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# The frequency of heterologous synapsis increases with aging in Robertsonian heterozygous male mice

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**Abstract** The house mouse is characterised by highly variable chromosome number due to the presence of Robertsonian (Rb) chromosomes. During meiosis in Rb heterozygotes, intricated chromosomal figures are produced, and many unsynapsed regions are present during the first prophase, triggering a meiotic silencing of unsynapsed chromatin (MSUC) in a similar mode to the sex chromosome inactivation. The presence of

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unsynapsed chromosome regions is associated with impaired spermatogenesis. Interestingly, in male mice carrying multiple Rb trivalents, the frequency of germ cell death, defective tubules, and altered sperm morphology decreases during aging. Here, we studied whether synapsis of trivalent chromosomes and MSUC are involved in this improvement. By immunocytochemistry, we analysed the frequency of unsynapsed chromosomes and of those positive to γH2AX (a marker of MSUC) labelling in spermatocytes of 3-, 5- and 7-month-old Rb heterozygotes. With aging, we observed a decrease of the frequency of unsynapsed chromosomes, of spermatocytes bearing them and of trivalents carrying γH2AX-negative unsynapsed regions. Our quantitative results show that both synapsis and MSUC processes are better accomplished during male aging, partially accounting for the improvement of spermatogenesis.

**Keywords** asynapsis · MSUC · meiosis · Robertsonian translocation · spermatogenesis · aging

**Abbreviations**

| | |
|---|---|
| ATR | ataxia telangiectasia and Rad3-related protein |
| BRCA | breast cancer |
| DABCO | 1, 4-Diazobicyclo-2,2,2-octane |
| DSB | Double Strand Break |
| CREST | Calcinosis Raynaud's Esophagus Sclerodactyly Telangiectasia |
| CS5 | Creative Suite 5 |
| CT | Close Trivalent |
| FITC | Fuorescein isothiocyanate |
| GCD | Germ Cell Death |
| IRCCS | Istituto di Ricovero e Cura a Carattere Scientifico |
| MSCI | Meiotic Sex Chromosome Inactivation |
| MSUC | Meiotic Silencing of Unsynaped Chromatin |
| MI | Metaphase I |
| MII | Metaphase II |
| OT | Open Trivalent |
| PBS | Phosphate Buffer Saline |
| POT | Partially Open Trivalent |
| Rb | Robertsonian |
| SC | Synaptonemal Complex |
| SYCP3 | Synaptonemal Complex Protein 3 |
| SUMO1 | small ubiquitin-related modifier-1 |
| TRITC | Tetramethylrhodamine isothiocyanate |
| ubiH2A | monoubiquitinated H2A histone |
| γH2AX | phosphorylated serine 139 of histone H2AX, H2A histone family, member X |

## Introduction

The house mouse (*Mus musculus domesticus*) is characterised by a highly variable chromosome number, from the standard, all telocentrics $2n=40$, to $2n=22$, the lowest described in feral populations (Capanna et al. 1976; Lehmann and Radbruch 1977; Redi and Capanna 1988; Searle 1993; Britton-Davidian et al. 2000). This extraordinary variability is produced either by Robertsonian translocation ((Rb) two telocentric chromosomes fuse at the centromeric regions producing a metacentric chromosome), or by whole arm reciprocal translocation (between Rb chromosomes or an Rb and a telocentric chromosome) (Capanna and Redi 1995; Pialek and Albrecht 2005).

