SPO11 is required for sex-body formation, and Spo11 heterozygosity rescues the prophase arrest of *Atm*$^{-/-}$ spermatocytes

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Summary

SPO11 introduces double-strand breaks (DSBs) that trigger the phosphorylation of H2AX during meiotic prophase. In mice, SPO11 is strictly required for initiation of meiotic recombination and synapsis, yet SPO11 is still considered to be dispensable for sex-body formation in mouse spermatocytes. We provide conclusive evidence showing that functional SPO11, and consequently recombination and synapsis, are required for phosphorylation of H2AX in the X-Y chromatin and for sex-body formation in mouse spermatocytes. We investigated the role in meiosis of the three kinases [ATM (ataxia telangiectasia mutated), ATR (ataxia-telangiectasia- and Rad-3-related) and DNA-PKcs (DNA-dependent-protein-kinase catalytic subunit)] known to phosphorylate H2AX in mitotic cells. We found that DNA-PKcs can be ruled out as an essential kinase in this process, whereas ATM is strictly required for the chromatin-wide phosphorylation of H2AX occurring in leptotene spermatocytes in response to DSBs. Remarkably, we discovered that *Spo11* heterozygosity can rescue the prophase-I-arrest characteristic of ATM-deficient spermatocytes. Characterization of the rescued *Atm*$^{-/-}$ *Spo11*$^{+/-}$ mutant indicates that ATM is dispensable for sex-body formation and phosphorylation of H2AX in this subnuclear domain. The co-localization of ATR, phosphorylated H2AX and the sex chromatin observed in the *Atm*$^{-/-}$ *Spo11*$^{+/-}$ mutant, along with ATR transcription kinetics during the first wave of spermatogenesis, confirm and expand recent findings indicating that ATR is the kinase involved in H2AX phosphorylation in the sex body.

Supplementary material available online at
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Key words: H2AX, ATM, ATR, SPO11, Sex body, Meiosis

Introduction

Meiosis is a specialized type of cell division that gives rise to haploid gametes. The reduction in chromosome number is achieved by undergoing one round of DNA replication (premeiotic S-phase) followed by two successive rounds of chromosome segregation (meiosis I and meiosis II). During meiosis I, chromosomes must find their cognate homolog, pair and undergo reciprocal recombination, leading to the establishment of physical connections between them (chiasmata), which (in conjunction with sister chromatid cohesion) ensure appropriate homolog segregation during anaphase I (Petronczki et al., 2003).

Heteromorphic sex chromosomes pose a challenge to the mechanisms that ensure accurate segregation of autosomes during meiosis I. Although largely diverged, murine X and Y chromosomes contain a 700 kb region of homology designated the pseudo-autosomal region (PAR) (Ellis and Goodfellow, 1989). In mouse spermatocytes, the sex chromosomes are able to pair, synapse and undergo reciprocal recombination, leading to chiasma formation in the PAR, thus ensuring their correct segregation during anaphase I (Anderson et al., 1999; Soriano et al., 1987). In order to deal with incomplete synapsis of the X-Y pair, mammals have adopted a strategy that involves seclusion of the sex chromosomes to a subnuclear compartment known as the sex body (or X-Y body). This subnuclear domain distinguishes the sex chromatin from the rest of the chromatin during pachynema (when the autosomes are completely synapsed) and diplonema. The sex body contains the X and Y chromosomes (which nucleate a unique array of proteins and post-transcriptionally modified histone variants) in a transcriptionally inactive chromatin configuration (for reviews, see Handel, 2004; Hoyer-Fender, 2003; Solari, 1974). Although its raison d'être remains a subject of debate, the most favored hypothesis is that sex-body formation might mask the incompletely synapsed X-Y pair from meiotic surveillance mechanisms (Handel, 2004; McKee and Handel, 1993).

Meiotic recombination is initiated early in prophase I by SPO11-dependent introduction of double-strand breaks (DSBs) throughout genomic DNA (Keeney, 2001). The resulting DNA ends are repaired by homologous recombination, with the eukaryotic RecA homologs RAD51 and DMC1 (meiosis specific) catalysing the invasion and strand-exchange reaction between non-sister chromatids on

homologous chromosomes. Disruption of *Spo11* in mice results in male and female infertility (Baudat et al., 2000; Romanienko and Camerini-Otero, 2000). SPO11 deficiency abolishes DMC1 and RAD51 focus formation, indicating an inability to initiate meiotic recombination. It also affects synapsis: axial elements develop normally but homologous chromosomes either fail to synapse or engage in non-homologous synapsis with multiple partners. These findings confirmed that, in mice as in yeast, initiation of recombination is required for synapsis (Baudat et al., 2000; Romanienko and Camerini-Otero, 2000).

Histone H2AX is a variant of the core histone H2A and is ubiquitously distributed throughout the genome. It has a characteristic C-terminal tail containing two SQ motifs that are subject to phosphorylation (Redon et al., 2002). Mitotic cells phosphorylate H2AX at serine 139 in response to DNA damage, inducing the formation of nuclear foci of phosphorylated H2AX (γH2AX) at the sites of damage. Hence, γH2AX can be used as a marker for DSBs (for reviews, see Fernandez-Capetillo et al., 2004; Pilch et al., 2003). Analysis of H2AX phosphorylation during mouse meiosis (Mahadevaiah et al., 2001) revealed that H2AX is phosphorylated throughout the chromatin in leptotene spermatocytes. This chromatin-wide γH2AX staining is absent from SPO11-deficient spermatocytes, indicating that the massive phosphorylation of H2AX occurring in leptonema is triggered by the SPO11-dependent introduction of DSBs (Mahadevaiah et al., 2001). As the breaks are processed by the recombination machinery and synapsis is established during zygonema, γH2AX staining gradually decreases and, by pachynema, it is undetectable on autosomal chromatin. During pachynema and diplonema, γH2AX staining is restricted to the sex body (Mahadevaiah et al., 2001). In *Spo11*$^{-/-}$ mice, zygotene-like spermatocytes have been reported to display a localized γH2AX signal that was interpreted to be on the sex chromosomes. Based on these observations, it was suggested that phosphorylation of H2AX in the sex body is independent of SPO11 and the generation of DSBs (Mahadevaiah et al., 2001), an idea widely accepted in the meiosis field (Baarends and Grootegoed, 2003; Fernandez-Capetillo et al., 2004; Handel, 2004).

Generation of a H2AX knockout (KO) mouse disclosed an essential role for H2AX in sex-body formation and normal prophase progression in males (Fernandez-Capetillo et al., 2003b). *H2AX*$^{-/-}$ female mice are fertile, whereas males suffer a severe prophase arrest (Celeste et al., 2002). In H2AX-defective spermatocytes the sex chromosomes fail to pair, form a sex body or undergo transcriptional silencing, suggesting that H2AX (or γH2AX) is essential for the chromatin-remodeling process that leads to sex-body formation and meiotic sex-chromosome inactivation (MSCI) (Fernandez-Capetillo et al., 2003b). A very recent report has shed further light on this issue by analysing the meiotic phenotype of *BRCA1*$^{\Delta 11/\Delta 11}$ *Trp53*$^{+/-}$ mice (Turner et al., 2004). The *BRCA1*$^{\Delta 11/\Delta 11}$ mutation abrogates recruitment of ATR (ataxia-telangiectasia- and Rad-3-related) to the X-Y chromatin and this correlates with a lack of γH2AX in the sex chromosomes and their inability to undergo MSCI, indicating that BRCA1/ATR-dependent phosphorylation of H2AX in the sex chromatin is required to induce MSCI (Turner et al., 2004).

In mitotic cells, three phosphoinositide-3-kinase-related kinases (PIKKs) have been shown to phosphorylate H2AX in response to DNA damage, ATM (ataxia telangiectasia mutated), ATR and DNA-dependent-protein-kinase catalytic subunit (DNA-PKcs) (Burma et al., 2001; Stiff et al., 2004; Ward and Chen, 2001). Even though there are some reports in the literature addressing the role of some of these PIKKs in phosphorylating H2AX in testes (Fernandez-Capetillo et al., 2003a; Hamer et al., 2004; Turner et al., 2005), no systematic study has yet been done to define the roles of these kinases in the phosphorylation of H2AX in mouse meiotic prophase. We have done a genetic analysis that indicates that DNA-PKcs can be ruled out as an essential player in this process, whereas ATM is strictly required for chromatin-wide phosphorylation of H2AX occurring during leptonema in response to SPO11-dependent DSBs. Given the previous report indicating that a *Spo11* mutation could dissociate chromatin-wide from sex-body-specific phosphorylation of H2AX (Mahadevaiah et al., 2001), we analysed the interplay between *Atm* and *Spo11* mutations. We found conclusive evidence that the γH2AX-containing chromatin domains observed in SPO11-deficient spermatocytes do not overlap with the X-Y chromatin, indicating that SPO11, and therefore recombination and synapsis, are required for sex-body formation in mouse spermatocytes. Unexpectedly, we found that *Spo11* heterozygosity can rescue the prophase-I arrest characteristic of ATM-deficient spermatocytes. The rescued *Atm*$^{-/-}$ *Spo11*$^{+/-}$ mutant allowed us to demonstrate that ATM is dispensable for sex-body formation and for phosphorylation of H2AX in this subnuclear domain. Co-localization of ATR and γH2AX to the sex chromatin in the *Atm*$^{-/-}$ *Spo11*$^{+/-}$ mutant, and the analysis of ATR transcription kinetics during the first wave of spermatogenesis complement and extend recent findings suggesting that ATR is the kinase involved in the phosphorylation of H2AX in the sex body (Turner et al., 2004).

