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E-type cyclins modulate telomere integrity in mammalian male meiosis

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Abstract

We have shown that E-type cyclins are key regulators of mammalian male meiosis. Depletion of cyclin E2 reduced fertility in male mice due to meiotic defects, involving abnormal pairing and synapsis, unrepaired DNA, and loss of telomere structure. These defects were exacerbated by additional loss of cyclin E1, and complete absence of both E-type cyclins produces a meiotic catastrophe. Here, we investigated the involvement of E-type cyclins in maintaining telomere integrity in male meiosis. Spermatocytes lacking cyclin E2 and one E1 allele (*E1+/-E2-/-*) displayed a high rate of telomere abnormalities but can progress to pachytene and diplotene stages. We show that their telomeres exhibited an aberrant DNA damage repair response during pachynema and that the Shelterin-complex proteins TRF2 and RAP2 were significantly decreased in the proximal telomeres. Moreover, the insufficient level of these proteins correlated with an increase of γ -H2AX foci in the affected telomeres, and resulted in telomere associations involving TRF1 and telomere detachment in later prophase-I stages. These results suggest that E-type cyclins are key modulators of telomere integrity during meiosis by, at least in part, maintaining the balance of Shelterin-complex proteins, and uncover a novel role of E-type cyclins in regulating chromosome structure during male meiosis.

Keywords

Meiosis; E-type cyclins; Cyclin E1; Cyclin E2; Meiosis control; Telomere; Telomere integrity; Shelterin complex; TRF1; TRF2

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Introduction

The family of E-type cyclins consists of two proteins, designated cyclin E1 and E2, which function during the G1 and S phases of the mitotic cell cycle. E1 and E2 exhibit high homology in their protein sequence (70% identity between the cyclin box and 47% between the overall sequence) (Hwang and Clurman 2005). Insight into their *in vivo* functions has been obtained from the generation of E1- and E2-deficient strains of mice (designated *E1*^{-/-} or *E2*^{-/-}, respectively) through targeted mutagenesis (Geng et al. 2007; Geng et al. 2003; Parisi et al. 2003). Mice lacking either protein were viable, although *E2*^{-/-} male mice were sub-fertile. Ablation of both E1 and E2 resulted in embryonic lethality due to placental failure. While the role of E-cyclins in mitotic somatic cells has been extensively studied, the molecular function of these proteins in the male germ line remains largely unknown, but is of great interest given the sub-fertility seen in male *E2*^{-/-} mice.

In contrast to their overlapping expression in somatic cells, we previously reported that E-type cyclins exhibit distinct patterns of expression in the germ line, notably in meiotic spermatocytes rather than in mitotic spermatogonia (Martinerie et al. 2014). Cyclin E1 expression is restricted to non-proliferating Sertoli cells and mid-pachytene to diplotene spermatocytes, where it is specifically localized along the axial element of the sex chromosomes. In contrast, cyclin E2 is restricted to spermatocytes during all stages of prophase I, suggesting that it may play a key role in male meiosis. Indeed, using constitutive and conditional knockout mice lines, we observed that cyclin E2-deficient (*E1*^{+/+}*E2*^{-/-}) males exhibited reduced fertility (Geng et al. 2003; Martinerie et al. 2014) resulting from meiotic defects in homologue pairing, synapsis and DNA repair (Martinerie et al. 2014). The relevance of both E-type cyclins for male meiosis is also underscored by our observations that the additional loss of one *E1* allele (*E1*^{+/-}*E2*^{-/-}) resulted in sterility, with severely disrupted spermatogenesis and enhanced meiotic defects (Martinerie et al. 2014). The complete absence of E-type cyclins in the male germ line resulted in a meiotic catastrophe, with most spermatocytes arrested at zygotene and early pachytene-like stages.

The most notable defects produced by loss of cyclin E2 function occurred at the chromosome ends. The conical thickening of the axial elements (AEs) at the chromosome ends, called synaptonemal complex attachment sites (SCAS), which are critical for tethering chromosomes to the nuclear envelope (NE) (Liebe et al. 2004), were significantly thinner than wild type spermatocytes (Martinerie et al. 2014). Additional depletion of cyclin E1 in the *E2*^{-/-} background further reduced the thickness of the SCAS. In addition, loss of cyclin E2 resulted in frequent end-to-end chromosome associations and the presence of γ H2AX foci at chromosome ends, which persisted until the diplotene stage. These combined observations suggested that the telomeres may be abnormal in spermatocytes deficient for E-type cyclins. Indeed, immuno-FISH analysis revealed severe defects in *E2*^{-/-} spermatocytes, which included extended telomeres and aberrant telomere associations (Martinerie et al. 2014). These defects increased with the additional depletion of *E1* alleles in the *E2*^{-/-} background. Almost all *E1*^{+/-}*E2*^{-/-} and *E1*^{-/-}*E2*^{-/-} spermatocytes showed chromosomes forming telomeric bridges between nonhomologous chromosomes. Moreover, ends of two or more individual synaptonemal complexes (SC) were tightly associated and occasionally led to end-to-end fusions that produced chromosome rearrangements.

