Mutations that affect meiosis in male mice influence the dynamics of the mid-preleptotene and bouquet stages


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ABSTRACT

Meiosis pairs and segregates homologous chromosomes and thereby forms haploid germ cells to compensate the genome doubling at fertilization. Homologue pairing in many eukaryotic species depends on formation of DNA double strand breaks (DSBs) during early prophase I when telomeres begin to cluster at the nuclear periphery (bouquet stage). By fluorescence in situ hybridization criteria, we observe that mid-preleptotene and bouquet stage frequencies are altered in male mice deficient for proteins required for recombination, ubiquitin conjugation and telomere length control. The generally low frequencies of mid-preleptotene spermatocytes were significantly increased in male mice lacking recombination proteins SPO11, MEI1, MLH1, KU80, ubiquitin conjugating enzyme HR6B, and in mice with only one copy of the telomere length regulator Terf1. The bouquet stage was significantly enriched in Atm−/−, Spo11−/−, Mei1m1/c/m1a/ Mlh1−/−, Terf1−/− and Hr6b−/− spermatogenesis, but not in mice lacking recombination proteins DMC1 and HOP2, the non-homologous end-joining DNA repair factor KU80 and the ATM downstream effector GADD45a. Mice defective in spermiogenesis (Tnp1−/−, Gmcl1−/−, Asm−/−) showed wild-type mid-preleptotene and bouquet frequencies. A low frequency of bouquet spermatocytes in Spo11−/−Atm−/− spermatogenesis suggests that DSBs contribute to the Atm−/−-correlated bouquet stage exit defect. Insignificant changes of bouquet frequencies in mice with defects...
in early stages of DSB repair (Dmc1<sup>−/−</sup>, Hop2<sup>−/−</sup>) suggest that there is an ATM-specific influence on bouquet stage duration. Altogether, it appears that several pathways influence telomere dynamics in mammalian meiosis.

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Introduction

Meiosis is a succession of two cell divisions that generates genetically diverse haploid gametes (or spores) to cope with the genome doubling at fertilization. During the extended prophase to the first meiotic division homologous chromosomes (homologues) first pair and then segregate from each other. Subsequently, without an intervening round of DNA replication, the meiosis II division segregates sister chromatids and thereby creates haploid gametes or spores. In the majority of sexually reproducing eukaryotes, homologous pairing starts after premeiotic DNA replication, when the DNA loops of sister chromatids have attached to proteinaceous axial elements (AEs/cores) [1] whose ends firmly attach to the nuclear envelope during leptotene stage [2,3]. During the same stage, meiotic recombination commences with breaks in double stranded DNA (DSBs) that are introduced by the conserved transesterase SPO11 [4–6]. DSBs are processed to create single stranded DNA ends that are instrumental for homology search and recognition in many organisms (for reviews see [7–9]).

Leptotene telomeres move along the nuclear envelope (NE) and accumulate in a limited region of the nuclear periphery (bouquet formation) concomitantly with the onset of homologue pairing at the leptotene/zygotene transition. Bouquet formation is thought to serve as a recombination-independent mechanism for the instigation of homologue interactions by bringing about the close proximity of chromosome ends (for review see, [10,11]). Telomere clustering may also contribute to telomere size adjustment that involves the recombination machinery [12]. Telomere function has been found important for recombination and synapsis [13,14]. In mammalian species, the duration of the bouquet stage, or chromosome polarization, varies between genders [15–17] suggesting that its duration, like the progress of DSB repair, is subject to surveillance, the nature of which is largely enigmatic.

In prophase I of the male mouse, telomere clustering occurs in a time window limited to the onset of zygotene, which only leaves a few such cells detectable (~0.5% of spermatocytes) in testes suspensions [2,18,19]. This feature is altered in mice deficient for ATM [20,21], a core element of the DNA damage surveillance machinery responsive to DNA damage by DSBs [22]. Atm<sup>−/−</sup> mice exhibit disrupted meiosis that relates to defects in DNA repair [23,24]. Atm<sup>−/−</sup> males show a dramatic increase in the frequency of bouquet stage spermatocytes among total spermatocytes [19,25], raising the possibility that exit from the bouquet stage is delayed in the absence of this DSB-responsive kinase and/or its signals. Furthermore, normal bouquet exit requires the downstream ATM effector histone H2 variant H2A.X [26], as absence of H2AX in mice induces, among other defects [27], a significant increase of the frequency of bouquet cells and the extension of telomere clustering into pachytene [18]. Meiotic telomere clustering is also extended, but to a lesser extent, in Sycep3<sup>−/−</sup> males that lack axial elements and are defective in recombination [2,28]. On the other hand, mice deficient in the SMC1β cohesin subunit are so far the only mammalian mutants with defective telomere attachment and bouquet formation [29].

Telomere clustering is also a fleeting event in other species with synaptic meiosis. Increased frequencies of bouquet meiocytes have been observed in meiotic cultures of budding yeast mutants that fail to form DSBs (spo11Δ; [30]), or have defects in recombinational repair (rad50S, rec8Δ) [31–33]. Similarly, Spo11 mutants of Sordaria undergo extended telomere clustering [34], suggesting that the absence of, or defects in recombination slows bouquet stage exit in these species. In the nematode C. elegans, the phenotype of chk-2 and syp-1 mutants suggests that chromosome polarization is regulated through DNA-damage responsive kinases [35]. In all, it appears that the installation of telomere clustering is independent of induction of recombination but that initiation or progress in DSB repair may in some way contribute to the timely resolution of meiotic telomere clustering [34,36].

