3′ UTR lengthening as a novel mechanism in regulating cellular senescence

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Cellular senescence has been viewed as a tumor suppression mechanism and also as a contributor to individual aging. Widespread shortening of 3′ untranslated regions (3′ UTRs) in messenger RNAs (mRNAs) by alternative polyadenylation (APA) has recently been discovered in cancer cells. However, the role of APA in the process of cellular senescence remains elusive. Here, we found that hundreds of genes in senescent cells tended to use distal poly(A) (pA) sites, leading to a global lengthening of 3′ UTRs and reduced gene expression. Genes that harbor longer 3′ UTRs in senescent cells were enriched in senescence-related pathways. Rras2, a member of the Ras superfamily that participates in multiple signal transduction pathways, preferred longer 3′ UTR usage and exhibited decreased expression in senescent cells. Depletion of Rras2 promoted senescence, while rescue of Rras2 reversed senescence-associated phenotypes. Mechanistically, splicing factor TRA2B bound to a core “AGAA” motif located in the alternative 3′ UTR of Rras2, thereby reducing the Rras2 protein level and causing senescence. Both proximal and distal poly(A) signals showed strong sequence conservation, highlighting the vital role of APA regulation during evolution. Our results revealed APA as a novel mechanism in regulating cellular senescence.

[Supplemental material is available for this article.]

Cellular senescence was originally described as a process that limits the proliferation of cultured human cells. After extensive proliferation, senescence occurs because of telomere shortening and loss in the absence of endogenous telomerase activity (Hayflick and Moorhead 1961; Olovnikov 1996). In addition to telomere erosion, many stimuli and stresses can cause cellular senescence, including DNA double-strand breaks, strong mitogenic signals, oxidative stress, and ectopic expression of cyclin-dependent kinase inhibitors (CDKIs) (Xue et al. 2004; Rodier and Campisi 2011; Li et al. 2013). Numerous morphological and molecular markers of senescent cells have been identified in recent decades, which include a flattened and enlarged cell morphology, increased senescence-associated β-galactosidase (SA-β-gal) activity, reduced proliferation rate, and expression of tumor suppressors, cell cycle inhibitors, and DNA damage markers (Dimri et al. 1995; Busuttil et al. 2003; Lopez-Otin et al. 2013; Munoz-Espin and Serrano 2014). Cellular senescence is viewed as an important mechanism for preventing cancer (Campisi et al. 2001). It is also involved in normal embryonic development and tissue damage (Munoz-Espin et al. 2013; Storer et al. 2013; Munoz-Espin and Serrano 2014). Removing senescent cells expands the healthy lifespan of mice (Baker et al. 2016). These results demonstrate the significance of cellular senescence.

A number of studies have shown that dramatic changes in the transcriptome and/or proteome accompany the phenotypic alterations of senescent cells (Kim et al. 2013b; Mazin et al. 2013; Waldera-Lupa et al. 2014; Wei et al. 2015) and that the development of senescence-associated phenotypes can be regulated by stage-specific gene expression modules (Kim et al. 2013b). Therefore, understanding the regulation of gene expression and the corresponding regulatory networks is crucial to dissecting the mechanism of cellular senescence. Alternative polyadenylation (APA) is recognized as a crucial contributor to the regulation of mammalian gene expression (Di Giammartino et al. 2011; Elkon et al. 2013; Chen et al. 2017; Tian and Manley 2017). Cleavage and polyadenylation of nascent RNA is essential for maturation of the vast majority of eukaryotic mRNAs and determines the length of 3′ UTRs (Sachs 1990). The process requires several cis-acting RNA elements and dozens of trans-factors (Millevoi and

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Vagner 2010). The key cis-element is a six-nucleotide (nt) motif (termed the poly[A] signal or PAS), the canonical form of which is “AAUAAA.” The PAS determines recognition and cleavage by the 3′ end-processing machinery (Proudfoot and Brownlee 1976). The mammalian 3′ end-processing machinery contains several subcomplexes as well as additional accessory factors which mediate the precise processing of mRNA precursors (pre-mRNAs) together (Di Giammartino et al. 2011; Elkon et al. 2013).