Although the origin of these defects is not known, one potential pathway modulated by E-type cyclins involves the protection of telomeres by the Shelterin complex. In somatic cells, the Shelterin complex, composed of TRF1, TRF2, RAP1, TIN2, TPP1, and POT1, generates the t-loop and capping of the chromosome ends, controlling telomere length and integrity by protecting them from being recognized as DNA damage (de Lange 2005; Martinez and Blasco 2011). Loss of Shelterin proteins, or even insufficient levels of its components, yields telomere deprotection (uncapping) and loss of inhibition of DNA repair proteins, resulting in telomere erosion (Cesare et al. 2013; de Lange 2005; Takai et al. 2003). Indeed, when chromosome ends retain insufficient TRF2 to inhibit the DNA damage repair (DDR) response and end joining, γ -H2AX foci form at the chromosome ends, telomeres are shortened, and chromosome fusions occur (Cesare et al. 2013). In addition, when only TRF1 is present at telomeric tracts of DNA, or when it is overexpressed in cells, the telomeres lose their individuality, with pairing of the telomeric sequences and association of different telomeres (Griffith et al. 1998; Lisaingo et al. 2014).

The Shelterin complex proteins TRF1, TRF2 and RAP1 are also components of meiotic telomeres (Cooper et al. 1998; Franco et al. 2002; Klutstein et al. 2015; Scherthan et al. 2000). In addition to its role in the Shelterin complex, TRF1 functions in attaching telomeres to the NE by association with the TERB1-SUN1-KASH5 complex in mouse spermatocytes (Shibuya et al. 2014). This interaction allows telomeres and chromosomes to move along the NE throughout prophase I (Scherthan et al. 1996; Tanemura et al. 2005), facilitating the pairing, synapsis and desynapsis of homologous chromosomes (Franco et al. 2002; Shibuya and Watanabe 2014).

In the present study we show that deficiency of E-type cyclins triggers a DNA damage signal at male meiotic telomeres, particularly at the proximal telomeres, a function quite unpredicted from the role of E-type cyclins during the mitotic cell cycle. The presence of γ -H2AX foci correlated with changes in the levels of the Shelterin complex proteins TRF2 and RAP1, a decrease that may result in unbalanced levels of TRF1 at telomeres and the induction of telomere associations and fusions, chromosome rearrangements and telomere detachment from the NE. Hence, the E-type cyclins play novel and fundamental roles to ensure the correct dynamic and behavior of chromosome structures, such as telomeres, during male meiosis.

Materials and Methods

Generation of *E1+/-E2-/-* male mice

E1-/-E2+/- (Geng et al. 2003) male mice were mated with *E1+/-E2-/-* females to obtain *E1+/-E2-/-* male mice. The use of animals was approved by Columbia University Medical Center Animal Care and Use Committee.

Spermatocyte spreads, squash preparations, and immunofluorescence

Spermatocyte spreads and squashes were generated following the procedures described by Manterola et al (Manterola et al. 2009). Briefly, for spread preparations, seminiferous tubules were isolated, placed in a petri dish and mechanically disaggregated using forceps.

Then, 80 to 200 μ l of 100mM sucrose was slowly added and mixed with the cells. From this suspension, 14 μ l was dropped onto a slide previously submerged in 1% paraformaldehyde (PFA), pH 9.2 and slowly spread throughout the entire slide. Then, the slides were slowly dried in a humid chamber for 3 hours, washed with Photo-Flo 0.08% in distilled water, dried and stored at -80°C until their use. In spermatocyte squash preparations, isolated seminiferous tubules were fixed in freshly prepared 2% formaldehyde in PBS (phosphate-buffered saline) pH 7.4 containing 0.05% Triton X-100 for 10 minutes at room temperature. After fixation, 3 to 4 tubules were placed on a slide and squashed using a coverslip, and immediately frozen in liquid nitrogen and stored at -80°C until their use. The slides were then placed in PBS and incubated with the following primary antibodies: mouse anti-SYCP3 1:200 (Abcam, Ab12452); rabbit anti-SYCP3 1:200 (Abcam, Ab15093); human anti-centromere 1:300 (Antibodies Incorporated, 15-235-0001); mouse anti-phospho-histone H2AX (Ser139) 1:1000, clone JWB301 (Upstate, 05-636); rabbit anti-TRF2 1:700 (Novus, NB110-57130); rabbit anti-TRF1 and rabbit anti-RAP1 both 1:500, kindly provided by Dr. Titia de Lange (The Rockefeller University, New York, NY, US); and mouse 13d4, recognizing all major LAP2 isoforms kindly provided by Dr. Manfred Alsheimer 1:300 (University of Würzburg; Würzburg, Germany). After rinsing in PBST (phosphate-buffered saline, 1% Tween-20), the slides were incubated with the appropriate secondary antibodies diluted 1:200 in PBS as follows: Alexa 488-conjugated donkey anti-rabbit IgG (H+L), Alexa 488-conjugated donkey anti-mouse IgG (H+L), Alexa 594-conjugated donkey anti-mouse IgG (H+L), Alexa 594-conjugated donkey anti-rabbit IgG (H+L), DyLight 594 goat anti-rabbit IgG F(ab')₂, Alexa 488-conjugated goat anti-mouse IgG, F(ab')₂, Alexa 350-conjugated donkey anti-mouse IgG. Slides were counter-stained with DAPI and mounted with Vectashield.

