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Shorter telomeres, accelerated ageing and increased lymphoma in DNA-PKcs-deficient mice

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Non-homologous end joining (NHEJ) is the principal repair mechanism used by mammalian cells to cope with double-strand breaks (DSBs) that continually occur in the genome. One of the key components of the mammalian NHEJ machinery is the DNA-PK complex, formed by the Ku86/70 heterodimer and the DNA-PK catalytic subunit (DNA-PKcs). Here, we report on the detailed life-long follow-up of DNA-PKcs-defective mice. Apart from defining a role of DNA-PKcs in telomere length maintenance in the context of the ageing organism, we observed that DNA-PKcs-defective mice had a shorter life span and showed an earlier onset of ageing-related pathologies than the corresponding wild-type littermates. In addition, DNA-PKcs ablation was associated with a markedly higher incidence of T lymphomas and infections. In conclusion, these data link the dual role of DNA-PKcs in DNA repair and telomere length maintenance to organismal ageing and cancer.

Keywords: DNA repair; DNA-PKcs; telomeres; ageing; cancer

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INTRODUCTION

DNA double-strand breaks (DSBs) occur in the genome as a consequence of oxidative energy metabolism, meiosis, DNA replication and V(D)J recombination as well as on exposure to external genotoxic agents such as ionizing radiation. To ensure cell viability, DSBs must be repaired correctly (Smith & Jackson, 1999). Eukaryotic cells have two main DSB repair pathways: homologous recombination (HR) and non-homologous end joining (NHEJ) (Jackson, 2002). In mammalian cells, NHEJ is the principal DSB repair mechanism involving the DNA-PK complex, formed by the Ku86/Ku70 heterodimer and the DNA-PK catalytic subunit (DNA-PKcs), as well as the XRCC4/ligase4 complex (Khanna & Jackson, 2001). Knockout mice deficient for any of the three components of the DNA-PK complex show severe immunodeficiency and increased sensitivity to ionizing radiation (Nussenzweig *et al*, 1996; Zhu *et al*, 1996; Gu *et al*, 1997; Gao *et al*, 1998; Taccioli *et al*, 1998). Interestingly, localization of Ku86 and DNA-PKcs at mammalian telomeres (Bianchi & de Lange, 1999; Hsu *et al*, 1999) suggests that the role of these proteins extends beyond NHEJ. In fact, analysis of Ku86- and DNA-PKcs-deficient mice showed that these proteins are essential for the capping function of the telomere (Bailey *et al*, 1999; Hsu *et al*, 2000; Samper *et al*, 2000; Gilley *et al*, 2001; Goytisolo *et al*, 2001). In keeping with a role for the DNA-PK complex in telomere length maintenance, Ku86 was reported to act as a negative regulator of telomerase (Espejel *et al*, 2002a), whereas DNA-PKcs cooperates with telomerase in telomere elongation (Espejel *et al*, 2002b).

Further studies are nonetheless necessary to elucidate in detail the role of these proteins at telomeres in the context of the organism. Here, we analyse DNA-PKcs-defective mice that had been aged in the animal facility to explore the role of DNA-PKcs in telomere length maintenance in the context of the ageing organism and to elucidate the functional implication of DNA-PKcs in cancer and longevity.

RESULTS AND DISCUSSION

Telomeric changes in aged DNA-PKcs$^{-/-}$ mice

We have recently shown that simultaneous ablation of DNA-PKcs and telomerase exacerbates telomere erosion (Espejel *et al*, 2002b). To determine whether the absence of DNA-PKcs alone affects telomere maintenance after continuous cell divisions during the life span of an organism, we measured telomere length in bone marrow (BM) cells obtained from aged wild-type and DNA-PKcs$^{-/-}$ mice with identical genetic backgrounds (Fig 1). The average telomere length in three independent aged DNA-PKcs$^{-/-}$ mice was 26.7 ± 0.24, 32.8 ± 0.29 and 26.3 ± 0.20 kb compared with 31.3 ± 0.24, 35.8 ± 0.27 and 35 ± 0.31 kb in age-matched wild-type controls analysed in parallel (Student's *t*-test, $P < 0.005$). When young DNA-PKcs$^{-/-}$

