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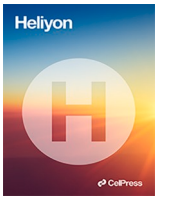

TRAF3IP2 drives mesenchymal stem cell senescence via regulation of NAMPT-mediated NAD biosynthesis

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ABSTRACT

Background: The cellular senescence of mesenchymal stem cells (MSCs) limits their application in regenerative medicine. This study aimed to clarify the role of TNF receptor-associated factor 3 interacting protein 2 (TRAF3IP2), a pro-inflammatory cytoplasmic adaptor protein, in regulating MSC senescence and to explore the potential mechanisms. **Methods**: MSC senescence was determined by senescence-associated β-galactosidase (SA-β-gal) staining. The expression of TRAF3IP2 and senescence-related proteins was detected by Western blotting. The nicotinamide adenine dinucleotide (NAD+) level and nicotinamide phosphoribosyl transferase (NAMPT) expression in MSCs was measured. **Results:** Compared with that in MSCs isolated from young donors (YMSCs), the expression of TRAF3IP2 was greatly increased in MSCs derived from aged donors (AMSCs). Overexpression of TRAF3IP2 accelerated YMSC senescence whereas downregulation significantly rescued cellular senescence. The protein level of NAMPT and the level of NAD+ were significantly decreased in AMSCs compared with YMSCs. Mechanistically, TRAF3IP2 induced MSC senescence via downregulation of NAMPT expression and NAD + level by inhibiting the AMPK signaling pathway. These effects were partially reversed by treatment with an AMPK or NAMPT activator. **Conclusion:** We revealed that TRAF3IP2 accelerated MSC senescence via downregulation of NAMPT-mediated NAD biosynthesis by mediation of the AMPK pathway, highlighting a novel means to rejuvenate senescent MSCs.

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1. Introduction

Over the past decades, the application of stem cell-based therapy in pre-clinical experiments and clinical trials has shown promising results for many diseases. Among the different types of stem cell, mesenchymal stem cells (MSCs) have been intensively studied due to their easy isolation and expansion ability, multipotency, and immunomodulatory effects [1,2]. Nonetheless MSCs derived from aged donors (AMSCs) undergo cellular senescence, as evidenced by increased cell size and decreased proliferative capacity and paracrine effects as well as gradual loss of functions. This has limited their therapeutic application [3–6]. We and others have revealed mounting evidence that, compared with MSCs derived from young donors (YMSCs), transplantation of AMSCs display impaired therapeutic efficacy for cardiovascular diseases [7–11]. Understanding the molecular mechanisms that underlie MSC senescence is essential when exploring plausible strategies to rejuvenate senescent MSCs and improve their therapeutic efficacy.

TNF receptor-associated factor 3 interacting protein 2 (TRAF3IP2) is a pro-inflammatory cytoplasmic adaptor protein that plays a critical role in regulating multiple diseases including cardiovascular and inflammatory diseases and tumor growth [12–14]. Importantly, TRAF3IP2 is involved in the regulation of cell proliferation in multiple cell lines including smooth muscle cells, cardiac fibroblasts and vascular endothelial cells [15–17]. It has been well documented recently that GATA4 induces cellular senescence and initiates a senescence-associated secretory phenotype (SASP) via activation of TRAF3IP2 in human fibroblasts [18]. More importantly, depletion of TRAF3IP2 partially abrogates GATA4-induced senescence, suggesting the TRAF3IP2 plays a critical role in regulating cellular senescence. Nevertheless whether and how TRAF3IP2 regulates MSC senescence have not been investigated.