Chromosome spreads were examined using a Nikon eclipse E800 microscope equipped with epifluorescence optics and the images were photographed with a high-definition cooled color camera head DS-Fi1c. All images were processed with Adobe Photoshop CS5 software. Squash preparations were examined using a confocal Zeiss LSM710 microscope. Z stacks, composed of 0.25 μ m optical sections, were obtained using the Zen 2011 software (Zeiss). Images were subsequently analyzed and processed using the public domain software ImageJ (NIH). As early, mid and late WT pachytene spermatocytes exhibited the same pattern of TRF1, TRF2 and RAP1 foci at the telomeres, a representative mid-pachytene cell is shown in Figures 2, 3 and 4.

Quantification of γ H2AX signal in chromosome segments

Chromosome length was measured using Image J. Using the segmented line tool, the chromosome length was selected and then measured using the Analyze/Measure command. Afterwards, the values were transferred to an Excel file for further analysis. Each chromosome length was then divided by 5 using the formula $s = x/5$, where x is the length of the measurement using Image J and s corresponds to segments numbered 1 to 5, each with an identical length (Figure 1B). Then, each s was identified in the chromosome and used to classify the presence of γ H2AX within the segment. As exemplified in Figure 1B, in order to better distinguish cytologically the segments containing the telomeres/centromeres from those adjacent to the telomeres, the first segment (which contains the centromere) and the

last segment were divided in half. Thus, the first segment which contains the centromere was subdivided into two subsegments of identical length, which were designated “proximal end” and “sub-proximal end”. Similarly, the last segment was subdivided into two sub-segments, designated “distal end” and “sub-distal end”. The segments covering the central part of the chromosome were designated “central”. Then, the number of segments with and without γ H2AX foci per chromosome was recorded and analyzed with Excel and GraphPad Prism 5. Four spermatocytes from early, mid and late pachynema from 3 different animals were analyzed and quantified. No differences in the distribution were observed for mid and late pachytene spermatocytes; thus quantifications were merged in one graph (Figure 1C, mid-late pachynema). Results represent mean $\pm$ standard deviation (SD). A statistical t-test was performed using a non-parametric Wilcoxon signed-rank test to determine the difference between the genotypes and the threshold of significance was set at 0.05. (GraphPad Prism 5, Graphpad Software, Inc., San Diego, CA).

Quantification of TRF1, TRF2 and RAP1 signal intensities in spermatocyte spread preparations

TRF1, TRF2, and RAP1 intensities were quantified by selecting the area of the protein signal in each telomere and measuring the intensity of the signal using the measurement/mean value tool in ImageJ (NIH). Both protein signal and background were measured and the final TRF1, TRF2, and RAP1 intensities were calculated by subtracting the background signal from the protein signal. Results represent the mean $\pm$ SD. Statistically significant differences between the genotypes were determined using a non-parametric Wilcoxon signed-rank test with the threshold of significance set at 0.05.

Quantification of TRF2 and γ H2AX signal intensities per telomere in spermatocyte squash preparations

TRF2 and γ H2AX intensities were quantified in confocal sections of spermatocyte squash preparations immunostained for SYCP3, TRF2 and γ H2AX. Each channel was captured and compiled into a stack using image J (preserving the individuality of each channel). For quantification, we excluded telomeres that formed clusters in the nucleus. The remaining individual telomeres were chosen at random from three mid and late pachytene spermatocytes. If the TRF2 and/or γ H2AX signals were present in more than one confocal section, the corresponding sections for each channel were merged into a single image. An average of 13 telomeres per spermatocyte was quantified for both TRF2 and γ H2AX signal. The intensity of each signal was determined by selecting the area of the protein signal in each telomere and measuring the intensity of the signal using the measurement/mean value tool in ImageJ (NIH). Both protein signal and background were measured and the final TRF2 and γ H2AX intensities were calculated by subtracting the background signal from the protein signal. The correlation coefficient (R) was calculated to define the strength of the relationship between TRF2 and γ H2AX intensities. The coefficient of determination was calculated to define the proportion of the variance of TRF2 intensity levels that is predictable from the intensity levels of γ H2AX signal. The coefficient of determination and graph were generated using GraphPad Prism 5 (Graphpad Software, Inc., San Diego, CA).

Results and Discussion

γ H2AX foci form at the ends of chromosomes in cyclin E-deficient pachytene spermatocytes

It has been proposed that there are two waves of H2AX phosphorylation during meiosis. The first occurs in leptotema in response to the generation of double strand breaks (DSBs) by Spo11 (Keeney et al. 2014; Mahadevaiah et al. 2001; Romanienko and Camerini-Otero 2000), which triggers the recruitment of DNA repair proteins to the sites of DSBs and the beginning of synapsis between homologous chromosomes. The second wave occurs during pachynema in response to chromosome asynapsis, resulting in the localization of γ H2AX in regions of unsynapsed chromosomes, such as the sex chromosomes. We had previously reported the presence of γ H2AX foci adjacent to the SC in autosomes, particularly at the ends of chromosomes, during the pachytene stage of E-type cyclin deficient spermatocytes (Martinerie et al. 2014). We now asked if the γ H2AX foci we observed in *E1+/-E2-/-* spermatocytes are a response of persistent unrepaired DSBs or if they are triggered by synapsis defects during the pachytene stage. We therefore analyzed the pattern and dynamics of γ H2AX localization during prophase I by immunodetection of γ H2AX, centromere proteins (to identify proximal from distal chromosome end), and SYCP3 (to identify AEs of the SC) in spermatocyte spread preparations from wild type (WT) and mutant mice. Although abnormal γ H2AX foci appeared in the absence of cyclin E2 alone, the number of cells exhibiting telomeric defects and the severity of these increased significantly with the additional deletion of one cyclin E1 allele in the E2 null background. Complete loss of cyclin E function in male germ cells resulted in arrest during early prophase I, with cells rarely surviving to early pachytene stages. Therefore, to enhance the numbers of cells with telomeric defects, all experiments were performed using testes from *E1+/-E2-/-* mice.