In mouse spermatogenesis, there is a peculiar change of nuclear topography at the onset of prophase I that can be visualized by centromere/telomere (tel/cen) FISH co-staining [37]. Such nuclei are characterized by a perinucleolar sheet of major satellite DNA and intranuclear telomeres and contain replicated repetitive target sequences. Since it is likely that they have completed premeiotic DNA replication, these have been termed mid-preleptotene spermatocytes (late preleptotene spermatocytes fail to exhibit SYCP3 threads but have perinuclear telomeres and major satellite clusters) [37]. Here, we used male mice with defects in the initiation and progression of recombinational DSB repair, ubiquitin conjugation, in telomere integrity and spermiogenic functions to investigate by molecular cytology whether and how bouquet and mid-preleptotene stage duration responds to these conditions. Specifically, we investigated mice with (i) absence of DSBs and recombination, i.e. Spo11<sup>−/−</sup> mice [38], or defects in recombination, like Mei1<sup>Δm1CS/m1CS</sup> (hereafter referred to as Mei1<sup>−/−</sup> [39]; Dmc1<sup>−/−</sup> [40,41]; Hop2<sup>−/−</sup> [42] and Mlh1<sup>−/−</sup> mice [43]), (ii) mice that are defective for the non-homologous end joining (NHEJ) repair pathway, i.e. SCID (severe combined immunodeficiency; [44] and Ku80 knockout mice [45]) and (iii) Gadd45α<sup>−/−</sup> mice that are deficient in the p53-dependent DNA damage response downstream of ATM [46,47]. Moreover, we examined mice deficient for ubiquitin conjugating enzyme HR6B that have damaged synaptonemal complexes (SCs) near telomeres and show defects in recombination [48], as well as Terf1<sup>−/−</sup> heterozygous mice that carry one inactivated gene encoding the TERF1 (TRF1) telomere repeat binding factor, as Terf1<sup>−/−</sup> homozygous mice experience embryonic lethality [49]. For comparison, we also analyzed Asm<sup>−/−</sup> [50], Gmcl1<sup>−/−</sup> (also known as mgcl-1) [51] and Tnp1<sup>−/−</sup> [95] mice, which all are infertile due to defects in sperm differentiation.
Materials and methods

Mice and testicular specimens

Mouse spermatocytes were obtained from testes of mice of about 4 weeks p.p. to exclude potential differences in the onset of the first meiotic wave. Genotyping was done as described in the references given in Table 2. Animals were anaesthetized and sacrificed by cervical dislocation. Testes were immediately resected and shock-frozen for 5 min in 2-methyl-butane (Sigma) on dry ice and stored at −80°C until further use [19]. Storage for more than 1 year did not adversely influence the performance of the samples in FISH or IF.

Irradiation

Mice were subjected to whole-body irradiation in a Nordion Gamma Cell 40 (137-Cs source). Mice received 3 Gy at a dose of 78 rad per minute. The irradiated animals were euthanized 26 h after treatment and their testes collected as described above.

Testes suspensions and fluorescence in situ hybridization

Preparation of testis suspensions containing structurally preserved nuclei for fluorescence in situ hybridization (FISH) and spermatocyte spreads, as well as FISH to telomeres and major satellite DNA of mouse pericentromeres were carried out as described [2]. An oligonucleotide specific to the major satellite carrying a 5′ Cy5 label and 7-mer biotin-T2AG3′ and biotin-C3TA2′ telomere repeat-specific DNA oligonucleotides were obtained from a commercial supplier (Invitrogen) and used as described above.

Immunofluorescence staining

The following antibodies were used: a monoclonal antibody against γ-H2AX (Biomol); an antiserum to testis-specific histone H1t [53], kindly provided by P. Moens (York University, York, ON, Canada); an antiserum to the SC protein SYCP3 [54] was a gift of C. Heyting (Wageningen University, Wageningen, Netherlands). The antisera were used at 1/1000 dilution in PBTG (PBS, 0.05% Tween 20, 0.2% bovine serum albumin, 0.1% gelatin). All antibodies were tested in individual staining reactions for their specificity and performance. Secondary antibodies to the primary ones, conjugated with different fluorochromes were from Dianova [19]. Immunostaining experiments were performed as described previously [2].

Microscopic evaluation

Microscopic evaluation was done by manually counting the cells in a Zeiss Axioskop I epifluorescence microscope (Carl Zeiss) equipped with a double band pass filter for green and red excitation (Chroma) and 63× and 100× Neofluar lens (C. Zeiss) [2]. Haploid, premeiotic and disrupted nuclei were excluded from the evaluation. All slides were evaluated by the same experienced investigator (B.L.) to minimize individual bias and because this type of analysis showed a high level of reproducibility in his hands. Images were recorded using the ISIS image analysis system (MetaSystems).

Statistics

For comparison of wild types and mutants, chi square tests were used. The Hochberg-Benjamini’s false discovery rate (FDR) procedure [46] was used for adjustment of p values, since this approach is more powerful than the Bonferroni method. Statistical significance was accepted, when the Hochberg-Benjamini-adjusted p value was less than 0.05. The FDR multiplies the i-th smallest p value by a factor m/i, where m is the total number of independent significance tests; m was set here to 36, the total number of genotypes tested for alterations in the two substages of prophase I and assuming that the “fold-increase” values between the two substages are not correlated (not all tests done are listed in Table 2). For example, several outcomes of p<0.0001 would be adjusted to <0.0036, <0.0018 etc., so that in any case p<0.005 is a valid assumption for a highly significant difference. For comparison of the tight bouquet frequencies, we used Poisson confidence intervals of Crow and Gardner [55], because of the low frequencies in the control mice.

Results and discussion

Mid-preleptotene and bouquet stages are rapidly traversed in wild-type spermatogenesis

The onset of first meiotic prophase sees a dramatic reorganization of nuclear topology and chromosome structure, which in mammals can be quantitatively monitored by centromere/telomere (cen/tel) FISH to testicular preparations (Fig. 1), a technique that facilitates the screening of a large number of cells required for bouquet analysis [2,19]. The conspicuous clustering of telomeres and pericentric major satellite DNA can be used to monitor the abundance of the bouquet stage (as the percentage of total spermatocytes) in mouse spermatogenesis, where tight clustering of peripheral telomeres only briefly installs at the leptotene/zygote transition coinciding with the onset of synaptic chromosome pairing [37]. As synopsis progresses in the wild-type telomere clustering becomes more relaxed and has disappeared by the end of zygote. Below we use the term ‘bouquet’ to address nuclei with telomeres clustered to one side of the nuclear periphery. This category includes nuclei with tight and more relaxed association of telomeres (Figs. 1C–E).

