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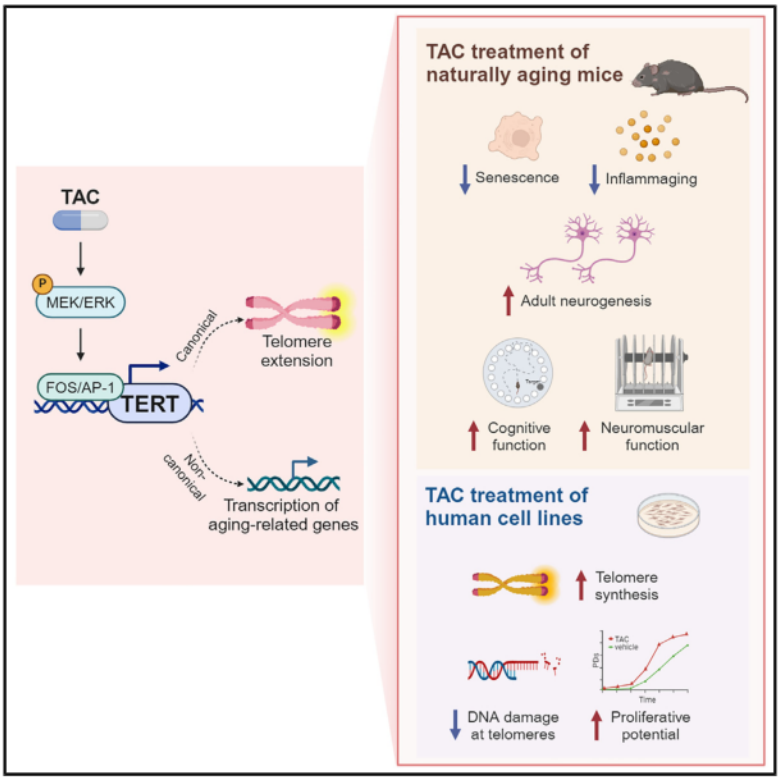
TERT activation targets DNA methylation and multiple aging hallmarks

Graphical abstract



Authors

Hong Seok Shim, Jonathan Iaconelli, Xiaoying Shang, ..., Michael J. Bollong, Peter G. Schultz, Ronald A. DePinho

Correspondence

rdepinho@mdanderson.org

In brief

This study identifies a small molecule (TAC) that restores physiological levels of TERT throughout aged tissues, resulting in the rejuvenation of multiple tissues. Specifically, TAC administration in very aged mice alleviates multiple aging hallmarks such as cellular senescence and systemic inflammation, promotes new neuron formation with improved cognitive ability, enhances neuromuscular function, and is well tolerated with no evidence of toxicity.

Highlights

- TERT has been linked directly or indirectly to all hallmarks of aging
- *TERT* gene is epigenetically repressed with onset of aging markers in all tissues
- TAC restores TERT levels to promote telomere maintenance and reprogram gene expression
- TAC in aged mice reduces senescence/inflammation and increases neurogenesis/cognition

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Article

TERT activation targets DNA methylation and multiple aging hallmarks

Hong Seok Shim,[1] Jonathan Iaconelli,[2] Xiaoying Shang,[1] Jiexi Li,[1] Zheng D. Lan,[1] Shan Jiang,[3] Kayla Nutsch,[2] Brittney A. Beyer,[2] Luke L. Lairson,[2] Adam T. Boutin,[1] Michael J. Bollong,[2] Peter G. Schultz,[2] and Ronald A. DePinho[1,4,*]

[1]Department of Cancer Biology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA
[2]Department of Chemistry, The Scripps Research Institute, La Jolla, CA 92037, USA
[3]Institute for Applied Cancer Science, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA
[4]Lead contact
*Correspondence: rdepinho@mdanderson.org
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SUMMARY

Insufficient telomerase activity, stemming from low *telomerase reverse transcriptase* (*TERT*) gene transcription, contributes to telomere dysfunction and aging pathologies. Besides its traditional function in telomere synthesis, TERT acts as a transcriptional co-regulator of genes pivotal in aging and age-associated diseases. Here, we report the identification of a TERT activator compound (TAC) that upregulates *TERT* transcription via the MEK/ERK/AP-1 cascade. In primary human cells and naturally aged mice, TAC-induced elevation of TERT levels promotes telomere synthesis, blunts tissue aging hallmarks with reduced cellular senescence and inflammatory cytokines, and silences $p16^{INK4a}$ expression via upregulation of DNMT3B-mediated promoter hypermethylation. In the brain, TAC alleviates neuroinflammation, increases neurotrophic factors, stimulates adult neurogenesis, and preserves cognitive function without evident toxicity, including cancer risk. Together, these findings underscore TERT's critical role in aging processes and provide preclinical proof of concept for physiological TERT activation as a strategy to mitigate multiple aging hallmarks and associated pathologies.

INTRODUCTION

Aging is characterized by time-dependent loss of physiological integrity and fitness in living organisms,[1,2] driven by multiple converging mechanisms leading to the accumulation of cellular damage and eventual decline in organ function and tissue homeostasis. Genetic and epigenetic alterations are the major contributors to functional and physiological decline with aging. Human and model organism studies have documented global changes in DNA damage and mutation, genomic instability, DNA methylation patterns, histone modifications, and chromatin remodeling with advancing age. These dynamic regulatory changes affect gene expression and protein functions governing diverse cellular pathways central to aging and age-related diseases.[3] In particular, age-associated epigenomic alterations can induce significant transcriptional drift and initiate cellular aging processes, implicating altered epigenetic signatures in aging pathophysiology and age-related diseases.[4,5] Notably, modulating chromatin modifier activity has shown promise in mitigating multiple aging risk factors and associated disease phenotypes on the organismal level.[6]

Telomere dysfunction is a primary hallmark of aging, causing molecular and cellular damage and serving as a driver or amplifier of the molecular circuitries driving the aging process and associated diseases.[1,2,7] Telomeres, specialized chromatin structures that preserve chromosomal integrity, undergo progressive attrition during aging-associated tissue renewal, leading to loss of their capping function. Telomere dysfunction itself is an aging hallmark and can also contribute to other hallmarks such as genome instability, stem cell exhaustion, mitochondrial dysfunction, and cellular senescence.[2,7] Telomere dysfunction has been causally implicated as a rate-limiting pathogenetic step in age-related diseases including premature aging (progeroid) syndromes, cardiovascular diseases, inflammatory bowel disease, pulmonary fibrosis, metabolic diseases, and neurological diseases.[2,7,8]

Telomerase, a ribonucleoprotein complex responsible for extending telomeres, maintains telomere length and genome integrity. Although essential for cell viability in normal healthy tissues, telomerase activity is tightly regulated in most adult somatic cells, primarily due to transcriptional repression of the core catalytic subunit of telomerase, telomerase reverse transcriptase (TERT). Indeed, defects in telomerase activity due to germline mutations in core telomerase components are linked to the premature loss of tissue renewal and premature death, such as dyskeratosis congenita,[9] aplastic anemia,[10] and idiopathic pulmonary fibrosis.[11] Conversely, enforced expression of TERT stabilizes endogenous telomeres, reduces senescence-associated markers, and restores the proliferative lifespan in mammals.[12–14] Moreover, in genetically engineered