Consistent with our previous observations, both WT and *E1+/-E2-/-* leptotene and early zygotene spermatocytes exhibited γ -H2AX throughout the chromatin of forming SCs (data not shown). In early pachynema, and after chromosome synapsis, γ H2AX localized not only in the sex chromosomes but also as small foci in the chromatin adjacent to the SC of the autosomes (Figure 1Aa,c). In addition, in WT chromosomes these foci tended to distribute in the regions adjacent to the chromosome ends and the central part of the chromosomes (Figure 1a, enlargement). In contrast, in *E1+/-E2-/-* spermatocytes γ -H2AX localized in the chromatin located in the chromosome ends, especially in the proximal ends where the centromere is located (Figure 1Ac, yellow arrows and enlargements). Interestingly, while in WT chromosomes the γ H2AX foci had almost disappeared by mid pachynema (Figure 1Ab, enlargements), in *E1+/-E2-/-* mid and late spermatocytes, γ H2AX foci remained concentrated at the chromosome ends, especially at the proximal ends (Figure 1Ad, yellow arrows, enlargements). This pattern persisted in the few diplotene spermatocytes observed in *E1+/-E2-/-* mice (data not shown). The persistence of γ H2AX foci in autosomes, particularly at the chromosome ends, throughout all prophase I suggested that these foci were more likely indicative of remaining unrepaired DSBs rather than having been formed in response to unsynapsed chromatin.

We then refined the different γ H2AX localization pattern in WT and *E1+/-E2-/-* chromosomes by quantifying its distribution throughout the length of the chromosome. By measuring the length of each chromosome in WT and *E1+/-E2-/-* spermatocytes ($n=36$ spermatocytes respectively), we defined 5 segments of identical size: two at the end and three in the middle of the chromosome. To better determine if the γ H2AX foci were at the chromosome ends or at regions adjacent to the ends, the two end segments were subsequently divided into two sub-segments of identical size, called “proximal end” and “sub-proximal end”, if they were in the segment containing the centromere; and “distal end” and “sub-distal end”, if they were in the end opposite the centromere. Thus, each chromosome contains one segment divided in “proximal end” and “sub-proximal end”, a segment called “central” (corresponding to the merge of the three middle segments), and one segment divided into “distal end” and “sub-distal end” (Figure 1B). Using this approach, we quantified the presence or absence of γ H2AX in each segment in each autosome in early, mid and late pachynema from WT and *E1+/-E2-/-* spermatocytes (Figure 1Ca,b). Since no differences were found in the distribution of γ H2AX in mid and late pachytene spermatocytes, we combined the data and presented them in one graph (Figure 1Cb). As γ H2AX was distributed throughout all chromatin in the sex chromosomes, the X and Y were not included in this quantification.

We found that in early pachynema, the pattern of γ H2AX detected in the sub-proximal, central and distal segments in each chromosome was similar in both WT and *E1+/-E2-/-* spermatocytes. However, γ H2AX foci in the sub-distal end in *E1+/-E2-/-* chromosomes decreased 19.3% (Figure 1Ca) and concomitantly, chromosomes with γ H2AX at the proximal end increased 38.6% compared with WT chromosomes (Figure 1Ca). This increase was even more dramatic in mid and late pachynema, where chromosomes with γ H2AX at the proximal end increased 57.9% and at the distal end increased 13.6% (Figure 1Cb). These results indicated that in *E1+/-E2-/-* spermatocytes, abnormal DNA damage accumulated in the ends of chromosome, preferentially in the proximal end, beginning from at least early pachynema, and persisting until late prophase I stages.

The γ H2AX foci are indeed present in the telomeres

To confirm that the γ H2AX foci located in the ends of chromosome were present in telomeres, we next examined the co-localization of γ H2AX foci and a component of the Shelterin complex, TRF1. To precisely localize these proteins in the ends of chromosomes, immunolabeling of γ H2AX and TRF1, along with SYCP3, was performed in three-dimensionally preserved squash preparations (Figure 2A). In WT pachytene spermatocytes, all telomeres were labeled with TRF1 and were devoid of γ H2AX (Figure 2Aa, enlargements). In contrast, in *E1+/-E2-/-* pachytene spermatocytes, 98.6% of the γ H2AX foci that were retained at the ends of chromosomes co-localized with TRF1 (Figure 2Ab, inset yellow arrows). During early and mid pachytene, both γ H2AX-positive and γ H2AX-negative telomeres were frequently localized at the nuclear periphery (Figure 3C). However, this pattern changed from mid pachynema to diplonema in that a subset of γ H2AX-positive telomeres were now located in the nuclear space (Table 1). These observations indicated that the deficiency of E-type cyclins correlated with telomeres being recognized as sites of DNA

damage during the pachytene stage, which affects negatively the dynamics of the telomeres in the meiotic nucleus.

