Premature aging with impaired oxidative stress defense in mice lacking TR4

Yi-Fen Lee,1,8 Su Liu,1,8 Ning-Chun Liu,1,8 Ruey-Sheng Wang,1 Lu-Min Chen,1,2 Wen-Yee Lin,1 Huei-Ju Ting,1 Hsin-Chiu Ho,1 Gonghui Li,1 Edward J. Puzas,1 Qiao Wu,3 and Chawnshang Chang1,2

1George Whipple Laboratory for Cancer Research, Departments of Pathology, Urology, and Orthopaedics, University of Rochester Medical Center, Rochester, New York; 2Sex Hormone Research Center, China Medical University/Hospital, Taichung, Taiwan; and 3Key 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 increased 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 (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 progeroid 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), Kit 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 multistress. 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.
Bone analyses. Mice were subjected to whole body X-ray in situ under anesthesia. Bone mineral density was quantified by dual-energy X-ray absorptiometry (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 with 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 H2O2 and 1 µl of 0.4 mmol/l FeSO4 were added and incubated at 37°C for 60 min. The reaction was electro-phoresed 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 discarded, 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 30 h for attachment. We then infected with retrovirus (vector or TR4 virus) for 24 h, washed with culture medium, and replaced with 200 µM H2O2 containing medium for 2 h. Cells without H2O2 treatment were recorded as day 0. After 2 h H2O2 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-dimethylthiazol-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 H2O2. 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 [1H]methyldimethyamine 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 H2O2 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 12 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 Ca2+-containing or Ca2+-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 H2O2 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 MgCl2, 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 RT2 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 RT2 Profiler PCR Array system (SuperArray Bioscience) according to the manufacturer’s instructions. DNsaitreated 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 RT2 First Strand kit and then combined with the RT2 qPCR Master Mix and added to lophylied primer pairs in the 96-well arrays. Thermal cycling was performed in a Bio-Rad iCycler. Relative gene expression levels were calculated using the ΔΔCt 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. 1A). 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. 1B) and an extramedullary hematopoiesis in the liver (Fig. 1C) 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-
3-mo-old TR4−/− mice showed no significant skeletal abnormalities (Fig. 1D) and had similar bone mineral density (BMD) compared with their littermate TR4+/+ mice (Fig. 1E), radiographs of 6-mo-old TR4−/− mice revealed a severe kyphosis (curvature of the spine; Fig. 1D), a landmark for aging bone. A reduction of BMD in 6- to 7-mo-old TR4−/− mice spine was revealed by DEXA scanning (Fig. 1E), 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. 2A), 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. 2B, 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 progeroid 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. 3A). However, after P4, TR4−/− MEFs gradually slowed down their growth rate, whereas TR4+/+ MEFs kept up a steady growth before P8 (Fig. 3B), which was confirmed by their cell cycle characteristics in which two-thirds of TR4−/− cells were arrested at

---

**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/match TR4+/+ mice. Differences in bone mineral density (BMD) of a particular sex and genotypes were analyzed by Student’s t-test *P < 0.05.

**Fig. 2. Increased reactive oxygen species (ROS) and oxidative damage in TR4−/− tissues.** A: 8-oxodeoxyguanosine (8-OxodG) staining was carried out in old (>2 mo) WT and KO mouse (>2 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 dichlorofluorescein staining and flow cytometric analyses. Shown are the mean fluorescence intensity ± SD (n = 5). *P < 0.05 vs. WT.
G2/M at P4, whereas less than one-third of TR4−/− MEFs were in G2/M 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. 3C, 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-beta-gal and found a significantly increased number of TR4−/− MEFs with positive SA-beta-gal staining (Fig. 3C, 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 H2O2 challenging (Fig. 4A), which suggests the involvement of TR4 in the ROS scavenging system. We then measured the single-stranded DNA breaks in MEFs with or without H2O2 treatment. As expected, the intrinsic as well as extrinsic (H2O2-induced) single-stranded DNA breaks were increased in TR4−/− MEFs compared with TR4+/+ MEFs (Fig. 4B), 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-
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. 4C, treatment with Fe2+/H2O2 caused plasmid DNA breaks, which would release SC forms (lane 1) into open circular (OC) forms (lanes 2 and 3); 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. 4C, lanes 4 and 6), and restoring TR4 via pBabe retrovirus infection into TR4−/− reduced the hydroxyl radical-induced DNA breaks (Fig. 4C, lane 5 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. 4D, 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

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 H2O2 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 PROCEEDURES. 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 Fe2+/H2O2 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 Fe2+/H2O2 treatment; lanes 3 and 4, Fe2+/H2O2-treated pUC19 in the presence of cellular proteins from TR4+/+ and TR4−/− MEFs; lanes 6 and 7, Fe2+/H2O2-treated pUC19 in the presence of cellular proteins from TR4+/+ MEFs and TR4−/− MEFs. D: WT and KO MEF cells were treated with NAC for 4 days and exposed to 250 μM H2O2 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 H2O2 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).
in TR4−/− was reduced under 4-day NAC treatment, whereas it had little effect on TR4+/+ cells (Fig. 4E, 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 H2O2 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 H2O2 formation is correlated with delayed aging and increased life span in p66Shc 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-responsiveness 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 1. Putative TR4 target genes in mouse DDR signaling**

<table>
<thead>
<tr>
<th>Gene Name (TR4-Upregulated Genes)</th>
<th>GeneBank access no.</th>
<th>Gene function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parp1</td>
<td>NM_007415</td>
<td>Base-excision repair</td>
</tr>
<tr>
<td>Rad51</td>
<td>NM_011234</td>
<td>Homologous recombination repair</td>
</tr>
<tr>
<td>Fen1</td>
<td>NM_007999</td>
<td>Other genes related to DNA repair</td>
</tr>
<tr>
<td>Polk</td>
<td>NM_012048</td>
<td>DNA polymerase ( \kappa ) (DNA directed)</td>
</tr>
</tbody>
</table>

---

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

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>GeneBank access no.</th>
<th>Gene function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR4-upregulated genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FancE</td>
<td>NM_007985</td>
<td>Oxidative stress defense</td>
</tr>
<tr>
<td>PdRx3</td>
<td>NM_007452</td>
<td>Antioxidant (peroxiredoxin)</td>
</tr>
<tr>
<td>Srxrd2</td>
<td>NM_013711</td>
<td>Antioxidant</td>
</tr>
<tr>
<td>Slc41a3</td>
<td>NM_027868</td>
<td>Antioxidant (peroxiredoxin)</td>
</tr>
<tr>
<td>TR4-downregulated genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gpx5</td>
<td>NM_010343</td>
<td>Antioxidants (glutathione peroxidases)</td>
</tr>
<tr>
<td>Gpx6</td>
<td>NM_145451</td>
<td>Antioxidants (glutathione peroxidases)</td>
</tr>
</tbody>
</table>

**FancE, faxoni anemia, complementation group C; PdRx3, peroxiredoxin 3; Srxrd2, 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 antioxidative NAC.

We identified expression level changes of certain genes in either DNA damage response signaling pathway or oxidative stress and antioxidative 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.

**DISCLOSURES**

The authors declare no conflict of interest.

**REFERENCES**


