Programmed phosphorylation of histone H2AX precedes a phase of DNA double-strand break-independent synapsis in mouse meiosis

Josefa Blanco-Rodrı´guez

Departamento de Biologı´a Celular, Facultad de Medicina, Universidad de Valladolid, Ramón y Cajal 7, 47005 Valladolid, Spain

Correspondence should be addressed to J Blanco-Rodrı´guez; Email: jblanco@med.uva.es

Abstract

Accurate homologue synapsis during meiosis is essential for faithful chromosome segregation and formation of viable gametes. The finding of Spo11-dependent gamma-H2AX (γH2AX) formation during leptotene and data on mutant mice have led to the notion that synapsis in mammals depends on meiotic DNA double-stranded break (DSB) repair. A second wave of ataxia telangiectasia mutated (ATM) and Rad3-related (ATR)-dependent γH2AX formation has been observed in Atm-null mice during zygotene, suggesting that this wave of phosphorylation also occurs in normal mice. Here I aimed to confirm and to analyse in deep this wave of phosphorylation. Immunostaining of spread spermatocytes shows that γH2AX accumulates on the short last axis stretches to pair. This accumulation appears within all the nuclei undergoing a specific step of late zygotene and disappears from every spermatocyte immediately after pairing completion. This γH2AX signal co-localises with ATR, is Spo11-independent and does not co-localise with free DNA 3'-end labelling. I conclude that ATR/γH2AX asynapsis signalling at the end of zygotene belongs to a physiologically programmed pathway operating at a specific meiotic step, and I propose that this pathway is involved in the triggering of a phase of DSB-independent chromosome pairing that leads to synapsis completion in normal mouse meiosis.


Introduction

Faithful execution of genetic recombination, chromosome pairing and synapsis is essential for ensuring the proper segregation of homologues during meiosis as well as the production of viable gametes harbouring intact haploid genomes. Meiotic recombination initiates with the generation of programmed DNA double-strand breaks (DSBs) by the SPO11 endonuclease (Keeney et al. 1997), which are repaired by homologous recombination. The ends of DSBs are resected to produce single-stranded DNA, at which RAD51/ DMC1 recombinases mediate strand exchange with homologous non-sister chromatids. These reciprocal recombination events create physical connections (chiasmata) that tether homologues together. Intimate alignment of homologues (pairing), the close association of paired chromosomes (synapsis) by synaptonemal complex (SC) formation and chiasmata form stable units or bivalents, thus ensuring the right orientation of homologues on the metaphase I spindle and their precise segregation to opposite poles during the first meiotic division (e.g. Burgoyne et al. (2009) and Handel & Schimenti (2010)).

The temporal and functional interdependence between DSB formation and synapsis is crucial to better understand the molecular mechanisms underlying genetic recombination. In Drosophila melanogaster, Caenorhabditis elegans and Bombyx mori females, DSB formation follows SC completion and Spo11 homologues are dispensable for synapsis, indicating that synapsis is DSB-independent in these animals (von Wettstein et al. 1984, Tsai & McKee 2011). In contrast, in most organisms, including mice, Spo11-dependent phosphorylation of the nucleosomal variant histone H2AX leading to gamma-H2AX (γH2AX) formation during leptotene (Mahadevaiah et al. 2001) and synapsis failure in spermatocytes defective in DSB processing (Pittman et al. 1998, Yoshida et al. 1998, Edelmann et al. 1999, de Vries et al. 1999, Kneitz et al. 2000, Kidane et al. 2010) have led to the concept that synapsis depends on DSB repair by homologous recombination (see Henderson & Keeney (2005), Zickler (2006) and García-Muse & Boulton (2007)). Nevertheless, considerable synapsis has been observed in Spo11-deficient spermatocytes, albeit between non-homologous chromosomes, indicating that synapsis in mice can also be initiated in the absence of recombination.
(Baudat et al. 2000, Romanienko & Camerini-Otero 2000). However, it is assumed that DSB-independent synapsis only occurs accidentally, as a consequence of a default pathway activated by the disruption or the absence of homologous synapsis after the zygotene/pachytene transition (Zickler & Kleckner 1999).

The PI3K kinases ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) are important players of surveillance systems (checkpoints) that monitor the fidelity of important meiotic events, phosphorylating downstream proteins such as H2AX. The leptotene wave of γH2AX that appears in response to DSB formation and that participates in the recruitment and accumulation of repair proteins at DSB sites depends on ATM, whereas ATR-dependent γH2AX formation mediates the meiotic silencing of unsynapsed chromosomes (reviewed in Burgoyne et al. (2007) and Burgoyne et al. (2009)). The analysis of Atm-deficient spermatocytes in a study of chromatin silencing revealed an ATR-dependent wave of H2AX phosphorylation targeted to unsynapsed chromosomes during zygote, and suggested that this second wave of γH2AX formation should also occur in normal mice (Turner et al. 2005). The failure of the accurate completion of chromosome pairing and synapsis can result in non-disjunction of homologues leading to subfertility and sterility or to the generation of aneuploid gametes (for a revision, see Handel & Schimenti (2010)). Therefore, to study γH2AX formation at this stage is important to improve our understanding of the mechanisms operating in chromosome synapsis in mice. Here I aimed to re-examine the dynamics of γH2AX in normal and Spo11-deficient mouse spermatocytes to confirm the occurrence of the proposed de novo wave of ATR-dependent H2AX phosphorylation and to analyse its significance in detail.

