Cellular senescence and organismal ageing in the absence of p21^{CIP1/WAF1} in *ku80*^{-/-} mice

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Ku80 is important in the repair of DNA double-strand breaks by its essential function in non-homologous end-joining. The absence of Ku80 causes the accumulation of DNA damage and leads to premature ageing in mice. We showed that mouse embryonic fibroblasts (MEFs) from *ku80*^{-/-} mice senesced rapidly with elevated levels of p53 and p21. Deletion of p21 delayed the early senescence phenotype in *ku80*^{-/-} MEFs, despite an otherwise intact response of p53. In contrast to *ku80*^{-/-}*p53*^{-/-} mice, which die rapidly primarily from lymphomas, there was no significant increase in tumorigenesis in *ku80*^{-/-}*p21*^{-/-} mice. However, *ku80*^{-/-}*p21*^{-/-} mice showed no improvement with respect to rough fur coat or osteopaenia, and even showed a shortened lifespan compared with *ku80*^{-/-} mice. These results show that the increased lifespan of *ku80*^{-/-} MEFs owing to the loss of p21 is not associated with an improvement of the premature ageing phenotypes of *ku80*^{-/-} mice observed at the organismal level.

Keywords: ageing; cellular senescence; Ku80; p21; p53

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INTRODUCTION

Ageing is a complex process that involves the decline of multiple systems required to maintain the homeostasis of an organism. Chronic accumulation of DNA damage (Vijg *et al*, 2005) and subsequent cellular senescence or apoptosis might deplete tissues of cells that are able to proliferate, thus compromising organ homeostasis and accelerating ageing (Campisi, 2005). Humans with defects in DNA repair such as those with Werner syndrome, present with features of premature ageing, and cells from these individuals also undergo accelerated senescence in culture

(Faragher *et al*, 1993). Furthermore, increased numbers of DNA damage foci have been observed in fibroblasts from old individuals (Scaffidi & Misteli, 2006) and there is evidence showing that senescent cells accumulate with age *in vivo* (Dimri *et al*, 1995). All of these findings are consistent with the concept that chronic accumulation of DNA damage correlates well with organismal ageing *in vivo*.

Ku80, which forms heterotrimers with Ku70 and DNA-PKcs (DNA-dependent protein kinase catalytic subunit), is essential in the non-homologous end-joining (NHEJ) pathway of DNA double-strand break repair (Lieber *et al*, 2004). Consistent with other animal models of DNA repair deficiencies, mice lacking Ku80 show premature ageing phenotypes, including kyphosis, skin atrophy and shortened lifespan (Vogel *et al*, 1999). Although deletion of p53 rescued the early senescence in *ku80*^{-/-} mouse embryonic fibroblasts (MEFs), *ku80*^{-/-}*p53*^{-/-} mice died of B-cell lymphomas by 2 months of age (Lim *et al*, 2000) making it difficult to assess the contribution of chronically activated p53 to the accelerated ageing phenotype of the *ku80*^{-/-} strain. p21^{CIP1/WAF1} (p21 hereafter) is an important downstream mediator of p53 in cell-cycle regulation and cellular senescence. Disruption of p21 results in the lifespan extension of normal human fibroblasts in culture (Brown *et al*, 1997), and overexpression of p21 alone is able to induce cellular senescence in both normal human fibroblasts and tumour cells (McConnell *et al*, 1998; Fang *et al*, 1999). To address the role of p21 in the premature senescence and ageing phenotype in *ku80*^{-/-} MEFs and mice, we generated *ku80*^{-/-}*p21*^{-/-} mice. We show that deletion of p21 rescued the early senescence phenotype in cultured *ku80*^{-/-} MEFs, whereas the p53 DNA damage response remained intact. Unlike the increased tumorigenesis reported in *ku80*^{-/-}*p53*^{-/-} mice, there was no increase of tumours in *ku80*^{-/-}*p21*^{-/-} mice. However, neither the lifespan nor the premature ageing phenotypes of *ku80*^{-/-} mice were improved by the loss of p21, indicating that p21 is not the main p53 response gene barrier to premature ageing in *ku80*^{-/-} mice.

