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Role of 2-5A-Dependent RNase-L in Senescence and Longevity

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Senescence is a permanent growth arrest that restricts the lifespan of primary cells in culture, and represents an *in vitro* model for aging. Senescence functions as a tumor suppressor mechanism that can be induced independent of replicative crisis by diverse stress stimuli. RNase-L mediates antiproliferative activities and functions as a tumor suppressor in prostate cancer, therefore, we examined a role for RNase-L in cellular senescence and aging. Ectopic expression of RNase-L induced a senescent morphology, a decrease in DNA synthesis, an increase in senescence-associated β-galactosidase activity, and accelerated replicative senescence. In contrast, senescence was retarded in RNase-L-null fibroblasts compared with wild-type fibroblasts. Activation of endogenous RNase-L by 2-5A transfection induced distinct senescent and apoptotic responses in parental and Simian virus 40-transformed WI38 fibroblasts, respectively, demonstrating cell type specific differences in the antiproliferative response to RNase-L activation. Replicative senescence is a model for *in vivo* aging; therefore, genetic disruption of senescence effectors may impact lifespan. RNase-L / mice survived 31.7% ($P<0.0001$) longer than strain-matched RNase-L$^{+/+}$ mice providing evidence for a physiological role for RNase-L in aging. These findings identify a novel role for RNase-L in senescence that may contribute to its tumor suppressive function and to the enhanced longevity of RNase-L / mice.

Keywords: RNase-L; senescence; apoptosis; 2′-5′-oligoadenylate; aging

Introduction

Eukaryotic cells have evolved mechanisms that block proliferation in response to genetic and epigenetic insults via growth arrest or cell death. These responses serve a common function, to prevent the introduction of potentially oncogenic mutations into the host genome, but exhibit distinct phenotypes including quiescence, senescence and apoptosis (Schmitt, 2003). Growth arrest can be either transient, in which quiescent cells retain the capacity to re-enter the cell cycle upon mitogenic stimulation, or permanent, in which cells enter a post-replicative but metabolically active state known as senescence. Senescence is a mechanism that restricts the replicative lifespan of primary cells in culture via telomere shortening, and represents an *in vitro* model for organismal aging (Hayflick, 1965) and reviewed by Campisi (2003) and Shay and Roninson (2004). Premature senescence can also be induced independent of replicative crisis by stimuli including DNA damage, oncogene expression and cell stress (Schmitt, 2003; Shay and Roninson, 2004). In contrast to quiescence and senescence in which cells remain viable, apoptosis results from the activation of genetically programmed catabolic pathways that lead to cell death. Importantly, identical insults can result in apoptosis or senescence depending on the cellular context (Schmitt, 2003; Shay and Roninson, 2004); this differential response may permit the development of therapeutic agents to selectively activate tumor-specific antiproliferative pathways.

Escape from constraints on cellular proliferation is a requisite step in malignant transformation; accordingly, apoptosis and senescence serve critical tumor suppressor functions. Although the antitumor function of apoptosis is well established, and has been exploited in therapeutic regimens, the role of senescence as a tumor suppressive mechanism *in vivo* has only recently been demonstrated. Specifically, studies in four independent systems determined that senescent cells were increased in premalignant lesions induced by oncogene activation, but were absent from malignant tumors that had escaped this proliferative block (Braig *et al*., 2005; Chen *et al*., 2005; Collado *et al*., 2005; Michaloglou *et al*., 2005). Consistent with a role for senescence in tumor suppression, the well-established tumor suppressors, p53 and

RB, and their upstream regulators (e.g. $p16^{INK4a}/p19^{ARF}$) and downstream effectors (e.g. $p21^{CIP}$, E2F) were identified as critical senescence mediators (Campisi, 2003). However, distinct profiles of gene expression are associated with senescence induced by different stimuli, and in specific cell types (Shelton *et al*., 1999; Yoon *et al*., 2004; Collado *et al*., 2005; Hardy *et al*., 2005), pointing out the need to better define the molecular components of the senescence response in specific physiological contexts.

Cytokines and growth factors function to maintain a balance between cell proliferation and growth arrest or apoptosis. Type 1 interferons (IFNα/β) were discovered as antiviral cytokines, that also induce potent antiproliferative and immunomodulatory activities. IFN induces a set of IFN-stimulated genes (ISGs) that mediate its biological activities (Stark *et al*., 1998). The 2-5A-dependent RNase-L pathway is among the best-characterized mediators of IFN action, and is comprised of two primary enzymatic activities (Silverman, 2003). A family of 2′-5′-oligoadenylate synthetases (OAS) are induced by IFN and, when activated by double-stranded RNA, polymerize adenosine triphosphate into, 2′-5′ linked, oligoadenylates (2-5A). 2-5A induces the dimerization and activation of a latent endoribonuclease, RNase-L, which cleaves single-stranded viral, messenger and ribosomal RNAs. RNase-L activity is attenuated by proteasomal degradation (Chase *et al*., 2003), 2-5A inactivation by cellular phosphatases and a 2′phosphodiesterase (Kubota *et al*., 2004), and by an RNase-L inhibitor, RLI (Bisbal *et al*., 1995). Experimental modulation of RNase-L expression and activity has confirmed its role in the antiviral, antiproliferative and proapoptotic activities of IFN (Hassel *et al*., 1993; Castelli *et al*., 1997; Zhou *et al*., 1997). RNase-L also exhibits antiproliferative effects independent of IFN, suggesting that it serves a broader role as a natural constraint on cell proliferation (Castelli *et al*., 1997; Zhou *et al*., 1997). Consistent with this prediction, a tumor suppressor function for RNase-L was recently discovered by mapping of the hereditary prostate cancer-1 susceptibility allele (*HPC1*) to the *RNASE-L* gene locus (Carpten *et al*., 2002), and by the association of RNase-L mutations with the disease (Casey *et al*., 2002).

