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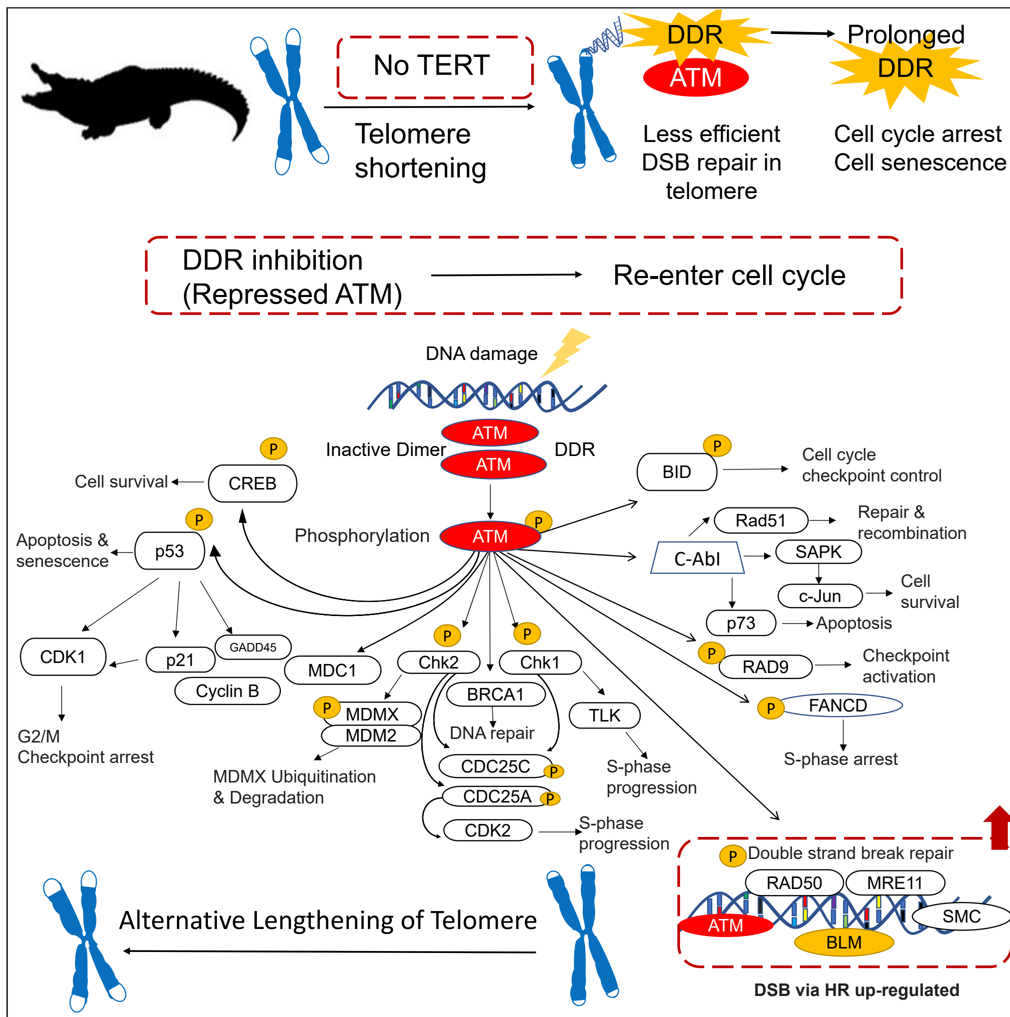

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Highlights

Alligator sinensis showed no telomere shortening; *TERT* expression is absent in adults

ATM experienced positive selection on *A. sinensis* and shared mutation ATM^{D1815N}

Repressed *ATM* expression with critical mutations suggested their fitness optimum shift

ALT-related genes were clustered in a high expression pattern in *A. sinensis*

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Alternative telomere maintenance mechanism in *Alligator sinensis* provides insights into aging evolution

Yu-Zun Guo,[1,5] Yi Zhang,[1] Qing Wang,[2] Jun Yu,[1] Qiu-Hong Wan,[1] Jun Huang,[3,4,*] and Sheng-Guo Fang[1,*]

SUMMARY

Lifespan is a life-history trait that undergoes natural selection. Telomeres are hallmarks of aging, and shortening rate predicts species lifespan, making telomere maintenance mechanisms throughout different lifespans a worthy topic for study. Alligators are suitable for the exploration of anti-aging molecular mechanisms, because they exhibit low or even negligible mortality in adults and no significant telomere shortening. Telomerase reverse transcriptase (*TERT*) expression is absent in the adult *Alligator sinensis,* as in humans. Selection analyses on telomere maintenance genes indicated that *ATM, FANCE, SAMHD1, HMBOX1, NAT10,* and *MAP3K4* experienced positive selection on *A. sinensis.* Repressed pleiotropic ATM kinase in *A. sinensis* suggests their fitness optimum shift. In ATM downstream, Alternative Lengthening of Telomeres (ALT)-related genes were clustered in a higher expression pattern in *A. sinensis,* which covers 10–15% of human cancers showing no telomerase activities. In summary, we demonstrated how telomere shortening, telomerase activities, and ALT contributed to anti-aging strategies.

INTRODUCTION

Natural selection is a process that modulates the genetics, physiology, and behavior of species, affecting many different traits; and longevity is one of them.[1–4] The disposable soma theory demonstrates that species lifespan is determined by longevity mechanisms that should be adjusted for an optimal compromise between effort in somatic maintenance and reproduction.[5–7] Low extrinsic mortality species afford more stress resistance investments that slow the intrinsic aging than high extrinsic mortality species.[8] Aging is a consequence of an increase in gene selective effect that influences the "theoretically inevitable" survival of early life and fecundity compared with those of late-life.[9] Moreover, senescence has several molecular characteristics, such as genomic instability, loss of protein homeostasis, and mitochondrial dysfunction.[10] Particularly, genomic stability of the chromosome ends, *i.e.* the telomeres, are hallmarks of aging.[11–13] The telomere is the DNA protein complex at the end of the chromosome in eukaryotic cells; telomere binding proteins together form a special "cap" structure, which has an essential role in preventing end-to-end fusion of chromosomes, maintaining the integrity of chromosomes, and controlling the cell-division cycle.[14,15] Telomere length shortens with age owing to incomplete DNA replication at its 3′ ends.[12,16,17] In addition, telomere dysfunction caused by short telomeres or telomere structural changes eventually results in chromosome instability and replicative cellular senescence.[18]

Nonetheless, a long telomere length is not consistent with long-living species. Humans have a longer lifespan with a telomere length of 5–15 kb in contrast to a shorter lifespan of mice, which have 20–50 kb of telomere length.[19,20] Of interest, a recent study revealed that telomere shortening rate is significantly related to aging, with slower telomere shortening associated with longer lifespans in various vertebrate species.[21] For example, in humans (maximum lifespan: 122.5 years), the telomere shortening rate is 70 bp/year,[22] whereas, in elephants (maximum lifespan: 65 years), flamingos (maximum lifespan: 60 years), vultures (maximum lifespan: 41.4 years), reindeer (maximum lifespan: 21.8 years), and mice (maximum lifespan: 4 years), the rates are 110, 110, 210, 530, and 7000 bp/year, respectively. In telomere maintenance, telomerase plays the role of reverse transcriptase in telomere elongation[23]; to activate or upregulate the telomerase reverse transcriptase (*TERT*) gene that encodes the catalytic component of telomerase can directly achieve telomere elongation.[24] Thus, whether telomeres actually determine the lifespan via telomere

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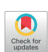

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maintenance mechanisms (TMMs) remains questionable. For example, telomere elongation via telomerase activity is known to counteract telomere attrition in human cancers and mouse somatic cells.[25–27] In the somatic cells of adult mice, telomerase activity is often high, whereas corresponding *TERT* expression levels are absent in somatic cells of humans.[28–31] *TERT* expression appears to be inactivated in long-living species such as humans with slow telomere shortening.[30,31] In contrast, active telomerase is associated with fast telomere shortening in mice.[21,27] Nonetheless, whether the telomere shortening status is critically related to TMM-related longevity in species without telomerase activity remains unclear.

