Sirt1 improves healthy ageing and protects from metabolic syndrome-associated cancer

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Genetic overexpression of protein deacetylase Sir2 increases longevity in a variety of lower organisms, and this has prompted interest in the effects of its closest mammalian homologue, Sirt1, on ageing and cancer. We have generated transgenic mice moderately overexpressing Sirt1 under its own regulatory elements (Sirt1-tg). Old Sirt1-tg mice present lower levels of DNA damage, decreased expression of the ageing-associated gene p16INK4a, a better general health and fewer spontaneous carcinomas and sarcomas. These effects, however, were not sufficiently potent to affect longevity. To further extend these observations, we developed a metabolic syndrome-associated liver cancer model in which wild-type mice develop multiple carcinomas. Sirt1-tg mice show a reduced susceptibility to liver cancer and exhibit improved hepatic protection from both DNA damage and metabolic damage. Together, these results provide direct proof of the anti-ageing activity of Sirt1 in mammals and of its tumour suppression activity in ageing- and metabolic syndrome-associated cancer.

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verexpression of protein deacetylase Sir2 in yeasts, flies and worms has the remarkable effect of extending longevity\textsuperscript{12}. Mammals have evolved a family of Sir2-related proteins known as sirtuins and composed of seven members (Sirt1-7), of which Sirt1 is the closest homologue to Sir2\textsuperscript{2}. However, little is known at present about the impact of mammalian Sirt1 on ageing\textsuperscript{3}. Regarding cancer, available genetic evidence supports a tumour-suppressor role of Sirt1\textsuperscript{4}. In particular, decreased Sirt1 activity accelerated the tumour-prone phenotype of p53-heterozygous mice\textsuperscript{5} and, reciprocally, overexpression of Sirt1 in lymphocytes delayed the development of radiation-induced lymphomas in p53-heterozygous mice\textsuperscript{6}. Moreover, Sirt1 binds and deacetylates \( \beta \)-catenin, thus cancelling its oncogenic transcriptional activity and decreasing intestinal tumour development\textsuperscript{7}. Although the above results are of obvious importance, the impact of systemic Sirt1 upregulation on ageing and on ageing-associated cancer remains to be determined. This is particularly relevant given the ongoing efforts to develop drugs that stimulate Sirt1 activity, including resveratrol and SRT1720\textsuperscript{8,9}. Regarding these latter compounds, it is controversial whether they activate Sirt1 directly\textsuperscript{10,11,12}. Alternatively, resveratrol may upregulate Sirt1 through indirect mechanisms that could involve AMPK\textsuperscript{13,14}.

It has been previously reported by others and us that transgenic mice systemically overexpressing Sirt1 (\(-3\)-fold) are protected from the physiological damage produced by a high-fat diet (HFD)\textsuperscript{15,16}. These observations are further reinforced by the phenotype of mice deficient in Dbc1, a negative regulator of Sirt1, which also shows systemic activation of Sirt1 and protection from HFD-induced damage\textsuperscript{17}. At a molecular level, protection against HFD has been reported to reflect the activity of Sirt1 as a negative regulator of nuclear factor-\( \kappa \)B (NF-\( \kappa \)B)\textsuperscript{18,19} and as a positive effector of PGC1\( \alpha \)\textsuperscript{20} and FoxO1\textsuperscript{21}. Chronic exposure to high levels of dietary fat results in a multi-systemic deterioration known as metabolic syndrome, which is characterized by insulin resistance, liver steatosis, atherogenic cardiovascular disease, dyslipidaemia and systemic inflammation, which may lead to fatal diseases such as liver cancer and heart failure\textsuperscript{22,23}. Given the prevalence of metabolic syndrome in the adult and aged human population\textsuperscript{24}, it is of interest to determine the impact of Sirt1 upregulation on metabolic syndrome-associated cancer.

In this study, we examine the ageing and longevity of Sirt1-tg mice, as well as their susceptibility to spontaneous cancer and to liver cancer associated with metabolic syndrome. Our results indicate that Sirt1 exerts antiageing activity in mammals, although this effect is not sufficiently potent to extend longevity, at least at the levels of overexpression achieved in our mice. Sirt1-tg mice are protected from the development of ageing-associated diseases such as glucose intolerance, osteoporosis and cancer. Moreover, Sirt1 provides a strong protection against liver cancer development in the context of metabolic syndrome.

