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Increased p53 activity does not accelerate telomere-driven ageing

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There is a great interest in determining the impact of p53 on ageing and, for this, it is important to discriminate among the known causes of ageing. Telomere loss is a well-established source of age-associated damage, which by itself can recapitulate ageing in mouse models. Here, we have used a genetic approach to interrogate whether p53 contributes to the elimination of telomere-damaged cells and its impact on telomere-driven ageing. We have generated compound mice carrying three functional copies of the p53 gene (super-p53) in a telomerase-deficient background and we have measured the presence of chromosomal abnormalities and DNA damage in several tissues. We have found that the *in vivo* load of telomere-derived chromosomal damage is significantly decreased in super-p53/telomerase-null mice compared with normal-p53/telomerase-null mice. Interestingly, the presence of extra p53 activity neither accelerates nor delays telomere-driven ageing. From these observations, we conclude that p53 has an active role in eliminating telomere-damaged cells, and we exclude the possibility of an age-promoting effect of p53 on telomere-driven ageing.

Keywords: p53; telomeres; ageing; DNA damage; tumour suppression

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INTRODUCTION

Ageing is produced by the accumulation of cellular damage that eventually results in tissue dysfunction (Hasty *et al*, 2003). Telomeres have emerged in recent years as an important source of damage, the harmful potential of which increases progressively with time (Blasco, 2003). Mammalian telomeres protect chromosomal ends from being recognized and processed as DNA double-strand breaks (de Lange, 2002). Telomeres are shortened with each cell division owing to incomplete replication of the DNA ends, and this progressive loss of telomere length is compensated in germline cells by the activity of telomerase (Blackburn, 2001). However, in adult somatic tissues, telomerase activity is insufficient and, accordingly, most cells and tissues experience telomere shortening in association with cell division and ageing (Blackburn, 2001). In this manner, telomeres are cell division counting devices that, on reaching a critically short length, impose an end to the replicative lifespan of individual cells. In addition to this, the rate of telomere loss per cell division is influenced by the presence of cellular stressors, such as oxidative damage, an aspect that further reinforces the involvement of telomeres in the onset of ageing (Von Zglinicki, 2003).

Critically short telomeres are dysfunctional and trigger DNA damage signalling pathways that may provoke senescence or apoptosis (Smogorzewska & de Lange, 2002; d'Adda di Fagagna *et al*, 2003; Takai *et al*, 2003). DNA repair mechanisms may incur in the infliction of further damage by ligating dysfunctional telomeres and, thus, generating interchromosomal end-to-end fusions and other chromosomal aberrations (Goytisolo & Blasco, 2002). The relevance of telomere dysfunction for ageing is supported by studies in telomerase-deficient mice, which show accelerated ageing (Herrera *et al*, 1999; Rudolph *et al*, 1999; Blasco, 2003; Leri *et al*, 2003). In addition, germline mutations in telomerase components underlie the human progeroid syndrome dyskeratosis congenita (Vulliamy *et al*, 2001), and it has been reported for humans and worms that there is a correlation between the telomere length of a given individual and its lifespan (Cawthon *et al*, 2003; Joeng *et al*, 2004). Altogether, present evidence strongly suggests that telomere damage is a relevant determinant of ageing.

The tumour suppressor p53 is an important sensor of dysfunctional telomeres (Chin *et al*, 1999; Gonzalez-Suarez *et al*, 2000; Smogorzewska & de Lange, 2002; Leri *et al*, 2003; Sharpless & DePinho, 2004). Absence of p53 enables proliferation