The evolution of aging occurs throughout diverse taxa. For example, vertebrates like giant tortoises, alligators, whales, elephants, humans and rougheye rockfishes, do not cluster in the same evolutionary branch despite their common long-living characteristic.[9,32–34] Their life-history traits could have convergently occurred in the outstanding performance of telomere maintenance, with slow telomere shortening rates. This convergence may result from selections in the same gene or by occurrence of the same mutational changes.[35,36] Ectothermic tetrapods like giant tortoises, crocodiles, and alligators are well suited to explore their molecular mechanisms of anti-aging since they exhibit little to no relationship between fecundity and aging, and low or even negligible mortality in adults.[37–40] Furthermore, alligators are the remaining species since the earliest crocodilians appeared 240 Mya.[41,42] Their anti-aging strategies and adaptations in longevity become a fair question to explore, as they are characterized by uncertain growth patterns, little to no relationship between bone texture change versus aging, negligible mortality and remarkable adaptations in stressful environments.[43,44] In cellular senescence mechanisms, accumulated cell cycle arrest exhibits the aging-related secretory phenotype in species individuals,[45,46] whereas the processes and mechanisms in crocodilians are not fully understood.[47] Therefore, exploring telomere shortening and maintenance mechanisms in alligators provides a better understanding of their aging and critical insights for anti-aging and longevity processes. In this study, we monitored telomere shortening status and explored the TMMs in *Alligator sinensis* and representative long-living species.

RESULTS

Telomere shortening and the *TERT* expression pattern cannot explain the aging of *A. sinensis*

We first measured the relative telomere lengths (rTLs) in all blood cells at different ages of the long-living *A. sinensis* and other four related species: *Nipponia nippon*, *Colombia livia*, *Pelodiscus sinensis* and *Xenopus laevis*. rTLs of *A. sinensis* increased with the age (β = 0.312, *p*-value = 0.015; Figure 1A). On the contrary, for *N. nippon*, *C. livia*, *P. sinensis*, and *X. laevis*, the rTLs decreased significantly with age (β = -0.32 to -0.43, *p*-values < 0.05; Figures 1B–1E). Higher telomerase activities are more acknowledged at telomere elongation in cell culture experiments[25–27]; therefore, to explore whether the more efficient telomere maintenance is owing to higher telomerase activities, we further measured the *TERT* expression levels of all blood cells at different ages of all five species. *A. sinensis TERT* expression was low since birth and was lower than detectable range at seven years (Figure 1A). As expected, for *N. nippon*, *C. livia*, *P. sinensis*, and *X. laevis*, the *TERT* expression levels decreased with increasing age (Figures 1B–1E). These results suggested that although the decrease in *TERT* expression is known to explain the telomere shortening and life span, an alternative mechanism shall be responsible for the extraordinary performance of telomere maintenance in *A. sinensis* throughout their extremely long lifespan.

Telomere maintenance genes under positive selection in alligators and modern long-living taxa

Lifespan is the one of most important life-history traits that can affect the generation time, offspring number, genetic diversity, mutation load, and selective efficacy.[48–53] Therefore, lifespan is commonly thought to be limited by natural selection.[9,52] Therefore, to explore the underlying genetic mechanism implicated in aging-related natural selection, we identified signatures of positive selection on 225 protein-coding genes of 38 species representing lifespan gradients (Tables S1 and S2), using phylogenetic analysis. The maximum lifespans of species were obtained from the Anage database.[38,54] We obtained a 22-mammal phylogenetic clade and a 16-non-mammal clade (Figure 2A). In the mammal clade, the maximum lifespan ranged from 4 years for *M. musculus* to 122.5 years for *Homo sapiens*, whereas in the non-mammal clade the maximum lifespan varied from 5.5 years for *Danio rerio* to 138 years for *Terrapene carolina triunguis* (Figure 2A).

Among all 225 genes studied, we identified genes under significant positive selection at the external branches, resulting in 79 genes for the non-mammal clade and 111 genes for the mammal clade

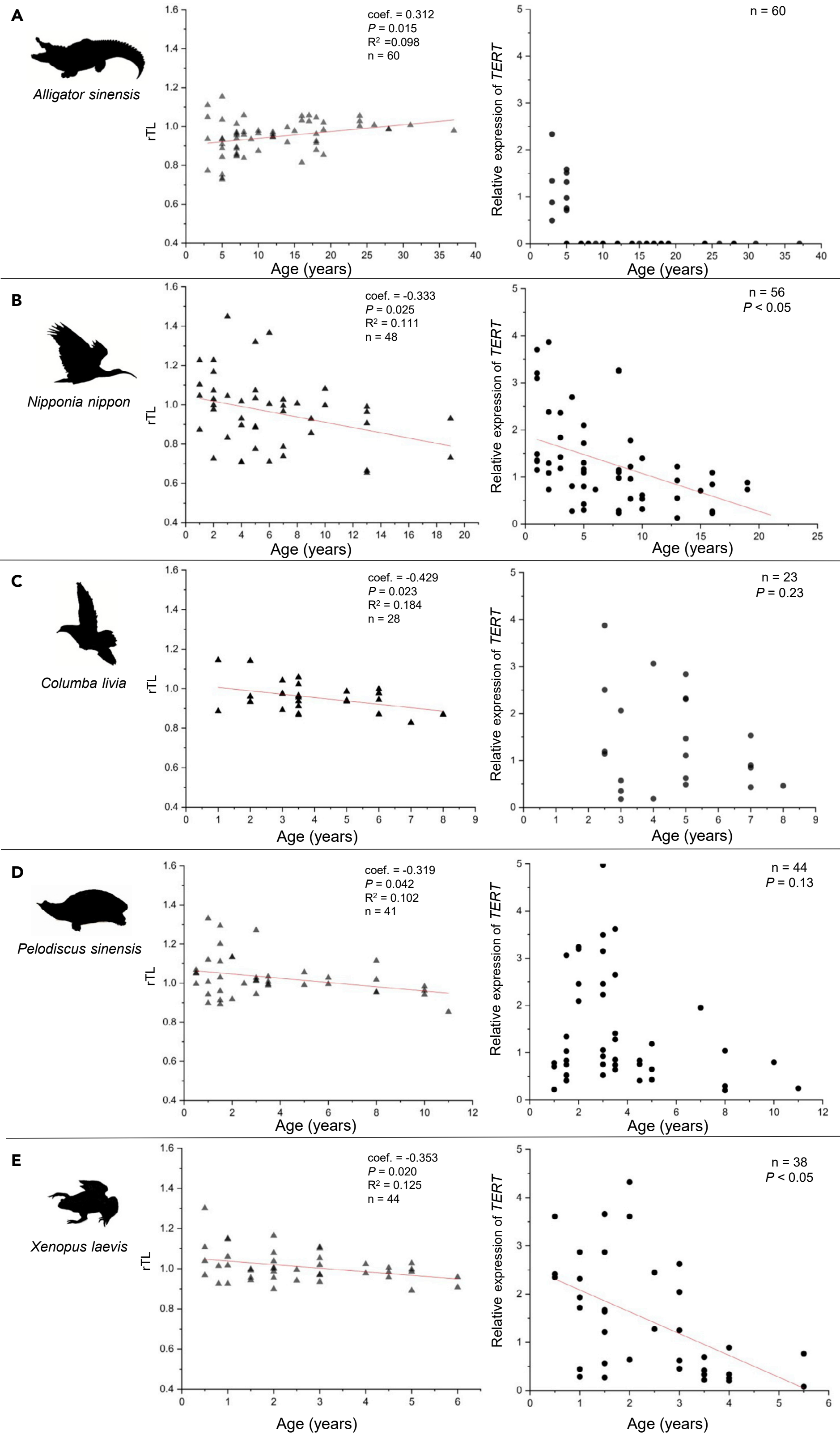
A
Alligator sinensis
coef. = 0.312
P = 0.015
R² =0.098
n = 60
rTL
Age (years)
n = 60
Relative expression of TERT
Age (years)
B
Nipponia nippon
coef. = -0.333
P = 0.025
R² = 0.111
n = 48
rTL
Age (years)
n = 56
P < 0.05
Relative expression of TERT
Age (years)
C
Columba livia
coef. = -0.429
P = 0.023
R² = 0.184
n = 28
rTL
Age (years)
n = 23
P = 0.23
Relative expression of TERT
Age (years)
D
Pelodiscus sinensis
coef. = -0.319
P = 0.042
R² = 0.102
n = 41
rTL
Age (years)
n = 44
P = 0.13
Relative expression of TERT
Age (years)
E
Xenopus laevis
coef. = -0.353
P = 0.020
R² = 0.125
n = 44
rTL
Age (years)
n = 38
P < 0.05
Relative expression of TERT
Age (years)

