Premature Aging in Mice Deficient in DNA Repair and Transcription

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One of the factors postulated to drive the aging process is the accumulation of DNA damage. Here, we provide strong support for this hypothesis by describing studies of mice with a mutation in *XPD*, a gene encoding a DNA helicase that functions in both repair and transcription and that is mutated in the human disorder trichothiodystrophy (TTD). TTD mice were found to exhibit many symptoms of premature aging, including osteoporosis and kyphosis, osteosclerosis, early greying, cachexia, infertility, and reduced life-span. TTD mice carrying an additional mutation in *XPA*, which enhances the DNA repair defect, showed a greatly accelerated aging phenotype, which correlated with an increased cellular sensitivity to oxidative DNA damage. We hypothesize that aging in TTD mice is caused by unrepaired DNA damage that compromises transcription, leading to functional inactivation of critical genes and enhanced apoptosis.

DNA damage, particularly oxidative lesions derived from normal metabolism, is thought to contribute to aging, but the mechanisms involved remain obscure (*1*–*4*). To counteract the effects of DNA damage, an intricate network of DNA repair pathways has evolved (*5*, *6*). One important pathway is nucleotide excision repair (NER), which removes helix-distorting damage including major ultraviolet (UV)-induced lesions, bulky chemical adducts, and some forms of oxidative damage (*7*). Xeroderma pigmentosum (XP) patients show the consequences of inherited defects in NER: sun (UV) hypersensitivity, cancer predisposition, accelerated aging of the skin, and, frequently, neurodegeneration (*8*).

Of the seven *XP* genes (*XPA*–*G*), *XPB* and *XPD* are exceptional because different mutations in these genes also cause Cockayne syndrome (CS) and a photosensitive form of the brittle hair disorder trichothiodystrophy (TTD) (*8*–*11*). TTD and CS are characterized by postnatal growth failure, progressive neurological dysfunction, impaired sexual development, skeletal abnormalities, and a strongly reduced life expectancy, but not cancer predisposition (*8*, *12*). A clue to the intriguing clinical heterogeneity linked with *XPB* and *XPD* mutations came with the discovery that these genes encode DNA helicase subunits of the transcription factor IIH (TFIIH) complex (*13*, *14*), which have dual functions: local opening of the DNA around a lesion during NER (*15*) and opening of the promoter DNA during transcription initiation (*16*). Thus, *XPB* and *XPD* mutations may not only compromise NER, causing photosensitivity, but may also affect transcription (*17*). To obtain insight into the complex pathophysiology of TTD, we generated mice carrying an *XPD* point mutation [Arg^{722}→Trp (R^{722}W)] found in TTD patients. TTD mice displayed many features of the human disease and partial defects in transcription and repair (*18*, *19*). Here, we report that TTD mice develop premature aging features caused by DNA damage.

Premature aging phenotype. Through regular observation of a large group of TTD and wild-type (wt) littermates (*20*), we noticed that TTD mice acquired an "aged" appearance beginning at ~3 months of age (Fig. 1). This, together with a shortened life-span (average <12 months, compared with >2 years for wt littermates, $P < 0.0001$), early cessation of development (see *18*), and cachectic dwarfism, prompted us to conduct a more systematic analysis of parameters indicative of premature aging.

TTD mice have brittle hair, the hallmark of TTD (*18*), that is normally pigmented (Fig.

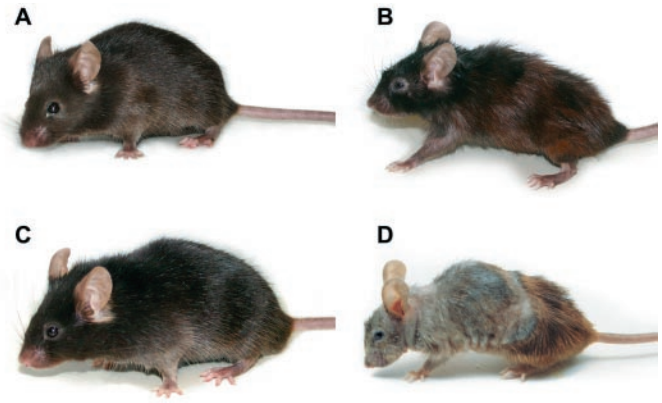

Fig. 1. TTD mice develop normally, then show a premature aging phenotype. Shown are wt (**A** and **C**) and TTD (**B** and **D**) mice at age ~3 months (A) and (B) and 15 to 16 months (C) and (D). Progeroid symptoms (cachexia and kyphosis) start to develop in TTD mice at age 3 to 4 months onward and become increasingly severe.

