the nuclear division cycle 80 (NDC80) network. The KMN network is localized at the kinetochore and is crucial for microtubule binding (Cheeseman et al., 2006; Foley et al., 2012; Varma et al., 2013). To ensure proper distribution of genetic material it is important that sister chromatids do not separate too early, before spindle checkpoint does not take place. This is ensured with cohesin complex, that holds sister chromatids together after synthesis (Nasmyth et al., 2000; van Heemst and Heyting, 2000).

In meiosis, when bi-orientation is achieved, tension is accumulated in chiasmata of two homologues. Tension in chiasmata is signal that proper attachment of chromosomes to spindle apparatus is achieved. This silences SAC and activates anaphase-promoting complex/cyclosome (APC/C), which allows cell to entry anaphase (Musacchio and Salmon, 2007). This is achieved through ubiquitylation and degradation of cyclin B (Glotzer et al., 1991) (kinase that regulates mitotic progression) and securin (Yamamoto, 1996).

Cyclin B is the main kinase that regulates the mitotic progression and securin is an inhibitor of enzyme that proteolytically degrades the cohesion complex, separase (Cohen-Fix et al., 1996; Funabiki et al., 1996; Holloway et al., 1993; King, 1995). In case of unattached or misaligned chromosome, SAC is triggered. Target of SAC is cell division control protein 20 (CDC20), co-activator of APC/C. When activated, SAC inactivates CDC20, which results in inhibition of APC/C and inhibition of securin degradation. This will disable anaphase entry (Li et al., 1997; Tang et al., 1998). The key step is formation of the mitotic checkpoint complex (MCC), heterotetramer formed through complex of CDC20 with MAD2, BUB1-related protein and BUB3 (Sudakin, 2001). To enable recruitment of all SAC components to the kinetochores, MPS1 kinase is required. MPS1 phosphorylates KNL1, which enables recruitment of BUB1 and BUB3 to the kinetochores (London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012). This allows recruitment of BUB3, BUBR1 (Rischitor et al., 2007; Vanoosthuyse et al., 2004) and MAD1 but the molecular mechanism of its recruitment is unknown.

Another protein that acts upstream in the SAC is the Aurora B, and it controls localization of other SAC proteins, which makes Aurora B and MPS1 proteins on top of the hierarchy of checkpoint proteins recruitment. In hierarchical recruitment, Aurora B and MPS1 are followed by BUB1-BUB3 complex, and afterwards by BUBR1-BUB3. On bottom of hierarchy is a

MAD1 and MAD2 heterotetramer (Yamagishi et al., 2012; Vigneron et al., 2004; Howell et al., 2004; Chen, 2002; Gillet, 2004; Heinrich et al., 2012; Sharp-Baker and Chen, 2001). For onset of mitotic progression, SAC needs to be silenced through dissociation of MCC, which is achieved by halting the MAD2 conversion due to loss of MAD1-MAD2 complex from the kinetochores. This is explained through various mechanism, which differ between organisms (Howell, 2001; Barisic, 2011; Gassmann et al., 2008; Gassmann et al., 2010). The silencing mechanism which is the most conserved is the one that includes the phosphatase activity of protein phosphatase 1 (Pinsky et al., 2009), whose localization is enabled through KNL1 (Rosenberg, 2011). Through its phosphatase activity, PP1 removes BUB1-BUB3 localized at kinetochores and that causes removal of MAD1-MAD2 as well. Another key role in the SAC silencing might have MPS1, since, after start of anaphase, degradation of MPS1 occurs which prevents checkpoint reactivation (Jelluma, 2010).

2.3.1 MAD protein recruitment

It has been established that each component in SAC is required in order to enable recruitment of proteins that act downstream from the previous one, but not all SAC proteins localized at kinetochores mean that SAC has been activated. The essential step in SAC activation is recruitment of the MAD1-MAD2 heterotetramer to the kinetochores (Kujit et al., 2014; Maldonado et al., 2011).

As previously mentioned, BUB1, acts upstream from MAD1-MAD2 and therefore is needed MAD1 localization to the kinetochore. The deterministic step in recruitment of BUB1, which allows also recruitment of MAD1, is phosphorylation mediated by MPS1. This makes MPS1 direct controller of the SAC activation (London and Biggins, 2014). The MAD2 protein exists in two conformations, the "open" form (O-MAD2) and the 'closed' form (C-MAD2) and the change of conformation from O-MAD2 to C-MAD2 is the key step in formation of MCC.