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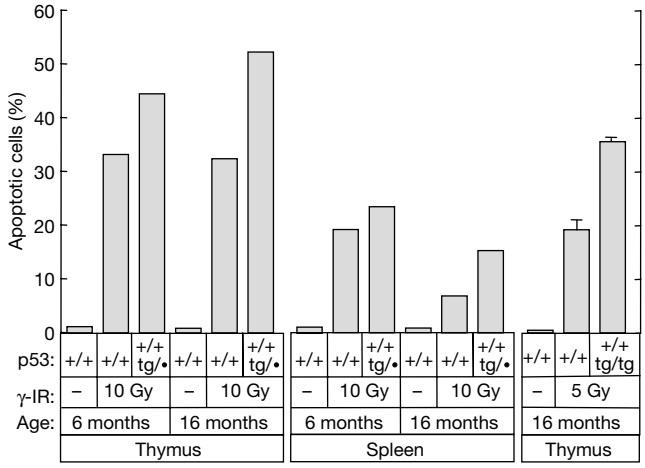

Fig 1 | Functionality of the p53 transgene in aged mice. Young (6 months) and old (16 months) mice of the indicated genotypes were left untreated or exposed to 5 or 10 Gy whole-body γ-irradiation (γ-IR). At 3 h post-irradiation, the thymus was extracted and thymocytes were analysed by flow cytometry to measure their DNA content and evaluate the percentage of apoptosis. Data correspond to individual mice, except the rightmost columns, which correspond to the average ± s.d. of three mice.

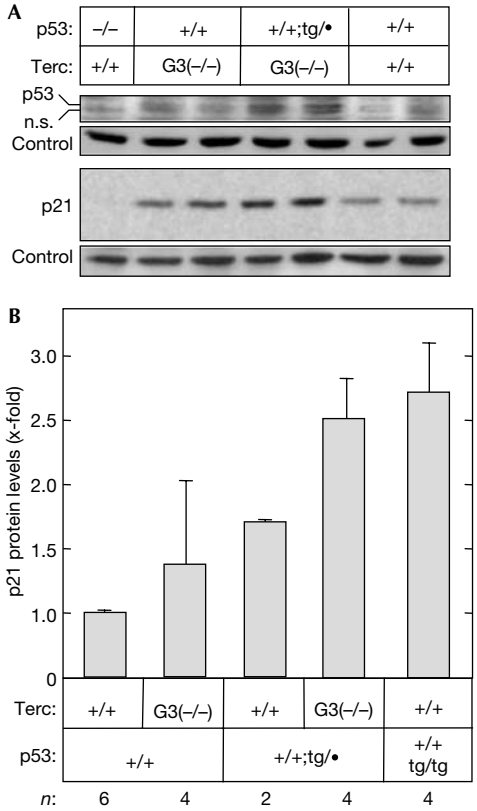

Fig 2 | Super-p53 mouse embryo fibroblasts respond to telomere dysfunction by activating the p53/p21 pathway. (**A**) Representative example of the levels of p53 and p21 in cell extracts from early-passage primary mouse embryo fibroblasts (MEFs) of the indicated genotypes. The band labelled as n.s. is nonspecific for p53. Bands from Ponceau S-stained western blots were used as loading control. The blots shown are representative of three experiments. (**B**) Quantification of p21 protein levels. Data are relative to p21 levels in wild-type MEFs. For each genotype, several independent MEF cultures (derived from different embryos) were assayed, indicated at the bottom of the figure (*n*). Data correspond to the average ± s.d.

in the face of telomere dysfunction and this translates into delayed telomere-driven phenotypes in p53-null/telomerase-null mice, such as testicular atrophy, and in higher incidence of spontaneous carcinomas compared with p53-null/telomerase-proficient mice (Chin *et al*, 1999; Artandi *et al*, 2000). Notably, however, the impact of p53 on telomere-driven ageing has remained unexplored. The analysis of ageing in p53-null or p53-heterozygous mice is not possible because these mice die of cancer at an early age. To circumvent this limitation, we have taken advantage of our recently generated super-p53 mice (Garcia-Cao *et al*, 2002). These mice carry a large genomic transgene containing an intact p53 gene that recapitulates in a quantitative manner the activity of a single endogenous allele. The availability of wild-type p53 and super-p53 mice, carrying respectively two and three gene doses of p53, allows the analysis of the impact of p53 on phenotypes that require long observation periods, such as ageing.