There is increasing recognition that the intracellular nicotinamide adenine dinucleotide (NAD+) level directly regulates multiple physiological and pathological processes including cellular senescence, cell biosynthesis and cell cycling [19,20]. The NAD + level is greatly reduced in senescent MSCs but supplementation with exogenous NAD + significantly postpones MSC senescence [21]. Nicotinamide phosphoribosyl transferase (NAMPT), the rate-limiting enzyme in the NAD salvaging pathway, plays an important role in regulating NAD + level [22]. Age-related decline of intracellular NAD+ is closely associated with downregulation of NAMPT expression [23]. Indeed, NAMPT expression is downregulated in MSCs isolated from aged rats and NAMPT overexpression ameliorates MSC senescence by improving the NAD + level [24]. Whether TRAF3IP2 mediates MSC senescence by regulating NAMPT-NAD + biosynthesis nonetheless remains unclear. We hypothesized that the regulatory effects of TRAF3IP2 on MSC senescence are related to NAMPT-NAD biosynthesis. In the current study, we showed that compared with YMSCs, TRAF3IP2 level was significantly increased in AMSCs and overexpression of TRAF3IP2 accelerated MSC senescence whereas downregulation significantly rescued cellular senescence. We also demonstrated that TRAF3IP2 accelerated MSC senescence by downregulating NAMPT-NAD + biosynthesis via inhibition of the AMPK pathway, revealing a potential novel means to rejuvenate MSCs.

2. Materials and methods

2.1. mRNA expression analysis

Five public mRNA expression microarray datasets were utilized to identify mRNA expression changes to TRAF3IP2 during the aging process in humans. They included (1) GSE137186 (GEO accession number) representing early and senescent passages of human BM-derived MSCs, (2) GSE97311 to compare human iPSC-derived MSCs (iMSCs) from fetal and aged individuals, (3) GSE37470 representing early and senescent passages of human BM-derived MSCs, (4) GSE35959 representing human MSCs from aging individuals and primary osteoporosis, and (5) GSE12274 to compare BM-MSCs from young and aged donors. The data of these studies were extracted from the GEO database. Expression data of TRAF3IP2 were selected for linear regression analysis.

2.2. Cell culture

Human YMSCs and AMSCs were cultured and characterized as in our previous report [7]. This procedure was approved by the research ethics board of Shanghai East Hospital (No. 2016-050). MSCs were cultured at 37 °C in DMEM/high glucose (Gibco, 11965084) medium containing 10% FBS (Life Technologies, 16000), 5 ng/ml EGF (PeProTech, AF-100-15) and 5 ng/ml FGF2 (PeProTech, 100-18B) in a humidified atmosphere with 5% CO_2. The MSCs were passaged at 3-day intervals. Both YMSCs and AMSCs at passage 3–4 were used in the current study. The ability of YMSCs, AMSCs, TRAF3IP2-YMSCs and TRAF3IP2-siRNA-AMSCs to differentiate into adipocytes or osteocytes was examined as previously reported [25].

2.3. SA-β-gal assay

MSC senescence was evaluated using a SA-β-gal staining kit (Beyotime, C0602) as previously described [26]. Briefly, MSCs with different treatments were cultured in 6-well plates. After washing with PBS three times, MSCs were fixed with formaldehyde for 20min and incubated with SA-β-gal staining solution at 37 °C without CO_2 overnight. Next, SA-β-gal positive cells (stained blue) were randomly captured from five different fields of view in each group. Finally, the percentage of senescent MSCs was calculated as the ratio of SA-β-gal-positive MSCs to total number of MSCs. Experiments were repeated at least three times.

2.4. Immunofluorescence staining

Immunofluorescence staining was performed as previously described [27]. Briefly, MSCs with different treatments were cultured in

a 24-well plate with glass cover slides and then fixed with 4% PFA for 30 min. After permeabilization with 0.2% Triton X-100 in PBS at room temperature for 30 min, cells were incubated at 4 °C overnight with Ki67 antibody (1: 100, Abcam, ab15580). Subsequently, cells were incubated with fluorescent secondary antibody at room temperature for 1 h. Finally, all samples were mounted with DAPI and photographed. The percentage of Ki67 positive MSCs was calculated. The experiments were repeated at least three times.

2.5. siRNA intervention

To knockdown TRAF3IP2 in AMSCs, TRAF3IP2-siRNA (RiboBio, China) and control siRNA (RiboBio, China) were used to transfect AMSCs using a Lipofectamine RNAiMAX Reagent Kit (Invitrogen, 13778–075) at a standardized MOI (multiplicity of infection) of 5 according to the manufacturer's instructions. After 72 h, the cells were collected and the silencing efficiency determined by Western blotting.