Interaction between MAD1 and BUB1 enables binding of C-MAD2 to the kinetochores, since MAD1 is the receptor for C-MAD2, forming a MAD1-MAD2 heterotetramer (Luo et al., 2008; Skinner et al., 2008). MAD1-MAD2 complex binds O-MAD2 from mitotic cytosol, which causes conversion of O-MAD2 to C-MAD2. C-MAD2 is the active conformation of MAD2 which can bind to Cdc20. This is known as 'MAD2 template' model (De Antoni et al., 2005; Vink et al., 2006).

Conversion of O-MAD2 to C-MAD2 is the deterministic step in activating the checkpoint. This was proven by tethering of C-MAD2 to kinetochores which resulted in checkpoint activation

(Kruse et al., 2014). This proves that MAD2 is in the bottom of SAC protein recruitment hierarchy and that BUB1-MAD1 interaction is essential for activation of MAD2.

2.3.2 Kinetochore 3F3/2 phosphoepitope

Bi-polar orientation is the only kind of MT-kinetochore attachment that allows proper chromosome segregation (Gonzalez et al., 1991). When it is applied, bi-polar orientation causes tension, which can be sensed by kinetochores. This was tested by micromanipulation experiments, done by Nicklas and Koch (1969), in meiosis I spermatocytes of insects. An experiment, which included applying tension to an unpaired X chromosome, showed that such condition forces anaphase entry of cell with improper chromosome segregation (Li and Nicklas, 1995).

The SAC proteins sense two types of irregularities, the lack of tension and improper MT-kinetochore attachment. The 3F3/2 phosphoepitope has shown to be present on unattached kinetochores and senses the lack of tension caused by incorrect MT-kinetochore attachment. This phosphoepitope is recognized by 3F3/2 monoclonal antibody and can be detected when immunofluorescence protocol is done in presence of phosphatase inhibitors (Gorbsky and Ricketts, 1993; Nicklas et al., 1998; Nicklas et al., 1995; Campbell and Gorbsky, 1995; Cyert et al., 1988). Staining with the 3F3/2 antibody showed that the 3F3/2 phosphoepitope is localized at the kinetochores of chromosomes that lack tension. When chromosomes are bi-oriented, the 3F3/2 phosphoepitope is not present at the kinetochores, but if metaphase cells are treated with microtubule drugs, like nocodazole or taxol, it appears at all kinetochores (Nicklas et al., 1995; Gorbsky and Ricketts, 1993).

These findings show that presence of the 3F3/2 phosphoepitope is regulated by tension forces at the kinetochores. Kinase that creates the 3F3/2 phosphoepitope regarding lack of tension present at the kinetochores is the Plk1 kinase (Ahonen et al., 2005). Staining of *Drosophila* and mammalian cells, showed that the 3F3/2 monoclonal antibody labels the centromere and centrosome and appears at prophase. With anaphase onset, labelling for 3F3/2 distinguishes (Sunkel, 1997; Gorbsky, unpublished results, Gorbsky and Ricketts, 1993).

2.4 *Spo11* gene

SPO11 is meiosis-specific protein, essential for meiotic recombination. During meiotic prophase I, it induces double strand breaks in the DNA, thereby initiating meiotic

recombination between homologous chromosomes. The *Spo11* gene encodes for Spo11 α and Spo11 β , which have different expression timing during prophase I.

Spo11 β -only mice have *Spo11 α* variant missing. Meiosis in female *Spo11 β* - only progresses normally, unlike in males. In *Spo11 β* -only male mice, approximately 70 % of spermatocytes X and Y chromosomes fail to pair and form chiasmata (Kauppi et al., 2011). Therefore, it is likely that efficient meiotic recombination between sex chromosomes, the X and the Y chromosome in wild type male mice, requires activity of SPO11 α .

3 Materials and Methods

For studies of localization of spindle assembly checkpoint proteins spermatocytes of transgenic *Spo11 β* - only and wild-type mice were used.