The presence of Rb chromosomes in heterozygosity has detrimental effects on spermatogenesis, giving rise to subfertility or complete sterility (Redi and Capanna 1988; Garagna et al. 1990, 2001b; Wallace et al. 1992, 2002; Everett et al. 1996; Hauffe and Searle 1998; Merico et al. 2003; Manterola et al. 2009; Nunes et al. 2011). During meiosis, Rb heterozygosities produce intricated prophase I figures, like long chains, rings or trivalents, which show mainly asynapsis, among several other synaptic defects (Johannisson and Winking 1994; Wallace et al. 2002; Manterola et al. 2009). In mammals, unsynapsed chromosome regions have been associated with impaired fertility. This association has been attributed to the presence of a pachytene checkpoint that recognises unrepaired double-strand breaks (DSBs) in unsynapsed chromosome regions and, accordingly, proceeds to the arrest of the cell cycle to allow DNA repairing or the elimination of the meiocyte (Burgoyne et al. 2009). To deal with this adversity, a number of proteins such as BRCA1, ATR, γH2AX, ubiH2A and SUMO-1 are recruited in the unsynapsed chromosome regions, triggering a meiotic silencing of unsynapsed chromatin (MSUC), similar to that observed during the meiotic sex chromosome inactivation process (Turner et al. 2005; Baarends et al. 2005; Sciurano et al. 2007; Kouznetsova et al. 2009; Manterola et al. 2009). However, deleterious effects may arise from the transcriptional silencing of genes located in regions subject to MSUC (Turner et al. 2006; Burgoyne et al. 2009; Saferali et al. 2010) of

impaired imprint of these same regions (Saferali et al. 2010) or of disturbed transcriptional inactivation of the sex chromosomes (Homolka et al. 2007) which could lead to a meiotic arrest and apoptosis of spermatocytes or of spermatids.

We have reported that, in mice heterozygous for eight multiple simple Rb chromosomes (i.e. Rb metacentrics that do not share any arm homology), pachytene spermatocytes carrying trivalents with unsynapsed regions were able to complete prophase and proceed to the metaphase I (MI) or metaphase II (MII) stage, when massive germ cell death (GCD) was observed (Merico et al. 2008; Manterola et al. 2009). Interestingly, a quantitative analysis of the efficiency of the meiotic process (based on the ratio between spermatids and spermatocytes) showed decreasing GCD frequencies in 3- (64%), 5- (63%) and 7-(58%)month-old mice (Merico et al. 2003). Also, dysfunction of spermatogenesis was found to vary among the three ages analysed, the oldest animals showing a lower frequency of defective seminiferous tubules and lower abnormal sperm morphology compared with younger mice (Merico et al. 2003).

To unravel possible mechanistic causes that could account for the differential efficiency of the meiotic process in these heterozygous Rb male mice, in the present study, we determined whether the pairing of homologous chromosomes and MSUC occurred differentially in individuals of different ages. Specifically, we quantified the frequency of (1) pachytene spermatocytes bearing unsynapsed chromosomes and their number *per* spermatocyte and (2) spermatocytes bearing chromosomes with different degree of unsynapsis and of unsynapsed trivalents with or without positive signal to a marker of MSUC (γH2AX protein).

## Materials and methods

### Mice

Male Rb heterozygous mice ($2n=32$, eight Rb chromosomes in a heterozygous state) were generated by mating females of the CD1 laboratory strain ($2n=40$; Charles River, Italy) and males of the Milano II race ($2n=24$; Gropp et al. 1982). Rb heterozygotes were grown up to 3, 5 and 7 months: Three males for each age were killed and then used for successive analysis. Mice were maintained at 22°C with a light/dark cycle of 12:12 h and fed ad libitum. Procedures involving the use of mice were approved by the animal ethics committee of the University of Pavia (Italy).

### Synaptonemal complex spreadings and immunostaining

Synaptomenal complex (SC) spreadings were prepared from cells of the seminiferous tubules of the left testis following Peters' drying-down procedure (Peters et al. 1997). Briefly, the albuginea membrane was removed, and seminiferous tubules were released in hypotonic solution for 30 min at room temperature (rt); small portions of tubules were disaggregated and washed in a sucrose solution 100 mM, pH 9.0; 60 μL of cellular suspension were transferred onto a glass slide previously covered with 100 μL of fixative solution (0.15% triton, 1% paraformaldehyde in water) and incubated in a humid chamber for 1 h at rt, washed in Photoflo 600 (0.13% in water for 2 min) and air-dried.

