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H2AX regulates meiotic telomere clustering

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The histone H2A variant H2AX is phosphorylated in response to DNA double-strand breaks originating from diverse origins, including dysfunctional telomeres. Here, we show that normal mitotic telomere maintenance does not require H2AX. Moreover, H2AX is dispensable for the chromosome fusions arising from either critically shortened or deprotected telomeres. However, H2AX has an essential role in controlling the proper topological distribution of telomeres during meiotic prophase I. Our results suggest that H2AX is a downstream effector of the ataxia telangiectasia–mutated kinase in controlling telomere movement during meiosis.

Introduction

Telomeres are not only critical components of somatic chromosomes, but also play a unique function during meiosis. Meiosis is a cellular differentiation program during which physiological double-strand breaks (DSBs) are created and repaired, giving rise to recombination events between parental chromosomes. During the first meiotic prophase, telomeres redistribute and cluster, forming a so-called “bouquet,” which may ensure proper homologue pairing before recombination (Loidl, 1990; Scherthan, 2001; Yamamoto and Hiraoka, 2001). The ataxia telangiectasia–mutated (ATM) kinase is required for transit through early prophase I (Pandita, 2002). In addition, ATM disruption has been found to alter telomere dynamics, leading to an accumulation of bouquet-stage nuclei with perturbed synapsis during zygotene (Pandita et al., 1999; Scherthan et al., 2000).

One of the immediate targets of the ATM kinase in response to DNA damage is the histone H2A variant H2AX (Redon et al., 2002). The analysis of H2AX-deficient mice has demonstrated a role for H2AX in a variety of responses to DSBs, including DNA repair, checkpoint signaling, and Ig class switching (Petersen et al., 2001; Bassing et al., 2002; Celeste et al., 2002; Fernandez-Capetillo et al., 2002; Reina-San-Martin et al., 2003). Similar to ATM-deficient cells, H2AX−/− cells senesce within a few passages in culture, and display an increased frequency of chromosomal aberrations (Celeste et al., 2002, 2003a). Moreover, H2AX−/− mice exhibit male-specific sterility, which is likely due to defects in chromatin remodeling during meiosis (Fernandez-Capetillo et al., 2003). Because of the strong correlation between defective DSB repair, genomic instability, and telomere dysfunction, we examined the role of H2AX in both mitotic and meiotic telomere maintenance.

Results and discussion

To determine whether H2AX regulates telomere length, we performed quantitative FISH (Zijlmans et al., 1997) on metaphase spreads derived from four independent sets of H2AX knockout and control mouse embryonic fibroblasts (MEFs). Although telomeres were slightly elongated in some of the H2AX−/− MEFs relative to H2AX+/+ isogenic cultures (Table I), this difference in telomere length was not statistically significant (t test, P > 0.1; at least 15 metaphases examined for each culture). Moreover, both genotypes displayed a similar heterogeneity in the frequency of telomere fluorescence intensities, indicating that H2AX deficiency did not modify the distribution of individual telomere lengths (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200305124/DC1). To rule out the possibility that the decreased proliferative capacity of H2AX−/− MEFs (Celeste et al., 2002) could bias the measurements of telomere lengths, we performed quantitative FISH in a variety of other primary cells including splenocytes, purified B cells, and lymph node T cells, derived from independent H2AX+/+ and H2AX−/− littermates (Table S1). None of the cell types showed a significant difference in telomere length (t test, P > 0.1; at least 15 metaphases examined for each culture).

Abbreviations used in this paper: ATM, ataxia telangiectasia mutated; DSB, double-strand break; MEF, mouse embryonic fibroblast; SC, synaptonemal complex; Terc, RNA component of telomerase.

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Thus, telomere dysfunction does not contribute significantly to H2AX deficiency. However, despite the high level of genomic instability in cells, we did not detect any significant increase in telomere lengths in B and T lymphocytes by flow cytometry 

To determine whether chromosomal aberrations arise in part from modifications in telomere structure, as is the case in numerous mouse models with defects in DSB repair (Goyti and Blasco, 2002), we analyzed individual metaphase spreads from four solo and Blasco, 2002), we analyzed individual metaphase spreads from four mice.