Materials and Methods

Mice

Spo11$^{-/-}$ mice have been previously described (C57BL/6 strain) (Romanienko and Camerini-Otero, 2000). *Atm*$^{-/-}$ mice (Barlow et al., 1996) were obtained from Jackson Laboratories (129S6/SvEvTac-*Atm*^{tm1Awb}/J) and maintained in our facilities. *Atm*-heterozygous mice were bred with *Spo11* heterozygotes to generate double heterozygotes, which were intercrossed to generate the required combinations of *Atm* and *Spo11* alleles, so *Atm*$^{-/-}$ *Spo11*$^{-/-}$ and *Atm*$^{-/-}$ *Spo11*$^{+/-}$ mice were of C57BL/6 × 129/Sv mixed background, and were compared with *Spo11*$^{-/-}$ and wild-type littermates. In order to minimize variability owing to strain background, experimental animals were compared with controls from the same litter (where possible) or from other litters from the same matings. DNA/PKcs-deficient mice were purchased from Jackson Laboratories (NOD.CB17-*Prkdc*$^{scid/scid}$/J). Mice were genotyped by PCR as previously described (Barlow et al., 1996; Romanienko and Camerini-Otero, 2000) using DNA from tail biopsies.

Mouse irradiation

Spo11$^{-/-}$ and *Spo11*$^{-/-}$ *Atm*$^{-/-}$ mice were subjected to whole-body irradiation in a Nordion Gamma Cell 40 (^{137}Cs source). Mice received 3 Gy (1 Gy = 100 rads) at a dose of 78 rads per minute. The irradiated animals were euthanized 1 hour after treatment and their testes immediately processed to prepare structurally preserved nuclei (SPNs), as described below. In SPNs, the three-dimensional structure of the spermatocyte nucleus is preserved. In this kind of preparation,

leptotene spermatocytes show nucleolar SCP3 aggregates (Dietrich et al., 1992; Prieto et al., 2004), which are usually lost during the spreading technique and are therefore not observed in spreads.

Spermatocyte preparations

For spermatocyte preparations 4-6-week-old mice were used. Spermatocyte surface-spread preparations were based on a previously described technique (Peters et al., 1997) with some modifications. Briefly, after removal of the tunica albuginea, the seminiferous tubules were minced in RPMI medium (Gibco) containing a cocktail of protease inhibitors (Complete EDTA-free, Roche). Large tissue pieces in the cell suspension were allowed to settle and the supernatant was transferred to a new tube and spun down. The pelleted cells were resuspended in a hypotonic solution (30 mM Tris-HCl, 50 mM sucrose, 17 mM trisodium citrate dihydrate, 5 mM EDTA, pH 8.2) for 30 minutes. Cells were then pelleted and resuspended in 100 mM sucrose, pH 8.2. Approximately 20 µl of this suspension were dropped on a glass slide containing a thin layer of fixative solution (2% paraformaldehyde, 0.15% Triton X-100, pH set to 9.2 using 10 mM sodium borate buffer solution, pH 9.2). Slides were placed for 2 hours in a humid chamber, washed three times for 1 minute in 0.4% Photo-Flo (Kodak) and air dried at room temperature. Specimens were stored at –70°C until further use.

Structurally preserved nuclei were prepared based on a previously published method (Scherthan et al., 2000a). Briefly, after removing the tunica albuginea, testes were minced in ice-cold RPMI medium (Gibco) containing a cocktail of protease inhibitors (Complete EDTA-free, Roche). Large tissue pieces were removed and a drop of cell suspension was mixed with three drops of fixative (3.7% paraformaldehyde, 0.1 M sucrose in PBS, pH 7.4) on silanized glass slides. Samples were allowed to air dry at 4°C and were stored at –20°C until further use.

For dual-color fluorescent in-situ hybridization (FISH), spermatocyte preparation was based on a previously described method (Henegariu et al., 2001). Briefly, spermatocytes were collected and pelleted as indicated above. Cells were then resuspended in 0.075 M KCl at 37°C for 20 minutes. After hypotonic treatment, the cells were spun down and resuspended in the fixative (methanol/acetic-acid, 3:1). Approximately 30 µl of this cell suspension were dropped on a glass slide that had been previously exposed to hot water vapor (3-5 seconds). Slides were again exposed to water vapor when the surface of the sample became grainy. The specimen was dried at room temperature and immediately processed for FISH.

Antibodies and DNA probes

The following primary antibodies were used: rabbit antibody against ATR at 1:100 (GeneTex); mouse antibody against γH2AX at 1:5000 (JBW301, Upstate Biotechnology); mouse antibody against γH2AX biotin conjugate at 1:5000 (JBW301, Upstate Biotechnology); rabbit antibody against γH2AX at 1:50 (Trevigen); mouse antibody against SCP3 and rabbit antibody against SCP3 at 1:200 (a gift from P. Moens, York University, UK); rabbit antibody against SCP1 at 1:200 (a gift from C. Höög, Karolinska Insitute); mouse antibody against ATM phosphorylated on Ser1981 (4526, Cell Signaling); rabbit antibody against ATM phosphorylated on Ser1981 (ab2888, Novus Biologicals); mouse antibody against BRCA1 at 1:10 (GH118, a gift from S. Ganesan, Dana-Farber Cancer Institute, Boston, MA); rabbit antibody against RAD51 (sc-8349, Santa Cruz Biotechnology). Secondary antibodies conjugated with Rhodamine Red-X, fluorescein isothiocyanate (FITC), Cy3 and Cy5 were from Jackson Immunoresearch, and those conjugated with AF350 were from Molecular Probes, as was the FITC-conjugated Neutravidin. All secondary antibodies were used at a dilution of 1:200. Fab fragments of affinity-purified secondary-antibody conjugates were from Jackson Immunoresearch.

Biotin-labeled mouse X and Y whole-chromosome paint probes and a biotin-FITC painting kit were used for FISH. An FITC-labeled X chromosome paint probe was combined with the biotin-labeled Y-chromosome paint probe for the dual-color detection (Cy3-FITC). All probes and detection reagents were from Cambio (Cambridge, UK).

Immunostaining

Slides were washed in PBST (0.1% Tween 20 in PBS) and blocked with antibody dilution buffer (3% bovine serum albumin, 10% goat serum in PBST) for 30 minutes at 37°C. Primary antibodies were then applied and incubated either overnight at 4°C or 60 minutes at 37°C. Controls were incubated with antibody dilution buffer without primary antibody. After three 5 minute washes in PBST, slides were incubated with the secondary antibodies for 60 minutes at room temperature. Following a new round of three PBST washes, the specimens were rinsed in 0.4% Photo-Flo and mounted in Vectashield medium (Vector Labs; with or without DAPI). All incubations were performed in a humid chamber. Spermatocyte staging was based on the changing morphology of autosomes and the XY pair as visualized by SCP3 (Plug et al., 1997) and SCP1 staining. For triple staining (Fig. 1A, Fig. 4A, Fig. 6), mouse antibody against SCP3 was combined with biotin-labeled mouse antibody against γH2AX and a third primary antibody raised in rabbit as follows. Mouse antibody against SCP3 was detected with a conjugated goat Fab fragment against mouse IgG and slides were subsequently incubated with biotinylated mouse antibody against γH2AX, followed by conjugated Neutravidin.

For quantification of the different γH2AX staining patterns, spermatocyte preparations were analysed from at least two mice from each genetic background; at least 50 nuclei (unless otherwise specified) of the appropriate stage were randomly selected and classified according to the criteria described in the text or figure legend.

Immuno-FISH

Combined immunostaining and FISH was based on techniques previously described (Kurz et al., 1996; Scherthan et al., 2000b). Briefly, spermatocyte preparations were washed in water and quenched in 0.5% glycin in PBS. Samples were permeabilized by subsequent incubation in 0.1 M HCl (10 minutes) and 0.5% Triton X-100, 0.5% saponin in PBS (10 minutes), equilibration in 20% glycerol in PBS (20 minutes) and final freezing in liquid nitrogen followed by immediate thawing in PBS. Preparations were then denatured in 70% formamide in 2× SSC (sodium chloride sodium citric acid) buffer (70-73°C) and hybridized with the biotin-labeled DNA probes at 37°C overnight. Probes were previously denatured, preannealed and applied following the provider recommendations. After post-hybridization washes (at 45°C) in 50% formamide in 1× SSC buffer, 1× SSC buffer and 0.05% Tween in 4× SSC, the X and Y chromatin was detected with FITC-avidin. Following washes in 0.05% Tween in 4× SSC buffer, the preparations were immunostained as described above.

For dual-color FISH, chromosome preparations were dehydrated in 100% ethanol for 5 minutes and dried at room temperature. Slides were then incubated in pepsin solution (0.01% pepsin in 10 mM HCl) for 2-3 minutes and washed in 2× SSC buffer. After a brief rinse in distilled water, samples were dehydrated by serial ethanol washing (70%, 90% and 100% ethanol) and air dried. Following an overnight aging at room temperature, the specimens and the probes were denatured, hybridized and detected according to the instructions of the manufacturer.