Recent efforts to identify senescence associated genes have determined that the upregulation of several ISGs, including the RNase-L activating enzyme, OAS, correlates with the induction of a senescent phenotype, whereas ISG repression is associated with cellular immortalization (Shou *et al*., 2002; Untergasser *et al*., 2002; Leszczyniecka *et al*., 2003; Yoon *et al*., 2004). These findings suggested a role for ISGs in cellular senescence, however the functional involvement in senescence has been demonstrated for only a few specific ISGs (e.g. IFI16 (Xin *et al*., 2004)). In light of the established antiproliferative activities of RNase-L and its role as a tumor suppressor, we investigated the possible involvement of RNase-L in cellular senescence. RNase-L expression or activation induced a senescent phenotype in human diploid fibroblasts (HDFs) whereas senescence was retarded in the absence of RNase-L. Moreover, activation of RNase-L by exogenous 2-5A resulted in distinct senescent and apoptotic responses in primary and immortalized HDFs, respectively, indicating that cellular factors can dictate the antiproliferative phenotype induced by RNase-L activation. Interestingly, RNase-$L^{-/-}$ mice exhibited a significantly extended lifespan as compared to strain-matched RNase-$L^{+/+}$ mice. These findings identify RNase-L as a novel regulator of the senescence response that impacts longevity in mice.

Results

Ectopic expression of RNase-L results in reduced DNA synthesis

RNase-L functions in diverse antiproliferative processes associated with IFN action (Hassel *et al*., 1993), differentiation (Bisbal *et al*., 2000) and apoptosis (Castelli *et al*., 1997; Zhou *et al*., 1997). To characterize the antiproliferative activity of RNase-L in the absence of exogenous agents, an RNase-L expression construct was microinjected into subconfluent Balb-c 3T3 cells. DNA synthesis was monitored by [^{3}H]-thymidine incorporation and autoradiography, and RNase-L expression was detected by immunostaining with antisera specific for the transgene-encoded protein. Cells were not synchronized before microinjection and [^{3}H]-thymidine addition; accordingly, both labeled and unlabeled nuclei were observed, indicative of the proportion of cells that traversed S phase during the course of the labeling. RNase-L expression was heterogeneous in the injected cells at the post-injection time examined, which may reflect an influence of cell cycle on its expression. Analysis of nuclear labeling and RNase-L expression in the same cells by bright field and fluorescence microscopy, revealed that RNase-L-expressing cells did not display nuclear labeling, suggesting that ectopic expression of RNase-L inhibited proliferation. In contrast, the nuclei of cells with low, background staining for RNase-L expression were strongly labeled (see arrowheads in Figure 1a). Control,

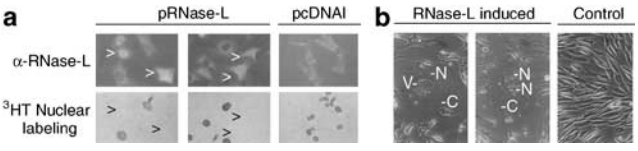

Figure 1 Ectopic expression of RNase L induces a senescent phenotype. (**a**) Microinjection of an RNase L expression plasmid reduces mitotic index. Balb c 3T3 cells were injected with plasmids as indicated, and labeled with [^{3}H] thymidine at 24 h post injection. Cells were then fixed for RNase L immunostaining, and auto radiography. Top and bottom panels are identical microscope fields viewed in fluorescence and bright light, and are representative of fields analysed in duplicate samples; note the lack of nuclear labeling in cells expressing RNase L (indicated by arrows). (**b**) Induction of RNase L results in a senescent morphology. Light micrographs of NIH 3T3 cells stably transfected with IPTG inducible RNase L or vector control cells 24 h after IPTG induction; N, nucleus; C, cytoplasm; V, vacuole.

vector-injected cells exhibited a higher frequency of nuclear labeling. Thus, ectopic expression of RNase-L resulted in a reduction in cells undergoing DNA synthesis, a property that is characteristic of quiescent and senescent cells.

RNase-L expression induces a senescent phenotype

The antiproliferative properties of RNase-L precluded the generation of cell lines that stably express transfected RNase-L, and transient transfections yield a heterogeneous population of cells with respect to RNase-L expression, thus limiting the analysis of its biological activities. Therefore, we utilized NIH-3T3 cells stably transfected with an isopropyl β-D-thiogalactopyranoside (IPTG)-inducible RNase-L expression construct to examine the effects of sustained RNase-L expression. As previously reported, IPTG treatment resulted in the induction of RNase-L protein within 24 h, and RNase-L expression induced apoptosis in the majority of cells (Castelli *et al*., 1997). However, in a subpopulation of cells, RNase-L induction resulted in a senescent phenotype. Specifically, these cells exhibited an increased cytoplasmic:nuclear volume and vacuolization, and a flattened, multinucleate morphology. In contrast, the mock induced cells retained a spindle shaped, fibroblast morphology (Figure 1b). These findings provide the first evidence of a link between RNase-L and a senescent phenotype.