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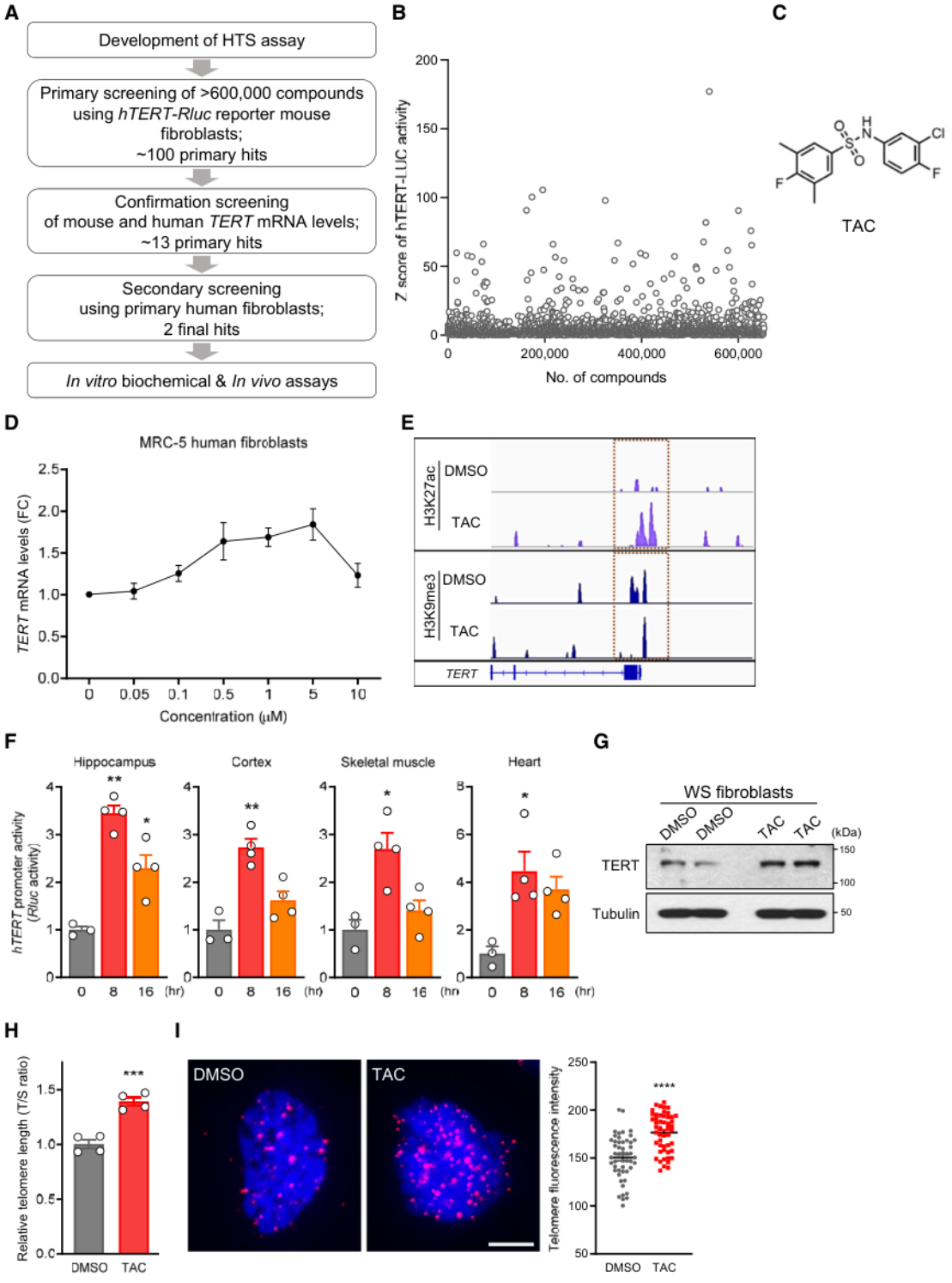

Figure 1. Identification of a small-molecule activator of TERT

(A) The workflow of high-throughput and confirmation screening strategy used to identify novel small-molecule TERT activators.

(B) Plate-based *Z* scores of hTERT-RLUC luminance measurements of all test compound screens in primary adult mouse fibroblasts of *hTERT-Rluc* transgenic mouse.

(C) Molecular structure of TAC.

(D) *TERT* mRNA levels in MRC-5 fibroblasts treated with the indicated concentration of TAC for 4 h.

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mice, TERT reactivation studies revealed the dual role of TERT in the reversal of aging via telomere synthesis in proliferative tissues as well as via gene expression modulation in post-mitotic tissues in a telomere-independent manner. Indeed, in post-mitotic neurons, restoration of physiological TERT levels improves capacity for neural stem cell regeneration, enhances synaptic plasticity, and preserves cognitive function in two models of Alzheimer's disease.[15,16] Accordingly, natural or synthetic compounds that induce TERT expression have been shown to promote cellular survival and metabolic fitness.[17–19] Although promising, the development of these compounds has been hampered by a lack of understanding of their downstream mechanisms of action. In addition, although numerous pathways regulate *TERT* gene transcription, the importance of each pathway and their associated promoter binding elements in regulating the transcription of *TERT* in human adult somatic tissues remains unclear.

In this study, we conducted a high-throughput screen through a library of approximately 653,000 compounds using mouse cells transgenic for the human *TERT* locus harboring a luciferase reporter element. We identified and characterized a small-molecule TERT-activating compound (TAC) that enhances *TERT* transcription in human and mouse adult somatic cells. We elucidated the mechanism of TAC-mediated *TERT* upregulation and explored its impact on regulatory factors governing aging, including $p16^{INK4a}$ methylation. TAC promoted tissue rejuvenation, including new neuron formation, and alleviated multiple aging hallmarks in aged mice, revealing the regenerative potential of adult tissues through physiological TERT activation.