Results

Spo11$^{-/-}$ spermatocytes do not phosphorylate H2AX in the sex chromatin and fail to form a canonical sex body

Two distinct patterns of γH2AX staining have been described during prophase I in wild-type mice (Mahadevaiah et al.,

2001). The first, characteristic of leptonema and early to mid-zygonema, shows chromatin-wide phosphorylation of H2AX, whereas the second exclusively stains the sex body during pachynema and diplonema. The primary goal of this work was to define the kinases responsible for these two distinct patterns of γH2AX staining. We reasoned that,

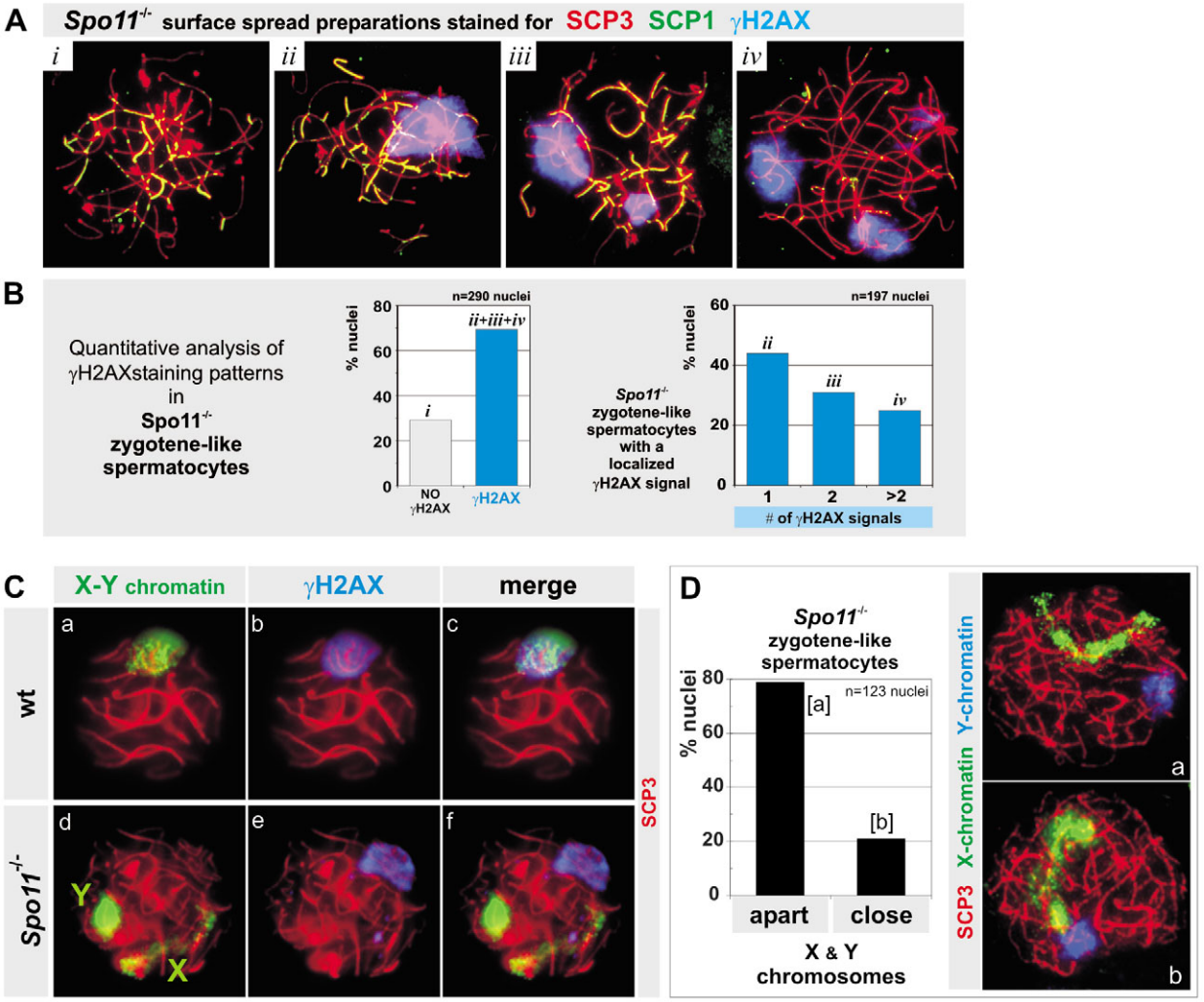


Fig. 1. *Spo11*$^{-/-}$ spermatocytes do not phosphorylate H2AX in the sex chromatin and fail to form a sex body. (A) Zygotene-like *Spo11*$^{-/-}$ spermatocytes display a wide range of γH2AX staining patterns. Analysis of *Spo11*$^{-/-}$ surface-spread preparations stained for SCP3, SCP1 and γH2AX revealed that a proportion of zygotene-like spermatocytes is devoid of γH2AX (i), whereas most nuclei contain one or more localized γH2AX chromatin domains. Examples are shown of nuclei containing one (ii), two (iii) or more (iv) γH2AX signals. (B) Quantitative analysis of γH2AX staining patterns observed in *Spo11*$^{-/-}$ zygotene-like spermatocytes. 350 nuclei were scored and classified according to their stage (based on SCP3 staining) and γH2AX staining pattern. Zygotene-like spermatocytes (*n*=290 nuclei, two animals) were classified as nuclei devoid of γH2AX (i) or nuclei displaying one or more localized γH2AX signals (ii-iv). These zygotene-like spermatocytes containing one or more localized γH2AX signals (*n*=197 nuclei) were further classified according to the number of γH2AX signals: one signal (ii), two signals (iii) and more than two signals (iv). Examples of each of these nuclei are displayed in Fig. 1Ai-iv. (C) The γH2AX signals frequently observed in zygotene-like spermatocytes are not on the sex chromatin. Combined FISH (whole-chromosome paint probes against the X and Y chromosomes are shown in green) and immunostaining (antibodies against SCP3 are shown in red and those against γH2AX in blue) performed on structurally preserved spermatocytes from wild-type (a-c) and *Spo11*$^{-/-}$ (d,f) mice (two sets of littermates). In wild-type spermatocytes, the X-Y chromatin contains γH2AX (c). In SPO11-deficient zygotene-like spermatocytes, even in those nuclei displaying a single γH2AX signal, this does not overlap with the X and Y chromatin. None of the 50 *Spo11*$^{-/-}$ nuclei analysed contained a γH2AX signal overlapping with either the X or the Y chromatin. Notice that the X chromosome appears extended and does not synapse with the Y chromosome. (D) *Spo11*$^{-/-}$ spermatocytes do not form a sex body. Combined SCP3 immunostaining and FISH (using differently labeled whole-chromosome probes recognizing the X and Y chromatin) was performed on methanol-acid-fixed *Spo11*$^{-/-}$ spermatocytes. 123 zygotene-like nuclei (two animals) were scored and classified according to the relative positions of the sex chromosomes as apart (a) or close (b). Almost 80% of SPO11-deficient zygotene-like nuclei displayed the X and Y chromosomes widely separated, whereas 21% contained the sex chromosomes close to one another. Nevertheless, in those nuclei containing the sex chromosomes in proximity, the X is always extended and barely contacting the Y on one end. This disposition clearly differs from the spatial configuration usually adopted by the X-Y chromosomes in the sex body in wild-type spermatocytes, providing further cytological evidence for the lack of sex body in SPO11-deficient spermatocytes.

if inactivating *Spo11* abolished chromatin-wide phosphorylation of H2AX without affecting sex-body-specific phosphorylation (Mahadevaiah et al., 2001), the *Spo11*-null background could be used to distinguish these two phosphorylation events and to help identify the kinases responsible for them.

When we analysed spermatocyte spreads from *Spo11* KO mice that we had generated (Romanienko and Camerini-Otero, 2000), we observed a wide range of γH2AX staining patterns in zygotene-like nuclei (Fig. 1Ai-iv). A quantitative analysis revealed that 70% of zygotene-like nuclei contained one or more localized γH2AX signals (Fig. 1B). Among these, 44% contained a unique γH2AX signal that could be presumed to be a sex body (Fig. 1Bii), whereas 31% presented two signals (one could argue these might be asynapsed X and Y chromosomes; Fig. 1Biii) and 25% presented more than two γH2AX signals (Fig. 1Biv). The fact that more than half of the nuclei displayed two or more localized γH2AX signals seemed inconsistent with the view that γH2AX decorates the sex body in *Spo11*$^{-/-}$ spermatocytes.

Next, we analysed whether the γH2AX signals observed in SPO11-deficient zygotene-like spermatocytes were actually overlapping with the sex chromatin. We combined FISH with immunostaining on structurally preserved nuclei using whole-chromosome paint probes to detect the X and Y chromatin and antibodies recognizing γH2AX and SCP3 (Fig. 1C). SCP3 staining allowed staging of spermatocytes, whereas the use of a sample-preparation technique that preserves the three-dimensional structure of the nucleus permitted unequivocal identification of overlapping signals. As expected, in wild-type spermatocytes the X and Y chromatin contained γH2AX (Fig. 1Ca-c). A pachytene spermatocyte is shown, but γH2AX staining of the sex chromatin was also verified in zygotene and diplotene spermatocytes (data not shown). When we analysed *Spo11*$^{-/-}$ spermatocytes, we found that, even in those nuclei containing a unique γH2AX signal, γH2AX is never detected on the X or Y chromatin (Fig. 1Cd-f). These results prove that the chromatin domain staining for γH2AX in SPO11-deficient spermatocytes does not correspond to the sex chromatin. Hence, it does not represent a canonical sex body, which would include the X and Y chromosomes as its defining components (the possibility that this domain might represent an ectopic or proto-sex body is evaluated in the Discussion). Furthermore, the X chromosome appears extended and does not synapse with the Y (Fig. 1Cd), a spatial configuration that differs completely from the one normally adopted by the X and Y chromosomes in the sex body. This abnormal spatial configuration of the sex chromosomes was verified by dual-color staining of the X and Y chromatin, which confirmed that the vast majority of *Spo11*$^{-/-}$ zygotene-like spermatocytes display extended X and Y chromosomes that are visibly separate from each other (Fig. 1Da).

These results clearly show that SPO11-deficient spermatocytes are unable to phosphorylate H2AX specifically in the sex chromatin and to form a sex body. Further analysis will be required to establish what triggers H2AX phosphorylation in certain chromatin domains in the *Spo11*$^{-/-}$ background, but it is clear that these γH2AX-domains do not involve the sex chromosomes and that the view that sex-body formation is independent of SPO11 and the formation of DSBs is seriously flawed.