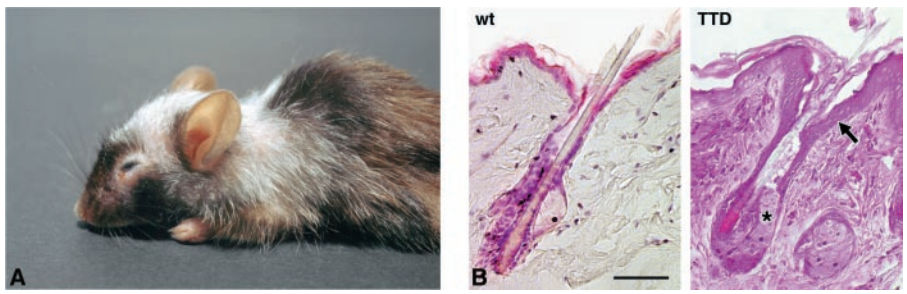

Fig. 2. Cutaneous symptoms of aging in TTD mice. (**A**) Typical example of early depigmentation in the fur of a 13-month-old black TTD mouse. No depigmentation was observed in age-matched wt mice [see table 1 in (*21*)]. (**B**) Follicular dilation and sebaceous gland hyperplasia (asterisk) in TTD compared to wt skin. Note the hyperkeratotic TTD epidermis (indicated by an arrow). The bar is ~100 μm.

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1B). However, they showed patchy depigmentation (Fig. 2A) earlier and more frequently than did wt littermates [table 1 in (*21*)]. Melanocytes were absent from grey skin patches, with foci of melanin granules in macrophages as found in normal greying (*22*). Young TTD mice also developed greasy hair and showed (benign) hyperplasia of the sebaceous gland (Fig. 2B), as observed in human aging (*23*).

The sexual behavior of most young female TTD mice appeared unimpaired (indicative of a normal hormonal status) and occasionally led to full-term pregnancy. TTD males were also fertile until at least 7 months of age [table 1 in (*21*)]; thus, initial sexual development per se is unimpaired. However, TTD females appeared to lose fertility over time, and to lose it early, because they never produced more than one litter and never after 6 months of age. TTD females ($n = 8$, age ~16 months) displayed ovarian dysfunction ranging from complete anovulation to sporadic, seemingly normal, ovulation (Fig. 3). There was no correlation between the severity of cachexia and the degree of anovulation, which suggests that the fertility defects were not due to nutritional problems. Rather, they resembled the fertility defects seen in aging rodents (*24*) and in menopausal women.

Although 2- to 4-month-old TTD mice showed no detectable skeletal abnormalities, radiographs of 14-month-old TTD mice revealed prominent kyphosis (curvature of the spinal column) (Fig. 4, A to D) and a generalized reduction in radiodensity of the skeleton, except for the skull. The mineral density of TTD vertebrae was 56% that of wt mice ($P < 0.01$), and the density of the TTD skull was 119% that of wt ($P < 0.05$, Fig. 4E). Osteosclerosis of the cranium and characteristic birdlike facies have been reported in TTD patients (*25*). The osteoporosis and concomitant kyphosis exhibited by TTD mice are hallmarks of aging in humans.

The most life-threatening symptom of TTD patients is failure to thrive, which leads to cachexia and, in turn, to a susceptibility to infections, which is a frequent cause of death (*12*). Cachexia in TTD mice was progressive, heterogeneous in onset and severity, and followed by premature death. At 6 months of age, TTD mice showed mild normochrome anemia [table 2 in (*21*)] and significantly decreased serum levels of the branched-chain amino acids (valine, leucine, and isoleucine) [table 3 in (*21*)], which is indicative of starvation (*26*). Anatomical, histological, and biochemical analysis indicated that starvation was not due to aberrant food uptake or malabsorbtion (*22*). The TTD mice did not show histological abnormalities in other vital organs such as the liver, kidney, or heart, except for an enlarged spleen, which might be related to the mild anemia.