RESULTS

p53 and p21 levels are elevated in *ku80*^{-/-} MEFs

As reported previously (Lim *et al*, 2000), *ku80*^{-/-} MEFs showed early senescence and reproducibly ceased to proliferate within 10 population doublings (PDs), whereas wild-type controls continued

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Fig 1 | Early senescence of *ku80*^{-/-} mouse embryonic fibroblasts correlates with elevated levels of p53 and p21. (A) Growth curves of MEFs starting at PD8 (which reflects time zero on the x axis), with genotypes as indicated. (B) Quantification of BrdU/SA-β-gal of WT and *ku80*^{-/-} MEFs at PD8. Mean ± s.d. reflects the results of three separate experiments from individual isolates. (C) Western blot showing levels of p53 and p21 in WT, *ku80*^{-/-}, *p53*^{-/-} (PD12) and *p21*^{-/-} (PD6) MEFs either untreated (-) or treated (+) with doxorubicin (Doxo; 100 ng/ml) for 20 h. (D) Quantifications of the levels of p53 and p21 were normalized to β-actin, and to basal levels of p53 and p21 in WT MEFs, respectively. Mean ± s.d. reflects the results of three separate experiments from individual isolates. For all the experiments, at least two sets of isolates for each genotype were assayed and similar results were obtained. BrdU, 5-bromodeoxyuridine; MEF, mouse embryonic fibroblast; PD, population doubling; SA-β-gal, senescence-associated β-galactosidase; WT, wild type.

to proliferate to about PD20 (Fig 1A; supplementary Fig 1 online). Signs of cellular senescence, which became apparent in *ku80*^{-/-} MEFs by PD8, included an enlarged and flattened morphology, lack of 5-bromodeoxyuridine (BrdU) incorporation and positive staining for senescence-associated β-galactosidase (SA-β-gal; Fig 1B; data not shown). *ku80*^{-/-} MEFs heterozygous for p21 also underwent early senescence with kinetics similar to those of *ku80*^{-/-} MEFs, showing that the loss of one allele of p21 does not extend the lifespan (Fig 1A). The early senescence of *ku80*^{-/-} MEFs is p53 dependent, as the absence of p53 in *ku80*^{-/-} MEFs has been reported to rescue premature senescence in these cells (Lim et al, 2000). Consistent with this study, *ku80*^{-/-} MEFs showed elevated basal levels of both p53 and p21 (Fig 1C,D). The elevated level of p21 in *ku80*^{-/-} MEFs must be p53 dependent, as levels of p21 in *p53*^{-/-} MEFs were low. The specificity of p53 and p21 proteins was verified by their absence in *p53*^{-/-} or *p21*^{-/-} MEFs and by their response to treatment with doxorubicin (Fig 1C,D). These results indicate that p53-dependent induction of p21 might be important in the early senescence phenotype of *ku80*^{-/-} MEFs.

Deletion of p21 delays early senescence in *ku80*^{-/-} MEFs

To study directly the role of p21 in the early senescence phenotype of *ku80*^{-/-} MEFs, we generated *ku80*^{-/-}*p21*^{-/-} MEFs. The absence of p21 in this complete knockout strain (Brugarolas et al, 1995) was confirmed by Western blot and PCR genotyping (Fig 1C; data not shown). As shown in Fig 2A, the loss of p21 restored the proliferative ability of *ku80*^{-/-} MEFs to a level comparable with that of wild-type

MEFs. These results were verified in experiments with another set of independently derived MEFs, including two more independently isolated *ku80*^{-/-}*p21*^{-/-} MEFs (supplementary Fig 1 online). Consistent with a previous report (Deng et al, 1995), *p21*^{-/-} MEFs initially grew more rapidly and achieved a higher density than wild-type MEFs (Fig 2A,B). In contrast to the early senescent phenotype of *ku80*^{-/-} MEFs, *ku80*^{-/-}*p21*^{-/-} MEFs remained morphologically indistinguishable from wild-type MEFs, actively incorporated BrdU and were mostly negative for SA-β-gal staining until beyond PD20 (Fig 2C). However, by PD26, a large fraction of *ku80*^{-/-}*p21*^{-/-} MEFs showed reduced proliferation and evidence of senescence, indicating that the deletion of p21 only delayed the onset of senescence in *ku80*^{-/-} MEFs.