ATM is responsible for chromatin-wide phosphorylation of H2AX during leptotene

Mammalian cells produce six PIKK family members, three of which (ATM, ATR and DNA-PKcs) have been shown to phosphorylate H2AX in mitotic cells in response to DNA damage and replication stress (Bakkenist and Kastan, 2004): ATM and DNA-PKcs in response to DSB-inducing agents such as ionizing radiation, X-rays, neocarzynostatin, etoposide and bleomycin (Burma et al., 2001; Stiff et al., 2004); and ATR in response to replication stress and ultraviolet damage (Ward and Chen, 2001).

First, we analysed the role of ATM and DNA-PKcs in the phosphorylation of H2AX during meiotic prophase by comparing the γH2AX staining patterns observed in mutant mice deficient in each of these PIKKs. Analysis of mice deficient in DNA-PKcs (*Prkdc*$^{scid/scid}$) did not reveal any differences in the γH2AX staining pattern with respect to the wild-type control (see Fig. S1 in supplementary material), ruling out any essential role for this kinase in H2AX phosphorylation during prophase I. This result is consistent

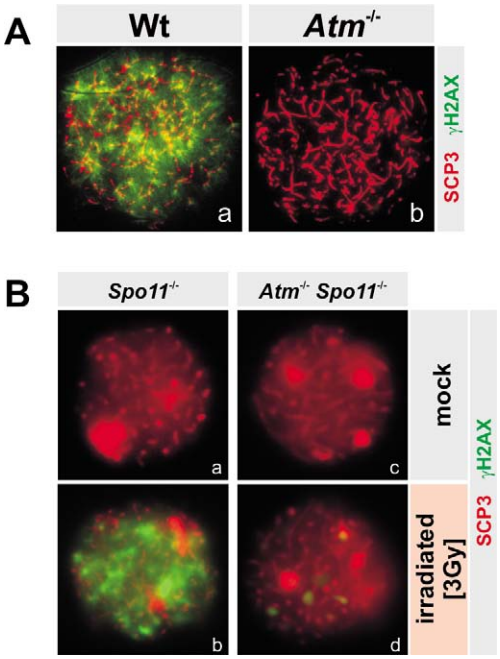


Fig. 2. ATM is strictly required for chromatin-wide phosphorylation of H2AX during leptotene. (A) Leptotene *Atm*$^{-/-}$ spermatocytes are devoid of γH2AX. Quantitative analysis of *Atm*$^{-/-}$ surface-spread preparations shows that 100% of the *Atm*$^{-/-}$ spermatocytes undergoing leptonema are devoid of γH2AX (n=64 nuclei, two animals). (B) Leptotene ATM-deficient spermatocytes can not phosphorylate H2AX in response to radiation-induced DSBs: *Spo11*$^{-/-}$ (a,b) and *Atm*$^{-/-}$ *Spo11*$^{-/-}$ (c,d) double-mutant mice were irradiated (3 Gy) or mock treated. 1 hour after irradiation, mice were sacrificed and their testes used to prepare structurally preserved nuclei, which were subsequently immunolabeled with antibodies against SCP3 (in red) and γH2AX (in green). 50 nuclei were scored for each condition. Irradiation-induced DSBs can restore H2AX phosphorylation in *Spo11*$^{-/-}$ spermatocytes undergoing leptotene (b), whereas, in the absence of functional ATM, no alternative PIKK can phosphorylate H2AX in leptotene spermatocytes in response to radiation-induced DSBs (d).The bright red spots represent nucleolar SCP3 aggregates.

with the fact that these mice, like other mutants deficient in functions required for non-homologous end joining, are fertile. In this respect, it has long been established that certain murine factors essential for non-homologous end joining are downregulated during leptotene and zygotene, ensuring that meiotic DSBs are repaired through homologous recombination (Goedecke et al., 1999).

When we performed a quantitative analysis of *Atm*$^{-/-}$ surface-spread preparations, we found that ATM deficiency completely abolishes phosphorylation of H2AX during leptonema (Fig. 2Ab, 100% of *Atm*$^{-/-}$ leptotene spermatocytes were devoid of γH2AX, 64 nuclei scored), indicating that ATM is strictly required for the chromatin-wide phosphorylation of H2AX observed in leptonema in response to DSBs introduced by SPO11. This result points to ATM as an essential player in the signal-transduction pathway orchestrating an efficient response to the programmed introduction of meiotic DSBs, and is consistent with the fact that *Atm*$^{-/-}$ mice are sterile because of a severe prophase arrest (Barlow et al., 1996).

We also found that leptotene spermatocytes deficient for ATM cannot phosphorylate H2AX in response to radiation-induced DSBs (Fig. 2B). As can be seen in Fig. 2Bb, γ-irradiation of *Spo11*$^{-/-}$ mice can restore H2AX phosphorylation in leptotene spermatocytes (1 hour after irradiation, all leptotene nuclei displayed chromatin-wide γH2AX, *n*=50). However, leptotene *Atm*$^{-/-}$ *Spo11*$^{-/-}$ double-mutant spermatocytes did not display chromatin-wide γH2AX staining after irradiation (Fig. 2Bd, none of the nuclei scored showed γH2AX staining, *n*=50), suggesting that neither of the other two PIKKs can substitute for the lack of ATM during leptotene (as will become evident in a later section, the inability of ATR to substitute efficiently for ATM at this stage might stem from the fact that it is weakly produced early in prophase I). Altogether, these results indicate that ATM is strictly required for the chromatin-wide phosphorylation of H2AX in leptotene spermatocytes in response to SPO11-dependent or irradiation-induced DSBs.

ATM is dispensable for phosphorylation of H2AX in the sex body

Next, we addressed the issue of H2AX phosphorylation in the sex body. The characteristic meiotic arrest displayed by the *Atm* KO mice (Barlow et al., 1996; Barlow et al., 1998) prevented us from drawing conclusions on the role of ATM in the sex body. We were able to find sporadic spermatocytes displaying a sex body decorated with γH2AX in *Atm*$^{-/-}$ spreads, suggesting that ATM might be dispensable for sex-body-specific phosphorylation of H2AX. Nevertheless, these were too infrequent (1 in 500 spermatocytes) to be used as robust evidence for sex-body phosphorylation in the absence of ATM.

In our search for a genetic background that would allow us to analyse the phosphorylation of H2AX in the sex body in the absence of functional ATM, we discovered an interesting

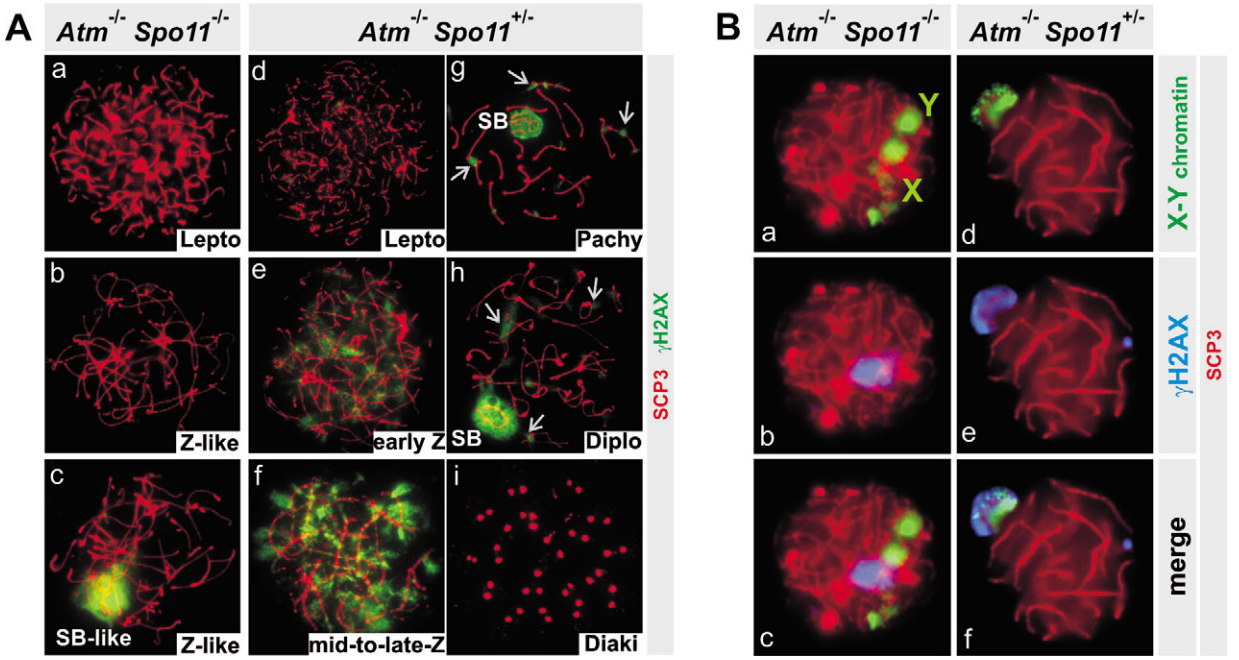


Fig. 3. The rescue of the prophase arrest of *Atm*$^{-/-}$ spermatocytes by *Spo11* heterozygosity reveals that ATM plays no essential role in the sex body. (A) A single copy of *Spo11* rescues the prophase-arrest characteristic of the ATM KO. Whereas inactivation of both copies of *Spo11* in an *Atm*$^{-/-}$ background results in a complete prophase arrest (and a meiotic phenotype indistinguishable to that of the *Spo11*$^{-/-}$ single mutant), *Spo11* heterozygosity rescues the prophase arrest of an ATM KO, allowing spermatocytes to complete prophase I. (B) ATM is dispensable for phosphorylation of H2AX in the sex body. Combined FISH (whole-chromosome paint probes against the X and Y chromosomes, in green) and immunostaining (antibodies against SCP3 in red and those against γH2AX in blue) performed on structurally preserved spermatocytes from *Atm*$^{-/-}$ *Spo11*$^{-/-}$ (a-c) and *Atm*$^{-/-}$ *Spo11*$^{+/-}$ (d-f) mice (two animals for each genetic background). As is the case for the *Spo11*$^{-/-}$ single mutant, none of the 50 nuclei scored for the *Atm*$^{-/-}$ *Spo11*$^{-/-}$ double-mutant strain contained a γH2AX signal overlapping with either the X or the Y chromatin. By stark contrast, in the rescued *Atm*$^{-/-}$*Spo11*$^{+/-}$ background, the X-Y chromatin consistently contains γH2AX. The γH2AX signal coincided with the X-Y chromatin in all of the 30 nuclei scored.

interplay between mutations in *Atm* and *Spo11*. In an $Atm^{-/-}$ background, both a wild-type complement and two null copies of *Spo11* result in severe prophase arrest but, remarkably, heterozygosity of *Spo11* rescues the prophase arrest.

