Premature aging with impaired oxidative stress defense in mice lacking TR4

Yi-Fen Lee, Su Liu, Ning-Chun Liu, Ruey-Sheng Wang, Lu-Min Chen, Wen-Jye Lin, Huei-Ju Ting, Hsin-Chiu Ho, Gonghui Li, Edward J. Puzas, Qiao Wu and Chawnshang Chang

Am J Physiol Endocrinol Metab 301:E91-E98, 2011. First published 26 April 2011; doi:10.1152/ajpendo.00701.2010

Am J Physiol Endocrinol Metab 301: E91–E98, 2011.
First published April 26, 2011; doi:10.1152/ajpendo.00701.2010.

Premature aging with impaired oxidative stress defense in mice lacking TR4

Yi-Fen Lee,[1]* Su Liu,[1]* Ning-Chun Liu,[1]* Ruey-Sheng Wang,[1] Lu-Min Chen,[1,2] Wen-Jye Lin,[1] Huei-Ju Ting,[1] Hsin-Chiu Ho,[1] Gonghui Li,[1] Edward J. Puzas,[1] Qiao Wu,[3] and Chawnshang Chang[1,2]

[1]*George Whipple Laboratory for Cancer Research, Departments of Pathology, Urology, and Orthopaedics, University of Rochester Medical Center, Rochester, New York;* [2]*Sex Hormone Research Center, China Medical University/Hospital, Taichung, Taiwan; and* [3]*Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, Xiamen University, Xiamen, China*

Submitted 23 December 2010; accepted in final form 20 April 2011

Lee YF, Liu S, Liu NC, Wang RS, Chen LM, Lin WJ, Ting H, Ho HC, Li G, Puzas EJ, Wu Q, Chang C. Premature aging with impaired oxidative stress defense in mice lacking TR4. *Am J Physiol Endocrinol Metab* 301: E91–E98, 2011. First published April 26, 2011; doi:10.1152/ajpendo.00701.2010.—Early studies suggest that *TR4* nuclear receptor is a key transcriptional factor regulating various biological activities, including reproduction, cerebella development, and metabolism. Here we report that mice lacking *TR4* ($TR4^{-/-}$) exhibited increasing genome instability and defective oxidative stress defense, which are associated with premature aging phenotypes. At the cellular level, we observed rapid cellular growth arrest and less resistance to oxidative stress and DNA damage in $TR4^{-/-}$ mouse embryonic fibroblasts (MEFs) in vitro. Restoring *TR4* or supplying the antioxidant *N*-acetyl-L-cysteine (NAC) to $TR4^{-/-}$ MEFs reduced the DNA damage and slowed down cellular growth arrest. Focused qPCR array revealed alteration of gene profiles in the DNA damage response (DDR) and anti-reactive oxygen species (ROS) pathways in $TR4^{-/-}$ MEFs, which further supports the hypothesis that the premature aging in $TR4^{-/-}$ mice might stem from oxidative DNA damage caused by increased oxidative stress or compromised genome integrity. Together, our finding identifies a novel role of TR4 in mediating the interplay between oxidative stress defense and aging.

testicular nuclear receptor 4; senescence

TESTICULAR NUCLEAR RECEPTOR 4 (*TR4*) belongs to the nuclear receptor superfamily (4) that regulates various genes via binding to AGGTCA-like direct-repeat motifs (23). Regulation of various genes in diverse pathways, for example, *apoE* (21), *Gata1* (42), *PEPCK* (25), and *CD36* (47), indicates that *TR4* plays vital roles in many important biological events. Knockout of *TR4* gene in mice ($TR4^{-/-}$) results in abnormal maternal behavior (8), impairment in male (32) and female (5) fertility, hypoglycemia (25), and neurological abnormalities (6), indicating *TR4*'s critical role in maintaining tissue homeostasis. Hence, loss of *TR4* might impair mouse physiological function in general, which might gradually lead to systematic declines over time. Indeed, $TR4^{-/-}$ mice suffered progeriod syndrome as demonstrated by shorter life span and signs of accelerating aging, including cachexia, graying hair, osteoporosis, and kyphosis, at a much younger age than their wild-type littermates.

Aging is characterized by the deterioration of physiological functions caused by the accumulation of stochastic damage to cellular macromolecules (26). Organisms are constantly bombarded by endogenous and environmental genotoxic insults such as reactive oxygen species (ROS), which cause damage to macromolecules, including DNA, and finally lead to genome instability. A number of theories have been put forth to explain the cause and mechanism of aging, and yet no single mechanism is sufficient to account for age-related frailty, disability, or diseases. In general, elevated oxidative stress, defective stress responses, and increased genomic instability are among the major causes that lead to accelerated aging (15).

Reducing stress tolerance is one characteristic of organism aging (33), during which cells become less resistant to stresses, and thus increased cellular senescence was triggered by the accumulated DNA damage to deplete the damaged cells (40). Remarkably, transgenic mice that overexpress mitochondrial catalase show a prolonged life span that is correlated with an increased ROS-scavenging activity (38). Also, loss of genome stability due to malfunctions in DNA repair machineries can have catastrophic consequences, including premature aging. This is supported by studies in premature aging mouse models with disruptions of genes involved in maintaining DNA integrity, such as *DNA-PKcs* (11), *Ku 80* (45), and *XPD* (10).

In our previous study, we have demonstrated that oxidative stress stimulates *TR4* expression via the stress responder *FOXO3a* (24), which has been associated with longevity in various organisms (19, 46). Furthermore, we also found that *TR4* expression level can be stimulated upon other various genotoxic stresses, including UV and gamma irradiation (Yan SJ, Lee YF, Ting HJ, Liu NC, Liu S, Yeh SD, and Chang C, unpublished observations). Thus, our findings have established *TR4*'s role in the regulation of the cellular response to multi-stresses. In this report, we demonstrate further that elevated ROS and increased genome instability contribute to the premature aging phenotypes in $TR4^{-/-}$ mice. Furthermore, we showed that the application of the antioxidant *N*-acetyl-L-cysteine (NAC) to $TR4^{-/-}$ mouse embryonic fibroblasts (MEFs) reduced the DNA damage and slowed down cellular growth arrest. Therefore, our report provides a novel mechanism that links *TR4*, stress defense, and aging.

EXPERIMENTAL PROCEDURES

Mice information generation, breeding, and genotyping of $TR4^{-/-}$ mice were described previously (8). The original $TR4^{-/-}$ mice were of C57/BL6/129 mixed background. $TR4^{-/-}$ mice were back-crossed to C57/BL6 mice for five generations and used in this study. Continuous back-cross to C57/BL6 background over six to seven generations caused embryonic lethality in $TR4^{-/-}$ mice.

Tissue preparation and histology. Mice were anesthetized with an overdose of pentobarbital sodium, and tissues were removed and fixed by immersion in 10% neutral buffered formalin. Tissues were cut into 5-μm sections, deparaffinized, and stained with haematoxylin and eosin by standard procedures.

* These authors contributed equally to this article.

Address for reprint requests and other correspondence: C. Chang, George Whipple Laboratory for Cancer Research, Depts. of Pathology, Urology, and Orthopaedics, Univ. of Rochester Medical Center, Rochester, NY 14620 (e-mail: chang@urmc.rochester.edu).

Bone analyses. Mice were subjected to whole body X-ray in situ under anesthesia. Bone mineral density was quantified by dual-energy X-ray absorptiomentry (DEXA) scanning.