RESULTS

The p53 transgene is functional in aged mice

The rapid and robust apoptotic response of lymphocytes to ionizing radiation is among the best-characterized biological readouts for p53 function *in vivo* (Lowe *et al*, 1993; Midgley *et al*, 1995). In this regard, we have previously reported that young super-p53 mice present enhanced thymocyte and splenocyte apoptosis following irradiation (Garcia-Cao *et al*, 2002). In the light of reports on ageing-associated silencing of certain transgenes (Cohn *et al*, 1991; Robertson *et al*, 1996; Sutherland *et al*, 1997; Guglielmi *et al*, 2003), we considered of relevance to assess the functionality of our p53 transgene in aged animals. To address this, we have examined the apoptotic response to γ-radiation in the thymus and spleen of wild-type and super-p53 mice of advanced age. As shown in Fig 1, radiation-induced apoptosis was higher in super-p53 mice (both $p53^{+/+;tg/\bullet}$ and $p53^{+/+;tg/tg}$) compared with wild-type ($p53^{+/+}$) mice regardless of age. These data show that the p53 transgene is not subject to ageing-associated silencing.

p53 reduces telomere-derived damage in MEFs

To explore the effect of increased p53 gene dosage in the particular context of telomere-derived damage, we crossed super-p53 mice (Garcia-Cao *et al*, 2002) into a genetic background lacking the RNA component of telomerase ($Terc^{-/-}$; Blasco *et al*, 1997). In human and mouse cells, telomere uncapping triggers p53 activation (Chin *et al*, 1999; Gonzalez-Suarez *et al*, 2000; Smogorzewska & de Lange, 2002; Leri *et al*, 2003). Accordingly, we found that super-p53 mouse embryo fibroblasts (MEFs) derived from late-generation (G3) telomerase-null mice (Terc-$G3^{-/-}$;$p53^{+/+;tg/\bullet}$) have higher basal levels of p53 and p21 than their p53 wild-type (Terc-$G3^{-/-}$;$p53^{+/+}$) counterparts (Fig 2A,B). Chromosomal fusions through the telomeres constitute the most prevalent and characteristic type of damage in

Table 1 | Increased p53 gene dosage reduces chromosomal aberrations in late-generation telomerase-null mice

	p53(+/+) Terc-G3(−/−)	p53(+/+;tg/•) Terc-G3(−/−)	*P*-value*
Primary MEFs†			
Signal-free ends/telomeres‡	369/3,160 (11.7%)	233/3,196 (7.3%)	<0.001
p–p fusions/metaphases (Robertsonian-like)	17/200 (8.5%)	5/200 (2.5%)	<0.01
q–q fusions/metaphases (dicentrics)	7/200 (3.5%)	1/200 (0.5%)	<0.05
p–q fusions/metaphases	2/200 (1.0%)	1/200 (0.5%)	NS
Total number of end-to-end fusions/metaphases	26/200 (13.0%)	7/200 (3.5%)	<0.001
7-month-old mice—splenocytes†			
Signal-free ends/telomeres‡	246/3,012 (8.2%)	238/3,192 (7.5%)	NS
p–p fusions/metaphases (Robertsonian-like)	12/100 (12.0%)	1/100 (1.0%)	<0.001
q–q fusions/metaphases (dicentrics)	1/100 (1.0%)	0/100 (0%)	NS
p–q fusions/metaphases	0/100 (0%)	0/100 (0%)	NS
Total number of end-to-end fusions/metaphases	13/100 (13.0%)	1/100 (1.0%)	<0.001
12-month old mice—splenocytes†			
Signal-free ends/telomeres‡	431/3,136 (13.7%)	364/3,084 (11.8%)	0.01
p–p fusions/metaphases (Robertsonian-like)	55/94 (58.5%)	23/95 (24.2%)	<0.001
q–q fusions/metaphases (dicentrics)	0/94 (0%)	1/95 (1.1%)	NS
p–q fusions/metaphases	0/100 (0%)	0/100 (0%)	NS
Total number of end-to-end fusions/metaphases	55/94 (58.5%)	24/95 (26.3%)	<0.001