Interestingly, the $Atm^{-/-}$ $Spo11^{-/-}$ double-KO mouse mimics the meiotic phenotype of a $Spo11^{-/-}$ single mutant, featuring an absolute lack of γH2AX during leptonema (Fig. 3Aa) and a subsequent arrest in a zygotene-like stage characterized by an inability to synapse homologous chromosomes (Fig. 3Ab,c). Most importantly, the localized γH2AX signal frequently observed in zygotene-like spermatocytes (Fig. 3Ac, SB-like) does not overlap with the X-Y chromatin, as evidenced by immuno-FISH analysis (Fig. 3Ba-c). Even though both $Atm^{-/-}$ and $Atm^{-/-}$ $Spo11^{-/-}$ mice display a severe arrest in prophase and are infertile, the double KO is a phenocopy of the $Spo11^{-/-}$ mutant; also, several features characteristic of the ATM deficiency, such as synaptonemal complex fragmentation and the very high levels of apoptosis, are suppressed, which suggests that the *Spo11* mutation is epistatic to *Atm*. This is in accordance with a recent report demonstrating epistasis of *Spo11* over *Atm* in murine oocytes (Di Giacomo et al., 2005).

In stark contrast to the severe arrest exhibited by the $Atm^{-/-}$ single mutant and the $Atm^{-/-}$ $Spo11^{-/-}$ double KO, we discovered that $Atm^{-/-}$ $Spo11^{+/-}$ spermatocytes can complete prophase I, as evidenced by the detection of spermatocytes in pachynema, diplonema and even diakinesis in surface-spread preparations (Fig. 3Ad-i). To date, we have examined spread preparations from six $Atm^{-/-}$ $Spo11^{+/-}$ animals, verifying the prophase rescue in all of them. Analysis of prophase-stage distribution in spreads from an $Atm^{-/-}$ $Spo11^{+/-}$ mouse and a wild-type littermate [29 days post partum (dpp)] has shown no significant differences between the genetic backgrounds in the proportion of nuclei undergoing the different stages of prophase (see Fig. S2 in supplementary material). A more in-depth analysis of the meiotic phenotype of the $Atm^{-/-}$ $Spo11^{+/-}$ mouse is presented in the Discussion. In terms of the γH2AX staining, as expected from the ATM deficiency, there is no chromatin-wide phosphorylation of H2AX during leptonema (Fig. 3Ad). Instead, phosphorylation seems to take place at later stages during mid- to late zygonema (Fig. 3Af), and to persist longer than in wild-type spermatocytes [notice the γH2AX 'eruptions' emanating from chromosome axes in pachytene and diplotene spermatocytes (Fig. 3Ag,h, arrows)]. Most importantly, during pachynema and diplonema, the sex body contains γH2AX (Fig. 3Ag,h, SB). Combined FISH and immunostaining confirmed that, in $Atm^{-/-}$ $Spo11^{+/-}$ pachytene spermatocytes, the localized γH2AX signal coincides with the X and Y chromatin (Fig. 3Bd-f), demonstrating that ATM is dispensable for phosphorylation of H2AX in the sex body.

ATR localizes to the sex body in $Atm^{-/-}$ $Spo11^{+/-}$ spermatocytes

Given that DNA-PKcs is not essential and ATM is dispensable for the phosphorylation of H2AX in the sex body, ATR remains the most likely candidate PIKK to account for this phosphorylation. The embryonic lethality resulting from ATR deficiency (Brown and Baltimore, 2000) precludes a genetic approach, yet localization of this kinase during wild-type prophase I seems to be consistent with a role for ATR in the phosphorylation of H2AX in the sex body. Previous reports have shown that ATR localizes to the cores of asynapsed autosomes during late zygotene and to the non-PAR axes of the X-Y pair as well as the X-Y chromatin, covering the whole sex body during late zygotene, pachytene and diplotene (Baart et al., 2000; Keegan et al., 1996; Moens et al., 1999). A recent report has shown that BRCA1-dependent recruitment of ATR to the X-Y chromatin is required in order to induce phosphorylation of H2AX in the sex body and transcriptional silencing of the sex chromosomes (Turner et al., 2004).

In the $BRCA1^{\Delta 11/\Delta 11}$ mutant, ATM does not seem to substitute for the kinase function of ATR in the sex body. However, given that BRCA1 and ATM can physically interact (Gatei et al., 2000) and that BRCA1 is required for the recruitment of previously activated ATM to the sites of DSBs after ionizing radiation (Kitagawa et al., 2004), it is formally possible that the $BRCA1^{\Delta 11/\Delta 11}$ mutation interferes with localization of ATM to the sex body, masking a role for ATM in phosphorylation of H2AX in this domain. In this respect, there is one report indicating that the phosphorylated form of ATM localizes to the sex body in wild-type spermatocytes (Hamer et al., 2004).

We analysed whether ATR localizes to the sex body in $Atm^{-/-}$ $Spo11^{+/-}$ spermatocytes, which contain γH2AX in the sex body in spite of the ATM deficiency. As can be seen (Fig. 4Ad-f), ATR localizes strictly to the γH2AX-stained sex body in pachytene spermatocytes (180/180 nuclei). Localization of ATR to the sex body is maintained during diplotene (data not shown). Our data showing localization of ATR to the sex body and γH2AX staining of this subnuclear compartment in the $Atm^{-/-}$ $Spo11^{+/-}$ mutant indicate that recruitment of ATR and the ensuing H2AX phosphorylation in the sex chromatin occur in an ATM-deficient background, ruling out any essential role for ATM in this process and providing further support to the findings by Turner et al. (Turner et al., 2004) suggesting that ATR might phosphorylate H2AX in the sex body. We also found that both a monoclonal antibody and a rabbit antiserum raised against the S1981-phosphorylated ATM peptide stain the sex body in $Atm^{-/-}$ $Spo11^{+/-}$ spermatocytes, indicating that the antibodies against the S1981-phosphorylated ATM peptide that are commonly used for immunoblots are not suitable for immunofluorescence studies (data not shown).

Turner et al. report (Turner et al., 2004) that, in those $BRCA1^{\Delta 11/\Delta 11}$ mutant spermatocytes in which BRCA1 does not localize to the X-Y chromosomes, ATR and γH2AX colocalize to non-sex-chromatin domains elsewhere in the nucleus. When we analysed ATR localization in $Spo11^{-/-}$ single-mutant and $Atm^{-/-}$ $Spo11^{-/-}$ double-mutant spermatocytes in which antibodies to γH2AX never stain the sex chromatin (Fig. 1Cf, Fig. 3Bc), ATR consistently localizes to the γH2AX-positive chromatin domain (Fig. 4Ag-l, 87/87 $Spo11^{-/-}$ and 112/112 $Atm^{-/-}$ $Spo11^{-/-}$ nuclei). There seems to be a correlation between targeting of ATR to a certain chromatin domain and the phosphorylation of H2AX in this region. In wild-type spermatocytes, it has been shown that BRCA1 localizes to the asynapsed cores of autosomes and the X-Y pair, and this is thought to recruit ATR to late-synapsing autosomes and to the sex body (Turner et al., 2004). We found that, in $Spo11^{-/-}$ and $Atm^{-/-}$ $Spo11^{-/-}$ spermatocytes, BRCA1 localizes to the chromosomal cores in the γH2AX-positive chromatin domains (see Fig. S3g-o in supplementary material, 34/34 $Spo11^{-/-}$ and 27/27 $Atm^{-/-}Spo11^{-/-}$). This result suggests that BRCA1