2.6. Western blotting

The proteins of MSCs with different treatments were extracted and their concentration measured. A total of 30 μg protein from each sample was loaded, separated by SDS/PAGE, and then transferred to a PVDF membrane. After blocking with 5% fat-free milk in TBST, the membrane was incubated at 4 °C overnight with the following antibodies: TRAF3IP2 (Proteintech, 26692-1-AP), NAMPT

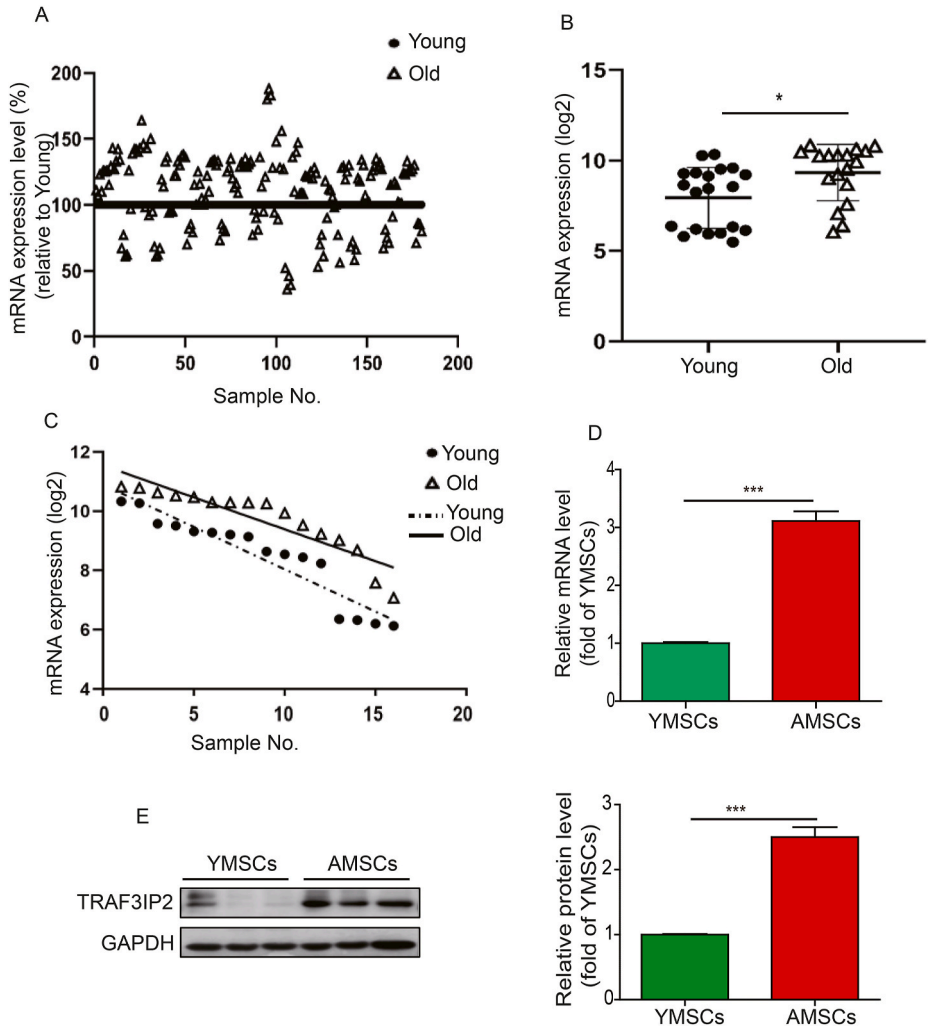

Fig. 1. Upregulation of TRAF3IP2 expression in AMSCs. (A) Scatter plot of mRNA expression of TRAF3IP2 (relative to young cells). (B) mRNA expression of TRAF3IP2 in young and old cells. (C) Linear regression of mRNA expression of TRAF3IP2 in young and old cells or tissue. (D) Quantitative analysis of TRAF3IP2 mRNA level in YMSCs and AMSCs. (E) Western blotting and quantitative analysis of TRAF3IP2 protein level in YMSCs and AMSCs (n = 3). All data were obtained from at least three independent experiments and each error bar represents the mean ± SEM. *$p < 0.05$, ***$p < 0.001$. The raw data of Fig. 1E is presented in Supplementary Fig. 3.