With the increasing application of high-throughput sequencing technologies, genome-wide studies indicate that most eukaryotic mRNA genes have multiple polyadenylation (pA) sites (Tian et al. 2005; Wang et al. 2008; Zheng and Tian 2014). Alternative pA sites can reside in the 3′-most exon or upstream regions and can give rise to multiple mRNA transcripts that contain different coding sequences, 3′ UTRs, or both (Di Giammartino et al. 2011; Elkon et al. 2013). Importantly, both microRNAs (miRNAs) and RNA binding proteins (RBPs) targeting 3′ UTRs are able to regulate translational efficiency, degradation, and subcellular localization of mRNA or protein (Di Giammartino et al. 2011; Berkovits and Mayr 2015; Tian and Manley 2017). It is well known that APA plays important roles in a wide range of biological processes such as cell differentiation (Ji et al. 2009; Mangone et al. 2010; Hilgers et al. 2011; Li et al. 2012; Ulitsky et al. 2012; Fu et al. 2016; Hu et al. 2017), cell proliferation (Sandberg et al. 2008; Elkon et al. 2012; Hoffman et al. 2016), cell/tissue identity (Zhang et al. 2005; Derti et al. 2012; Smibert et al. 2012; Ni et al. 2013), and carcinogenesis (Mayr and Bartel 2009; Fu et al. 2011; Lin et al. 2012; Xia et al. 2014). However, whether APA is involved in senescence-associated gene expression and contributes to cellular senescence remains to be answered.

We therefore examined the potential role and possible mechanism of APA in cellular senescence by applying our polyadenylation sequencing (PA-seq) approach (Ni et al. 2013) in two cellular senescence models, the passage of mouse embryonic fibroblasts (MEFs) and aortic vascular smooth muscle cells of rats (rVSMCs) at different ages.

**Results**

Global lengthening of 3′ UTRs couples with decreased gene expression in senescent cells

To determine whether APA plays a role during cellular senescence, we first used MEFs undergoing replicative senescence in vitro and having population doubling (PD) times of 6, 8, 10, and 11 passages (Fig. 1A; Supplementary Fig. S1; Dinri et al. 1995; Parrinello et al. 2003; Tian and Li 2014; Tijges et al. 2014). We applied RNA sequencing (RNA-seq) and PA-seq (Ni et al. 2013) to discern the relationship between gene expression and 3′ UTR length patterns in these cells. After confirming the reliability of called pA sites, such as genomic location distribution, poly(A) signal enrichment, and overlap with poly(A)_DB (Supplementary Figs. S2, S3; Supplementary Table S1), the effective 3′ UTR length (or weighted mean of 3′ UTR length) was used to estimate the relative trend of pA site usage and changes in 3′ UTR length according to our previous method, which took pA site location, the distance to a stop codon, and tag number into consideration (Ni et al. 2013). Effective 3′ UTRs showed a global lengthening trend during MEFs senescence (Fig. 1B), indicating a tendency to use the distal pA sites. To further evaluate the changes in 3′ UTR length at the level of individual genes, we compared effective 3′ UTR length in later passaged (PD8, PD10, and PD11) cells with that in the earlier passaged (PD6) cells using different cut-offs. Cells of later passages always had many more genes with a longer 3′ UTR compared with cells of the earlier passage (Fig. 1C). Moreover, the number of genes with a lengthened 3′ UTR gradually increased from PD8 to PD11, while the number of genes with a shortened 3′ UTR continuously decreased (Fig. 1C). The same trend of global 3′ UTR lengthening was further confirmed by adopting a different methodology, the relative usage of distal polyadenylation sites (RUD) index (Ji et al. 2011), using separate RNA-seq data from the same MEFs used in PA-seq (Supplemental Figs. S4, S5; Supplementary Table S2). Biological replicates of PD11 MEFs had more genes with a longer 3′ UTR compared with those of PD6 MEFs (Supplemental Fig. S6; Supplementary Table S3). Together, these results demonstrate the global lengthening of 3′ UTRs in senescent MEFs.

To determine whether cell cycle affects the length of 3′ UTRs during senescence, PD6 MEF cells were subjected to serum starvation to drive them into G0 phase (Gustincich and Schneider 1993). The global pattern of 3′ UTR length in G0 cells was most similar to that in PD6 compared with other passages (Supplemental Figs. S8A, S7; Supplemental Tables S1, S2), implying that the global lengthening of 3′ UTRs resulted from senescence rather than from cell cycle alterations.