Entry into prophase I, on the other hand, can be deduced from the frequency (percentage of this substage among total spermatocytes) of so-called ‘mid-preleptotene’ nuclei that upon cen/tel FISH display a conspicuous layer of major satellite DNA/heterochromatin lining the nuclear envelope, while a subset of telomeres locates in the nuclear interior (Fig. 1B) [37]. It should be noted that our mid-preleptotene category is only detectable by cen/tel FISH and will be a substage of the classic preleptotene stage that follows premeiotic S-phase up to the point where AEs develop (leptonema) [56, 57]. We used the frequency of mid-preleptotene and bouquet stage nuclei, as deduced from the percentage of these cells among total
spermatocytes, to determine the impact of the disruption of recombinational DSB repair and other chromosome related functions on meiotic centromere and telomere dynamics during earliest prophase I.

First, we determined the percentage of mid-preleptotene and bouquet spermatocytes among total spermatocytes of wild-type mice from different strains by cen/tel FISH to testes suspensions. The use of testes suspensions facilitates FISH scoring of a large number of nuclei directly down the microscope [2], which is required for the study of such a short-lived stage in fixed preparations. In our hands, FISH analysis of testes suspensions proved superior to tissue sections where closely-spaced and overlapping nuclei often complicate the recognition of bouquet stage spermatocytes (H.S., unpublished). Cen/tel FISH analysis of wild-type mice (e.g. C57BL6, 129SvJae/ Black Swiss) revealed that testis suspensions on average contain 0.56% (±0.42 SD) mid-preleptotene spermatocytes and 0.42% (±0.16 SD) bouquet spermatocytes among total spermatocytes (n=33,631; Table 1).

Tight telomere clustering (Figs. 1C, D), which occurs only at the leptotene/zygotene transition, was observed in 0.07% (±0.02 SD) of total wild-type spermatocytes (n=33631). Similar frequencies were obtained when mid-preleptotene and bouquet frequencies of testes suspensions of the different mouse strains were considered individually (Table 1). The values obtained also match with earlier cen/tel FISH analysis [2,37,58] and corroborate the view that mid-preleptotene and bouquet stages are short-lived in wild-type males. To exclude potential genotype differences in mutant analyses, we subsequently compared mutant frequencies with the respective isogenic wild-type values (Tables 1 and 2). Statistical evaluation was done using Hochberg-Benjamini’s false discovery rate (FDR) procedure for adjustment of p values [59], since this approach is more powerful than the Bonferroni method (see Materials and Methods).

Mice deficient for recombination proteins show increased frequencies of mid-preleptotene and bouquet stage spermatocytes

Next, we analyzed mutants that have impaired fertility and failure to induce or complete recombinational DSB repair (Table 2). Where possible, the percentage of mid-preleptotene and bouquet spermatocytes was determined among at least 4000 successfully hybridized spermatocyte nuclei per genotype that were stained by cen/tel FISH (see Materials and Methods). An increased frequency (%) relative to the isogenic wild-type frequency was interpreted to indicate an extension of that particular substage [2,60]. Previous cen/tel FISH analyses in Lmna−/− mice, which lack A-type lamins, revealed significantly elevated mid-preleptotene frequencies but wild-type-like bouquet frequencies [58]. On the other hand, Sycp3−/− mice, which lack AEs because of deficiency for the SYCP3 (SCP3) AE protein [28], display wild-type mid-preleptotene but significantly increased percentage of bouquet spermatocytes [2]. The increases were highly significant.
The bouquet stage has resolved, i.e. in early pachytene and like Lmna using the statistics of this investigation (not shown).

<table>
<thead>
<tr>
<th>Table 1 – Frequencies of mid-preleptotene, total and bouquet spermatocytes in different wild-type strains</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT mice</strong></td>
</tr>
<tr>
<td>Spoo11+/+Atm+/+</td>
</tr>
<tr>
<td>Hop2 WT</td>
</tr>
<tr>
<td>Snc1b WT</td>
</tr>
<tr>
<td>Average C57BL/6b</td>
</tr>
<tr>
<td>Lmna WT</td>
</tr>
<tr>
<td>Gadd45a WT</td>
</tr>
<tr>
<td>Tnp1 (3814) WT</td>
</tr>
<tr>
<td>Atn WT</td>
</tr>
<tr>
<td>Gmc1 WT</td>
</tr>
<tr>
<td>Dmc1 WT</td>
</tr>
<tr>
<td>Average Bl6x129/SVc</td>
</tr>
<tr>
<td>FVB/N</td>
</tr>
<tr>
<td>1295V/PAS</td>
</tr>
<tr>
<td>C.B17</td>
</tr>
<tr>
<td>12956/SVEvTac</td>
</tr>
</tbody>
</table>

- Tight bouquets are those whose telomere signals are tightly associated, while total bouquets include telomeres distributed more widely over approximately one third of the NE.