*Fisher's exact test; NS, not significant ($P > 0.05$). †For each genotype, data for chromosome fusions were obtained from two independent cultures of primary MEFs, or from two littermate mice of the indicated age, as corresponds. ‡Data for signal-free ends are given as the frequency of chromosome ends without detectable telomere signal relative to the total number of chromosome ends analysed.
MEFs, mouse embryo fibroblasts.

telomerase-deficient cells (Blasco *et al*, 1997). To determine the consequences of p53 activity on telomere-derived damage, we compared the incidence of telomere fusions in primary Terc-G3$^{-/-}$ MEFs with wild-type or super-p53 gene dosage. As shown in Table 1, combined telomere fluorescence *in situ* hybridization (FISH) and cytogenetic analyses showed that the p53 transgene significantly reduced both the number of chromosomes with critically short telomeres ('signal-free ends') and the incidence of chromosomal fusions through the telomeres ('end-to-end fusions'). From these data, we conclude that genetically increased p53 function translates into a significant reduction in the amount of viable cells with telomere-derived DNA damage.

p53 reduces telomere-derived damage in the organism

To extend the above observations, we obtained splenocytes from late-generation (G3) telomerase-null mice and we examined the integrity of telomeres and chromosomes in *ex vivo* metaphases. For these analyses, we used Terc-G3$^{-/-}$ mice of two different ages: 7 months of age, when ~75% of the Terc-G3$^{-/-}$ mouse colony is alive; and 12 months of age, when less than 50% of the Terc-G3$^{-/-}$ mouse colony is alive (Fig 4). Telomere length was significantly shorter in 12-month-old than in 7-month-old Terc-G3$^{-/-}$ mice, regardless of the presence or absence of the extra gene copy of p53 (data not shown). Terc-G3$^{-/-}$ mice with wild-type p53 (Terc-G3$^{-/-}$;p53$^{+/+}$) presented a considerable amount of telomere-derived damage in the spleen ('signal-free ends' and 'end-to-end fusions') at 7 months of age and this was remarkably exacerbated at 12 months of age (Table 1). Importantly, Terc-G3$^{-/-}$;p53$^{+/+;tg/•}$ splenocytes showed a clear reduction in the amount of telomere-derived damage, and this was particularly significant at 12 months of age, as reflected by a diminished incidence of critically short telomeres and chromosomal fusions (Table 1). These data indicate that a modest increase in the activity of p53 (from the normal complement of two copies to three copies) has a measurable and significant effect reducing the load of telomere-damaged cells in the spleen.

We wished to extend the above concept to other adult cell types. For this, we measured γ-H2AX foci as a surrogate marker of telomere-derived damage. Previous investigators have shown that dysfunctional telomeres trigger the formation of γ-H2AX foci (d'Adda di Fagagna *et al*, 2003; Takai *et al*, 2003; Hao *et al*, 2004) and, moreover, that there is an age-dependent increase in the number of cells containing γ-H2AX foci (Sedelnikova *et al*, 2004). We analysed intestinal sections from 9-month-old mice of the relevant genotypes and we measured the percentage of γ-H2AX-positive epithelial cells (Fig 3A,B). Telomerase-null (Terc-G3$^{-/-}$;p53$^{+/+}$) mice showed a significant increase in the percentage of γ-H2AX-positive cells compared with telomerase-proficient (Terc$^{+/+}$;p53$^{+/+}$) mice, thus validating the use of this marker as a measurement of telomere-derived damage. Importantly, telomerase-null mice carrying an extra copy of p53 (Terc-G3$^{-/-}$;p53$^{+/+;tg/•}$) had levels of γ-H2AX-positive cells similar to those in telomerase-proficient (Terc$^{+/+}$;p53$^{+/+}$ and Terc$^{+/+}$;p53$^{+/+;tg/•}$) mice (Fig 3B). These observations were corroborated