**Results**

**Normal longevity and decreased spontaneous cancer.** To evaluate the impact of Sirt1 on mammalian ageing, we analysed two independent lines of Sirt1 transgenic mice carrying different, but overlapping genomic DNA segments (Supplementary Fig. 1a). These transgenics, named hereafter Sirt1-tgA\textsuperscript{1} and Sirt1-tgB, overexpress \(-3\)-fold Sirt1 mRNA across all tissues tested (Supplementary Fig. 1b), and present detectably higher levels of Sirt1 protein (Supplementary Fig. S2a–c), as well as an increase in hepatic Sirt1 activity measured by the lower levels of acetylated lysine 310 of p65RelA (Supplementary Fig. S2c), a known target of Sirt1 deacetylase activity\textsuperscript{24}. In agreement with their similar levels of Sirt1 upregulation, both transgenic lines displayed similar phenotypes regarding protection from HFD, such as, decreased adipose tissue inflammation (Supplementary Fig. S3a), improved glucose tolerance (Supplementary Fig. S3b and ref. 15), and protection from hepatic steatosis (Supplementary Fig. S3c and ref. 15), as well as increased sensitivity to lipopolysaccharide shock under standard diet (Supplementary Fig. S4 and ref. 15).

To determine the impact of Sirt1 upregulation on longevity, we followed up cohorts of the two lines, together with their corresponding wild-type (WT) controls. All cohorts had the same uniform but hybrid genetic background (C57BL6/CBA 87.5:12.5\%). The corresponding survival curves are represented by either separating the two lines or pooling them together. Pooling allowed us to achieve a cohort size per genotype and sex of \( n > 40 \), which provides sufficient statistical power to reliably detect differences greater than 10\% in mean lifespan\textsuperscript{25}. The survival curves of Sirt1-tg (A + B) and WT (A + B) were indistinguishable for both male and female mice (Fig. 1a). When male and female mice were grouped together, the number of WT mice and tg mice was \( n > 100 \) and, again, no differences were detected in survival (Fig. 1a). Moreover, no significant differences were found between Sirt1-tg mouse and their corresponding WT controls when each line was analyzed separately (Fig. 1a). We conclude that systemic threefold upregulation of Sirt1 has no detectable impact, neither detrimental nor beneficial, on mouse longevity.

Detailed histopathological analyses of old moribund mice revealed a lower incidence of malignant tumours in Sirt1-tg mice, compared with WT mice of both lines (Fig. 1b). Remarkably, the suppressive effect of Sirt1 on malignant tumours was restricted to carcinomas and sarcomas (mostly osteosarcomas), whereas it had no effect on the incidence of lymphomas and histiocytic lymphomas (the latter also known as histiocytic sarcomas) (Fig. 1c). It is important to mention that most spontaneous carcinomas and sarcomas were in general of small size (as was the case of liver or lung carcinomas) or affected non-vital organs (such as Harderian gland carcinomas in the eyes or osteosarcomas in the limbs). Therefore, despite their malignancy, most carcinomas and sarcomas were at a stage of dissemination that is unlikely to be the primary cause of death. This is in contrast to spontaneous lymphomas, which had similar incidences in WT and tg mice, and were highly aggressive and the likely cause of death. In summary, these results indicate that Sirt1 upregulation provides protection from spontaneous carcinomas and sarcomas, but not from spontaneous lymphomas. Conceivably, the lack of effect of Sirt1 on spontaneous lymphomas could mask a putative effect of Sirt1 on longevity.

**Improved healthy ageing.** To examine the impact of Sirt1 on ageing, we began by examining a physiological parameter related to metabolic syndrome, namely, ageing-associated glucose intolerance\textsuperscript{26}. Young (3 months old) WT and Sirt1-tg mice under a standard diet showed similar performance in glucose-tolerance tests\textsuperscript{27,28} (see also Supplementary Fig. S3b). Interestingly, at 1.5 years of age, WT mice already showed evidence of glucose intolerance, whereas transgenic Sirt1 mice preserved a significantly better glucose uptake (Fig. 2a). These results indicate that Sirt1 confers protection against ageing-associated metabolic damage under standard diet and, together with previous data on mice under an HFD\textsuperscript{15–17}, reinforce and extend the concept that Sirt1 is a general protector against metabolic damage.