TRF1 levels are normal in pachytene spermatocytes

TRF1 is known to be a fundamental player in the establishment of the Shelterin complex and thus, in telomere homeostasis (Yoo et al. 2014). Although TRF1 does not contribute significantly to the end protection functions of the Shelterin complex (Zimmermann et al. 2014), it plays a key role in maintaining telomere length, and in the formation of telomere associations in mitosis and meiosis (Cooper et al. 1998; Miller et al. 2006). It has been further shown that unbalanced levels of TRF1 in telomeres, produced by experimental overexpression or observed in cancer cells, results in telomeric bridges and aggregates containing TRF1 protein and telomeric DNA (Lisaingo et al. 2014; Munoz et al. 2009). We have previously reported that one of the most striking meiotic phenotypes observed in E-type cyclin deficient spermatocytes is a high frequency of telomeric bridges between non-homologous chromosomes (Martinerie et al. 2014). To investigate the origin of the DNA damage produced at the telomeres and the previously observed telomere defects, we determined the localization pattern and levels of TRF1 at the telomeres during pachynema.

Immunolabeling for TRF1, centromere proteins, and SYCP3 in spread preparations of WT and *E1+/- E2-/-* pachytene spermatocytes revealed that TRF1 localized as defined foci in the ends of all chromosomes (Figure 2Ba,b, enlargements). We also quantified the levels of TRF1 in both proximal and distal telomeres (Figure 2C). No significant differences were observed between telomeres from WT and *E1+/- E2-/-* pachytene spermatocytes. Interestingly, we also observed the localization of TRF1 connecting telomeres of two or more chromosomes (Figure 2Bb white arrow) or in fused telomeres (Figure 2Bb, blue arrow). This pattern of localization has been described at telomeres when unbalanced levels of TRF1 are present (Lisaingo et al. 2014) and resembled the telomeric defects we previously reported using FISH (Martinerie et al. 2014). These results showed that levels of TRF1 at the telomeres were not affected by deficiency of E-type cyclins, but its localization pattern might be affected, forming stretches of DNA and bridges between chromosomes.

The presence of γ H2AX foci at the telomeres is correlated with reduced levels of TRF2 at the telomeres

We have mentioned above that a proper balance of the proteins in the Shelterin complex is essential for the maintenance of telomere integrity and function (Cesare et al. 2013; de Lange 2005; Doksani et al. 2013; Sfeir and de Lange 2012). Insufficient levels of TRF2 have been reported to result in a failure to inhibit ATM from phosphorylating H2AX and thus, prevent telomeres from being recognized as DNA damage (Cesare et al. 2013). As a consequence, aberrant activation of the DDR response results in non-homologous end joining, telomere fusions, and chromosome rearrangements (de Lange 2005; Dimitrova et al. 2008), defects we previously reported in E-type cyclin deficient spermatocytes (Martinerie et al. 2014).

Therefore, we next asked if the distribution and levels of TRF2 were affected by deficiency of E-type cyclins by immunolocalization in pachytene spermatocyte spread preparations. In

WT spermatocytes, TRF2 localizes as well-defined foci at each chromosome end (Figure 3Aa, enlargements). In contrast, in *E1+/-E2-/-* spermatocytes, the signal of TRF2 at the telomeres was notably lower compared to WT, particularly at the proximal ends (Figure 3Ab, yellow arrows, enlargements). Notably, immunodetection of TRF2 concomitantly with γ H2AX and SYCP3, revealed that telomeres with low levels of TRF2 were positive for γ H2AX (Figure 3B, yellow arrows). This observation was confirmed by quantifying the signal intensity of both TRF2 and γ H2AX in each telomere. That is, in *E+/-E2-/-* pachytene spermatocytes, TRF2 and γ H2AX levels have a strong inverse relationship, as indicated by a correlation coefficient of -0.95 . This indicated that the decrease of TRF2 levels was strongly correlated with the increase of γ H2AX observed at the telomeres. Moreover, 86% of the total variation in TRF2 intensity levels can be explained by the inverse linear relationship between TRF2 and γ H2AX levels (coefficient of determination R^2 0.86) (Figure 3C). The decrease of TRF2 was not equal in both telomeres. While TRF2 levels were reduced 38.9% in the distal telomeres, its reduction was more pronounced in the proximal telomeres, where it decreased 53.7% compared with WT telomeres (Figure 3D). This decrease of TRF2 levels in the telomeres during pachynema, particularly in the proximal telomeres, could be associated with the aberrant presence of γ H2AX foci and the DDR response in the affected telomeres.

Deficiency of E-type cyclins also alters the levels of the Shelterin complex protein RAP1 in the telomeres

To investigate if other components of the Shelterin complex are also altered by deficiency of E-type cyclins, we next studied the localization pattern and telomeric levels of RAP1. RAP1, an essential binding partner of TRF2 that relies on its interaction with TRF2 for its binding to the telomeres (Palm and de Lange 2008), represses homology-directed repair at chromosome ends (Martinez et al. 2010; Sfeir et al. 2010). We found that in both WT and *E1+/-E2-/-* spermatocytes, RAP1 localized as defined foci at each chromosome end (Figure 4Aa-b). However, in *E1+/-E2-/-* spermatocytes, the RAP1 foci at the proximal telomeres were notably less intense than those observed in WT chromosomes (Figure 4b, yellow arrows, enlargements). In fact, quantification of the RAP1 signals at proximal telomeres revealed a 29.84% decrease compared with WT telomeres (Figure 4B).

