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RESEARCH ARTICLE

Reduced MAD2 levels dampen the apoptotic response to non-exchange sex chromosomes and lead to sperm aneuploidy

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ABSTRACT

In meiosis, non-exchange homologous chromosomes are at risk for mis-segregation and should be monitored by the spindle assembly checkpoint (SAC) to avoid formation of aneuploid gametes. Sex chromosome mis-segregation is particularly common and can lead to sterility or to aneuploid offspring (e.g. individuals with Turner or Klinefelter syndrome). Despite major implications for health and reproduction, modifiers of meiotic SAC robustness and the subsequent apoptotic response in male mammals remain obscure. Levels of SAC proteins, e.g. MAD2, are crucial for normal checkpoint function in many experimental systems, but surprisingly, apparently not in male meiosis, as indicated by the lack of chromosome segregation defects reported earlier in *Mad2*$^{+/-}$ spermatocytes. To directly test whether MAD2 levels impact the meiotic response to mis-segregating chromosomes, we used *Spo11*β-only^{mb} mice that are prone to non-exchange X-Y chromosomes. We show that reduced MAD2 levels attenuate the apoptotic response to mis-segregating sex chromosomes and allow the formation of aneuploid sperm. These findings demonstrate that SAC protein levels are crucial for the efficient elimination of aberrant spermatocytes.

KEY WORDS: Apoptosis, Aneuploidy, Sex chromosomes, Meiosis, Spindle assembly checkpoint, MAD2, Mouse

INTRODUCTION

In order to generate haploid gametes (ova and sperm in mammals), replicated chromosomes undergo two rounds of cell division: meiosis I and meiosis II. In preparation for the first meiotic division, homologous chromosomes (homologs) pair and recombine, align at the metaphase plate and then segregate into two daughter cells. At least one crossover (reciprocal exchange between homologs) is formed per chromosome in meiosis I. Crossovers then mature into chiasmata. These physical connections between each homolog pair are essential for correct homolog alignment at metaphase, and for the fidelity of chromosome segregation. Incorrect homolog segregation results in abnormal chromosome number, i.e. aneuploidy, in the daughter cells.

Homologs without a crossover (non-exchange chromosomes) are at risk for mis-segregation. Non-exchange chromosomes should be monitored by the spindle assembly checkpoint (SAC), an evolutionarily conserved signaling cascade that delays anaphase onset in cells that contain chromosomes not properly aligned on the metaphase plate. First identified in mitotic cells (Hoyt et al., 1991; Li and Murray, 1991), the SAC was shown subsequently to be also operational in meiotic cells (Shonn et al., 2000). In meiosis, the SAC can remain activated in the presence of kinetochores that are unoccupied by microtubules, or if chromosome pairs lack inter-homolog tension due to either mono-oriented or achiasmate homologs (Nicklas et al., 2001). The SAC-dependent delay allows attempts for error correction; in budding yeast this delay is dependent on Mad3 (BubR1 in mammals) as the 'meiotic timer', and on Mad1 and Mad2 via their role in non-exchange chromosome surveillance (Cheslock et al., 2005; Tsuchiya et al., 2011). Persistent SAC activation in mammalian spermatocytes typically leads to an apoptotic response and to elimination of the cell(s) where proper homolog alignment was not achieved; this has been best demonstrated in *Mlh1*$^{-/-}$ mice (Eaker et al., 2002), where MLH1, a central crossover-associated protein (Hunter and Borts, 1997), has been knocked out and in which, consequently, nearly all chromosomes are achiasmate (Baker et al., 1996; Edelmann et al., 1996). *Mlh1*$^{-/-}$ spermatocytes face elimination by the SAC at meiosis I (Eaker et al., 2002); however, the genetic requirements for this response remain unexplored. Depending on what fraction of spermatocytes experience homolog alignment problems, this apoptotic response may or may not lead to infertility (Sutcliffe et al., 1991; Davisson and Akeson, 1993; Eaker et al., 2001, 2002; Barchi et al., 2008; see Faisal and Kauppi, 2016 for a summary).

Some 10-30% of fertilized human oocytes are aneuploid, but the vast majority of them will not produce viable embryos (Hassold and Hunt, 2001). The first-ever description of an aneuploid individual was that of an XXY male (Jacobs and Strong, 1959). Indeed, we now know that sex chromosome aneuploidies are among the most common chromosomal abnormalities in live-born individuals and are accompanied by clinical phenotypes, most notably Turner and Klinefelter syndromes (45, X and 47, XXY karyotypes, respectively; reviewed by Hall et al., 2006). Half of XXY trisomies originate from paternal meiosis I (Hall et al., 2006; MacDonald et al., 1994; Thomas and Hassold, 2003), and most of these are caused by crossover failure between the X and Y chromosomes (Hassold et al., 1991; Thomas and Hassold, 2003).

In males, sex chromosome recombination is particularly challenging due to the fact that it is restricted to a small region of sequence homology between the X and Y chromosomes, called the pseudo-autosomal region (PAR) (Simmler et al., 1985; Perry et al., 2001). For successful PAR recombination, a specific isoform of the meiotic double-strand break catalyst SPO11 (Baudat et al., 2000; Keeney et al., 1997) is needed (Kauppi et al., 2011). Of its two main isoforms, SPO11α and SPO11β (Keeney et al., 1997, 1999; Romanienko and Camerini-Otero, 1999; Bellani et al., 2010), the former is dispensable for autosomal double-strand break formation and recombination but is required for efficient X-Y recombination (Kauppi et al., 2011, 2012). Depending on the genetic background, between 30 and 50% of spermatocytes of mice lacking SPO11α

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(*Spo11*β-only mice) accomplish PAR pairing and recombination (Kauppi et al., 2011; Faisal and Kauppi, 2016). We have shown previously that *Spo11*β-only males on a mixed genetic background (*Spo11*β-only^{mb}), despite having 50% of pachytene spermatocytes with X-Y pairing failure, are able to maintain sperm production and fertility (Faisal and Kauppi, 2016). Nevertheless, we also observed many apoptotic metaphase I cells in *Spo11*β-only^{mb} testes, suggesting that spermatocytes with non-exchange X-Y chromosomes trigger a SAC response and, consequently, are eliminated (Faisal and Kauppi, 2016). Thus, this presumably SAC-mediated quality control helps to eliminate spermatocytes with achiasmate (non-exchange) chromosomes at metaphase I, similar to observations made by Eaker and colleagues in a mouse model with heterozygous Robertsonian translocations (Eaker et al., 2001).