A longer 3′ UTR region could provide more opportunities for regulation by miRNAs and/or RBPs, which would influence mRNA and/or protein abundance at the post-transcriptional level (Mayr and Bartel 2009; Zheng and Tian 2014). In line with this hypothesis, a global decline in gene expression was observed for genes preferring distal pA sites during senescence (Supplemental Fig. S8A, C–E). In contrast, control genes with a single pA site or favoring proximal pA sites during senescence did not show such a trend (Supplemental Fig. S8B, F, G). Meanwhile, changes in APA and gene expression at the individual gene level were also analyzed by comparing PA-seq data from PD11 and PD6 MEFs using TSI (tandem 3′ UTR isofrom switch index), a higher value of which indicates 3′ UTR lengthening (Fu et al. 2011; Li et al. 2012). Genes with a significantly lengthened 3′ UTR outnumbered those containing a shortened one (Fig. 1C, D; Supplemental Fig. S9). Within 3′ UTR lengthened genes, more genes showed reduced expression than elevated expression (P < 5.6 × 10^−12, binomial test) (Fig. 1D, right half), while no such difference was observed for genes with shorter 3′ UTRs (Fig. 1D, left half). These data suggest that global 3′ UTR lengthening is associated with decreased gene expression during cellular senescence.

To explore whether APA-induced global lengthening of 3′ UTRs occurred in other senescence systems, we performed PA-seq in VSMCs derived from young and aged rats (Supplemental Table S4). The results showed that senescent rVSMCs and MEFs had a similar ratio between numbers of genes with longer and shorter 3′ UTRs (Supplemental Fig. S9). A significant overlap of genes with longer 3′ UTR usage was observed in these two senescence models (Supplemental Figs. S10, S11). The observation that more genes with lengthened 3′ UTRs tended to be down-regulated was also verified in rVSMCs (P < 1.5 × 10^−5, binomial test) (Fig. 1E). Together, the results indicate that APA-mediated 3′ UTR lengthening is involved in gene expression regulation in multiple cellular senescence systems.

**Genes preferred distal pA sites in senescent cells enrich in senescence-associated pathways**

To further understand the correlation between pA site selection and cellular senescence, we performed pathway analysis on genes...
preferring distal pA sites in PD11 compared with PD6 MEFs. Genes containing a significantly longer 3′ UTR in PD11 compared with PD6 MEFs were enriched in pathways that are highly pertinent to cellular senescence (Fig. 1F), as were the genes whose 3′ UTRs progressively lengthened in four different passages of MEFs (PD6, PD8, PD10, and PD11) (Supplemental Fig. S12). We also found similar enrichment in senescence-associated pathways between old and young rVSMCs (Fig. 1G). Notably, four of the shared pathways in senescent MEFs and rVSMCs, including ubiquitin-mediated proteolysis, the Wnt signaling pathway, cell cycle, and regulation of the actin cytoskeleton (Fig. 1F,G), are linked to cellular senescence (Amberg et al. 2012; Chandler and Peters 2013; Deschenes-Simard et al. 2014; Hofmann et al. 2014). Further examination of the genes preferring distal pA sites in mouse and rat revealed that these two species also possessed common genes undergoing APA regulation (Fig. 1H), which may serve as good candidates to study the function of 3′ UTR lengthening in cellular senescence.

**APA-induced longer 3′ UTR of Ras2 reduces protein production and promotes cellular senescence**

To identify candidate genes that can affect cellular senescence through alternate pA site usage, we applied the following criteria: (1) favoring distal pA site usage both in senescent mouse and rat cells; (2) belonging to the shared four senescence-associated pathways (Fig. 1H); and (3) exhibiting decreased expression during

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**Figure 1.** Global lengthening of 3′ UTRs in senescent cells. (A) SA-β-gal staining for MEFs of PD6, PD8, PD10, and PD11 passages. (B) Box plot for Z-score transformed effective 3′ UTRs across PD6, PD8, PD10, and PD11 MEFs. (C) Number of genes with lengthened and shortened effective 3′ UTRs in senescent MEFs (PD11 vs. PD6). (D) Scatter plot between TSI and expression changes for genes with significantly longer or shorter 3′ UTRs in senescent MEFs (PD11 vs. PD6). TSI denotes tandem UTR isoform switch index (see Methods). The red and blue histograms represent up- and down-regulation (1.5-fold) in senescent cells, respectively. (E) Scatter plot between TSI and expression changes for genes with significantly longer or shorter 3′ UTRs in rVSMCs (old vs. young), similar to that in D. (F,G) Pathways significantly enriched (P < 0.01, Fisher’s exact test) for genes tended to use distal pA sites (TSI > 0 in D and E) in senescent MEFs (F) and VSMCs derived from old rats (G). The shared senescence-related pathways are marked in red text. (H) Overlap of genes in the shared pathways between senescent MEFs and rat VSMCs. The number of genes is shown in each grid and shared gene names are in brackets.
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More importantly, overexpression of RRAS2 (Fig. 2P) was able to re-