Figure 1. rTL measurements for *Alligator sinensis*

Nipponia nippon, *Colombia livia*, *Pelodiscus sinensis*, and *Xenopus laevis* using monochrome multiplex quantitative PCR assay (MMQPCR), and relative expression of *TERT* was measured by RT-qPCR in individuals of different ages for Chinese alligator (*A. sinensis*), (B) crested ibis (*N. nippon*), (C) rock dove (*C. livia*), (D) softshell turtle (*P. sinensis*), and (E) African clawed frog (*X. laevis*). Each point represents a different individual. Note that scales of RT-qPCR are fixed for clearer observation of variations.

(Tables S3 and S4). Particularly, six genes were found to experience positive selection in *A. sinensis*: *ATM*, *FANCE*, *SAMHD1*, *HMBOX1*, *NAT10*, and *MAP3K4*; and 13 genes in *Alligator mississippiensis*: *BLM*, *RFC5*, *PRKCQ*, *MAPK3*, *MEIOB*, *PRKDC*, *NABP2*, *PRKCA*, *CBX3*, *TNKS1BP1*, *FANCE*, *CHEK1* and *ERCC1*. Some identified genes were related to double-strand break repair (DSB), such as *ATM* and *BLM*. To explore whether these selected genes are related to large lifespan species, we sorted 106 telomere maintenance genes commonly under positive selection among three or more species based on their median values of the maximum lifespan across the species harboring these genes. Eleven genes putatively experienced positive selection based on the top 10% median values of the maximum lifespan's distribution (>61 years; Table S5), such as the ataxia telangiectasia mutated (*ATM*) in *A. sinensis*, *Gorilla gorilla*, *Tursiops truncatus*, *Chrysemys picta bellii*, and *Varanus komodoensis*; meiosis-specific cohesin subunit SA3 (*STAG3*) in *Balaenoptera musculus*, *Loxodonta africana*, *Macaca mulatta*, and *Equus caballus*; chromobox protein homolog 3 (*CBX3*) in *A. mississippiensis*, *C. abingdonii* and *V. komodoensis*; and DNA-dependent protein kinase catalytic subunit (*PRKDC*) in *A. mississippiensis, C. abingdonii*, *V. komodoensis* and *C. picta bellii* (Figure 2B).

Conserved mutations among long-living species potentially change protein activity

Genetic selection can increase the frequency of positive mutation; therefore, we further evaluated point mutations present in the telomere maintenance genes under positive selection. We found 73 amino acid replacements were identified in *Alligator* spp. among ATM, PRKDC, protein kinase C theta (PRKCQ), *CBX3*, chaperonin containing TCP1 subunit 2 (CCT2), and tankyrase 1 binding protein (TNKS1BP1) (Table S6). These genes play a common role in DSB repairs, whereas ATM,[55,56] PRKDC,[57,58] and TNKS1BP1[59] are serine and threonine kinases for DNA damage response (DDR). CCT2 is a chaperonin-containing T-complex (TRiC) component that mediates the folding of WRAP53/TCAB1, subsequently regulating telomere maintenance.[60]

To further explore whether the conservation of these amino acids changes protein chemical properties, Sorting Intolerant From Tolerant (SIFT)[61,62] was used to test whether these substitutions affected protein functions based on UniRef90.[63] We found that most substitutions, such as D1815N, S1816G and T3031I in ATM and S904G, S3229A and V3961T in PRKDC (Figure 3) of *A. sinensis* and extreme long-living species, were predicted to affect protein functions (Scores < 0.05) (Table S7). In addition, the mutations $CCT2^{D177N}$ in *Alligator* spp. and giant turtles, and $CCT2^{Q380K}$ in *Alligator* spp. may increase the capability of chromatin- and DNA-binding when the hydrophilic amino acid residues are presented on protein surfaces. In *Alligator* spp. and *C. abingdonii*, the ATM replaced serine or threonine in S1816G and T3031I, respectively (Figure 3), were notable substitution sites, substitutions at which potentially decrease serine- or threonine-protein kinase activities.[64,65] S904G, S3229A and V3961T mutations in PRKDC kinase and $PRKCQ^{A536S}$ in *Alligator* spp. (Figure 3) may affect protein kinase activities. Notably, the ATM^{D1815N} mutation was commonly shared by long-living mammals and non-mammals, covering *Alligator* spp., *B. musculus, Orcinus orca, T. truncatus*, *C. picta bellii* and *T. carolina triunguis*. Ancestral state inference of ATM^{D1815N} indicated that at the 1815 site the ancestral amino acid was aspartic acid, whereas asparagine was presented in modern species of extreme long-live (Figure 4A). The replacements of asparagine residues possibly change the local electrostatic field upon ATM protein surface. The aspartic acid residue with negatively charged side chain (pI = 2.77) was replaced by asparagine, a hydrophilic amino acid residue (pI = 5.41), which may increase nucleotide-binding efficiency (Figure 4B).

ATM expression is repressed, but Alternative Lengthening of Telomeres (ALT) related genes were clustered in a higher expression pattern in *A. sinensis*

To explore expression patterns of ATM in *A. sinensis*, we sorted *ATM* expression patterns within a normal distribution of total expression in *A. sinensis,* and compared them with those of *N. nippon, C. livia, X. laevis* and *P. sinensis*. The RNA samples were extracted from whole blood cells around the sexual maturity time of each species (see STAR methods). *ATM* expression distributions in *A. sinensis* were less than 10% (logged FPKM = -0.720; *p*-value = 0.071) within the whole expressed genes, which is lower than that in *N. nippon*

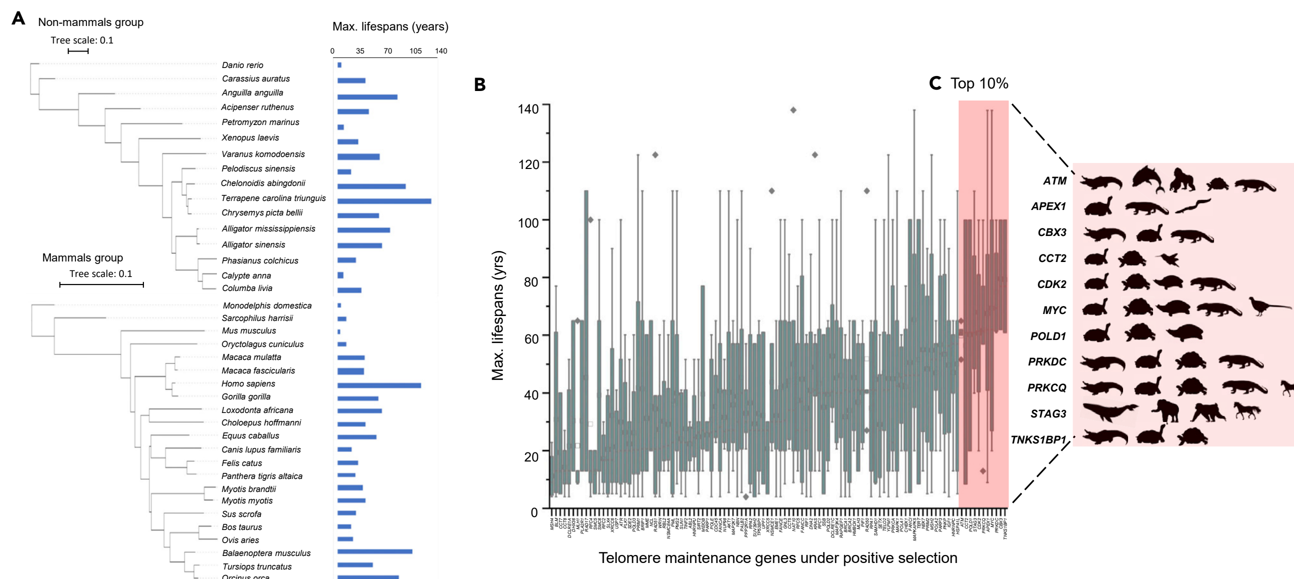

Figure 2. Positive selection analyses of telomere maintenance-related genes

(A) Phylogenetic relationships for selective pressure analyses in telomere maintenance genes among 38 species, including reptiles, birds, amphibians, fish and cyclostomes (clade one); and mammals (clade two). Maximum lifespans of each species are shown in the bar chart.