Using the statistics of this investigation (not shown), Sycp3−/− and Lmna−/− mutants both undergo apoptosis in stages after the bouquet stage has resolved, i.e. in early pachytene or equivalent stages [2,28,58]. Together, these findings suggest that an increased frequency in one stage does not lead to a similar or inverse effect in the other stage, which may relate

Table 2 – Fold increase in mid-preleptotene and bouquet spermatocytes in spermatogenesis of knockout mice relative to respective isogenic wild type

<table>
<thead>
<tr>
<th>Genotype (isogenic WT see Table 1)</th>
<th>% mPL spermatocytes: (fold increase over isogenic wild type)</th>
<th><strong>p value</strong></th>
<th>% total bouquet spermatocytes: (fold increase over isogenic wild type)</th>
<th><strong>p value</strong></th>
<th>H1t-IF</th>
<th>n</th>
<th>Mutant Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atm−/−(11)</td>
<td>1.34 (1.2)</td>
<td>0.62*</td>
<td>9.55 (13.7)/4.33</td>
<td>&lt;0.0036*</td>
<td>–</td>
<td>4022</td>
<td>[21]</td>
</tr>
<tr>
<td>Spoo11−/−(1)</td>
<td>1.96 (3.3)</td>
<td>0.025*</td>
<td>1.89 (2.4)/0.62</td>
<td>0.096*</td>
<td>–</td>
<td>4025</td>
<td>[38]</td>
</tr>
<tr>
<td>Spoo11−/−(2)</td>
<td>1.72 (1.9)</td>
<td>0.020*</td>
<td>1.95 (5.1)/0.55</td>
<td>&lt;0.0036*</td>
<td>–</td>
<td>4008</td>
<td>[64]</td>
</tr>
<tr>
<td>Spoo11−/− IR(3),f</td>
<td>0.95 (1)</td>
<td>0.93*</td>
<td>2.81 (7.4)/0.67</td>
<td>&lt;0.0036*</td>
<td>–</td>
<td>4021</td>
<td>[64]</td>
</tr>
<tr>
<td>WT IR(3)</td>
<td>0.32 (0.7)</td>
<td>0.44</td>
<td>0.64 (3.3)/0.22</td>
<td>0.0399</td>
<td>+</td>
<td>4079</td>
<td>[64]</td>
</tr>
<tr>
<td>Spoo11−/−Atm−/−(7)</td>
<td>0.75 (0.8)</td>
<td>0.46*</td>
<td>1.42 (1.9)/0.42</td>
<td>0.0066*</td>
<td>–</td>
<td>4022</td>
<td>[100]</td>
</tr>
<tr>
<td>Mel1−/−(2)</td>
<td>1.51 (1.8)</td>
<td>0.019*</td>
<td>2.02 (3.1)/0.72</td>
<td>0.0072*</td>
<td>–</td>
<td>4053</td>
<td>[39]</td>
</tr>
<tr>
<td>Dmc1−/−(6)</td>
<td>3.24 (1.8)</td>
<td>0.089*</td>
<td>1.19 (1)/0.10</td>
<td>0.98*</td>
<td>–</td>
<td>4044</td>
<td>[40]</td>
</tr>
<tr>
<td>Hop2−/−(2)</td>
<td>1.49 (1.6)</td>
<td>0.088*</td>
<td>2.27 (0.7)/n.d.</td>
<td>0.58*</td>
<td>–</td>
<td>4101</td>
<td>[42]</td>
</tr>
<tr>
<td>Mlk3−/−(2)</td>
<td>1.56 (3.4*)</td>
<td>&lt;0.0036</td>
<td>1.38 (3.7)/0.52</td>
<td>&lt;0.0036</td>
<td>+</td>
<td>4045</td>
<td>[43]</td>
</tr>
<tr>
<td>PrkdEcked/eckid (10)</td>
<td>1.19 (1.6)</td>
<td>0.092</td>
<td>0.72 (2.4)/0.12</td>
<td>0.016</td>
<td>+</td>
<td>4021</td>
<td>[101]</td>
</tr>
<tr>
<td>Ku80−/−(7)</td>
<td>1.16 (2.5*)</td>
<td>&lt;0.0036</td>
<td>0.42 (1.1)/0.05</td>
<td>0.83</td>
<td>+</td>
<td>4048</td>
<td>[87]</td>
</tr>
<tr>
<td>Gadd45a−/−(4)</td>
<td>0.87 (1.8)</td>
<td>0.33</td>
<td>0.32 (0.5)/0.1</td>
<td>0.34</td>
<td>+</td>
<td>4029</td>
<td>[46]</td>
</tr>
<tr>
<td>Terf1−/−(9)</td>
<td>2.05 (3.3*)</td>
<td>&lt;0.0036</td>
<td>1.00 (2.7)/0.07</td>
<td>&lt;0.0036</td>
<td>+</td>
<td>4098</td>
<td>[49]</td>
</tr>
<tr>
<td>Hr69−/−(39)</td>
<td>1.95 (2.3*)</td>
<td>&lt;0.0036</td>
<td>1.29 (3.7)/0.27</td>
<td>&lt;0.0036</td>
<td>+</td>
<td>4041</td>
<td>[48]</td>
</tr>
<tr>
<td>Asm1−/−(5)</td>
<td>0.42 (1.0)</td>
<td>1</td>
<td>0.52 (1.6)/0.22</td>
<td>0.59</td>
<td>+</td>
<td>4036</td>
<td>[94]</td>
</tr>
<tr>
<td>Gmc1−/−(5)</td>
<td>0.27 (0.7)</td>
<td>0.54</td>
<td>0.45 (1.6)/n.d.</td>
<td>0.35</td>
<td>+</td>
<td>4041</td>
<td>[51]</td>
</tr>
<tr>
<td>Tnp1−/−(7)</td>
<td>0.52 (1.1)</td>
<td>0.80</td>
<td>0.35 (0.9)/0.10</td>
<td>0.84</td>
<td>+</td>
<td>4027</td>
<td>[95]</td>
</tr>
</tbody>
</table>

Superscripts numbers in column 1 refer to the indexed respective wild-type frequencies shown in Table 1.