3.1 Mice used in the research

Spindle assembly checkpoint proteins were observed in mouse spermatocytes in metaphase I. Before being able to determine any localization, it was necessary to conduct optimization of several existing protocols. Optimization experiments were done on wild type mouse cells, minimum 3 weeks of age. Detection of spindle assembly checkpoint proteins by immunofluorescence, which was done after performing all required optimizations, was performed on spermatocytes of transgenic *Spo11 β* -only mice.

3.2 Testes dissection

All protocols performed in this research were done on chromosome spreads or chromosome squashes. In order to prepare chromosome spreads and squashes, male mice were sacrificed and testes were dissected. Wild type and *Spo11 β* - only mice, both minimum 3 weeks old, were used.

Mice were sacrificed using carbon dioxide, followed by cervical dislocation. Incisions were made in the abdominal area and fat pads were located. By pulling on the fat pads, testes could be visualized, and isolated by cutting them off from the fat pads. Testes were placed in a Falcon tube containing Minimum Essential Media supplemented with Complete EDTA-free protease inhibitor, and stored on ice. In order to avoid protein degradation, storing on ice should be as short as possible, before performing further steps of the protocol.

3.3 Preparation of chromosome spreads

The preparation of chromosome spreads was done in order to be able to perform immunofluorescence protocols, which enable SAC proteins to be visualized. Reagents needed are listed in Table 2.

Table 2. List of used substances and concentrations for Chromosome Spread Preparation protocol.

Reagent	Concentration
sucrose	0,1 M
Paraformaldehyde (pH 9,2)	1 %
Minimum Essential Media (MEM)	50 mL
Protease inhibitor tablet (Roche)	1 tablet per 50 mL of MEM
Triton-X	0.1 %
Ilfotol (Ilford)	0.4 %

Mouse was sacrificed, testes were dissected, and stored in Falcon tube, in MEM containing Complete EDTA-free protease inhibitor tablet. In a dry weigh boat, testes's tunica albuginea was removed and after that testes were chopped thoroughly with a flat razor blade. In order to collect the cells, MEM containing protease inhibitor tablet was added into weigh boat and the cell suspension was transferred into a Falcon tube. The weigh boat was rinsed with 0.5 mL more MEM in order to collect remaining cells in the same Falcon tube, which was then inverted 10 times by hand and placed on ice to allow for large tissue pieces to sediment. Supernatant contained wanted spermatocytes and it was pipetted in volume of 1 mL into each Eppendorf tube of 1,5 mL volume, which were then centrifuged for 5 minutes at 5800x G. During the centrifugation, 65 μ L of 1 % paraformaldehyde (containing 0.1 % Triton-X 100) was applied onto each glass slide and was evenly distributed.

After centrifugation, the supernatant was removed and spermatocyte pellet could be seen at the bottom of the Eppendorf tube. Each pellet was resuspended with a pipette in 40 μ L of 0,1 M sucrose. Prepared suspension of sucrose and spermatocytes was applied onto slides, 20 μ L per slide. Slides were placed in a humid chamber and incubated at room temperature for 2-3 hours.

When incubation was done, slides were washed once in wetting agent Ilfotol (Ilford) and afterwards were additionally rinsed, also using Ilfotol, with a transfer pipette, aiming above any visible clumps of cells. Slides were afterwards air dried and stored at -80°C.

3.4 Squash protocol

As well as chromosome spread, another chromosome preparation is the squash method. Unlike chromosome spread, with squash method 3D structure of nucleus is preserved, which is often needed for research. One wild-type testis is sufficient for preparation of 60 squash slides. This

protocol is for method for one testis. Dissected testes were rinsed with 1x PBS and then decapsulated. In a weigh boat on ice, tubules from testis were minced and afterwards collected into a Falcon tube. Cell suspension was centrifuged at 1000 rpm for few minutes. Supernatant was discarded and cell pellets were resuspended in 2 % PFA/ 0.05 % Triton-X/1xPBS. 5 μ L of cell suspension was applied onto previously ethanol-cleaned slide and coverslip was added.

After cover slips were lightly pressed, slides were frozen by immersing them in liquid nitrogen for 1 minute. Cover slips were removed and 3 washes, each per 5 minutes, were performed in 1xPBS. Slides were afterwards rinsed with distilled water, and then with 4 % PhotoFlo. Slides were then air dried and placed in -80°C freezer for long-term storage.

3.5 Immunofluorescence

3.5.1 Immunoincubation protocol

For immunoincubation, B/ABD buffer was used as a blocking buffer and it was prepared by dissolving following reagents in 1x PBS. The list of reagents is found in Table 3.