PolyA-seq data from human tissues confirmed the existence of

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2007; Muck et al. 2008; Cardus et al. 2013; Zhang et al. 2014).

human umbilical vein endothelial cells (HUVECs) (Kim et al.

these data indicate that RRAS2 plays a crucial role in delaying

sion, and DNA replication (Fig. 2T,U; Supplemental Table S5).

nescence-relevant biological processes, including cell cycle, cell ade-

considered, with the idea that longer 3′ UTRs tended to display a decreased mRNA abund-

tance. To investigate how APA-induced increase in Rras2 3′ UTR length contributes to gene expression changes, we evaluated the RNA degradation rate of Rras2 transcripts containing long and short 3′ UTRs. After blocking transcription followed by qRT-PCR, we found that Rras2 transcripts with a longer 3′ UTR were less stable than those with a shorter 3′ UTR (Fig. 2F). We then inserted the shorter 3′ UTR (represented as 3′ UTR_S) and the longer 3′ UTR (3′ UTR_L) with a mutated proximal PAS of Rras2 into a dual-
nescence reporter system, respectively. The reporter gene contain-

3′ UTR_L exhibited significantly reduced luciferase activities compared with that containing 3′ UTR_S (Fig. 2G), implying that the APA-induced longer 3′ UTR of Rras2 down-regulated RRAS2 protein abundance.

To determine whether decreased levels of RRAS2 trigger sen-

escence-associated phenotypes, we depleted RRAS2 with two short hairpin RNAs (shRNA-mediated knockdown; Rras2-KD) in mouse NIH3T3 cells (Fig. 2H; Supplemental Fig. S14A) and observed delayed cell proliferation and increased SA-β-gal staining (Fig. 2I, J; Supplemental Fig. S14B,C). Cell cycle analysis showed that RRAS2-depletion reduced the percentage of S phase cells (Fig. 2K, L). Cdkn1a, which encodes cyclin-dependent kinase inhibitor 1A (P21), showed increased expression upon Rras2-KD (Fig. 2M).

Moreover, Rras2-KD in primary MEFs has similar effects to those in NIH3T3 cells (Fig. 2N; Supplemental Fig. S15). In addition, reintroduction of RRAS2 (Rras2-Rescue) into Rras2-KD cells rescued the expression level of RRAS2 (Fig. 2O) and gave rise to reduced SA-β-gal signals (Fig. 2Q). Through RNA-seq profiling of Rras2-KD and Rras2-Rescue NIH3T3 cells, we found that altered RRAS2 expression caused expression changes in a variety of genes (Fig. 2S). Gene Ontology (GO) and pathway analysis revealed that those genes showing opposite expression trends were enriched in senescence-relevant biological processes, including cell cycle, cell adhesion, and DNA replication (Fig. 2T,U; Supplemental Table S5). More importantly, overexpression of RRAS2 (Fig. 2P) was able to re-

verse the SA-β-gal staining (Fig. 2R) and rescue Cdkn1a expression levels in senescent MEFs (Supplemental Fig. S16). Taken together, these data indicate that RRAS2 plays a crucial role in delaying cellular senescence.

To address whether APA in Rras2 is evolutionarily conserved between rodents and human, we verified the existence of its two 3′ UTRs in both human embryonic kidney (HEK) 293T cells and human umbilical vein endothelial cells (HUVECs) (Kim et al. 2007; Muck et al. 2008; Cardus et al. 2013; Zhang et al. 2014). Our PA-seq data from 293T cells (Ni et al. 2013) and public PolyA-seq data from human tissues confirmed the existence of both 3′ UTRs in human RRAS2 (Fig. 3A). The two 3′ UTRs were validated by 3′ RACE (Fig. 3B), and their PCR products were cloned and sequenced by the Sanger method to confirm the existence of poly(A) or poly(T) sequence at the end of the amplicon (Supplemental Fig. S17). Of note, senescent HUVEC cells also fa-

vored the distal pA site (Fig. 3C), which generated less protein (Fig. 3D,E). Knockdown of RRAS2 (Fig. 3F–I) increased SA-β-gal activity (Fig. 3J), slowed cell proliferation rate (Fig. 3K,L), and induced Cdkn1a expression in both 293T cells and HUVECs (Fig. 3M,N). These findings indicate an evolutionarily conserved mechanism that longer 3′ UTR of Rras2 causes reduced protein production and then results in senescence in both rodents and hu-

man cells.