Results

γH2AX signalling of asynapsed autosomes occurs at a substage of late zygotene in normal mice

γH2AX was monitored in well-spread spermatocytes from mid–late zygotene to the zygotene/pachytene transition, using 4’,6-diamidino-2-phenylindole (DAPI) and SYCP3 staining for meiosis substaging as described previously (Plug et al. 1998, Turner et al. 2001). The observation of these spermatocytes confirmed the occurrence of the previously proposed de novo zygote wave of H2AX phosphorylation in normal mice. The resultant γH2AX labels the last autosome stretches to pair and the sex chromosomes (Fig. 1). Most late zygotene spermatocytes (93%, n = 47, three animals) displayed a variable number of heterogeneously γH2AX-stained chromatin domains. Nevertheless, some of them (6%) showed their few asynapsed axis stretches totally devoid of γH2AX labelling (Fig. 1). The analysis of these domains indicated that γH2AX during late zygotene is highly dynamic. Thus, before pairing completion, the new wave of phosphorylation causes massive γH2AX accumulation on the short stretches of the autosome axes remaining unpaired as well as on the sex chromosomes (Fig. 1B and C). Because chromosome pairing and synapsis are asynchronous (Guitart et al. 1985, Scherthan & Schönborn 2001), H2AX phosphorylation on these last autosomes stretches to pair as well as their dephosphorylation after pairing completion are also asynchronous. These asynchronies cause the coexistence of different levels of phosphorylation/ dephosphorylation that results in the various staining intensities shown by different chromatin domains within the same nucleus: 1) intensely stained chromatin domains covering the short stretches of unpaired axial elements (AEs), usually seen at the ends of the autosomal bivalents; 2) domains showing various weaker staining intensities either on unpaired AEs or on paired lateral elements (LEs) and 3) synapsed LEs totally devoid of phosphorylation (Fig. 1B and C). γH2AX labelling starts to appear on the X chromosome when nearly all the autosomes were already paired and preceded the Y chromosome labelling, although both the X and Y chromosomes appear labelled before pairing (Fig. 1B and C), that always occurs after the autosome pairing completion at the zygotene/pachytene transition, when the sex chromosomes appear intensely immunostained (Fig. 1D). In most spermatocytes at the zygote/pachytene transition, the γH2AX asynapsis signal is restricted to the sex chromosomes and has disappeared from the already paired autosomes, indicating that it begins to fade as soon as pairing is completed (Fig. 1D). That is, this stereotyped distribution of γH2AX staining appears in most spermatocytes at late zygote and disappears immediately after synapsis completion at the zygote/pachytene transition (Fig. 1B, C and D), implying that it is a short-lived substage of late zygote, which takes place just at the end of zygote.

Zygote wave of γH2AX asynapsis signalling is Spo11-independent

The zygote wave of γH2AX shows a similar behaviour to that of the first wave of phosphorylation, given that both the leptotene wave (Mahadevaiah et al. 2001) and the zygote wave analysed here appear before synapsis and disappear with pairing progression. This similarity between the two waves of H2AX phosphorylation poses the possibility that this chromatin modification may play a role in promoting chromosome synapsis. Nevertheless, in contrast to the leptotene wave, the zygote wave is preserved in Atm-null mice (Turner et al. 2005), indicating that this second wave of γH2AX formation is independent of the first wave appearing at leptotene in response to the meiotic DSBs. As indicated above, extensive DSB-independent synapsis, albeit non-homologous synapsis, occurs in Spo11-null mice.
(Keeney et al. 1999, Romanenko & Camerini-Otero 2000) and γH2AX mediates chromatin silencing of unsynapsed axes in these animals (Mahadevaiah et al. 2001, 2008, Bellani et al. 2005). Therefore, γH2AX-immunostained Spo11−/− spermatocytes were also analysed, aiming to study the relationship between the dynamics of H2AX phosphorylation and DSB-independent pairing (Fig. 2). Well-spread Spo11-null spermatocytes (n=195) were scored and classified on the basis of their DAPI staining patterns and on the length and thickness of SYCP3-stained AEs and/or LEs following previously established criteria (Turner et al. 2001, Ashley et al. 2004; Table 1, Fig. 2A). Then, γH2AX staining patterns appearing at different stages of pairing progression were analysed in detail (Fig. 2B, C, D, E, F and G) to monitor the evolution of H2AX phosphorylation from the end of leptotene (early spermatocytes that show fully developed AEs, observed as continuous SYCP3-stained thin filaments, and that look like asynaptic zygotene spermatocytes, L/Z) to the pachytene-like stage (spermatocytes showing extensive synapsis and formation of LEs, observed as thicker SYCP3-stained filament, P).

A close observation of Spo11-deficient spermatocytes at L/Z revealed two different γH2AX staining patterns (Table 1), one (8.8%, n=195) displaying a variable number of intensely stained areas that were always seen on broken AEs (averaging 1.4 areas per cell) and another (9.6%, n=195) showing a granular weak staining throughout the nucleus plus an average number of 10.3 moderately stained small areas (Fig. 2B). These tiny γH2AX-labelled areas were analysed in detail, scored and classified according to their morphology and their...
different relationships to AEs in ten randomly picked nuclei as: 1) irregularly shaped areas on broken AEs (an average number of 1.2 areas per nucleus), 2) arrays of foci roughly forming open circles around one single axis (an average of 3.1 per nucleus) and 3) irregularly shaped areas that seem to embrace two or more AEs together (an average of 5.6 per nucleus), with these areas being either open or closed around AEs (Fig. 2B).

Nuclei showing variable rates of AE/LE SYCP3-stained filaments were considered as zygotene-like or areas that seem to embrace two or more AEs together (an average of 5.6 per nucleus), with these areas being either open or closed around AEs (Fig. 2B).