MAD2 (encoded by the *Mad2l1* gene, henceforth referred to simply as *Mad2*) is a highly conserved component of the SAC machinery. It functions in both mitosis and meiosis (Lara-Gonzalez et al., 2012; Sun and Kim, 2012), and localizes to kinetochores in mouse spermatocytes (Kallio et al., 2000). In mammals, its complete loss (i.e. *Mad2*$^{-/-}$) results in embryonic lethality (Dobles et al., 2000). Although *Mad2*$^{+/-}$ mice are viable, produce litters and only develop tumors after long latency (Dobles et al., 2000), several studies have shown that *Mad2* heterozygosity causes abnormal cellular phenotypes. *Mad2* haploinsufficiency in mammals is most evident in mitotic cells, where it leads to increased aneuploidy (Michel et al., 2001; Jeganathan and van Deursen, 2006). However, the absence of aneuploidy in *Mad2*$^{+/-}$ spermatocytes (Jeganathan and van Deursen, 2006) suggests that MAD2 levels do not influence SAC fidelity in male meiosis. This was a surprising finding, especially given that *Mad2* heterozygous females show accelerated meiotic progression and meiosis I chromosome mis-segregation in >20% of oocytes (Niault et al., 2007).

Here, we explore the impact of MAD2 levels on SAC fidelity in spermatogenesis when non-exchange chromosomes are present. We provide evidence that normal MAD2 levels are essential for a stringent apoptotic and SAC response to non-exchange sex chromosomes, and for preventing X-Y aneuploidy in sperm. In contrast, in mice with widespread achiasmy *Mad2* heterozygosity had no detectable impact on spermatogenic progression or on the apoptotic response. Collectively, these observations reveal that spermatocytes with individual non-exchange chromosome pairs – but not those with severe crossover defects – can occasionally escape the SAC and subsequently form aneuploid sperm.

RESULTS

Mad2 heterozygote mice exhibit reduced MAD2 levels

To assess how *Mad2* heterozygosity impacts MAD2 protein expression, we compared MAD2 protein levels in testis, liver and kidney of *Mad2*$^{+/-}$, *Mad2*$^{+/-}$*Spo11*β-only^{mb} and *Mad2*$^{+/-}$*Mlh1*$^{-/-}$ mice with those in *Mad2*$^{+/+}$ mice. Consistent with previous reports (Michel et al., 2001; Schvartzman et al., 2011), *Mad2* heterozygosity resulted in reduced MAD2 protein levels (Fig. 1A and Fig. S1). On average, MAD2 protein levels in *Mad2*$^{+/-}$ and *Mad2*$^{+/-}$*Spo11*β-only^{mb} mice were 42% and 44% of controls, respectively (Fig. 1A and Table S1). Similarly, *Mad2*$^{+/-}$*Mlh1*$^{-/-}$ males showed reduced MAD2 levels (Fig. S1C, not quantified).

Mad2 heterozygotes have smaller testes

To assess whether reduced MAD2 levels affect testis weight, we determined testis weight/body weight ratios (i.e. relative testis weight) in *Mad2*$^{+/-}$ mice. Relative testis weight in *Mad2*$^{+/-}$ animals was reduced compared with controls (Fig. 1B), and *Mad2*$^{+/-}$*Spo11*β-only^{mb} testes were even smaller (Fig. 1Bi,ii). Testis sizes of *Mad2*$^{+/+}$*Spo11*β-only^{mb} and *Mad2*$^{+/-}$*Spo11*β-only^{mb} mice were similar to each other, indicating that the *Mad2* genotype did not affect testis weight of *Spo11*β-only^{mb} mice. We also measured relative testis weight of *Mlh1*$^{-/-}$ animals. Both *Mad2*$^{+/+}$*Mlh1*$^{-/-}$ and *Mad2*$^{+/-}$*Mlh1*$^{-/-}$ mice had substantially smaller testes than controls, and there was no significant difference in testis weight between these two genotypes (Fig. 2A).

Testis morphology

To determine whether *Mad2* heterozygosity affects testis cellularity, we examined the morphology of seminiferous tubules in histological sections. Testis morphology of *Mad2*$^{+/-}$ mice was similar to control males (Fig. 1C), although somewhat more atrophic seminiferous tubules were observed (Fig. 1D). In both *Spo11*β-only^{mb} and *Mad2*$^{+/-}$*Spo11*β-only^{mb} males, the majority of seminiferous tubules appeared normal. Nevertheless, both *Spo11*β-only^{mb} and *Mad2*$^{+/-}$*Spo11*β-only^{mb} testes displayed more atrophic seminiferous tubules (Fig. 1Ciii,iv) than controls (15.7% and 15.5% of tubules were atrophic, respectively; Fig. 1D).

Spermatogenesis in both *Mad2*$^{+/+}$*Mlh1*$^{-/-}$ and *Mad2*$^{+/-}$*Mlh1*$^{-/-}$ mice progressed normally to stage XII of the seminiferous epithelium, i.e. to metaphase I. However, no haploid cells were detected in these mice (Fig. 2). Spermatocytes are likely arrested at metaphase I and eliminated by apoptosis, which probably causes the observed atrophy of the seminiferous tubules. Our data on *Mad2*$^{+/+}$*Mlh1*$^{-/-}$ mice is consistent with a previous *Mlh1*$^{-/-}$ mice study (Eaker et al., 2002), and reduced MAD2 level (i.e. *Mad2*$^{+/-}$*Mlh1*$^{-/-}$ males) did not provide any detectable rescue of testis cellularity.

Prophase I progression

To evaluate prophase I progression in *Spo11*β-only^{mb} (both *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$), *Mad2*$^{+/-}$ and control mice, we used indirect immunofluorescence with antibodies against synaptonemal complex components SYCP3 and SYCP1 on spermatocyte chromosome preparations. All showed a similar abundance of cells at different stages of meiosis I (Fig. 3A). These observations indicate that there are no overt defects in spermatogenic progression prior to metaphase I in any of the genotypes.

The X-Y chromosome pairing defect in *Mad2*$^{+/-}$*Spo11*β-only^{mb} mice

To examine whether disruption of one *Mad2* allele affects sex chromosome pairing in *Spo11*β-only^{mb} mice, we scored X-Y pairing frequency in pachytene spermatocytes of *Mad2*$^{+/-}$*Spo11*β-only^{mb} mice. As previously seen in *Spo11*β-only^{mb} spermatocytes (Faisal and Kauppi, 2016), we found two distinct classes of pachytene stage nuclei: those with X and Y chromosomes paired (Fig. 3Bi); and those with X and Y chromosomes separated (Fig. 3Bii). X and Y chromosomes were paired in 52% of *Mad2*$^{+/-}$*Spo11*β-only^{mb} pachytene nuclei (Fig. 3C), which was indistinguishable from *Spo11*β-only^{mb} mice (Faisal and Kauppi, 2016). This is in contrast to control mice, where X and Y PARs were paired in 97% of pachytene nuclei.