The results obtained with TRF2 and RAP1 suggest that the balance of the Shelterin complex proteins at telomeres was altered by the combination of the depletion of E2 and deficiency of cyclin E1, and that proximal, rather than distal telomeres, were more affected. We suggest that as a consequence, insufficient levels of TRF2 and thus, RAP1, in the telomeres could alter the protection of the telomeres. This in turn could activate a DDR response and allow the formation of the telomeric bridges, non-homologous associations and chromosome rearrangements that are observed in E-type cyclin deficient spermatocytes (Martinerie et al. 2014).

Defects in *E1+/-E2-/-* pachytene spermatocytes also involve loss of telomere tethering in the nuclear envelope

We next asked if the loss of telomere integrity observed in *E1+/-E2-/-* spermatocytes affected the telomere tethering in the NE using three-dimensionally preserved spermatocyte

squash preparations immunolabeled with TRF1, LAP2 and γ H2AX. LAP2 (Lamina-associated polypeptide 2) was used to identify the nuclear envelope. As expected, in WT nuclei all telomeres were tethered in the NE (Figure 4Aa, green arrows). In *E1+/-E2-/-* spermatocytes, however, we detected 47.1% of the nuclei containing multiple TRF1 signals aggregated between chromosomes located in the nuclear space (Figure 4Ab, 4Ba; Table 1). Indeed, 40.3% of the untethered telomeres also exhibited γ H2AX signal (Figure 4Ab, light blue arrow; Table 1). Chromosomes with detached telomeres, which were rarely observed in early stages of prophase I, were frequently found in the few spermatocytes that achieved late stages (Table 1). This suggested that telomere detachment from the NE could be, in part, a consequence of the loss of telomere integrity and telomere damage produced in earlier meiotic stages with loss of E-type cyclin function.

Interestingly, we also found that 14.2% of the detached telomeres retained residual LAP2 (Figure 4Ba,b, white arrows; Table 1), indicating that the integrity of the NE was compromised by the telomere detachment. A similar defect has been described in CDK2 null spermatocytes (Viera et al. 2015). These cells exhibited alterations in the nuclear envelope and detached telomeres in the nuclear space that were associated with the LINC complex protein SUN1 and with residual NE proteins (Viera et al. 2015). We previously reported that both cyclin E1 and E2 interact with CDK2 and drive its localization in telomeres during pachynema and diplonema. Thus, it is not surprising that *E1+/-E2-/-* and *CDK2-/-* spermatocytes share these telomere detachment and NE defects, suggesting that the role of E-type cyclins in telomere stability and function might be, at least in part, mediated by complex formation with CDK2.

Importance of telomeres in meiotic progression

The organization and integrity of telomeres is of special importance during meiosis, not only to allow crucial meiotic events, but also to preserve the genomic integrity of the gamete. Our observations in the present study suggest that E-type cyclins have a key role in modulating telomere integrity by, at least in part, maintaining a proper balance of the Shelterin complex proteins at the telomeres. Loss of this balance, as a result of insufficient levels of TRF2 and RAP1, might induce telomere deprotection and a subsequent DDR response. Although the mechanisms by which E-type cyclins modulate the levels of TRF2 and RAP1 remain to be determined, clearly the resulting telomere deprotection altered the integrity of telomeres and chromosomes and resulted in non-homologous telomere associations and chromosome rearrangements. It is of particular interest that the loss of integrity at the telomeres is not random; rather it seems to be associated with the position of the centromere. That is, our results showed that deficiency of the E-type cyclins affected the proximal more than the distal telomere and that changes in the levels of TRF2 and RAP2 were involved. We thus suggest that TRF2 is a key player in the telomere instability defects produced by deficiency of E-type cyclins. Additionally, although RAP1 does not participate in telomere attachment and bouquet formation in mammalian meiosis (Scherthan et al. 2011), its deficiency might be related to the retention of γ H2AX foci at the telomeres.

Why does loss of the E-type cyclins more severely affect the proximal telomere?

It has been shown in fission yeast that during meiosis, telomeres create a microenvironment during bouquet formation that promotes centromere organization (Klutstein et al. 2015). Indeed, the telomere-centromere proximity provided by the bouquet is believed to be essential for the organization of centromeres by telomeres. Whether a similar mechanism operates during mammalian meiosis remains to be determined. Mouse chromosomes are unique in that all chromosomes are telocentric (Capanna and Castiglia 2004). Thus, the proximal telomere, which is close to the centromere, could be more involved in organizing the centromere than the distal telomere. The close contact of the proximal telomere with the centromere could then dictate the creation of a particular microenvironment that determines a distinct telomere metabolism during male mammalian meiosis. Defective telomeres could alter this microenvironment and the subsequent metabolism of the telomeres, especially the proximal ones. Although we did not detect overt centromeric defects, a more detailed analysis might uncover such defects in E-type cyclin deficient spermatocytes.

When are the Shelterin-complex proteins being modulated by E-type cyclins?

Cyclin E2 is normally expressed in spermatocytes as early as their entry into meiosis and in the absence of cyclin E2, cyclin E1 is expressed at similar stages (Martinerie et al. 2014). Although our present analyses were focused on mid and late prophase I stages, we recognize that the absence of E-type cyclins from the beginning of meiosis could also be affecting telomeres as early as leptotema and zygotema, but this remains to be determined. However, defects in telomere integrity in early pachytene were readily detected and became more severe in later stages of the prophase I, including the observation of a subset of γ H2AX-positive telomeres that were no longer at the nuclear periphery, but were rather in the nuclear space. The permanent clustering of the proximal telomeres by the chromocenters throughout all prophase I would seem to suggest that both early and late functions for E-type cyclins could be involved in generating a special microenvironment during all stages, modulating telomeres in more than a single manner.