A triple immunostaining was performed on the same preparations in order to detect the axial elements (SYPC3) of the synaptonemal complex, the centromeres (CREST serum), and the phosphorylated serine 139 of histone H2AX. Immunostaining was performed overnight at 37°C simultaneously with the following primary antibodies: rabbit *anti*-rabbit SYCP3 (1:15,000, generous gift from Dr. Christa Heyting, Wageningen University, The Netherlands), human CREST serum (1:6,000; generous gift of Claudia Alpini, Laboratorio di analisi chimico cliniche, IRCCS San Matteo, Pavia, Italy), mouse anti-mouse γH2AX (1:10,000; Upstate, cat. n. 05–636). Slides were washed three times in phosphate buffered saline (PBS)/0.1% Tween 20 and then incubated with the appropriate secondary antibodies (anti-rabbit IgG FITC-conjugated, 1:10,000; anti-human IgG Cy5 conjugated, 1:3,000; anti-mouse IgG TRITC-conjugated, 1:10,000) for 1 h at 37°C. Slides were then washed three times in PBS/0.1% Tween 20 and counterstained with Hoechst 33342 (1 μg/μL) for 10 min and mounted in DABCO antifading. SC spreadings were examined with an Olympus Provis fluorescence microscope, and distinct FITC, TRITC, Cy5, and Hoechst images were taken with a Photometrics CH-350/A camera controlled by the IP Lab software (Scananalytics, Inc.) and merged using IP Lab and ADOBE Photoshop CS5 softwares. For each individual, at least 25 pachytene spermatocytes were considered following the staging schedule of meiotic prophase progression in the mouse (Moses 1980).

## Results

The pairing and the morphology of autosomes and sex chromosomes were determined using immunostaining against SYCP3, a protein component of the SC axial elements. As expected, at the pachytene stage, we observed the presence of the sex bivalent, eight trivalents and three telocentric bivalents (Fig. 1, a). Each trivalent was formed by the association of a metacentric chromosome with its two homologous telocentrics. Based on the degree of synapsis observed (Fig. 1, b–g), trivalents were classified into three main groups: (1) closed trivalents (CTs), in which short segments of the proximal regions of the telocentric chromosomes were involved in heterologous synapsis (Fig. 1, b–c); (2) partially open trivalents (POTs), in which heterologous synapsis involved long proximal segments of telocentrics (Fig. 1, d–e); and (3) open trivalents (OTs), in which one or both proximal segments of telocentrics were in complete asynapsis (Fig. 1, f–g). POTs and OTs showed unsynapsed segments either negative or positive to γH2AX labelling (Fig. 1, d'–g'); CTs positive in the short proximal segments of the telocentrics (Fig. 1, c–c") were very rare.

To quantify the fraction of spermatocytes carrying trivalents with synaptic defects, we have evaluated the frequency of middle/late pachytene cells carrying fully