A complete NER defect dramatically enhances the severity of the TTD phenotype. In view of the dual function of XPD, the accelerated aging features could be a result of impaired transcription, impaired NER, or a combination of the two. A DNA repair defect alone seemed unlikely because TTD patients and mice have considerable residual NER activity (*19*), whereas XP-A patients and mice, who have a complete NER defect, do not display premature aging (*8*, *27*, *28*).

To examine whether TTD aging was due to a defect in transcription, independent of NER status, we crossed TTD mice with mice carrying an *XPA* null allele (*27*). Combined homozygosity for XPA and TTD was found to be compatible with normal embryogenesis but was associated with increased neonatal lethality [table 4 in (*21*)], which suggests that the combined mutations reduced the tolerance of mice to stress. The surviving double-mutant mice exhibited a retarded but steady growth in the first 1.5 weeks, but failed to gain further weight after 2 to 3 weeks, and developed dramatically runted growth and extreme cachexia resulting in a severely shortened life-span of only 22 days ($n = 10$)

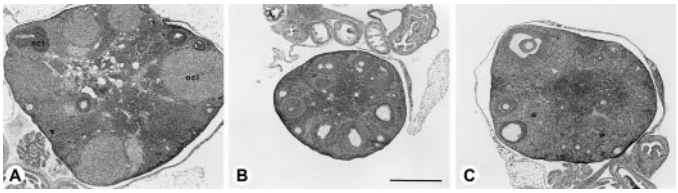
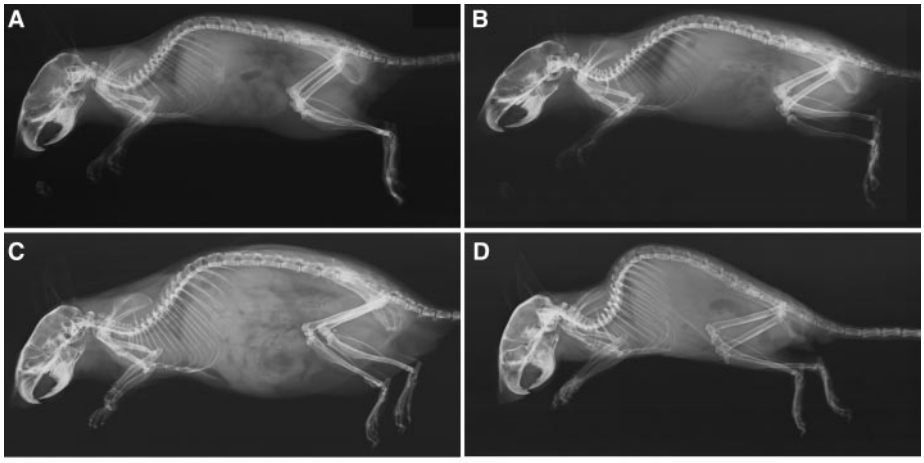


Fig. 3. Heterogeneous ovarian dysfunction in TTD females. (**A**) Ovary from a wt control animal at oestrus. Note the presence of old and new corpora lutea. (**B** and **C**) Ovaries from TTD females. Two groups of TTD animals could be discerned on the basis of ovarian histology. In the first group (three animals), no signs of an active oestrus cycle were found (B). Ovaries were very small and contained immature preantral and small antral follicles, but no preovulatory follicles. In addition, little interstitium and absent corpora lutea implied complete anovulation. In these animals, a copulatory plug was never observed, probably resulting from the absence of an oestrus cycle. (C) In the other group (five TTD females), a broad range of ovarian dysfunction ranging from complete anovulation to sporadic normal ovulation was found (note the presence of antral follicles with new corpora lutea, but absence of corpora lutea from previous cycles, indicating infrequent ovulation). Ncl, newly formed corpus luteum; ocl, old corpus luteum. The bar is 500 μm.