Reduced MAD2 level partially attenuates the apoptotic response in *Spo11*β-only^{mb} mice

To query whether spermatocytes are arrested at metaphase I stage and eliminated by apoptosis, we performed combined TUNEL and anti-H3Ser10 antibody staining (see Materials and Methods for details). In control males, H3Ser10-positive seminiferous tubules

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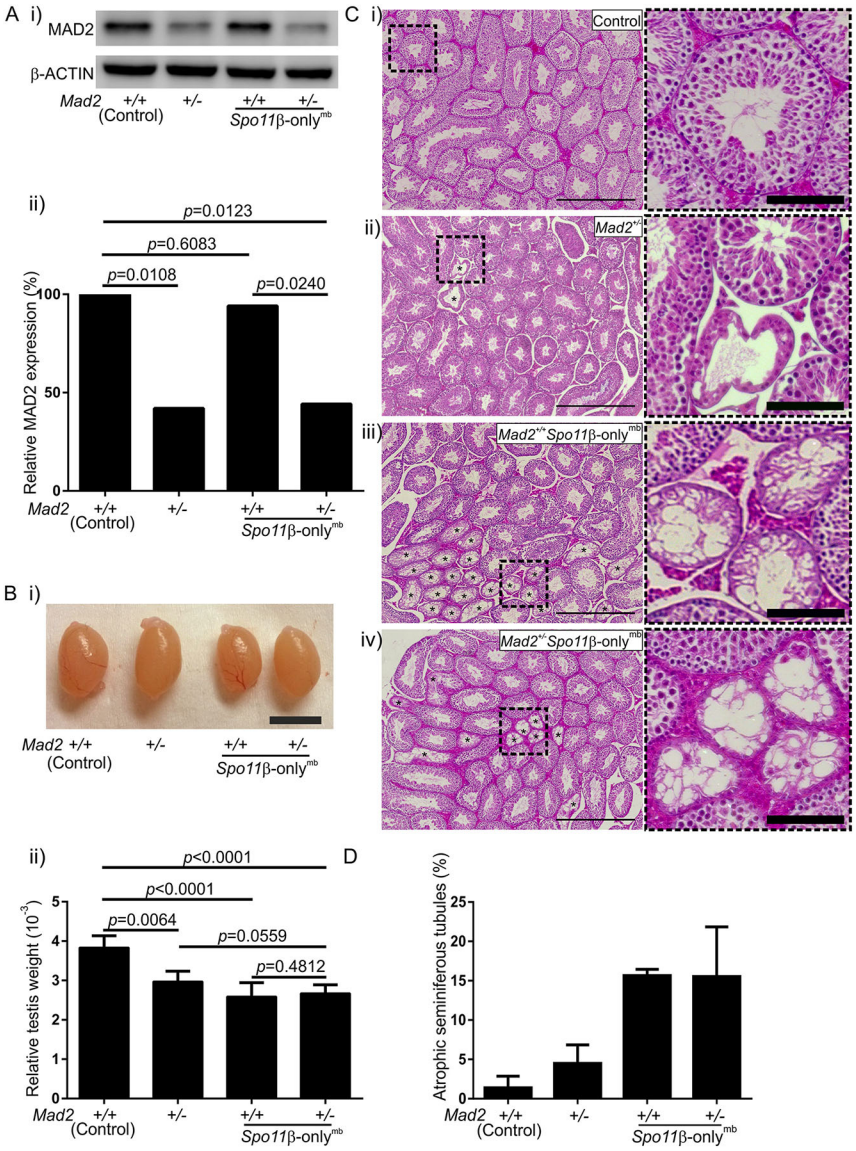

Fig. 1. Effects of *Mad2* heterozygosity on MAD2 protein levels and on testis morphology. (A) MAD2 protein levels. (i) Western blot with an antibody against MAD2 on whole-testis lysates of mice of the indicated genotypes. β-Actin was used as loading control. (ii) Quantification of MAD2 expression from two independent experiments. n=2 for each genotype. P values are from unpaired t-tests. See also Fig. S1 and Table S1. (B) Testis weights. (i) Testes from mice of the indicated genotypes. Both $Mad2^{+/+}Spo11$β-only^{mb} and $Mad2^{+/-}Spo11$β-only^{mb} testes are smaller than controls. Scale bar: 5 mm. (ii) Quantification of relative testis weights. n=5 for control, n=3 for $Mad2^{+/-}$ and n=12 for both $Mad2^{+/+}Spo11$β-only^{mb} and $Mad2^{+/-}Spo11$β-only^{mb} mice. Data presented are mean±s.d. P values are from unpaired t-tests. (C) Histological morphology of testes from mice of the indicated genotypes. Asterisks mark atrophic seminiferous tubules (see Materials and Methods for scoring criteria). Scale bars: 500 µm (left panels); 50 µm (right panels). (D) Quantification of atrophic seminiferous tubules. A total of 846 seminiferous tubules for control (number of mice, n=5), 1408 for $Mad2^{+/-}$ (n=3), 642 for $Mad2^{+/+}Spo11$β-only^{mb} (n=2) and 1188 for $Mad2^{+/-}Spo11$β-only^{mb} (n=3) were counted. Data presented are mean±s.d.

were rarely TUNEL positive; in $Mad2^{+/-}$ males, one-fifth of seminiferous tubules were positive for both markers (Fig. 4B and Table S2). In $Mad2^{+/-}Spo11$β-only^{mb} mice, the majority of H3Ser10-positive seminiferous tubules were also TUNEL positive (Fig. 4B and Table S2); compared with our earlier observations in $Mad2^{+/+}Spo11$β-only^{mb} mice (Faisal and Kauppi, 2016), we noted no drastic reduction in the percentage of double-positive tubules. Both $Mad2^{+/+}Mlh1^{-/-}$ and $Mad2^{+/-}Mlh1^{-/-}$ testes contained many seminiferous tubules positive for both TUNEL and H3Ser10, and *Mad2* heterozygosity did not affect the number of TUNEL-positive cells per double-positive tubule either (Fig. S2C).

Next, we quantified the number of TUNEL-positive spermatocytes within individual H3Ser10-positive seminiferous tubules. Strikingly, the mean number of TUNEL-positive spermatocytes per H3Ser10-positive tubule was substantially reduced in $Mad2^{+/-}Spo11$β-only^{mb} mice compared with $Spo11$β-only^{mb} mice with wild-type MAD2 levels (Fig. 4C; mean number of TUNEL-positive spermatocytes 7.4 and 11.5 per tubule, respectively). We conclude that a subset of spermatocytes in $Spo11$β-only^{mb} and $Mad2^{+/-}Spo11$β-only^{mb} are eliminated by an apoptotic response at metaphase I, and that reduced MAD2 decreases the number of apoptotic cells.