Another common feature of ageing is the development of osteoporosis, a process that can be promoted by NF-\( \kappa \)B\textsuperscript{29,30} the activity of which is increased during ageing\textsuperscript{31}. In agreement with the reported ability of Sirt1 to inhibit NF-\( \kappa \)B\textsuperscript{15,17,32}, we observed that Sirt1-tg mice preserved normal bone density values at old age (2.5 years), whereas WT mice showed a significant decline in bone density at this age (Fig. 2b). Additional indicators of fitness at old age were obtained by measuring skin regeneration (wound-healing assay, Fig. 2c) and neuromuscular coordination (tightrope assay, Fig. 2d), both of which suggested a better performance by Sirt1-tgA mice (no effect at this level was detected in Sirt1-tgB mice).

To evaluate ageing at a molecular level, we measured two well-established markers of ageing, namely, the levels of p16\textsuperscript{INK4a} mRNA\textsuperscript{33} and the accumulation of nuclear foci of DNA damage proteins\textsuperscript{28,29}. 
In the case of p16 Ink4a, its transcript levels in liver increased >15-fold in old WT mice compared with young ones (2 years versus 3 months), whereas this increase was significantly attenuated to ~7-fold in Sirt1-tg animals (Fig. 2e). Moreover, the number of cells with 53BP1 DNA damage foci in the liver was reduced in old Sirt1-tg mice compared with WT mice of the same age (Fig. 2f). Collectively, the analysis of ageing-associated pathologies (glucose intolerance, osteoporosis, decreased wound healing, impaired neuromuscular coordination) and molecular markers of ageing (p16 Ink4a, DNA damage) support the concept that Sirt1 possesses anti-ageing activity in mammals.

Decreased metabolic syndrome-associated liver cancer. After analysing the effect of Sirt1 on health and cancer during ageing, we wanted to further extend the cancer studies to a model relevant for metabolic syndrome, a condition that affects up to one-quarter of the human population after middle age19,20. The array of diseases comprised by metabolic syndrome can be initiated by a high dietary intake and, in the case of liver, results in fatty liver, which can be followed by cirrhosis and finally cancer18,30. To model metabolic syndrome-associated cancer, we treated mice with a single injection of a hepatic-specific carcinogen, diethylnitrosamine (DEN), followed by continued exposure to HFD. To decrease inter-individual variation due to the hybrid genetic background, all the following assays were performed in mice that had been backcrossed for eight generations with C57BL6 mice and therefore were >99% C57BL6. Furthermore, it is relevant to note that DEN is a poor carcinogen on its own in mice of a C57BL6 genetic background31. In fact, most of our WT mice (6 of 11 = 55%) were completely free of liver tumours at 11 months after treatment with DEN under standard diet conditions. Importantly, all WT mice treated with DEN and under HFD for 11 months developed multiple tumours (on an average 10 tumours) that were quantified and measured in live mice by microcomputed tomography (microCT) (Fig. 3a). Notably, the corresponding Sirt1-tg littermate mice presented a significantly lower incidence and burden of liver tumours (Fig. 3a). On necropsy, the differences in tumour number and size between WT and Sirt1-tg livers were dramatic (Fig. 3b) and histological analyses confirmed that all tumours, both in WT and tg mice, corresponded to liver carcinomas (Fig. 3c). We questioned whether the strong protection observed against DEN/HFD-induced hepatocarcinogenesis was also present in other protocols of chemical carcinogenesis. However, when we induced fibrosarcomas with 3-methyl-cholanthrene (3MC), no differences were observed between WT and Sirt1-tg mice (Fig. 3d). This differential cancer protection between hepatocytes and fibroblasts correlates with the effect of Sirt1 on DNA damage in the same cell types (see below). We conclude that moderate upregulation of Sirt1 strongly suppresses metabolic syndrome-associated liver cancer.