Immunofluorescence staining of 8-oxodeoxyguanosine. The assessment of 8-oxodeoxyguanosine (8-OxodG) followed the protocol with small revision (41). Liver tissues were prefixed by the addition of 2% (wt/vol) paraformaldehyde (in PBS at pH 7.4) for 15 min after the tissues were washed with PBS, fixed and permeabilized with ice-cold methanol for 15 min, and rehydrated in PBS before blocking with PBS containing 10% (wt/vol) normal goat serum (NGS). The blocking solution was washed off with PBS containing 0.2% (wt/vol) NGS. DNA damage was visualized with avidin-conjugated FITC (1:200 in PBS for 1 h) under fluorescence microscopy. The percentage of the 8-OxodG-containing cells per 500 cells was counted, and the average percentage was achieved from a total of 2,000 cells for each sample.

Generation of MEF cells. We removed the heads and all of the internal organs from E14.5 embryos and rinsed with PBS, added 5 ml of DMEM, passed through a 22-gauge needle a few times to mince tissues, and then allowed the fibroblasts to attach to the culture flask for 24 h and changed the medium to remove unattached cells and debris. MEFs at *passage 0* (P0) would form a confluent monolayer after 2–3 days. Cells were then trypsinized and subcultured for genotyping and experiments. All of the experiments were finished before *passage 4* (P4).

Protective effect of cellular protein on pUC19 DNA damage. To determine the protective effects of cellular proteins on plasmid DNA, 5 μl of 0.25 μg/μl pUC19 DNA was incubated with 5 μg of cellular proteins from $TR4^{-/-}$ or $TR4^{+/+}$ MEFs (PBS was used as control). Then, 1 μl of 6 mmol/l H_2O_2 and 1 μl of 0.4 mmol/l $FeSO_4$ were added and incubated at 37°C for 60 min. The reaction was electrophoresed on an agarose gel, and DNA damage evaluations were based on the loss of supercoiled (SC) monomer.

Cell cycle profiling. MEFs from $TR4^{-/-}$ or $TR4^{+/+}$ mice were collected and fixed with 70–75% EtOH at 4°C for ≥12 h. Cells were then centrifuged at 1,000 rpm for 7 min at 4°C, the supernatant was decanted, and 1 ml of RNase (1 mg/ml in 1× PBS) was added for 30 min. Cells were then incubated with 500–1,000 μl of propidium iodide (20 μg/ml) and analyzed by flow cytometry.

Growth assay (MTT). We seeded 2,000 cells in 96-well plates and waited ≥12 h or overnight for attachment. We then infected with retrovirus (vector or *TR4* virus) for 24 h, washed with culture medium, and replaced with 200 μM H_2O_2 containing medium for 2 h. Cells without H_2O_2 treatment were recorded as *day 0*. After 2 h H_2O_2 treatment, we washed cells with culture medium and replaced with the fresh medium and harvested cells on *days 1*, *3*, and *5* for 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. To determine the cells' sensitivity to stress, MEFs from $TR4^{-/-}$ and $TR4^{+/+}$ mice were seeded and treated with various doses of γ-irradiation or 2 h of H_2O_2. We harvested the cells on *day 3* to determine cell growth by MTT assay. The survival rate was determined as the ratio between treated and nontreated groups.

Measurement of DNA single-stranded breaks. A DNA precipitation assay was used for DNA strand breaks detection. Confluent MEFs from $TR4^{-/-}$ and $TR4^{+/+}$ mice were labeled with 0.25 μCi/ml [^{3}H]methylthymidine for 24 h, and then the cells were washed thoroughly with PBS and supplied with serum-free medium in the presence and absence of 250 μM H_2O_2 for another 30 min. The cells were washed and lysed with lysis buffer (10 mM Tris·HCl, 10 mM EDTA, 50 mM NaOH, and 2% SDS, pH 12.4), followed by addition of KCl (12 mM) for 10 min at 65°C, followed by a 5-min cooling and precipitation period on ice. A DNA protein K-SDS precipitate was formed under these conditions, from which low-molecular-mass broken DNA was released. DNA fragments were recovered in the supernatant from a 10-min centrifugation at 200 *g* at 10°C and transferred into a liquid scintillation vial containing 1 ml of 50 mM HCl. The precipitated pellet (intact double-stranded DNA) was solubilized with water at 65°C. Radioactivity was determined by scintillation counter. The amount of double-stranded DNA remaining was calculated for each sample by dividing the dpm value of the pellet by the total dpm value of the pellet plus supernatant and multiplying by 100. In control cells (cells incubated in Ca^{2+}-containing or Ca^{2+}-free EGTA), the level of total double-stranded DNA was ~75%. Pretreatment with the various chelators did not affect this level.

Measurement of intracellular ROS by flow cytometry. The production of intracellular ROS was detected by flow cytometry using dichlorofluorescein diacetate (DCFH-DA). The MEFs from $TR4^{-/-}$ and $TR4^{+/+}$ mice were cultured in 35-mm tissue culture dishes. When cells reached 80% confluence, they were incubated with 10 μM DCFH-DA together with 250 μM H_2O_2 for 30 min at 37°C in the dark, washed once with PBS, detached by trypsinization, collected by centrifugation, and suspended in PBS prior to flow cytometry. For the control group, the medium was changed to serum-free medium and incubated with 10 μM DCFH-DA and followed by flow cytometric analyses, and the amount of cellular ROS levels was quantified by the fluorescence of dichlorofluorescein density.

Senescence-associated β-galactosidase assay. Briefly, cells were washed in phosphate-buffered saline and fixed in 2% formaldehyde-0.2% glutaraldehyde. Then the cells were washed and incubated at 37°C overnight with fresh senescence-associated β-galactosidase (SA-β-Gal) stain solution [1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 40 mM citric acid-sodium phosphate (pH 6.0), 150 mM NaCl, 2 mM $MgCl_2$, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide]. All visualization and photography of cells were performed on a Leica microscope with Sony digital imaging.

Pathway-focused RT^2 Profiler PCR Array. The relative expression of 84 DNA damage signaling-related genes and 84 oxidative stress defense-related genes was evaluated using the mouse DNA damage signaling pathway and oxidative stress and antioxidant defense signaling pathway RT^2 Profiler PCR Array system (SuperArray Bioscience) according to the manufacturer's instructions. DNase-treated total RNA was purified from cultured MEF cells, and cDNA was generated by reverse transcription from 1 μg of total RNA from each sample using the RT^2 First Strand kit and then combined with the RT^2 qPCR Master Mix and added to lyophilized primer pairs in the 96-well arrays. Thermal cycling was performed in a Bio-Rad iCycler. Relative gene expression levels were calculated using the $\Delta\Delta C_T$ method with normalization to the average expression level of five common genes (*ACTB*, *B2M*, *GAPDH*, *HPRT*, and *RPL13A*).

RESULTS

$TR4^{-/-}$ mice display premature aging phenotypes. We first found that almost all $TR4^{-/-}$ mice developed cachexia by 1 mo, characterized by severe weight loss, prominent reduction of fat tissue, and weakness (8). Cachexia in $TR4^{-/-}$ mice was progressive, heterogeneous in severity, and followed by premature death. Generally, $TR4^{-/-}$ mice had much shorter life span compared with *TR4* wild-type ($TR4^{+/+}$) and heterozygote ($TR4^{+/-}$) mice. Seventy percent of $TR4^{-/-}$ mice died before 4 mo, and >90% of the $TR4^{-/-}$ mice (72 out of 77 $TR4^{-/-}$ mice) could not live >1 yr (Fig. 1*A*). More than 90% of $TR4^{+/+}$ and $TR4^{+/-}$ mice survived over this period of study, and the average life span of C57BL/6J mice exceeds 2 yr (13).