Figure 2 The zygotene wave of γH2AX asynapsis signalling is preserved in Spo11−/− spermatocytes. (A) Quantitative analysis of γH2AX staining patterns shown by Spo11−/− spermatocytes scored and classified according to DAPI counterstaining (grey/blue) and SYCP3 immunostaining (red). L/Z, leptotene/zygotene-like; Z, zygotene-like; lZ, late zygotene-like; Z/P, zygotene/pachytene transition; P, pachytene-like. (B) Leptotene-/zygotene-like spermatocyte showing fully developed but asynaptic AEs with several small γH2AX-stained areas that signal one broken axis (arrowhead and inset) or that encircle or tie asynapsed axes together (arrows and insets in the combined panel). (C) Zygote-like spermatocyte with moderate γH2AX staining covering nearly the whole nucleus. Only a few AEs (arrow) are devoid of γH2AX labelling. Some LEs show total (arrowhead) or partial (inset) dephosphorylation associated with pairing progression. (D) Zygote-like spermatocyte showing several irregularly shaped γH2AX areas of various staining intensities mainly localised on asynapsed axes (arrows and insets 1 and 2). LEs devoid of γH2AX labelling (⁎) or still showing small stained chromatin regions (arrowhead) are also present. (E) Spermatocyte mimicking the zygotene/pachytene transition. γH2AX signals mainly appear on AEs (insets), although a few small labelled areas appear related to LEs. (F) Pachytene-like spermatocyte. One intensely stained γH2AX chromatin domain signals a few stretches of AEs (insets). This domain has been termed the pseudo-sex body because it mimics that which forms the inactivated XY body in normal pachytene spermatocytes. (G) Correlation of the length of asynapsed axes with the measurements of γH2AX-stained areas in spermatocytes from the late zygotene-like to pachytene-like stages. Scale bar = 5 μm.
pachytene-like spermatocytes. The analysis of these spermatocytes indicated that the γH2AX staining patterns change with pairing progression in these nuclei. Spermatocytes showing low levels of pairing (<35% of the total length of the chromosome axes) display a granular weak-to-moderate staining covering nearly the whole nucleus (10.7%, n=195, Table 1, Fig. 2C). A closer observation of these cells demonstrated that γH2AX labelling seems to wrap most AEs and LEs. Nevertheless, some LEs displaying heterogeneous γH2AX staining intensities along their length as well as a few unlabelled AEs and LEs were also observed within these cells (Fig. 2C). Most zygotene-like spermatocytes showing higher levels of chromosome pairing (>35%) seemed to show similar γH2AX staining patterns and, in contrast to L/Z spermatocytes and to spermatocytes showing lower levels of pairing, they display a variable number of irregularly shaped and heterogeneously stained chromatin domains. Analysis of these nuclei revealed that the relationship between γH2AX chromatin domains and asynapsed or synapsed axes evolves with pairing progression (Table 1, Fig. 2D and E). Thus, zygotene-like nuclei with an average of 35–50% pairing of the total length of the AEs (6.2%, n=195) showed γH2AX staining clearly concentrated on a few (typically one to four) irregularly shaped chromatin areas that were always found on unsynapsed axes. These areas were of different sizes and often showed various staining intensities. Nevertheless, most unsynapsed axes within these nuclei do not show γH2AX signalling. Zygote-like spermatocytes containing higher levels of paired chromosomes (50–70%) and centromeres clustered in a few subdomains (12.3%, n=195) exhibited γH2AX staining concentrated in an average number of 9.1 irregularly shaped γH2AX signals of quite different sizes and staining intensities (Fig. 2D). Most signals were localised on asynapsed axes, whereas LEs were usually devoid of γH2AX staining. Nevertheless, some asynapsed AEs (averaged 3.7) totally devoid of γH2AX labelling can also be observed in these spermatocytes and a few paired axes (averaged 1.4) appear to be covered by smaller and more weakly stained areas (Table 1, Fig. 2D). Nuclei containing 60–80% of paired axes, but already displaying brightly stained centromeres, as in normal spermatocytes after pairing completion (7.6%, n=195), also display a variable number of γH2AX areas of different staining intensities, similarly to those appearing in nuclei showing lower levels of pairing. Nevertheless, asynapsed axes totally devoid of γH2AX signalling were less frequently observed (averaging 0.7) within these nuclei. That is, the main difference found between zygote and zygote-like spermatocytes is that in normal spermatocytes, the observation of AEs remaining asynapsed but devoid of the corresponding γH2AX signal is restricted to an extremely short period of time, whereas in Spo11-null mice, asynapsed AEs devoid of signalling appear to be dispersed in nuclei bearing 35–80% pairing, given that non-homologous synapsis requires frequent partner switches and involves several bivalents.

As non-homologous pairing advances, most unsynapsed AEs are seen labelled and γH2AX disappears from some synapsed LEs. The staining pattern shown by these spermatocytes mimics that found in wild-type spermatocytes at the zygotene/pachytene transition or at pachytene (Fig. 2E, F and G). Thus, nuclei displaying non-homologous pairing ranging from 80 to 95% (26.2%, n=195), γH2AX are typically restricted to the few stretches of axes remaining asynapsed, and they exhibit a staining pattern that parallels that found in