(B) Distributions of 106 telomere maintenance genes under selection by the median values of the maximum lifespan distribution across the species, in which the external branch exhibited significant signatures of positive selection. Boxes order follows median values of the maximum lifespan from low to high. (C) The top 10 percent of median values of maximum lifespan distribution are highlighted in red, showing identified genes and related species.

(logged FPKM = 0.801; *p*-value = 0.485), *C. livia* (logged FPKM = -0.131; *p*-value = 0.238), *P. sinensis* (logged FPKM = 0.378; *p*-value = 0.386), and *X. laevis* (logged FPKM = -0.5; *p*-value = 0.137), the four TERT positive species (Figure 5B). *ATM*, as a pleiotropy gene, regulates multiple signaling pathways covering DSB repair, cell cycle checkpoint, apoptosis and senescence (Figure 5A).[66–68] Repressed *ATM* expression in *A. sinensis* together with critical mutations in ATM conservative regions suggested their fitness optimum shift.[69] Clustering analysis of the expression of telomere maintenance genes indicated that the genes thought to be required for alternative lengthening of telomeres (ALT) were clustered in a higher expression pattern; for example, *BLM*, *SMC6*, *FANCA* and *TPP1* were the only high expression cluster in *A. sinensis* (Figure 5C). In ATM downstream pathways, BLM RecQ helicase and structural maintenance of chromosomes 6 (SMC6) are required ALT proteins for homologous recombination. Upregulated BLM RecQ helicases were correlated with ALT by DSB repair via homology recombination[70] whereas Fanconi anemia group A (FANCA) are required for the recombination-dependent restart of stalled telomeric DNA replication.[71] TPP1 is part of POT1-TPP1 telomere complex and stabilizes POT1, promoting efficient telomere maintenance as a component of the telomere shelterin complex.[72,73] ALT was found in 10–15% of human cancers in contrast to 85–90% TERT-positive cancers.[74,75]

DISCUSSION

Telomere dynamics are related to aging, but telomere length depends on diverse factors, including telomerase activity, genetic factors, hormone characteristics reflecting gender, environmental factors such as UV-radiation and oxidative stress, exposure to diseases and socio-ecological variables that may be used as bio-markers of aging and stress exposure.[76–80] Large variations of telomere length usually occur in the early-life of species, which may become a driver for natural selection.[81] In this case, more sensitive and younger individuals may have different telomere lengths and maintenance dynamics, and these individuals may not be fully captured owing to premature death compared with other individuals in the same population, which underlines the difficulty of having relevant patterns in the comparison between species. Therefore, we measured the telomere length together with the evaluation of the telomere maintenance ability of species, such as telomerase activity or ALT to link telomere maintenance and longevities. Although telomerase is commonly known to elongate telomeres, *A. sinensis* obviously has a different TMM as the *TERT* expression was below detection levels in the adult stage and no significant telomere shortening was observed. Similar results were also reported in *Mus musculus* and humans; whereas the telomere

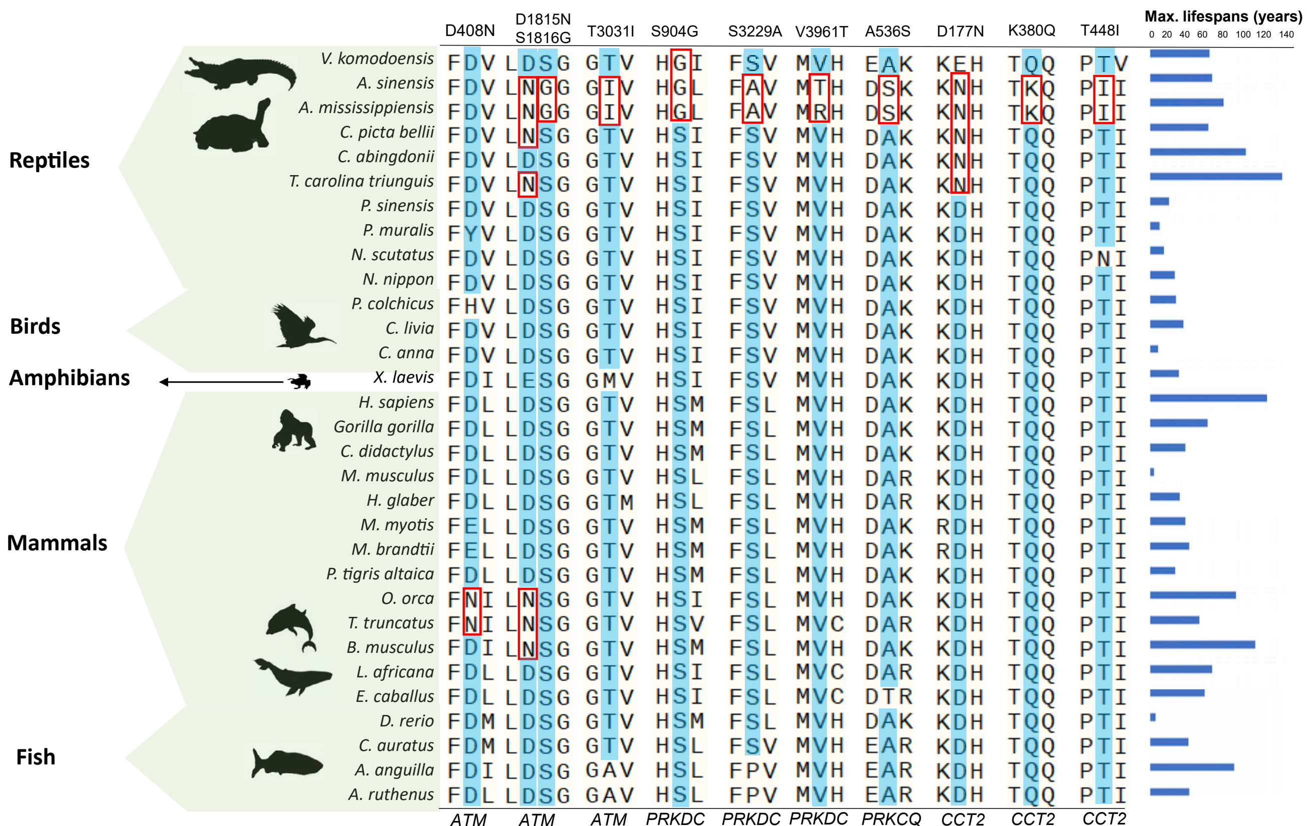

Figure 3. Sequence alignment of ATM

PRKDC, PRKCQ and CCT2 in *Alligator* spp. and other representative species of reptiles, birds, amphibians, fish and mammals. Notable amino acid replacements (chemical property change) were marked with red rectangles.

shortening rate was 100-fold higher in *M. musculus* than in humans,[20,22] no expression of *TERT* could be detected in the adult stage of the latter.[23,28,29] Telomerase is active during early human development, but transcriptional silencing occurs between 12 and 18 weeks of gestation.[30,31] ATM could play an essential role in long-living species and was found to experience positive selection in *A. sinensis* and many long-living taxa (longevity > 61 years). DSB repair pathways may be strongly related to telomere maintenance without telomerase.[55,82] For example, the TMM of 85–90% of human cancer cells is via telomerase,[83–85] but 10–15% of cancers show no telomerase activities, and their TMM via ALT[74,75,86] and is achieved by DSB repair via homology recombination.[71,84,87] The chief mobilizers that activate DSB repair signaling, the ATM kinase, and the assembled double-strand repair proteins, MRE11 and RAD50[55,56] are potentially involved in DSB repair via homology recombination. The same ATM^{D1815N} mutation occurred in extremely long lifespan groups of marine mammals, giant tortoises and *Alligator* spp. In this case, *A. sinensis* and other long-living species with a similar longevity phenotype resulted from the same genetic change through parallel evolution.[35,36] Independently evolved long-lived species have similar traits and genetic adaptations of anti-aging mechanisms, such as TP53 in anti-cancer mechanisms, IGF1 in metabolism, and DSB repair mechanisms.[9,32,88,89] We further suggested that telomere maintenance via the DSB repair mechanism in long-lived vertebrates was strongly related to their extraordinary telomere maintenance.