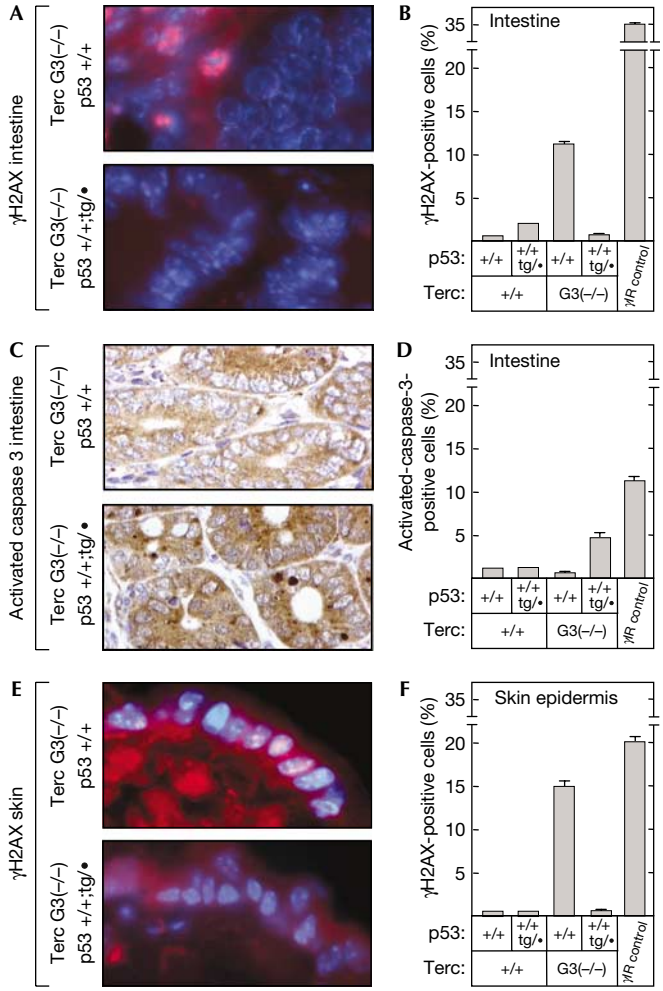

Fig 3 | Super-p53 mice with short telomeres have reduced levels of DNA-damaged cells and present higher levels of apoptosis. (**A**) Illustrative example of cryostat sections of the small intestine from 9-month-old mice of the indicated genotypes assayed for the presence of γ-H2AX foci. (**B**) Quantification of the percentage of γ-H2AX-positive cells. (**C**) Illustrative example of paraffin-embedded sections of the small intestine from 9-month-old mice of the indicated genotypes assayed for the presence of active caspase 3. (**D**) Quantification of the percentage of active caspase 3-positive cells. (**E**) Illustrative example of cryostat sections of the skin from 9-month-old mice of the indicated genotypes assayed for the presence of γ-H2AX foci. (**F**) Quantification of the percentage of γ-H2AX-positive cells. Quantifications shown in (**B**,**D**,**F**) correspond to the average ± s.d. of two mice. As a positive control, two wild-type mice were γ-irradiated with 10 Gy and killed 3 h after irradiation.

in epidermal cells (Fig 3E,F), thus confirming and extending the role of p53 in eliminating telomere-damaged cells. The cells of the intestinal epithelium are prone to undergo apoptosis on infliction of damage. A prediction from the above observations is that the number of apoptotic cells should be increased in super-p53/telomerase-null (Terc-G3$^{-/-}$;p53$^{+/+;tg/•}$) mice compared with normal-p53/telomerase-null (Terc-G3$^{-/-}$;p53$^{+/+}$) mice. Indeed, the levels of activated caspase 3 in the intestine of Terc-G3$^{-/-}$;p53$^{+/+;tg/•}$ mice were significantly higher than those in Terc-G3$^{-/-}$;p53$^{+/+}$ mice (Fig 3C,D), thus providing a mechanistic explanation for the decreased number of γ-H2AX-positive cells in super-p53/telomerase-null mice. Together, our data on splenocytes, intestinal epithelium and epidermis indicate that an increment of p53 function mitigates the accumulation of telomere-derived DNA damage in the organism.