Hepatic protection from carcinogenic damage. Sirt1-tg mice present a lower accumulation of ageing-associated DNA damage in the liver (see above Fig. 2f), and other investigators have found that Sirt1 contributes to maintain genomic stability4,15. On the basis of these observations, it can be hypothesized that Sirt1-mediated protection against DEN/HFD-induced liver cancer reflects a dual impact of Sirt1, decreasing the damage produced first by DEN and then by HFD. Although the protection of Sirt1 from HFD-induced damage is well substantiated15–17 (see also Supplementary Fig. S3), nothing is known about the impact of Sirt1 on DEN-induced liver damage. Treatment of mice with DEN under standard feeding conditions triggers a well-known cascade of events in the liver that include DNA damage and apoptosis of centrilobular hepatocytes, production of proinflammatory cytokines and compensatory proliferation32. Serum levels of alanine transaminase 48 h after
DEN injection already indicated a significant protection from liver injury in Sirt1-tg mice in standard diet (Fig. 4a). Immunohistological examination of livers 48 h after DEN showed intense nuclear staining of Sirt1 restricted to the centrilobular regions of the liver (around the central veins) and absent in the periportal regions (around the portal triad) (Fig. 4b and panoramic views in Supplementary Fig. S5a). Treatment of in vitro-cultured cells with genotoxic agents recruits Sirt1 to the chromatin6, and we questioned whether this also happens in vivo on treating with DEN. Indeed, we observed that DEN increased the amount of chromatin-bound Sirt1 in liver extracts, without detectably affecting the total amount of Sirt1 (Fig. 4c). After observing that Sirt1 actively responds to DEN by concentrating in the nucleus and stably binding to chromatin, we examined the impact of Sirt1-tg on the liver on treatment with DEN under standard feeding conditions. Livers from WT mice showed a strong DNA damage response in centrilobular regions, accompanied by high levels of apoptosis and compensatory proliferation (Fig. 4d and panoramic views in Supplementary Fig. S5b). Importantly, and in sharp contrast, DEN-treated Sirt1-tg mice showed significantly lower levels of damage, apoptosis and compensatory proliferation (Fig. 4d and panoramic views in Supplementary Fig. S5b). DEN is known to require bioactivation by cytochrome CYP2E134, and we asked whether Sirt1 could be exerting its protective effect by directly affecting DEN metabolism. To address this question, we measured CYP2E1 mRNA levels in control or DEN-treated livers. DEN treatment decreased CYP2E1 levels as previously reported34, but no differences in its levels could be observed between WT and Sirt1-tg mice, thus suggesting that Sirt1 does not affect DEN bioactivation (Fig. 4e). Next, we wondered whether Sirt1 protection against DNA damage was also detectable in fibroblasts; however, detailed kinetic analyses of γH2AX in individual cells upon treatment with neo-carzinostatin could not detect a better repair in Sirt1-tg compared with WT fibroblasts (Supplementary Fig. S6). Our assays suggest a cell-type specificity in the effects of Sirt1 on DNA damage, being more protective in hepatocytes than in fibroblasts. However, the assays and DNA damage agents used here preclude a direct comparison and this point remains to be clarified in future studies. Therefore, overexpression of Sirt1 at the levels achieved in our mouse lines (threefold) significantly protects hepatocytes, but not


**Discussion**

In this study, we provide direct genetic evidence for the anti-ageing activity of Sirt1 in mammals. We have found that moderate upregulation of Sirt1 expression (threefold) improves healthy ageing but not longevity. Importantly, old Sirt1-tg mice are partially protected from the development of pathologies typically associated with ageing, such as glucose intolerance, osteoporosis and poor wound healing. This improved maintenance of physiological fitness at old ages is accompanied by a decreased expression of molecular markers of ageing in liver, particularly p16 mRNA levels and nuclear foci of DNA damage proteins. However, the overall impact on ageing under standard ad libitum feeding conditions was not sufficiently potent to extend longevity. Given the beneficial impact of Sirt1 on ageing, it is tempting to speculate that attaining levels of Sirt1 activity higher than those achieved by us, either genetically or through the use of pharmacological activators, could lead to an extension of the lifespan. Moreover, considering that there are seven different sirtuins in mammalian organisms, it is also possible that several sirtuins must be targeted concurrently to produce lifespan extension.