E-type cyclins, telomere integrity and chromosome movement

Dynamic chromosome movement is a characteristic of mid-late prophase I stages and has been suggested to be involved in the elimination of unwanted chromosomal associations (Koszul and Kleckner 2009). Therefore, it is possible that the instability and damage in the mutant telomeres in early pachytene compromised the strength of the telomere to drive chromosome movements. As consequence, the damage expanded, resulting in more γ H2AX at the telomeres, and damaged telomeres were then unable to maintain the association to the NE and resist forces from the cytoskeleton. As consequence, the tethering breaks and telomeres retained NE proteins in their nuclear space. Loss of telomere integrity could also affect the dynamic behavior of telomeres and chromosomes during meiosis, by changing the normal chromosome interactions and grouping. Our current model suggests that telomere detachment is a secondary effect produced by loss of telomere integrity but that chromosome movement might also be affected.

We do not know if the mis-regulation of the balance of Shelterin complex proteins is a direct downstream target of E-type cyclins, possibly complexed with CDK2, or if it reflects an

indirect function mediated by other downstream targets. It will be relevant to determine if other proteins involved in telomere-maintenance are also affected by the loss of E-type cyclin function in spermatocytes. Similarly, it will be of interest to examine the dynamics of other proteins which, upon depletion, produce telomere defects similar to those we have observed, such as *Terb1* and *SMC1 β* , which have been proposed to play a role in telomere stabilization during meiosis (Adelfalk et al. 2009; Biswas et al. 2013).

In summary, we have provided evidence that points to novel functions of the E-type cyclins during the progression of male mammalian meiosis and which are quite distinct from their canonical functions proposed in mitotic cells. In particular, we provide evidence that uncovers a heretofore unanticipated role for cyclin E1 and E2 in maintaining the integrity of such critical chromosome structures as telomeres, disruption of which modulates the normal occurrence of key meiotic events.

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Figure 1. Deficiency of E-type cyclins produces abnormal DNA damage at the ends of the chromosomes, preferentially at the proximal end

(A) Localization of γ H2AX (green), centromeric proteins (CEN) (white), and SYCP3 (red) during the pachytene stage in wild type (WT) (a-b) and *E1+/- E2-/-* (c-d) spermatocyte spread preparations. XY indicates the sex body. Enlargements to the upper right of each panel display the chromosomes demarcated by the white rectangle in the full image. The bottom panel corresponds to chromosomes shown above but only γ H2AX and CEN localization is depicted, to facilitate the visualization of the γ H2AX foci. (a,c) Early pachytene spermatocytes. (a) γ H2AX localizes in the sex chromosomes (XY) but also in the chromatin close to the SC in synapsed regions (enlargement). (c) In *E1+/- E2-/-* spermatocytes, γ H2AX also localizes at chromosome ends (yellow arrows, enlargement) as well as in other chromosome regions. (b,d) Mid pachytene spermatocytes. (b) In WT autosomes, very few γ H2AX foci persist in the chromatin close to the SC. (d) In *E1+/- E2-/-* autosomes, γ H2AX foci persist in the chromatin, mainly at the chromosome ends and notably in the proximal ends (yellow arrows, enlargement). (B) Scheme for localizing and quantifying the distribution of γ H2AX foci. Top panel: example of an early pachytene WT chromosome (demarcated by the white rectangle in panel A(a) immunolocalized for γ H2AX (green), CEN (white), and SYCP3 (red). The bottom panel corresponds to the chromosome

shown above but only γ H2AX and CEN localization is depicted. Five discrete equal segments were defined along the chromosome length. The first and last segments were divided in two sub-segments of equal length and named proximal (p) (yellow) and sub-proximal (s-p) (orange), if they were in or next to the centromere; and sub-distal (s-d) (blue) and distal (d) (green), if they were in the opposite end of the chromosome from the centromere. The other three segments were designated central (c) (white). These segments were used to define the distribution of γ H2AX foci along the chromosome length. (C) Distribution of the γ H2AX foci along the chromosome segment per spermatocyte, displayed as a percentage of the total number of segments (defined in B) from WT (black bars) and *E1+/- E2-/-* (dotted bars) chromosomes. Four spermatocytes each from early, mid and late pachynema from 3 different animals were analyzed and quantified (total $n=36$). Error bars indicate standard deviation. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 2. γ H2AX foci co-localize with TRF1 at the telomeres in *E1+/-E2-/-* pachytene spermatocytes, although the levels of TRF1 are normal

(A) Immunolocalization of γ H2AX (blue), TRF1 (red) and SYCP3 (grey) in squash preparations of pachytene spermatocytes. (a,b) Confocal images of WT (a) and *E1+/-E2-/-* (b) mid pachytene nuclei. XY indicates the sex body. Small panels to the upper right of the main figures enlarge the ends of chromosomes positive for TRF1 localization, demarcated by the white rectangles in a and b. Lower small panels correspond to the chromosomes ends shown above, but only γ H2AX and TRF1 localization is depicted. (a) WT nucleus. All telomeres are devoid of γ H2AX. (b) In cyclin E-deficient nuclei, a subset of telomeres