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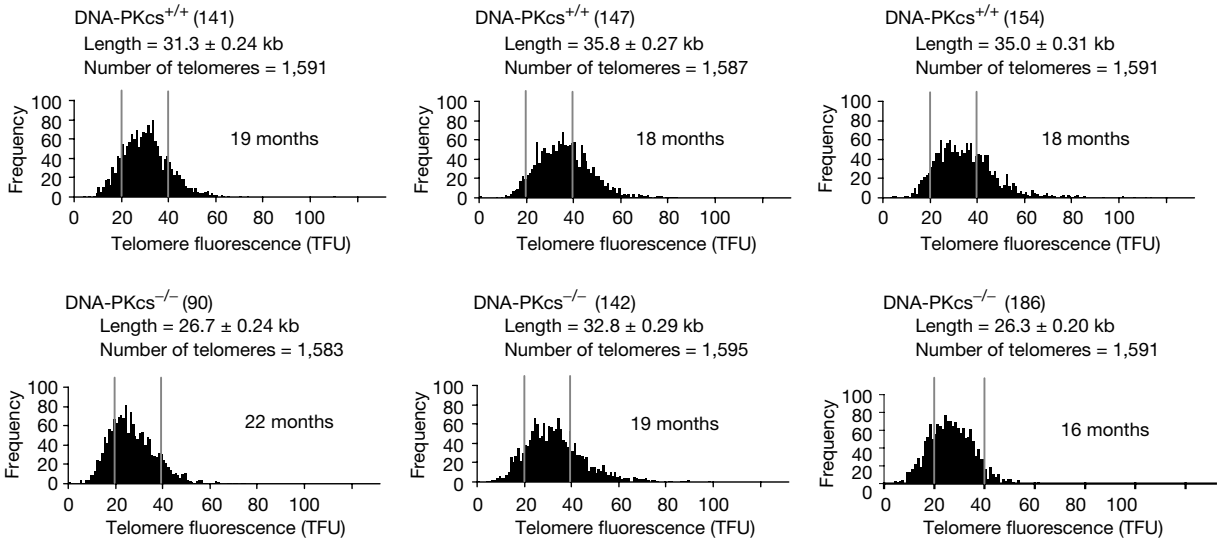


Fig 1 | Telomere length distribution in BM cells from aged wild-type and DNA-PKcs$^{-/-}$ mice. One telomere fluorescence unit (TFU) corresponds to 1 kb of TTAGGG repeats. Average telomere length, standard deviation and total number of telomeres analysed from three different aged wild-type (DNA-PKcs$^{+/+}$) and G1 DNA-PKcs$^{-/-}$ mice are shown. Numbers in parentheses identify individual mice.

and wild-type mice were compared, however, the differences in telomere length did not reach statistical significance, in agreement with previous results obtained with mouse embryonic fibroblasts (MEFs) (Gilley *et al*, 2001; Goytisolo *et al*, 2001). These data indicate that the continuous absence of DNA-PKcs function during the life of a mouse results in progressive telomere shortening and, importantly, this becomes evident at old ages.

To determine whether DNA-PKcs ablation provokes spontaneous occurrence of telomere fusions in the aged organism, we studied the involvement of telomeres in chromosomal aberrations spontaneously arising in BM cells. In particular, we looked at telomere fusions, as well as at telomere associations (TAs) (see Methods). Analysis of metaphases from aged wild-type and DNA-PKcs$^{-/-}$ mice showed no fusions involving telomeres (data not shown), in contrast to what has been reported previously for DNA-PKcs$^{-/-}$ MEFs, which showed increased end-to-end fusions involving telomere sequences (Gilley *et al*, 2001; Goytisolo *et al*, 2001). A possible explanation for this apparent discrepancy is that MEFs are more sensitive to factors that produce DSBs, such as oxygen, or have a weaker checkpoint response to telomeric defects than BM cells. As shown in Fig 2, we nonetheless observed a significant increase in TAs in BM cells from aged DNA-PKcs$^{-/-}$ mice compared with age-matched wild-type mice (0.053 and 0.013 TAs per metaphase, respectively; Fisher's exact test, $P<0.01$). The increased frequency of TAs suggests that lack of DNA-PKcs compromises proper telomere capping in the ageing organism.

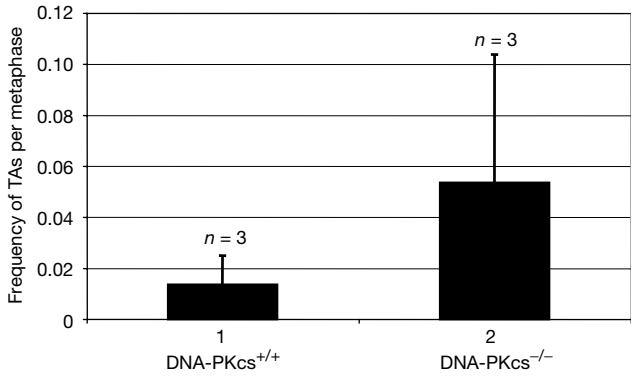


Fig 2 | TAs in BM cells from aged wild-type and G1 DNA-PKcs$^{-/-}$ mice. Averages of frequency of TAs per metaphase from three different aged wild-type (DNA-PKcs$^{+/+}$) and DNA-PKcs$^{-/-}$ mice are shown.