RESULTS

Identification of a small-molecule activator of TERT expression

Given the established benefits of genetic TERT activation on diseases of aging,[2,14,16] we sought to identify small molecules that could induce transient expression of the human and mouse *TERT* gene in somatic cells. To this end, we developed a cell-based high-throughput screening (HTS) assay to enable large-scale screening of small molecules that modulate *in vivo* transcriptional activity of the human *TERT* transgene in adult mouse ear fibroblasts (Figures 1A, 1B, and S1A). These cells were derived from mice transgenic for a 160-kb bacterial artificial chromosome (BAC) that contains the *hTERT* gene, in which a *Renilla* luciferase (*Rluc*) reporter cassette was inserted into the *hTERT* initiation codon and its neighboring loci. This human *hTERT* reporter transgene is capable of mirroring and recapitulating the key transcriptional and functional aspects of the regulation of *TERT* gene expression in normal mouse somatic tissues, thus providing a useful tool for preclinical drug screening.[20] Following an initial screen of 653,000 compounds from the California Institute for Biomedical Research (Calibr)'s library, approximately 100 hits were further characterized by measuring reporter bioluminescence, yielding several TACs capable of modestly inducing the transcription activity of *TERT* gene (Figure S1B). Immunoblot analysis demonstrated that the screening hit TAC induced the highest level of protein expression, and therefore it was chosen for further analyses (Figure S1C). TAC treatment resulted in a dose-dependent induction of *TERT* mRNA in primary human fibroblasts MRC-5 (Figures 1C and 1D). Consistent with TAC-induced *TERT* gene expression, TAC treatment led to the accumulation of the active enhancer/promoter mark H3K27ac and loss of repressive mark H3K9me3 upstream of the transcriptional start site of the *TERT* gene (Figure 1E), indicating that TAC can override the repressive chromatin state of the human *TERT* locus. Moreover, TAC (intraperitoneal [i.p.] injection of 6 mg/kg) increased human *TERT* gene expression across multiple tissues of *hTERT-Rluc* transgenic mice, including brain, heart, and skeletal muscle (Figure 1F). Further analysis of cell-type-specific effects in primary cells revealed that TAC (0.5 μM) can trigger expression of endogenous *Tert* gene in both proliferating and post-mitotic cells within the physiological range (Figures S1D–S1F). We also assessed TAC activity in primary Werner syndrome (WS) fibroblasts that normally undergo rapid senescence that can be reversed by enforced hTERT expression.[21] We found that TAC (0.5 μM) was able to induce TERT expression in WS fibroblasts (Figures 1G and S1G). In this WS model, both quantitative PCR and fluorescence *in situ* hybridization (FISH) analyses showed that long-term TAC treatment led to an increase in endogenous telomere length relative to the control group (Figures 1H and 1I). TAC-treated WS fibroblasts also exhibited a marked reduction in telomere dysfunction-induced DNA damage foci (TIFs) and an enhancement in proliferative potential relative to the vehicle control group (Figures S1H and S1I), suggesting that DNA damage at short telomeres is repaired efficiently by TAC/TERT-mediated telomere addition and that human primary fibroblasts retain the proliferative capacity. Further, our comprehensive survey demonstrates that TAC upregulates human *TERT* transcription independently of cell or tissue types.

(E) The chromatin occupancy of active enhancer/promoter mark H3K27ac and repressive histone mark H3K9me3 in the *TERT* gene of vehicle- or TAC-treated MRC-5 fibroblasts.

(F) *hTERT* promoter activity in the indicated tissues of *hTERT-Rluc* reporter transgenic mice at the indicated time points (h) post-injection (i.p.) of 6 mg/kg TAC (n = 3∼4 per group, two-way ANOVA with Tukey's multiple comparisons test).

(G) Immunoblots for the indicated endogenous proteins in vehicle- or TAC-treated primary WS fibroblasts. A tubulin was used as a loading control.

(H) Relative telomere length of primary WS fibroblasts treated with vehicle or TAC. Relative telomere length was determined as the ratio of telomere repeat copy numbers to single copy gene *36B4* copy number measured by quantitative PCR (n = 4 per group, two-tailed unpaired t test).

(I) Left, representative FISH images for telomeres (red) in interphase nuclei of vehicle- or TAC-treated WS fibroblasts. Right, quantification of telomere FISH. Each value represents average intensity of each nuclei (n = 50 nuclei per group, two-tailed unpaired t test) scale bars, 10 μm. Data are mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

See also Figure S1.

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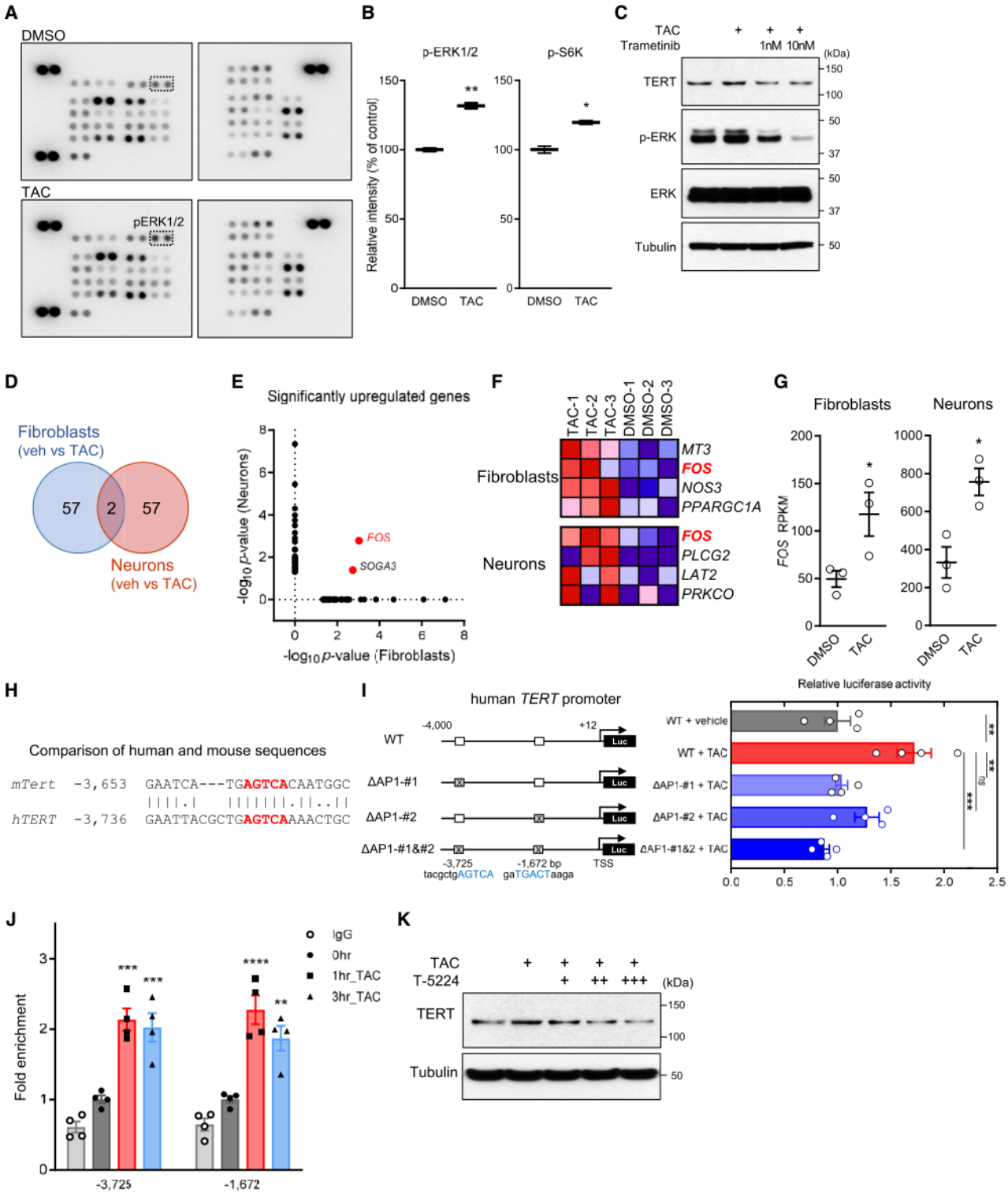

Figure 2. TAC activates the MEK/ERK/AP-1 cascade to upregulate *TERT* transcription

(A) Alteration of cellular signaling in TAC-treated MRC-5 cells. MRC-5 cells were treated with vehicle or TAC for 0.5 h, and the cell lysates were subjected to the human phospho-kinase array.