RNase-L induces senescence and reduces in vitro *proliferative lifespan*

Senescence was first described as a mechanism that limits the replicative capacity of primary cells in culture (Hayflick, 1965); therefore, we determined the effect of RNase-L expression on the proliferative properties of HDFs that exhibit a finite proliferative lifespan. RNase-L was stably introduced into early passage parental WI38, and Simian virus 40 (SV40)-immortalized SV-WI38, HDFs by retroviral transduction. Expression of the myc-tagged RNase-L transgene was readily detected in the transduced cells, and, in contrast to cell lines examined to date (Diaz *et al*., 1997; Malathi *et al*., 2004), stable RNase-L expression in WI38 HDFs did not induce apoptosis (not shown and see Figure 4). To determine if RNase-L expression altered the replicative lifespan of the cells, RNase-L-transduced and vector control cells were passaged at a constant inoculum, and the population doubling level (PDL) was calculated at each passage. Cells were considered senescent when they failed to double in 2 weeks. When the population doubling time of the RNase-L-transduced WI38 HDFs began to decline (PDL 15), cells were stained for senescence-associated β-galactosidase (β-gal) activity. Counting of the stained cells revealed that 62% of RNase-L-transduced WI38 HDFs were β-gal positive as compared to only 18% in the vector control cells. In contrast, no β-gal staining was observed in either the RNase-L- or vector-transduced SV-WI38 cells (Figure 2a). To address the possibility that RNase-L may induce

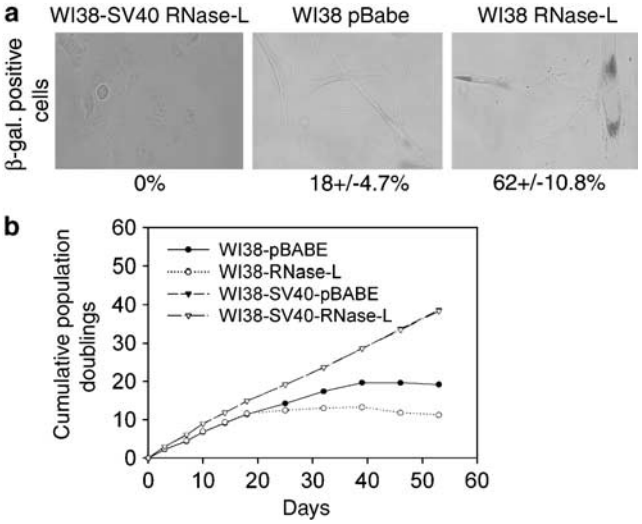

Figure 2 Ectopic expression of RNase L induces senescence associated β gal activity and reduces replicative lifespan. (**a**) WI38 derived cells were stained for β gal activity when the WI38 RNase L cells began to exhibit proliferative arrest (PDL 15). β gal positive cells were counted in 10 fields of replicate cultures to quantify staining. (**b**) Cells were counted at each passage, and the cumulative PDL was calculated. The curves for the cumulative population doublings of WI38 SV40 pBABE and WI38 SV40 RNase L coincide in the graph.

β-gal expression independent of senescence, we determined the consequences of RNase-L expression on the *in vitro* proliferative lifespan of WI38 cells. Replicative senescence was accelerated by nearly 3 weeks in the RNase-L WI38 as compared to vector control cells (Figure 2b). The vector control cells senesced at PDL $\sim$20 similar to the parental WI38 cells. Thus, ectopic expression of RNase-L markedly reduced the lifespan of WI38 HDFs suggesting that it serves a functional role in senescence; however, RNase-L expression did not result in an antiproliferative stimulus that was sufficient to induce senescence in immortalized SV-WI38 cells.

Senescence is delayed in RNase-L-deficient fibroblasts

To examine the relationship between endogenous RNase-L and senescence, we utilized embryonic fibroblasts derived from mice with a targeted disruption of the RNase-L gene (Zhou *et al*., 1997). Mouse embryonic fibroblasts (MEFs) undergo senescence at a much earlier PDL than primary HDFs, accordingly, wild-type (WT) and RNase-L $^{/}$ MEFs were analysed for β-gal positive senescent cells after 10 population doublings (Figure 3). Cell counts from three independent cultures revealed that, at the same passage, 92% of the WT MEFs exhibited β-gal staining, whereas only 27% of the RNase-L $^{/}$ cells were β-gal positive (Figure 3a). The RNase-L $^{/}$ MEFs continued to proliferate for two to three additional passages after the WT cells had senesced (Figure 3b). These findings indicate that the absence of a functional RNase-L protein retards the onset of senescence.