An important observation derived from the analyses of old moribund Sirt1-tg mice is that they are partially protected against spontaneous carcinomas and sarcomas, but not from lymphomas. Other investigators have reported that Sirt1 protects against lymphomas in p53-heterozygous mice. It is conceivable that Sirt1 has a stronger protective effect against lymphomas with a high degree of genetic instability, such as those developed in p53-heterozygous mice, whereas, spontaneous lymphomas develop less aggressively late in life and are presumably less dependent on protection from acute DNA damage. In the case of spontaneous carcinomas, it is possible that Sirt1 could exert its protective effect through deacetylation and inhibition of β-catenin, which is an oncogene generally associated with epithelial cancers. Together, these factors could explain, at least in part, the differential effect of Sirt1 on ageing-associated carcinomas and sarcomas, versus lymphomas.

Caloric restriction (CR) is a well-known manipulation with the ability to delay ageing and increase lifespan. It has been proposed that the beneficial effects of CR in mice are mediated, at least in part, through Sirt1. In this regard, it is interesting to note that our Sirt1-tg mice present an improved glucose tolerance at old age, which is a characteristic feature of CR-treated mice. Further studies are necessary to address the similarities between Sirt1-tg mice under ad libitum conditions and CR-treated mice. In this context, it is also worth to point out the similarities between the phenotypes of our Sirt1-tg mice and the reported effects of the small natural compound resveratrol on mice, which upregulates Sirt1 activity, perhaps indirectly through effects mediated by AMPK. Mice chronically fed with resveratrol are protected from metabolic syndrome induced by HFD and present improved healthy ageing without affecting longevity. Interestingly, although resveratrol did not protect from spontaneous cancers, our Sirt1-tg mice were partially protected from these cancers.

The role of Sirt1 in protection against metabolic syndrome is solidly established. On this basis, we have developed a novel carcinogenic protocol to model metabolic syndrome-associated cancer. Metabolic syndrome leads over time to liver steatosis, followed by cirrhosis, which, in turn, is the main risk factor for the development of liver carcinoma. Specifically, this new experimental cancer model consists of a single injection of the hepatic-specific carcinogen DEN, followed by continued exposure to HFD. This protocol produced a 100% incidence of liver carcinomas in WT C57BL6 mice. Importantly, Sirt1-tg littermate mice were dramatically protected. We show here that the resistance to liver cancer in Sirt1-tg mice is due to a dual protective effect both from the initial acute damage elicited by the carcinogen (DEN) and from the chronic damage produced by HFD. In agreement with previous reports implicating Sirt1 in the maintenance of genomic stability, we demonstrate here that Sirt1-tg mice are protected from DEN-triggered DNA damage in hepatocytes in vivo. These results reinforce the association between metabolic syndrome and liver cancer, and demonstrate the critical role of Sirt1 in protection from these pathologies. In this context, it is of relevance to mention that recent analyses of Sirt1 expression in various human cancers have found decreased Sirt1 expression in liver cancer, but not in most other human malignancies. Together, our observations demonstrate that chronic and systemic upregulation of Sirt1 is beneficial for health and cancer.
**Note added in proof:** A similar liver cancer mouse model initiated by DEN and promoted by HFD has been reported while this work was under consideration (Park, E.J., Lee, J.H., Yu, G.-Y., He, G., Ali, S.R., Holzer, R.G., Osterreicher, C.H., Takahashi, H. & Karin, M. *Cell* 140, 197–208 (2010)).