(B) Quantification of p-ERK1/2 and p-S6K from the phospho-kinase array in (A) (n = 2 per group, two-tailed unpaired t test).

(C) TERT and ERK levels in MRC-5 cells treated with TAC and/or trametinib, a selective MEK inhibitor. A tubulin was used as a loading control.

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TAC activates the MEK/ERK/AP-1 cascade to directly upregulate *TERT* gene transcription

To gain insight into the downstream signaling cascades and *TERT* promoter elements linked to TAC-induced TERT expression, we first analyzed the phosphorylation profiles of kinases and their substrates, using phospho-kinase arrays. We found that phosphorylation of extracellular signal-regulated kinase (ERK) and its downstream effector S6 kinase was consistently increased by TAC treatment in primary human MRC-5 cells (Figures 2A and 2B). Accordingly, inhibition of ERK with the MEK inhibitor trametinib abolished TAC-induced ERK phosphorylation and TERT upregulation in MRC-5 cells (Figures 2C and S2A).

Next, we investigated transcriptomic changes across different somatic cell types to elucidate potential transcription factors and their binding elements linking ERK activation to *TERT* transcriptional control. Through transcriptome profiling of human normal fibroblasts and induced pluripotent stem cell (iPSC)-derived neurons, we observed that acute treatment with TAC (0.5 μM for 1 h) resulted in the significant induction of only a limited number of common genes in both human cell types (Figure 2D). Strikingly, only two genes, *FOS* and *SOGA3*, exhibited significant upregulation in both cell types following treatment (≥2-fold cutoff; $p < 0.05$), with *FOS*—encoding a major constituent of the activator protein 1 (AP-1) transcriptional complex—identified as the gene most significantly regulated by TAC in both cells (Figures 2E–2G). In addition, TAC treatment did not elicit any significant change in the global translation rate (Figure S2B), providing evidence that TAC treatment specifically induces *TERT* expression and its downstream transcriptional effects without affecting general mRNA translation.

The *FOS* gene encodes a critical component of AP-1 transcription factor complex, which binds to specific *cis*-acting elements on gene promoters stimulating the expression of these target genes.[22] We identified two AP-1 binding sites residing within the 4-kb upstream regulatory region of the human *TERT* gene, with one of these binding motifs well conserved between human and mouse (Figures 2H and 2I). To explore whether AP-1 motifs are required for *TERT* promoter activation, we constructed a human *TERT* promoter-luciferase reporter containing 4-kb upstream sequences from the transcription start site of the *TERT* gene. Following transient transfection of MRC-5 cells with these luciferase reporter constructs, we stimulated the cells with TAC. Consistent with our observation of endogenous TERT levels, TAC induced *TERT* reporter activity in human fibroblasts (Figure 2I). Of note, single and double deletions, including the AP-1 binding motif common in the promoters of both mouse and human, significantly abolished TAC-induced *TERT* promoter activity (Figure 2I), highlighting that the conserved AP-1 *cis*-element has a more prominent function in inducing *TERT* transcription compared with the other. We next assessed whether the AP-1 complex is specifically recruited to the *TERT* promoter in response to TAC treatment in their endogenous chromatin context. Chromatin immunoprecipitation followed by real-time quantitative PCR (ChIP-qPCR) analysis showed that TAC treatment led to recruitment of endogenous FOS, a subunit of AP-1 complex, to two AP-1 binding motifs in the endogenous *TERT* promoter (Figure 2J). A selective AP-1 inhibitor T-5224 that specifically blocks FOS/AP-1 binding to DNA without affecting their expression[23] impaired TAC-induced expression of TERT (Figures 2K and S2C). Together, these data indicate that TAC specifically activates the transcriptional activation of *TERT* via the MEK/ERK/AP-1 pathway.

TAC attenuates multiple aging hallmarks *in vivo*

The capacity of genetic TERT reactivation to rejuvenate prematurely aged mice with telomere dysfunction[15,24] prompted us to evaluate whether TAC-mediated TERT induction could similarly attenuate organismal aging in naturally aged mice with intact telomeres. Intraperitoneal administration of TAC, followed by the time-dependent quantification of TAC levels by mass spectrometry-based pharmacokinetics, demonstrated favorable plasma exposure for TAC ($T_{1/2}$: 0.568 h, AUC: 285 h•ng/mL) (Figures S3A and S3B). Notably, TAC exhibited central nervous system (CNS) exposure, with approximately 2-fold partitioning of the compound in the CNS relative to plasma. This CNS exposure corresponded to an increase in the signaling cascade upstream of *TERT* transcription within 0.5–3 h post-administration, mirroring the rapid induction of *hTERT* promoter activity observed in brain tissues of TAC-treated transgenic mice (Figure 1F). TAC was cleared from the plasma by 3 h, with plasma levels tracking those in the brain (Figures S3C and S3D).

To further investigate transcriptomic changes following TAC treatment, we performed RNA sequencing (RNA-seq) of peripheral blood mononuclear cells (PBMCs) from 12-month-old mice. Consistent with previous *in vivo* experiments, TERT protein levels were significantly increased in PBMCs from middle-aged mice treated with TAC (Figure S4A). Following a 1-week treatment course (daily i.p. injection of 6 mg/kg/day), transcriptome

(D) Venn diagram showing the overlap of significantly upregulated genes upon TAC treatment in human fibroblasts and neurons (≥2-fold cutoff; $p < 0.05$).
(E) Scatterplot comparing the statistical significance (p values) of DEGs in human fibroblasts and neurons. Red dots indicate the genes significantly upregulated in both cells ($n = 3$ per group).
(F) RNA-seq heatmap of genes upregulated upon TAC treatment in human MRC-5 fibroblasts and human iPSC-derived neurons ($n = 3$).
(G) Quantification of the expression of *FOS* genes in human MRC-5 fibroblasts and human iPSC-derived neurons treated with vehicle or TAC ($n = 3$ per group, two-tailed unpaired t test).
(H) Sequence comparison of a putative FOS binding site in human and mouse *TERT* 5′-UTR region.
(I) Schematic representation and transcriptional activity of human −4-kb *TERT* promoter-Luc reporter constructs and deletion mutants in MRC-5 cells ($n = 4$ per group, two-way ANOVA with Tukey's multiple comparisons test). Putative AP-1 binding sites are boxed.
(J) c-FOS ChIP-qPCR enrichment at the endogenous promoter region of *TERT* in MRC-5 cells after TAC treatment ($n = 4$ per group, two-way ANOVA with Sidak's multiple comparisons test).
(K) TERT levels in MRC-5 cells treated with TAC and/or T-5224, a selective c-FOS/AP-1 inhibitor. Data are mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; ns, not significant.
See also Figure S2.