Sperm production

To evaluate whether reduced MAD2 has any effect on the amount of sperm produced, we examined the histology of cauda epididymides in mice of all the genotypes. Numerous spermatozoa were present in epididymides of $Mad2^{+/-}$ and $Mad2^{+/-}Spo11$β-only^{mb} mice (Fig. S3). As in $Spo11$β-only^{mb} males, the sperm density in $Mad2^{+/-}Spo11$β-only^{mb} males appeared somewhat lower compared with controls (Fig. S3, not quantified). Nevertheless, both $Mad2^{+/-}$ (consistent with previous reports by Niault et al., 2007; Schvartzman et al., 2011; Sotillo et al., 2007) and $Mad2^{+/-}Spo11$β-only^{mb} males were fertile, and pups sired by them developed normally. $Mad2^{+/+}Mlh1^{-/-}$ mice had no sperm in the cauda epididymis, as expected (Fig. S3v). *Mad2* heterozygosity did not, even partially, ameliorate the azoospermia of $Mlh1^{-/-}$ mice: as in $Mad2^{+/+}Mlh1^{-/-}$ males, epididymides in $Mad2^{+/-}Mlh1^{-/-}$ mice lacked sperm completely (Fig. S3vi).

Ploidy assessment on *Spo11*β-only^{mb} mice sperm

To assess meiotic chromosome segregation outcome, we quantified sex chromosome aneuploidy in epididymal spermatozoa of control, $Mad2^{+/-}$, $Mad2^{+/+}Spo11$β-only^{mb} and $Mad2^{+/-}Spo11$β-only^{mb} mice using fluorescent *in situ* hybridization (Fig. 5). A fluorescent

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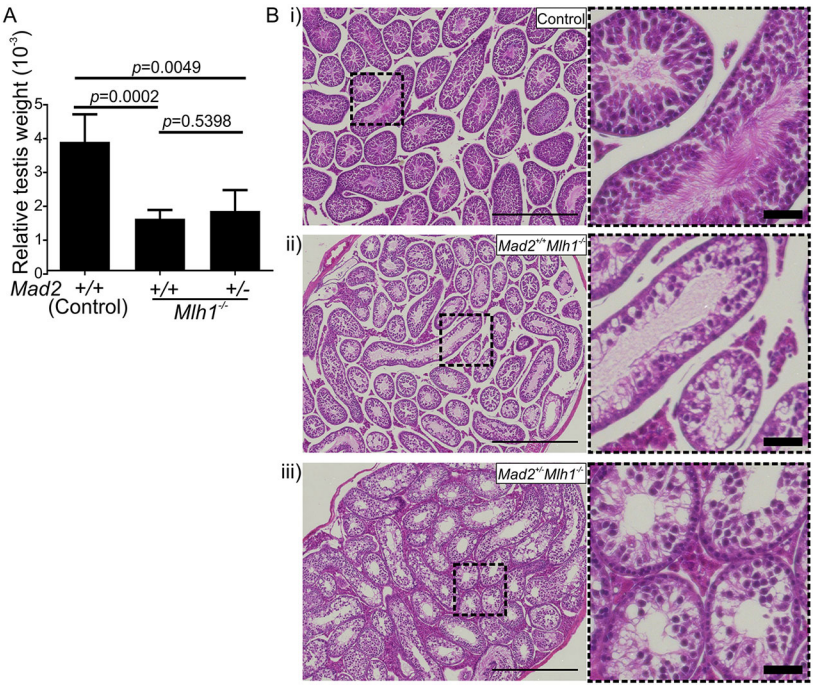

Fig. 2. Spermatogenic progression of *Mlh1*$^{-/-}$ mice. (A) Relative testis weights of the indicated genotypes. *n*=7 for control, *n*=6 for *Mad2*$^{+/+}$*Mlh1*$^{-/-}$ and *n*=4 for *Mad2*$^{+/-}$*Mlh1*$^{-/-}$ mice. *P* values are from unpaired *t*-tests. Data presented are mean±s.d. (B) Testis section morphology of control (i), *Mad2*$^{+/+}$*Mlh1*$^{-/-}$ (ii) and *Mad2*$^{+/-}$*Mlh1*$^{-/-}$ (iii) mice. Scale bars: 500 µm (left panels); 50 µm (right panels). See also Fig. S3 and S4.

in situ hybridization probe for chromosome 18 served as internal control for successful hybridization; no genotype-dependent autosomal aneuploidy was observed in the sperm analyzed (Table S3). In control, *Mad2*$^{+/-}$ and *Spo11*β-only^{mb} mice, only sperm haploid for the X or Y chromosomes were seen (Fig. 5B). This is in contrast to *Spo11*β-only^{mb} males heterozygous for *Mad2*, which produced a small but detectable quantity (1.6%) of sperm aneuploid for sex chromosomes (Fig. 5B). Aneuploid sperm were either disomic or nullisomic for sex chromosomes (Fig. 5Aiii,iv). We conclude from this experiment that males predisposed to segregation problems of individual homolog pairs (here, the sex chromosomes) can produce aneuploid gametes when MAD2 levels are reduced.

DISCUSSION

In the presence of homologs that have not achieved proper biorientation during meiosis, the SAC component MAD2 plays an important role in delaying the cell cycle, allowing time for reorientation attempts (Shonn et al., 2003). *Mad2* heterozygosity has been shown to reduce SAC fidelity in murine embryonic fibroblasts and human Hct cells (Michel et al., 2001), budding yeast mitosis (Barnhart et al., 2011) and mouse oocytes (Niault et al., 2007), but not male meiosis (Jeganathan and van Deursen, 2006). This apparent discrepancy could be due to differences in SAC function that exist in mitosis versus meiosis and/or in male versus female meiosis (Kallio et al., 2000; Homer, 2006; Lara-Gonzalez et al., 2012; Sun and Kim, 2012), or because *Mad2*$^{+/-}$ mice are not a sensitive enough system to reveal this effect.