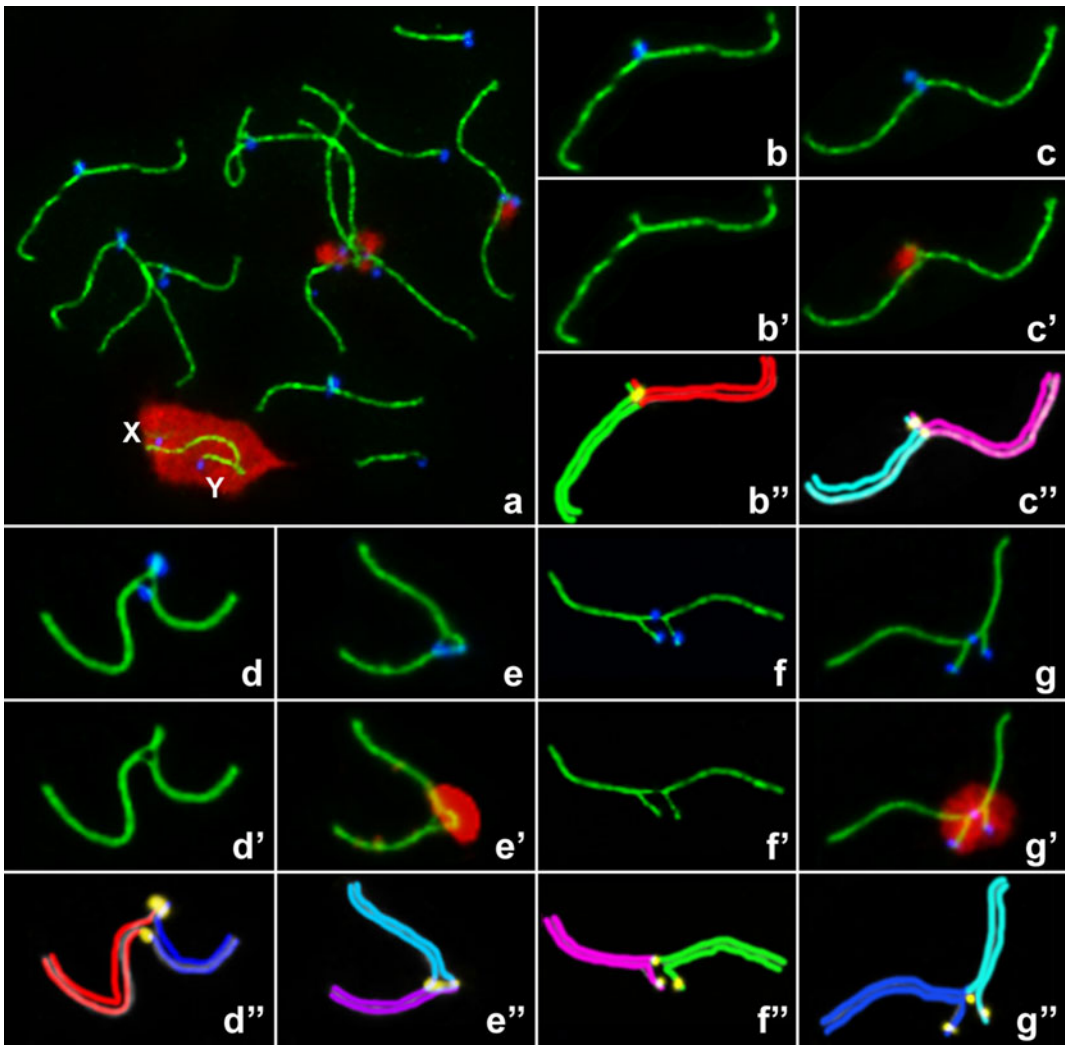


**Fig. 1** Immunolocalization of γH2AX (*red*), SYCP3 (*green*) and centromeres (*blue*). γH2AX-positive signals are localised on the sex body (*a*), on closed (CTs, although extremely rare; *c'*), partially open (POTs, *e'*) or open (OTs, *g'*) trivalents. POTs (*d'*) and OTs (*f'*) have also been found devoid of γH2AX immunostaining

synapsed trivalents (those classified into the CT group) and spermatocytes carrying one or more trivalents with unsynapsed regions (Table 1). The frequency of spermatocytes bearing trivalents of the CT type only was very low (6.40%) in 3-month-old mice but increased with age reaching 53.90% in 7-month-old mice. As a consequence, the frequency of spermatocytes carrying unsynapsed trivalents decreased with age, and spermatocytes with a high number (i.e., five or six) of unsynapsed trivalents were not found in the oldest animals (Table 1). Nevertheless, the frequency of spermatocytes with synaptic defective trivalents remained high in mice of all ages (93.60% in 3-month-old mice, 64.90% in 5- and 46.10% in 7-month-old animals).

Since trivalents showed different degrees of asynapsis, we then evaluated the frequency of CTs, POTs and OTs in spermatocytes of animals of the three different ages. We found an increase of frequency of CTs that was correlated with a decrease of that of both POTs and OTs during aging (Fig. 2a). Furthermore, the decrease of the frequency of POTs was more pronounced compared with that of OTs, being the POTs/OTs ratio 0.53, 0.38 and 0.25 in 3-, 5- and 7-month-old mice, respectively. The more marked decreased frequency of POTs, trivalents in which heterologous synapsis in the distal segments of telocentrics might likely be still in progress, suggests changes in the dynamics of synapsis during aging. These data, together with those reported in Table 1, indicate that the process of synapsis is better accomplished in older animals.