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analysis revealed a reduction in aging gene signatures including cell-cycle arrest and PML body, which are established key cellular changes associated with aging PBMCs of middle-aged C57BL/6 mice (Figure 3A). Specifically, relative to vehicle-treated controls, TAC-treated PBMCs exhibited repression of $p16^{Ink4a}$, a key driver and biomarker of *in vivo* senescence and aging, as well as other senescence-associated secretory phenotype (SASP) components—*Il-1α*, *Il-1β*, *Mmp-3*, and *Vegf* (Figure 3B). Conversely, TAC treatment concurrently induced signatures of organism growth and natural killer cell activation (Figure 3C), which are known to decline with age.[25] Across a wide range of tissues, a 1-week course of TAC treatment decreased expression of the classical senescence marker $p16^{Ink4a}$, but not $p21^{Cip1}$, in the brain, skeletal muscle, kidney, heart, and liver of middle-aged (about 10- to 12-month-old) mice (Figures 3D and S4B–S4D). TAC-mediated repression of $p16^{Ink4a}$ was abolished in age- and sex-matched *Tert*-null mice (Figure 3E), indicating the requirement of TERT for TAC-induced silencing of $p16^{Ink4a}$.

To determine the molecular mechanism of TAC-mediated $p16^{Ink4a}$ repression, we examined known regulators governing $p16^{Ink4a}$ transcription. Transcriptomic analysis of the brains of adult $Tert^{+/+}$ and first-generation (G1) $Tert^{-/-}$ mice (with intact telomeres)[16] showed significant and specific reduction in the expression of *Dnmt3b* (Figures S4E and S4F), a DNA methyltransferase responsible for the hypermethylation of $p16^{Ink4a}$ promoter.[26] Quantitative RT-PCR analysis of $Tert^{+/+}$, $Tert^{+/-}$, and $Tert^{-/-}$ mouse brains confirmed marked downregulation of *Dnmt3b* levels in $Tert^{+/-}$ mice, which was further reduced in $Tert^{-/-}$ mice (Figure 3F), indicating a positive correlation between TERT levels and *Dnmt3b* expression *in vivo*. Correspondingly, ChIP sequencing (ChIP-seq) analysis demonstrated that TERT bound to the *DNMT3b* promoter in human iPSC-derived neurons (Figure 3G). These results are consistent with previous findings that TERT can act as a transcriptional modulator not only in highly proliferating cells but also in terminally differentiated cells.[16,27]

Given that *de novo* DNA methylation mediated by DNMT3b is associated with repression of $p16^{INK4a}$ transcription,[26] we examined whether TAC treatment could induce hypermethylation of the $p16^{Ink4a}$ promoter *in vivo*. Real-time methylation-specific PCR (MSR) was used to detect the methylation of CpG islands flanking the translation start site of murine $p16^{Ink4a}$,[28,29] revealing a significant increase in methylated CpG sites at the promoter region of $p16^{Ink4a}$ in middle-aged mouse tissues after TAC treatment compared with vehicle controls (Figure 3H). Furthermore, chronic administration of TAC for 6 months reduced senescence cell burden as well as the production of pro-inflammatory interleukin (IL)-1β and IL-6 SASP factors in multiple tissues of naturally aged (about 26- to 27-month-old) mice (Figures 3I and S4G–S4J). Thus, TAC-driven TERT upregulation reduces age-dependent tissue senescence and regulates major genetic drivers of cellular senescence, including $p16^{Ink4a}$ and SASP components.

Chronic TAC administration ameliorates brain aging

Genetic reactivation of TERT can restore the neurogenic and cognitive capacities in rodent models of premature aging and Alzheimer's disease and can increase levels of mature brain-derived neurotrophic factor (BDNF), a key molecule promoting the growth, survival, and differentiation of newborn neurons in the normal mouse brain.[14–16] Consistent with these reports, immunoblotting and mature BDNF (mBDNF)-specific enzyme-linked immunosorbent assay (ELISA) demonstrated a marked increase in the levels of mBDNF as well as TERT in adult hippocampus following 1-week administration of TAC (Figures S5A–S5C).

To gain further insight into the impact of TAC on gene expression programs, we performed RNA-seq analysis of microdissected hippocampal tissues from middle-aged (about 12- to 14-month-old) C57BL/6 mice treated with vehicle or TAC for 3 weeks (daily i.p. injection of 6 mg/kg/day). Remarkably, TAC treatment induced a significant increase in the expression of genes positively associated with adult hippocampus neurogenesis and brain function,[30–35] including *Eomes*, a driver of immune cell development and neurogenesis; *Dlk1*, a mediator of adult hippocampal neurogenesis and cognition; glial cell line-derived neurotrophic factor (*Gdnf*), a neurotrophic factor; doublecortin (*Dcx*), a marker for newborn neurons; *Sox1/2*, critical determinants of adult neurogenesis; *Gdf11*, a rejuvenation factor; and *Fos*, an *in vivo* marker of neural activity (Figure 4A). Moreover, pathway enrichment analysis revealed that TAC treatment resulted in the activation of pathways associated with synaptic potential, axon guidance, hippocampus/stem cell development, telomere maintenance, neuroblast proliferation, dopaminergic neurogenesis/CNS neuron differentiation, and mitogen-activated protein kinase (MAPK) family signaling cascades in the hippocampus of middle-aged mice (Figure 4B). Conversely, cytokines and inflammatory response, whose over-activation or dysregulation is known to contribute to age-related diseases and aging itself,[36] were downregulated with TAC administration (Figure 4B). Notably, immunohistochemical and immunofluorescent analyses demonstrated that TAC treatment for 4 weeks markedly increased the number of DCX-expressing newborn neurons in the hippocampal dentate gyrus (DG) (Figures 4C and 4D), indicating that TAC can indeed enhance the regenerative capacity of the aged brain. Further confirmation of TAC-induced adult neurogenesis was obtained with another marker of immature neurons, the polysialylated form of the neural cell adhesion molecule (PSA-NCAM), revealing a higher number of DCX^+ $PSA\text{-}NCAM^+$ immature neurons in TAC-treated mice relative to controls (Figures 4E and S5D). No change in body weight was detected between the two groups throughout the study (Figure S5E). Thus, pharmacological activation of TERT induces rejuvenation-associated gene signatures and stimulates adult neurogenesis in the hippocampus of adult mice.

Chronic neuroinflammation is a pervasive feature of the aging brain and is thought to contribute to diminished brain function. Microglia, the resident immune cells of the brain, are the primary players in neuroinflammation, and microglial activation is considered a hallmark of neuroinflammation seen in most brain aging and neurodegenerative conditions.[37,38] TAC administration significantly attenuated the levels of IBA1-positive activated microglia in the brains of aging mice relative to vehicle-treated controls (Figure 4F). TAC-treated animals displayed a prominent reduction in the cell density and soma size of IBA1-positive microglia in the hippocampus. Consistent with a crucial role for activated microglia as the primary source of pro-inflammatory cytokines in the brain,[39] the age-related increases in expression