---

### Table 1 Percentage of Spo11-null spermatocytes with different γH2AX staining patterns.

<table>
<thead>
<tr>
<th>Centromere DAPI staining pattern</th>
<th>Pairing (%)</th>
<th>Stage-like</th>
<th>γH2AX staining pattern</th>
<th>In percent (n=195)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clustered in a few subdomains</td>
<td>0</td>
<td>L/Z</td>
<td>Typically one to three intensely stained areas on broken AEs</td>
<td>8.8</td>
</tr>
<tr>
<td>Clustered in a few subdomains</td>
<td>0</td>
<td>L/Z</td>
<td>Granular weak staining throughout the nucleus plus some few stained areas (Fig. 2B)</td>
<td>9.6</td>
</tr>
<tr>
<td>Clustered in a few subdomains</td>
<td>&lt;35%</td>
<td>Z</td>
<td>Granular moderate staining throughout the nucleus (Fig. 2C)</td>
<td>10.7</td>
</tr>
<tr>
<td>Clustered in a few subdomains</td>
<td>35–&lt;50%</td>
<td>Z</td>
<td>One to four stained signals, always localised on some AEs</td>
<td>6.2</td>
</tr>
<tr>
<td>Clustered in a few subdomains</td>
<td>50–&lt;70%</td>
<td>Z</td>
<td>Several signals of diverse sizes and intensities, most of them on AEs but a few on LEs (Fig. 2D)</td>
<td>12.3</td>
</tr>
<tr>
<td>Separated into multiple subdomains</td>
<td>60–80%</td>
<td>IZ</td>
<td>Several signals of various sizes and intensities, most of them signalling most asynapsed axes</td>
<td>7.6</td>
</tr>
<tr>
<td>Separated into multiple subdomains</td>
<td>&gt;80–&lt;95%</td>
<td>Z/P</td>
<td>Several signals, most of them signalling every asynapsed axis (Fig. 2E)</td>
<td>26.2</td>
</tr>
<tr>
<td>Separated into multiple subdomains</td>
<td>&gt;95%</td>
<td>P</td>
<td>One intensely stained area on asynapsed AEs (Fig. 2F)</td>
<td>18.4</td>
</tr>
</tbody>
</table>

Spread spermatocytes from Spo11-null mice were immunostained for γH2AX, scored and classified on the basis of DAPI and SYCP3 staining (Turner et al. 2001, Ashley et al. 2004). L/Z, leptotene/zygotene (fully developed axial elements just before starting synapsis); Z, zygotene; lZ, late zygotene; Z/P, zygotene/pachytene transition; P, pachytene.
normal spermatocytes at the zygotene/pachytene transition (Fig. 2E). From non-homologous 95% pairing onwards (18.4%, n=195), γH2AX labelling usually accumulates on one intensely stained area that contains the asynapsed axis stretches appearing in these spermatocytes, termed the pseudo-sex body because it resembles the inactivated XY chromatin domain apparent in normal pachytene spermatocytes (Fig. 2F and G). In these nuclei, there is a significant correlation between γH2AX-stained areas and the length of the asynapsed axes (r=0.98, P<0.01, n=30), analysed in randomly chosen nuclei previously classified as late zygotene-like to pachytene-like spermatocytes using Pearson’s correlation test (Fig. 2G), indicating that, as in normal mice, there is a close correlation between the asynapsed axes and γH2AX labelling in these cells and that the dynamics of H2AX phosphorylation/dephosphorylation in Spo11-deficient mice is similar to that in normal mice at these meiotic stages, which is consistent with the concept that the second wave of H2AX phosphorylation that appears at late zygotene in normal mice is preserved in Spo11-deficient mice.

**Late zygotene γH2AX labelling co-localises with ATR**

Because phosphorylation of H2AX during meiotic inactivation of the sex chromosomes and of the unsynapsed regions of autosomes from mutant spermatocytes and from translocation carriers during pachytene depends on the ATR kinase (Turner et al. 2004, 2005), ATR immunostaining was combined with γH2AX immunostaining to confirm that ATR is involved in the zygotene wave of phosphorylation analysed here (Fig. 3). The observation of these spermatocytes indicated that ATR forms dense aggregates which co-localise with γH2AX chromatin domains either in wild-type nuclei exhibiting the characteristic staining patterns of spermatocytes at the end of zygotene (Fig. 3A) or at pachytene (Fig. 3B), when both signals remain limited to the sex body area. The observation of spread Spo11-deficient spermatocytes indicated that, as in the wild-type mouse, ATR appears at every γH2AX-stained chromatin region (Fig. 3C and D).

**Zygotene wave of ATR/γH2AX asynapsis signalling is physiologically programmed**

Data on spread spermatocytes described above, as well as the observation of seminiferous tubule sections (Blanco-Rodríguez 2009), indicated that γH2AX asynapsis signalling appears as part of a programmed pathway operating at this developmental step. If this was the case, γH2AX would appear on asynapsed axes in every spermatocyte undergoing a specific step of development. During mammalian spermatogenesis, all spermatocytes originated from the same spermatogonium are kept together by persistent cytoplasmic bridges undergoing together every event of the meiotic programme. Therefore, to test whether the appearance of ATR/γH2AX signalling at late zygotene is a

---

**Figure 3** γH2AX and ATR co-localise in spread spermatocytes from wild-type (Wt) and Spo11−/− mice. (A) Wt spermatocyte exhibiting the γH2AX and ATR staining patterns that characterise the end of zygotene. (B) Wt pachytene spermatocyte showing the sex body area labelled by γH2AX and ATR. (C) Spo11−/− spermatocyte exhibiting multiple irregularly γH2AX-stained areas co-localising with ATR. (D) Pachytene-like spermatocyte from a Spo11−/− mouse showing an intensely stained pseudo-sex body where γH2AX co-localises with ATR. Scale bar=5 μm.
physiologically programmed event occurring at a specific developmental step, ATR/γH2AX immunostaining was performed in squashed preparations of spermatogenic cells at stages XI–XII, which contain early spermatocytes at late zygotene and at the zygotene/pachytene transition (Fig. 4). The analysis of these preparations showed that numerous ATR/γH2AX-positive chromatin domains appear within all the nuclei of late zygotene spermatocytes undergoing together the epithelial stage XI (Fig. 4A). In the same way, squashed spermatocytes at the zygotene/pachytene transition appearing at spermatogenic stage XII displayed ATR/γH2AX-positive structures, judged to be the sex chromosomes that appear labelled before the sex body formation at this developmental step (Fig. 4B). That is, as suggested by the distribution of γH2AX in spread spermatocytes and in testis sections (Blanco-Rodríguez 2009), the analysis of squashed nuclei evidenced that the late zygotene wave of H2AX phosphorylation operates in every spermatocyte passing through a specific developmental step, demonstrating that this zygotene wave of ATR/γH2AX signalling is always activated before pairing completion.