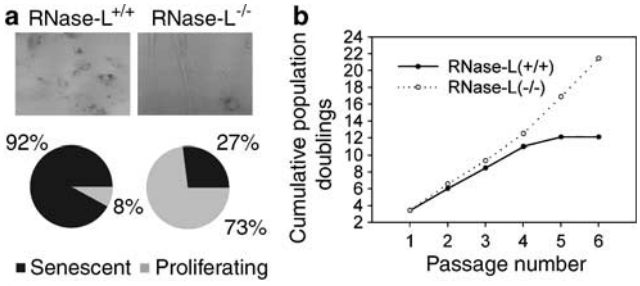

Figure 3 Senescence is delayed in RNase L $^{-/-}$ MEFs. (**a**) RNase L $^{-/-}$ and strain matched RNase L$^{+/+}$ MEFs were cultured until the RNase L$^{+/+}$ MEFs began to exhibit a senescent phenotype (PDL $\simeq$10), and then stained for β gal activity; a light micrograph of a typical field is shown. β gal positive cells were quantified by counting replicate fields in three independent cultures, and the mean was expressed as a percentage of the total cells counted. (**b**) RNase L $^{-/-}$ and $^{+/+}$ MEFs were counted at each passage and the PDL was determined.

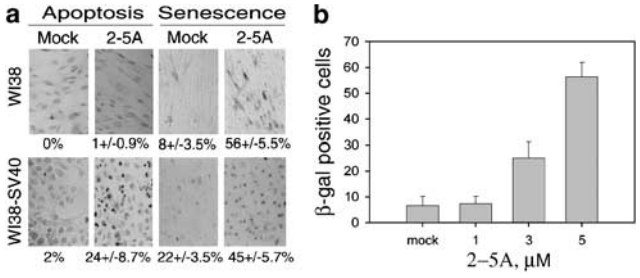

Figure 4 RNase L activation results in senescence and apoptosis in primary and immortalized HDFs, respectively. (**a**) WI38 and SV WI38 cells were transfected with 5 μM 2 5A tetramer for 90 min; apoptotic cells were labeled by TUNEL assay, and senescent cells were stained for β gal activity. Light micrographs of typical fields are shown, and the percentage of senescent and apoptotic cells was determined by counting. (**b**) WI38 HDFs were transfected with 2 5A tetramer at the concentrations indicated for 90 min, then incubated with fresh medium and stained for β gal activity after 36 h; β gal positive cells were quantified by counting as indicated in the graph.

RNase-L activation induces premature senescence

Ectopic RNase-L expression induced distinct responses of senescence in HDFs (Figure 2) and apoptosis in established cell lines (Castelli *et al*., 1997) that may reflect a fundamental difference in the response of primary and immortalized cells to antiproliferative stimuli. To determine if RNase-L activation by exogenous 2-5A induced senescence in primary HDFs, and if this response differed in SV40-immortalized cells in which the critical senescence mediators p53 and pRb are inactivated (White and Khalili, 2004), 2-5A was transfected into parental and SV-WI38 cells. Remarkably, 2-5A transfection of primary WI38 HDFs induced a dose-dependent, eightfold increase in β-gal staining (Figure 4a and b), indicating that primary HDFs remain viable, albeit senescent, following RNase-L activation. In contrast, SV-WI38 cells displayed a high basal level of senescent cells consistent with recent reports of elevated senescence in premalignant cells (Braig *et al*., 2005; Chen *et al*., 2005; Collado *et al*., 2005; Michaloglou *et al*., 2005); however, 2-5A transfection resulted in only a twofold increase in senescent cells (Figure 4a, right panel). To determine if the reduction in 2-5A-induced senescence in SV-WI38 as compared to WI38 cells reflected a distinct, apoptotic response, we analysed the induction of apoptosis following 2-5A transfection by TdT-mediated dNTP nick end labeling (TUNEL) assay. Interestingly, no TUNEL-positive cells were detected in 2-5A-transfected WI38 cells, whereas SV-WI38 cells displayed a significant increase in apoptotic cells (Figure 4a, left two panels). Thus, an apoptotic response to 2-5A transfection distinguished immortalized SV-WI38 cells from their parental cells, and suggested that intrinsic cellular factors can modulate the antiproliferative phenotype induced by 2-5A.

Physiological impact of RNase-L on longevity

The proliferative capacity of primary cells in culture inversely correlates with the *in vivo* age and lifespan of the donor organism, and senescent cells accumulate in aged tissues, providing evidence for senescence as an *in vitro* model for organismal aging (Campisi, 2003). To determine if the involvement of RNase-L in cellular senescence resulted in an altered lifespan of RNase-L $^{-/-}$ mice, aging colonies were established. Specifically, 82 male RNase-L $^{-/-}$ mice and 98 strain-matched male WT mice were housed in a pathogen-free environment, and monitored for 152 weeks. To ensure that our analysis reflected the natural lifespan of the mice, animals that died from injury or disease were censored in the study. Remarkably, Kaplan–Meier analysis revealed a significant increase in the mean survival time of RNase-L $^{-/-}$ mice (108 ± 5.6 weeks) compared to WT mice (82 ± 4.6 weeks). The age at which survival probability was 50% (T50), and the maximum survival age (Tmax) were increased by 28 and 16%, respectively, in the RNase-L $^{-/-}$ mice. Thus, the absence of RNase-L resulted in a significant 31.7% ($P < 0.0001$) increased lifespan as compared to the WT mice (Figure 5 and Table S1), and suggests a novel role for RNase-L in longevity.