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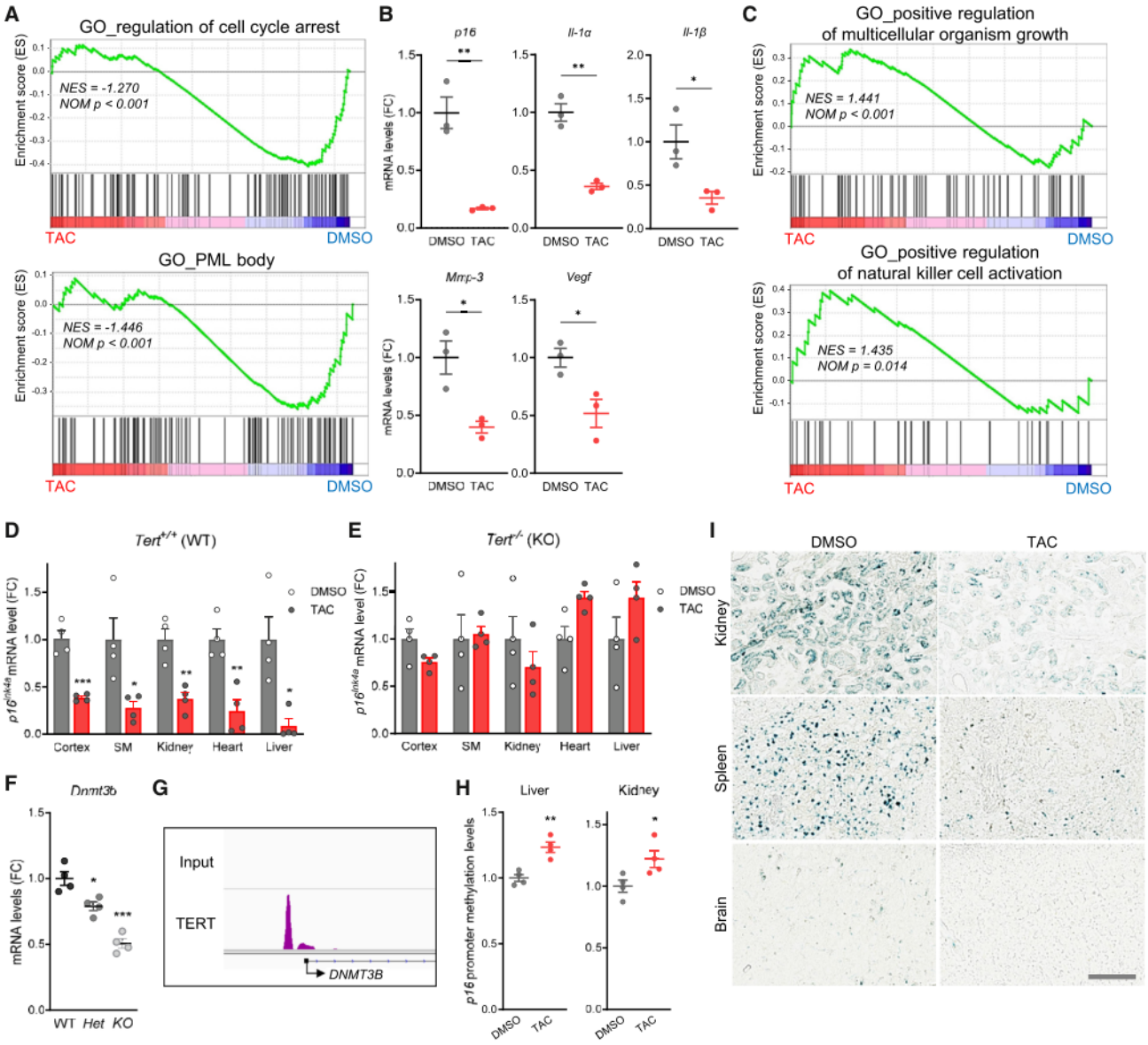

Figure 3. TAC attenuates diverse aging hallmarks *in vivo*

(A) GSEA plots showing downregulated GO pathways in the PBMC of TAC-treated C57BL/6 mice relative to vehicle-treated controls (n = 4 per group; 10∼12 months old).

(B) mRNA levels of senescence-related genes downregulated in TAC-treated PBMCs, compared with control (n = 3 per group, two-tailed unpaired t test).

(C) GSEA plots showing upregulated GO pathways in the PBMC of TAC-treated mice relative to vehicle-treated controls.

(D and E) $p16^{Ink4a}$ mRNA levels in the multiple tissues of TAC-treated wild-type ($Tert^{+/+}$) (D) or *Tert*-KO ($Tert^{-/-}$, first-generation [G1]) (E) mice relative to each control group (n = 4 per group; 10∼12 months old, two-tailed unpaired t test).

(F) mRNA levels of *Dnmt3b* gene in wild-type ($Tert^{+/+}$), *Tert* heterozygous ($Tert^{+/-}$), and *Tert* homozygous KO ($Tert^{-/-}$, G1) mouse brains (n = 4 per group, two-way ANOVA with Tukey's multiple comparisons test).

(G) TERT occupancy in the *DNMT3B* gene of human iPSC-derived neurons.

(H) $p16^{Ink4a}$ promoter methylation levels in the liver and kidney tissues of vehicle- or TAC-treated C57BL/6 mice (n = 4 mice per group; 10∼12 months old, two-tailed unpaired t test).

(I) Representative images of SA-β-gal staining in the tissues of vehicle- or TAC-treated aged C57BL/6 mice (n = 4, 26∼27 months old). Data are mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

See also Figures S3 and S4.

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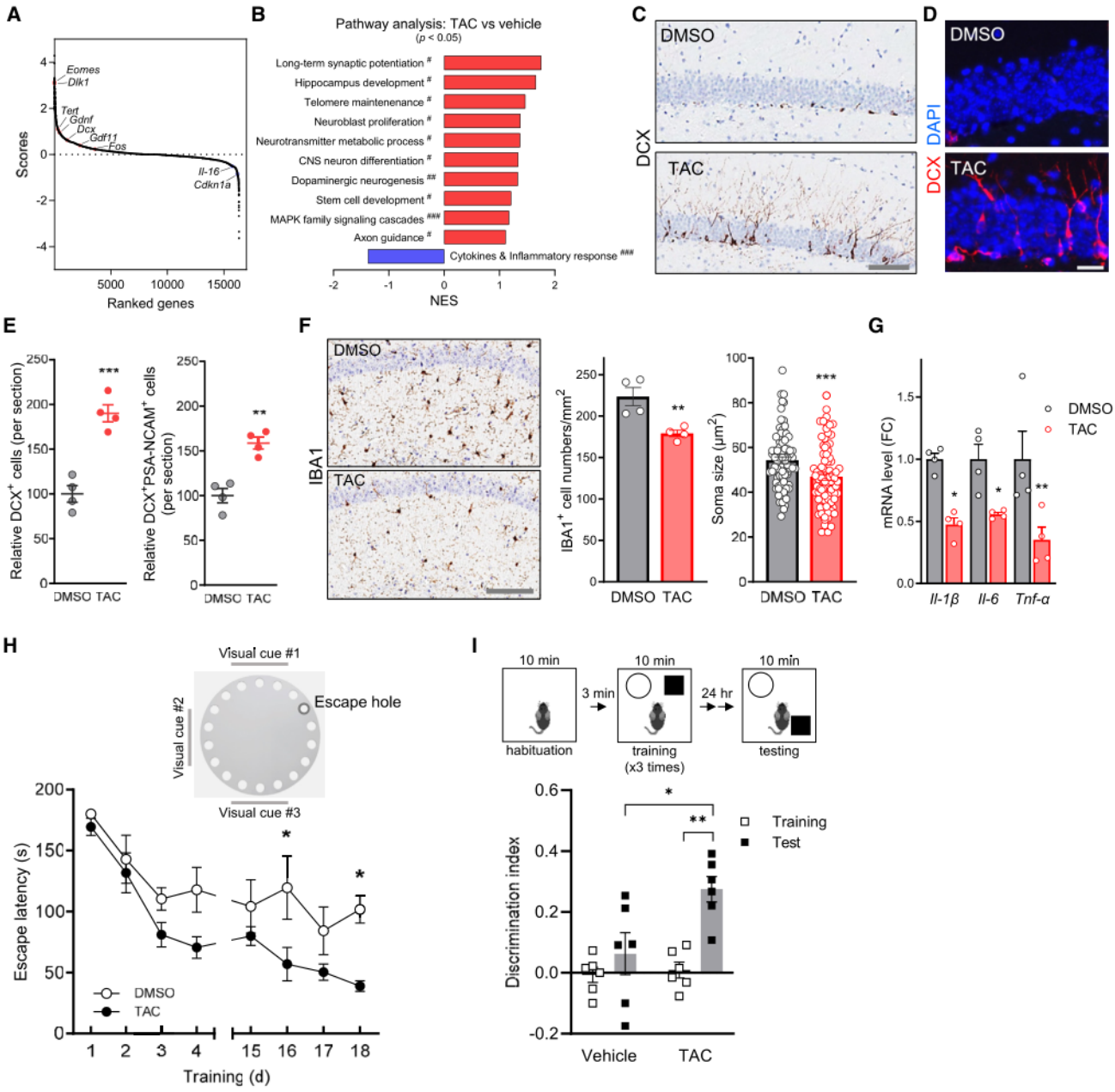