Table 3. B/ABD buffer recipe for immunoincubation protocol (reagents are dissolved in 1x PBS).

Reagent	Concentration
BSA	0,2 %
Gelatin	0,2 %
Tween 20	0,05 %

Slides were blocked in a Coplin jar in B/ABD buffer for 30 to 45 minutes. Afterwards, primary antibodies were diluted in B/ABD buffer and 100 μ L of such dilution was applied per slide. Slides were covered with a piece of parafilm and stored in a humid chamber in a cold room, at +4 °C over night. The next day, the piece of parafilm was removed and 4 washes in a Coplin jar containing B/ABD buffer were performed. Each wash was done for a duration of 5 minutes. Secondary antibodies were diluted in B/ABD buffer and 100 μ L of the dilution was applied per slide, which was then covered with a piece of parafilm and incubated in a humid chamber at +37 °C for 1 hour. After incubation, 4 washes of 5 minutes each in B/ABD buffer were performed. Slides were drip-dried, 10 μ L of Vectashield containing 5 μ g mL⁻¹ DAPI was added and slides were then covered with a cover slip, which was then attached to the slide using clear nail polish. In Table 4 the primary antibodies used and their dilutions are listed. To detect primary antibodies, secondary antibodies with Alexa Fluor dyes (Life technologies) were used.

Table 4. Primary antibodies and their dilutions.

Antibody	Company	Dilution
Mouse SYCP3	Santa Cruz	1:500
Goat SYPC3	Santa Cruz	1:500
Rabbit SYCP3	Abcam	1:1000
Goat SYCP1		1:400
Rabbit H3Ser10		1:500
Mouse MAD2	Abcam	1:500
Rabbit MAD2		1:100
Rabbit BUB1	Abcam	1:200
Mouse gamma-tubulin		1:500
Mouse 3F3/2 phosphoepitope		1:3000

3.5.2 Immunofluorescence for 3F3/2 phosphoepitope

Staining for 3F3/2 phosphoepitope required a different protocol. Instead of B/ABD buffer, 1x MBS containing 0.2 % BSA was used as a blocking solution. Reagents needed to prepare this blocking solution can be found in Table 5.

Table 5. Reagents used for preparing the blocking solution.

Substance	Concentration
MOPS	10mM
NaCl	150mM
BSA	0.2 %

Before continuing with the protocol, pH of the solution was adjusted to 7.3. The blocking solution was boiled for 10 minutes and afterwards Phosphostop was added, and solution was stirred and sterile-filtered. Per 10 mL of blocking solution, 1 tablet of Phosphostop was added. Slides were blocked in blocking solution, and antibodies were diluted in it as well. After applying antibodies, the slides were incubated in a humid chamber in a cold room overnight at +4°C. The next day, three washes of 10 minutes each in 1x MBS containing 0.05 % Tween-20. Secondary antibodies and further procedures were performed the same way as in the immunoincubation protocol described above.

3.6 Imaging

Microscopy was performed on Zeiss Axio Imager.Z2 upright epifluorescence microscope at the Biomedicum Imaging Unit, University of Helsinki.

3.7 Image processing and analysis

Acquired images of metaphase I cells were processed in Fiji software, image processing package.

4 Results and Discussion

Aim of this experiment is to determine localization of MAD2 and 3F3/2 phosphoepitope proteins in case of improper microtubule to kinetochores attachment. This can be examined via immunostaining protocol for named proteins and the kinetochore protein SYCP3.

Primary plan was to develop common staining protocol for 3F3/2 phosphoepitope and MAD2 that will enable visualizing both proteins together with SYCP3 on same slide. This included experiments with different incubation times and different blocking solution. Since all experiments ended with unsatisfying results, due to high backgrounds and low visibility of protein signal, main experiment in the end was done by separate staining for MAD2 and 3F3/2 phosphoepitope on two separated slides.

To be able to observe unusual protein signals on slide containing *Spo11 β* -only spermatocytes, control was stained as well, containing wild type mouse spermatocytes and both results were compared. For this experiment, metaphase I cells were studied and it was important to be able to differentiate stages of meiosis. It is known that in metaphase all chromosomes are aligned in equatorial line, but if the equatorial line will be seen as a plate depends from which angle the cell is being observed. In order to be able to recognize metaphase cells no matter from which angle the cell is being observed, separate immunofluorescence experiment for SYCP3 and α -Tubulin was done, based on knowledge that on chromosomes aligned in equatorial line, microtubule-kinetochore attachments are achieved Following Figure 3 shows spermatocytes in metaphase.