Discussion

A functional role for RNase-L in replicative senescence

Endogenous constraints on cell proliferation represent logical end points for therapeutic intervention in the treatment of human malignancies. Accordingly, much effort has focused on dissecting the molecular components of antiproliferative pathways. Indeed, the induction of apoptosis by chemotherapeutic agents has been extensively studied, however, relatively little is known about the senescent response and its potential utility in cancer therapy (Schmitt, 2003; Shay and Roninson, 2004). Recent studies have demonstrated the presence of senescent cells in premalignant, but not malignant, tumors providing evidence for the tumor suppressive function of senescence *in vivo*

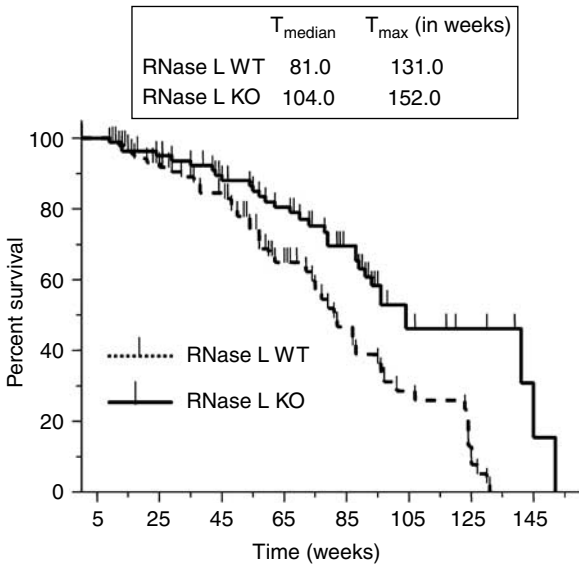

Figure 5 RNase L / mice exhibit enhanced longevity. Kaplan Meier survival curve: C57BL/6 mice with a pure genetic back ground of RNase L$^{+/+}$, *n* 98, and RNase L / , *n* 82 were monitored for a period of 152 weeks and analysed for their survival probability using product limit method of Kaplan and Meier as described (Materials and methods). Solid and broken lines show the survival of RNase L / and WT mice respectively. Data from deaths due to infection or injury were not included in the study; the deaths of individual mice are depicted as (I) in the graph, and this data is provided in Supplementary Table S3. Tmax and T50 in weeks are shown in the box insert.

(Braig *et al*., 2005; Chen *et al*., 2005; Collado *et al*., 2005; Michaloglou *et al*., 2005). The current challenge is to identify the molecular mediators of senescence, and determine how they are regulated in diverse oncogenic settings. IFN and ISGs are implicated in the senescent response (Kulaeva *et al*., 2003; Xin *et al*., 2004), and RNase-L is an established mediator of the antiproliferative activity of IFN (Hassel *et al*., 1993; Zhou *et al*., 1997), and functions as a tumor suppressor (Carpten *et al*., 2002; Casey *et al*., 2002); therefore, we examined the potential role for RNase-L in senescence.

Ectopic expression of RNase-L induced a proliferative arrest and a senescent phenotype in murine and human cells (Figures 1–3). *In vitro* lifespan was reduced in RNase-L overexpressing cells and increased in RNase-L / cells (Figures 2 and 3), providing evidence of a functional role for RNase-L in replicative senescence. In addition, 2-5A transfection activated RNase-L and resulted in telomere shortening, a hallmark of replicative senescence (data not shown). A detectable increase in RNase-L was not observed in senescent cells (data not shown), suggesting that small, transient changes in RNase-L expression may be sufficient to initiate the senescence program. OAS is upregulated in senescence, and may function to activate RNase-L-induced senescence in the absence of changes in RNase-L expression (Kulaeva *et al*., 2003; Yoon *et al*., 2004).

Activation of endogenous RNase-L induces senescent and apoptotic responses

Cellular stresses including treatment with chemotherapeutic agents, oncogene activation, and oxidative damage induce senescent or apoptotic responses depending on the cell type and physiological context (Schmitt, 2003; Shay and Roninson, 2004). 2-5A transfection induces apoptosis in prostate cancer cell lines, and this response was reduced in normal prostate epithelia which may have reflected a senescent outcome (Malathi *et al*., 2004). 2-5A treatment and RNase-L activation are associated with cellular stress responses, including inhibition of protein synthesis and the activation of stress kinases, that may contribute to its senescence-inducing activity (Iordanov *et al*., 2000; Li *et al*., 2004). Remarkably, 2-5A transfection resulted in an exclusively senescent response in WI38 HDFs, but shifted to a more apoptotic response in immortalized SV-WI38 cells. The transcription factors p53 and pRb are critical mediators of senescence and apoptosis that are inactivated by Large-T antigen in SV40-transformed SV-WI38 cells (White and Khalili, 2004). The reduced senescent response in SV-WI38 cells suggests that 2-5A-induced senescence was mediated, in part, by p53 and pRb transcriptional pathways. 2-5A mediated activation of RNase-L is predicted to primarily impact messenger RNA stability. However, a recent microarray analysis reported that 2-5A transfection resulted in the induction of more than twice as many genes as were repressed, suggesting that the biological activities of RNase-L are mediated by both RNA degradation and gene induction (Malathi *et al*., 2005). A comparison of gene expression in cells that exhibit senescent and apoptotic responses to 2-5A will provide a better understanding of the molecular basis of these outcomes.