**Discussion**

The main objectives of this study were primarily to confirm whether a wave of H2AX phosphorylation occurs in normal mice at zygotene and to analyse the dynamics of the appearance/disappearance of the resultant γH2AX. The observation of spread spermatocytes shows that, as previously suggested in a study of meiotic silencing by the analysis of Atm-null spermatocytes (Turner et al. 2005), a de novo wave of H2AX phosphorylation takes place during zygotene in normal mice. The resultant γH2AX covers the previously unlabelled last axis stretches to pair, which are mostly located at the ends of the autosomal axes. Given that γH2AX labelling has been found on all the axes where pairing has not yet occurred in late zygotene spermatocytes, these autosomal ends should correspond to the proximal ends, consistent with the localisation of chromosome centromeres in mice and the recent finding that centromeres are invariably the last regions to pair.

**Free DNA 3′-ends are not detected at sites of γH2AX asynapsis signalling**

Spo11-independent H2AX phosphorylation followed by the pairing of homologues might be the response to DSBs introduced within the last chromosome stretches to pair by a different endonuclease operating at late zygotene. To rule out this possibility, tissue sections were used for the detection of free DNA 3′-end combined with γH2AX immunostaining. The analysis of these sections showed that both signals had quite different distributions in the nuclei of early spermatocytes appearing in these preparations in seminiferous tubules at stages XI (Fig. 5A) and XII (Fig. 5B) and that only a few nuclei (0.3%, n=243) showed small areas of partial co-localisation (Fig. 5A), indicating that free DNA 3′-ends are not present in most γH2AX asynapsis signals appearing in spermatocytes at late zygotene and at the zygotene/pachytene transition.

![Figure 4](https://www.reproduction-online.org)  
Several ATR/γH2AX signals appear in every spermatocyte at a specific developmental step. (A) Squashed spermatocytes from spermatogenic stage XI, assessed by the presence of a diplotene nucleus (arrow). Several γH2AX and ATR signals co-localise within every early spermatocyte (small nuclei and inset). (B) Squashed spermatocytes from spermatogenic stages XI–XII. Early spermatocytes (small nuclei and insets) show intensely stained structures judged to be the sex chromosomes appearing apart (X, Y) or paired (XY) within the nuclei at the zygotene/pachytene transition. Diplotene nuclei (arrow) and metaphase I (arrowhead) spermatocytes are also apparent. Scale bar= 5 μm.
in these animals (Bisig et al. 2012, Qiao et al. 2012). The appearance of γH2AX coincides with the massive accumulation of ATR and both proteins spread throughout the chromatin associated with all the axes remaining asynapsed within every nucleus ending zygote, in agreement with the concept that phosphorylation of H2AX at these chromatin domains is Atr-dependent. These localisation patterns are consistent with previous studies on ATR immunolocalisation and with the known BRCA1-dependent accumulation of ATR on unsynapsed chromatin as well as with the involvement of Atr-dependent H2AX phosphorylation in the triggering of meiotic silencing (Keegan et al. 1996, Moens et al. 1999, Turner et al. 2004). However, as most studies have focused on mutant mice, there is a broad agreement that Atr-dependent γH2AX formation on autosomes only occurs to signal synaptic errors, mainly caused by delayed or defective DSB processing (Edelmann et al. 1999) or by autosomal translocations (Mahadevaiah et al. 2001, Sciurano et al. 2007), and that it is part of a default pathway leading to either non-homologous synapsis or meiotic silencing (see, for instance, Turner et al. 2005 and Mahadevaiah et al. 2008). Instead, the detailed analysis of the dynamic of H2AX phosphorylation/dephosphorylation presented here supports the idea that most ATR/γH2AX signals do not necessarily mark the impairment of DSB repair or synaptic errors, given that, as indicated by the analysis of either squashed preparations (this study) or seminiferous tubule sections (Blanco-Rodríguez 2009), they are found within all the nuclei undergoing the end of zygote and disappear from every normal spermatocyte at the zygote/pachytene transition, when homologous pairing has been completed. As the different stages of the spermatogenic cycle are characterised by typical cellular associations of several germ cell progenies, each at a specific step of development, differentiating in a synchronised manner, the appearance/disappearance of the ATR/γH2AX signals can be observed in all the early spermatocytes undergoing these specific steps of differentiation in every seminiferous tubule section and in every squash preparation at the corresponding epithelial stages (Blanco-Rodríguez 2009 and this study). The analysis of spread nuclei reveals that these ATR/γH2AX signals selectively accumulate on the short last axis stretches to pair at the end of zygote and disappear from these locations as soon as pairing is completed. As a consequence, these signals are not observed at the zygote/pachytene transition, just following the end of zygote. The notion that γH2AX dephosphorylation occurs immediately after pairing completion is also clearly inferred from the fact that most ending zygote nuclei show intensely stained domains on axis stretches where pairing has not yet occurred, coexisting with weakly stained domains either on unpaired AEs or on paired LEs. Given the known...
asynchrony of chromosome pairing (Guitart et al. 1985, Scherthan & Schönborn 2001), these weakly stained domains should correspond to either ATR/γH2AX still being accumulated on unpaired AEs or disappearing from recently paired LEs. Therefore, the γH2AX staining pattern appearing in ending zygotene nuclei as well as the fact that the ATR/γH2AX accumulations are not present at the zygotene/pachytene transition are clearly consistent with a close linkage between ATR/γH2AX and chromosome pairing. Taken together, observing the staining patterns in tubule sections and squashes, as well as in spread-staged spermatocytes, it is difficult to believe that all these ATR/γH2AX signals correspond to accidental delays of DSB processing or to synopsis errors appearing within all the nuclei as part of a default pathway. Rather, the appearance of ATR/γH2AX on the distal axis ends where pairing has not yet occurred and its disappearance as soon as pairing has been completed are consistent with the idea that ATR plays a role in sensing late pairing regions in ending zygotene nuclei and activates a physiologically programmed pathway that leads to autosome pairing completion, thus contributing to finishing the bivalent formation at the end of zygotene. BRCA1-mediated ATR localisation during pachytene on asynapsed axes that fail to synapse has been proposed to be DSB dependent (Perera et al. 2004). However, despite that the unsynapsed Y chromosome is subject to BRCA1/ATR-mediated silencing, DSB markers on the Y chromosome axis are rarely seen (Turner et al. 2004, 2005, 2006). The observation of large γH2AX foci in about 30% of pachytene and diplotene nuclei from normal mice also led the authors to suggest that these foci could correspond to delayed or unregulated DSB repair events (Chicheportiche et al. 2007), although they did not rule out other interpretations. It might be argued that these large γH2AX foci might be related to the appearance of ATR/γH2AX accumulation on unpaired axes. Nevertheless, it is difficult to think that these large foci are related in some way to the ATR/γH2AX signals found on the last axis stretches to pair. This is because, first, the large γH2AX foci, described by Chicheportiche et al. (2007), are Atr-independent and, second, they are localised on chromatin loops (Chicheportiche et al. 2007). In addition, as indicated above, the analysis of the time course of the ATR/γH2AX signals carried out in either tubule sections or squashes as well as in spread cells shows that in normal spermatocytes, the ATR/γH2AX signals disappear from the autosomes as soon as pairing is completed at the zygote/pachytene transition. The idea that the accumulation of ATR on unsynapsed axes plays a role in the completion of chromosome pairing was previously proposed by Moens et al. (1999) based on the ATR distribution and on the finding that it does not co-localise with RAD51/DMC1 recombinase foci, the sites of DSB processing by homologous recombination. Interestingly, the XRCC6 (KU70) protein, which plays a critical role in DSB repair by non-homologous end joining, is not expressed in zygote spermatocytes (Goedcke et al. 1999). Accordingly, here I show that the zygote wave of ATR/γH2AX asynapsis signalling is preserved in Spo11 zytogene-like spermatocytes and that it does not co-localise with free DNA 3’-ends, indicating that it is Spo11-independent and that it does not appear in response to DSBs which might have been introduced by an endonuclease different from Spo11. Interestingly, γH2AX immunostaining is not seen at these locations in a small fraction of late zygote cells judged to pertain to a short developmental step that precedes the occurrence of the zygote wave of asynapsis signalling. In this regard, it is important to take into account that both the Atm-independency (Turner et al. 2005) and Spo11-independency (this study) of the second wave of γH2AX formation originated at the end of zygote also indicate that it cannot depend on the first wave of γH2AX occurring during leptotene in response to meiotic DSB formation.