Loss of p21 abrogates G1 arrest in *ku80*^{-/-} MEFs

Senescent *ku80*^{-/-} MEFs (PD10) showed a lower S-phase fraction and a higher G2-phase fraction compared with wild-type MEFs, suggesting a biphasic arrest at both the G1 and G2 phases (Fig 3A). The increase in G2-phase cells is consistent with DNA damage checkpoint activation (Chan et al, 2000). Although p21 has been reported to function in both the G1 and G2 checkpoints (Bunz et al, 1998), the loss of p21 function in *ku80*^{-/-} MEFs primarily abrogated the G1 checkpoint, as shown by a marked shift of cells from the G1 to the G2 phase in late passage (PD26) *ku80*^{-/-}*p21*^{-/-} MEFs (Fig 3B). It is noted that there was increased tetraploidy in senescent *ku80*^{-/-}*p21*^{-/-} MEFs (Fig 3B), which probably reflects the additive effects of DNA damage and G1 checkpoint abrogation

Fig 2 | Effects of p21 deletion on growth properties of *ku80*^{-/-} mouse embryonic fibroblasts. (A) Growth curves of MEFs starting at PD8, with genotypes as indicated. Of note is that *p21*^{-/-}, WT and *ku80*^{-/-}*p21*^{-/-} MEFs showed sub-populations associated with a loss of p53, which bypassed senescence and eventually took over the culture, as reflected by the accelerated phase on their growth curves. (B) Saturation densities of MEFs. MEFs (1 × 10⁴) at PD4 were plated in six 60-mm petri dishes and the medium was changed every 3 days. At 1 week, three dishes were stained with crystal violet, and representative images are shown on the left. Cell numbers from parallel dishes are shown on the right. Mean ± s.d. reflects results of 3 separate experiments from individual MEFs. (C) Representative images of BrdU (brown)/SA-β-gal (blue) double staining and cell morphology after 22 days in culture; PDs are extrapolated from growth curves. Quantifications for BrdU or SA-β-gal staining are shown on the right. Mean ± s.d. reflects the results of three separate experiments from individual isolates. For all the experiments, at least two sets of isolates for each genotype were assayed and similar results were obtained. BrdU, 5-bromodeoxyuridine; MEF, mouse embryonic fibroblast; PD, population doubling; SA-β-gal, senescence-associated β-galactosidase; WT, wild type.

from the loss of both Ku80 and p21. The spontaneous immortalization of MEFs can be caused either by the mutation of p53 or by the loss of p19^{ARF} expression (Sherr, 1998). To exclude the possibility that the delayed senescence of *ku80*^{-/-}*p21*^{-/-} MEFs was the consequence of selection for variants lacking the function of p53 or p19^{ARF}, basal and induced levels of p53 and p19^{ARF} were determined in MEFs at different population doublings. The response of p53 remained intact in *ku80*^{-/-}*p21*^{-/-} MEFs to at least PD20, and no loss of p19^{ARF} was detected (Fig 3C).