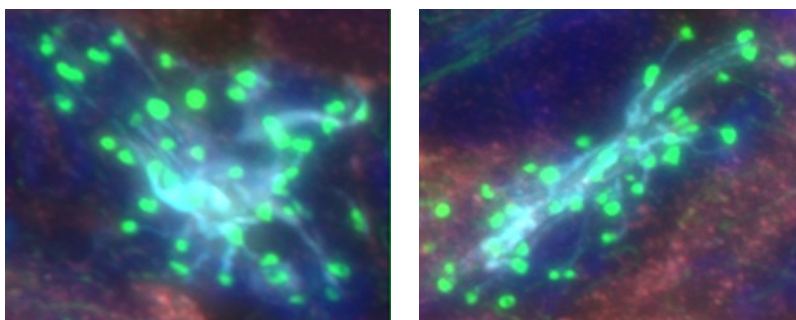


Figure 3. Metaphase I cells stained for SYCP3 (green channel) and α -Tubulin (cyan channel).

In this images characteristic shape and size of kinetochores stained for SYCP3 in metaphase I can be recognized, and microtubules attached to them.

4.1 3F3/2 phosphoepitope and SYCP3 staining

In Figure 4 control cell stained for 3F3/2 phosphoepitope (green channel), SYCP3 (red channel) and DAPI (blue channel) is shown.

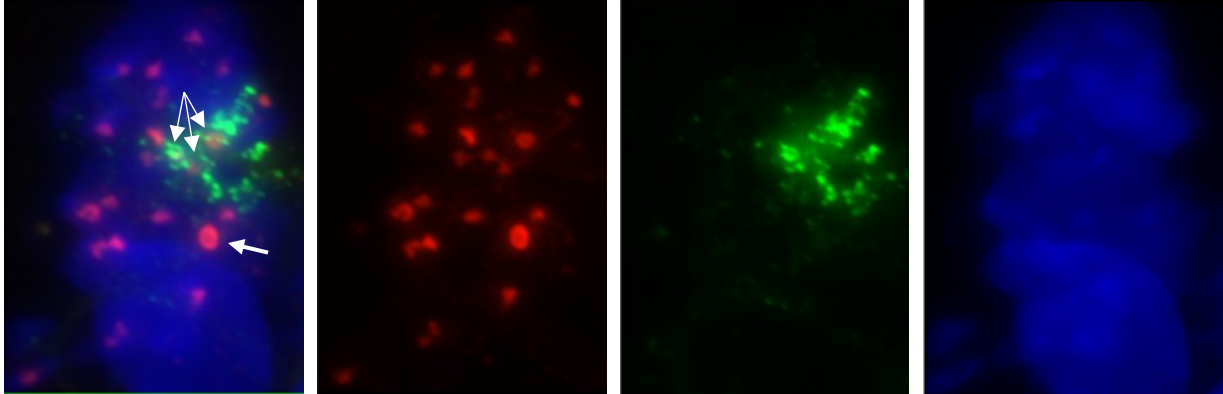


Figure 4. Control cell stained for 3F3/2 phosphoepitope (green channel), SYCP3 (red channel) and DAPI (blue channel). First image shows all three channels merged together.

Examining obtained signals in green channel, area of big dirt is observed, and in that area signals cannot be considered as genuine. In red channel, few signals are presented as large circular dots, which does not resemble signal given by SYCP3. Signals not considered as genuine are marked with an arrow.

Figure 5. shows *Spo11 β* -only cell stained for 3F3/2 phosphoepitope (green channel), SYCP3 (red channel) and DAPI (blue channel).