In order to identify whether the synaptic behaviour was different within the spermatocyte population carrying unsynapsed trivalents and at different ages, we evaluated the frequency of spermatocytes bearing POTs, OTs or both configuration types (POTs + OTs) (Fig. 2b). OTs bearing spermatocytes were the most numerous within the population of meiocytes carrying unsynapsed trivalents, their frequency increasing with aging (Fig. 2b). An opposite trend was observed for POTs + OTs bearing spermatocytes, the frequency of which decreased from 32.3% to 18.8% and 16.7% in 3-, 5- and 7-month-old mice, respectively. POTs bearing spermatocytes were more frequent in 5- compared with 3-month-old heterozygotes, whereas they were not detected in the oldest animals. These data suggest that the dynamics of synapsis of Rb trivalents varies among spermatocytes and at different ages.

We have previously reported that the unsynapsed pericentromeric regions of the trivalents may not always be positive to γH2AX antibody (Manterola et al. 2009) (Fig. 1, d and f). Here, we determined the frequency of CTs, OTs or POTs either positive or negative to γH2AX labelling. As expected, almost all CTs were negative for γH2AX labelling at all ages (99.54%, 99.57% and 98.84% in 3-, 5- and 7-month-old heterozygotes, respectively). In 5- and 7 month-old animals, almost all POTs and OTs were positive to γH2AX antibody whereas, in 3-month-old heterozygotes, trivalents were more

**Table 1** Number and frequency of middle/late pachytene spermatocytes carrying trivalents either partially or fully synapsed in 3-, 5- and 7-month-old Rb heterozygotes

| Rb heterozygotes | | | | | | |
|---|---|---|---|---|---|---|
| Number of unsynapsed trivalents | Age (months) | | | | | |
| | 3 | | 5 | | 7 | |
| | *n* | % | *n* | % | *n* | % |
| 0 | 5 | 6.40 | 53 | 35.10 | 90 | 53.90 |
| 1 | 25 | 32.00 | 68 | 45.03 | 60 | 35.92 |
| 2 | 17 | 21.80 | 18 | 11.92 | 12 | 7.18 |
| 3 | 10 | 12.80 | 8 | 5.30 | 3 | 1.80 |
| 4 | 12 | 15.30 | 4 | 2.65 | 2 | 1.20 |
| 5 | 6 | 7.60 | 0 | 0.00 | 0 | 0.00 |
| 6 | 2 | 2.50 | 0 | 0.00 | 0 | 0.00 |
| Total | 78 | 100.00 | 151 | 100.00 | 167 | 100.00 |