- Increase of mid-preleptotene (mPL) and bouquet spermatocyte frequencies over corresponding isogenic wild-type frequencies (see Table 1).
- p values adjusted according to the Hochberg-Bejamini method (see Materials and methods).
- Tight bouquets are those whose telomere signals are tightly associated, while total bouquets include telomeres distributed more widely over one third of the NE.
- H1t expression as determined by IF to testes suspensions (see Fig. 2).
- p values of the LR-test taking into account a reduction factor of 0.5 for spermatocytes in the rec− mutants that are H1t-negative (H1t-IF−) due to a demise of spermatocytes in mid-pachytene (see respective ref.).
- Bouquet frequency (%) in irradiated Spoo11−/− tests suspensions is significantly increased over untreated Spoo11−/− from the same strain.

Asterisks mark significant differences according to χ² statistics: *p<0.05; **p<0.005 (see Materials and methods). n.d., not detected.
to the fact that we analyzed 4-week-old mice that displayed non-synchronous meiosis and showed no arrest in the stages monitored.

Spermatogenesis in other recombination-defective mice is often disrupted after an altered zygotene-like stage (based on AE/SC morphology) with apoptosis usually occurring during stage IV of spermatogenesis that is equivalent to mid pachytene [24,61]. The loss of post-mid-pachytene spermatocytes in mutants with stage IV apoptosis will inflate the number of earlier substages among the remaining spermatocytes of a testis suspension and relative to wild type, which in turn complicates comparisons between data derived from testes with undisrupted versus arrested spermatogenesis. To compensate for such a deviation in the mutants studied, and because mid-preleptotene and bouquet stage spermatocytes I do not express histone H1t [19], we investigated for H1t expression by immunofluorescence (IF). H1t is detectable from mid-pachytene (post stage IV) onwards and marks progression of meiotic differentiation beyond this substage [53,62,63]. IF staining for histone H1t immunofluorescence was, in our hands, absent from testis suspensions of the Atm−/−, Spo11−/− [64], Mei1−/−, Dmc1−/− and Hop2−/− recombination mutants (Fig. 2; Table 2), which agrees with stage IV apoptosis in these mutants [24, 61]. It is of interest that the mice of Baudat et al. [38] express H1t [24], while the Spo11−/− mice of Romanienko and Camerini-Otero [64], failed to show H1t expression in our assay (Table 2). This discrepancy may relate to a progression further into prophase I in the former mice due to strain differences. While differences with the Barchi study cannot be excluded for technical reasons, a difference in telomere cluster resolution between the two strains (see below) also points to a strain-specific prophase I progression in the studied Spo11−/− mice.

Histone H1t-expressing cells make up to ∼50% of spermatocytes in a testes suspension [59] and mid-preleptotene and bouquet stages are found in the H1t-negative spermatocyte fraction [19]. Thus, we compensated for loss of H1t-expressing late prophase I stages in the recombination mutants with spermatogenesis arrest and stage IV apoptosis by adjusting the wild-type mid-preleptotene and bouquet frequencies with that of knockout mice by multiplying the number of wild-type spermatocytes with a correction factor of 0.5 before comparing them to isogenic mutant values (doubling the frequency of the monitored stages yielded the same results, but was not followed for statistical reasons).

**SPO11 is required for rapid passage through mid-preleptotene and bouquet stages**

Meiotic recombination is initiated by DSBs that are introduced into leptotene DNA by the conserved SPO11 transesterase (for review see [6,8]). Cen/tel FISH analysis of testes suspensions of two different Spo11−/− mouse strains [38,64] disclosed increased frequencies of mid-preleptotene nuclei in testes suspensions of both Spo11 mutant strains analyzed (Table 2). This increase, with or without correcting for loss of post mid-pachytene spermatocytes, was significant relative to wild type (Table 2) suggesting that the normal transition of the post-replicative mid-preleptotene stage benefits from presence of SPO11.

An increased frequency of bouquet nuclei has been observed in Spo11 mutants of budding yeast [32] and Sordaria [34] that both lack DSBs and recombination, suggesting a link between DSB formation and the exit from meiotic telomere clustering. Previous investigation showed that telomere functions influence synapsis and recombination in mice [14]. In Spo11−/− mice we observed a 2.4- or 5.1-fold increase (depending on strain background) in the frequency of bouquet spermatocytes relative to corrected wild type (Table 2). When comparing the frequencies of spermatocytes with tight telomere clustering (which only occurs at the onset of zygotene), we noted a similar increase in both mutants (Fig. 3). This suggests that duration of the bouquet stage in the mouse also responds to absence of DSBs and/or the SPO11 protein. The latter possibility deserves further investigation, e.g. in a catalytically dead Spo11 mutant.

Fig. 2 – Histone H1t-IF staining (red) on wild-type (A) and Hop2−/− (B) testicular suspensions. H1t immunofluorescence is only detected in the wild type. The small nuclei represent haploid round spermatids (arrows) and the large nuclei spermatocytes (arrowheads). (B) Hop2−/− spermatogenesis, like that of all other rec− mutants, lacked H1t IF (except for Mlh1−/−) and haploid cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
We also noted that the Spo11−/− mice of Romanienko and Camerini-Otero [64] displayed a significant 5.1-fold increase over corrected wild type, while the 2.4-fold increase of bouquet spermatocytes in the Spo11−/− mice of Baudat et al. [38] was insignificant, indicating strain-dependent aspects with respect to bouquet stage extension. Slight strain-dependent differences have also been noted in another study [24]. However, relative to average wild type, both strains showed highly significantly increased bouquet frequencies (not shown). Thus, like in other species, commencement of meiotic telomere clustering and recombination are independent events in mammalian meiosis. Furthermore, the fold increase in Spo11−/−-induced bouquet stage frequency mirrors the situation in Spo11-mutant meiosis of fungi [32,34], which suggests that DSBs contribute to rapid passage of the bouquet stage in the mouse too.

The relatively moderate increase of bouquet frequencies as compared to Atm or H2ax (now termed H2afx) mutants [Fig. 3 and below; [18]) suggests that DSB formation per se is not a major requirement for exit from the mouse bouquet stage.