Loss of p21 fails to rescue *ku80*^{-/-} ageing phenotypes

As the loss of p21 delayed the onset of senescence of *ku80*^{-/-} MEFs in culture, we sought to determine whether the loss of p21 also extended the lifespan of *ku80*^{-/-} mice. The median lifespan of *ku80*^{-/-} mice was 22 weeks (Fig 4A), which is similar to that observed in a previous study (Lim et al, 2000). The loss of one p21 allele changed neither the proliferative capacity of *ku80*^{-/-} MEFs (Fig 1A) nor the overall well-being of *ku80*^{-/-} mice; thus, the survival data for *ku80*^{-/-}*p21*^{+/+} and *ku80*^{-/-}*p21*^{-/-} mice were

Fig 3 | Cell-cycle profiles and p53 responses in *ku80*^{-/-}*p21*^{-/-} mouse embryonic fibroblasts. Representative cell-cycle profiles of (A) *Ku80*^{+/+} and *ku80*^{-/-} MEFs with or without p21 at PD10, and (B) in WT and *ku80*^{-/-}*p21*^{-/-} MEFs at PD26. Mean ± s.d. reflects the results of three separate experiments from individual MEFs. Two sets of MEFs for each genotype were assayed with similar results (**P* < 0.05). (C) Western blot analysis of the response of p53 in *ku80*^{-/-}*p21*^{-/-} MEFs with increasing PDs. Cells were either untreated (–) or treated (+) with doxorubicin (Doxo; 300 ng/ml) for 20 h, and levels of p53 and p19 are shown; β-actin was used as a loading control. MEF, mouse embryonic fibroblast; PD, population doubling; WT, wild type.

combined. Although *ku80*^{-/-}*p21*^{-/-} mice survived to adulthood, they failed to live longer than *ku80*^{-/-} mice and, in fact, their lifespan was significantly shortened (median lifespan was 11 weeks; Fig 4A). However, unlike *ku80*^{-/-}*p53*^{-/-} mice (Lim et al, 2000), *ku80*^{-/-}*p21*^{-/-} mice remained largely free of tumours (Table 1).

Consistent with the early senescence phenotype of *ku80*^{-/-} MEFs *in vitro*, *ku80*^{-/-} mice had fewer proliferating cells in their hair follicles than wild-type mice and showed a rough fur phenotype; however, the absence of p21 failed to improve these premature ageing phenotypes in *ku80*^{-/-} mice (Fig 4B; supplementary Fig 2A,B online). No increase in apoptosis was detected in the hair follicles in *ku80*^{-/-} or *ku80*^{-/-}*p21*^{-/-} mice (data not shown). We also investigated whether the loss of p21 improved osteopaenia in *ku80*^{-/-} mice, another premature ageing phenotype that is reflected by the early appearance of kyphosis and/or closure of the growth plate. This phenotype was not observed until after 20 weeks of life in *ku80*^{-/-} mice, similar to previously reported results (Li et al, 2007). By 30 weeks, more than 50% of *ku80*^{-/-} mice and 3 out of 4 *ku80*^{-/-}*p21*^{-/-} mice analysed showed kyphosis (Fig 4B); by contrast, kyphosis was not observed in wild-type or *p21*^{-/-} mice until after 1 year. *ku80*^{-/-} mice also showed an early closure of the growth plate in the distal femur, reflected by reduced chondrocyte numbers compared with those of wild-type or *p21*^{-/-} mice. *ku80*^{-/-}*p21*^{-/-} mice showed comparable chondrocyte numbers as *ku80*^{-/-} mice, implying a similar rate of growth plate closure (Fig 4C). In addition, *ku80*^{-/-} mice showed low body weight (Vogel et al, 1999). Although low body weight is not considered to be premature ageing phenotype of *ku80*^{-/-} mice because they are born smaller, the absence of p21 also failed to improve this phenotype (supplementary Fig 2C online). Taken together, the loss of p21 failed to rescue several premature ageing phenotypes in *ku80*^{-/-} mice.