(Proteintech, 11776-1-AP), p21 (Santa Cruz, SC-271532), p16 (Proteintech, 10883-1-AP), and GAPDH (Santa Cruz, SC-137179). Next, the membrane was washed with TBST and incubated for 1hr at room temperature with horseradish peroxidase-conjugated secondary antibodies (1:10,000; Santa Cruz). Finally, the membrane was exposed to radiography film in a dark room and protein blots quantified using ImageJ software.

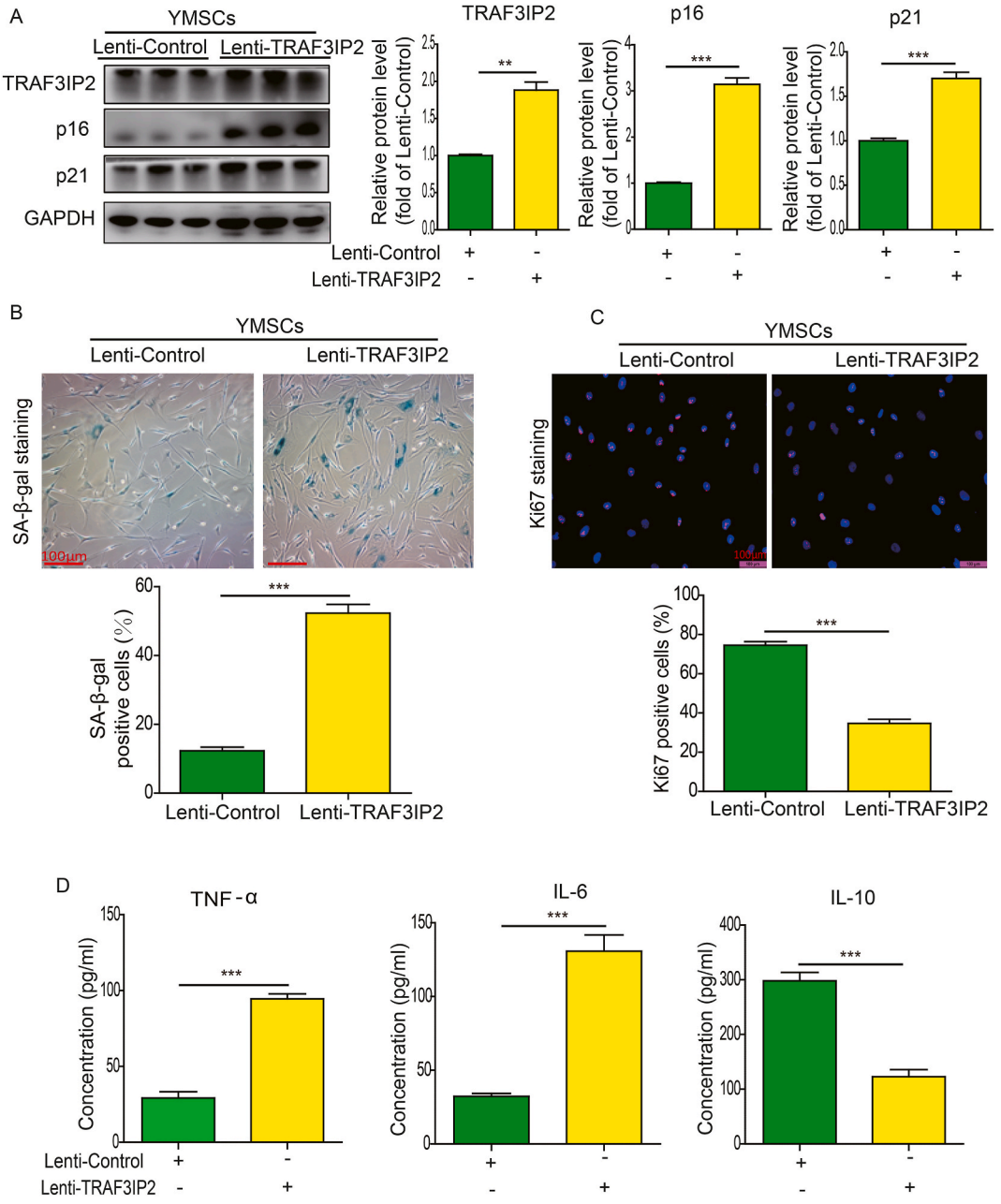

Fig. 2. Overexpression of TRAF3IP2 induces YMSC senescence. (A) Western blotting and quantitative analysis of TRAF3IP2, p16 and p21 expression in YMSCs transfected with control-lentivirus or TRAF3IP2-lentivirus. (B) Representative images and quantitative analysis of SA-β-gal staining in control-lentivirus- or TRAF3IP2-lentivirus-treated YMSCs. (C) Representative images and quantitative analysis of Ki67 immunostaining in control-lentivirus- or TRAF3IP2-lentivirus-treated YMSCs. (D) The concentration of TNF-α, IL-6 and IL-10 in CdM isolated from control-lentivirus- or TRAF3IP2-lentivirus-treated YMSCs. All data were obtained from at least three independent experiments and each error bar represents the mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$. The raw data of Fig. 2A is presented in Supplementary Fig. 3.

2.7. NAD + measurement

Intracellular NAD + concentration of MSCs from different groups was measured using a NAD+/NADH Quantification Kit (Colorimetric) (ab65348; Abcam) as previously described [28]. Briefly, a total of 2 × 10^5 MSCs was collected and lysed with NAD+/NADH extraction buffer. Next, the supernatant was used for NAD+/NADH measurement using an NAD+/NADH assay kit. Subsequently, NAD + content was determined according to the standard curve created by NADH standards. Finally, the data were normalized to the total cell number.

