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SUPPLEMENTARY TABLES

Table S1. Delayed aging of s-Arf/p53 mice.

|                                 | all mice     | wild type       | s-p53          | s-Arf          | s-Arf/p53       |
|                                 | average lifespan ± S.E.M. | 113.8 ± 2.4 | 116.7 ± 2.2 (p = 0.87) | 117.0 ± 4.3 (p = 0.478) | 130.7 ± 4.5 (p = 0.008) |
|                                 | median lifespan | 117.9         | 117.9          | 125.7          | 136.6 (+16%)    |

|                                 | cancer-free mice | wild type       | s-p53          | s-Arf          | s-Arf/p53       |
|                                 | mean lifespan ± S.E.M. | 107.8 ± 4.4 | 118.0 ± 3.1 (p = 0.145) | 124.8 ± 5.4 (p = 0.021) | 135.1 ± 4.5 (p = 0.002) |
|                                 | median lifespan | 110.3        | 118.7          | 133.3          | 137.4 (+25%)    |

Data for mean lifespan are the arithmetic mean of the lifespan in weeks of individual mice. Median lifespan corresponds to the lifespan that divides the population of data in two equal parts. The number of mice in the top part are: wt, n=104; s-p53, n=89; s-Arf, n=52; s-Arf/p53, n=17. The number of mice in the bottom part are: wt, n=49; s-p53, n=47; s-Arf, n=27; s-Arf/p53, n=14. Statistical significance was assessed using the Student’s t-test. Note that these n values refer to the actual number of mice dying of ageing or cancer (valid deaths). These numbers are slightly lower than the n values of Fig. 2A and S3 because not all the mice used in the Kaplan-Meier curves have reached the valid endpoint of death by ageing or cancer (some were still alive at the time of writing this report and others were censored because were used for experimentation).
Table S2. Differential loss of Arf or p53 in spontaneously immortalized MEFs

<table>
<thead>
<tr>
<th>genotype of parental cultures (number of independent cultures)</th>
<th>loss of Arf b</th>
<th>loss of p53 b</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type (n=52)</td>
<td>55 %</td>
<td>45 %</td>
</tr>
<tr>
<td>super-Arf (n=21)</td>
<td>5 %</td>
<td>95 %</td>
</tr>
<tr>
<td>super-p53 (n=10)</td>
<td>100 %</td>
<td>0 %</td>
</tr>
<tr>
<td>super-Arf/p53 (n=9)</td>
<td>66 %</td>
<td>33 %</td>
</tr>
</tbody>
</table>

a Cultures were passaged according to the 3T3 protocol. After reaching senescence, cultures were maintained until signs of growth and then were passaged again according to the 3T3 protocol. Fully immortalized cultures were analyzed for the status of Arf and p53.

b The status of Arf was determined by immunoblot and the status of p53 was determined by immunoblot before and after treatment of the cells with doxorubicin.

SUPPLEMENTARY FIGURES

Figure S1. Hypothesis. Cellular damage is thought to be at the origin of both cancer and aging. The Arf/p53 module is well known as a tumor suppressor because of its ability to detect cellular damage, hence its anti-cancer activity. The hypothesis tested in the present work consists in the possibility that Arf/p53 could also impact on the cellular damage that causes aging. If this is the case, the Arf/p53 module simultaneously provides anti-cancer and anti-aging activity.
Figure S2. Increased gene dosage of Arf and p53 translates into increased levels of Ink4b, Arf, Ink4a, p53 and p21.

(A) Liver samples (n = 4-7 for each age group and genotype) were analyzed for the expression of Ink4b, Arf and Ink4a by quantitative real-time PCR. PCR data were normalized to β-actin expression and are expressed relative to gene expression levels in young wt mice. Values correspond to the mean of 2^ΔCt values relative to young wt mice. Error bars represent the relative S.D. of the Ct values. Student’s t test was performed with the ΔΔCt values (***, p<0.001).
(B) Protein extracts from early-passage MEFs of the indicated genotypes were used for immunoprecipitation against p53, followed by immunoblot against p53 (see Materials and Methods). As a loading control, 10 μg of the total extracts used for the immunoprecipitation were immunoblotted against actin. Quantification by densitometry is shown in the graph at the bottom.

(C) Spleen samples (total protein extracts) from 2 month old mice of the indicated genotypes were assayed for protein levels of p53, p21 and β-actin by Western blot. The presence of non-specific cross-reacting band is indicated with an asterisk. Quantification by densitometry is shown in the graph at the bottom. Values are relative to wt and correspond to the mean ± S.E.M.

Figure S3. Effect of increased gene dosage of Arf and p53 on tumor spectrum. Wild type (n=96), s-p53 (n=77), s-Arf (n=42) or s-Arf/p53 (n=15) mice were sacrificed when they showed overt signs of poor health, such as reduced activity or dramatic weight loss, and analyzed for the presence of malignancies. Data are given as the percentage of mice that presented malignant tumors, which were grouped as lymphomas, histiocytic sarcomas, hemangiosarcomas, and carcinomas. Benign tumors are not considered. Statistical significance was calculated using the Fisher’s Exact test relative to wt (#, p<0.1; *, p<0.05; ***, p<0.001).
Figure S4. Effect of increased gene dosage of Arf or p53 on lifespan.

(A) Cohorts of mice of the indicated genotypes were followed up for a period of 175 weeks. The figure shows a Kaplan-Meier representation of the survival of the indicated mouse strains.