ku80^{-/-} *p21*^{-/-} mice show no increased tumorigenesis

Most *ku80*^{-/-}*p53*^{-/-} mice die within 2 months almost exclusively because of pre-B-cell lymphomas (Lim et al, 2000); by contrast, more than 50% of *ku80*^{-/-}*p21*^{-/-} mice survived for more than 3 months (Fig 4A). To gain insights into the causes of death of *ku80*^{-/-}*p21*^{-/-} mice, 14 moribund or dead mice from the *ku80*^{-/-}*p21*^{-/-} group were autopsied, and the gross and histological evidence of tumours compared with 20 moribund or dead *ku80*^{-/-} mice. We observed that infection accounted for most cases of death, with facial and visceral abscesses being the most frequent pathology in both *ku80*^{-/-} and *ku80*^{-/-}*p21*^{-/-} mice (Table 1). The infections were most likely opportunistic, as these mice were maintained in a pathogen-free environment (Li et al, 2007). Of note is that ageing phenotypes of *ku80*^{-/-} mice have been shown to be independent of chronic inflammation caused by immune deficiency and infections (Holcomb et al, 2007). Some mice also had to be euthanized because of rectal prolapse and dehydration caused by diarrhoea (Table 1).

The pre-B-cell lymphomas in *ku80*^{-/-}*p53*^{-/-} mice usually manifest themselves as splenomegaly (Lim et al, 2000); however, spleens in adult *ku80*^{-/-}*p21*^{-/-} mice were generally atrophied as they were in *ku80*^{-/-} mice (Fig 5A,B). There was a significant increase in apoptosis in the spleens and other lymphoid tissues of *ku80*^{-/-}*p21*^{-/-} mice compared with *ku80*^{-/-} mice (Fig 5C; data not shown), which is consistent with the effects of p21 loss in *atm*^{-/-} mice (*atm* for ataxia telangiectasia mutated; Wang et al, 1997). Of note is that spontaneous apoptosis in MEFs with all genotypes was similar (data not shown), presumably because the primary response to Ku80 loss in MEFs is growth arrest rather than apoptosis. We observed 2 lymphomas and 1 squamous cell carcinoma among 17 wild-type mice, and 3 fibrosarcomas, 2 lymphomas and 1 rhabdomyosarcoma among 30 *p21*^{-/-} mice. Some mice were lost to observation, and therefore the

Fig 4 | Effects of p21 deletion on ageing phenotypes of *ku80*^{-/-} mice. (A) Survival curves (100% × number of mice alive after each week/total number of mice at 2 weeks) for mice with each genotype. (B) Comparison of the appearance of 32-week-old mice with each genotype; note the rough fur coat and kyphosis in *ku80*^{-/-} and *ku80*^{-/-}*p21*^{-/-} mice. (C) Comparison of the distal femoral epiphysis in mice with each genotype. The cortical wall and part of the diaphysis are also shown. Chondrocyte numbers in the growth plates from two mice between 32 and 34 weeks old for each genotype were counted, and the quantification is shown on the right. Scale bar 200 μm.

Table 1 | Causes of death in *ku80*^{-/-} and *ku80*^{-/-}*p21*^{-/-} mice

Cause of death	<i>ku80</i> ^{-/-} (n = 20) Cases/total (%)	<i>ku80</i> ^{-/-} <i>p21</i> ^{-/-} (n = 14) Cases/total (%)
Infections		
Superficial	7/20 (35)	4/14 (29)
Visceral	8/20 (40)	4/14 (29)
Dehydration	3/20 (15)	3/14 (21)
Tumors	1/20* (5)	2/14 ^{‡,§} (14)
Rectal prolapse	1/20 (5)	1/14 (7)

*Lymphoma; [‡]lymphoma and histiocytic sarcoma; [§]P = 0.21, compared with the *ku80*^{-/-} group.

tumour incidence might be underestimated. Nonetheless, the tumour spectrum in *p21*^{-/-} mice was similar to that reported previously (Martín-Caballero *et al*, 2001). Among 20 *ku80*^{-/-} mice, we identified 1 lymphoma (40 weeks) two tumours were found among 14 *ku80*^{-/-}*p21*^{-/-} mice. Pathological analysis showed one tumour to be a histiocytic sarcoma (37 weeks), and the other to be a lymphoma (9.6 weeks) in the thymus (Table 1). Medulloblastomas are seen in most mice lacking both NHEJ repair functions and p53, such as in *lig4*^{-/-}*p53*^{-/-} mice (*lig4* for DNA Ligase IV; Lee & McKinnon, 2002) or

rag1^{-/-}*ku80*^{-/-}*p53*^{-/-} mice (*rag1* for recombination activating gene 1; Holcomb *et al*, 2006). Thus, we subjected the brains of 8 *ku80*^{-/-}*p21*^{-/-} mice, which had survived for varying periods from 2 to 8 months, to gross and histopathological analysis for any brain tumours, and none were observed. All of these findings indicate that the loss of p21 function did not increase tumorigenesis in *ku80*^{-/-} mice.