To test how reduced (50% of wild type) MAD2 levels impact SAC fidelity in mouse spermatogenesis, we used three mouse lines with different propensities to non-exchange chromosomes. *Mad2*$^{+/-}$ testes displayed a small but detectable increase in TUNEL-positive cells (Fig. 4B), which was also reflected in histology (Fig. 1C,D). The cause is unclear; one possibility is that upon reduced MAD2 levels, the MAD2-mediated SAC signaling cascade in metaphase I and/or metaphase II, or in prometaphase of either, is misregulated. Regardless, and consistent with previous observations (Jeganathan and van Deursen, 2006), we found no evidence for sperm aneuploidy induced by *Mad2* heterozygosity in these otherwise genetically intact mice (Fig. 5B). This could reflect the fact that mouse chromosomes in male meiosis are not normally prone to achiasmy; univalent frequency at diplonema has been estimated to be just 1.1% (Roig et al., 2010). Thus, in wild-type mice, a partially 'leaky' SAC would have little consequence for sperm ploidy. In *Spo11*β-only^{mb} mice with wild-type MAD2 levels, despite frequent X-Y separation at pachynema, no sperm with X-Y aneuploidy were detected. This indicates that, in the presence of a normally functioning SAC, spermatocytes with non-exchange sex chromosomes are efficiently eliminated by the apoptotic response, in agreement with earlier studies (Burgoyne et al., 1992; Odorisio et al., 1998). However, the high elimination stringency appears to be at least partially dependent on strain background: in XY^{Sxr} mice (with a mixed genetic background of C3H/HeH and 101/H strains) with 64% of X-Y achiasmy, 3.7% of sperm were X-Y aneuploid (Ashley et al., 1994). This indicates some leakiness of the (wild-type) SAC in this setting, and contrasts with our current observations in the C57BL/6-129/Sv background of 50% X-Y achiasmy and 0% aneuploidy. As *Mad2*$^{+/-}$*Spo11*β-only^{mb} mice displayed an attenuated apoptotic response despite frequent non-exchange X-Y chromosomes, a key issue was whether this was accompanied by sperm aneuploidy. A small but significant percentage of sperm were aneuploid for sex chromosomes (Fig. 5 and Table S3), indicating that a less stringent SAC is generated upon reduced MAD2 levels. Previously, a lowered genetic dose of *Bubr1* (but not *Mad2* or other key SAC genes) was found to modify chromosome segregation fidelity in male meiosis (Jeganathan and van Deursen, 2006). Here, for the first time, we provide evidence that MAD2 levels are also crucial for SAC stringency and for the elimination of spermatocytes with abnormal chromosome number. This effect was revealed by the *Spo11*β-only^{mb} mouse model with its propensity for X-Y segregation defects, and was undetectable in *Mad2*$^{+/-}$ heterozygous but otherwise genetically intact mice (Jeganathan and van Deursen, 2006; Fig. 5B). However, *Mad2* heterozygosity provided no rescue of meiotic progression in

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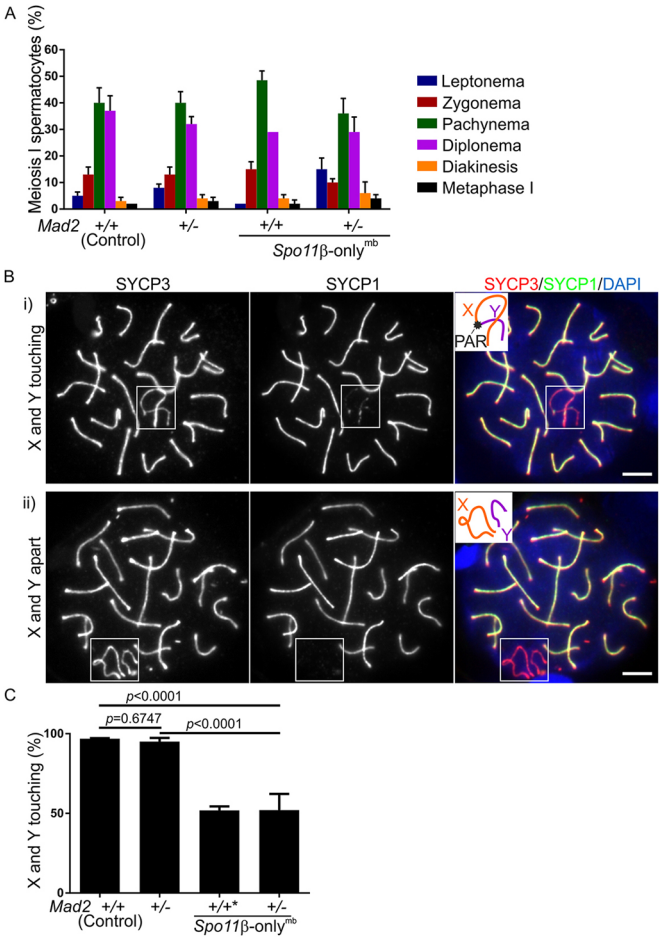

Fig. 3. Early meiosis I progression. (A) Quantification of meiosis I sub-stages in spermatocytes of mice with the indicated genotypes. Two animals from each genotype were used in the quantification and 100 spermatocytes per animal were scored. Data presented are mean±s.d. (B) Sex chromosome pairing at pachynema. Nuclei were stained with anti-SYCP3 (red), anti-SYCP1 (green) and DAPI (blue). Two spermatocyte nuclei are shown from the same *Mad2*$^{+/-}$*Spo11*β-only^{mb} mouse, one with X and Y PARs touching (i) and the other with X and Y chromosomes apart (ii). Insets in i and ii show a schematic representation of sex chromosome configuration in the same nuclei. Scale bars: 5 µm. (C) Quantification of X-Y pairing in pachynema in mice of the indicated genotypes. Data for *Mad2*$^{+/+}$*Spo11*β-only^{mb} mice have been published previously (Faisal and Kauppi, 2016) and are marked with an asterisk. A minimum of two mice of each genotype was used. *n*=63 pachynema for control, *n*=60 for *Mad2*$^{+/-}$ and *n*=78 for *Mad2*$^{+/-}$*Spo11*β-only^{mb} mice. Data presented are mean±s.d. and *P* values are from two-tailed Fisher's exact test.

Mlh1$^{-/-}$ mice, where virtually all chromosomes, not just sex chromosomes, fail to form crossovers. This implies that the presumably much stronger SAC response in this setting cannot be alleviated by reduced MAD2 levels. Our experiments, performed on fixed cells, do not directly address how some spermatocytes with non-exchange sex chromosomes escape SAC surveillance. We speculate that there is a critical threshold for MAD2 levels in the meiotic SAC response (as demonstrated in mitotic cells, Collin et al., 2013). This notion, when combined with stochastic cell-to-cell variation in MAD2 levels, could lead to a situation where in some *Mad2*$^{+/-}$ spermatocytes, there is not a sufficient amount of MAD2 recruited to kinetochores to maintain a functional SAC response. In the future, assessment of spermatogenic progression and aneuploidy in a testis-specific conditional *Mad2* knockout mouse model would be highly informative. As MAD2 also has a role in regulating prometaphase I and metaphase I duration (Tsuchiya et al., 2011), it is further possible that a small subset of spermatocytes prematurely enters anaphase when MAD2 levels are reduced (such as in *Mad2*$^{+/-}$*Spo11*β-only^{mb} mice). Nevertheless, the vast majority (>90%) of *Mad2*$^{+/-}$*Spo11*β-only^{mb} spermatocytes with non-exchange X-Y chromosomes must still have been 'caught' by SAC surveillance and eliminated from the meiotic pool, because the percentage of aneuploid sperm is much lower than that of spermatocytes with X-Y recombination defects. In budding yeast, Mad3 (BUBR1 in mammals) is particularly important for segregating non-exchange chromosomes (Cheslock et al., 2005), and in mouse oocytes, BUBR1 is required both for establishing stable microtubule-to-kinetochore attachments and for persistent SAC activity (Touati et al., 2015). Thus, examining sperm aneuploidy in *Bubr1*$^{+/-}$*Spo11*β-only^{mb} mice in the future would be of high interest, as achiasmate sex chromosome segregation in these animals may be further compromised. In *Bubr1* hypomorphic mice with drastically reduced protein levels, 5% of secondary spermatocytes display abnormal chromosome numbers (Baker et al., 2004; Jeganathan and van Deursen, 2006).