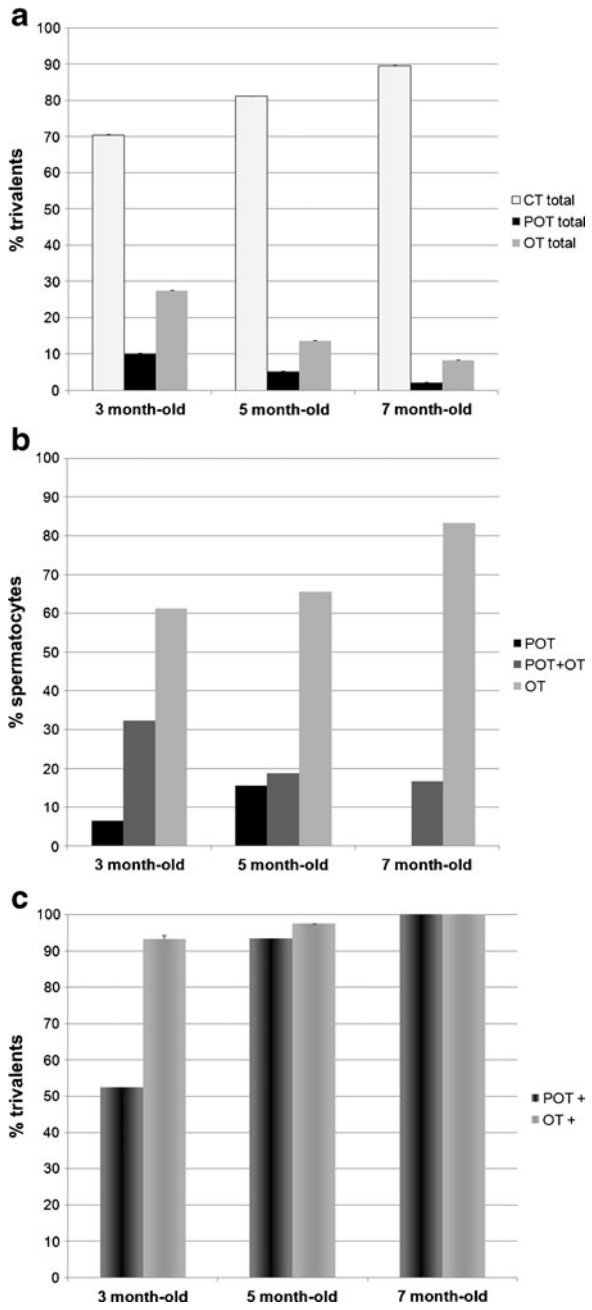


**Fig. 2** Frequency of trivalents±standard error (**a**, **c**) and of spermatocytes carrying unsynapsed trivalents (**b**) in Rb mice at the three ages considered. Trivalents are classified according to the synapsis condition in the heterologous regions: CTs close trivalents; POTs partial open trivalents; OTs open trivalents. In **c**, frequency of CTs, POTs and OTs positive to γH2AX immunolabelling

heterogeneous in the response to γH2AX labelling within the heterologous region. In fact, about 52% of POTs and 93% of OTs had positive signal (Fig. 2c).

These analyses showed that, in younger individuals, trivalents have higher variability in the synapsis and MSUC behaviour compared with older individuals. In fact, in the latter animals, spermatocytes carried unsynapsed trivalents of the POTs + OTs or OTs type-only and the unsynapsed regions were always positive to γH2AX antibody.

While γH2AX positive labelling was found to vary on autosomes, depending on the type of asynapsis (POTs or OTs) and on the age of the animal, the XY body was positive in 100% of the spermatocytes analysed at all ages.

## Discussion

The purpose of this study was to determine whether relevant processes that take place during the prophase stage of meiosis I, i.e. synapsis of homologous chromosomes and MSUC (monitored as positive labelling of unsynapsed regions using γH2AX antibody), differentially occur in Rb heterozygous male mice of different ages.

A first result of our work is the indication that the process of synapsis is more defective in younger heterozygotes. This is evidenced by higher frequency of pachytene spermatocytes still bearing unsynapsed trivalents in 3- (93.6%) compared with both 5- (64.9%) and 7-(46.1%) month-old mice. The analysis of trivalent configurations in these pachytene spermatocytes also confirmed improvement of synapsis with aging. This improvement is substantiated by a decreased frequency of both POTs and OTs and an increased frequency of CTs, although in older mice POTs tend to be progressively less frequent in relation to OTs. These data indicate that synaptic adjustment is better accomplished with aging, a phenomenon never described before. We do not have an explanation for this unexpected finding. We can speculate that there might be a "tempo" for the process of synapsis to be correctly established. Synapsis and DNA repair processes are dependent on one another. This may explain why, in older mice, synapsis is improved and the frequency of MSUC-negative unsynapsed chromosomes decreases. This suggests that the DNA repair "tempo" could be affected with aging. As a consequence, we hypothesise that differences in the timing of DNA repair response activity could explain the improved synapsis observed in older mice. Compared with older animals,

in younger mice, the less efficient synapsis ends with the production of a higher frequency of spermatocytes with unsynapsed trivalents. These spermatocytes are subsequently eliminated during MI and MII, when massive GCD is observed (Merico et al. 2003, 2008; Manterola et al. 2009). A decrease of meiocyte GCD was also observed during aging in hybrids obtained by breeding *Graomys griseoflavus* × *Graomys centralis* (Rodriguez et al. 2010), another example which might support the hypothesis that improvement of synapsis might be achieved with time.