ATM as a pleiotropy gene acts at the center of multiple downstream pathways and modulates many ATM-dependent pathways covering DSB repair, checkpoint arrest, cellular senescence, and apoptosis.[68,90,91] Genetic adaptations in pleiotropy are expected to be strongly stabilizing because it influences many separate fitness optima.[92,93] In contrast to *ATM* expression in the other four representative species, the *ATM* of *A. sinensis* expressed in a down-regulated level together with critical mutations in conservative sequence regions, which reflects their fitness optimum shift, and could dominate the benefits throughout the multiple downstream pathways, such as anti-aging mechanisms and stabilizing their telomere lengths. In *ATM*

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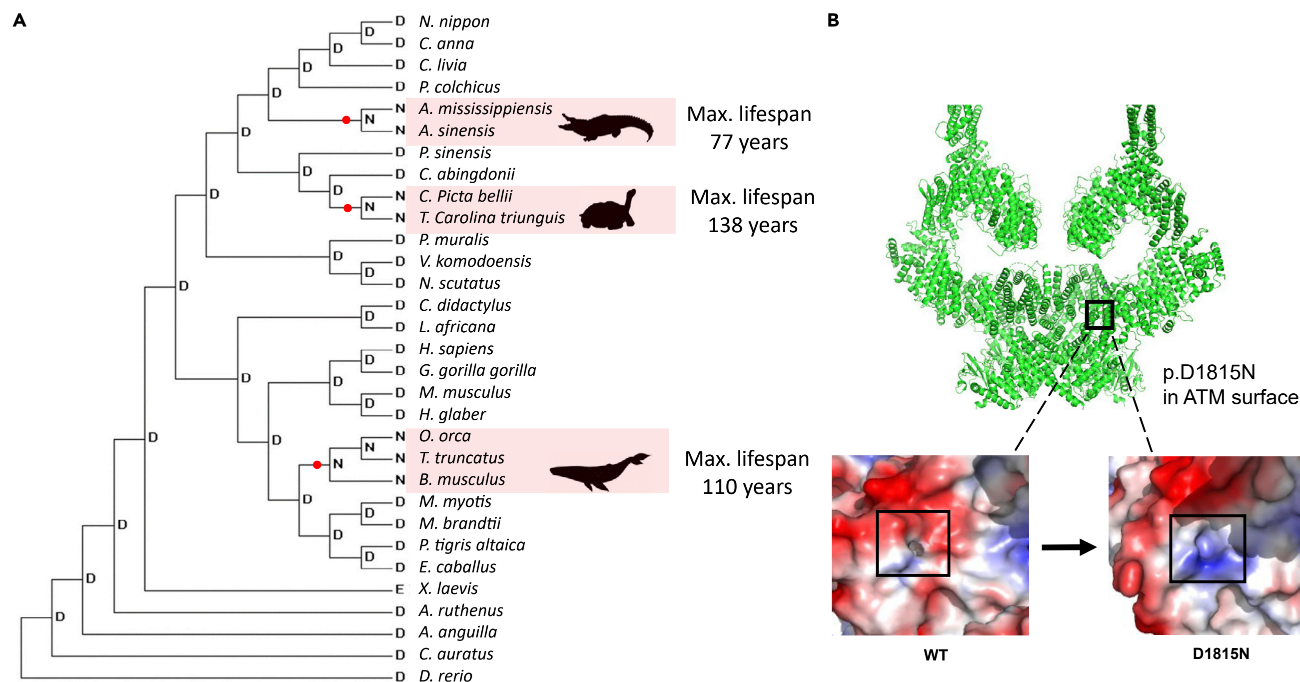


Figure 4. ATM^{D1815N} evolution and protein function analyses

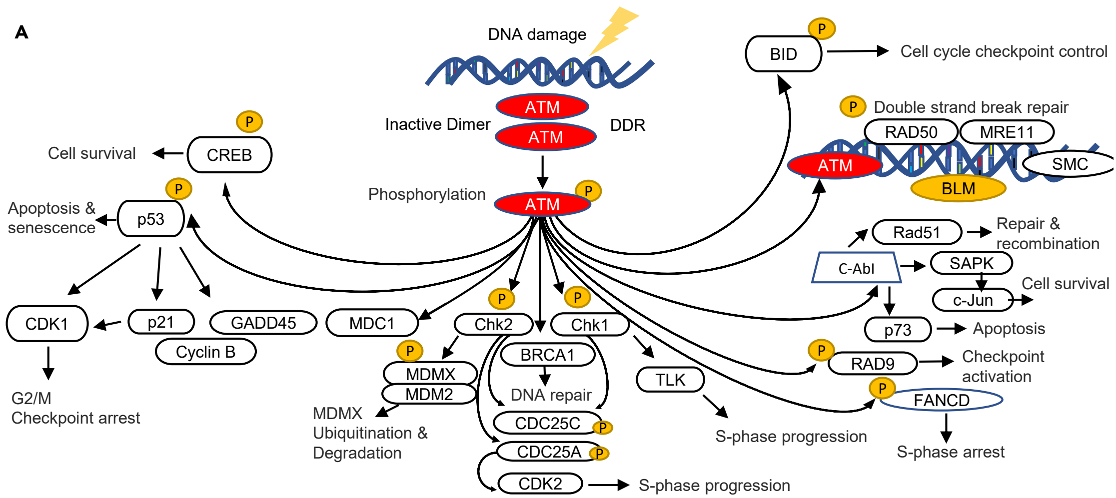
(A) Ancestral ATM^{D1815N} state reconstruction in *Alligator* spp., extremely long-living tortoise, marine mammals and representative species of reptiles, birds, amphibians, fish and mammals.

(B) Notable structural variances ATM protein surface upon ATM^{D1815N} mutation. The *A. sinensis* ATM predicted electrostatic surfaces based on the homology model of the D1815N residues are highlighted. Negatively charged areas are depicted in red, whereas positively charged areas are depicted in blue. The positions of variants ATM^{D1815N} were marked on the ATM homology model of *A. sinensis*.

downstream, the up-regulated *BLM* suggested that the enhancement of homology recombination plays an essential role in TMMs of *A. sinensis*.

How does ATM kinase affect senescence? Researchers have shown that telomere shortening caused by telomere protective structure or capping factor loss is similar to the one-ended DSBs mechanism and causes DNA damage response (DDR).[94,95] ATM is a DDR signal kinase that activates DSBs repair signaling and assembles double-strand repair proteins.[55,56] However, DSB repair is very less efficient when DDR occurs in telomeres and triggers persistent DDR.[96–98] Prolonged DDRs in telomeres are usually correlated with cell senescence.[99,100] In contrast, inhibiting DDR signal kinases (ATM, ATR, CHK1, and CHK2) causes senescent cells to re-enter the cell cycle.[94,100,101] In contrast to other representative species, the down-regulated expression of ATM kinase in *A. sinensis* may strongly suggest that their anti-aging mechanism was related to inhibition of DDR signal kinases to prevent accumulated cell cycle arrest.[64,65] Extremely long-living reptiles, such as *Alligator* spp., *V. komodoensis* and giant tortoises may affect the protein kinase activities by replacing serine or threonine, such as in S1816G and T3031I of ATM or S904G and S3229A of PRKDC, which potentially reduce phosphorylation levels of these DDR signal kinases.