Telomeric changes in progeny of DNA-PKcs$^{-/-}$ mice

We derived successive generations (G1–G4) of DNA-PKcs$^{-/-}$ mice and measured telomere fluorescence on metaphases derived from BM cells and meiocytes from the different generations of young (4–6 months old) DNA-PKcs$^{-/-}$ mice and age-matched wild-type controls. We found that in DNA-PKcs$^{-/-}$ mice, the average telomere length decreased progressively with increasing generations (Fig 3). In particular, G1 DNA-PKcs$^{-/-}$ mice ($n=2$) showed a mean telomere length of 49.9 ± 0.30 kb, G3 mice ($n=3$) of 45.3 ± 0.23 kb and G4 mice ($n=3$) of only 43.9 ± 0.22 kb, compared with a mean telomere length of 51.3 ± 0.31 kb in wild-type animals ($n=2$). The difference in the average telomere length between G1 DNA-PKcs$^{-/-}$ and wild-type animals was not statistically significant, concurring with earlier reports (Gilley *et al*, 2001; Goytisolo *et al*, 2001; Espejel *et al*, 2002b). However, there was a highly significant difference in telomere length in late-generation DNA-PKcs$^{-/-}$ mice (G3 and G4) as compared with wild-type mice (Student's *t*-test, $P<0.005$). Generation-related telomere shortening was also evident when we compared G3 with G4 DNA-PKcs$^{-/-}$-deficient animals (Student's *t*-test, $P<0.005$). Quantitative fluorescence *in situ* hybridization (Q-FISH) assay on testis sections (Fig 4) further confirmed these results because we

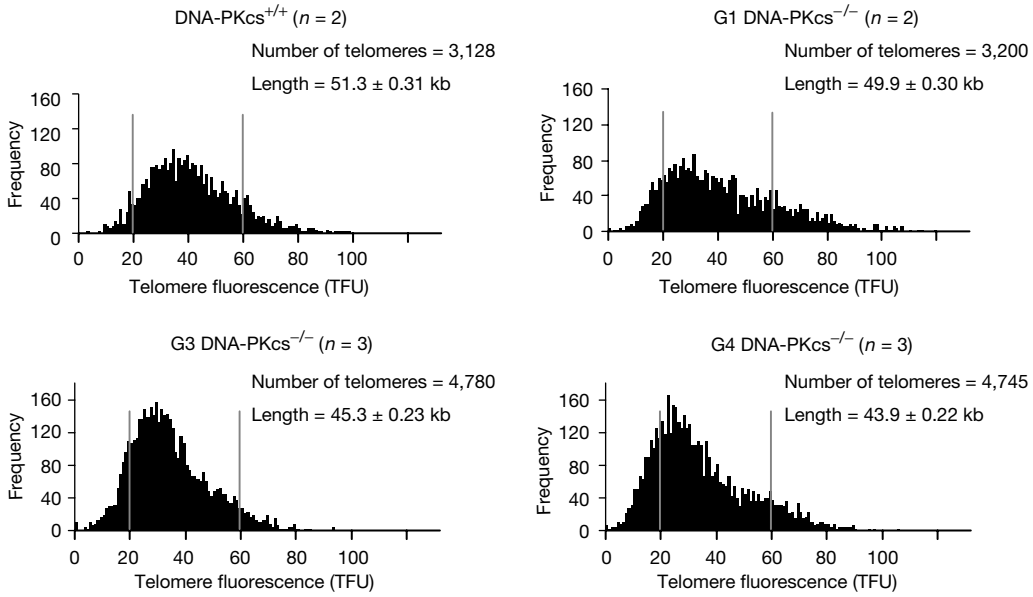


Fig 3 | Telomere length distribution in BM cells from wild-type and successive generations of DNA-PKcs$^{-/-}$ mice. One telomere fluorescence unit (TFU) corresponds to 1 kb of TTAGGG repeats. Average telomere length, standard deviation and total number of telomeres analysed from two or three different young (4–6 months) wild-type (DNA-PKcs$^{+/+}$), and G1, G3 and G4 DNA-PKcs$^{-/-}$ mice are shown.