Meiosis in male mice is known for its stringent quality control. This contrasts with females, where the SAC can be satisfied when most – but not all – homolog pairs are correctly aligned (Kolano et al., 2012; Lane et al., 2012). We show here that *Spo11*β-only^{mb} males, with ∼50% dissociated sex chromosomes, produce only haploid sperm when MAD2 levels are intact, but when MAD2 levels are compromised, aneuploid sperm are formed at a low but detectable frequency (see Fig. 6 for proposed model on the SAC response). Unfortunately, in human males both X-Y segregation and SAC surveillance appear to be substantially less efficient, as indicated by observations that defective X-Y pairing often results in aneuploidies (Hassold et al., 1991; Thomas et al., 2000). There is substantial inter-individual variability: first, in the inherent propensity to X-Y recombination defects; and second, in the stringency with which meiocytes defective for sex chromosome recombination are eliminated (Ferguson et al., 2007). Furthermore, an inverse correlation between the frequency of sex chromosome recombination and X-Y disomic sperm was reported in healthy men (Ferguson et al., 2007), underscoring the importance of SAC surveillance in eliminating human spermatocytes with chromosome (especially X-Y) segregation defects. Like MAD2, other SAC component levels can fluctuate *in vivo* due to a wide variety of factors, such as mutational inactivation, overexpression, microRNA-mediated fine-tuning of gene expression (Tambe et al., 2016) and epigenetic mechanisms. Fluctuations in SAC protein levels can endanger human male meiosis and increase the risk of sperm aneuploidy, especially for sex chromosomes.

MATERIALS AND METHODS

Mice and genotyping

*Spo11*β-only^{mb} mice, on a mixed genetic background of C57BL/6 and 129/Sv strains, were derived from crosses of *Mad2* knockout (Dobles et al., 2000) and *Spo11*β-only mice (Kauppi et al., 2011), and have been described previously (Faisal and Kauppi, 2016). All *Spo11*β-only^{mb} mice are homozygous for the *Spo11*β_B transgene [i.e. *Tg*(*Xmr-Spo11*β_B)$^{+/+}$] and mutant-homozygous for endogenous *Spo11* (i.e. *Spo11*$^{-/-}$). The *Spo11*β_B transgene is under the control of a meiosis-specific promoter and has been described previously (Kauppi et al., 2011). The full genotype for animals referred to here as '*Spo11*β-only^{mb} mice' is *Mad2*$^{+/+}$*Spo11*$^{-/-}$ *Tg*(*Xmr-Spo11*β_B)$^{+/+}$. *Mlh1* knockout mice (Edelmann et al., 1996) were of the B6.129-*Mlh1*^{tm1Rak} strain (strain 01XA2, National Institutes of Health, Mouse Repository, NCI-Frederick), also on a C57BL/6 and 129/Sv mixed genetic background. The genotype of control mice varied depending

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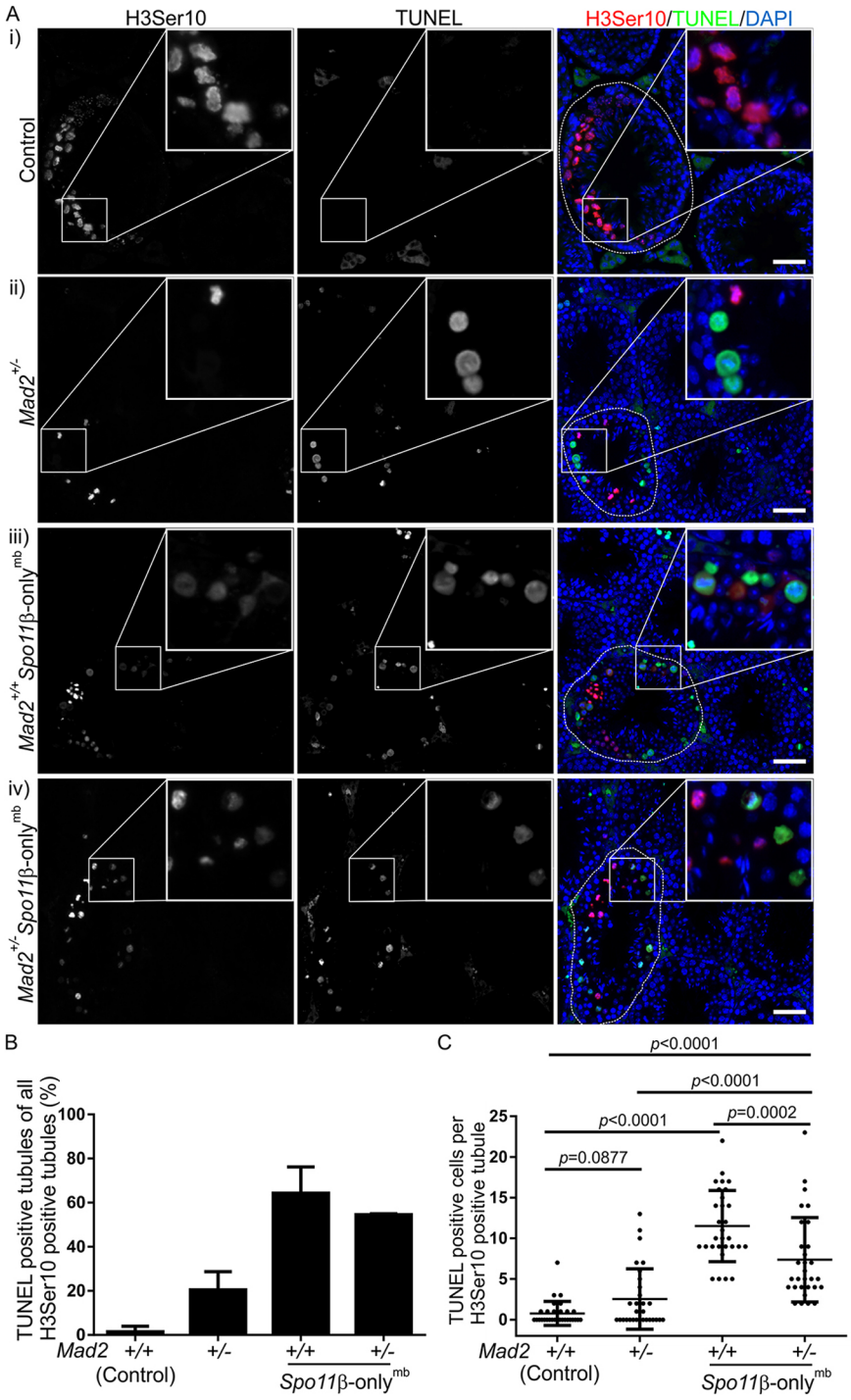