On the other hand, why does *A. sinensis* not activate TERT to counteract telomere attrition? DNA repair mechanisms are highly adapted in long-living species, which may be because of their extraordinary telomere maintenance. These species have improved mechanisms to avoid the telomere shortening effects of environmental factors, such as ultraviolet irradiation and oxidative stress, whereas these mechanisms are not present in short-living species.[102–105] For instance, long-living primates can repair UV-induced damage, whereas long-living rodent species show higher DNA repair rates than short-living rodents.[106] Thus, short-lived species have less efficient DNA repair mechanisms; therefore, telomerase activities may become necessary to slow down their fast telomeres shortening. On the other hand, long-living species show anti-cancer adaptations.[107–109] High telomerase activities might not be an advantageous TMM for long-living species and can provide extra opportunities for tumor cell activation.[85,110,111] Downregulated *TERT* expression is typically observed in long-living birds to protect against tumor development.[112] In


A
DNA damage
ATM
ATM
Inactive Dimer
DDR
Phosphorylation
ATM
P
BID
Cell cycle checkpoint control
Double strand break repair
RAD50
MRE11
ATM
SMC
BLM
Cell survival
CREB
Apoptosis &
senescence
p53
Rad51
Repair &
recombination
C-Abl
SAPK
Cell survival
c-Jun
CDK1
p21
GADD45
MDC1
Chk2
Chk1
p73
Apoptosis
Cyclin B
MDMX
MDM2
BRCA1
DNA repair
RAD9
Checkpoint
activation
TLK
G2/M
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FANCD
S-phase progression
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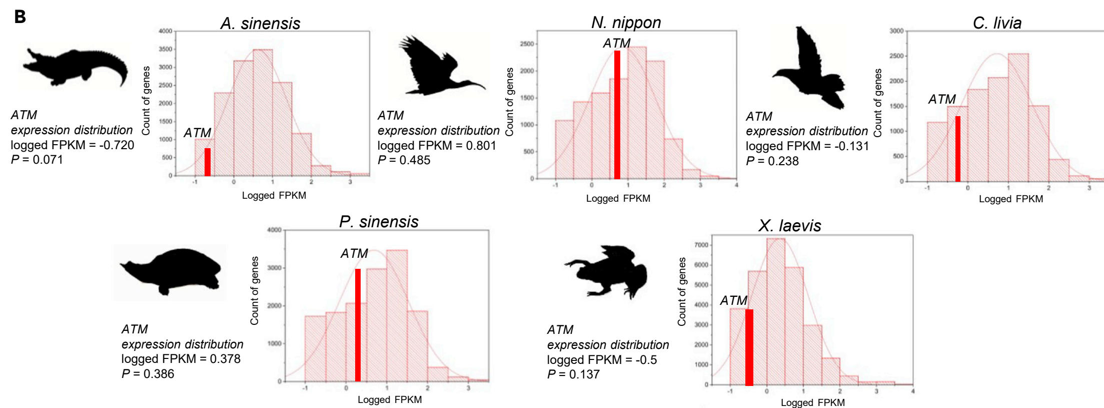
S-phase progression


B
A. sinensis
ATM
expression distribution
logged FPKM = -0.720
P = 0.071
N. nippon
ATM
expression distribution
logged FPKM = 0.801
P = 0.485
C. livia
ATM
expression distribution
logged FPKM = -0.131
P = 0.238
P. sinensis
ATM
expression distribution
logged FPKM = 0.378
P = 0.386
X. laevis
ATM
expression distribution
logged FPKM = -0.5
P = 0.137
Count of genes
Logged FPKM

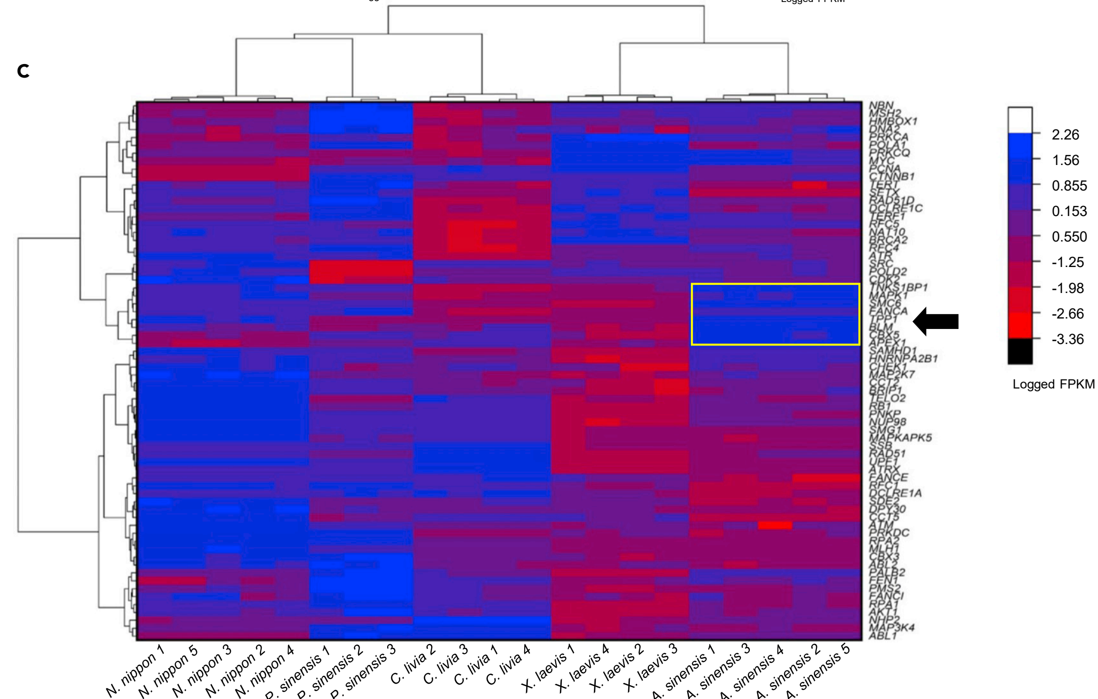

C
2.26
1.56
0.855
0.153
0.550
-1.25
-1.98
-2.66
-3.36
Logged FPKM
N. nippon 1
N. nippon 5
N. nippon 3
N. nippon 2
N. nippon 4
P. sinensis 1
P. sinensis 2
P. sinensis 3
C. livia 2
C. livia 3
C. livia 1
C. livia 4
X. laevis 1
X. laevis 4
X. laevis 2
X. laevis 3
A. sinensis 1
A. sinensis 3
A. sinensis 4
A. sinensis 2
A. sinensis 5

Figure 5. *ATM* expression distribution in relation to other telomere maintenance genes

(A) ATM-dependent pathways involved in infrared radiation (IR)-induced DNA damage, covering DSB repair, cell cycle checkpoint, apoptosis, and senescence.

(B) Distribution of log10 FPKM values in *ATM* based on the whole RNA-seq data of *A. sinensis, N. nippon, C. livia, P. sinensis*, and *X. laevis.*

(C) Gene expression clustering analysis of telomere maintenance genes in log10 FPKM values among *A. sinensis, N. nippon, C. livia, P. sinensis*, and *X. laevis.*

most common human cancers, somatic mutations in the proximal promoter region of human *TERT* are thought to be noncoding.[113–117] Therefore, dominant *TERT* inhibition could be an anti-tumor adaptation in the TMM evolution of extreme long-living species.

Limitations of the study

As cross-sectional studies only reveal individual differences, a telomere shortening trend was not observed in long-living species possibly because of the limited scale of ages of individual samples. The maximum lifespans of species are much higher than their average lifespans, but the scarcity of older individuals limited the sample size in this study.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - DNA extraction, MMQPCR, and relative telomere length (rTL) analysis
  - RNA extraction, cDNA library construction, and sequencing
  - RNA-seq analysis
  - Telomerase reverse transcriptase (TERT) gene expression
  - Phylogenetic, evolutionary, and structural analyses of telomere maintenance genes
- QUANTIFICATION AND STATISTICAL ANALYSIS
- ADDITIONAL RESOURCES

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105850.