**DSBs induce prolonged telomere clustering in absence of ATM**

Inactivation of the ATM DNA-damage responsive kinase leads to the most dramatic increase in percentage of bouquet spermatocytes with respect to all mouse mutants analyzed for telomere clustering to date, suggesting that ATM is a major factor required for rapid exit from the bouquet stage in the male mouse (reviewed in [60]). Like Spo11−/− mice, Atm−/− mice suffer from impaired gametogenesis and undergo stage IV apoptosis but with a more severe phenotype [23,24]. To see whether DSBs contribute to bouquet extension in absence of ATM, we analyzed prophase I entry and bouquet stage duration by cen/tel FISH to testis suspensions in Atm−/− and Atm−/−Spo11−/− mutants. In the strain background studied in this investigation, we noted a >10-fold Atm−/−-induced bouquet increase over corrected wild-type values (Table 2). Previous investigation in the C57BL/6X129/Sv mixed background revealed a ~20-fold increase in bouquet spermatocytes [19,25]. However, tight bouquet frequencies between the two strains were similar (3.2% and 2.2%; Fig. 3) and highly significantly increased relative to wild type, suggesting that the impact of the Atm mutation is similarly affecting tight bouquet formation in both strains, while there is a difference in the delayed telomere cluster resolution between the two strains, also indicating a strain difference in prophase I progression in this mutant.

To determine the contribution of DSBs to telomere cluster arrest in the Atm−/− condition, we next investigated the double mutant. The Atm−/−Spo11−/− heterozygote was similar to the Spo11−/− single mutant (not shown). The Atm−/−Spo11−/− double mutant displayed only a 1.9-fold increase of total bouquet spermatocytes relative to corrected wild type (Table 2). This is significantly less than the 13.7-fold and the 5.1-fold increase in the isogenic Atm−/− and Spo11−/− single mutant, respectively. Similar frequencies of tight bouquet spermatocytes were observed in the Atm−/−Spo11−/− and Spo11−/− mutants (Table 2, Fig. 3) indicating that the Spo11 mutation is epistatic to Atm. These results suggest that DSBs are required to elicit the Atm−/−-dependent accumulation of bouquet spermatocytes in male mouse mutants.

Like Atm−/− mice, Dmc1−/− mice show severe gametogenesis defects [23,24]. However, Dmc1−/− spermatogenesis displayed...
wild-type bouquet frequencies, suggesting that ATM contributes to a telomere-specific feature of DSB repair that in particular influences telomere dynamics. Interestingly, in somatic cells, ATM has been shown to directly interact with the telomere repeat binding protein TERF2, which alters the read out of the DNA damage response [65].

**Exogenous DSBs extend telomere clustering in mice**

The observations above and previous studies raised the possibility that bouquet stage exit duration could be coupled with progression of DSB repair [15,32,34]. If so, we reasoned that delivery of exogenous DSBs should extend the short-lived bouquet stage of male mice. To test for this possibility, we exposed wild-type and Spo11−/− mice that fail to form programmed DSBs, to 3 Gy of γ-irradiation. This dose, which is well below the lethal dose of 8 Gy and is tolerated by rodent spermatocytes [66], will deliver approximately 120 DSBs to a mammalian nucleus [67,68]. Testes were collected 26 h post-irradiation, which should give ample time either for bouquet exit or extension, given that the minimal time window for telomere clustering in the mouse may comprise ~2 h. The latter being a rough estimate based on a prophase of 336 h [69] and a mean wild-type bouquet frequency of 0.5% of total spermatocytes (Table 1). More accurate timing, however, will require further analysis.

Cen/tel FISH to testes suspensions from γ-irradiated wild-type and Spo11−/− testes disclosed a highly significant increase of the percentage of bouquet stage spermatocytes relative to non-irradiated isogenic Spo11+/+ or Spo11−/+ mice, while the percentage of mid-preleptotene frequencies was similar (Table 2). Comparing the frequencies of bouquet nuclei with tight telomere clustering revealed closely matching values in irradiated and non-irradiated mice, while the former contained significantly more nuclei with relaxed telomere clustering. This indicates that the time spent in the later phase of the bouquet stage is extended by exogenous DSBs, being consistent with a timely entry but delayed exit from telomere clustering. Moreover, we observed partially improved synapsis and substantial histone H2AX phosphorylation in the γ-irradiated Spo11−/− spermatocytes (Fig. 4), which suggests that exogenous DSBs can at least partially ameliorate prophase I defects of the Spo11 mutation, which is reminiscent of the situation induced by treatment of Spo11−/− mice with the DSB-seeding drug Cisplatin [64].

Our observations indicate that the delivery of unscheduled, exogenous DSBs, and possibly their repair, is capable to significantly extend bouquet stage duration in spermatogenesis of the male mouse. Alternatively, there is the possibility that irradiation may have caused a general extension of prophase I substages; however, we consider this possibility unlikely, since we observed similar mid-preleptotene frequencies and tight bouquet frequencies in irradiated and non-irradiated testes of wild-type and Spo11−/− mice (Table 2, Fig. 3). Furthermore, at the dose applied rodent spermatocytes efficiently repair irradiation-induced DSBs and do not undergo apoptosis [66,70,71].

In contrast to the situation in the mouse testis, irradiation of Sordaria spo11 mutants induced a reduction of the mutation-dependent high bouquet frequencies [34], which indicates a difference to the mammalian situation, which could relate to differences in prophase I duration and a high steady state level of bouquet frequency in Sordaria (5–10%, [34]), as compared the ~0.4% of wild-type mouse spermatogenesis (this investigation). In the mouse, the DSB-induced increase of bouquet spermatocyte frequencies is interpreted as a prolonged maintenance of the bouquet stage when exogenous DSBs are seeded into spermatocytes by irradiation, which is likely to induce an additional requirement for time for their processing. In Sordaria, DSB repair proceeds much faster and thus such requirements may not be readily evident. Maintenance of the bouquet stage may also protect chromosomes when these harbor DSBs in a stage that sees vigorous nuclear motility [33,72].