Besides the shorter life span, $TR4^{-/-}$ mice also acquired an aged appearance at an earlier stage. By 6 mo, most $TR4^{-/-}$ mice have gray hair (Fig. 1*B*) and an extramedullary hematopoiesis in the liver (Fig. 1*C*) that is often found in aging mice and the PolgA mutant premature aging mouse model (43).

Furthermore, bone condition was examined for age-associated changes in $TR4^{+/+}$ and $TR4^{-/-}$ mice. Although 2- to

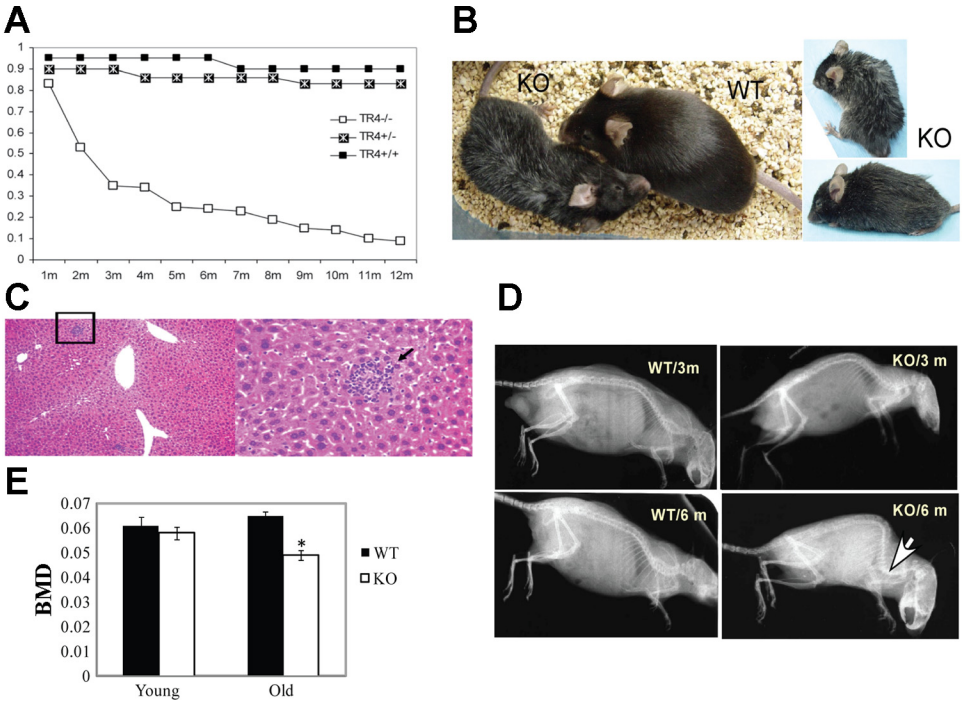

Fig. 1. Premature aging phenotypes in testicular nuclear receptor 4 ($TR4$)$^{-/-}$ mice. *A*: 1-yr survival rate between $TR4^{+/+}$, $TR4^{+/-}$, and $TR4^{-/-}$ mice (n = 74 for $TR4^{+/+}$ and $TR4^{+/-}$ mice, n = 77 for $TR4^{-/-}$ mice). *B*: general appearance of $TR4^{-/-}$ [knockout (KO)] mice. Gray hair and hunchback were seen in 6-mo-old $TR4^{-/-}$ female mice compared with age-matched $TR4^{+/+}$ [wild-type (WT)] mice. *C*: extramedullary hematopoiesis in 6-mo-old $TR4^{-/-}$ mice liver. *D*: radiograph of 3- and 6-mo-old $TR4^{-/-}$ and $TR4^{+/+}$ mice showed skeletal abnormalities in aging $TR4^{-/-}$ mice. Six-month-old $TR4^{-/-}$ mice display curvature of the spinal column (kyphosis). Pictures from *B*–*D* are the representative pictures from ≥3 pairs of WT and KO mice. *E*: dual-energy X-ray absorptiometry scan analyses of young (2–4 mo; n = 4) and old male $TR4^{-/-}$ mice (n = 5) at the age of 6–7 mo compared with age/sex-matched $TR4^{+/+}$ mice. Differences in bone mineral density (BMD) of a particular sex and genotypes were analyzed by Student's t-test $^*P < 0.05$.

3-mo-old $TR4^{-/-}$ mice showed no significant skeletal abnormalities (Fig. 1*D*) and had similar bone mineral density (BMD) compared with their littermate $TR4^{+/+}$ mice (Fig. 1*E*), radiographs of 6-mo-old $TR4^{-/-}$ mice revealed a severe kyphosis (curvature of the spine; Fig. 1*D*), a landmark for aging bone. A reduction of BMD in 6- to 7-mo-old $TR4^{-/-}$ mice spine was revealed by DEXA scanning (Fig. 1*E*), although there was no difference of BMD in the skull between $TR4^{-/-}$ and $TR4^{+/+}$ (data not shown).

Taken together, Fig. 1, *A*–*E*, demonstrated that $TR4^{-/-}$ mice developed a segmental progeroid syndrome characterized by the early onset of premature aging phenotypes and shorter life span.

Increasing genome instability and ROS in $TR4^{-/-}$ mice. Mammalian aging is characterized by the functional decline caused by accelerated accumulation of somatic damage to macromolecules. To examine the level of macromolecular damage in $TR4^{-/-}$ mice, the 8-OxodG level was assayed. 8-OxodG is a major form of oxidatively modified DNA that increases with age and can lead to a variety of diseases and the age-associated decline of physiological functions (29). The level of 8-OxodG in the liver tissue of old $TR4^{-/-}$ mice was 50% higher than that in the littermate $TR4^{+/+}$ mice (Fig. 2*A*), suggesting that TR4 is involved in the regulation of redox homeostasis or the defense against ROS. This is consistent with our previous finding that *TR4* mRNA was induced directly by *FOXO3a* under hydrogen peroxide treatment (24).

Among all sources of somatic damage in organisms, ROS, the by-products of oxidative phosphorylation, are considered the main insult (37). To further investigate the possible implication of TR4 in ROS homeostasis, ROS levels were measured in liver extracts from $TR4^{-/-}$ and $TR4^{+/+}$ mice. As shown in Fig. 2*B*, ROS level in the liver extracts from $TR4^{-/-}$ mice was significantly higher than that from their $TR4^{+/+}$ littermates, indicating that TR4 plays essential roles in regulating redox homeostasis.