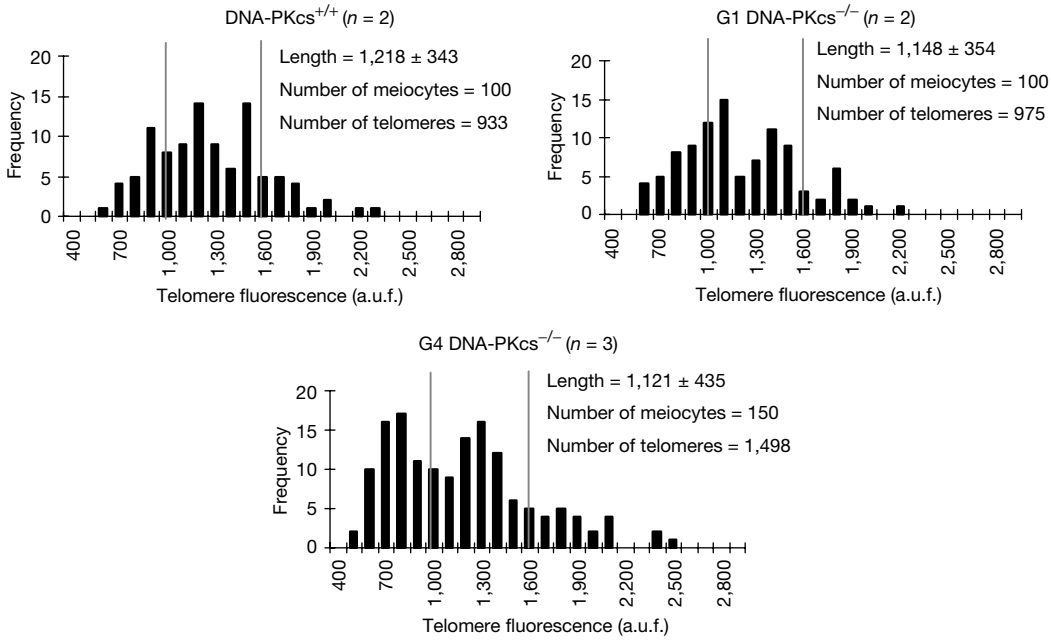


Fig 4 | Telomere length determination in meiotic cells on testis sections from wild-type and successive generations of DNA-PKcs$^{-/-}$ mice. Average telomere fluorescence in arbitrary units (a.u.f.), standard deviation and total number of telomeres analysed from two or three different wild-type (DNA-PKcs$^{+/+}$), and G1 and G4 DNA-PKcs$^{-/-}$ mice are shown.

found no difference in the average telomere length between G1 DNA-PKcs$^{-/-}$ and age-matched wild-type controls but a significant decrease in telomere fluorescence in G4 DNA-PKcs$^{-/-}$ meiotic cells compared with those from age-matched wild-type controls (Student's *t*-test, $P = 0.04$). In the case of DNA-PKcs-deficient BM cells, we calculated a rate of telomere loss of 1.85 kb per mouse generation. Thus, successive generations of DNA-PKcs$^{-/-}$ mice undergo a progressive telomere length reduction despite the fact that these mice have telomerase activity, indicating that endogenous telomerase is not sufficient to compensate for the loss of telomeric sequences in the absence of DNA-PKcs.

To further address the issue whether this progressive telomere shortening in successive generations of DNA-PKcs$^{-/-}$ mice provokes spontaneously occurring chromosomal aberrations, we analysed BM metaphases from each mouse strain by Q-FISH. No telomere fusions were detected in any of the genotypes analysed, except for one single telomere fusion out of 300 metaphases analysed in the case of G3 DNA-PKcs$^{-/-}$ mice ($n=3$, data not shown). The apparent lack of end-to-end telomere fusions with increasing generations of DNA-PKcs-deficient mice is in agreement with the finding that DNA-PKcs ablation was not associated with the occurrence of signal-free ends, that is, in the Q-FISH assay we did not observe chromosomes that had lost their telomeres and, thus, would be substrates for end-to-end fusions (Fig 3). Nonetheless, we observed a significant increase in TAs in the different generations of DNA-PKcs$^{-/-}$ mice as compared with wild-type controls (Fig 5), which is consistent with increased TAs due to DNA-PKcs deficiency in aged mice (Fig 2).