The relative timing of DSB formation and chromosome synapsis can shed light on the molecular mechanism of genetic recombination and, therefore, it is a subject of intense study. The finding of Spo11-dependent γH2AX during leptotene (Mahadevaiah et al. 2001) and reduced or abnormal SC formation in DSB repair-defective mutants (Barlow et al. 1998, Pittman et al. 1998, Celeste et al. 2002) supports that in mice, homologous synapsis depends on meiotic DSB repair. However, Spo11 spermatocytes display considerable non-homologous synapsis (Baudat et al. 2000, Romanienko & Camerini-Otero 2000), supporting the view that DSB repair is indeed required for homologue recognition, but additional mechanisms account for synapsis. Actually, as shown in this study, the main difference between zygote and zygote-like spermatocytes is that in normal spermatocytes, the asynapsed AEs remain devoid of γH2AX during an extremely short period of time, whereas in Spo11-null mice, the asynapsed AEs devoid of γH2AX labelling appear dispersed in nuclei bearing 35–80% pairing, which is a logical consequence of the frequent partner switches as well as the involvement of several bivalents required in non-homologous synapsis. Therefore, as the timing of pairing is an individual feature of every bivalent (Guitart et al. 1985, Scherthan & Schönborn 2001), non-homologous synapsis should be delayed compared with homologous synapsis, and asynchrony should also be greater than that in homologous synapsis. Nevertheless, unsynapsed AEs that remain devoid of γH2AX labelling also decrease in conjunction with progression of the spermatocyte development. The observation of ATR/γH2AX signalling on unsynapsed stretches that disappear after aberrant non-homologous synapsis in Spo11−/− mice is in agreement with previously published data (Mahadevaiah et al. 2008) and supports the concept that the absence of DSBs does impede homologue recognition yet.
synapsis occurs after ATR/γH2AX asynapsis signalling. Accordingly, in DSB repair-defective mice, such as Atm^{−/−}, H2ax^{−/−} and Dmc1^{−/−} as well as in those without a fully functional MRE11 complex, synapsis occurs between non-homologous chromosomes (Barlow & Hulten 1998, Pittman et al. 1998, Celeste et al. 2002, Cherry et al. 2007). The observation of extensive synapsis in the most advanced Spo11 spermocytes is consistent with the fact that they survive until spermatogenic stage IV (mid-pachytene in normal mice) and express markers of early to mid-pachytene (Barci et al. 2005). Therefore, the most important alteration of these spermocytes seems to be the failure of homologue finding, indicating that this finding is Spo11-dependent, whereas synopsis is not. The distribution of ATR/γH2AX in Spo11^{−/−} spermocytes shown in this study also supports the idea of ATR/γH2AX playing a role in driving DSB-independent chromosome synapsis. In spite of important advances, how homologues identify each other in mammals remains poorly understood. Data on Spo11-deficient yeast (Bhuiyan & Schmekel 2004) and mouse (Baudat et al. 2000, Mahadevaiah et al. 2008) support the concept stated above that the repair of Spo11-dependent DSBs allows chromosomes find their homologous partners. Synapsis might then occur by the polymerisation of the SC components along AEs that become together. Therefore, in the absence of DSB repair, homologues are not able to identify each other, but SC formation still occurs between the closest chromosomes. The physical proximity of AEs playing a key role in promoting the SC assembly is consistent with the fact that the clustering of telomeres (bouquet formation) at the onset of zygote is critical in chromosome synapsis (reviewed in Scherthan (2009)) as well as with the recent proposal that SYCP3 plays a role in promoting homologous centromere pairing (Bisig et al. 2012). As in mouse centromeric regions are always the last to pair (Bisig et al. 2012, Qiao et al. 2012), it is likely that centromeric heterochromatin could interfere with pairing progression at the proximal ends. Structural changes caused by H2AX phosphorylation might contribute to making easier the physical proximity of AEs to promote the establishment of centromere pairing and/or to provide a docking site to facilitate the recruitment of the SC components, as it occurs with cohesins, which are recruited to meiotic DSB sites in a γH2AX-dependent manner (Únal et al. 2004), thus contributing to the assembly of AEs during leptotene (Eijpe et al. 2000, Pelttari et al. 2001, James et al. 2002). Actually, a role in synopsis completion in male spermatocytes has been found for the cohesin subunit RAD21L, a recently identified kleisin that localises along AEs and LEs and, as H2AX, exhibits sexual dimorphism in fertility (Celeste et al. 2002, Herrán et al. 2011). From this point of view, the arrays of γH2AX foci, which are shown for the first time in this study and which seem to embrace two or more AEs together in Spo11^{−/−} asynaptic zygote spermatocytes, might play a role in promoting non-homologous synopsis. Against this reasoning, it could be argued that an apparently normal synapsis occurs between autosomes in H2AX-deficient spermatocytes (Celeste et al. 2002). However, several points should be taken into consideration in this regard. First, it is important to take into account that the role played by H2AX in DSB repair is beyond a doubt but, in spite of it, H2ax^{−/−} mice present a surprisingly relatively mild phenotype (Celeste et al. 2002), indicating that redundant and default pathways exist to accomplish such an important function. Second, early-pachytene H2ax^{−/−} spermocytes frequently exhibit hallmarks of apoptosis (Celeste et al. 2002). Therefore, it is likely that those spermocytes that have not been able to complete synapsis have been removed and, therefore, they cannot be observed. Third, the X and Y chromosomes either fail to pair or appear to be erroneously paired to autosomes in these H2AX-deficient nuclei (Celeste et al. 2002), indicating that, as I am proposing here, H2AX plays a role in chromosome pairing, at least in the chromosome stretches that are the last to pair, such as the PAR region in the X and Y chromosomes. Furthermore, most H2ax^{−/−} cells exhibit extensive SC fragmentation, as shown by SYCP3 immunostaining (Celeste et al. 2002), pointing to a role of H2AX in the correct localisation of the SYCP3 protein. Interestingly, as indicated above, the involvement of SYCP3 in promoting homologous centromere pairing has recently been proposed by Bisig et al. (2012), establishing a relationship between H2AX and centromere pairing, the last regions to pair in mice (Bisig et al. 2012, Qiao et al. 2012). The surprisingly relatively mild phenotype presented by H2ax-null spermocytes could be explained because ATR might signal downstream effectors or because other members in the pathway might share with H2AX some of their functions, as it actually occurs in DNA repair (Celeste et al. 2003).