RNase-L modulates aging in vivo

Cellular senescence functions as a tumor suppressor mechanism by inducing a permanent growth arrest that is associated with cellular aging *in vitro* and *in vivo*. Indeed, the same genes that mediate senescence also function in tumor suppression (Campisi, 2003; Shay and Roninson, 2004), providing a mechanistic link between cancer, long known to be an age-associated disease and aging. Senescence-associated tumor suppressor genes, designated 'gatekeepers', are thought to protect the organism from cancer during its younger, reproductive years, but to exhibit detrimental effects that contribute to aging in older individuals (Campisi, 2003). Consistent with this relationship between senescence, aging and cancer, senescent cells are more prevalent in aged tissues, and secrete growth factors and proteases resulting in a pro-oncogenic environment (Campisi, 2003). As a tumor suppressor that functions in apoptosis and senescence, RNase-L fits the definition of a gatekeeper gene. In this capacity, genetic disruption of RNase-L in mice is predicted to increase the *in vivo* lifespan. In fact, RNase-L / mice survived 31.7% longer than strain-matched WT mice (Figure 5, Table S1) providing evidence for a physiological role for RNase-L in aging. In agreement with this finding, an

age-dependent increase in IFN-β and OAS in human fibroblasts (Tahara *et al*., 1995), and in RNase-L from mouse tissues was reported previously (Floyd-Smith and Denton, 1988). Importantly, mice that died from injury or disease were excluded from this study to insure the analysis reflected a natural lifespan rather than an increased susceptibility to disease associated with deletion of the RNase-L gene. RNase-L $^{/}$ mice did not die of tumors suggesting that other insults, in addition to the loss of this tumor suppressor, are required for tumorigenesis. Longevity is a complex phenotype that is estimated to be 48–79% heritable in mice, and a number of genes that impact lifespan have been identified (Quarrie and Riabowol, 2004). The specific role of RNase-L in aging and how it may impact other aging-associated genes requires further investigation.

Tumor cells retain the capacity to undergo senescence, thus inducers of senescence represent a new class of tumor suppressor (Schmitt, 2003). Our results identify a novel role for RNase-L in senescence that may contribute to its tumor suppressive function, and to the enhanced longevity of RNase-L $^{/}$ mice.

Materials and methods

Kaplan Meier survival analysis
RNase L $^{/}$ and strain matched RNase L$^{+/+}$ mice were housed in a pathogen free facility, and no difference was observed in the growth or health of the RNase L $^{/}$ and $^{+/+}$ mice (Zhou *et al*., 1997). The survival of RNase L $^{/}$ ($n=82$) and $^{+/+}$ ($n=98$) mice was monitored for a period of 152 weeks. Mice that died from disease or injury were excluded from the longevity study. The product limits method of Kaplan and Meier (Kaplan EL, 1958) was used to estimate the survival function S defined as:

$$\hat{S}(t) = \prod_{t_i \leqslant t} \left(1 \quad \frac{d_i}{n_i}\right)$$

where t_i is duration of study at point i, d_i is number of deaths up to point i and n_i is number of individuals at risk just before t_i. S is the product (P) of these conditional probabilities. Analysis was performed using SPSS 14.0 software (see Supplementary data Tables S1 S3).

Cell culture
Embryos derived from RNase L $^{/}$ and strain and age matched WT mice that had been backcrossed for 10 generations to C57/Bl6 mice were used to prepare primary MEFs (Zhou *et al*., 1997). Cells were cultured at 37°C in a humidified chamber of 95% air, 5% CO_2 and passaged twice a week at a constant inoculum of 6.7×10^4 cells/cm^2.

WI38 HDFs and SV40 immortalized WI38 cells (SV WI38; Coriell Cell Repository, Camden, NJ, USA) were cultured in minimum essential medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (20% FBS for SV WI38), 2 mM L glutamine, 2 × essential, non essential amino acids and vitamin solution (Invitrogen, Carlsbad, CA, USA). Cells were passaged twice a week at a constant inoculum of 1.3×10^3 cells/cm^2. Swiss 3T3 and NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM L glutamine and 1% antibiotic antimycotic solution.

Retroviral vectors and gene transduction
The human RNase L complementary DNA (cDNA) containing an amino terminus c myc epitope tag was cloned into the retroviral vector, pBabe puro. pBabe RNase L or vector control was transfected into the Bosc23 packaging cell line using Lipofectamine plus as per the manufacturers instructions (Invitrogen, Carlsbad, CA, USA) and virus containing supernatants were collected. WI38 and SV WI38 cells were infected with the pBabe RNase L, or pBabe virus and stable cell lines were selected by growth in 3 μg/ml puromycin.