Impact of DNA-PKcs ablation on organismal fitness

We observed a reduced viability in the DNA-PKcs$^{-/-}$ mouse colony compared with that of the wild-type colony. The 50% survival for DNA-PKcs$^{-/-}$ mice was only 13 months compared with 19.5 months in the case of wild-type controls (Fig 6). In addition, only 3% of the DNA-PKcs$^{-/-}$ mice were alive 2 years after birth compared with 23% of the wild-type mice (Fig 6). To determine the causes of premature death in the DNA-PKcs$^{-/-}$ colony, animals that showed signs of poor health, such as reduced activity, weight loss or bristly hair, were killed and subjected to histopathological analysis. We found that the main causes of death could be attributed to thymus lymphoma, infection and intestinal atrophy.

Lymphomas were detected in both wild-type and DNA-PKcs$^{-/-}$ mice above 1 year of age, which is in agreement with the view that this type of malignancy is common in old mice (Smith *et al*, 1973). In particular, 5 out of 17 wild-type (24.4%) and 2 out of 18 DNA-PKcs$^{-/-}$ mice (11.1%) showed lymphoma at the time of death (Fig 7A). However, whereas no lymphomas were observed in young wild-type mice (less than 1 year old), 4 out of 41 (9.8%) young DNA-PKcs$^{-/-}$ mice developed full-blown lymphomas (Fig 7A). Strikingly, 75% of the lymphomas in DNA-PK-deficient mice affected the thymus (Fig 7B), suggesting that DNA-PKcs$^{-/-}$ mice are more susceptible to developing thymic lymphoma than wild-type cohorts. This observation is in agreement with the previously reported occurrence of thymic lymphoma in DNA-PKcs$^{-/-}$ mice on an FVB/N background (Jhappan *et al*, 1997); other previous works, however, did not report on the occurrence of lymphoma in DNA-PKcs$^{-/-}$ mice (Gao *et al*, 1998; Taccioli *et al*, 1998) or described a lower incidence (Kurimasa *et al*, 1999).

Furthermore, we found signs of pneumonia or hepatitis at the time of death in young (<1 year of age) and aged (>1 year of age) DNA-PKcs$^{-/-}$ mice at a frequency of 12.1% (5/41) and 66.6% (12/18), respectively (Fig 7C). In contrast, none of the young and only 5.9% (1/17) of aged wild-type mice developed these pathologies. As our animal colony is housed in a specific pathogen-free environment, these diseases are likely to be caused by endogenous intestinal pathogens (see below) particularly affecting DNA-PKcs$^{-/-}$ mice, which are immunodeficient (Gao *et al*, 1998; Taccioli *et al*, 1998).

As mentioned above, DNA-PKcs$^{-/-}$ mice showed intestinal atrophy. Four out of 41 DNA-PKcs$^{-/-}$ mice younger than 1 year of age (9.7%) and 1 out of 18 DNA-PKcs$^{-/-}$ mice older than 1 year of age (5.5%) showed a marked intestinal atrophy at the time of death, whereas wild-type mice were never affected by this degenerative pathology (Fig 7D). This phenotype could be explained by the fact that intestinal epithelium is a rapidly proliferating tissue, and an elevated DNA replication rate without repair could cause accumulation of genomic alterations that reduce cell growth and viability.

To further investigate whether DNA-PKcs ablation accelerates ageing, we studied two hallmarks of ageing, namely abnormally increased convexity in the curvature of the thoracic spine (lordokyphosis) and loss of bone density. Skeletal X-ray analysis of 6-month-old animals (Fig 8A,B) revealed a reduction of the mean spine angle from 104.5±0.7° ($n=2$) in wild-type mice to

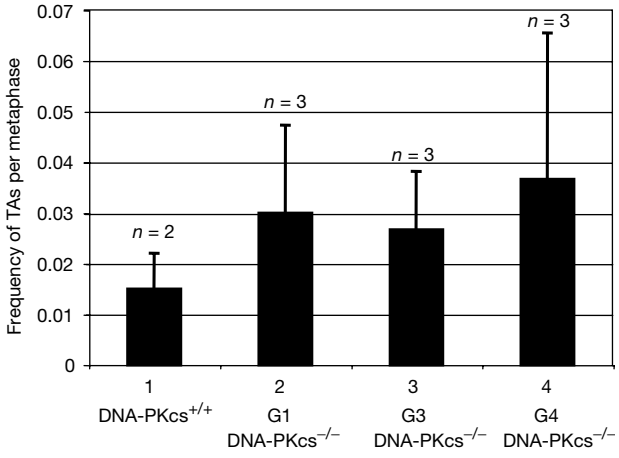


Fig 5 | TAs in BM cells from wild-type and successive generations of DNA-PKcs$^{-/-}$ mice. Averages of frequency of TAs per metaphase from two or three different wild-type (DNA-PKcs$^{+/+}$), and G1, G3 and G4 DNA-PKcs$^{-/-}$ mice are shown.