Fig. 4. Attenuation of apoptotic response in *Mad2*$^{+/-}$*Spo11*β-only^{mb} testes. (A) Testis sections containing seminiferous tubules were stained for apoptosis by TUNEL (green), using an antibody against H3Ser10 (red) and DAPI (blue). Insets show magnified views of regions of seminiferous tubules (areas within the white frames in larger image). Dashed lines in the rightmost panel outline the approximate boundaries of H3Ser10 seminiferous tubules. Scale bars: 50 µm. (B) Quantification of TUNEL-positive tubules within all H3Ser-positive seminiferous tubules. TUNEL-positive tubules are common in both *Spo11*β-only^{mb} and *Mad2*$^{+/-}$*Spo11*β-only^{mb} testes; *n*=62 H3Ser10-positive seminiferous tubules for control, *n*=50 for *Mad2*$^{+/-}$ and *n*=55 for *Mad2*$^{+/-}$*Spo11*β-only^{mb} testes. Data presented are mean±s.d. Data for *Mad2*$^{+/+}$*Spo11*β-only^{mb} mice have been published previously (Faisal and Kauppi, 2016) and are shown here for ease of comparison. (C) Number of TUNEL-positive cells per H3Ser10-positive seminiferous tubule cross-section in the indicated genotypes. Each dot shows the number of TUNEL-positive cells within one H3Ser10-positive seminiferous tubule. Data presented are mean±s.d. Testis sections from three animals per genotype are scored in B and C. *P* values are from Mann-Whitney *U*-test. See also Table S2.

on availability within the same litter or closely related litters as experimental animals. For *Spo11*β-only^{mb} mice, *Mad2*$^{+/+}$*Spo11*$^{+/+}$*Tg*(*Xmr-Spo11*β_B)$^{+/+}$, *Mad2*$^{+/+}$*Spo11*$^{+/-}$*Tg*(*Xmr-Spo11*β_B)$^{+/+}$ and *Mad2*$^{+/+}$*Spo11*$^{+/-}$*Tg*(*Xmr-Spo11*β_B)$^{+/-}$ genotype males served as controls. For *Mlh1*$^{-/-}$ mice, *Mad2*$^{+/+}$*Mlh1*$^{+/+}$ and *Mad2*$^{+/+}$*Mlh1*$^{+/-}$ mice were used as controls. All experiments included at least two animals per group, except the western blot experiment in testis, liver and kidney of *Mad2*$^{+/-}$ mice (Fig. S1A), performed on only one pair of mice. All cytological and histological experiments were performed on tissues derived from 8- to 12-week-old males, and each experiment was repeated at least twice. No significant inter-individual differences were found within the groups. Details of the genotyping procedure, as well as PCR primers and cycling conditions for *Mad2*, endogenous *Spo11*, the *Tg*(*Xmr-Spo11*β_B) transgene and *Mlh1* alleles have been published previously (Baudat et al., 2000; Dobles et al., 2000; Kauppi et al., 2011; Pussila et al., 2013) and are listed in the supplementary Materials and Methods. All applicable national and institutional guidelines (Animal Experiment Board in Finland and Laboratory Animal Centre of the University of Helsinki, respectively) for the care and use of animals were followed.

Western blot

Tissue lysate was prepared according to a published protocol (Kallio et al., 2000) with modifications (see supplementary Materials and Methods for details) and lysate concentrations were measured using Pierce BCA kit (Thermo Scientific). Antibodies against MAD2 (Santa Cruz, catalog number sc-6329), β-actin (Sigma, catalog number A5441) and GAPDH

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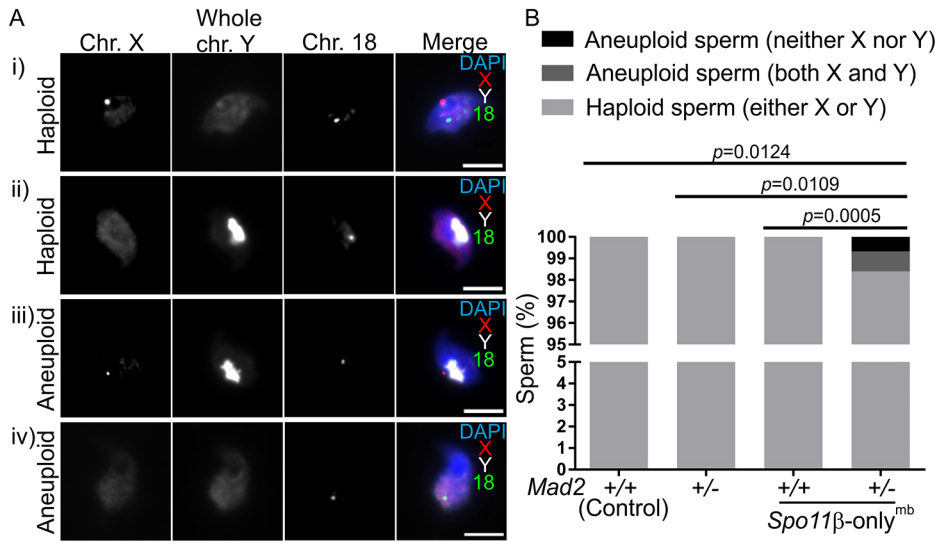

Fig. 5. *Spo11*β-only^{mb} males produce aneuploid sperm when MAD2 levels are reduced. (A) Sperm smears from cauda epididymis were stained by fluorescence *in situ* hybridization (FISH) using a chromosome X point probe (red), a Y whole chromosome paint probe (white), a chromosome 18 point probe (green) and DAPI (blue) (see Materials and Methods for details). For all cells, the chromosome 18 probe served as an internal control for the success of FISH. Shown are four different classes of sperm found in *Mad2*$^{+/-}$*Spo11*β-only^{mb} epididymis: (i) haploid sperm with one X chromosome signal, (ii) haploid sperm with one Y chromosome signal, (iii) aneuploid sperm showing XY disomy and (iv) X-Y nullisomic sperm. Scale bars: 2 µm. (B) Sex chromosome ploidy in sperm. *n*=350 sperm for control, *n*=400 for *Mad2*$^{+/-}$, *n*=700 for *Mad2*$^{+/+}$*Spo11*β-only^{mb} and *n*=750 for *Mad2*$^{+/-}$*Spo11*β-only^{mb} mice. Sperm smears from at least two independent mice per genotype were used for quantification. *P* values are for the comparison of aneuploid cell numbers between the genotypes (two-tailed Fisher's exact test). X-Y aneuploid sperm were seen only in *Mad2*$^{+/-}$*Spo11*β-only^{mb} mice (mean 1.59%±standard deviation 0.23%). See also Table S3.