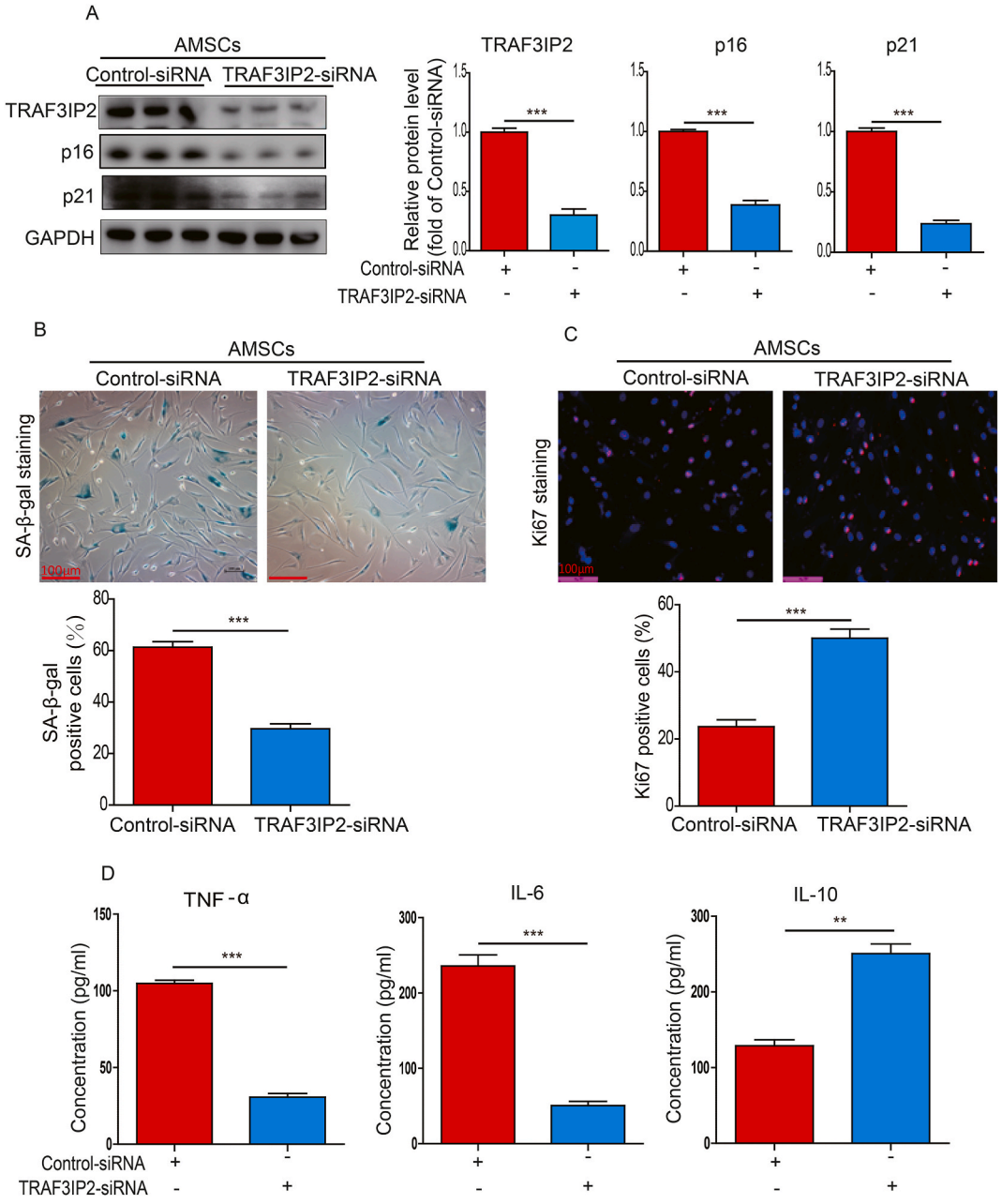

Fig. 3. Knockdown of TRAF3IP2 rejuvenates AMSC senescence. (A) Western blotting and quantitative analysis of TRAF3IP2, p16 and p21 expression in AMSCs transfected with control-siRNA or TRAF3IP2-siRNA. (B) Representative images and quantitative analysis of SA-β-gal staining in control-siRNA or TRAF3IP2-siRNA -treated AMSCs. (C) Representative images and quantitative analysis of Ki67 immunostaining in control-siRNA or TRAF3IP2-siRNA-treated AMSCs. (D) Concentration of TNF-α, IL-6 and IL-10 and TNF-α in CdM isolated from control-siRNA or TRAF3IP2-siRNA-treated AMSCs. All data were obtained from at least three independent experiments and each error bar represents the mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$. The raw data of Fig. 3A is presented in Supplementary Fig. 3.

2.8. Lentiviral construct packaging and infection

The lentiviral plasmid constructs for TRAF3IP2 were purchased from GenePharma (Suzhou, China). The lentiviruses were packaged by transfecting 293 T cells using the lentiviral packaging system that contained the recombinant lentiviral transfer TRAF3IP2 plasmid as previously reported [29]. After 48 h of culture, the supernatant of transfecting 293 T cells was harvested, concentrated, and titered. Next, the virus was used to infect YMSCs with a confluence of 70–80% at an MOI of 10 with polybrene (8 μg/ml). Finally, transduction efficiency was examined by Western blotting.

2.9. Enzyme-linked immunosorbent assay (ELISA)