Early onset of senescence in TR4-deficient cells. To better study the possible mechanisms leading to the progerorid phenotypic changes in $TR4^{-/-}$ mice at the cellular level, we examined the growth characteristics of primary MEFs from $TR4^{-/-}$ and $TR4^{+/+}$ embryos. At early passages (P1–P3), both $TR4^{-/-}$ and $TR4^{+/+}$ MEFs grew well, and their proliferation capacity did not show any noticeable difference (data not shown). Consistent with this, there was no difference between their cell cycle profiles at early passages (Fig. 3*A*). However, after P4, $TR4^{-/-}$ MEFs gradually slowed down their growth rate, whereas $TR4^{+/+}$ MEFs kept up a steady growth before P8 (Fig. 3*B*), which was confirmed by their cell cycle characteristics in which two-thirds of $TR4^{-/-}$ cells were arrested at

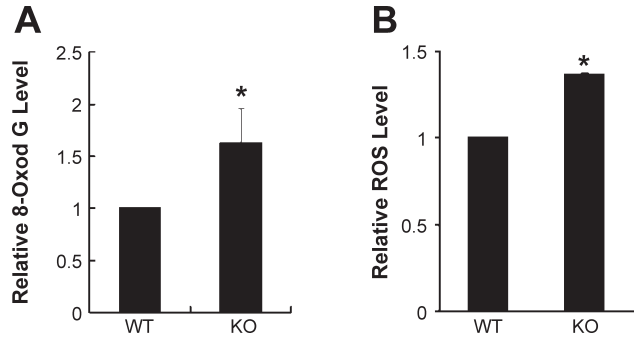

Fig. 2. Increased reactive oxygen species (ROS) and oxidative damage in $TR4^{-/-}$ tissues. *A*: 8-oxodeoxyguanosine (8-OxodG) staining was carried out in old (>12 mo) WT and KO mouse (>12 mo) liver tissues, as described in EXPERIMENTAL PROCEDURES. The number of 8-OxodG-positive cells/500 cells was counted, and the percentage was calculated. A total of 2,000 cells were counted from each mouse. Shown are the average percentage ± SD (n = 4). *B*: tissue ROS levels were quantified in liver extracts from WT and KO mice by dichorofluorescein staining and flow cytometric analyses. Shown are the mean fluorescence intensity ± SD (n = 5). $^*P < 0.05$ vs. WT.

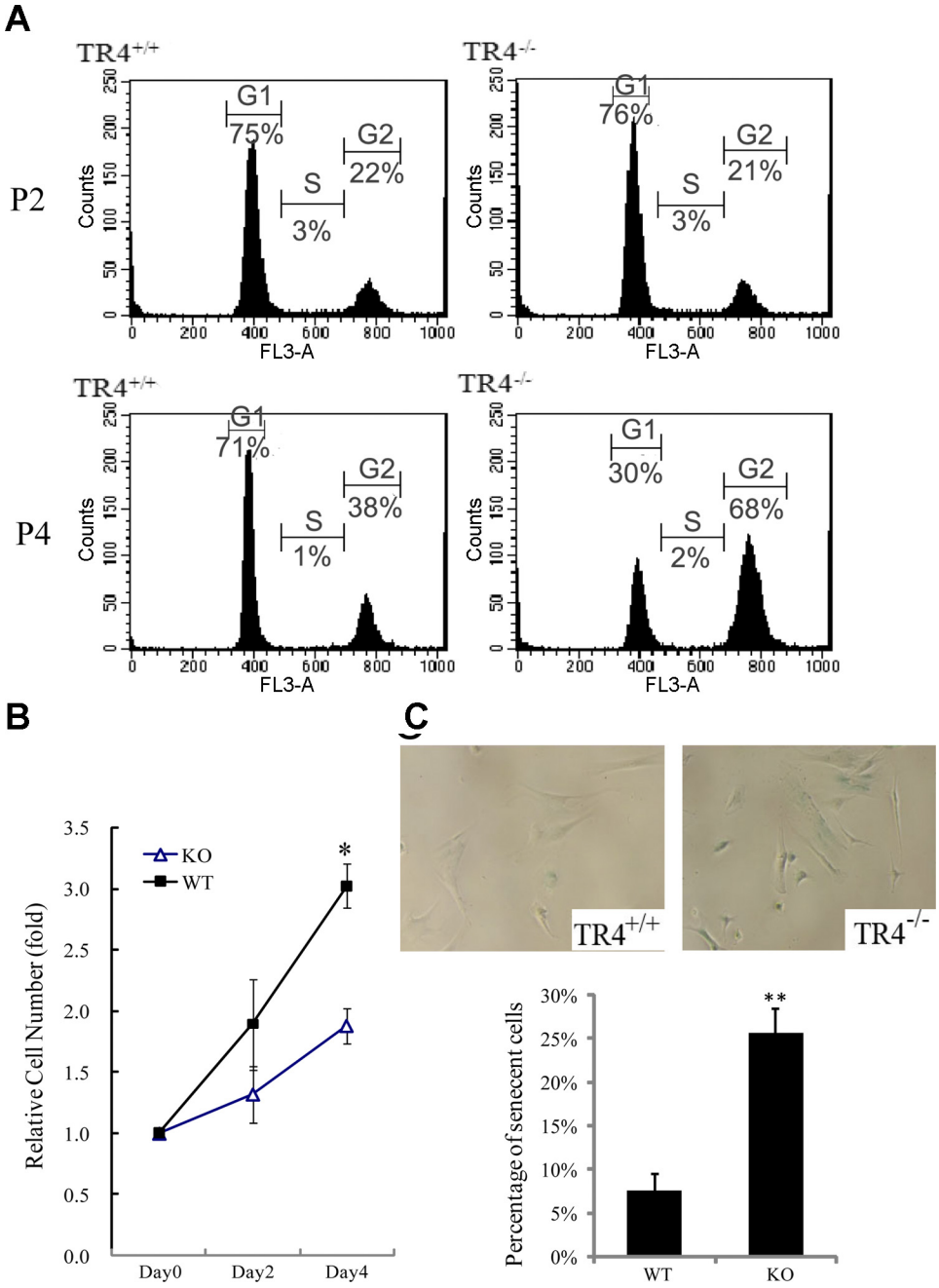

Fig. 3. Growth arrest and early onset of cellular senescence in *TR4*$^{-/-}$ mouse embryonic fibroblasts (MEFs). *A*: cell cycle profile analysis of *passages 2* (P2) and *4* (P4; MEFs from *TR4*$^{-/-}$ and *TR4*$^{+/+}$ mice). *TR4*$^{-/-}$ display an early G_2/M arrest in P4, whereas P4 *TR4*$^{+/+}$ MEFs showed a normal cell cycle distribution. *B*: the growth rate of MEFs from *TR4*$^{+/+}$ (WT) and *TR4*$^{-/-}$ (KO) was examined by 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The MEFs, from both genotypes at P5, were seeded and harvested at indicated days. The growth rate was calculated as the ratio to *day 0*. Three independent experiments were carried out, and the representative results are shown. Shown are means ± SD (*$P < 0.05$ vs. WT). *C*: P5 *TR4*$^{+/+}$ and *TR4*$^{-/-}$ MEFs were subjected to senescence-associated β-galactosidase (SA-β-Gal) staining, as described in EXPERIMENTAL PROCEDURES (*top*). Cellular senescence was indicated by the blue-colored staining. The percentage of SA-β-Gal staining positive cells was counted. Representative pictures from *TR4*$^{+/+}$ and *TR4*$^{-/-}$ MEFs are shown (*bottom*). **$P <$ 0.01 vs. WT.

G_2/M at P4, whereas less than one-third of *TR4*$^{+/+}$ MEFs were in G_2/M (Fig. 3*A*) at P4.