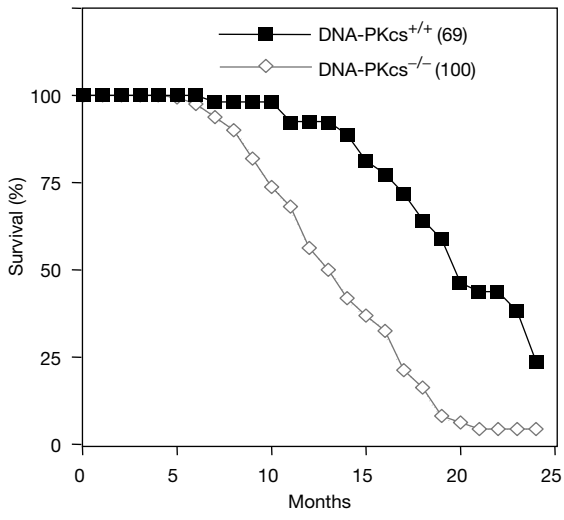
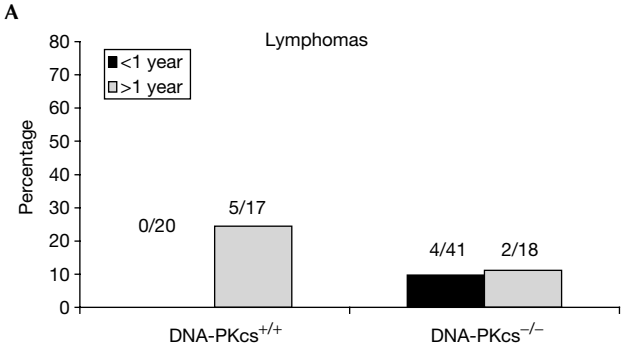


Fig 6 | Survival of wild-type and G1 DNA-PKcs$^{-/-}$ mice. The total number of mice from each genotype is indicated.



B

Age	Genotype	Type of lymphoma	Number of mice with lymphoma
<1 year	DNA-PKcs$^{-/-}$	Thymus	3/4 (75%)
		Multicentric	1/4 (25%)
>1 year	Wild type	Multicentric	2/5 (40%)
		Nodes and lung	1/5 (20%)
		Spleen, nodes and thymus	1/5 (20%)
		Intestine	1/5 (20%)
	DNA-PKcs$^{-/-}$	Thymus	1/2 (50%)
		Spleen	1/2 (50%)

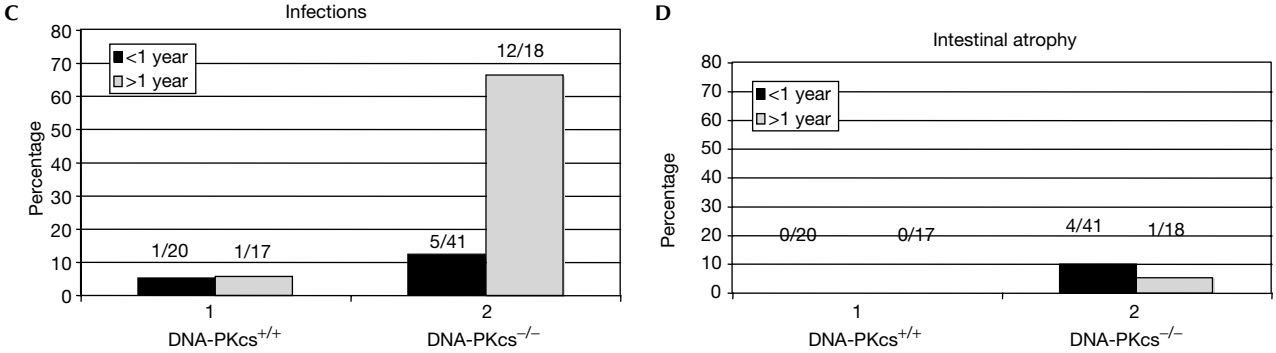


Fig 7 | Pathologies of wild-type and G1 DNA-PKcs$^{-/-}$ mice at the time of spontaneous death at different ages: (**A**) percentage that presented lymphomas; (**B**) classification of lymphomas; (**C**) percentage that presented infections; and (**D**) percentage with intestinal atrophy. The total population for wild-type (DNA-PKcs$^{+/+}$) mice was 20 animals younger than 1 year and 17 animals older than 1 year; in the case of DNA-PKcs$^{-/-}$ mice, 41 animals were younger than 1 year and 18 were older than 1 year. The proportion of mice that showed the specific lesion at necropsy is indicated above each bar of the graph.