DISCUSSION

The onset of senescence of cells in culture seems to be influenced by exogenous as well as endogenous factors. For example, the cellular lifespan of wild-type MEFs can be extended significantly by culturing them in 3%, as opposed to 20%, oxygen, which is associated with reduced oxidative stress (Parrinello *et al*, 2003). However, these same authors reported that the lifespan of *ku80*^{-/-} MEFs in culture, which have a severe endogenous DNA damage phenotype, is not increased even at low oxygen concentration (Parrinello *et al*, 2003). Species differences can also influence lifespan in culture. MEFs with p21 loss of function show a delayed onset of senescence (Pantoja & Serrano, 1999), whereas human fibroblasts with p21 loss of function have been reported to completely bypass senescence (Brown *et al*, 1997). Here, we have shown that *ku80*^{-/-} MEFs exhibited elevated basal levels of p53 and p21, presumably in response to chronic DNA damage associated with Ku80 loss of function. The role of p21 in this premature senescence phenotype was demonstrated by the ability of

Fig 5 | Effects of p21 deletion on lymphoid tissue in *ku80*^{-/-} mice. (A) Spleen/body weight ratio from mice with various genetic backgrounds at 16 weeks. (B) HE staining of spleen tissue from each group; note the loss of splenic follicles in *ku80*^{-/-} compared with *Ku80*^{+/+} mice. (C) TUNEL staining of spleen tissue was performed on paraffin sections (three mice for each genotype). Quantification of three high-power fields (HP) per spleen is shown on the right. HE, haematoxylin and eosin; TUNEL, TdT-mediated dUTP nick-end labelling.

ku80^{-/-}*p21*^{-/-} MEFs to show increased lifespan comparable with that of wild-type MEFs under the same culture conditions. We observed that senescent *ku80*^{-/-}*p21*^{-/-} MEFs are arrested primarily at the G2/M phase, suggesting that G2 checkpoints can also be used in senescence programming.

Although deletion of p21 in culture delayed senescence in *ku80*^{-/-} MEFs, the loss of p21 failed to extend the lifespan of *ku80*^{-/-} mice. In fact, the lifespan of *ku80*^{-/-}*p21*^{-/-} mice was significantly shortened compared with that of *ku80*^{-/-} mice. The compromised lifespan of *ku80*^{-/-}*p21*^{-/-} mice could not be attributed to the increased tumorigenesis found in *ku80*^{-/-}*p53*^{-/-} mice (Lim et al, 2000), as the tumour incidence in *ku80*^{-/-}*p21*^{-/-} mice was low. Loss of function of Lig4, an enzyme involved in NHEJ repair similar to Ku80, results in embryonic lethality (Frank et al, 2000), while *lig4*^{-/-}*p53*^{-/-} mice survive to adulthood but exhibit a high frequency of lymphoma (Frank et al, 2000) as do *ku80*^{-/-}*p53*^{-/-} mice. *lig4*^{-/-} mice possessing a p53R172P mutant, which is defective in p53 apoptotic but not growth arrest functions (Rowan et al, 1996), also survive to adulthood and show some suppression of the early lymphomagenesis seen with complete loss of p53 function (Van Nguyen et al, 2007). These findings, together with our present results, suggest that p53 cell-cycle arrest and apoptosis functions can act independently as barriers to lymphomagenesis. In fact, the absence of p21 has been reported to delay the onset of lymphoma in *atm*^{-/-} mice by making them more sensitive to a p53 apoptotic response (Wang et al, 1997). Consistent with these findings, we observed increased

levels of apoptosis in the spleens of *ku80*^{-/-}*p21*^{-/-} mice, which might not only act to inhibit tumorigenesis but also to exacerbate the compromised immune and haematopoietic systems in these mice (Rossi et al, 2007).