Conditioned medium (CdM) from YMSCs, AMSCs, and YMSCs with overexpression of TRAF3IP2 or AMSCs with TRAF3IP2-siRNA treatment was prepared as previously described [30]. The concentration of SASP-related factors in the CdM, including interleukin-6 (IL-6), IL-10 and tumor necrosis factor-α (TNF-α), was measured by ELISA. The experiments were repeated at least three times.

2.10. Statistical analysis

Data are expressed as mean ± SEM. Statistical analyses were performed using Prism 5.0 (GraphPad Software). Differences between two groups were assessed using unpaired Student's t-test and between multiple groups using one-way ANOVA followed by the Bonferroni test. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Expression of TRAF3IP2 is upregulated in AMSCs

To illustrate the relationship between TRAF3IP2 and senescence, we first determined the gene expression of TRAF3IP2 from five publicly available microarray datasets representing young versus old/senescent cells and tissue (GSE137186, GSE97311, GSE37470, GSE35959, and GSE12274). In all five datasets, we analyzed the mRNA expression of TRAF3IP2 during the aging process. A scatter plot for mRNA expression (relative to young) of TRAF3IP2 is shown in Fig. 1A. The mRNA expression of TRAF3IP2 was significantly increased in old cells or tissue compared with young (Fig. 1B). Linear regression of mRNA expression in the old cells/tissue was greater than that in the young samples (Fig. 1C). These results prompted us to examine the mRNA and protein level of TRAF3IP2 in YMSCs and AMSCs. RT-PCR revealed that the mRNA level of TRAF3IP2 was much higher in AMSCs than YMSCs (Fig. 1D). Moreover, the protein level of TRAF3IP2 was greatly increased in AMSCs compared with YMSCs (Fig. 1E). These findings indicated that the expression of TRAF3IP2 was upregulated in AMSCs and that TRAF3IP2 is involved in MSC senescence.