Splicing factor TRA2B represses RRAS2 protein level through binding to its alternative 3′ UTR and contributes to cellular senescence

We reasoned that cis-elements recognized by either miRNAs or RBPs in the alternative 3′ UTR of Rras2 contribute to decreased RRAS2 protein production. To this end, we divided the 3′ UTR of Rras2 into four regions by deleting sequences of different lengths (shown as R1−R4 in Fig. 4A) and inserted them separately into a luciferase reporter, which was then transected into mouse cells. Subsequent luciferase assays demonstrated significantly reduced luciferase activity from the construct containing a long 3′ UTR (re-

sulting from mutated proximal PAS, labeled “M”) compared with the short 3′ UTR (labeled “S”) and the R1–R4 constructs (Fig. 4B). Thus, the sequence of last 148 base pairs (bp) located in the alternative 3′ UTR of Rras2 contains key motifs or elements essential for protein production.

To further narrow the range of key elements, four more re-

porter constructs harboring the truncated 3′ UTRs (presented as R5–R8 in Fig. 4A) were generated. The construct lacking a 34-bp el-

ement showed significantly reduced luciferase activity, indicating that this element is critical for down-regulation of protein produc-

tion (Fig. 4A,B). No miRNA binding sites were predicted in this 34-

bp region; therefore, we searched RBPsnap (Paz et al. 2014) for potential RBPs that might recognize this element and identified four RBPs (SRSF2, ZC3RB1, TRA2B, and MBLN1). Following individual knockdown of the four RBPs, we measured the luciferase activities of S and M constructs (Fig. 4C). Depletion of TRA2B, a known splicing factor involved in mRNA processing, cell proliferation, and migration (Yang et al. 2015), eliminated the difference in luciferase activities between the S and M fragments-containing reporter constructs (Fig. 4C), suggesting that TRA2B played an important role in down-regulating protein production. These data implied that the 34-bp element in the 3′ UTR of Rras2 is required for TRA2B-mediated down-regulation of RRAS2.

The “AGAA” element may serve as the core sequence of the TRA2B binding motif (Fig. 4D; Grellscheid et al. 2011). Considering that “AGAA” shared 1 nt with the downstream PAS “AATTA” in the alternative 3′ UTR of Rras2, we mutated the first 3 nt to avoid disruption of the PAS (“AGAA” to “CCCA”) (designated Mut3 in Fig. 4D). All 4 nt were also mutated (“AGAA” to “CCCC”) (denoted Mut4 in Fig. 4D) to validate the effects that are dependent on the whole core sequence. According to luciferase reporter assays, the Mut3 construct partially restored luciferase activity compared with that for M, while Mut4 showed a higher capability of restoring the repressed luciferase activity than Mut3 (Fig. 4D). To explore whether TRA2B directly binds to the alternative 3′ UTR of Rras2, we carried out RNA immunoprecipitation coupled with both regular and quantitative PCR (RIP-PCR and RIP-qPCR) assays. The results showed an enrichment of TRA2B-binding signal (Fig. 4E), suggesting direct binding of TRA2B to the alternative 3′ UTR of Rras2. To further evaluate the role of “AGAA,” we moved this motif to two upstream positions in the
Figure 2. Down-regulation of Rras2 through 3′ UTR lengthening promotes cellular senescence in mouse cells. (A) PA-seq track of proximal and distal pA site usage in PD6 and PD11 MEFs. Raw tag numbers reflected by both width and height of each pA peak are indicated in parentheses. (B) Changes of distal and proximal pA site usage between PD6 and PD11 MEF cells detected by 3′ RACE. The 3′ RACE strategy is shown in the upper panel. F and R indicate forward and reverse primers, respectively. PCR product sizes for distal and proximal pA sites are shown in parentheses. (C) The Rras2 gene has increased usage of the distal pA site in MEF PD11 compared to PD6 detected by qRT-PCR. (D, E) qRT-PCR (D) and Western blot (E) of Rras2 expression in PD6 and PD11 MEFs. Gapdh serves as the internal control. (F) Stability comparison of Rras2 isoforms with 3′ UTR_S and 3′ UTR_L in mouse fibroblast cell line NIH3T3. (G) Luciferase activity from a reporter system containing 3′ UTR_S or 3′ UTR_L. (H) qRT-PCR (upper panel) and Western blot (lower panel) validation of Rras2 knockdown in NIH3T3 cells. (I) Proliferation rate evaluation of NIH3T3 cells without (Ctrl) and with Rras2 knockdown (Rras2-KD, sh979) by Cell Counting Kit-8 (CCK-8). (J) SA-β-gal staining in NIH3T3 cells without (Ctrl) and with Rras2 knockdown (Rras2-KD). (K) FACS analysis of four replicate samples showed a lower percentage of Rras2-KD NIH3T3 cells in S phase compared with control cells. (L) Percentage of cells in S, G1, and G2/M phase in one replicate. (M,N) Rras2 depletion promoted Cdkn1a expression in mouse NIH3T3 cells (M) and MEFs (N) evaluated by qRT-PCR. (O,P) Western blot validations of Rras2 rescue in Rras2-KD NIH3T3 cells (O) and Rras2-overexpression (OE) in senescent MEFs (P). GAPDH serves as the internal control. (Q) Higher SA-β-gal staining caused by reduced Rras2 expression was rescued by overexpression of Rras2 (Rras2-OE) in mouse NIH3T3 cells. (R) Rras2-OE reversed senescence in primary MEF cells as evaluated by SA-β-gal staining assay. (S) Heat map of log2-transformed expression ratio of differentially expressed genes shared by Rras2-KD and Rras2-Rescue cells compared with the control NIH3T3 cells. Red and green denotes increased and decreased expression, respectively. The numbers of down- and up-regulated genes are shown on the left. (T,U) Enrichment of all differentially expressed genes shown in S for biological process (T) and the KEGG pathway (U), as determined by DAVID. (∗∗∗) P < 0.001, (∗∗) P < 0.01, (∗) P < 0.05, two-tailed t-test.
alternative 3' UTR of Rras2. The “AGAA” motif at a more proximal position did not repress protein production (Supplemental Fig. S18), implying that the “AGAA” motif in the alternative 3' UTR of the Rras2 gene was required but not sufficient to repress protein levels. Together, these data suggest that the “AGAA” motif within the alternative 3' UTR of Rras2 is essential for TRA2B binding and repression of RRAS2 protein production.