exhibit γ H2AX signal co-localizing with TRF1 in the nuclear periphery (yellow arrows, enlargement). (B) TRF1 (green), centromeric proteins (CEN) (white) and SYCP3 (red) in mid pachytene chromosome spreads of WT and *E1+/- E2-/-* spermatocytes. XY indicates the sex chromosomes. As above, upper panels enlarge the chromosomes demarcated by the white rectangle in (a) and (b). Lower panels correspond to chromosomes shown above but only TRF1 and CEN localization is depicted. (a) WT mid pachytene spermatocyte with defined TRF1 signal in all telomeres (enlargement). (b) *E1+/- E2-/-* mid pachytene spermatocyte exhibit telomeres with TRF1 in the telomeres (enlargements). In some chromosomes, TRF1 localizes forming bridges between two or more chromosomes (white arrow) or fused telomeres (blue arrow). (C) Quantification of the intensity of TRF1 signal in proximal and distal telomeres in both WT (black bars) and *E1+/- E2-/-* (dotted bars) chromosomes. $n=36$ spermatocytes. Error bars indicate standard deviation.

Figure 3. The presence of γ H2AX foci at the telomeres is correlated with reduced levels of TRF2, mainly in the proximal telomeres

(A) Localization of TRF2 (green), centromeric proteins (CEN) (white) and SYCP3 (red) in mid pachytene spermatocyte spread preparations. Small panels to the right of the main image highlight the chromosomes demarcated by white rectangles in (a) and (b). Upper panel of each set shows localization of SYCP3 (red), TRF2 (green), and CEN (white) while the lower panels depict only CEN and TRF2 signal. XY indicates the sex chromosomes. (a) WT pachytene spermatocyte with defined TRF2 signal in all telomeres. (b) *E1+/- E2-/-* spermatocyte with reduced signal of TRF2 at the telomeres. Yellow arrows indicate telomeres with significantly reduced TRF2 signal, mainly in the proximal telomeres. B)

Confocal image of a *E1+/-E2-/-* mid pachytene spermatocyte squash preparations immunolabeled with SYCP3 (grey), TRF2 (red) and γ H2AX (blue). XY indicates the sex body. Telomeres with low levels of TRF2 are correlated with the presence of γ H2AX foci (yellow arrows, upper enlargement), while telomeres with normal TRF2 levels are devoid of γ H2AX foci (bottom enlargement, green arrow). Enlargements show only TRF2 and γ H2AX signal. C) Quantification of the intensity of TRF2 signal in relation to the intensity of γ H2AX foci in the telomeres of *E1+/-E2-/-* pachytene spermatocytes. R^2 indicates the coefficient of determination between the signal intensity of TRF2 and γ H2AX. D) Quantification of the intensity of TRF2 signal in proximal and distal telomeres of WT (black bars) and *E1+/-E2-/-* (dotted bars) chromosomes. $n= 36$ spermatocytes. Error bars indicate standard deviation. *** $p < 0.001$.

Figure 4. The Shelterin complex protein RAP1 levels also decrease in the proximal telomeres of $E1^{+/-} E2^{-/-}$ pachytene spermatocytes

(A) Localization of RAP1 (green), centromeric proteins (CEN) (white) and SYCP3 (red) in pachytene spermatocyte spreads. Panels on the right enlarge chromosomes demarcated by the white rectangles in (a) and (b). Upper figures depict immunolocalization of all three proteins while the lower panels depict only CEN and RAP1 signal on those chromosomes shown above. XY indicates the sex chromosomes. (a) WT pachytene spermatocyte with defined RAP1 signal in all telomeres. (b) Deficiency of E-type cyclins results in a significant reduction in RAP1 signal in the proximal telomeres (yellow arrows). B) Quantification of the intensity of RAP1 signal in proximal and distal telomeres of WT (black bars) and $E1^{+/-} E2^{-/-}$ (dotted bars) chromosomes. $n = 36$ spermatocytes. Error bars indicate standard deviation. *** $p < 0.05$.

Figure 5. Deficiency of E-type cyclins results in telomere detachment from the nuclear envelope in late pachytene spermatocytes

(A) Confocal images corresponding to the center of the nuclei of late pachytene spermatocyte squash preparations immunolabeled with TRF2 (red), γ H2AX (blue), and LAP2 (green). (a) WT nucleus with all telomeres tethered at the nuclear envelope (green arrows). (b) *E1+/- E2-/-* nucleus with detached and clumped telomeres that are positive for γ H2AX foci present in the nuclear space (light blue arrow), and other telomeres tethered to the NE and positive for γ H2AX signal (orange arrow). Asterisk in panel A(b) indicates the sex body. (B) Confocal images corresponding to the center of nuclei from *E1+/- E2-/-* late pachytene spermatocyte squash preparations immunolabeled with TRF2 (red) and LAP2 (green). (a,b) Detached telomeres present in the nuclear space retain residual LAP2 from the NE (white arrow). Green arrow indicates telomeres still tethered at the NE.

TABLE 1

Percentage of spermatocytes with detached telomeres and characteristics of the detached telomeres.

	Early pachytene spermatocytes		Mid pachytene to diplotene spermatocytes	
	Wild type	<i>E1+/-E2-/-</i>	Wild type	<i>E1+/-E2-/-</i>
<u>Present in the nuclear space</u>	0	1.8	0.9	47.1
<u>γH2AX-positive</u>	0	1.8	0	40.3 [*]
Retaining LAP2	0	0	0	14.2 [*]

^{*}represents a percentage from the total spermatocytes with detached telomeres (47.1%)