In summary, here I confirm that a zygote wave of H2AX phosphorylation occurs in normal mice and show that the resultant γH2AX appears on previously unlabelled axis stretches that remain asynapsed at very late zygote and spreads through several chromatin sites that are ATR-labelled. Then, both ATR and γH2AX disappear from the autosomes immediately after pairing completion at the zygote/pachytene transition. The appearance/disappearance of ATR/γH2AX signalling occurs at several sites in every normal spermatocyte undergoing these meiotic steps. I also show that ATR/γH2AX accumulation is Spo11-independent and does not co-localise with the detection of free DNA 3’-ends. Taken together, these data indicate that in normal male meiosis, a de novo wave of Atr-dependent γH2AX formation is physiologically programmed at a specific step of late zygote to signal the last chromosome axis stretches to pair. In contrast to the leptotene wave, this zygote wave is DSB independent.
Nevertheless, mirroring the leptotene signal, this signal also disappears after chromosome pairing, supporting the notion that it plays a role in the triggering of a phase of DSB-independent pairing and synopsis completion. It is likely that γH2AX serves as a platform for recruitment and retention not only of some DNA repair proteins (recently reviewed in Bekker-Jensen & Mailand (2010)), but also of some pairing mediators. Alternatively, this chromatin modification might cause structural changes in the asynapsed regions which contribute to leading distant homologue stretches to become closer, thus driving their physical interaction to facilitate DSB repair and pairing. I propose a model where ATR/γH2AX signalling at the end of zygotene is involved in promoting a second phase of synopsis by a DSB-independent pathway operating before the triggering of meiotic silencing, this silencing only proceeding after synopsis failure (Fig. 6). Much research on that field remains to be done, but elucidating the molecular mechanisms concerning this second phase of synopsis will contribute to have a better understanding of how bivalents are formed to allow the proper segregation of homologues and the formation of viable gametes.

Materials and Methods

Animals

Normal young male mice (n = 4) were on a random-bred MF1 background and housed under conventional, controlled standard conditions. Mice homozygous for the null allele Spo11<sup>tm1M</sup> (n = 2), together with wild-type littermates (n = 2), were as described previously (Baudat et al. 2000). All procedures were approved and performed in accordance with the guidelines of Valladolid University’s Animal Care and Use Committee.