To further confirm the contribution of TRA2B-RRAS2 regulation to cellular senescence, we overexpressed TRA2B in 293T cells, which show a higher usage of the distal pA site in RRAS2 and can serve as an appropriate cell model to perform the test, given that TRA2B bound to the alternative 3' UTR of Rras2 (Fig. 3A). As expected, ectopic expression of TRA2B led to decreased RRAS2 protein level (Fig. 4F,G). Notably, neither RNA stability (Fig. 4H, I) nor mRNA steady-state levels of RRAS2 (Fig. 4J) showed considerable changes upon TRA2B overexpression, suggesting that the reduced RRAS2 protein level was caused by repressed translation through binding of TRA2B to the alternative 3' UTR. Up-regulation of TRA2B led to a reduced proliferation rate (Fig. 4K), higher SA-β-gal staining level, and increased CDKN1A expression (Fig. 4L,M). Recovery of RRAS2 expression by additional overexpression of RRAS2 in TRA2B-overexpressed cells attenuated the CDKN1A expression and SA-β-gal staining to basal levels (Fig. 4N,O). Taken together, these data demonstrated that binding of TRA2B to the alternative 3' UTR of Rras2 results in decreased RRAS2 expression, which in turn accelerated cellular senescence.

Key elements involved in APA regulation of Rras2 are evolutionarily conserved

Since Rras2 prefers the distal pA site in senescent mouse, rat, and human cells (Figs. 2A–C, 3C; Supplemental Fig. S13), it would be
interesting to have an evolutionary view on related $Rras2$ sequences. Comparative genomic analysis revealed that the 3′ UTR of $Rras2$ was extremely conserved in representative species from galliformes to primates (Supplemental Fig. S19), implying that APA-mediated 3′ UTR lengthening during cellular senescence was of evolutionary significance. Notably, all listed animals contained a canonical PAS (AA/TTAAA) near the proximal pA site (Fig. 4P). This strongly indicated that APA-regulated $Rras2$ expression plays a crucial role during evolution. Sequence adjacent to the distal pA site within the 3′ UTR of $Rras2$ also contained the canonical PAS “ATTAAA” and the TRA2B binding motif “AGAA,” which were conserved from birds to mammals (Fig. 4P), indicating that a strong selection pressure is likely to have driven the molecular evolution for APA-mediated regulation of APA in $Rras2$.