We also observed an increased number of *TR4*$^{-/-}$ MEFs with flattened and enlarged morphology, a feature typically associated with senescence, after P4 (Fig. 3*C*, *top*). Senescence as a stress response to environmental insults, DNA damage, or telomere shortening (1) is a common feature among the in vitro cultured primary cells from individuals with progeroid syndromes (3). To determine whether *TR4*$^{-/-}$ MEFs underwent an early onset of senescence, we stained both *TR4*$^{-/-}$ and *TR4*$^{+/+}$ MEFs for the senescence biomarker endogenous SA-β-gal and found a significantly increased number of *TR4*$^{-/-}$ MEFs with positive SA-β-gal staining (Fig. 3*C*, *bottom*). Together, our data suggest that *TR4* deficiency leads to an early onset of senescence.

Higher cellular levels of ROS and increased DNA damage in TR4$^{-/-}$ *MEFs could be rescued by restoration of TR4.* To investigate the cause of the early onset of senescence in *TR4*$^{-/-}$ MEFs, we assessed the levels of ROS and oxidative DNA damage in MEF cells. *TR4*$^{-/-}$ MEFs did display higher cellular ROS levels than *TR4*$^{+/+}$ MEFs after H_2O_2 challenging (Fig. 4*A*), which suggests the involvement of *TR4* in the ROS scavenging system. We then measured the single-stranded DNA breaks in MEFs with or without H_2O_2 treatment. As expected, the intrinsic as well as extrinsic (H_2O_2-induced) single-stranded DNA breaks were increased in *TR4*$^{-/-}$ MEFs compared with *TR4*$^{+/+}$ MEFs (Fig. 4*B*), suggesting a protective role of *TR4* against DNA damage. To validate the involvement of *TR4* in cellular stress defense, we transfected functional *TR4* into *TR4*$^{-/-}$ MEFs and found that restoring func-

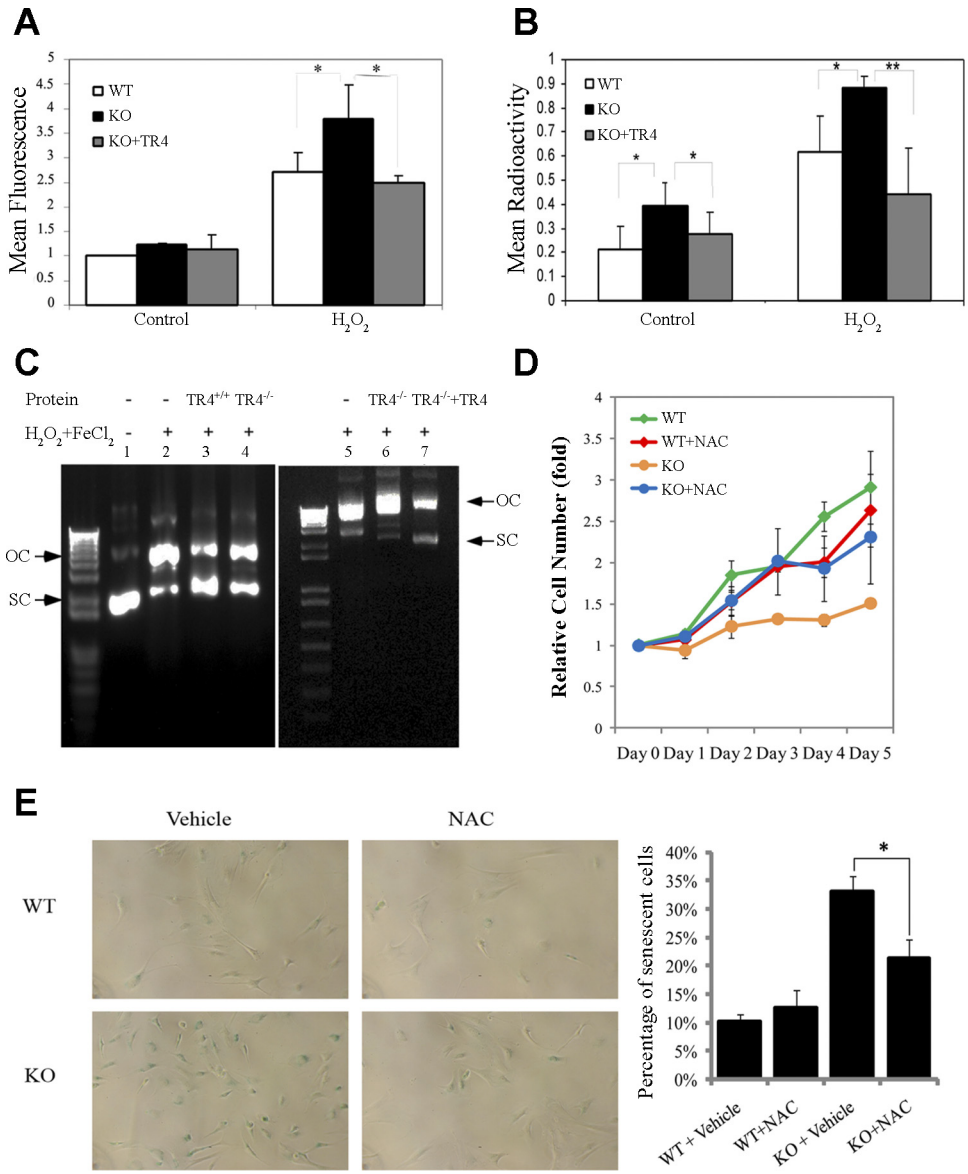

Fig. 4. Higher cellular levels of ROS, increased DNA single-stranded breaks, and growth arrest in $TR4^{-/-}$ MEFs could be rescued by *TR4* or the antioxidant reagent *N*-acetyl-L-cysteine (NAC). *A*: $TR4^{+/+}$ (WT) MEFs and $TR4^{-/-}$ (KO) MEFs transfected with vector or pBabe-*TR4* MEFs were treated with 250 μM H_2O_2 or vehicle control and examined for cellular ROS levels by flow cytometry. Three independent experiments were carried out, and the representative results are shown. Shown are the mean radioactivity ± SD (*$P < 0.05$ vs. control). *B*: single stranded DNA breaks in $TR4^{+/+}$ MEFs and $TR4^{-/-}$ MEFs transfected with vector or pBabe-*TR4* MEFs were compared by DNA precipitation, as described in EXPERIMENTAL PROCEDURES. Three independent experiments were carried out, and the representative results are shown. Shown are the mean fluorescence intensity ± SD (*$P < 0.05$ vs. control; **$P < 0.01$ vs. control). *C*: protective effects on pUC19 plasmid DNA break caused by hydroxyl radical produced by the Fe^{2+}-H_2O_2 system. Electrophoresis was carried out on a 0.8% agarose gel. *Lane 1*, control pUC19 DNA; *lanes 2* and *5*, DNA breaks on pUC19 by Fe^{2+}-H_2O_2 treatment; *lanes 3* and *4*, Fe^{2+}- H_2O_2-treated pUC19 in the presence of cellular proteins from $TR4^{+/+}$ and $TR4^{-/-}$ MEFs; *lanes 6* and *7*, Fe^{2+}-H_2O_2-treated pUC19 in the presence of cellular proteins from $TR4^{-/-}$ MEFs and *TR4*-restored $TR4^{-/-}$ MEFs. *D*: WT and KO MEF cells were treated with NAC for 4 days and exposed to 250 μM H_2O_2 for 30 min and switched back to normal medium. After different periods of time, cell viability was measured by MTT assay. The relative surviving cell numbers compared with control, without H_2O_2 treatment, were calculated and plotted. *E*: MEFs from $TR4^{+/+}$ and $TR4^{-/-}$ embryos were cultured and maintained in vitro and treated with NAC or vehicle for 4 days before SA-β-Gal staining. Representative pictures from 3 independent experiments are shown at *left*. Quantification results are shown at *right*. Three independent experiments were carried out; means ± SD are shown (*$P < 0.01$ vs. control).

tional *TR4* into $TR4^{-/-}$ MEFs reduced both endogenous and H_2O_2-induced ROS and single-stranded DNA breaks significantly (Fig. 4, *A* and *B*). These results suggested that *TR4* is involved in the antioxidative stress system, one of the major defenses against genotoxic insults to maintain genome integrity, and this might be one of the mechanisms through which *TR4* contributes to longevity.