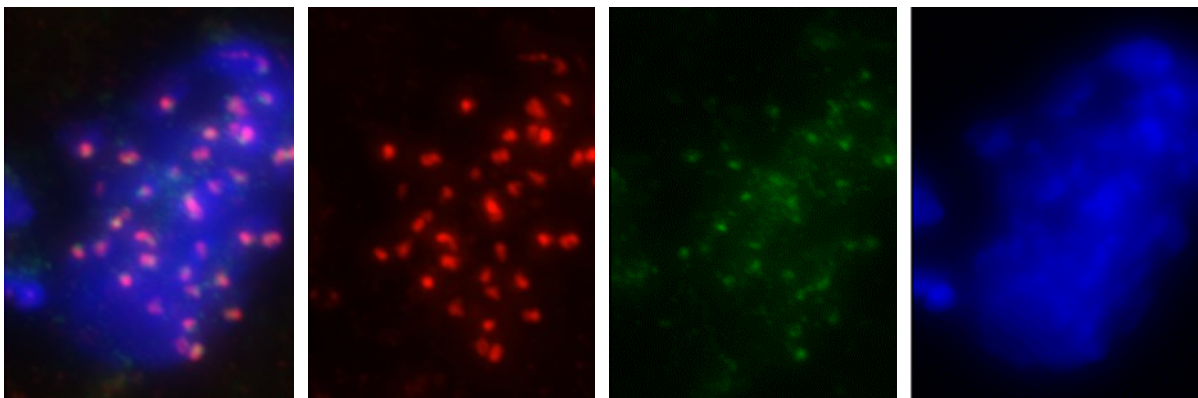


Figure 5. *Spo11 β* -only cell stained for 3F3/2 phosphoepitope (green channel), SYCP3 (red channel) and DAPI (blue channel). First image shows all three channels merged together.

In first *Spo11 β* -only cell, all the provided signals look genuine. There is a minor background when looking at green channel, but the background is distinguishable from signal given by proteins. Also, in image where all channels are merged the green background is noticeable. Due to this, all given protein signals can be taken in consideration as genuine.

Figure 6 shows another *Spo11β*-only cell in metaphase I stained for 3F3/2 phosphoepitope (green channel), SYCP3 (red channel) and DAPI (blue channel).

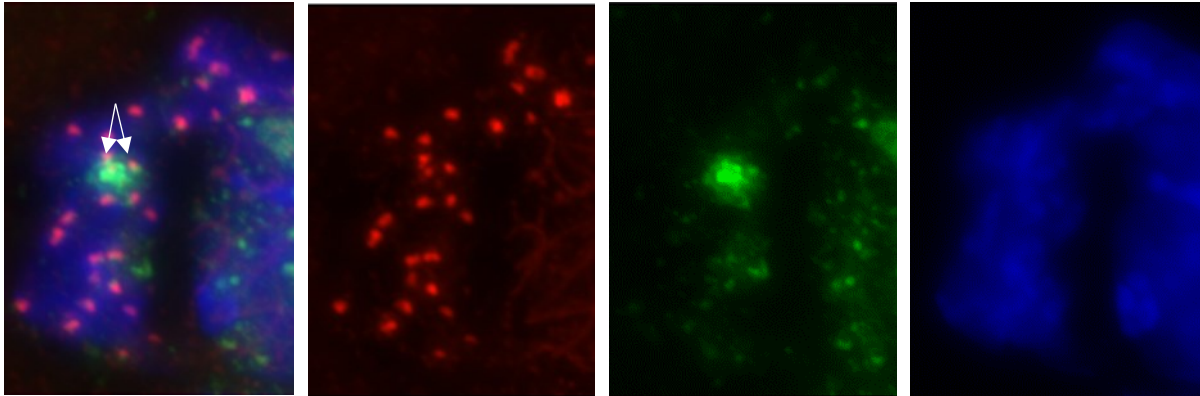


Figure 6. *Spo11β*-only cell stained for 3F3/2 phosphoepitope (green channel), SYCP3 (red channel) and DAPI (blue channel).

There is of a very strong signal in green channel, which is clearly an area of high background, so the signals in this part can be ignored. Other signals appear normal and their quantified values as average.

Comparing obtained signals from all three cells, it can be observed that protein signals in green channel, MAD2 signal, suit location of SYCP3 signal and therefore, it can be concluded that MAD2 colocalizes with SYCP3.

4.2 MAD2 quantification

In Figure 7 control cell stained for MAD2 (green channel), SYCP3 (red channel) and DAPI (blue channel) can be seen.