The presence of POTs and OTs at all ages suggests that synapsis may differentially initiate and proceed in different trivalents. One interpretation is that heterologous synapsis of the telocentrics within each trivalent, which is usually initiated at distal regions, is a very difficult process to achieve. Perhaps, this is due to the spatial requirements that homologues must overcome when they move close to each other within the nuclear space (Garagna et al. 2001a; Manterola et al. 2009). However, once heterologous synapsis is initiated at the distal regions, it might differentially progress towards proximal regions in mice of different ages, as documented by the increase in the frequency of CTs and a decrease of POTs and OTs in 5- and 7- compared with 3-month-old Rb heterozygotes. On the basis of these results, we hypothesise that, during aging, the observed synapsis improvement might be achieved by a faster establishment of heterologous synapsis and/or an increase of synapsis speed along the whole trivalent. This improvement of synapsis is reflected by a lower frequency of spermatocytes that carry unsynapsed trivalents in older mice. However, when synapsis is not achieved, trivalents are predominantly of the OTs type, as shown in 7-month-old Rb heterozygotes.

A second result of our study is related to the MSUC response. In a previous paper, we reported the presence of unsynapsed regions of trivalents negative for a number of MSUC markers (Manterola et al. 2009). This feature was mainly found in early pachytene spermatocytes. In this study, we confirmed the absence of an MSUC marker in unsynapsed trivalents in middle and late pachytene cells, and we showed that the frequency of spermatocytes carrying unsynapsed trivalents negative to γH2AX labelling decreases with age. These data suggest that MSUC might be more effective in older than in younger mice. Thus, in 3-month-old heterozygotes, only 52% for POTs and 93% for OTs had γH2AX-positive signals on the unsynapsed regions, a frequency that increased in 5- and 7-month-old mice. Based on these results, we suggest that, in younger mice, a consistent fraction of trivalents with unsynapsed regions, mainly of the POT type, either escaped from MSUC or were γH2AX-negative because they might be engaged in a slow process of synaptic adjustment. Delayed meiotic progression, as that we described in a previous paper using the same type or Rb mice (Manterola et al. 2009), could favour synaptic adjustment of γH2AX-negative POTs, in which a small region is still unsynapsed. This might be more difficult to occur in OT-negative-bearing spermatocytes, in which a relevant region of the proximal chromosome ends is not synapsed. In fact, in older animals, unsynapsed trivalents are almost all of the OT type. The

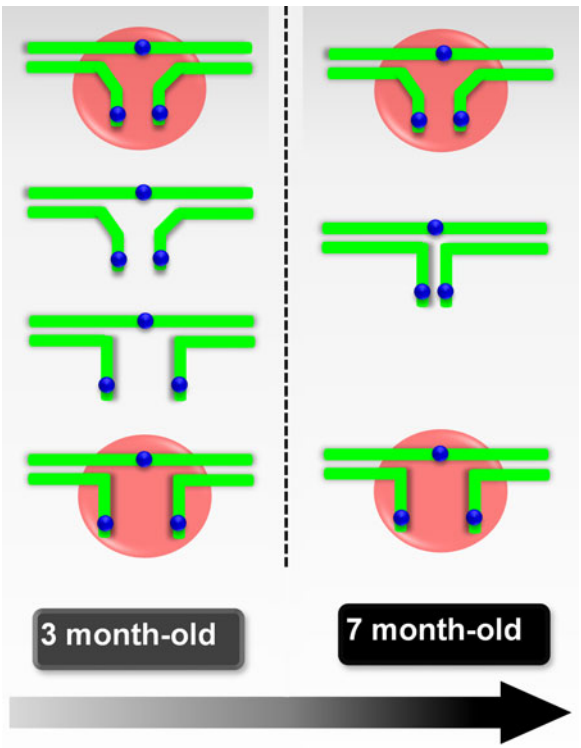


**Fig. 3** Synapsis and MSUC improvement during aging. In younger mice, in most trivalents, the heterologous region is either unsynapsed or partially synapsed, and it may or not show a γH2AX MSUC marker. In the oldest mice, most trivalents have the heterologous regions in synapsis, and those that are not are always γH2AX-positive. During aging, both POTs and OTs, either positive or negative to the MSUC marker, may complete their synapsis achieving a CT conformation. Also, γH2AX-negative POTs and OTs may acquire the MSUC marker. *Green lines*, axial elements of the synaptonemal complex; *blue circles*, centromeres; *red circles*, chromatin positive to γH2AX labelling, marker of MSUC