To further dissect the anti-ROS actions of *TR4*, we tested whether *TR4* can promote cellular scavenging ability by examining the *TR4* capacity for protecting DNA damage in vitro. pUC19 DNA plasmids were treated with hydroxyl radicals to induce DNA breaks and then incubated with cellular proteins from $TR4^{+/+}$ and $TR4^{-/-}$ MEFs. As shown in Fig. 4*C*, treatment with Fe^{2+}/H_2O_2 caused plasmid DNA breaks, which would release SC forms (*lane 1*) into open circular (OC) forms (*lanes 2* and *5*); the protective effects against DNA breaks from SC into OC forms were found in the presence of $TR4^{+/+}$ MEF protein extracts (*lane 3*). In contrast, there was a less protective effect in the presence of $TR4^{-/-}$ MEF protein extracts in which mainly OC forms were present (Fig. 4*C*, *lanes 4* and *6*), and restoring *TR4* via pBabe retrovirus infection into $TR4^{-/-}$ reduced the hydroxyl radical-induced DNA breaks (Fig. 4*C*, *lane 7* vs. *lane 6*). These data support the hypothesis that *TR4* promotes the anti-ROS defense capacity via mediating ROS-scavenging pathways and maintenance of DNA integrity.

Senescence and growth arrest in $TR4^{-/-}$ MEFs could be rescued by NAC treatment. To verify the link between increasing ROS and growth arrest in $TR4^{-/-}$ MEFs, we examined whether supplying antioxidants into $TR4^{-/-}$ cells could rescue cells from the early onset of growth arrest. As shown in Fig. 4*D*, continuing supplementation with NAC released $TR4^{-/-}$ MEFs from growth arrest, and the growth rate of $TR4^{-/-}$ MEFs under NAC treatment was comparable with $TR4^{+/+}$ MEFs without NAC. NAC, a precursor for glutathione, can be found naturally in foods and is a powerful antioxidant. Recently, it was found to attenuate senescence in *Prx II*$^{-/-}$ MEF cells (14), suppress lymphoma, and increase longevity in Atm-deficient mice (35). Furthermore, the number of SA-β-Gal-positive senescent cells

Table 1. *Putative TR4 target genes in mouse DDR signaling*

Gene Name (*TR4*-Upregulated Genes)	DDR Pathway PCR Array	
	GeneBank access no.	Gene function
Parp1	NM_007415	Base-excision repair
Rad51	NM_011234	Homologous recombination repair
Trex1	NM_011637	Editing mismatched 3′-termini
Fen1	NM_007999	Other genes related to DNA repair
Polk	NM_012048	

TR4, testicular nuclear receptor 4; DDR, DNA damage response; Parp1; poly (ADP-ribose) polymerase family, member 1; Rad51, RAD51 homolog (*S. cerevisiae*); Trex1, 3′ repair exonuclease 1; Fen1, flap structure-specific endonuclease 1; Polk, polymerase κ (DNA directed).

in $TR4^{-/-}$ was reduced under 4-day NAC treatment, whereas it had little effect on $TR4^{+/+}$ cells (Fig. 4*E*, representative pictures at *left*, quantification at *right*). These results indicated that the early onset of senescence in $TR4^{-/-}$ MEFs was caused by accumulation of oxidative damage, and supplementation with NAC can slow down ROS-induced cell growth arrest in $TR4^{-/-}$ MEFs.

Alteration of the expression level of the genes involved in ROS and DNA damage response pathways in $TR4^{-/-}$ MEFs. The above data have clearly shown that *TR4* is involved in cellular oxidative stress defense and maintenance of genome integrity. In an effort to explore the molecular mechanism through which *TR4* regulates these cellular events to maintain genome integrity, we screened for *TR4* targeted genes in two specific pathways, oxidative stress and antioxidant pathway and DNA damage signaling pathway, with the focus quantitative PCR array. It is not surprising that we found that a number of the genes express differently between $TR4^{+/+}$ and $TR4^{-/-}$ MEFs (Tables 1 and 2). What caught our interest is that some of the genes have been linked to aging in previous studies. For example, *Fen1* mutation yeast displays premature aging (18), and *Parp1* is an important determinant in telomere regulation and thus might play a role in the aging process (2). Further investigation is needed to clarify which specific genes are truly responsible for the impaired genome integrity in $TR4^{-/-}$ mice.

DISCUSSION

We report that $TR4^{-/-}$ mice suffered premature aging with a deficient oxidative stress defense system and compromised genomic integrity. Aging is characterized by the deterioration of physiological functions caused by loss of stress defenses and increased genomic instability (15, 26). Reducing stress tolerance is one hallmark of organism aging (9), and discoveries of the association between longevity and stress resistance in yeast (44), flies (39), and cells of mice (36) support the "multiplex resistance mechanisms" hypothesis that life span augmentation is proportional to the level of resistance to environmental stresses (30). Manipulations that boost multi-stress defense pathways usually lead to extension of both mean and maximal life span (31, 48). Together with our previous findings that *TR4* is implicated in cellular response to different kinds of stress, such as oxidative stress (24), UV, and γ-irradiation (Yan SJ, Lee YF, Ting HJ, Liu NC, Liu S, Yeh SD, and Chang C, unpublished observations), our study provides a promising link between *TR4*, cellular stress defenses, and aging.

What is the molecular mechanism(s) underlying the accelerated aging in $TR4^{-/-}$ mice? Our results imply there that might be more than one mechanism behind this phenomenon. Expression of *TR4* in $TR4^{-/-}$ MEFs reduced the endogenous ROS levels and defended exogenous H_2O_2 insults as well as slowed down the cellular growth arrest, suggesting that *TR4* is involved in the ROS-scavenging defense system. $TR4^{-/-}$ mice displayed higher levels of single-stranded DNA damage, and focused quantitative PCR array revealed the reduced expression of genes in DNA damage response and anti-ROS pathways in $TR4^{-/-}$ MEFs, suggesting that *TR4* is involved in maintaining genome stability. All these different levels of defense systems form an intricate pathway network of maintaining genome integrity, which is a major factor in longevity (12, 26). More in-depth studies are essential to fully understand *TR4* roles in this genomic integrity maintenance network.

ROS status, determined by the balance between ROS production and scavenging, is critical for organism longevity, and pathways that control the ROS would be critical to determine the longevity (12). Mitochondria are one of the free radical generation sites in cells (22). Decreased mitochondrial H_2O_2 formation is correlated with delayed aging and increased life span in $p66^{Shc}$ knockout mice (28). Interestingly, we have found mitochondrial dysfunction with electron transport chain complex I deficiency in skeletal muscle of $TR4^{-/-}$ mice (Liu S, Lee, YF, Chou S, Uno H, Li G, Brookes P, Massett MP, Wu Q, Chen LM, and Chang C, unpublished observations). It has been reported that a complex I defect induces ROS release in primary open-angle glaucoma patients (16). Is it possible that the complex I defect accounts for the increased oxidative stress in $TR4^{-/-}$ mice and finally leads to premature aging? Further investigation is needed to find out the exact cause of the increased oxidative stress in $TR4^{-/-}$ mice.