(Abcam, catalog number ab181602) were used as primary antibodies at 1:500, 1:5000 and 1:10,000 dilutions, respectively. Either infrared (680 and 800 from Li-Cor) or HRP-conjugated secondary antibodies (Bio-Rad and Invitrogen) were used, images were captured with a Li-Cor Odyssey Fc Imaging system and further processed with Li-Cor Image Studio Lite software (version 5.2) and CorelDraw (version X7). MAD2 expression was normalized to respective loading control, and then mean values from each genotype were normalized to mean MAD2 expression from controls.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Faisal and Kauppi, 2016) with slight modifications (see supplementary Materials and Methods for details). Seminiferous tubules containing less than half the normal number of spermatocytes were scored as atrophic seminiferous tubules. A total of three sections from each testis were used when scoring atrophic seminiferous tubules.

Evaluation of meiosis I progression

Meiosis I stages were identified using antibodies against synaptonemal complex proteins SYCP3 (Santa Cruz Biotechnology, catalog number: sc-74569) and SYCP1 (Novus Biologicals, catalog number: NB300-229) on spermatocyte chromosome preparations (see supplementary Materials and Methods for details). The combination of these two antibodies allows identification of prophase I sub-stages (leptonema, zygonema, pachynema, diplonema, diakinesis and metaphase). Alexa Fluor 488- and 568-conjugated secondary antibodies (Invitrogen) were used at 1:200 dilutions. Pairing of X and Y chromosomes at pachynema was scored based on their physical contact at the PAR: 'X-Y apart' was scored when X

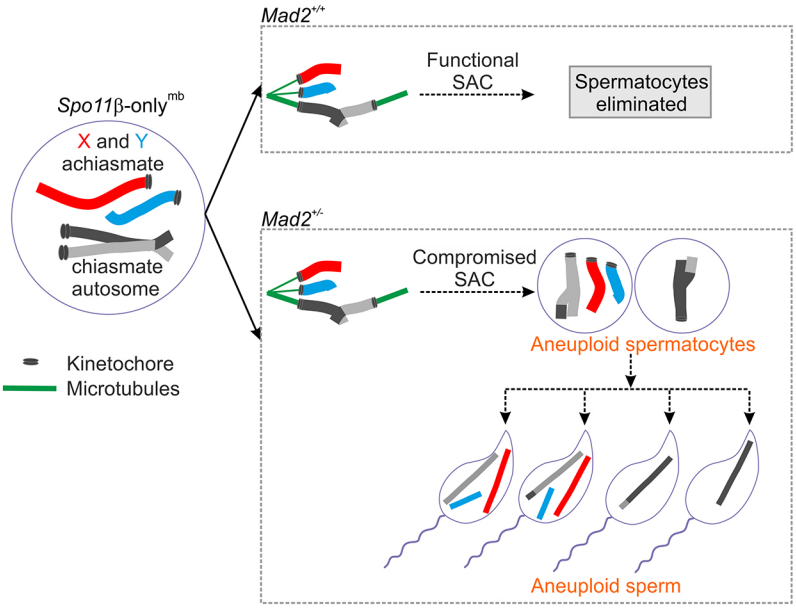

Fig. 6. Summary model of meiotic SAC fidelity in *Mad2*$^{+/+}$ and *Mad2*$^{+/-}$ males in the presence of non-exchange sex chromosomes. When MAD2 levels are reduced, cells with individual non-exchange chromosome pairs can occasionally escape the SAC and lead the formation of aneuploid sperm. When MAD2 is intact (i.e. *Mad2*$^{+/+}$) in *Spo11*β-only^{mb} animals, all aneuploid spermatocytes are likely eliminated by a functional SAC. See also Fig. S4.

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and Y chromosomes were clearly separated from each other based on SYCP3 staining.

Combined TUNEL and immunofluorescent staining of testis sections

Details of the combined terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and immunofluorescent staining can be found in the supplementary Materials and Methods. TUNEL staining allows the identification of apoptotic cells, and the anti-H3Ser10 antibody in the testis marks metaphase I spermatocytes. Seminiferous tubules containing five or more TUNEL-positive cells were considered TUNEL-positive tubules. Similarly, seminiferous tubules with five or more H3Ser10-positive spermatocytes were scored as H3Ser10 positive. An antibody against phosphorylated histone H3Ser10 (1:500, Millipore, catalog number 06-570) was used as primary antibody, and Alexa-Fluor 568-conjugated IgG (Invitrogen) served as a secondary antibody.

Fluorescence *in situ* hybridization

Preparation of epididymal sperm smears and detailed conditions for fluorescence *in situ* hybridization (FISH) are described in the supplementary Materials and Methods. Probes were denatured and incubated as described previously (Kauppi et al., 2011), with the same chromosome X and chromosome 18 BAC-based FISH probes. A nick translation kit (Abbott Molecular, catalog number 07J00-001) was used for labeling of the FISH probes. X chromosome probe was orange dUTP (Abbott Molecular, catalog number 02N33-050) conjugated and chromosome 18 probe was biotin-conjugated. The biotin-conjugated chromosome 18 probe was further incubated with Alexa Fluor 647-conjugated streptavidin (Thermo Scientific). For Y chromosome FISH, a fluorescein-conjugated mouse chromosome Y painting probe was used (Empire Genomics, catalog number MCEN-Y-10-GR).

Scoring of sperm ploidy

As sperm are haploid, euploid cells should display one sex chromosome (either X or Y) FISH signal, and one chromosome 18 FISH signal. To distinguish between technical FISH failure and genuine X-Y aneuploidy, only sperm nuclei with a clear chromosome 18 FISH signal were included in sex chromosome ploidy scoring.

Statistical tests

Statistical tests were performed using GraphPadPrism software (Version 6). The following tests were performed to calculate *P* values: unpaired *t*-tests in Figs 1Aii,Bii and 2A, Fisher's exact test in Fig. 3C, and Mann-Whitney *U*-tests in Fig. 4C and Fig. S2C.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.K.; Methodology: I.F., L.K.; Software: I.F.; Validation: I.F.; Formal analysis: I.F., L.K.; Investigation: I.F.; Resources: L.K.; Writing - original draft: I.F., L.K.; Writing - review & editing: I.F., L.K.; Visualization: I.F.; Supervision: L.K.; Project administration: I.F., L.K.; Funding acquisition: L.K.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.149492.supplemental

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