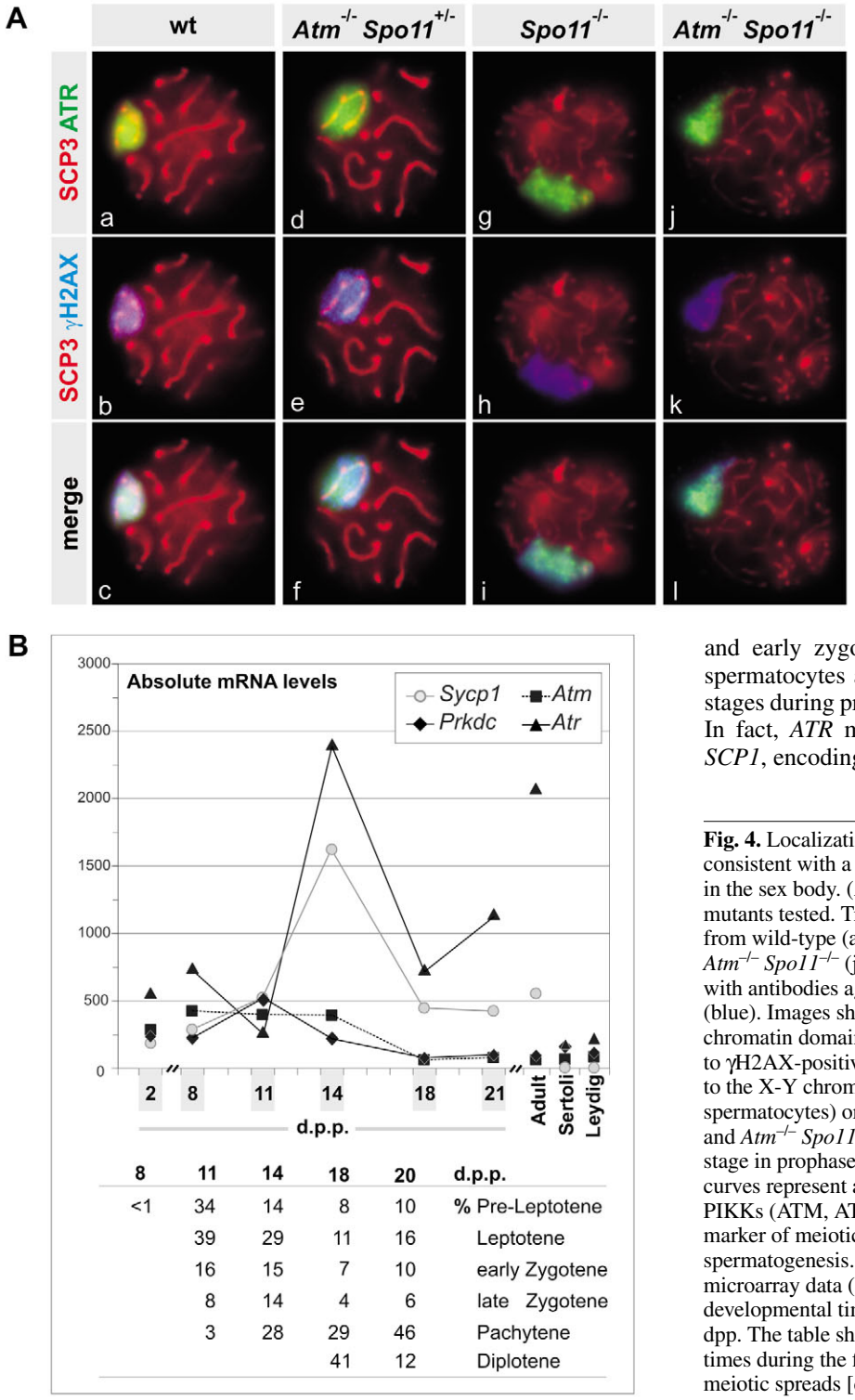
participates in recruitment of ATR to these ectopic sites in the mutants, just as it targets the kinase to the sex body in wild-type spermatocytes.

Expression pattern of PIKKs during meiotic prophase-I progression

We looked at the expression patterns of the genes encoding ATM, ATR and DNA-PKcs during meiotic progression in order to see whether the conclusions drawn on the basis of cytological data were reinforced by the kinetics of expression of these kinases during the first wave of spermatogenesis. Our analysis of previously published (Schultz et al., 2003) microarray data (Fig. 4B) shows the absolute mRNA levels for each kinase throughout prophase-I progression.

ATM mRNA levels display stable maximal values in pre-leptonema and early prophase stages (leptonema, zygonema), which seem to decrease as spermatocytes progress into pachynema and diplonema. The timing of *ATM* expression is consistent with the important role ATM appears to play in chromatin-wide H2AX phosphorylation during leptonema according to our cytological data as well as previous reports (Barlow et al., 1998). The rather flat curve might be accounted for by the mechanism of ATM activation, which involves autophosphorylation of dormant dimer subunits to release active ATM monomers (Bakkenist and Kastan, 2003).

By contrast, the kinetics of *ATR* expression displays a sharp increase in mRNA levels peaking around late-zygonema and early-to-mid pachynema (14 dpp). Fig. 4B shows that *ATR* mRNA levels increase almost tenfold between 11 dpp (when 89% of the cells are pre-leptotene, leptotene and early zygotene nuclei) and 14 dpp (when 42% of spermatocytes are in late zygonema and pachynema), the stages during prophase when the sex body can first be seen. In fact, *ATR* mRNA expression kinetics mimics that of *SCP1*, encoding the component of the central element that



8	11	14	18	20	d.p.p.
<1	34	14	8	10	% Pre-Leptotene
	39	29	11	16	Leptotene
	16	15	7	10	early Zygotene
	8	14	4	6	late Zygotene
	3	28	29	46	Pachytene
			41	12	Diplotene

Fig. 4. Localization and transcription kinetics of ATR are consistent with a role for ATR in the phosphorylation of H2AX in the sex body. (A) ATR colocalizes with γH2AX in all of the mutants tested. Triple labeling of structurally preserved nuclei from wild-type (a-c), $Atm^{-/-}$ $Spo11^{+/-}$ (d-f), $Spo11^{-/-}$ (g-i) and $Atm^{-/-}$ $Spo11^{-/-}$ (j-l) mice (two sets of littermates were analysed), with antibodies against SCP3 (red), ATR (green) and γH2AX (blue). Images show the focal plane in which the γH2AX-chromatin domain can be visualized. ATR consistently localizes to γH2AX-positive chromatin domains, whether these correspond to the X-Y chromatin (as in wild-type and $Atm^{-/-}$ $Spo11^{+/-}$ spermatocytes) or to non-sex-chromatin regions (as in $Spo11^{-/-}$ and $Atm^{-/-}$ $Spo11^{-/-}$ mutants). (B) *ATR* transcription peaks at the stage in prophase when the sex body can first be visualized. The curves represent absolute levels of the mRNAs encoding the three PIKKs (ATM, ATR and DNA-PKcs) and SCP1 (Sycp1, as a marker of meiotic progression) during the first wave of spermatogenesis. Data were extracted from previously published microarray data (Schultz et al., 2003). Triplicates from each developmental time point were averaged and normalized to 1 dpp. The table shows prophase-stage distribution at different times during the first wave of spermatogenesis determined on meiotic spreads [data from Goetz et al. (Goetz et al., 1984)].

completes the synaptonemal complex during zygonema progression and maintains homologs in close proximity during pachynema (Meuwissen et al., 1992). This upregulation of *ATR* transcription is not surprising given that control of cellular ATR kinase activity does not involve conformational changes affecting its kinase activity but seems to rely primarily on recruitment of the kinase to sites of damage (Bakkenist and Kastan, 2004).

The kinetics of transcription of *ATR* probably account for the fact that, in the $Atm^{-/-}$ $Spo11^{+/-}$ mutant, H2AX is not phosphorylated during leptonema but γH2AX staining gradually increases as spermatocytes progress through zygonema (Fig. 3Ad-f). The low levels of *ATR* expression during early prophase might also explain the inability of $Atm^{-/-}$ $Spo11^{-/-}$ double-mutant spermatocytes to phosphorylate H2AX during leptonema in response to radiation-induced DSBs (Fig. 2Bd), and the complete lack of γH2AX observed in leptotene $Atm^{-/-}$ spermatocytes (Fig. 2Ab).

Discussion

SPO11, recombination and synapsis are required for sex-body formation in mouse spermatocytes

Previous reports seemed to indicate that H2AX phosphorylation in the sex body was independent of SPO11 and might therefore be triggered by some aspect of sex-body formation rather than meiotic DSBs (Mahadevaiah et al., 2001). An underlying implication of this idea is that sex chromosomes might rely on a mechanism independent of sequence homology and/or recombination to drive their pairing and/or synapsis. Our immuno-FISH analysis clearly shows that the γH2AX-positive chromatin domains frequently observed in SPO11-deficient spermatocytes do not overlap with the X-Y chromatin. Furthermore, in $Spo11^{-/-}$ spermatocytes, the X and Y chromosomes are extended, asynapsed and apart in the vast majority of zygotene-like spermatocytes. These results indicate that the current view that a sex body can be formed in mouse spermatocytes even in the absence of functional SPO11 (Baarends and Grootegoed, 2003; Fernandez-Capetillo et al., 2004; Handel, 2004) is incorrect. Altogether, our data indicate that, in SPO11-deficient spermatocytes, the inability to initiate meiotic recombination affects the sex chromosomes and the autosomes to the same extent in their ability to find its cognate homolog, pair and synapse.

Our findings show that SPO11-deficient spermatocytes are unable to specifically phosphorylate H2AX in the sex chromatin and to form a sex body. Because SPO11-deficient spermatocytes are arrested in a zygotene-like stage, it might be argued that lack of sex-body formation is due to the fact that spermatocytes never reach the stage in which the sex body is actually visualized. This argument has been used to dispute the idea that recombination initiation is required for synapsis in mice. Nevertheless, in wild-type spermatocytes, pairing and alignment of homologous chromosomes occurs during leptonema/early-zygonema and γH2AX is detected on the sex chromatin throughout zygonema (during mid- to late zygonema, as γH2AX staining progressively disappears from the autosomal chromatin, the X and Y chromosomes are always decorated with γH2AX). Our immuno-FISH data clearly show that SPO11-deficient spermatocytes are unable to pair and synapse the sex chromosomes in leptonema and zygonema [the same holds true for autosomes, as evidenced by FISH using paint probes against chromosomes 18 and 9 (data not shown)] and that zygotene-like nuclei do not phosphorylate H2AX in the sex chromatin. Given that these two processes occur during leptonema and zygonema, stages that are attained by $Spo11^{-/-}$ spermatocytes, this data clearly argue in favor of the idea that DSB formation and initiation of recombination are required for synapsis of both autosomes and sex chromosomes in mouse spermatocytes.