**Methods**

**Animal experimentation.** The Sirt1-tgB line was generated following the same protocol as described for the generation of the Sirt1-tgA line. Both bacterial artificial chromosomes (BAC) were obtained from CHORI (identification numbers: RP23-119G23 for tgA line and RP24-306L15 for tgB line) and purified using the Large-Construct kit (Quiagen). For transgenesis, Sirt1-containing bacterial artificial chromosomes were digested with the restriction enzyme PI-SceI, thus linearizing them in the vector at a position adjacent to the T7 end of the genomic inserts. Pronuclei of fertilized oocytes, derived from intercrosses between (C57BL6×CBA)F1 mice, were injected with ~2 pl of a DNA solution (~1 ng ml⁻¹) containing linearized bacterial artificial chromosomes. The resulting offspring was analysed for the presence of transgenes using PCR reactions that amplify sequences from each BAC vector (primers tgA-F 5′-AGATAGTTCACCGGGGTGAGAA-3′; tgA-R 5′-TTCGGTCGAAGAGTATCTGGTG-3′; tgB-F 5′-ACTCTTTAACCGGCCCTTACAAG-3′; tgB-R 5′-TCCTGTGGATCTACCCACTAGTCA-3′).

All mice were housed at the serum pathogen-free barrier area of the Spanish National Cancer Research Center (CNIO), Madrid. Mice were treated in accordance with Spanish Laws and the Guidelines for Human Endpoints for Animals Used in Biomedical Research. Mice were observed on a daily basis and killed when they showed signs of morbidity or overt tumours. The genetic background of the mice used in the ageing assays was 87.5% C57BL6 and 12.5% CBA (after two backcrosses). For the rest of the experiments, >99% C57BL6 (after eight backcrosses).
mice were used. Mice were fed either with a standard chow diet (Teklad 2018) or with HFD (Research Diets D12451, 45% kJ from fat) when indicated.

For the glucose tolerance test, WT and tg mice on both diets were subjected to 6h of fasting and injected intraperitoneally (i.p.) with 2g kg⁻¹ glucose, and glucose levels in serum were determined using a Glucocard strip (A. Menarini Diagnostics).

For the wound-healing assay, wounds were made in the back skin using 4 mm biopsy punches, and healing was measured after 48h by two researchers blinded to the experiment, with the following semiquantitative scoring: 0 for well healed, dry and almost closed wounds; 2 for moist and overtly opened wounds; and 1 for intermediate ones. Osteoporosis was assessed in femurs of live mice on a microCT system (eXplore Vista PET/CT, GE Healthcare), analysing the values obtained from scans acquired with 40 μm resolution (100 μm slice thickness, 0.5 mm voxel size, 300 μm field of view, 120 kV, 156 μA). Tumour sizes were measured by their largest transversal area using eXplore Vista software.

**Mouse tumour models.** For the DEN/HFD-tumorigenesis model, 16-day-old male mice were injected with 5mg kg⁻¹ DEN, and then fed on HFD (see above) since weaning. Tumour detection and measurement were performed by microCT in living mice as described for the osteoporosis measurements, except that, in this case, mice were intravenously injected with the iodinated contrast agent Iopamiro (Bracco). Tumour sizes were measured by their largest transversal area using eXplore Vista software.

**Protein analyses.** For isolation of hepatocytes, livers were collected and sliced into small pieces with surgical blades and then incubated at 37°C for 30 min to separate cells from the liver tissue. Cells were then processed for Western blotting.

**Histological and immunohistochemical methods.** A detailed histopathological analysis of aged moribund mice was performed by one of the authors (Cañamero, a trained pathologist). Liver sections from DEN-treated mice were stained with the following antibodies: anti-Sirt1 (ab12193, Abcam, 1:2,000), anti-Actin (AC-15, Sigma, 1:5,000) and anti-β-H3 (ab791, Abcam, 1:1,000), anti-Ac(K310)-p65 (ab1791, Abcam, 1:1,000), anti-β-actin.

**Statistical analyses.** Kaplan–Meier curves were compared using the log-rank test. Cancer incidences were compared using Fisher’s exact test. All other measurements were compared using the two-tailed Student’s t-test.

**References**


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Author contributions
D.H. performed most of the experiments, contributed to data analysis, discussion and writing the paper; M.M.M. performed all the mouse manipulations; M.C. performed all pathological analyses; F.M. performed all the imaging analyses by microCT; B.M.-P. performed the DNA repair assays; O.F.C. designed and supervised the DNA damage data; M.S. designed and supervised the study, secured funding, analysed the data and wrote the paper. All authors discussed the results and commented on the paper.

Additional information
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