p53 has no impact on telomere-driven ageing

The biological end point of telomere dysfunction in somatic cells is a generalized proliferative defect culminating in diverse symptoms of tissue atrophy that become particularly evident in highly proliferative tissues such as the intestine, spleen and testis (Lee *et al*, 1998; Herrera *et al*, 1999; Rudolph *et al*, 1999). Histopathological analysis of moribund mice of successive generations (G1–G3) of telomerase-deficient wild-type and super-p53 genotypes showed essentially the same incidence and spectrum of degenerative pathologies (Table 2; a complete histopathological analysis is available as supplementary information online). The only exception to this was the incidence of testicular atrophies, which were moderately, but significantly, increased in the case of old super-p53 mice in a telomerase-proficient background. This effect of the p53 transgene was not noticeable in telomerase-deficient mice, probably owing to the dominant effect of telomerase deficiency, which results in high incidence of testicular atrophies (Table 2). Together, these data indicate that increased p53 function does not have an appreciable impact on the tissue renewal defects associated to telomere dysfunction.

Finally, we measured the effect of increased p53 function on the lifespan in the context of telomere-driven ageing. Observation of telomerase-null cohorts for more than two and half years confirmed, as described in previous work (Herrera *et al*, 1999; Espejel *et al*, 2002a,b), that progressive telomere shortening along successive generations (G1–G3) was paralleled by a corresponding progressive decrease in lifespan (Fig 4). Also, in the case of telomerase-proficient mice, we confirmed that increased p53 gene dosage has no effect on lifespan (Fig 4; Garcia-Cao *et al*, 2002). Remarkably, the gene dose of p53 did not affect the lifespan of the different mouse colonies at increasing generations in the absence of telomerase (Fig 4). Regarding the impact of p53 on spontaneous tumour development, super-p53 mice have a significantly lower incidence of spontaneous malignant tumours (supplementary Table 2 online; Garcia-Cao *et al*, 2002). In this regard, it should be noted that the lower incidence of malignant tumours is nonetheless insufficient to extend the lifespan, suggesting that spontaneous malignancies are probably not the primary cause of death in these aged mice. In the case of telomerase-deficient mice, the incidence of spontaneous carcinomas in our colonies was too low to detect significant effects (supplementary Table 2 online). In summary, we conclude that extra p53 activity does not accelerate telomere-driven atrophies or ageing.

DISCUSSION

Here, we provide evidence that super-p53 mice have an enhanced response to telomere dysfunction. We have observed that late-generation super-p53/telomerase-null MEFs express higher protein levels of p53 and p21 than their normal-p53/telomerase-null counterparts. This observation reinforces the concept that p53 is a sensor of telomere damage (Introduction). An important issue of relevance for ageing is whether the activation of p53 in

Table 2 | Increased p53 function does not affect atrophic lesions associated with telomere-driven ageing*

	Terc genotype							
	Terc(+/+)		G1 Terc(−/−)		G2 Terc(−/−)		G3 Terc(−/−)	
	p53 genotype							
Atrophic tissue	(+/+)	(+/+; tg/•)	(+/+)	(+/+; tg/•)	(+/+)	(+/+; tg/•)	(+/+)	(+/+; tg/•)
Spleen	5/32 (16%)	11/65 (17%)	3/9 (33%)	1/9 (11%)	4/15 (27%)	4/15 (27%)	5/12 (42%)	3/14 (21%)
Intestine	0/32 (0%)	0/65 (0%)	7/9 (78%)	5/9 (56%)	13/15 (87%)	13/15 (87%)	10/12 (83%)	12/14 (86%)
Testis	0/18 (0%)	5/29† (17%)	2/4 (50%)	4/5 (80%)	6/8 (75%)	8/11 (73%)	5/6 (83%)	6/9 (67%)
Ovary	0/14 (0%)	7/37 (19%)	1/5 (20%)	0/4 (0%)	1/7 (14%)	0/4 (0%)	1/6 (17%)	2/5 (40%)

*Mice of the indicated genotype were killed when they showed signs of poor health, such as reduced activity or cachexia, and subjected to exhaustive histopathological analysis. The table shows data only for those tissues that presented signs of atrophy with high frequency (for more details, see the supplementary information online).
†$P<0.05$ versus wild-type controls (Fisher's exact test).