The question still remains as to what is triggering H2AX phosphorylation in one or more confined chromatin domains in the absence of SPO11 and meiotic DSBs. Is this an ectopic or proto-sex body? In other words, are other chromatin regions normally associated with the sex chromatin in the sex body in wild-type spermatocytes, and inactivation of *Spo11* simply prevents the sex chromosomes from joining this differentiated chromatin domain? We analysed whether XMR, another sex-body-specific marker (Escalier and Garchon, 2000), localized to the γH2AX-positive chromatin domain in $Spo11^{-/-}$ spermatocytes but we found that only 10% of the nuclei displayed partial overlap of this sex-body-specific marker with γH2AX-chromatin (data not shown). Given that certain nuclear factors are sequestered in nucleoli during prophase in budding yeast (San-Segundo, 1999), we also explored the possibility that γH2AX might be localizing to the nucleoli, by staining spermatocytes with antibodies against the nucleolar protein nucleolin. We could detect several nucleolar regions in $Spo11^{-/-}$ zygotene-like spermatocytes but none of them localized to the γH2AX-labeled chromatin domains (data not shown), ruling out this possibility.

Further analysis of the $Spo11^{-/-}$ mutant will be required to identify the cues driving phosphorylation of H2AX in confined chromatin domains in the absence of SPO11 and meiotic DSBs, and whether these involve a specific set of chromosomes. Strict localization of ATR to these γH2AX-labeled chromatin domains seems to implicate ATR in these 'ectopic' phosphorylation events, whereas localization of BRCA1 to the chromosomal cores of these domains suggests a BRCA1-dependent recruitment of ATR to these sites.

Distinct roles for ATM and ATR during prophase I

In terms of defining the kinases involved in phosphorylating H2AX during meiosis, we have shown that DNA-PKcs does not play an essential role, whereas ATM is strictly required for chromatin-wide phosphorylation of H2AX during leptonema. It was not surprising to unveil such a central role for ATM in meiosis, given that human patients suffering from ataxia telangiectasia and *Atm*-null mice are sterile (Barlow et al., 1996). In $Atm^{-/-}$ male mice, meiotic abnormalities are cytologically evident as early as leptonema, with the RAD51 and DMC1 recombinases showing reduced localization to synaptonemal complexes (Barlow et al., 1998). As spermatocytes progress into zygonema, they display alterations in synaptonemal complex morphology ranging from axial gaps to extensive synaptonemal complex fragmentation. These abnormalities result in a severe prophase arrest and a significant increase in the levels of apoptosis (Barlow et al., 1996; Barlow et al., 1997; Barlow et al., 1998). Our results are consistent with a very early role for ATM in mediating the

response to SPO11-dependent DSBs introduced during leptonema.

The fact that, in the *Atm*$^{-/-}$ background, a normal complement of *Spo11* results in complete prophase arrest, just one copy rescues the arrest and two null alleles of *Spo11* result in a *Spo11*$^{-/-}$ phenocopy (this work) can be interpreted as evidence that the meiotic phenotype of the *Atm*$^{-/-}$ mutant is a consequence of a defect in the response to meiotic DSBs. An early stage of the response to meiotic DSBs is phosphorylation of H2AX (Mahadevaiah et al., 2001) and, in mitotic cells at least, γH2AX has been shown to be essential for the recruitment of cohesin to large chromatin domains around the sites of damage, thus contributing to recombinational repair (Unal et al., 2004). However, the severe meiotic phenotype of the *Atm* KO can not be attributed to the lack of H2AX phosphorylation during leptonema because *H2AX*$^{-/-}$ spermatocytes seem to repair DSBs on schedule during prophase (Fernandez-Capetillo et al., 2003b) and the phenotypes of ATM and H2AX KO mice differ substantially (Barlow et al., 1996; Barlow et al., 1998; Fernandez-Capetillo et al., 2003b). Therefore, ATM might be required per se to achieve efficient DSB repair or to phosphorylate one or more

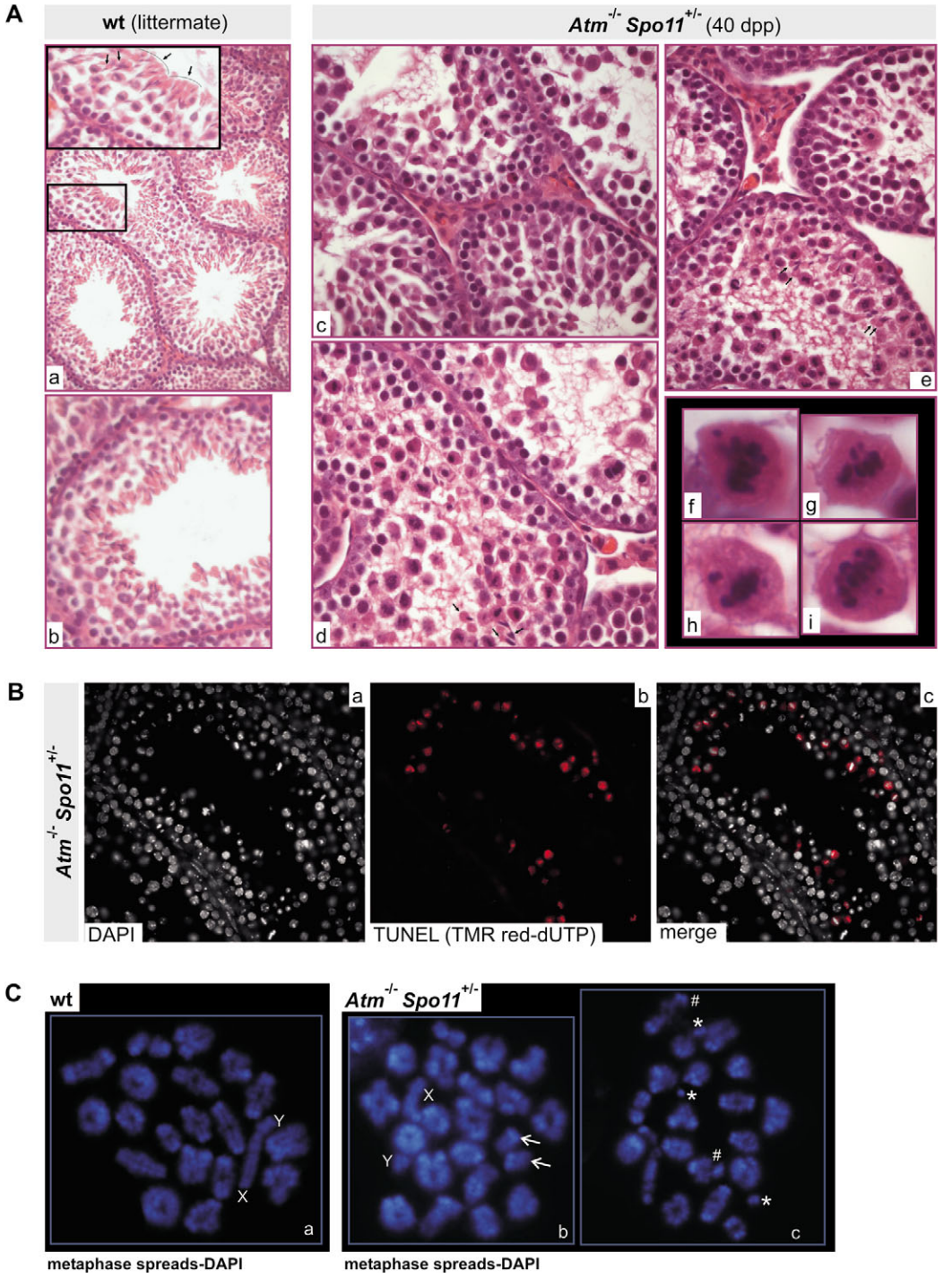


Fig. 5. *Atm*$^{-/-}$ *Spo11*$^{+/-}$ testis sections show severe defects in spermatogenesis and substantial levels of apoptosis. (A) *Atm*$^{-/-}$ *Spo11*$^{+/-}$ testes show severe defects in spermatogenesis in spite of the prophase-I rescue. Paraffin-embedded testis sections from wild-type (a,b) and *Atm*$^{-/-}$*Spo11*$^{+/-}$ (c-i) mice stained with hematoxylin and eosin. In wild-type sections, many round or elongated spermatids are found in the most lumenal part of the tubules. By contrast, in *Atm*$^{-/-}$ *Spo11*$^{+/-}$ testes, very few tubules contain elongated spermatids (d,e, arrows) and, even in positive tubules, only groups of two to 15 spermatids can be found. Examples of aberrant metaphases are frequently observed (f-i). (B) Metaphases frequently stain positive for TUNEL in *Atm*$^{-/-}$ *Spo11*$^{+/-}$ testes sections. Apoptotic cells were detected by TUNEL on paraffin-embedded testes sections. The DNA was stained with DAPI. (C) Aberrant metaphases of the first meiotic division can be observed in metaphase spreads from the *Atm*$^{-/-}$ *Spo11*$^{+/-}$ mutant. Metaphase spreads show metaphases of the first meiotic division containing autosomal univalents (marked with arrows), chromosomal fragments [asterisk (*)], achiasmatic sex chromosomes and broken bivalents [hash (#)].