3.2. Overexpression of TRAF3IP2 accelerates YMSC senescence

To determine the role of TRAF3IP2 in the regulation of MSC senescence, we first treated YMSCs with TRAF3IP2-lentivirus to overexpress TRAF3IP2. As shown in Fig. 2A, TRAF3IP2-lentivirus treatment significantly enhanced TRAF3IP2 expression and improved expression of senescence-related markers p16 and p21 in YMSCs (Fig. 2A). SA-β-gal staining showed that the number of SA-β-gal-positive cells was significantly increased in TRAF3IP2-YMSCs compared with YMSCs (Fig. 2B). Ki67 staining demonstrated that overexpression of TRAF3IP2 inhibited the proliferative capacity of YMSCs as evidenced by the decreased number of Ki67-positive cells (Fig. 2C). Overexpression of TRAF3IP2 also significantly improved the secretion of IL-6 and TNF-α in YMSCs and inhibited that of IL-10 (Fig. 2D). We also evaluated the adipogenic and osteogenic differentiation ability of YMSCs and TRAF3IP2-YMSCs. Compared with YMSCs, TRAF3IP2-YMSCs exhibited an enhanced adipogenic differentiation ability but decreased osteogenic differentiation ability as evidenced by Oil Red O staining and Alizarin Red staining, respectively (Fig. S1). Collectively, these data showed that overexpression of TRAF3IP2 accelerated progression of YMSCs to a senescent state.

3.3. Knockdown of TRAF3IP2 rejuvenates senescent AMSCs

To further verify the relationship between TRAF3IP2 and MSC senescence, we used siRNA to knockdown TRAF3IP2 in AMSCs. As shown in Fig. 3A, TRAF3IP2-siRNA treatment greatly downregulated TRAF3IP2, p16 and p21 protein expression in AMSCs (Fig. 3A). Moreover, SA-β-gal activity (Fig. 3B) was greatly decreased and number of Ki67-positive cells increased (Fig. 3C) in AMSCs. More importantly, TRAF3IP2-siRNA treatment inhibited the secretion of IL-6 and TNF-α in AMSCs and enhanced that of IL-10 (Fig. 3D). We further determined that TRAF3IP2-siRNA treatment greatly downregulated adipogenic differentiation ability of AMSCs but upregulated that for osteogenic differentiation (Figure S2). These results demonstrated that knockdown of TRAF3IP2 rejuvenated senescent MSCs.

3.4. TRAF3IP2 regulates MSC senescence via mediation of NAMPT–NAD biosynthesis

Several studies have shown that NAMPT–NAD biosynthesis plays a critical role in regulating cellular senescence [31,32]. To determine whether TRAF3IP2 regulates MSC senescence via mediation of NAMPT–NAD biosynthesis, we first examined the NAMPT