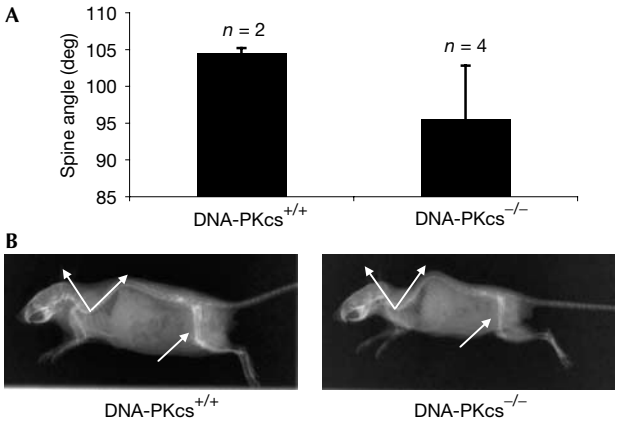


Fig 8 | Lordokyphosis in G1 DNA-PKcs$^{-/-}$ mice. (**A**) Average and standard deviation of spine angle from two 6-month-old male wild-type (DNA-PKcs$^{+/+}$) and four different age-matched DNA-PKcs$^{-/-}$ mice are indicated. A narrowing of the angle indicates an increase in lordokyphosis. (**B**) Representative X-ray radiographs of 6-month-old male wild-type and DNA-PKcs$^{-/-}$ mice.

95.5±7.3° ($n=4$) in DNA-PKcs$^{-/-}$ mice. Visual inspection of the X-ray films also pointed to a decrease of bone density in DNA-PKcs$^{-/-}$ mice (Fig 8B; see arrow). Similarly to lymphoma development, the elevated spine convexity and decreased bone density are alterations that can already be observed in young DNA-PKcs$^{-/-}$ mice (6 months of age). Taken together, these results point to an early onset of ageing as a direct consequence of DNA-PKcs ablation.

Finally, it should be noted that DNA-PKcs$^{-/-}$ cohorts were smaller than age-matched wild-type mice (Fig 9). In particular, young (4–8 months old) DNA-PKcs$^{-/-}$ males show an average body weight reduction of 17% compared with age-matched controls, which further increased to 30% in older males (>8 months). Similarly, the average weight reduction of young and old DNA-PKcs$^{-/-}$ females was 14% and 8%, respectively (Fig 9). These results are of particular interest, as work from other laboratories did not provide specific data on growth defects in their DNA-PKcs$^{-/-}$ cohorts (Gao *et al*, 1998; Taccioli *et al*, 1998; Kurimasa *et al*, 1999). However, we cannot exclude the possibility that the implication of DNA-PKcs deficiency in growth may be related to intestinal atrophy (see above).

Conclusions

Previous studies from our laboratory and others showed that, apart from its well-established role in DSB repair (reviewed in Khanna & Jackson, 2001), DNA-PKcs is essential for telomere capping in cultured MEFs (Gilley *et al*, 2001; Goytisolo *et al*, 2001; Espejel *et al*, 2002b). It can be anticipated, therefore, that the biological

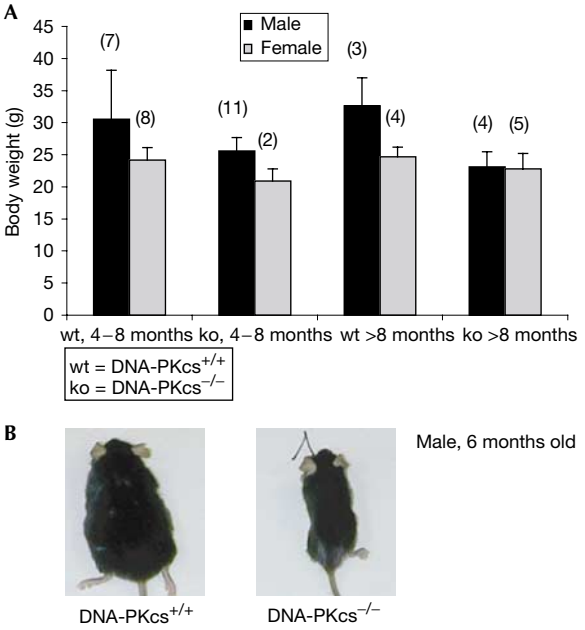


Fig 9 | Growth defects in G1 DNA-PKcs$^{-/-}$ mice. (**A**) The average body weights of wild-type and DNA-PKcs$^{-/-}$ males and females at the indicated ages are shown. The total number of mice is indicated above each graph (numbers in parentheses) (wt, wild type, that is, DNA-PKcs$^{+/+}$; ko, DNA-PKcs$^{-/-}$). (**B**) Representative images of age-matched (6 months) male wild-type and DNA-PKcs$^{-/-}$ mice.