Figure 4. Chronic TAC administration ameliorates brain aging

(A) A ranked list of genes in the observed transcriptional data from TAC-treated mouse hippocampi relative to control (n = 3 mice per group).

(B) Pathways enriched (red) or depleted (blue) in TAC-treated hippocampi. Resource categories: #, Gene Ontology; ##, WikiPathways; ###, Reactome.

(C and D) Representative images of DCX immunoreactivity by immunohistochemistry (C) or immunofluorescence (D) in the dentate gyrus of middle-aged (10- to 12-month-old) mice that were treated with vehicle and TAC for 1 month. Scale bars, 100 and 25 μm, respectively.

(E) Quantifications of DCX^+ and DCX^+ $PSA\text{-}NCAM^+$ cells in both groups (n = 4 per group, two-tailed unpaired t test).

(F) Representative images of IBA1-labeled microglia (left) and quantifications of microglial density (middle) and cell soma size (right) in the hippocampus of middle-aged (10- to 12-month-old) mice that were treated with vehicle and TAC for 1 month (n = 4 [$IBA1^+$ cell numbers] and 80 [soma size], respectively, per group; two-tailed unpaired t test). Scale bars, 100 μm.

(G) mRNA levels of pro-inflammatory cytokines *Il-1β*, *Il-6*, and *Tnf-α* in the hippocampus of middle-aged mice (10∼12 months old) treated with vehicle or TAC for 1 month (n = 4 per group, two-way ANOVA with Sidak's multiple comparisons test).

(H) Escape latency in Barnes maze trials over training days for aged (26- to 27-month-old) mice that were treated with vehicle or TAC for 6 months (n = 6 per group, two-way ANOVA with Sidak's multiple comparisons test).

(I) Discrimination index for vehicle- or TAC-treated aged (26- to 27-month-old) mice in the novel-location recognition test (n = 6 per group, two-way ANOVA with Tukey's multiple comparisons test). Data are mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

See also Figure S5.

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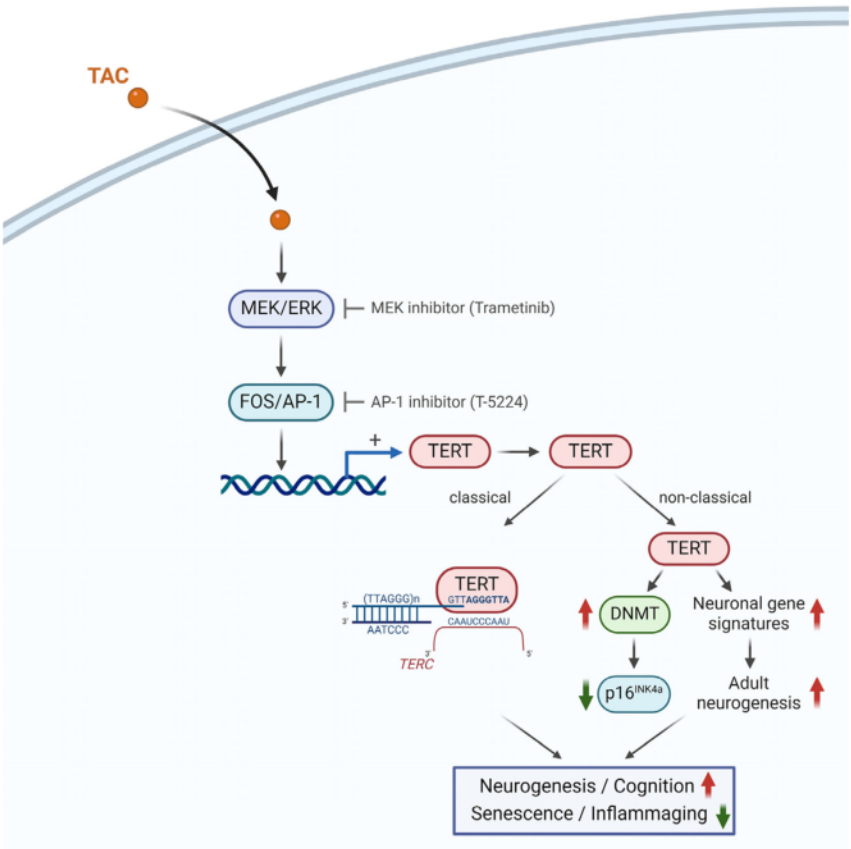

Figure 5. Schematic illustration of TAC/TERT-driven anti-aging effects

The expression of TERT, a catalytic subunit of telomerase, is tightly suppressed in normal somatic cells. A novel small-molecule TAC can trigger transcriptional activation of somatic *TERT* expression via activation of MEK/ERK/AP-1 signaling cascade. Somatic TERT induction not only reduces tissue senescence by silencing $p16^{INK4a}$ through DNMT3B-mediated promoter hypermethylation and inflammation but also enhances adult neurogenesis and cognitive function by promoting hippocampal transcriptomic signatures.

of pro-inflammatory cytokines including *Il-1β*, *Il-6*, and tumor necrosis factor alpha (*Tnf-α*) were also attenuated in TAC-treated hippocampus relative to vehicle-treated controls (Figure 4G).

TAC-induced neurogenesis and reduced neuroinflammation prompted us to assess the role of TAC in modulating memory in aged mice approaching end of life. Naturally aged (about 20- to 21-month-old) C57BL/6 mice were randomized into TAC and vehicle treatment groups. Following a 6-month treatment course (i.p. injection of either vehicle or 6 mg/kg/day of TAC, 3 times a week), aged mice (26∼27 months old) that were treated with TAC showed improved performance on three hippocampal-dependent cognitive tests—Barnes maze, Y-maze, and novel-location recognition tasks (Figures 4H, 4I, S5F, and S5G). In addition, TAC-treated aged mice showed improved rotarod performance and grip strength (Figures S5H and S5I, respectively), which are known to decline with advancing age,[40] consistent with improved motor coordination and muscle strength. Together, these findings demonstrate that the modest TAC-induced increase in endogenous TERT levels improves not only hippocampal-dependent cognitive function but also enhances neuromuscular function in aged mice without any overt adverse consequences.