Microinjection, [^{3}H] thymidine labeling and immunostaining
Balb/c 3T3 cells were grown on coverslips until approximately 50% confluence. All cells contained within a circular field inscribed on the coverslip were microinjected with the pcDNA1 RNase L expression construct (pRNase L) or empty vector as described (Smith *et al*., 1986). At 24 h post injection, cells were labeled with 5 μCi [^{3}H] thymidine (Amersham, Piscataway, NJ, USA) for an additional 18 h, then fixed and immunostained for RNase L. The primary antibody to human RNase L (Dong and Silverman, 1995) was used at a 1:1000 dilution in 0.5% bovine serum albumin/phosphate buffered saline, and RNase L was visualized using the secondary antibody, Cy3 conjugated donkey anti mouse immunoglobulin G (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) at a 1:2000 dilution. Air dried coverslips were mounted on slides and autoradiography was performed as described (Mulcahy *et al*., 1985). RNase L immunostaining and nuclear labeling were visualized by fluorescence and bright field microscopy, respectively, and quantified by counting.

2 5A Transfection
Cells seeded at 7.5×10^3 cells/cm^2 in a chamber slide were transfected with tetramer 2 5A ($p_3A(2'p5'A)_3$) at the concentrations indicated for 90 min using Ca^{2+} phosphate. Cells were then cultured for 36 h before staining for senescence or apoptosis.

Senescence associated β *gal staining*
Cells were seeded at 3×10^4 cells/well in a 4 cm^2 chamber slide (Nalge Nunc, Naperville, IL, USA) 48 h before senescence associated β gal staining according to manufacturer's protocol (Cell Signaling, Beverly, MA, USA). Cells which stained blue, were considered positive for β Gal activity (Dimri *et al*., 1995).

Apoptosis staining (TUNEL assay)
Apoptotic cells were stained according to the manufacturer's protocol (Calbiochem, La Jolla, CA, USA). After labeling, cells were immediately counterstained with methyl green solution for detection of non apoptotic cells. Nuclei stained black or green were considered apoptotic or non apoptotic, respectively.

Acknowledgements

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References

Bisbal C, Martinand C, Silhol M, Lebleu B, Salehzada T. (1995). Cloning and characterization of a RNAse L inhibitor. A new component of the interferon regulated 2 5A pathway. *J Biol Chem* **270**: 13308 13317.

Bisbal C, Silhol M, Laubenthal H, Kaluza T, Carnac G, Milligan L *et al*. (2000). The 2′ 5′ oligoadenylate/RNase L/ RNase L inhibitor pathway regulates both MyoD mRNA stability and muscle cell differentiation. *Mol Cell Biol* **20**: 4959 4969.

Braig M, Lee S, Loddenkemper C, Rudolph C, Peters AH, Schlegelberger B *et al*. (2005). Oncogene induced senescence as an initial barrier in lymphoma development. *Nature* **436**: 660 665.

Campisi J. (2003). Cancer and ageing: rival demons? *Nat Rev Cancer* **3**: 339 349.

Carpten J, Nupponen N, Isaacs S, Sood R, Robbins C, Xu J *et al*. (2002). Germline mutations in the ribonuclease L gene in families showing linkage with HPC1. *Nat Genet 2000* **30**: 181 184.

Casey G, Neville PJ, Plummer SJ, Xiang Y, Krumroy LM, Klein EA *et al*. (2002). RNASEL Arg462Gln variant is implicated in up to 13% of prostate cancer cases. *Nat Genet* **32**: 581 583.

Castelli JC, Hassel BA, Wood KA, Li XL, Amemiya K, Dalakas MC *et al*. (1997). A study of the interferon antiviral mechanism: apoptosis activation by the 2 5A system. *J Exp Med* **186**: 967 972.

Chase BI, Zhou Y, Xiang Y, Silverman RH, Zhou A. (2003). Proteasome mediated degradation of RNase L in response to phorbol 12 myristate 13 acetate (PMA) treatment of mouse L929 cells. *J Interferon Cytokine Res* **23**: 565 573.

Chen Z, Trotman LC, Shaffer D, Lin HK, Dotan ZA, Niki M *et al*. (2005). Crucial role of p53 dependent cellular senescence in suppression of Pten deficient tumorigenesis. *Nature* **436**: 725 730.

Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M *et al*. (2005). Tumour biology: senescence in premalignant tumours. *Nature* **436**: 642.

Diaz G, Rivas C, Esteban M. (1997). Activation of the IFN inducible enzyme RNase L causes apoptosis of animal cells. *Virology* **236**: 354 363.

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C *et al*. (1995). A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc Natl Acad Sci USA* **92**: 9363 9367.

Dong B, Silverman RH. (1995). 2 5A dependent RNase molecules dimerize during activation by 2 5A. *J Biol Chem* **270**: 4133 4137.

Floyd Smith G, Denton JS. (1988). Age dependent changes are observed in the levels of an enzyme mediator of interferon action: a (2′ 5′)A(n) dependent endoribonuclease. *Proc Soc Exp Biol Med* **189**: 329 337.

Hardy K, Mansfield L, Mackay A, Benvenuti S, Ismail S, Arora P *et al*. (2005). Transcriptional networks and cellular senescence in human mammary fibroblasts. *Mol Biol Cell* **16**: 943 953.

Hassel BA, Zhou A, Sotomayor C, Maran A, Silverman RH. (1993). A dominant negative mutant of 2 5A dependent RNase suppresses antiproliferative and antiviral effects of interferon. *EMBO J* **12**: 3297 3304.

Hayflick L. (1965). The limited *in vitro* lifetime of human diploid cell strains. *Exp Cell Res* **37**: 614 636.