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DECLARATION OF INTERESTS

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Raw data (RNA-seq) of *Alligator sinensis 1*	This paper.	Sequences data accession No.(SRA) SRX18535475;
Raw data (RNA-seq) of *Alligator sinensis 2*	This paper.	Sequences data accession No.(SRA) SRX18523405;
Raw data (RNA-seq) of *Alligator sinensis 3*	This paper.	Sequences data accession No.(SRA) SRX18523014;
Raw data (RNA-seq) of *Alligator sinensis 4*	This paper.	Sequences data accession No.(SRA) SRX18521814;
Raw data (RNA-seq) of *Alligator sinensis 5*	This paper.	Sequences data accession No.(SRA) SRX18521813
Raw data (RNA-seq) of *Nipponia nippon 1*	This paper.	Sequences data accession No.(SRA) SRX18547918;
Raw data (RNA-seq) of *Nipponia nippon 2*	This paper.	Sequences data accession No.(SRA) SRX18545429;
Raw data (RNA-seq) of *Nipponia nippon 3*	This paper.	Sequences data accession No.(SRA) SRX18544770;
Raw data (RNA-seq) of *Nipponia nippon 4*	This paper.	Sequences data accession No.(SRA) SRX18537049;
Raw data (RNA-seq) of *Nipponia nippon 5*	This paper.	Sequences data accession No.(SRA) SRX18535756.
Raw data (RNA-seq) of *Columba livia* 1	This paper.	Sequences data accession No.(SRA) SRX18637365;
Raw data (RNA-seq) of *Columba livia* 2	This paper.	Sequences data accession No.(SRA) SRX18634217;
Raw data (RNA-seq) of *Columba livia* 3	This paper.	Sequences data accession No.(SRA) SRX18633397;
Raw data (RNA-seq) of *Columba livia* 4	This paper.	Sequences data accession No.(SRA) SRX18632104
Raw data (RNA-seq) of *Pelodiscus sinensis 1*	This paper.	Sequences data accession No.(SRA) SRX18631083
Raw data (RNA-seq) of *Pelodiscus sinensis 2*	This paper.	Sequences data accession No.(SRA) SRX18549066
Raw data (RNA-seq) of *Pelodiscus sinensis 3*	This paper.	Sequences data accession No.(SRA) SRX18549036
Raw data (RNA-seq) of *Xenopus laevis 1*	This paper.	Sequences data accession No.(SRA) SRX18637368
Raw data (RNA-seq) of *Xenopus laevis 2*	This paper.	Sequences data accession No.(SRA) SRX18637570
Raw data (RNA-seq) of *Xenopus laevis 3*	This paper.	Sequences data accession No.(SRA) SRX18661685
Raw data (RNA-seq) of *Xenopus laevis 4*	This paper.	Sequences data accession No.(SRA) SRX18641864

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
Primers for GC-clamp *GAPDH* in for MMQPCR, see Table S10	This paper	N/A
Primers for *TERT* and *GADPH* primers for qRT-PCR see Table S12	This paper	N/A
Software and algorithms		
Origin version 2018	Moberly et al.[135]	N/A
Prank	Löytynoja et al.[128]	N/A
Snapgene version 6.1.2	https://www.snapgene.com/	
RAxML version 8	Stamatakis et al.[130]	N/A
Gblocks version 0.91b	Castresana et al.[129]	N/A
Sorting Intolerant from Tolerant (SIFT)	Ng and Henikoff[61]; Sim et al.[62]	N/A
Reconstruct Ancestral State in Phylogenies (RASP)	Yu et al.[133]	N/A
Other		
TRIzol LS reagent extraction kit	Invitrogen, Carlsbad, CA, USA	2107B
NEBNext Ultra Directional RNA Library Prep Kit for Illumina	New England Biolabs, Ipswich, MA, USA	E7420
TaKaRa PrimeScript RT Reagent Kit	Takara,shiga,Japan	RR037A
TB Green® Premix	Takara,shiga,Japan	RR820B (A × 2)
CFX Manager Real-time PCR System	Bio-Rad Laboratories, Hercules, CA, USA	1855201
NanoDrop 8000 Spectrophotometer	Thermo Fisher Scientific	ND-8000-GL

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yu-Zun Guo, email: yuzun78@163.com.

Materials availability

This study did not generate new unique reagents.

Data and code availability

RNA-seq data have been deposited at Sequence Read Archive (SRA) data in NCBI and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The total blood cells of 221 individuals were sampled for rTL analyses, and that of another 221 individuals were sampled for *TERT* expression analyses. These individuals were from *A. sinensis* and four representative species (*N. nippon*, *C. livia*, *P. sinensis* and *X. laevis*). Blood samples from *A. sinensis* were obtained from the Changxing Yinjiabian Chinese Alligator Nature Reserve, China. Individuals from 0–7 years of age were reared in separate ponds. At seven years of age, individuals implanted with microchips were released into the main ponds; therewith, the accurate age of elderly individuals was known. For *N. nippon*, blood samples were collected from the Xiaozhu Lake Crested Ibis Breeding Base, Deqing County, China. Microchips were attached to individuals at a known age before release into the wild; therefore, their accurate ages were known after the recapture of elderly individuals. *C. livia*, *P. sinensis* and *X. laevis* were provided by animal farms (Tables S8 and S9) with legal breeding licenses, as permitted by the Animal Ethics Committee of Zhejiang University, China. The keepers identified the individuals' ages. All blood samples were collected after obtaining ethical approval and permission from the Animal Ethics Committee of Zhejiang University, China (ZJU20200142).

All blood samples were stored immediately in liquid nitrogen for further DNA and RNA extractions. Blood cells were collected for transcriptome analyses from the individuals around their sexual maturity. *A. sinensis* samples were collected from seven-year-old individuals (five individuals: one male and four females, female sexual maturity: 2191 days), *N. nippon* from three-year-old individuals (five individuals: three males and two females, sexual maturity: 2–4 years), *C. livia* from five-month-old individuals (four individuals: two females and two males, sexual maturity: 140 days), *P. sinensis* from four-year-old individuals (three individuals: one male and two females, sexual maturity: 4 years), and *X. laevis* from six-month-old individuals (four individuals: one male and three females, female sexual maturity: 183 days). All blood samples were collected after obtaining ethical approval and permission from the Animal Ethics Committee of Zhejiang University, China (ZJU20200142).

METHOD DETAILS

DNA extraction, MMQPCR, and relative telomere length (rTL) analysis

Genomic DNA of whole blood cells was extracted using a GENEray DNA extraction Kit (Shanghai, China), following the manufactures instructions. Telomeres from blood cells were evaluated to account for differences in telomere lengths in multiple cell types, as Demanelis et al.[12] revealed that telomeres from blood cells can effectively reflect the individual telomere status. Sample quality was assessed by electrophoresis on 1% agarose gels. DNA concentrations were measured using a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and standardized to 20 ng/μL for use in the monochrome multiplex quantitative PCR (MMQPCR).