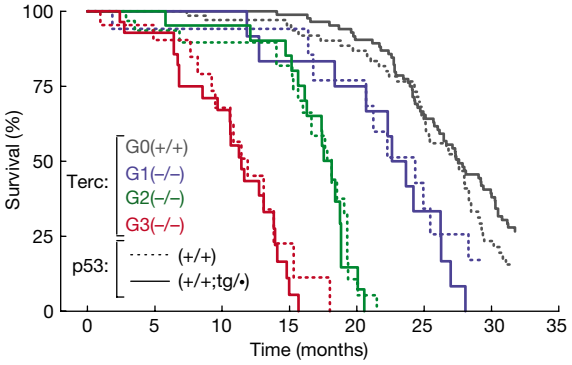

Fig 4 | Increased p53 function does not affect telomere-driven ageing. Cohorts of successive generations of telomerase-deficient mice (G0, black lines; G1, blue lines; G2, green lines; G3, red lines) either wild-type for p53 (p53(+/+), dashed lines) or super-p53 (p53(+/+;tg/•), solid lines) were followed up for a period of 32 months. The figure shows a Kaplan–Meier representation of the survival of the following groups of mice: G0-Terc(+/+)p53(+/+), $n=68$; G0-Terc(+/+)p53(+/+;tg/•), $n=105$; G1-Terc(−/−)p53(+/+), $n=17$; G1-Terc(−/−)p53(+/+;tg/•), $n=15$; G2-Terc(−/−)p53(+/+), $n=31$; G2-Terc(−/−)p53(+/+;tg/•), $n=21$; G3-Terc(−/−) p53(+/+), $n=22$; G3-Terc(−/−)p53(+/+;tg/•), $n=28$.

response to telomere damage results in the elimination of damaged cells or, alternatively, in the accumulation of damaged cells. We have observed that increased p53 function correlates with reduced presence of damaged cells in the spleen, intestine and skin, while at the same time we could demonstrate an increased apoptosis in the intestine. Together, these data demonstrate that, in the context of telomere dysfunction, the primary consequence of enhancing p53 function is a more efficient elimination of telomere-damaged cells.

We have previously shown that ageing in telomerase-proficient mice is not affected by increasing either the number of p53 alleles (Garcia-Cao *et al*, 2002) or the number of alleles of ARF, a p53 activator (Matheu *et al*, 2004). In the present work, we have further challenged the putative impact of p53 on ageing by focusing on telomere-driven ageing, thus minimizing the contribution of other types of damage that could operate in the case of spontaneous ageing. Importantly, in the absence of telomerase, super-p53 mice aged with the same kinetics and pathological spectrum as p53 wild-type mice. These results, together with our previous data on super-p53 mice and recent data on mice with extra ARF or hypomorphic Mdm2, negate a role for p53 in ageing, both spontaneous and telomere driven (Garcia-Cao *et al*, 2002; Matheu *et al*, 2004; Mendrysa *et al*, 2006). Although increased gene dosage of p53 has no impact on telomere-driven ageing, it is important to note that it has a detectable impact, decreasing the load of telomere-derived damage. This suggests that the reduction in damaged cells, although significant at a cellular level, is not of sufficient magnitude to delay the multi-organ failure characteristic of ageing. Finally, it must be emphasized that our data are not in conflict with the fact that aberrant, non-regulated, p53 activity could accelerate ageing. Indeed, mouse models vastly over-expressing p53 or carrying truncated forms of p53 present severe organ atrophies (Nakamura *et al*, 1995; Godley *et al*, 1996) or premature ageing (Tyner *et al*, 2002; Maier *et al*, 2004).