Squash and spread preparations

Squashed (Page et al. 1998) and surface-spread (Peters et al. 1997) spermatocytes were prepared as described previously, using 2% paraformaldehyde containing 0.02% SDS, pH 8, as the fixative. To analyse germ cells that maintain their spatial organisation, 2 mm pieces of tubules were collected according to their stage-specific light absorption pattern, as described by Kotaja et al. (2004), prior to squashing.

Paraffin-embedded sections

For the analysis of testis sections, testes were excised, decapsulated and fixed directly in cold 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4, and then processed for paraffin embedding and sectioning according to standard procedures. Orthogonal sections across the seminiferous tubules (5 µm) were mounted in aminopropyltriethoxy silane-coated slides (Sigma Chemical Co.).

Immunofluorescent staining

In testis sections, epitope retrieval was carried out by boiling deparaffinised sections for 12 min (3 × 4), in 10 mM citrate buffer, pH 6.0, using a microwave oven at 350 W power output. After PBS washes, spread spermatocytes or tissue sections were incubated in PBS containing 0.15% BSA and 0.1% Tween-20 (PBT) for 60 min before incubation overnight at 4°C with primary antibodies diluted in PBT. Mouse monoclonal anti-γH2AX antibody (Upstate/Millipore, Billerica, MA, USA; JBW301, Cat no. 05-636) was used at 1:3000, rabbit polyclonal anti-SYCP3 (Ortega et al. 2003) at 1:500 and goat polyclonal anti-ATR (Santa Cruz Biotechnology, Inc., N-19, Cat no. SC-1887) at 1:50. After washes, primary antibodies were detected by incubating slides for 2 h in the following secondary antibodies diluted in PBT: donkey anti-mouse Alexa Fluor 488 and donkey anti-rabbit Alexa Fluor 594 (Invitrogen), both diluted at 1:500. Following PBS washes, preparations were counterstained with DAPI, washed, air-dried and mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA).

Labelling of free DNA 3’-ends

Deparaffinised tissue sections were treated with 1 µg/ml proteinase K in 10 mM Tris–HCl, pH 7.4–8, during 8 min at 37°C in a humidified chamber and subjected to in situ cell fractionation by incubating the slides with protein extraction...
buffer (50 mM HEPES, pH 7.5, 1 mM EDTA, pH 8.3, 10 mM NaF, 10 mM β-glycerol phosphate, 0.5% Triton X-100, 1 M NaCl, 2 M urea, 0.05% SDS in 1 ml of water) during 15 min to allow the detection of non-apoptotic free DNA 3'-ends. Then, TUNEL was performed using the In Situ Cell Death Detection Kit from Roche (Cat no. 1 684). After PBS washes, sections were incubated with the TUNEL reaction mixture (TdT solution plus dUTP solution) for 60 min at 37 °C in a humidified atmosphere in the dark, using the TdT solution and the dUTP solution as provided and following the manufacturer’s directions. To achieve the appropriate labelling of non-apoptotic free DNA 3'-ends, numerous previous experiments were carried out to fit the proper time of incubation as well as the concentration of Triton X-100, NaCl, urea and SDS in the protein extraction buffer (see Fig. S1 of the supplementary material, see section on supplementary data given at the end of this article). For combined γH2AX detection, samples were further processed following the fluorescence immunostaining protocol. Adjacent sections were used for a periodic acid-Schiff staining to verify the spermatogenic stages. At least four sections per animal were analysed.

Imaging and data analysis

Image capture and analysis were performed using a Zeiss Axiophot microscope equipped with epifluorescence, a Plan-Neofluar 100×/1.3 oil-immersion objective (Carl Zeiss, Inc., Jena, Germany) and a computer-assisted (Spot, RT Color, Diagnostic Instruments, Inc., Sterling Heights, MI, USA) cooled CCD (1520×1080 pixels) camera. Each fluorochrome was captured separately as a 16-bit source image. Spermatogenic stages in tubule sections were identified following the criteria established previously (Leblond & Clermont 1952, Blanco-Rodríguez 2009). Spread spermatocytes were first classified based on the DAPI staining pattern, as described in Turner et al. (2001). Then, SYCP3 immunostaining was used to label the AEs (observed as continuous SYCP3-stained thin filaments) and the LEs (observed as thicker SYCP3-stained filaments) of the SC for meiosis substaging (Plug et al. 1998). Specific features such as the length and thickness of AEs/SCs and the presence of non-homologous synapsis were applied as supplementary data for Spo11-deficient cells, also following previously established criteria (Ashley et al. 2004, Mahadevaiah et al. 2008). After establishing the γH2AX staining pattern, it was used as an additional marker for cell identification. Digital images were processed to increase the visibility of immunostained structures and to reduce the background, using the Spot analysis software and Adobe Photoshop 8.0.1 (Adobe Systems Incorporated).

The length of the asynapsed axes and the total γH2AX-labelled area per nucleus were determined in randomly selected Spo11−/− spermatocytes of the appropriate stage (n = 30) using the ImageJ [http://www.rsbiinfo.nih.gov/ij] image analysis software package. The area of γH2AX detection threshold was set on the mean staining intensity plus 2× the s.d. Correlation analysis between the asynapsed axes and the γH2AX-stained areas was carried out using Pearson's correlation test.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-12-0326.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work has been supported by the Spanish Ministry of Health (FIS 02/1886 to J Blanco-Rodríguez) and by the Regional Government of Castilla y León (VA23/03 to J Blanco-Rodríguez).

Acknowledgements

We thank Francesca Cole (M Jasin Laboratory) for providing spermatocyte spreads and Ana Cadavez Pedro for help with some steps of immunofluorescence staining.

References


Blanco-Rodríguez J 2009 γH2AX marks the main events of the spermatogenic process. Microscopy Research and Technique 72 823–832. (doi:10.1002/jemt.20730)


www.reproduction-online.org


Received 23 August 2012
Accepted 2 October 2012