(B) The survival curves of the same mouse cohorts shown in panel (A) were redrawn after excluding those animals that had developed malignant tumors at the time of death. The statistical significance of the differences was assessed using the logrank test. For additional analyses see Table S1. In addition, it is worth to mention that the survival (total and cancer-free) of s-Arf/p53 mice was significantly different from s-p53 mice (p < 0.01), but was not significantly different from s-Arf mice (p > 0.05).
Figure S5. Effect of increased gene dosage of Arf and p53 on the incidence of aging-related pathologies.

Wild type (n=96), s-p53 (n=77), s-Arf (n=42) or s-Arf/p53 (n=15) mice were sacrificed when they showed overt signs of poor health, such as reduced activity or dramatic weight loss, and subjected to a detailed histopathological analysis. Pathologies were grouped in the indicated classes. Statistical significance was calculated using the Fisher’s Exact test (#, p<0.1; *, p<0.05; ***, p<0.001).
Figure S6. Effect of increased gene dosage of Arf and p53 on the onset of aging-related pathologies.
Values correspond to the average onset time of the pathologies shown in Fig. S5. Data are mean values ± S.E.M., statistical significance was calculated using the Student’s t test (#, p<0.1; *, p<0.05; **, p<0.01; ***, p<0.001).
Figure S7. Example of the determination of γ-H2AX-positive cells in liver cryosections of old (>24 mo.) mice.
Figure S8. Body weight, metabolism, IGF-1 levels and telomere length in s-Arf/p53 mice.

(A) Body weight of male and female mice was determined at the indicated time points and is given as mean value ± S.E.M. Student’s t test was used to show that there was no significant (p>0.05) difference in body weight between wild-type and s-Arf/p53 mice. The number of mice weighted are as follows. For female wt, the number of mice in
each group was 11 (5 weeks) and 12 (8, 12, and 15 weeks), 3 (0.5 to 1 years) and 10 (>2 years). For female s-Arf/p53, the number of mice in each group was 7 (5 weeks), 4 (8 weeks), 6 (12 weeks), 3 (15 weeks), 3 (0.5 to 1 years) and 4 (>2 years). For male wt, the number of mice was 7 (5 weeks), 8 (8 weeks), 9 (12 and 15 weeks), 3 (0.5 to 1 years) and 21 (>2 years). For male s-Arf/p53, the number of mice in each group was 5 (5 weeks), 4 (8 weeks), 3 (12 weeks), 2 (15 weeks), 3 (0.5 to 1 years) and 12 (>2 years).

(B) Wild type (n=14) and s-Arf/p53 (n=4) mice were housed in metabolic cages to determine intake of food and water, as well as, output of faeces and urine. Data are mean value ± S.E.M., Student’s t test was used to show that there was no significant (p>0.05) difference in these metabolic parameters between wt and s-Arf/p53 mice.

(C) Serum levels of IGF-1 in retro-orbital cavity blood from aged (≥24 months) wild type (n=19) and s-Arf/p53 (n=11) mice were determined by a competitive enzyme immunoassay. Data are mean value ± S.E.M., Student’s t test was used to show lack of significant (p>0.05) difference between wt and s-Arf/p53 mice.

(D) Telomere length was determined by Q-FISH analysis of interphase nuclei in cryosections from livers from old (≥24 months) wild type (n=3) or s-Arf/p53 (n=5) mice. The total number of nuclei analyzed wt and s-Arf/p53 nuclei was 4797 and 6108, respectively. Data are given as arbitrary units of average telomere fluorescence per nucleus and are mean values ± S.E.M., Student’s t test was used to show lack of significant (p>0.05) difference between wt and s-Arf/p53 mice.

Figure S9. Examples of the determination of ROS in splenocytes by DCF fluorescence detected by FACS.
Figure S10. Example of the determination of malondialdehyde (MDA) in liver extracts by HPLC.

Figure S11. Example of the determination of protein carbonyl groups in total proteins from liver using the “OxyBlot” protein oxidation kit (Intergen). The example corresponds to young (3 mo.) mice. The “OxyBlot” is shown at the left and the same gel stained with Ponceau S is at the right. Photographs of the gels were scanned and used for quantification.
Figure S12. Expression of p66Shc and Sirt1 in the liver of young (3 mo.) mice (n=4) determined by qRT-PCR. Values correspond to the mean of $2^{\Delta\Delta C_t}$ values relative to wt mice. Error bars represent the relative S.D. of the $\Delta C_t$ values. Student’s t test was performed with the $\Delta C_t$ values and indicated that the differences were not significant.

Figure S13. Effect of aging on the expression of the indicated genes in the liver of wt mice (n=4) determined by qRT-PCR. Values correspond to the mean of $2^{\Delta C_t}$ values relative to young mice. Error bars represent the relative S.D. of the $\Delta C_t$ values. Student’s t test for each age group compared to young mice was performed with the $\Delta C_t$ values (***, p<0.001).
Figure S14. Examples of the determination of reduced glutathione (GSH) in liver extracts by HPLC. Every sample was spiked with an internal calibration standard consisting in γ-glutamyl-glutamate.

Figure S15. Brain levels of reduced glutathione. Reduced glutathione (GSH) was determined by high-performance liquid chromatography in brain samples. The number of samples used for young mice were: wild type (n=4), s-p53 (n=3), s-Arf (n=4) or s-Arf/p53 (n=4); and for old mice: wild type (n=5), s-p53 (n=1), s-Arf (n=3) or s-Arf/p53 (n=9). Values are expressed relative to the corresponding wt value. Data are mean values ± S.E.M., Student’s t test for each age group is relative to wt (#, p<0.1; *, p<0.05).