**Discussion**

In this study, we discovered that APA-mediated 3′ UTR lengthening played a role in cellular senescence. As exemplified by $Rras2$ (Fig. 5), we extended the functional importance of APA to the aging field and provided a novel perspective for understanding the mechanism underlying cellular senescence. However, the factors controlling 3′ UTR lengthening during cellular senescence need to be further explored. Given that certain core factors in the 3′ processing machinery were known to play a role in APA regulation (Takagaki et al. 1996; Zheng and Tian 2014), we therefore surveyed a set of core factors controlling 3′ UTR lengthening during cellular senescence. Most of these factors underwent a trend of decreased expression during MEFs senescence (Supplemental Fig. S20), consistent with

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**Figure 4.** Binding of TRA2B to the alternative 3′ UTR of $Rras2$ causes cellular senescence via reduced $RRAS2$ expression. (A) Schematic diagram of the truncation strategy to identify key cis regulatory elements. M denotes full length 3′ UTR with mutated proximal poly(A) signal. S represents 3′ UTR using the proximal pA site. R1–R4 refer to four truncated 3′ UTR fragments constructed for the first round screen. R5–R8 denote four additional constructs for the second-round screen. (B) Luciferase assays for all constructs indicated in A. (C) Luciferase assay screen for key trans-acting factors in candidate RBP knockdown cells. (ns) Not significant. (D) Luciferase assay in mouse cells transfected with constructs containing mutations introduced in the potential region containing the key cis-element. “AGAA” is the core binding motif of TRA2B (Grellscheid et al. 2011). (E) TRA2B binding to the alternative 3′ UTR of $Rras2$ evaluated by RIP-PCR and RIP-qPCR. The PCR primer pair (F and R, forward and reverse, respectively) was designed to span the $Rras2$ UTR of interest. Comparative genomic analysis revealed that the 3′ UTR of $Rras2$ was extremely conserved in representative species from galliformes to primates (Supplemental Fig. S19), implying that APA-mediated regulation of $Rras2$ is of evolutionary significance. Notably, all listed animals contained a canonical PAS (AA/TTAAA) near the proximal pA site (Fig. 4P). This strongly indicated that APA-regulated $Rras2$ expression plays a crucial role during evolution. Sequence adjacent to the distal pA site within the 3′ UTR of $Rras2$ also contained the canonical PAS “ATTAAA” and the TRA2B binding motif “AGAA,” which were conserved from birds to mammals (Fig. 4P), indicating that a strong selection pressure is likely to have driven the molecular evolution for TRA2B-mediated regulation of APA in $Rras2$. [Graphs and images are not fully transcribed due to limitations in the text model. Additional analysis would be required for a complete understanding.]
recent findings that up-regulation of polyadenylation factors was associated with 3′ UTR shortening (Mayr and Bartel 2009; Xia et al. 2014). These results provided a possible mechanistic explanation for 3′ UTR lengthening during cellular senescence and deserved further investigation.

Genes preferring distal pA sites showed a global lengthening of the 3′ UTR and a trend of decreased mRNA levels in both senescent MEFs and rVSMCs (Fig. 1), supplementing the observation that genes favoring proximal pA sites tended to have increased mRNA levels in cancer cells (Mayr and Bartel 2009; Xia et al. 2014). We also found that many genes displayed opposite pA site usage preference in senescent cells compared with cancer cells. Eighty-two and 166 genes that favored proximal pA sites in seven tumor types were prone to use distal pA sites in senescent MEFs and rVSMCs, respectively (Supplemental Fig. S21). In addition, 35 genes preferring shorter 3′ UTRs in multiple cancer cells tended to use longer 3′ UTRs in both senescent MEFs and rVSMCs (Supplemental Fig. S21). These findings supported a model that interaction between condition-specific trans-acting factors and dynamic changes in 3′ UTR length determined by APA could contribute to opposite biological processes, such as cellular senescence and tumor development.

There are two major categories of cellular senescence, developmentally programmed senescence and stress-induced premature senescence (SIPS) (Munoz-Espin and Serrano 2014). Here, we showed that MEFs in replicative senescence underwent global 3′ UTR lengthening. VSMCs derived from old rats were likely to undergo a combination of replicative senescence and varieties of stress-induced senescence. Whether SIPS itself will induce global 3′ UTR lengthening needs to be further determined. Thus, more senescence models are required to fully understand the prevalence and functional relevance of 3′ UTR lengthening. In conclusion, our results provide evidence that APA contributes to the regulation of gene expression during cell senescence in multiple species, implying APA-regulated gene expression may be evolutionarily conserved.