As an additional quantitative measurement, we analyzed telomere lengths in B and T lymphocytes by flow cytometry FISH (Rufer et al., 1998), which confirmed the lack of telomere fusions in H2AX-deficient MEFs (Celeste et al., 2002, 2003a). In contrast to DNA ligase IV, H2AX was not essential for fusions arising from deprotected telomeres, H2AX+/+ and H2AX−/− MEFs were infected with a TRF2 dominant-negative–expressing retrovirus (TRF2ΔATM) or with the corresponding vector pLIPC (Karseder et al., 1999). Following the strategy used to assess the role of the NHEJ factor DNA ligase IV in telomere fusions (Smogorzewska et al., 2002), MEFs were generated in a p53-deficient background, which partially alleviates the growth defects in primary H2AX−/− MEFs (Celeste et al., 2002, 2003a). In contrast to DNA ligase IV, H2AX was not essential for fusions arising from TRF2 dominant-negative infection (Fig. 1, B and C). In 30 metaphases examined by telomere FISH, we observed a total of 43 telomere fusions in H2AX−/− p53−/− MEFs, compared with 29 fusions in H2AX+/+ p53−/− MEFs. Thus, although H2AX appears to modulate NHEJ (Downs et al., 2000; Bassing et al., 2003; Celeste et al., 2003a), H2AX is not required for chromosome fusions arising from either shortened or structurally deprotected telomeres.

During mouse meiosis, telomeres reposition along the nuclear periphery to create a characteristic bouquet configuration. This clustering of chromosome ends generally occurs at the leptotene/zygotene transition (Scherthan, 2001), coincident with the initiation of homologous DSB repair (for review see Hunter et al., 2001). To date, the only protein that has been implicated in the regulation of the bouquet stage in mammals is the ATM kinase (Pandita et al., 1999). To determine whether the ATM target H2AX is involved in meiotic telomere dynamics, we investigated telomere and centromere behavior by FISH (Scherthan et al., 1996) in wild-type and H2AX-deficient testes preparations from 4-wk-old mice (Fig. 2 A). The analysis of structurally preserved spermatocyte nuclei revealed similar frequencies of preleptotene spermatocytes (1.0 vs. 1.6%) in wild-type and mutant testes suspensions (based on 2,772 wild-type and 2,567 mutant nuclei), respectively, with the difference.

### Table 1. Quantitative FISH analysis of telomere length

<table>
<thead>
<tr>
<th>Litter</th>
<th>Genotype</th>
<th>Chromosome arm</th>
<th>Telomere length [A.U. (SD)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+/-</td>
<td>p</td>
<td>1285 (352)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>q</td>
<td>1509 (429)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>1397 (389)</td>
</tr>
<tr>
<td>B</td>
<td>+/-</td>
<td>p</td>
<td>1320 (356)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>q</td>
<td>1550 (421)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>1435 (388)</td>
</tr>
<tr>
<td>C</td>
<td>+/-</td>
<td>p</td>
<td>1335 (277)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>q</td>
<td>1601 (546)</td>
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<tr>
<td></td>
<td></td>
<td>Total</td>
<td>1648 (476)</td>
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<tr>
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<td>+/-</td>
<td>p</td>
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<tr>
<td></td>
<td></td>
<td>q</td>
<td>1699 (658)</td>
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<tr>
<td></td>
<td></td>
<td>Total</td>
<td>1584 (624)</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>p</td>
<td>1457 (568)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>q</td>
<td>1807 (695)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>1632 (631)</td>
</tr>
</tbody>
</table>

| aAt least 15 metaphases were used per analyzed cell culture.  
| bH2AX−/−; +/-, H2AX+/+. A.U., arbitrary units; SD, standard deviation.
being statistically insignificant (P = 0.1; χ² and Fisher test; Fig. 2 B). However, we noted a 20-fold increase in the frequency of H2AX<sup>++</sup> bouquet-stage nuclei (H2AX<sup>++</sup>, 6%; wild-type, 0.4%; based on 2,567 mutant and 2,772 wild-type spermatogenic nuclei), with the differences being highly significant (P < 0.0001; χ² and Fisher test; Fig. 2 B). To determine the stages in which elevated levels of bouquet nuclei accumulate, we combined immunostaining of the telomere-associated protein TRF1 with that of SCP3 (Lammers et al., 1994), a component of the axial/lateral element of the synaptonemal complex (SC; Fig. 2 C). Three-dimensional microscopy revealed that TRF1 signals capped the ends of axial/lateral elements that clustered at the nuclear envelope. Strikingly, many of the structurally preserved H2AX<sup>++</sup> prophase I nuclei displayed a bouquet topology with telomeres clustered in a limited nuclear envelope region from early leptotene until early pachytene, with long U-shaped SCs emanating from the clustered telomeres (Fig. 2 C). The occurrence of telomere clustering as early as leptotene and its maintenance up to late zygotene/pachytene stages contrasts with wild-type spermatogenesis of adult mice, where telomere clustering occurs only in a limited time window during the leptotene/zygotene transition (Scherthan et al., 1996). In testes suspensions of wild-type mice, bouquet-stage cells are generally detected at an average frequency of 0.2–0.8%, which underlines the short-lived nature of this stage in spermatogenesis (Scherthan et al., 1996, 2000). Thus, the significant increase in bouquet
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hadevaiah et al., 2001), we found that phosphorylation in response to Spo11-mediated DSBs (Ma...