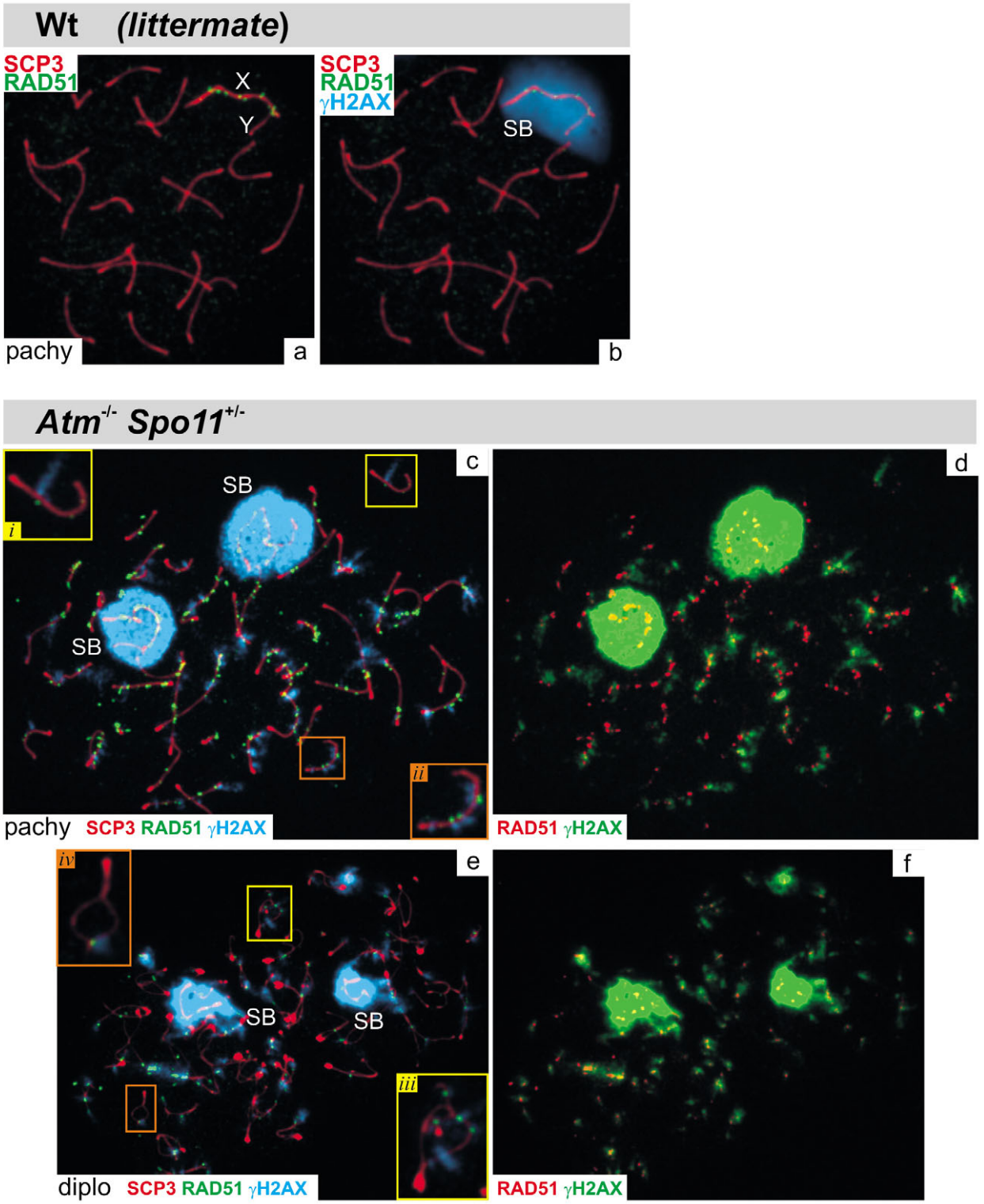


Fig. 6. In *Atm*$^{-/-}$ *Spo11*$^{+/-}$ spermatocyte spreads, pachytene and diplotene nuclei frequently display RAD51/DMC1 foci on the cores in chromosome regions containing γH2AX on the chromatin loops. Spermatocyte spreads from *Atm*$^{-/-}$ *Spo11*$^{+/-}$ mice and wild-type littermates were stained for SCP3, RAD51 and γH2AX. (c,e) Triple staining of two pachytene (c) and two diplotene (e) *Atm*$^{-/-}$ *Spo11*$^{+/-}$ nuclei. (d,f) Rad51 and γH2AX staining of the same nuclei shown in c,e. In wild-type spreads, RAD51/DMC1 foci can be detected in leptotene and zygotene spermatocytes; by pachynema, hardly any foci remain on the synaptonemal complex of autosomal bivalents, but some foci can be frequently observed on the asynapsed X axial element (Moens et al., 1997; Tarsounas et al., 1999) (a,b). In the *Atm*$^{-/-}$ *Spo11*$^{+/-}$ mutant, we frequently observe RAD51/DMC1 foci on the chromosomal cores of pachytene (c) and diplotene (e) nuclei, suggesting a delay in either the introduction of DSBs or in the ensuing strand invasion and exchange process catalysed by these recombinases. Rad51 foci are located on the cores of chromosomal regions containing γH2AX (also see d,f).

downstream substrates besides H2AX that might be essential for the repair process.

The discovery that *Spo11* heterozygosity can rescue the prophase arrest of *Atm*-null spermatocytes has allowed us to demonstrate that ATM is dispensable for phosphorylation of H2AX in the sex body. It also permitted analysis of ATR localization in a genetic background that, in spite of the ATM deficiency, progresses through prophase, phosphorylates H2AX on the sex chromatin and forms a sex body. The fact that $Atm^{-/-}$ $Spo11^{+/-}$ spermatocytes contain ATR and γH2AX in the sex body demonstrates that ATM is dispensable for phosphorylation of H2AX in the sex chromatin and for sex-body formation, complementing and extending results from a previous report indicating that ATR is probably the kinase involved in phosphorylating H2AX in this subnuclear domain (Turner et al., 2004).

Spo11 heterozygosity rescues the prophase-I arrest in ATM-deficient spermatocytes

We have uncovered an unusual interplay between mutations in the *Atm* and *Spo11* genes. In the absence of functional ATM, both a normal complement of *Spo11* and two null copies of *Spo11* result in complete prophase arrest (Barlow et al., 1997) (Fig. 3Ab,c), whereas *Spo11* heterozygosity allows ATM-deficient spermatocytes to complete prophase I (Fig. 3Ad-i). Nevertheless, spermatogenesis is arrested at a later stage in $Atm^{-/-}$ $Spo11^{+/-}$ mutants, as evidenced by the significantly reduced number of elongated spermatids observed in testis sections. Less than 5% of tubule sections contain elongated spermatids, and even positive tubules contain very few spermatids in comparison with the wild-type control (fewer than 15 vs more than 100 spermatids per tubule normally observed in wild-type sections) (Fig. 5Ac-e). Preliminary results indicate a partial arrest in metaphase I (Fig. 5Ac-i), with a substantial proportion of metaphases staining positive for TUNEL (terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling) in paraffin-embedded tissue sections (Fig. 5B). Metaphase spreads (Fig. 5C) show metaphases of the first meiotic division containing autosomal univalents (arrows), chromosomal fragments [asterisks (*)], achiasmatic sex chromosomes and broken bivalents [hash (#)]. Altogether, these findings probably explain the infertility of these mice. So far, none of the five males and four females has been able to produce offspring after three to four months (their lifespan is reduced by the ATM defect).

How can half the complement of *Spo11* rescue the prophase arrest? The most straightforward explanation is that either the number of breaks or the timing with which they are introduced is affected in such a way as to permit a kinase that normally acts later in prophase to substitute for the lack of ATM. That is, fewer and possibly later breaks might allow this alternative kinase to complement the absence of ATM more efficiently than in the $Spo11^{+/+}$ context. Given the expression kinetics of ATR and the well documented cross-talk between the signaling pathways regulated by ATR and ATM, ATR might very well be this alternative kinase. In fact, in the $Atm^{-/-}$ $Spo11^{+/-}$ background, in spite of the absolute lack of γH2AX observed during leptonema (Fig. 3Ad), H2AX is eventually phosphorylated as spermatocytes progress into zygonema (Fig. 3Ae,f). The kinetics of transcription of *ATR* show low levels of expression early in prophase followed by a tenfold increase as spermatocytes progress into late zygonema and pachynema, which is consistent with ATR being able to substitute for ATM as spermatocytes approach mid- to late zygonema and enter pachynema.

Another ATM substrate (Chk2), which becomes phosphorylated around mid-pachynema in a wild-type background, remains unmodified in $Atm^{-/-}$ and $Atm^{-/-}Spo11^{-/-}$ testes but is phosphorylated in the $Atm^{-/-}Spo11^{+/-}$ mutant (P.J.R. and R.D.C.-O., unpublished), providing further evidence that an alternative kinase comes into play in this genetic background.

Even though *Spo11* heterozygosity allows ATM-deficient spermatocytes to undergo prophase I, preliminary data indicate that they suffer a lack of coordination between DSB repair, synapsis and prophase progression. Spermatocytes seem to proceed into late prophase with unrepaired DSBs, as evidenced by the persistence of γH2AX eruptions in pachytene and diplotene spermatocytes (Fig. 3Ag,h, arrows). We have also observed persistent RAD51/DMC1 foci over the chromosomal cores of pachytene and diplotene spermatocytes, suggesting again that DSBs are not introduced and/or repaired on schedule in the $Atm^{-/-}$ $Spo11^{+/-}$ mutant (Fig. 6c-f).

The rescue of the ATM KO by a single copy of *Spo11* was surprising given that $Spo11^{+/-}$ mice do not have any evident meiotic phenotype. It will be interesting to find out whether transcription and/or expression of *Spo11* are significantly reduced or delayed in $Spo11^{+/-}$ meiocytes and whether this affects meiotic progression (an abnormal distribution of spermatocytes in the different prophase stages might indicate delays in meiotic progression). Differences in meiotic progression observed between the $Spo11^{+/-}$ and the rescued $Atm^{-/-}$ $Spo11^{+/-}$ mice might shed some light on the role of ATM in responding to DSBs as well as surveying and/or regulating prophase progression.

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