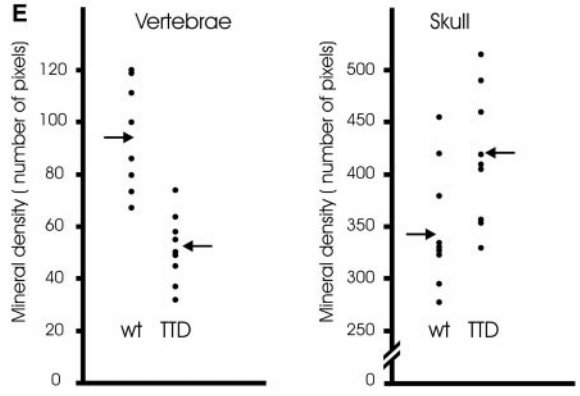


Fig. 4. Skeletal abnormalities in aging TTD mice. Radiograph of a wt (**A** and **C**) and TTD (**B** and **D**) mouse at age 2 months (A) and (B) and 14 months (C) and (D). Old, but not young, TTD mice display curvature of the spinal column (kyphosis), decreased mineral density in vertebrae and limbs, and increased mineral density of the skull. (**E**) Quantitation (*21*) of mineral density of the vertebrae and skull in 14-month-old wt and TTD mice. The average for skull and vertebral density (arrows) was significantly different from wt ($P < 0.01$). Head circumference and length of the tibia were not significantly different (*22*).

(Fig. 5). The life-span could not be extended by providing newly lactating mothers to the pups. Most double mutants showed a disturbed gait, suggesting that neurodysfunction was more pronounced than in the TTD mice, and all double mutants developed spinal kyphosis indicative of osteoporosis. Two XPA/TTD double mutants escaped juvenile death and lived to 4 and 12 months of age. Pathological analysis did not reveal defects in any organ, except for complete absence of body fat, including subcutaneous fat (Fig. 5C). The cachexia in the XPA/TTD mice resembled the progressive pathology of the TTD mutants in manifestation and as a cause of death, but it developed at a vastly accelerated rate. Surprisingly, other typical TTD characteristics were also much more pronounced in XPA/TTD mutants: excessive epidermal hyperkeratosis and severe dilation of hair follicles (Fig. 5C). This suggests that the complete absence of NER enhances the transcriptional insufficiency thought to be responsible for the cutaneous abnormalities (*18*).