Iordanov MS, Paranjape JM, Zhou A, Wong J, Williams BR, Meurs EF *et al*. (2000). Activation of p38 mitogen activated protein kinase and c Jun NH(2) terminal kinase by double stranded RNA and encephalomyocarditis virus: involvement of RNase L, protein kinase R, and alternative pathways. *Mol Cell Biol* **20**: 617 627.

Kaplan EL MP. (1958). Nonparametric estimation from incomplete observations. *J Am Stat Assoc* **53**: 457 481.

Kubota K, Nakahara K, Ohtsuka T, Yoshida S, Kawaguchi J, Fujita Y *et al*. (2004). Identification of 2′ phosphodiesterase, which plays a role in the 2 5A system regulated by interferon. *J Biol Chem* **279**: 37832 37841.

Kulaeva OI, Draghici S, Tang L, Kraniak JM, Land SJ, Tainsky MA. (2003). Epigenetic silencing of multiple interferon pathway genes after cellular immortalization. *Oncogene* **22**: 4118 4127.

Leszczyniecka M, Su ZZ, Kang DC, Sarkar D, Fisher PB. (2003). Expression regulation and genomic organization of human polynucleotide phosphorylase, hPNPase(old 35), a Type I interferon inducible early response gene. *Gene* **316**: 143 156.

Li G, Xiang Y, Sabapathy K, Silverman RH. (2004). An apoptotic signaling pathway in the interferon antiviral response mediated by RNase L and c Jun NH2 terminal kinase. *J Biol Chem* **279**: 1123 1131.

Malathi K, Paranjape JM, Bulanova E, Shim M, Guenther Johnson JM, Faber PW *et al*. (2005). A transcriptional signaling pathway in the IFN system mediated by 2′ 5′ oligoadenylate activation of RNase L. *Proc Natl Acad Sci USA* **102**: 14533 14538.

Malathi K, Paranjape JM, Ganapathi R, Silverman RH. (2004). HPC1/RNASEL mediates apoptosis of prostate cancer cells treated with 2′,5′ oligoadenylates, topoisomerase I inhibitors, and tumor necrosis factor related apoptosis inducing ligand. *Cancer Res* **64**: 9144 9151.

Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM *et al*. (2005). BRAFE600 associated senescence like cell cycle arrest of human naevi. *Nature* **436**: 720 724.

Mulcahy LS, Smith MR, Stacey DW. (1985). Requirement for ras proto oncogene function during serum stimulated growth of NIH 3T3 cells. *Nature* **313**: 241 243.

Quarrie JK, Riabowol KT. (2004). Murine models of life span extension. *Sci Aging Knowledge Environ* **31**: re5.

Schmitt CA. (2003). Senescence, apoptosis and therapy cutting the lifelines of cancer. *Nat Rev Cancer* **3**: 286 295.

Shay JW, Roninson IB. (2004). Hallmarks of senescence in carcinogenesis and cancer therapy. *Oncogene* **23**: 2919 2933.

Shelton DN, Chang E, Whittier PS, Choi D, Funk WD. (1999). Microarray analysis of replicative senescence. *Curr Biol* **9**: 939 945.

Shou J, Soriano R, Hayward SW, Cunha GR, Williams PM, Gao WQ. (2002). Expression profiling of a human cell line model of prostatic cancer reveals a direct involvement of interferon signaling in prostate tumor progression. *Proc Natl Acad Sci USA* **99**: 2830 2835.

Silverman RH. (2003). Implications for RNase L in prostate cancer biology. *Biochemistry* **42**: 1805 1812.

Smith MR, DeGudicibus SJ, Stacey DW. (1986). Requirement for c ras proteins during viral oncogene transformation. *Nature* **320**: 540 543.

Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. (1998). How cells respond to interferons. *Annu Rev Biochem* **67**: 227 264.

Tahara H, Kamada K, Sato E, Tsuyama N, Kim JK, Hara E *et al*. (1995). Increase in expression levels of

interferon inducible genes in senescent human diploid fibroblasts and in SV40 transformed human fibroblasts with extended lifespan. *Oncogene* **11**: 1125 1132.

Untergasser G, Koch HB, Menssen A, Hermeking H. (2002). Characterization of epithelial senescence by serial analysis of gene expression: identification of genes potentially involved in prostate cancer. *Cancer Res* **62**: 6255 6262.

White MK, Khalili K. (2004). Polyomaviruses and human cancer: molecular mechanisms underlying patterns of tumorigenesis. *Virology* **324**: 1 16.

Xin H, Pereira Smith OM, Choubey D. (2004). Role of IFI 16 in cellular senescence of human fibroblasts. *Oncogene* **23**: 6209 6217.

Yoon IK, Kim HK, Kim YK, Song IH, Kim W, Kim S *et al.* (2004). Exploration of replicative senescence associated genes in human dermal fibroblasts by cDNA microarray technology. *Exp Gerontol* **39**: 1369 1378.

Zhou A, Paranjape J, Brown TL, Nie H, Naik S, Dong B *et al.* (1997). Interferon action and apoptosis are defective in mice devoid of 2′,5′ oligoadenylate dependent RNase L. *EMBO J* **16**: 6355 6363.