wild-type spermatocytes, which exhibit massive H2AX...

ing that meiotic DSB-triggered...

Figure 2. H2AX inactivation results in aberrant telomere clustering during meiotic prophase I. (A) Telomere (red)—centromere (green) FISH patterns in structurally preserved spermatocytes. (I) Premiotic nuclei with numerous internal telomere and satellite DNA clusters (focal plane at nuclear equator). (II) Preleptotene nuclei with peripheral satellite DNA clusters (focal plane at nuclear equator). (III) Two bouquet nuclei each with clustered telomeres (leptotene/zygotene; focal plane at nuclear top). (IV) Pachytene nuclei with dispersed peripheral telomeres and satellite DNA clusters (focal plane at nuclear equator). (B) Frequency of preleptotene and bouquet spermatocytes, with the latter being dramatically increased in the H2AX knockout; see Results for details. (C) Immunofluorescence of axial/lateral cores (SCP3, red) and telomeres (TRF1, green) in structurally preserved H2AX+/− nuclei (DAPI, blue). (I) Early leptotene nucleus with a tight telomere cluster at a sector of the nuclear periphery and SCP3 speckles. (II) More advanced leptotene with short SCP3 threads and clustered telomeres. (III) Two late zygotene/pachytene nuclei with more relaxed telomere clustering near the nuclear top and U-shaped SCs that extend into the nuclear lumen. (IV) Pachytene nuclei with meandering SCs and telomeres dispersed around the nuclear periphery. Bar, 10 μm.

frequencies in the H2AX knockouts as compared with age-matched controls suggests that the absence of H2AX leads to an extended bouquet stage. Moreover, in contrast to wild-type spermatocytes, which exhibit massive H2AX phosphorylation in response to Spo11-mediated DSBs (Mahadevaiah et al., 2001), we found that γ-H2AX staining was largely absent in ATM−/− leptotene/zygotene-stage spermatocytes (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200305124/DC1), therefore demonstrating that meiotic DSB-triggered γ-H2AX formation is dependent on ATM. These results place H2AX downstream of ATM in the signal transduction pathway that orchestrates meiotic telomere clustering.

The initiation of telomere clustering appears to be a default reaction because it occurs in the absence of synapsis, homologous chromosomes, and/or recombination (for review see Scherthan, 2001). However, the accumulation of bouquet-stage meiocytes in DSB and SC-deficient yeast or worm meiosis (Trelles-Sticken et al., 1999; MacQueen et al., 2002) suggests that the resolution of telomere clustering is triggered upon completion of synapsis and/or repair. Consistent with this, both H2AX−/− and ATM−/− mice display an accumulation of spermatocytes with persistence of bouquet topology. The fact that bouquet-type arrangements in H2AX-deficient spermatocytes are observable up to pachytene suggests that the increased telomere clustering observed in ATM-deficient cells may be directly related to impaired phosphorylation of H2AX, rather than being an indirect consequence of the early leptotene/zygotene arrest. According to this view, ATM facilitates telomere-promoted homologue pairing via phosphorylation of H2AX, thereby coordinating clustering with the initiation of DSB repair. The dissolution of meiotic telomere clustering would then depend on the dephosphorylation of γ-H2AX, which may signal the completion of DSB repair and/or induce changes in higher order chromatin structure (Fernandez-Capetillo et al., 2003). Because the exit from the bouquet stage is coordinated with completion of DSB repair (Trelles-Sticken et al., 1999; MacQueen et al., 2002), the elevated telomere clustering in H2AX−/− spermatocytes may therefore reflect an altered repair capacity of the H2AX knockout spermatocytes.