Similarly to reports from patients with progeroid syndromes or premature aging mouse models (17, 20, 27), we observed an early onset of senescence in $TR4^{-/-}$ MEFs. Senescence, a state of irreversible cellular growth arrest, is one of the key cellular-responsive programs when cells are exposed to stress (3). How senescence contributes exactly to aging is unclear yet, although it has been commonly accepted that accumulation of senescent cells in organisms, when a certain threshold is reached, might compromise tissue function, and senescence may also impair the regenerative potential of stem cells (7). The early onset of senescence in $TR4^{-/-}$ MEFs might be a result of the excessive

Table 2. *Putative TR4 target genes in mouse oxidative stress and antioxidant defense signals*

Gene Name	Oxidative Stress and Antioxidant Defense PCR Array	
	GeneBank access no.	Gene function
TR4-upregulated genes		
Fancc	NM_007985	Oxidative stress defense
Prdx3	NM_007452	Antioxidant (peroxiredoxin)
Txnrd2	NM_013711	Antioxidant
Slc41a3	NM_027868	Antioxidant (peroxiredoxin)
TR4-downregulated genes		
Gpx5	NM_010343	Antioxidants (glutathione peroxidases)
Gpx6	NM_145451	Antioxidants (glutathione peroxidases)

Fancc, fanconi anemia, complementation group C; *Prdx3*, peroxiredoxin 3; *Txnrd2*, thioredoxin reductase 2; *Slc41a3*, solute carrier family 41, member 3; *Gpx5*, glutathione peroxidase 5; *Gpx6*, glutathione peroxidase 6.

oxidative stress since it can be attenuated by supplying the antioxidant NAC.

We identified expression level changes of certain genes in either DNA damage response signaling pathway or oxidative stress and antioxidant defense signaling pathways in *TR4*$^{-/-}$ mice. Some of the genes have been linked to aging in previous studies, including *Fen1* (18) and *PARP1* (2). However, more questions have emerged. How does *TR4* regulate these genes? Are they responsible for the premature aging in *TR4*$^{-/-}$ mice? How does *TR4* work through/with these genes in normal aging? Further investigation is needed to discover answers to all these questions. Restoration of target genes into *TR4*$^{-/-}$ cells might be essential to validate their roles in the *TR4*-regulated cellular oxidative stress defense network.

Unfortunately, no matter how closely the segmental progeroid syndrome in *TR4*$^{-/-}$ mice resembles the "natural" aging process, the *TR4*$^{-/-}$ mouse model, like all other progeria mice models (34), does not really represent the true normal aging process. Examination of *TR4* expression levels and/or activity throughout the life span in healthy people would hold the key to the understanding of the roles of *TR4* and its downstream factors in the natural aging process.

In conclusion, our study of the progeroid syndrome in *TR4*$^{-/-}$ mice opens up many new avenues for exploration and provides a useful experimental model for further dissecting the molecular basis of aging. In the future, the identification of upstream regulator(s) and/or activators to control *TR4* actions will be extremely important and rewarding tasks for revealing the secrets of controlling the fate of life.

GRANTS

This work was supported by NIH-RO1-DK-063212 (C. Chang), ACS-RSG-06-123-01-CNE (Y.-F. Lee), NIH-CA-127548 (Y.-F. Lee), and Taiwan Department of Health Clinical Trial and Research Center of Excellence Grant DOH99-TD-B-111-004.

DISCLOSURES

The authors declare no conflict of interest.