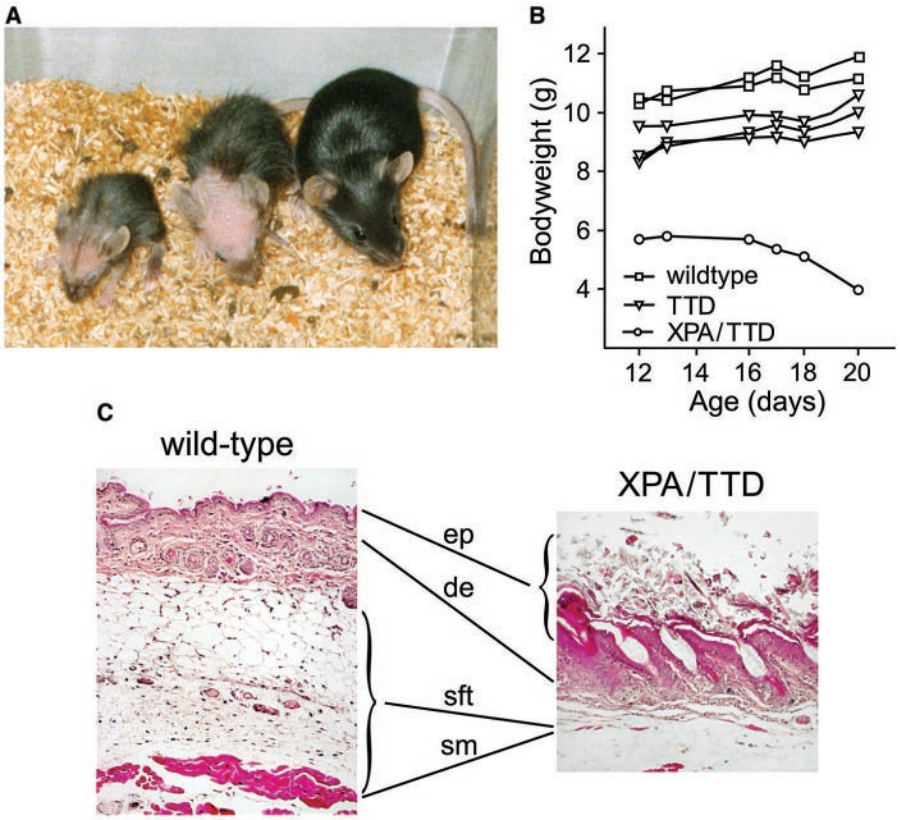


Fig. 5. Phenotype of XPA/TTD double-mutant mice. (**A**) Photograph of a 3-week-old XPA/TTD double-mutant (left), TTD (middle), and XPA (right) mouse. After normal embryogenesis, double mutants develop severe growth retardation, neurologic abnormalities, kyphosis (indicative of osteoporosis), and extreme cachectic appearance. (**B**) Body weight of wt, TTD, and XPA/TTD double-mutant littermates. Note the very mild growth delay of TTD mice and the severe growth retardation of the XPA/TTD mouse, with typical loss of body weight preceding death within ~3 weeks. (**C**) Histology of wt and XPA/TTD skin. XPA/TTD skin exhibits a dramatically enhanced phenotype of severe dilation of the hair follicles, massive hyperkeratosis, and complete absence of subcutaneous fat tissue. ep, epidermis; de, dermis; sft, subcutaneous fat tissue; sm, skeletal muscle. The bar is ~200 μm.

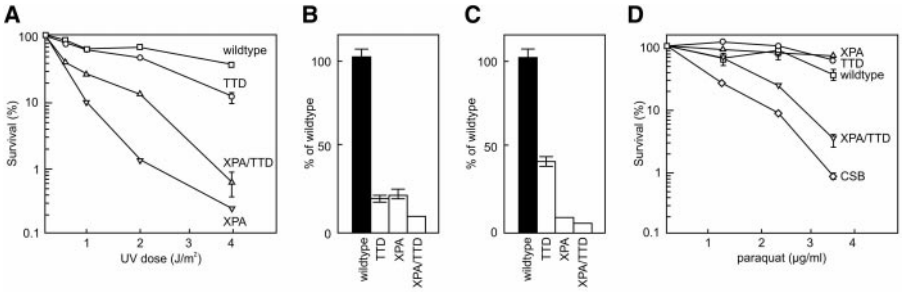


Fig. 6. DNA repair functions of MEFs from TTD, XPA, and XPA/TTD double-mutant embryos. (**A**) UV sensitivity of MEFs of the indicated genotypes. (**B**) UV-induced DNA repair synthesis and (**C**) recovery of RNA synthesis after UV-irradiation of MEFs of the indicated genotype (expressed as % of wt activity). (**D**) Hypersensitivity of XPA/TTD cells to oxidative damage. MEFs of the indicated genotype were exposed to increasing concentrations of paraquat for 3 days. Three wt and two XPA/TTD double-mutant MEF cell lines have been tested in ≥3 experiments with identical results [see (*21*) for methods].

TTD/XPA double-mutant cells are hypersensitive to oxidative stress. These findings suggest that unrepaired DNA lesions of endogenous origin aggravate the TTD symptoms in double-mutant mice. Although oxidative DNA damage is primarily –(but not exclusively) repaired by the base-excision repair pathway rather than by NER (*6*), we focused on this type of endogenous damage, because it has already been implicated in aging. As expected, experiments measuring survival, DNA repair synthesis, and the recovery of RNA synthesis after UV exposure all showed that the partial repair deficiency of TTD was converted to the total NER defect of XPA (Fig. 6, A to C). Sensitivity to oxidative injury was determined by exposure of cells to a continuous low dose of paraquat for 3 days (*21*, *29*, *30*). Although XPA and TTD single-mutant cells showed a survival curve similar to that of wt cells, XPA/TTD double mutants were clearly more sensitive (Fig. 6D), showing a survival curve similar to that of Cockayne Syndrome group B (CSB)–deficient fibroblasts, which are completely deficient in repair of transcription-blocking lesions (*7*). The TTD/XPA and CSB cells were also hypersensitive to a fractionated dose of x-irradiation (*22*). The synergistic effect of the XPA/TTD double mutant both at the organismal level and in terms of sensitivity to oxidative damage provides evidence for a causal link between DNA damage and the dramatically enhanced aging features.