consequences of DNA-PKcs deficiency will be governed by compromised cell viability resulting from a pronounced synergism between unrepaired DNA breaks and uncapped telomeres. In fact, in the context of the organism, we demonstrate that DNA-PKcs ablation provokes telomere shortening and an increased frequency of TAs with age. These results agree with our previous finding that, in the absence of telomerase activity, DNA-PKcs abrogation accelerates the rate of telomere loss (Espejel *et al*, 2002b), thus supporting a role for DNA-PKcs in telomere length maintenance. Importantly, we show *in vivo* data suggesting that this correlates with a shorter life span due to an increased incidence of thymic lymphomas as well as an earlier onset of age-related pathologies. In conclusion, the present report links the dual role of DNA-PKcs in DNA repair and telomere length maintenance to organismal ageing and cancer.

METHODS

Mice. The generation of DNA-PKcs$^{-/-}$ mice on a C57BL/6 genetic background was described elsewhere (Taccioli *et al*, 1998). Wild-type and DNA-PKcs$^{-/-}$ mice were obtained from heterozygous crosses. To obtain successive generations of DNA-PKcs$^{-/-}$ mice, homozygous mice of each generation were intercrossed. Mice were housed in the barrier area at the CNIO, in which pathogen-free procedures are used in all mouse rooms. Quarterly health monitoring reports were negative for all pathogens according to the recommendations from the Federation of European Laboratory Animal Science Association (FELASA).

Histological analysis. Mouse tissues were fixed in 10% paraformaldehyde and embedded in paraffin. Sections (5 μm) were stained with Harris haematoxylin and eosin according to standard procedures. For X-ray analysis, mice were anaesthetized with an intraperitoneal injection of ketamine (75 μg per gram body weight) and medetomidine (1 μg per gram body weight) before examination in a lateral position using a Philips Diagnostic 93 Medio 65-CP-H set at 2.0 m.a.s. and 40 kV.

Q-FISH on metaphase chromosomes. Fresh BM BM samples were obtained by flushing femora with sterile phosphate-buffered saline. BM was dispersed immediately in RPMI 1640 medium plus 10% fetal calf serum using a 24-gauge needle. BM cells (5×10^6) were cultured for 72 h in Myelocult 5300 medium (StemCell Technologies, Vancouver, BC, Canada) supplemented with 15% WEHI-3B cell-conditioned medium as a source of interleukin 3 (IL-3). Metaphase cells were prepared using colcemide treatment and dropped onto slides wetted with 45% acetic acid before hybridization with the telomeric PNA-Cy3 probe (PE Biosystems), and analysed by Q-FISH as described previously (Herrera *et al*, 1999). Telomere fluorescence was analysed using the TFL-telo program kindly provided by Peter Lansdorp (British Columbia Cancer Center, Vancouver, Canada). To minimize intra-experimental variations of telomere length determinations, samples used to compare different genotypes and mouse generations with each other were processed in a single batch and analysed side by side.

Q-FISH scoring of chromosomal abnormalities. BM cell metaphases (100 each) were scored for chromosomal aberrations by superimposing the telomere image on the DAPI chromosome image using the TFL-telo software. We define telomere fusions as chromosomes joined at their telomeres and showing at least two overlapping telomeric signals and TAs as chromosomes with four distinct telomere signals but aligned less than one-half chromatid apart (Goytisolo *et al*, 2001).

Q-FISH on testis sections. Whole testes were fixed in 10% formaldehyde for 2 h and paraffin embedded following standard methods. Testis sections from mice of each genotype were hybridized with a PNA-tel probe, and telomere length was determined as described above. For average telomere fluorescence calculations, 50 meiotic nuclei per mouse obtained from at least two mice per genotype were captured at ×100 magnification and the telomere fluorescence was integrated using spot IOD analysis using the TFL-telo program.

Statistical analysis. To assess statistical significance, Student's *t*-test values were calculated using the Microsoft Excel software. Where indicated, Fisher's exact test was performed using the Instat software.

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