absence of unsynapsed regions negative to γH2AX labelling and the concomitant increase of CTs in older animals might be explained by the spreading of the synaptonemal complex from adjacent homologous regions that more easily drives heterologous synapsis (Schoenmakers et al. 2008) in those regions that are negative to MSUC markers. On the other hand, the presence of unrepaired meiotic DSBs might prevent synapsis (Schoenmakers et al. 2008; Manterola et al. 2009) of POTs and OTs as witnessed by the finding that in 7-month-old mice unsynapsed trivalents are all γH2AX-positive.

It is not known why a certain fraction of trivalents carrying unsynapsed chromosome regions are γH2AX-negative, a condition already encountered in *Spo-11* null pachytene spermatocytes (Mahadevaiah et al. 2008). A possible exhausting of MSUC factors cannot be considered a cause in our mouse model, as the degree of unsynapsis is lower than in other mouse models (Mahadevaiah et al. 2008; Kouznetsova et al. 2009). Moreover, sex bodies were always found positive to γH2AX labelling at all ages independently from the number of trivalents (positive or negative to γH2AX labelling) that were present in the meiocyte. It might also be that those POTs or OTs that are negative to γH2AX labelling do not escape MSUC, but instead they might be recognised by an, as yet, unknown MSUC factor/s acting during late pachytene/early diplotene stages, or, as in the fungus *Neurospora crassa*, by components of the RNAi machinery (Shiu et al. 2001; Shiu and Metzenberg 2002). However, we favour the idea that these trivalents appear negative because, having already accomplished the processes of DNA recombination/repair, they have not completely achieved the process of synapsis. Alternatively, synaptic adjustment might entail a phase of desynapsis that is not accompanied by MSUC initiation.

It has been proposed that asynapsis and MSUC may have dramatic consequences for meiosis progression. Silencing of autosomal genes that are crucial either for pachytene or subsequent spermatid survival (Turner et al. 2006) or of imprint defects (Saferali et al. 2010) might exert detrimental effects on spermatogenesis. Also, MSUC can interfere with the correct X chromosome inactivation, thus contributing to spermatogenesis impairment (Homolka et al. 2007). The quantitative results that we have obtained show that both synapsis and MSUC processes are better accomplished during male aging. In older animals, more trivalents complete synapsis and those that do not achieve it are always positive to the MSUC marker that we used (Fig. 3). This meiotic behaviour could partially account for the improvement of spermatogenic performance with age. However, while the variations in GCD rank from 64% to 58% between 3- and 7-month-old mice, the reduction of unsynapsed or MSUC-negative trivalents is much higher. This suggests that if there is a relationship between GCD and synapsis performance, improvement of synapsis has little effect on meiosis progression in Rb heterozygotes. Thus, spermatogenic failure in these mice is unlikely a consequence of a pachytene checkpoint. The presence of trivalents, while little affecting the prophase of meiosis, exerts its detrimental effects at later stages, mainly MI and MII (Merico et al. 2008; Manterola et al. 2009). A delay of anaphase initiation, due to difficulties of trivalents to achieve a correct orientation on the meiotic spindle, may trigger metaphase checkpoints (Nicklas et al. 1995, 2001; Eaker et al. 2001) leading to GCD. On the other hand, it must be remarked that pericentromeric heterochromatin is one of the main structural constituents of the unsynapsed regions. Therefore, the chromatin modifications that are produced during MSUC, reflected by the presence of γH2AX, would not necessarily have a negative impact on the cell transcriptional status and, as a consequence, on meiosis progression towards cell division (Manterola et al. 2009; Searle 1993). In addition, unsynapsed chromosome regions negative to γH2AX might contribute with correct gene expression to the progression of spermatocytes toward MI and MII.

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