REFERENCES

1. **Ben-Porath I, Weinberg RA.** When cells get stressed: an integrative view of cellular senescence. *J Clin Invest* 113: 8–13, 2004.
2. **Beneke S, Cohausz O, Malanga M, Boukamp P, Althaus F, Bürkle A.** Rapid regulation of telomere length is mediated by poly(ADP-ribose) polymerase-1. *Nucleic Acids Res* 36: 6309–6317, 2008.
3. **Campisi J, d'Adda di Fagagna F.** Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol* 8: 729–740, 2007.
4. **Chang C, Da Silva SL, Ideta R, Lee Y, Yeh S, Burbach JP.** Human and rat TR4 orphan receptors specify a subclass of the steroid receptor superfamily. *Proc Natl Acad Sci USA* 91: 6040–6044, 1994.
5. **Chen LM, Wang RS, Lee YF, Liu NC, Chang YJ, Wu CC, Xie S, Hung YC, Chang C.** Subfertility with defective folliculogenesis in female mice lacking testicular orphan nuclear receptor 4. *Mol Endocrinol* 22: 858–867, 2008.
6. **Chen YT, Collins LL, Uno H, Chang C.** Deficits in motor coordination with aberrant cerebellar development in mice lacking testicular orphan nuclear receptor 4. *Mol Cell Biol* 25: 2722–2732, 2005.
7. **Collado M, Blasco MA, Serrano M.** Cellular senescence in cancer and aging. *Cell* 130: 223–233, 2007.
8. **Collins LL, Lee YF, Heinlein CA, Liu NC, Chen YT, Shyr CR, Meshul CK, Uno H, Platt KA, Chang C.** Growth retardation and abnormal maternal behavior in mice lacking testicular orphan nuclear receptor 4. *Proc Natl Acad Sci USA* 101: 15058–15063, 2004.
9. **d'Adda di Fagagna F.** Living on a break: cellular senescence as a DNA-damage response. *Nat Rev Cancer* 8: 512–522, 2008.
10. **de Boer J, Andressoo JO, de Wit J, Huijmans J, Beems RB, van Steeg H, Weeda G, van der Horst GT, van Leeuwen W, Themmen AP, Meradji M, Hoeijmakers JH.** Premature aging in mice deficient in DNA repair and transcription. *Science* 296: 1276–1279, 2002.
11. **Espejel S, Martín M, Klatt P, Martín-Caballero J, Flores JM, Blasco MA.** Shorter telomeres, accelerated ageing and increased lymphoma in DNA-PKcs-deficient mice. *EMBO Rep* 5: 503–509, 2004.
12. **Finkel T, Holbrook NJ.** Oxidants, oxidative stress and the biology of ageing. *Nature* 408: 239–247, 2000.
13. **Forster MJ, Morris P, Sohal RS.** Genotype and age influence the effect of caloric intake on mortality in mice. *FASEB J* 17: 690–692, 2003.
14. **Han YH, Kim HS, Kim JM, Kim SK, Yu DY, Moon EY.** Inhibitory role of peroxiredoxin II (Prx II) on cellular senescence. *FEBS Lett* 579: 4897–4902, 2005.
15. **Hasty P, Campisi J, Hoeijmakers J, van Steeg H, Vijg J.** Aging and genome maintenance: lessons from the mouse? *Science* 299: 1355–1359, 2003.
16. **He Y, Leung KW, Zhang YH, Duan S, Zhong XF, Jiang RZ, Peng Z, Tombran-Tink J, Ge J.** Mitochondrial complex I defect induces ROS release and degeneration in trabecular meshwork cells of POAG patients: protection by antioxidants. *Invest Ophthalmol Vis Sci* 49: 1447–1458, 2008.
17. **Herbig U, Ferreira M, Condel L, Carey D, Sedivy JM.** Cellular senescence in aging primates. *Science* 311: 1257, 2006.
18. **Hoopes LL, Budd M, Choe W, Weitao T, Campbell JL.** Mutations in DNA replication genes reduce yeast life span. *Mol Cell Biol* 22: 4136–4146, 2002.
19. **Hwangbo DS, Gersham B, Tu MP, Palmer M, Tatar M.** Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. *Nature* 429: 562–566, 2004.
20. **Keyes WM, Wu Y, Vogel H, Guo X, Lowe SW, Mills AA.** p63 deficiency activates a program of cellular senescence and leads to accelerated aging. *Genes Dev* 19: 1986–1999, 2005.
21. **Kim E, Xie S, Yeh SD, Lee YF, Collins LL, Hu YC, Shyr CR, Mu XM, Liu NC, Chen YT, Wang PH, Chang C.** Disruption of TR4 orphan nuclear receptor reduces the expression of liver apolipoprotein E/C-I/C-II gene cluster. *J Biol Chem* 278: 46919–46926, 2003.
22. **Kujoth GC, Hiona A, Pugh TD, Someya S, Panzer K, Wohlgemuth SE, Hofer T, Seo AY, Sullivan R, Jobling WA, Morrow JD, Van Remmen H, Sedivy JM, Yamasoba T, Tanokura M, Weindruch R, Leeuwenburgh C, Prolla TA.** Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. *Science* 309: 481–484, 2005.
23. **Lee YF, Lee HJ, Chang C.** Recent advances in the TR2 and TR4 orphan receptors of the nuclear receptor superfamily. *J Steroid Biochem Mol Biol* 81: 291–308, 2002.
24. **Li G, Lee YF, Liu S, Cai Y, Xie S, Liu NC, Bao BY, Chen Z, Chang C.** Oxidative stress stimulates testicular orphan receptor 4 through forkhead transcription factor forkhead box O3a. *Endocrinology* 149: 3490–3499, 2008.
25. **Liu NC, Lin WJ, Kim E, Collins LL, Lin HY, Yu IC, Sparks JD, Chen LM, Lee YF, Chang C.** Loss of TR4 orphan nuclear receptor reduces phosphoenolpyruvate carboxykinase-mediated gluconeogenesis. *Diabetes* 56: 2901–2909, 2007.
26. **Lombard DB, Chua KF, Mostoslavsky R, Franco S, Gostissa M, Alt FW.** DNA repair, genome stability, and aging. *Cell* 120: 497–512, 2005.
27. **Martin GM, Oshima J.** Lessons from human progeroid syndromes. *Nature* 408: 263–266, 2000.
28. **Migliaccio E, Giorgio M, Mele S, Pelicci G, Reboldi P, Pandolfi PP, Lanfrancone L, Pelicci PG.** The p66shc adaptor protein controls oxidative stress response and life span in mammals. *Nature* 402: 309–313, 1999.
29. **Mikkelsen L, Bialkowski K, Risom L, Løhr M, Loft S, Møller P.** Aging and defense against generation of 8-oxo-7,8-dihydro-2′-deoxyguanosine in DNA. *Free Radic Biol Med* 47: 608–615, 2009.
30. **Miller RA.** Cell stress and aging: new emphasis on multiplex resistance mechanisms. *J Gerontol A Biol Sci Med Sci* 64: 179–182, 2009.
31. **Moskovitz J, Bar-Noy S, Williams WM, Requena J, Berlett BS, Stadtman ER.** Methionine sulfoxide reductase (MsrA) is a regulator of antioxidant defense and lifespan in mammals. *Proc Natl Acad Sci USA* 98: 12920–12925, 2001.
32. **Mu X, Lee YF, Liu NC, Chen YT, Kim E, Shyr CR, Chang C.** Targeted inactivation of testicular nuclear orphan receptor 4 delays and disrupts late meiotic prophase and subsequent meiotic divisions of spermatogenesis. *Mol Cell Biol* 24: 5887–5899, 2004.

33. **Murakami S, Salmon A, Miller RA.** Multiplex stress resistance in cells from long-lived dwarf mice. *FASEB J* 17: 1565–1566, 2003.
34. **Puzianowska-Kuznicka M, Kuznicki J.** Genetic alterations in accelerated ageing syndromes. Do they play a role in natural ageing? *Int J Biochem Cell Biol* 37: 947–960, 2005.
35. **Reliene R, Schiestl RH.** Antioxidants suppress lymphoma and increase longevity in Atm-deficient Mice. *J Nutr* 137, *Suppl* 1: 229S–232S, 2007.
36. **Rudolph KL, Chang S, Lee HW, Blasco M, Gottlieb GJ, Greider C, DePinho RA.** Longevity, stress response, and cancer in aging telomerase-deficient mice. *Cell* 96: 701–712, 1999.
37. **Salmon AB, Richardson A, Perez VI.** Update on the oxidative stress theory of aging: does oxidative stress play a role in aging or healthy aging? *Free Radic Biol Med* 48: 642–655, 2010.
38. **Schriner SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, Coskun PE, Ladiges W, Wolf N, Van Remmen H, Wallace DC, Rabinovitch PS.** Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science* 308: 1909–1911, 2005.
39. **Sepulveda S, Shojaeian P, Rauser CL, Jafari M, Mueller LD, Rose MR.** Interactions between injury, stress resistance, reproduction, and aging in Drosophila melanogaster. *Exp Gerontol* 43: 136–145, 2008.
40. **Serrano M, Blasco MA.** Putting the stress on senescence. *Curr Opin Cell Biol* 13: 748–753, 2001.
41. **Struthers L, Patel R, Clark J, Thomas S.** Direct detection of 8-oxodeoxyguanosine and 8-oxoguanine by avidin and its analogues. *Anal Biochem* 255: 20–31, 1998.
42. **Tanabe O, Shen Y, Liu Q, Campbell AD, Kuroha T, Yamamoto M, Engel JD.** The TR2 and TR4 orphan nuclear receptors repress Gata1 transcription. *Genes Dev* 21: 2832–2844, 2007.
43. **Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE, Bohlooly-Y M, Gidlöf S, Oldfors A, Wibom R, Törnell J, Jacobs HT, Larsson NG.** Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* 429: 417–423, 2004.
44. **Van Raamsdonk JM, Hekimi S.** Deletion of the mitochondrial superoxide dismutase sod-2 extends lifespan in Caenorhabditis elegans. *PLoS Genet* 5: e1000361, 2009.
45. **Vogel H, Lim DS, Karsenty G, Finegold M, Hasty P.** Deletion of Ku86 causes early onset of senescence in mice. *Proc Natl Acad Sci USA* 96: 10770–10775, 1999.
46. **Willcox BJ, Donlon TA, He Q, Chen R, Grove JS, Yano K, Masaki KH, Willcox DC, Rodriguez B, Curb JD.** FOXO3A genotype is strongly associated with human longevity. *Proc Natl Acad Sci USA* 105: 13987–13992, 2008.
47. **Xie S, Lee YF, Kim E, Chen LM, Ni J, Fang LY, Liu S, Lin SJ, Abe J, Berk B, Ho FM, Chang C.** TR4 nuclear receptor functions as a fatty acid sensor to modulate CD36 expression and foam cell formation. *Proc Natl Acad Sci USA* 106: 13353–13358, 2009.
48. **Yu BP, Chung HY.** Stress resistance by caloric restriction for longevity. *Ann NY Acad Sci* 928: 39–47, 2001.