The rTL was measured using the MMQPCR method as described by Cawthon,[118] and referring to Morinha's guidelines for the telomere qPCR method.[119] The ratio of the telomeric sequence to a single-copy gene is the T/S value, which was assayed in the same plate to reduce measurement error during qPCR analysis. DNA samples were assayed using TB Green Premix (TaKaRa, Shiga, Japan) and telomere primers telg (5′-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3′) and telc (5′-TGTTAGGTATCCCTAT CCCTATCCCTATCCCTATCCCTAACA-3′). The single-copy gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) primers with GC-clamp (Table S10) were used as control. All primers were diluted to 900 nM. Melt-curve analysis showed no primer-dimer detected. MMQPCR was performed in an initial reaction volume of 20 μL, containing 10 μL of TB Green Premix (TaKaRa), 6 μL nuclease-free water, 1 μM forward and reverse primers, and 2 μL DNA (the negative controls with 2 μL ribonuclease-free water). MMQPCR was performed on a CFX Manager Real-time PCR System (Bio-Rad Laboratories, Hercules, CA, USA) with the following protocol: 95 °C for 15 min; 2 cycles at 94 °C for 15 s and 49 °C for 15 s; and 32 cycles at 94 °C for 15 s, 62 °C for 10 s, 74 °C for 15 s (for telomere amplification), or at 84 °C for 10 s and 88 °C for 15 s (for GC-clamped single-copy gene amplification). The reference samples' serial dilutions (5 × dilutions, from 100 to 0.032 ng/μL) were used to generate standard curves to measure amplification efficiency. A sample containing 20 ng/μL DNA was used for calibration. Each experimental sample was assayed three times; therefore, the average of three T/S values became the rTL of the sample (coefficient of variation < 0.05; otherwise, the data were excluded from analyses). CFX Manager (Bio-Rad 3.1 Standard Edition Optical System Software) was used to export raw data, and LinRegPCR[120] was used to calculate amplification efficiencies. The reaction efficiencies of each species are listed in Table S11. Values for rTL were calculated as described by Pfaffl.[121]

RNA extraction, cDNA library construction, and sequencing

Total RNA extraction was performed using a TRIzol LS reagent extraction kit (Invitrogen, Carlsbad, CA, USA). Each blood sample (30 μL) was mixed with 1 mL TRIzol LS reagent and subjected to a 30 min vortex, and following procedures were performed accordingly to the manufacturer's instructions. RNA quality was assessed by electrophoresis on 1% agarose gels. RNA concentrations were quantified using a NanoDrop 8000 Spectrophotometer. RNA from *A. sinensis* samples were collected from seven-year-old individuals (five individuals, female sexual maturity: 2191 days), *N. nippon* from three-year-old individuals (five individuals, sexual maturity: 2–4 years), *C. livia* from five-month-old individuals (four individuals, sexual maturity: 140 days), *P. sinensis* from four-year-old individuals (three individuals, sexual maturity: 4 years), and *X. laevis* from six-months-old individuals (four individuals, female sexual maturity: 183 days) that were selected for further complementary DNA (cDNA) library construction and sequencing. Briefly, 3 μg RNA of each sample was used for strand-specific cDNA library construction using a NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA), according to the manufacturer's protocol. Library quality was checked using a Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA).

Clustered and index-coded samples were generated using a TruSeq PE Cluster Kit v3-cBot-HS on a cBot Cluster Generation System (Illumina, San Diego, CA, USA). Library sequencing was performed on an Illumina Novaseq platform, and 150 bp paired-end reads were generated. Raw reads in fastq format were pre-processed using in-house Perl scripts available with Biomarker Technologies Co. Ltd. (Beijing, China). Adapters, poly-N sequences, and low-quality reads were removed from analysis before calculating the Q20, Q30, and GC contents of the remaining reads for subsequent analyses.

RNA-seq analysis

RNA-sequencing (RNA-seq) pre-processed reads were mapped to the reference genomes of *A. sinensis* (GCA_000455745.1), *N. nippon* (GCA_000708225.1), *C. livia* (GCA_000337935.2), *P. sinensis* (GCA_000 230535.1), and *X. laevis* (GCA_001663975.1) in GenBank assemblies using Hisat2 (v2.0.5).[122] Transcripts were assembled and quantified using StringTie (v1.3.3b). The numbers of reads mapped to each gene were counted using featureCounts (v1.5.0-p3). Fragments per kilobase million (FPKM) and the log10 transformation of the FPKM values for telomere maintenance genes were calculated according to the genome annotations.

Telomerase reverse transcriptase (TERT) gene expression

Total RNA extracted for RNA-seq was also used for gene expression analysis. One microliter of RNA in a final volume of 20 μL was reverse-transcribed (RT) using a TaKaRa PrimeScript RT Reagent Kit. RT-qPCR was performed in 10 μL containing 5μLTB Green Premix, 0.5 μL forward and reverse primers, 1 μL cDNA (NTCs with 1 μL ribonuclease-free water), and 3 μL ribonuclease-free water. Cycling was controlled using a CFX Manager PCR System as follows: 95 ºC for 3 min, followed by 40 cycles of 94 ºC for 10 s, the primer-specific annealing temperature for 30 s (Table S12), and fluorescence acquisition. Primers were designed using the Premier Primer 5.0 software. All primers showed amplification efficiencies of 0.95–1.05 (R^2 > 0.98), in which the primer's efficiency was highly dependent on their specificities. The $2^{-\Delta\Delta Ct}$ method was used to quantify *TERT* expression.[123] Single reference genes (group standard deviation of $C_t < 0.2$) were selected for each species.[124]

Phylogenetic, evolutionary, and structural analyses of telomere maintenance genes

In total, 38 vertebrates were used in the selective pressure analyses, showing the maximum lifespan gradient of 1–10 years in five species, 11–20 years in four species, 21–30 years in six species, five species in 31–40 years, four species in 41–50 years, six species in 51–70 years, and seven species more than 70 years), which covered 16 orders of mammal and 14 orders of non-mammal vertebrates (Table S2). The ratios ensure less than 1.5:1 between mammals and non-mammal species in each lifespan interval during species selection. Telomere maintenance genes were functionally analyzed using the Bioconductor package[125] in R to determine their gene ontology (GO) enrichment. In total, 225 genes were chosen for the positive selection test (Table S1), as described in Foley study,[126] which were combined with 45 target genes from Morgen et al. study.[127]

OrthoFinder (v2.4.0) was used for ortholog searching of all 225 genes from mammals and non-mammal vertebrates. After 1:1 orthologs were identified, Prank[128] was used for protein-coding sequence search and single-copy gene family alignments, and large insertions/deletions were removed from alignments by Gblocks.[129] In total, 115 orthologs were identified for mammals, and 87 core orthologs were found in non-mammal species. Phylogenetic trees of mammals and non-mammal species were constructed using the Maximum Likelihood method with RAxML.[130] Positive selection analysis was carried out using the Codeml software in PAML package[131] implemented in the python pipeline "OH-SNAP" (Optimized High-throughput Snakemake Automation of PAML; available at https://github.com/batlabucd/OHSNAP), which required inputs of phylogenetic trees, alignments, leading branches labeled in taxa, and set-up models. Leading branches were set for all mammal and non-mammal species in parallel. OH-SNAP_CHECK was performed for system checking before OH_SNAP_RUN_CLUSTER analysis for positive selection in the branch-site model (model A versus model null).[132] A likelihood ratio test and χ^2 with one degree of freedom were used to detect significance in the two tests q-values were corrected by the FDR method ($q < 0.05$).

Sequence alignment analyses were performed by using the Snapgene software to identify mutations and important residues of ATM, APEX1, CBX3, CCT2, CDK2, MYC, POLD1, PRKDC, PRKCQ, STAG3 and TINKS1BP1 of species among *A. sinensis, A. mississippiensis, V. komodoensis, C. picta bellii, C. abingdonii,*

T. carolina triunguis, P. sinensis, P. muralis, N. scutatus, N. nippon, P. colchicus, C. livia, C. anna, X. laevis, H. sapiens, G. gorilla, C. didactylys, M. musculus, H. glaber, M. myotis, M. brandtii, P. tigris, O. orca, T. truncates, B. musculus, L. africana, E. caballus, D. rerio, C. auratus, A. anguilla, and *A. ruthenus* in Table S13. Sorting Intolerant from Tolerant (SIFT)[61,62] was used to test affected protein function in substitutions of amino acid in the proteins by UniRef90. Reconstruct Ancestral State in Phylogenies (RASP)[133] was used to reconstruct phylogenetic relationship in amino acid ATM^{D1815N} mutations among *Alligator* spp. and the representative species above. The homology models of *Alligator* spp. were built using the most similar template for the NMR structure of ATM (PDB code: 5NP0, resolution: 5.70 Å) proteins and the SWISS-MODEL software.[134] Graphical analysis in the heatmap were generated with cluster method in 'ward' and cluster distance in 'Euclidean' by Origin 2018.[135]

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification and statistical analysis were performed by Origin 2018.[135]

ADDITIONAL RESOURCES

A total of 38 vertebrates' reference genomes used for comparative genomics analyses were listed in Table S2, and that of protein coding genes for sequence alignment analyses were listed in Table S13.