**Bouquet stage duration only responds to certain steps in recombination**

To see whether defective DSB repair processing impacts on bouquet stage duration, we investigated mid-preleptotene and bouquet stage frequencies in knockout mice with defects at different steps in recombinational DNA repair.

Mei1 is a gene required for completion of meiosis in both genders of the mouse [39]. Similar to Spo11−/− mice, spermatocytes of the Mei1−/− mouse cannot form DSBs, are defective in recombination and synopsis, and arrest during an extended zygotene-like stage [73]. Spermatocytes of Dmc1−/− mice form DSBs but are defective in their processing, display residual synopsis and undergo apoptotic arrest after an aberrant zygotene-like stage during stage IV of spermatogenesis [40].

The MLH1 mismatch repair protein, on the other hand, forms foci at the sites that correspond in number and position to location of crossovers [43,74,75]. Both female and male Mlh1−/− mice are infertile due to a failure to form reciprocal crossovers and normal spindles at the meiotic division, leading to cell death in the metaphase I stage [43,76]. GADD45a is a protein downstream of ATM that is required for maintenance of genome stability [47,77].

Cen/tel FISH to testes suspensions of the mice described above revealed that Mei1−/− and Mlh1−/− mice both display significantly increased mid-preleptotene and bouquet frequencies, while Dmc1−/−, Hop2−/− and Gadd45−/− mice are similar to wild type (Table 2, Fig. 3). Mei1−/− mice display similar defects in meiosis like Spo11−/− mice [73,78], which extended also to our analysis as we observed similarly increased frequencies of bouquet spermatocytes, both tight and more relaxed ones (Table 2, Fig. 3).

Mlh1−/− mice displayed significantly increased mid-preleptotene and bouquet stage frequencies among total spermatocytes relative to wild type (Table 2, Fig. 3), which is the case with and without correcting for apoptotic events (not shown). As Mlh1−/− cells die in metaphase I, and we only analyzed prophase I spermatocytes in our assay, we consider a correction for the obtained MLH1 frequencies as not necessary for the cen/tel-FISH assay. Absence of mismatch repair function of MLH1 has been shown to cause faulty S-phase checkpoint control [79]. Such a defect in premeiotic S-phase may lead to carry-over of DNA lesions, which could underlie the delayed passage through...
the mid-preleptotene stage. Alternatively/additionally, some yet unknown requirement for MLH1 may contribute to the observed defect. In this respect, it is noteworthy that MLH1 has been localized to pericentric heterochromatin of meiotic chromosomes in a Pms2−/− background [80]. Heterochromatin undergoes a dynamic redistribution during mid-prelep-

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Fig. 4 - Immunofluorescent staining for γ-H2AX (red) and SYCP3 (green) in spread wild type (WT), Spo11−/−, irradiated wild type (WT IR) and irradiated Spo11−/− (Spo11−/− IR) spermatocytes. (Ai) WT leptotene spermatocyte with weak SYCP3 staining and strong overall γ-H2AX staining. (Aii) WT early zygotene spermatocyte with large patches of γ-H2AX staining and SYCP3 threads. γ-H2AX patches persist at some SCs. The inset shows a non-spread bouquet spermatocyte at the nuclear equator with telomeres (green) clustered to the upper right (asterisk) and large patches of γH2AX. Scale bar: 5 μm. (Aiii) Early zygotene Spo11−/− nucleus with a few small γ-H2AX foci and long unpaired SYCP3-positive axial elements and clusters. (Bii) Advanced zygote Spo11−/− nucleus showing unpaired AEs persistent SYCP3 clusters and one large γ-H2AX patch (see [24] for details). (C) Irradiated WT spermatocyte showing the same γ-H2AX and SYCP3 pattern as non-irradiated leptotene spermatocytes. (Cii) Irradiated early zygotene WT spermatocyte with overall patchy γ-H2AX staining and SYCP3 threads. (Ciii) Irradiated leptotene spermatocyte with γ-H2AX staining of the XY body and some additional small radiation-induced γ-H2AX foci. (D) γ-Irradiated Spo11−/− spermatocytes. (Di) Leptotene Spo11−/− nucleus with several distinct γ-H2AX foci, SYCP3 AEs and a few SYCP3 patches. (Dii) Irradiated early zygotene Spo11−/− nucleus with several distinct γ-H2AX patches and SYCP3 threads reminiscent of wild-type zygote patterns. (Diii) Irradiated zygote-like Spo11−/− spermatocyte showing advanced synopsis and large γ-H2AX patches. Note the absence of SYCP3 patches and increase of nucleoid diameter in the irradiated zygote Spo11−/− spermatocytes. Scale bar: 10 μm.
tonema [37]. The increase in MLH1−/− bouquet frequencies, on the other hand, makes it attractive to speculate that the crossover pathway is linked to bouquet exit.

Interestingly, we observed that DMC1, HOP2 and GADD45a are dispensable for telomere cluster resolution as the respective mutants show wild-type-like frequencies (Table 2), with the statistical differences being non-significant using the isogenic or averaged wild-type frequencies. Particularly the wild-type bouquet frequency of Dmc1−/− mice distinguishes them from Atm−/− mice that otherwise show similarly severe gametogenesis defects [23,24]. The low bouquet frequency observed in the Hop2 mutant could indicate problems in formation of the telomere cluster, but conclusive evidence into this direction requires the evaluation of larger numbers of spermatocytes. In any case, our observations suggest that the Dmc1 and Hop2 mutants may fail to set the condition/s that couple telomere dynamics to recombinational DNA repair. Still, this and previous investigations revealed that DSBs in combination with ATM or H2AX deficiency dramatically alter bouquet stage duration [18,25], while delayed bouquet stage exit was much less pronounced in HR6B ubiquitin-conjugating protein and TERF1 telomere protein contribute to normal chromosome dynamics in prophase I