A sub-population of *ku80*^{-/-}*p21*^{-/-} mice, which survived for more than 20 weeks, allowed us to compare the ageing phenotypes with those of *ku80*^{-/-} mice. In such survivors, the loss of p21 neither altered the accelerated rate of growth plate closure nor improved other *ku80*^{-/-} premature ageing phenotypes such as rough fur coat or rectal prolapse (Li et al, 2007). By contrast, abrogation of the senescence programme induced by the loss of p21 function led to a delay in organismal ageing without an increase of tumorigenesis in the *Terc*^{-/-} (telomerase RNA component) premature ageing mouse model (Choudhury et al, 2007). The various effects of p21 loss of function on the lifespan of *Terc*^{-/-} mice compared with *ku80*^{-/-} mice might originate from different severities of ageing phenotypes in *Terc*^{-/-} and *ku80*^{-/-} mice. Although the loss of telomerase activity gradually impairs rapidly proliferating compartments, such as small intestinal crypts and haematopoietic stem cells, Ku80 loss of function impairs V(D)J recombination and lymphocyte development (Nussenzweig et al, 1996), as well as depleting the haematopoietic stem-cell population (Rossi et al, 2007). Consequently, *ku80*^{-/-} mice are born smaller and nearly half die within the first 2 weeks of life (Lim et al, 2000). p21 has also been reported to have a function in the maintenance of haematopoietic stem cells, as its loss initially increases the

proliferative potential but rapidly exhausts this population (Cheng *et al*, 2000). Premature cellular senescence in culture has often been shown to correlate well with mammalian premature ageing syndromes and is recognized as a counterpart of organismal ageing (Campisi, 2005). However, although the loss of p21 in the *ku80*^{-/-} model impedes premature senescence in culture, it fails to rescue overall organismal ageing, presumably because of the combined adverse effects of loss of function of these two genes.

METHODS

Generation of *ku80*^{-/-} *p21*^{-/-} mice. *p21*^{-/-} and *ku80*^{-/-} mice on a C57BL6 × 129 mixed background were described previously (Brugarolas *et al*, 1995; Nussenzweig *et al*, 1996). Intercrosses among *Ku80*^{+/-} *p21*^{+/-} and *Ku80*^{+/-} *p21*^{-/-} mice were used to generate *ku80*^{-/-} *p21*^{+/-}, *ku80*^{-/-} *p21*^{+/-} and *ku80*^{-/-} *p21*^{-/-} mice, as well as their wild-type and *p21*^{-/-} counterparts. All mice were maintained in a pathogen-free environment. The frequency of birth compared with the expected ratio was 76.8% for *ku80*^{-/-} and 86% for *ku80*^{-/-} *p21*^{-/-} newborns.

Generation and culture of MEFs. The generation and culture of MEFs are described in detail in the supplementary information online. To determine growth curves, 1.5 × 10⁵ cells at passage 4 (approximated as PD8) or passage 1 (approximated as PD2) were plated onto 60 mm plates and then transferred on confluence to minimize the possible selection of more densely growing variants. Population doubling was calculated with the formula: PD = log(n2/n1)/log2, where n1 is the number of cells seeded and n2 is the number of cells recovered. PD are used to reflect cumulative population doublings.

Flow cytometry. Cells were stained with propidium iodide using the CycleTEST Plus DNA reagent kit (340242, Becton Dickinson, San Jose, CA, USA), and analysed using the CellQuest 3.2 software.

BrdU and SA-β-gal double staining. Details are described in the supplementary information online.

Western blotting, immunohistochemistry and TdT-mediated dUTP nick end labelling staining. Details are described in the supplementary information online.

Supplementary information is available at *EMBO reports* online (<http://www.emboareports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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