DISCUSSION

This study highlights the significant regenerative capacity of aging organ systems as well as the ability to pharmacologically modulate aging hallmarks during natural aging. We report the discovery of a novel small-molecule telomerase activator that induces the physiological expression of *TERT* in both human and mouse somatic tissues. Our findings reinforce the view that TERT exerts anti-aging activity not only by preserving telomere integrity but also by modulating gene expression and cellular signaling pathways governing cellular survival, senescence, neurogenesis, and stress resistance, among other processes. TERT levels are tightly regulated in normal adult somatic cells and further repressed in aged tissues and in the Alzheimer's disease brain.[16] The age-associated repression of *TERT* is reminiscent of other key longevity-associated genes, where epigenetic modifications altering chromatin structure and accessibility accumulate in the *cis*-regulatory sequences during aging, leading changes in the transcriptional regulation of genes governing aging.[41,42] TAC effectively counteracts the epigenetic silencing of the *TERT* gene in adult somatic cells, thereby influencing the expression of genes governing aging hallmarks. Notably, TAC treatment significantly reduces the presence of senescent cells in diverse tissues along with DNMT3B-mediated repression of the master cellular mortality gene $p16^{Ink4a}$, in a TERT-dependent manner. Additionally, long-term administration of brain-penetrant TAC in aged mice reduces neuroinflammation and inflammatory cytokines, enhances rejuvenation-associated gene signatures, promotes adult neurogenesis, and preserves cognitive function without overt side effects. Thus, physiological TERT activation holds promise for assuaging aging phenotypes during natural aging in mice (Figure 5).

In the setting of aging and chronic disease, small-molecule therapeutics may offer advantages over biologics due to their lower immunogenicity and cost-effectiveness.[43,44] TAC possesses a low molecular weight (<400 Da) and lipophilicity that favor drug uptake across all tissues, including the CNS.[45] Particularly encouraging is TAC's ability to alleviate age-associated increase in pro-inflammatory cytokines (*Il-1β*, *Il-6*, and *Tnf-α*) and induction of the key neurotrophic factors (BDNF and GDNF) governing synaptic plasticity and memory. This encourages clinical testing of TAC as a neuroprotective agent in normal aging and

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in neurodegenerative diseases, such as Alzheimer's disease, or as a countermeasure for chemotherapy-induced neurological dysfunction. Indeed, our recent study provided additional genetic evidence that somatic TERT maintenance restrains the pathological hallmarks of Alzheimer's disease, including amyloid pathology, dendritic spine deficits, and cognitive decline.[16] In addition, our current analyses provide novel insights into the mechanism by which TAC and TERT impact the regulation of pivotal aging genes. Our biochemical and cell-based assays show that TAC fine-tunes MEK/ERK/FOS signaling pathway, resulting in transient upregulation of *TERT* expression in the physiological range. Along these lines, it is intriguing that ERK signaling has been linked to the cognitive-enhancing effects of plant secondary metabolites, such as curcumin and apigenin.[46,47] Although sustained hyperactivation of MEK/ERK signaling is a prerequisite for malignant transformation or progression, the fine-tuning of this pathway activation by natural bioactive compounds, such as flavonoids, appears to promote physiological responses of gene transcription/translation and cell growth/survival and in turn advance human health as neuroprotectants and cognitive enhancers.[48–51] Also, it cannot be ruled out that additional molecular mediator(s) might be involved in TAC-driven *TERT* transactivation since most biologically active small molecules exhibit polypharmacology (that is, simple molecules are more likely to bind to multiple protein targets).[52] Future studies are warranted to identify the precise target(s) of TAC, using chemical proteomics and subsequent structural and functional validation studies. Nevertheless, our findings provide ample evidence that the ERK/AP-1 cascade functions as a prime genetic switch regulating TAC-induced somatic *TERT* expression and that fine-tuning of the ERK-AP-1-TERT signaling axis induces beneficial effects on biological aging.

Somatic *TERT* de-repression via TAC administration confers cellular and organismal benefits via canonical and non-canonical telomerase functions. Physiological activation of somatic TERT expression and activity not only sustains cellular replicative potential via telomere maintenance but also enhances tissue-level physiology by modulating multiple aging-relevant pathophysiological processes, including senescence and inflammation. The multifaceted organismal and functional benefits may arise from a combination of TERT's canonical and non-canonical functions. To discern the specific contributions of these functions, further experiments using cells or animals deficient in telomerase enzymatic activity such as telomerase RNA component (*TERC*) knockout (KO) models will be necessary.

Long-term anti-aging treatments necessitate ensuring safety in the chronic settings. It is encouraging, however, that long-term TAC treatment was well tolerated without any overt or histological adverse effects including carcinogenesis. Nonetheless, additional safety studies in non-human primates are warranted. Along these lines, it is worth noting that TAC may reduce cancer incidence, given that insufficient telomerase resulting in telomere dysfunction coupled with loss of p53-dependent DNA checkpoint control is a major driver of chromosomal instability and cancer genesis in the aged.[53] Correspondingly, TERT reactivation in aged inducible-TERT mice was shown to reverse aging phenotypes without causing an increase in cancer.[15] Together, these observations strengthen the case for testing small-molecule TERT activators capable of inducing transient physiological upregulation of TERT expression. Moreover, although the half-life of TAC is short, TERT protein half-life is 2–3 h, and its actions can be more enduring via such actions as DNMT3B-mediated silencing of the $p16^{INK4a}$ promoter. In this light, the engagement of diverse aging mechanisms by TERT makes TAC treatment a viable strategy for aging per se and for specific associated age-related diseases, particularly those characterized by impaired telomerase/telomere function.

Limitations of the study

The study identifies a TAC capable of restoring physiological TERT levels via activation of MEK/ERK signaling on a conserved AP-1 binding element in the *TERT* promoter. TAC modulates key age-related regulatory factors such as $p16^{INK4a}$ and DNMT3b, mitigates aging phenotypes such as senescence and inflammation, and stimulates neurogenesis with improved cognition and neuromuscular function. Despite these encouraging findings, the study faces several limitations. First, although TAC offers a variety of health benefits, its potential impact on lifespan extension remains to be determined. Second, although TAC exhibits favorable drug-like properties without evident toxicity, the study did not explore dose optimization and dosing schedules, nor did it determine the maximum tolerated dose. Further, the drug's metabolites and their potential liabilities were not assessed. Third, although prolonged TAC administration was well tolerated in mice, differences in drug metabolism across species necessitate thorough toxicity studies in non-human primates and human subjects to assess safety, potentially requiring further drug modifications and formulation development. Last, although the maintenance of TERT levels in the physiological range transiently would preserve telomere function and reduce cancer incidence, it would be prudent to carefully monitor cancer incidence in clinical trials assessing sustained TAC administration.

STAR★METHODS

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