HR6B (UBE2B), a ubiquitin-conjugating enzyme, is homologous to yeast RAD6/UBC2 and involved in post replication DNA repair and sporulation in yeast [88]. In the mouse, HR6B is required for male fertility [89], with male Ube2b−/− mice (for simplicity called Hr6b−/− hereafter) being infertile due to sperm defects, while spermatocytes show altered chromatin and SC structure near telomeres as well as increased levels of recombination as deduced from MLH1 focus analysis [48]. Cen/tel FISH to Hr6b−/− testes suspensions revealed a highly significant increase in mid-preleptotene bouquet spermatocyte frequencies (Table 2). Both tight and more relaxed telomere cluster frequencies were significantly increased (Fig. 3), suggesting that HR6B ubiquitin-conjugating activity is also involved in promoting progression through mid-preleptotene and bouquet stages. Since Hr6b−/− spermatocytes are recombination proficient, one possibility is that the compromised SC structure at Hr6b−/− telomeres alters bouquet stage duration. It will hence be interesting to learn about the structure of meiotic Hr6b−/− telomeres at the EM level. Furthermore, it cannot be ruled out at this stage that there may be a slowed transit through the monitored prophase I stages.

Generation of a mouse that lacks the telomere length regulator protein TERF1 (also known as TRF1) [90] revealed that homozygous Terf1 mutation disrupts embryonic development, suggesting a role for TERF1 in addition to telomere metabolism [49]. TERF1 has been shown to interact with ATM [91] and defective telomeres induce a DNA damage response that involves the ATM pathway [65]. Investigation of Terf1−/− heterozygous mice by cen/tel FISH revealed significantly increased mid-preleptotene and bouquet stage frequencies (Table 2). Particularly bouquet stage spermatocytes with more relaxed telomere clustering were significantly enriched in Terf1−/− mice (Fig. 3) indicating a slowed bouquet exit in these mice. Synopsis and telomere/nuclear envelope attachment was undisturbed (data not shown). The extended mid-preleptotene and bouquet stages in Terf1−/− mice indicate that TERF1 copy number is important for a wild-type passage through these stages, one possibility being that TERF1 heterozygosity may alter early prophase I progression; dysfunctional telomeres in somatic cells can elicit a cell cycle response [92,93]. Alternatively, an altered protein composition of the meiotic telomere could be involved. Clearly, an issue that deserves more analysis.

Normal prophase I transit in mice with spermiogenic defects

Finally, we investigated knockout mice with defects in sperm chromatin remodeling or membrane alterations, (Asm−/−, Gmcl-1−/− and Tnp1−/−). Asm knockout mice lack the acid sphingomyelinase [94] and are reduced in fertility due to membrane defects in haploid cells [50]. Mice lacking the transition protein 1 (TNP1) are infertile due to a failure in sperm chromatin remodeling [95,96]. Gmcl-1 (mgcl-1) is the mouse ortholog of the Drosophila germ cell-less (gcl-1) gene. The protein localizes to the nuclear envelope through its
interaction with LAP2-β (lamina-associated polypeptide 2-beta), and Gmcl-1−/− mice display defective sperm chromatin condensation due to faulty expression of transition proteins and protamines [51]. LAP2β is a nuclear envelope-associated protein that could play a role in envelope structure and chromatin attachment. However, cen/te FISH to spermatocytes of Asm−/−, Gmcl-1−/− and Tnp1−/− showed normal telomere localization patterns as well as mid-preleptotene and bouquet spermatocyte frequencies (Table 2). Only in Asm−/− spermatogenesis there was a significant increase in tight but not of total bouquet frequencies. Altogether, it appears that defects in genes that only affect sperm differentiation seem not to grossly alter entry and progression through early prophase I substages, at least in the mice investigated here.

Conclusions

Meiotic telomere redistribution in mammals seems to be highly dynamic and the mid-preleptotene and/or bouquet stage is extended in the presence of disruptions in recombination or other functions of meiotic chromosome metabolism. With regard to DSB-repair defects, we observed significantly increased frequencies of mid-preleptotene spermatocytes in male mice lacking Spo11, MEI1, MLH1, KU80 DNA repair proteins, suggesting that the earliest stage of prophase I is extended in absence of these functions. It could be that these mutations alter the formation of pre-recombination chromatin that is thought to support homologous recombination [97–99].

Significantly elevated bouquet frequencies, on the other hand, were evident in ATM, Spo11 and MLH1-deficient mice, suggesting that bouquet exit is somehow coordinated with DSB repair, like has been suggested for Sordaria [34]. In the mouse it seems that proteins which function in early (SPO11, MEI1, ATM) or late (MLH1) steps of recombinational DSB repair, as well as delivery of exogenous DSBs, can delay telomere cluster exit. However, the absence of other molecules (DMC1, HOP2, NHEJ repair proteins) fails to set the stage for such a response. In comparison to the phenotype of the spo11−/−Y153F mutant of Sordaria [34] or the re3A cohesin mutant of budding yeast [33] the increased frequencies of bouquet stage spermatocytes in the responsive KO mice are still moderate. The only exception being the Atm−/− mutant that, in combination with physiological DSBs, elicits a major bouquet exit defect. ATM for instance may mediate its instigative role on telomere cluster resolution in part through its downstream effector histone H2AX [18] and possibly through an interaction with the telomere complex component TERF2 [65]. Furthermore, mutations in genes that influence chromosome and telomere metabolism (Hr6b, Terf1) also led to extended bouquet duration. Thus, it appears that several pathways can influence meiotic telomere dynamics in the mouse. It will thus be interesting to further unravel the regulatory puzzle at the mammalian meiotic telomere.

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References

[18] O. Fernandez-Capetillo, B. Liebe, H. Scherthan,


[27] A. Storlazzi, S. Tesse, S. Garnano, F. James, N. Kleckner, A. Butler, X. He, R.E. Gordon, H.S. Wu, S. Gatt,