**Methods**

**Cell isolation, cultivation, and total RNA extraction**

Primary MEFs were isolated from embryos of a 12.5- to 14-d pregnant C57BL/6 mouse according to a previously described method (Todaro and Green 1963). Details were described in Supplemental Methods. Total RNA was isolated using TRIzol reagent (Invitrogen for both MEFs and rVSMCs from rats of different ages (2 wk and 2 yr).

**SA-β-gal staining, cell proliferating rate assay, and cell cycle analysis**

SA-β-gal activity (Dimri et al. 1995) was monitored in mouse (MEFs, NIH3T3) and human (293T, HUVECs) cells using a senescence detection kit (BioVision, cat. no. K320-250). Mouse (NIH3T3) and human (293T, HUVECs) cells with or without candidate gene knockdown were cultured and assayed for cell proliferation using a Cell Counting Kit-8 (CCK-8) according to the vendor’s instructions (Dojindo). Details of SA-β-gal staining and cell cycle analysis are described in Supplemental Methods.

**PA-seq and RNA-seq library construction, mapping, and peak calling**

The PA-seq libraries were constructed according to our established protocol (Ni et al. 2013). The dUTP-based strand-specific RNA-seq libraries were constructed by following a previously described protocol (Parkhomchuk et al. 2009). Both types of libraries were sequenced using an Illumina HiSeq2000 platform in a paired-end 2 × 101-bp manner. Processed raw data were aligned to the mouse genome (version mm9) or rat genome (version rn5) using TopHat2 (Kim et al. 2013a). Detailed peak calling of pA sites and expression analysis were included in Supplemental Methods. All called pA clusters in MEFs are listed in Supplemental Table S6.
Comparison of APA profiles between MEFs of different passages

The tandem 3′ UTR isoform switch index values were calculated according to a published approach (Fu et al. 2011; Li et al. 2012). Genes with expression fold-change ≥ 1.5 and FPKM ≥ 1 in both samples are shown in Figure 1, D and E. The genes with a P-value cut-off of 0.05 (corresponding to a false discovery rate [FDR]) estimated by the Benjamini–Hochberg method with R software (R Core Team 2015) were considered to have significantly changed APA among the different passages. Specifically, an FDR ≤ 0.05 with a positive TSI (Li et al. 2012) implies a lengthening 3′ UTR across the different passages of cellular senescence; an FDR ≤ 0.05 with a negative TSI implies a shortening 3′ UTR. All APA genes with detailed information, including expression value, effective 3′ UTR length, and RUD index (Ji et al. 2011), are included in Supplemental Table S7. Comparison of lengthening or shortening of 3′ UTRs is included in Supplemental Table S8.

Pathway enrichment analysis

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Huang da et al. 2009) was used for the pathway enrichment analysis, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was selected.

Western blotting and qRT-PCR

Primary antibodies (RRAS2, 1:1000, cat. no. ab209078, Abcam; GAPDH, 1:2000–3000, cat. no. sc-32233, Santa Cruz Biotechnology; CDKN2A, 1:500–1000, cat. no. sc-1207, Santa Cruz Biotechnology) targeting proteins of interest were used for the Western blot. A detailed description is included in Supplemental Methods. Total RNA used for expression quantification was reverse-transcribed into cDNA using random primers, and then mRNA levels were quantified by qRT-PCR and normalized to that of Gapdh (Roche LightCycler). For PA site usage quantification, RNAs were reverse-transcribed with oligo(dT) primer, followed by PCR with two pairs of primers (proximal and distal) targeting different regions of the cDNAs. Specifically, the region targeted by the proximal pair is common to both APA isoforms and the region targeted by the distal pair is unique to the longer isoform. qPCR signals from the proximal and distal pairs of primers were compared to indicate the relative expression of the two isoforms. All primer sequence information is listed in Supplemental Table S9.

RNA stability, RNA immunoprecipitation, and luciferase assay

For evaluation of RNA stability, cells were treated with actinomycin D (5 μg/mL) for 0, 2, 4, 6, 8, 10, and 24 h. After the indicated time, total RNAs were isolated and analyzed by qRT-PCR. Primer sequences are shown in Supplemental Table S9. RNA immunoprecipitation (RIP) was performed according to a previously published protocol with minor modification (Zhao et al. 2008). The Dual-Luciferase Reporter 1000 Assay System (Promega) was carried out to evaluate the impact of protein production for different lengths of 3′ UTRs. Detailed information of RIP-PCR and the luciferase assay are described in Supplemental Methods.

Data access

The raw PA-seq and RNA-seq data from this study have been submitted to the NCBI Sequence Read Archive (SRA); https://www.ncbi.nlm.nih.gov/sra/ under accession number SRP065821.

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