Discussion. The fact that TTD mice develop normally until adulthood indicates that the phenotype we describe here is not the result of aberrant development but rather reflects bona fide aging. Because TTD is associated with several features of normal aging, it can be considered a segmental progeroid disorder (*31*). In comparison to other progeroid disorders, such as Werner, Cockayne, and Bloom syndromes (*32*, *33*), TTD is associated with much faster aging. Patients with the XPD $R^{722}W$ mutation mimicked in the mouse did not live longer than 5 years (*34*). This interpretation is reinforced by the recent description of two TTD sisters who were less than 5 years old but were described as looking prematurely aged (*35*). Moreover, many TTD symptoms overlap with those seen in CS, a well-characterized progeroid condition (*8*, *17*).

Several observations suggest that DNA is

a critical target in relation to premature aging. Telomere shortening has been implicated in the aging phenotype of highly proliferative tissues (*36*–*39*). Cells from patients with Werner, Cockayne, and Bloom syndromes display genome instability that is caused by defects in DNA helicases (*40*–*42*) similar to the XPB and XPD helicases affected in TTD. Our work on TTD mice, and particularly on XPA/TTD double-mutant mice, highlights the role of DNA damage, repair, and transcription in the onset of premature aging. Interestingly, CSB and Cockayne Syndrome group A mice, which have a defect in the repair of transcription-blocking lesions (transcription-coupled repair) but normal global genome NER (*43*), exhibit the same dramatic TTD/XPA double-mutant phenotype when crossed with XPA mutants (*22*). However, when the NER defect is incomplete—as in the cases of TTD/CSB and TTD/XPC double mutants (which have some residual repair)—the enhancement of the TTD features is less pronounced (*22*). These results suggest that the residual repair activity in TTD and in the latter double mutants can still cope with the low level of endogenous damage and thus prevent most of the deleterious effects. Thus, the level of residual repair appears to modulate the severity and rate of CS/TTD aging symptoms. In the complete absence of any NER and transcription-coupled repair, death can occur within 3 weeks.

The candidate lesions that trigger the onset of aging in TTD are most likely those that arise from endogenous insults, block transcription, and serve as substrates for NER. Although some alkylating and cross-link lesions fall into this category, we favor 5′,8-purine cyclodeoxynucleotides (*44*, *45*) and other oxidative damage (*46*). First, a causal relation between oxidative stress and aging has long been suspected. For example, caloric restriction experiments have implicated the generation of reactive oxygen species and oxidative damage derived from cellular metabolism in the pathogenesis of aging (*1*). Second, defective transcription-coupled repair of oxidative DNA injury is thought to trigger the onset of CS symptoms (*46*–*48*). Third, we find that the TTD/XPA double-mutant mouse embryo fibroblasts (MEFs) are hypersensitive to x-rays and paraquat. However, other types of DNA lesions may also be important. TTD cells are not sensitive to paraquat or x-rays, suggesting a role for other lesions in TTD aging. Endogenously generated DNA cross-links are thought to be involved in the cellular and hepatic senescence phenotype of mice deficient in the repair gene *ERCC1* (*49*), and a previously undescribed premature aging syndrome in man has been traced to mutations in the ERCC1/XPF complex (*22*). Finally, mice defective in the double-strand break-repair protein Ku86 exhibit features of early aging (*50*). Together, these observations indicate that DNA damage–induced genome dysfunction underlies the aging process.

What is the molecular mechanism underlying the premature aging in TTD mice? We propose that DNA damage persists longer and accumulates in TTD mice because the XPD mutation impairs not only global genome NER but also transcription-coupled repair of any lesions that stall elongating RNA polymerase II. Because XPD is also thought to function in removal of the blocked polymerase (*5*–*7*, *46*), the stalled RNA polymerase II complex may persist longer in TTD, in turn preventing repair (*46*, *51*, *52*). Conceivably, this would cause gene inactivation and trigger apoptosis (*53*–*55*), leading to functional decline and depletion of cell renewal capacity. Both cell death and impaired cell functioning may underlie the aging phenotype in TTD. In support of this model is recent work showing that mice expressing a hyperactive p53 mutant also exhibit accelerated aging (*56*) that is likely to be due to increased apoptosis. Obviously, any events causing gene inactivation or cell death such as telomere attrition, chromosomal instability, and increased levels of oxidative damage might accelerate aging.

In conclusion, our data strongly support the DNA damage theory of aging and suggest a significant role of transcription decay and subsequent cell death in its pathophysiology. The TTD mice may also prove to be a useful experimental model for further dissecting the molecular basis of aging.

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