Like many other mouse models with defects in DSB repair and/or telomere maintenance, absence of H2AX is associated with growth defects, radiation sensitivity, genomic instability, and cancer predisposition (Bassing et al., 2002, 2003; Celeste et al., 2002, 2003a). Although a number of DNA repair proteins play essential roles in maintaining telomere structure, we have found that H2AX is largely dispensable for somatic telomere maintenance. In principle, this could be explained by the fact that H2AX is not required for the recruitment of damage sensors to DNA lesions, and therefore, the cellular response to unprotected chromosome ends may proceed normally in its absence (Celeste et al., 2003b). However, H2AX is essential for the proper spatial rearrangement of chromosome ends during the first meiotic prophase. Further analysis will be necessary to dissect the role of meiotic telomere clustering and its dissolution with respect to homologue pairing and DSB repair.

Materials and methods

Mice and cell lines

Generation of H2AX+/−, ATM−/−, and Terc−/− mice have been described previously (Barlow et al., 1996; Blasco et al., 1997; Celeste et al., 2002). E13.5 MEFs were obtained from intercrossing mice following standard procedures, and H2AX−/−p53−/− MEFs are described elsewhere (Celeste et al., 2003a). For all experiments, littermates were compared. B lymphocytes were isolated using CD19 microbeads (Miltenyi Biotec), and were stimulated with LPS or LPS+IL4 as described previously (Celeste et al., 2002). Splenocytes or lymph node–derived B and T lymphocytes were stimulated with either LPS or Con A, respectively.
Analysis of telomere lengths and fusions

Quantitative FISH analysis using a Cy3-labeled (CCCTAA) peptide nucleic acid probe (Applied Biosystems) was performed as described previously (Zijlma et al., 1997; Hande et al., 1999). Telomere length measurements were performed on at least 15 metaphases for each cell type. DAPI chromosome and Cy3 telomere images were acquired with a constant exposure time that ensured all captured fluorescent signals were within the linear range. All the images from matched littermate samples were acquired blindly and in parallel on the same day. To correct for differences in the microscope settings and hybridization efficiencies, the fluorescence intensity of Cy3-labeled fluorescent beads (Molecular Probes, Inc.) was used to normalize intensities from different experiments. Quantitative analysis of telomere fluorescence was performed with the TFL Telo software, which allows for a proper identification and editing of individual telomere intensities (a gift from Dr. Peter Lansdorp). Statistical analysis of the measured telomere intensities was performed with Microsoft Excel 2000 (Microsoft Corp.) and Probit (BBN Technologies) softwares. Chromosomal aberrations, including breaks and telomere fusions, were scored by examining DAPI and telomeric images from at least 65 metaphases derived from cultures of H2AX+/Terc+/− (G0), H2AX+/Terc−/− (G0), H2AX+/Terc−/+ (G5), and H2AX−/−Terc−/− (G5) MEFs (a total of 417, 355, 357, and 346 metaphases were examined, respectively, for each genotype).

Retroviral infection and plasmids

pLPC-puro and pLPC-TRF2−/−/−/− retroviral vectors have been described previously (Karlesder et al., 1999). For retroviral infection, Phoenix α cells (American Type Culture Collection) were seeded at 5 × 105 cells/10-cm dish, and 20 μg of each plasmid was transfected using CaPO4. 5 h after transfection, the cells were washed with PBS and the medium was replenished. A 10-ml supernatant was collected 72 h after transfection, passed through a 0.45-μm filter, and supplemented with polybrene at 4 μg/ml. MEFs were seeded 24 h before infection at 8 × 105 cells/10-cm dish. For infection, MEFs were overlaid with virus-containing medium, and centrifuged for 1.5 h at 5,000 rpm. Cells were split into three 10-cm dishes 24 h after infection, and the medium was replaced by DME/15% FCS containing 2 μg puromycin per ml. Metaphases were prepared 96 h after selection.

Testicular preparations and bouquet analysis

Testes suspensions containing structurally preserved nuclei for simultaneous analysis of synaptonemal complexes of the rat is related to X-linked lymphoproliferative disease. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200305124/DC1.

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