In summary, we demonstrate here that p53 has an active role in eliminating telomere-damaged cells *in vivo*, and that moderately increased levels of regulated p53 do not affect telomere-driven ageing.

METHODS

Mice and primary mouse cells. The generation of super-p53 mice carrying one ($p53^{+/+;tg/•}$) or two ($p53^{+/+;tg/tg}$) extra copies of p53 has been described previously (Garcia-Cao *et al*, 2002). Successive generations of telomerase-proficient and telomerase-deficient mice were generated by crosses of super-p53 ($p53^{+/+;\ tg/•}$) mice with a mouse strain deficient for the RNA component of telomerase ($Terc^{-/-}$; Blasco *et al*, 1997). From these crosses, the two basic genotypes compared in this study were obtained: $Terc^{-/-}$;$p53^{+/+;tg/•}$ and $Terc^{-/-}$;$p53^{+/+}$. Their genetic background was largely (>75%) C57BL6. Mice were housed in a pathogen-free barrier area. On signs of morbidity, mice were killed in accordance with the Guidelines for Humane Endpoints for Animals Used in Biomedical Research. Primary MEFs were prepared from day 13.5 embryos, as previously described (Palmero & Serrano, 2001). Cells were cultured under atmospheric oxygen pressure in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal

bovine serum (Sigma) and 1% penicillin G/streptomycin sulphate (Sigma).

Immunoblots. Immunoblots were carried out according to standard procedures. The antibodies used were as follows: for detection of p53, monoclonal antibody PAb240 (Calbiochem); for p21, polyclonal antibody C-19 (Santa Cruz). Secondary antibodies were either horseradish peroxidase-linked anti-rabbit (Amersham) or anti-mouse (DAKO). Detection was carried out by chemiluminescence using the ECL detection system (Amersham).

Chromosome analysis. Metaphase spreads from MEFs prepared from littermate embryos were scored for end-to-end fusions by superimposing the telomere image (quantitative-FISH (Q-FISH)) on the chromosome image (4,6-diamidino-2-phenylindole (DAPI)) using the TFL-telo software as described (Samper *et al*, 2000). Telomere fluorescence was quantified using the TFL-telo software kindly provided by Peter Lansdorp (British Columbia Cancer Center, Vancouver, Canada). For Q-FISH analysis of spleen sections, whole spleens were fixed in 10% formaldehyde for 2 h and paraffin embedded following standard methods. Spleen sections were hybridized with a PNA-tel probe and the telomere signals in meiotic nuclei were captured at $\times 100$ magnification and analysed using the TFL-telo program.

Immunohistochemical analysis. Histological sections were carried out according to standard procedures. Apoptotic cells were identified using monoclonal antibodies against the active form of caspase 3 (AF835, R&D Systems) and visualized using peroxidase-coupled secondary antibodies and 3,3′-diaminobenzidine tetrahydrochloride as chromogen. Nuclei were counterstained with haematoxylin. Photomicrographs were obtained with a Leica DM LB microscope.

For the detection of γ-H2AX-positive cells in intact tissue, OCT-embedded cryostat sections of small intestine (6 μm thick) were placed on slides, air-dried at ambient temperature and fixed in acetone for 2 min. Slides were then air-dried, permeabilized with 0.1% Triton X-100 and blocked for 45 min at 37 °C with 5% BSA. Subsequently, samples were incubated for 1 h with an anti-phospho-histone H2AX monoclonal antibody (1:500 in 2% BSA; Upstate Biotechnology), rinsed with PBS and incubated with a Cy3-labelled secondary antibody (Molecular Probes) according to standard procedures. Nuclei were counterstained with DAPI. Slides were mounted with coverslips using Vectashield/DAPI (Vector Laboratories) and examined under a fluorescence microscope (Leitz DMRB; Leica).

Quantifications were carried out, in a blind manner, by one of the authors (A.T.-L.). A minimum of 200 epithelial cells, intestinal or epidermal, were counted per mouse, distributed in at least two randomly chosen microscope fields.

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

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