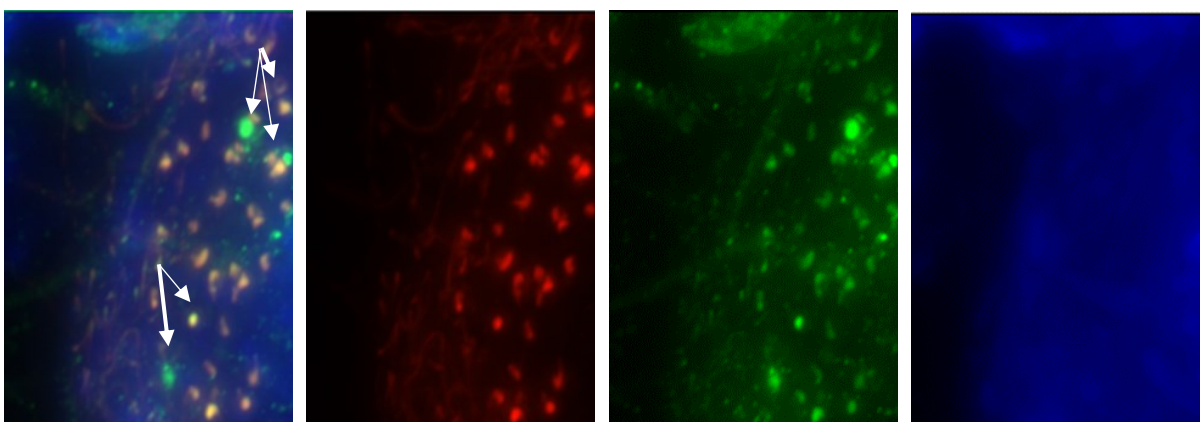


Figure 7. Control cell stained for MAD2 (green channel), SYCP3 (red channel) and DAPI (blue channel). First image shows all three channels merged together.

Examining green channel, a few signals do not appear as genuine and therefore can be ignored. In red channel no background signal is observed. Signals that are not genuine are marked with an arrow.

In figure 8 *Spo11β*-only cell stained for MAD2 (green channel), SYCP3 (red channel) and DAPI (blue channel) can be seen.

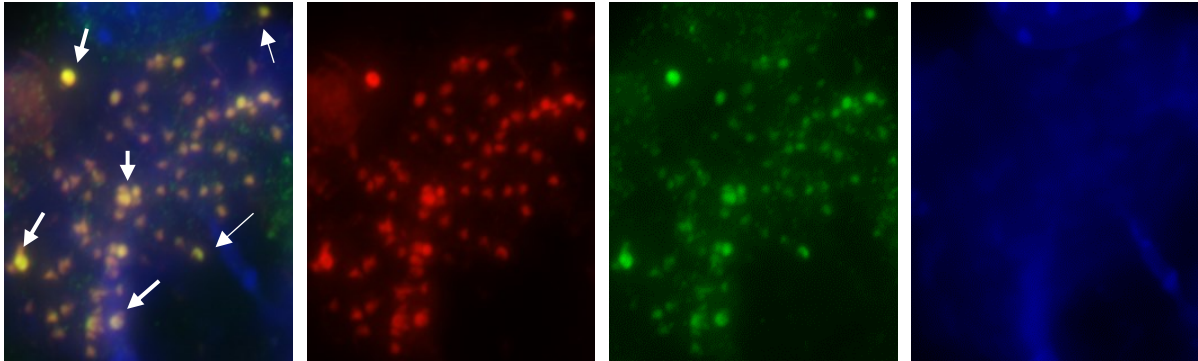


Figure 8. *Spo11β*-only cell stained for MAD2 (green channel), SYCP3 (red channel) and DAPI (blue channel). First image shows all three channels merged together.

Based on number of chromosomes in this one image, it can be concluded that those are two metaphase I cells next to each other. Signals marked with an arrow give circular, uncharacteristic shape for stained protein, and it is concluded those signals are not genuine, and therefore are ignored.

Observing signals provided from SYCP3 and MAD2, it can be recognized the signal positions match and that SYCP3 and MAD2 are colocalized in metaphase I.

5 Conclusion

Based on acquired results, following can be concluded:

1. Immunostaining protocol for MAD2 was successfully modified and enabled visualization of MAD2 protein.
2. MAD2 colocalizes with SYCP3 in metaphase I.
3. It can be also observed that 3F3/2 phosphoepitope colocalizes with SYCP3 in metaphase I.

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