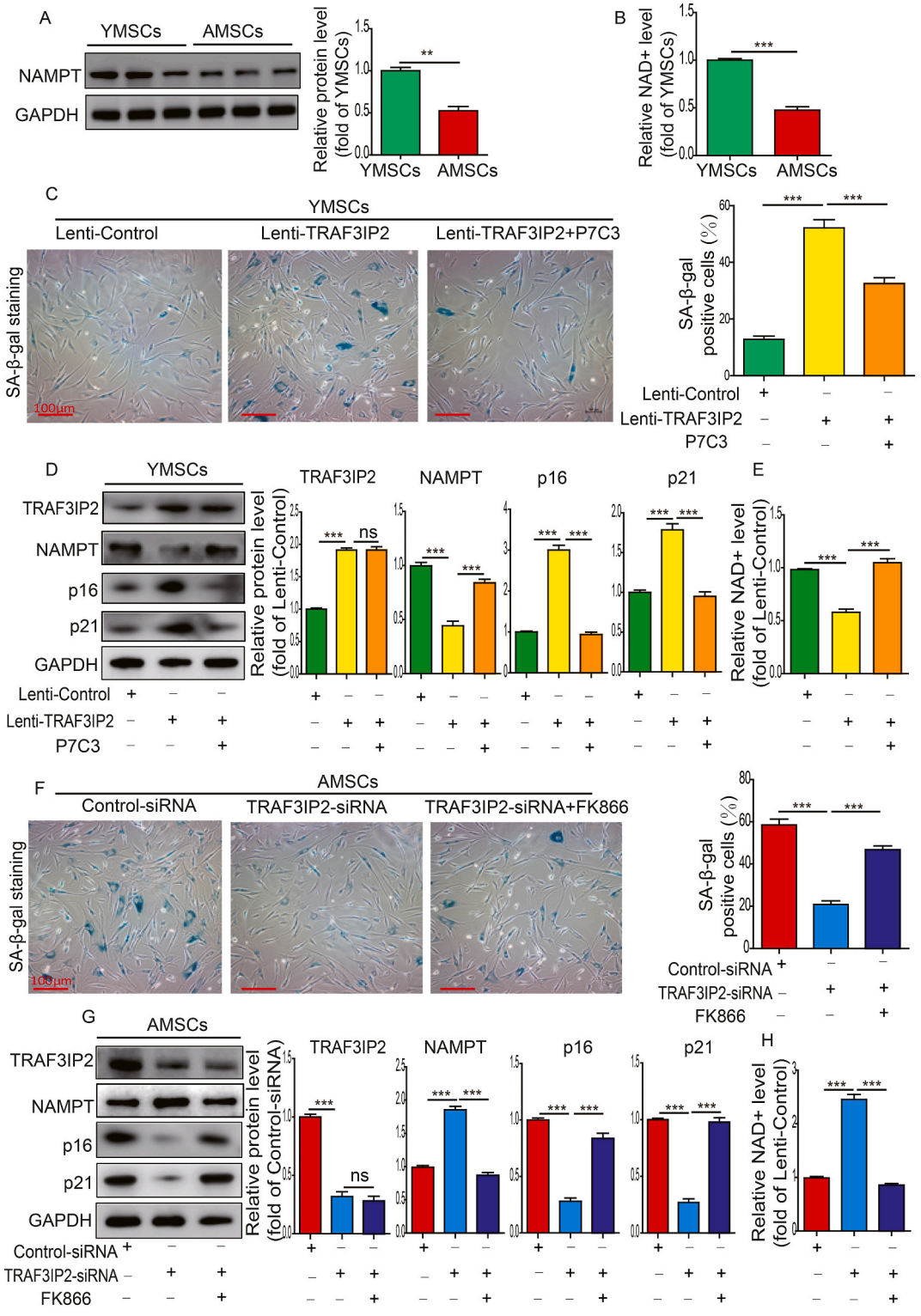

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Fig. 4. TRAF3IP2 regulates MSC senescence via mediation of NAMPT–NAD biosynthesis. (A) Western blotting and quantitative analysis of NAMPT protein level in YMSCs and AMSCs. (B) Quantitative analysis of the intracellular NAD + concentration in YMSCs and AMSCs. (C) Representative images and quantitative analysis of SA-β-gal staining in control-lentivirus, TRAF3IP2-lentivirus or TRAF3IP2-lentivirus + P7C3-treated YMSCs. (D) Western blotting and quantitative analysis of NAMPT, p16 and p21 protein level in control-lentivirus, TRAF3IP2-lentivirus or TRAF3IP2-lentivirus + P7C3-treated YMSCs. (E) Quantitative analysis of the intracellular NAD + concentration in Control-lentivirus, TRAF3IP2-lentivirus or TRAF3IP2-lentivirus + P7C3-treated YMSCs. (F) Representative images and quantitative analysis of SA-β-gal staining in Control-siRNA, TRAF3IP2-siRNA or TRAF3IP2-siRNA + FK866-treated AMSCs. (G) Western blotting and quantitative analysis of NAMPT, p16 and p21 protein level in control-siRNA, TRAF3IP2-siRNA or TRAF3IP2-siRNA + FK866-treated AMSCs. (H) Quantitative analysis of the intracellular NAD + concentration in control-siRNA, TRAF3IP2-siRNA or TRAF3IP2-siRNA + FK866-treated AMSCs. All data were obtained from at least three independent experiments and each error bar represents the mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$. The raw data of Fig. 4A, D, G is presented in Supplementary Fig. 4.

expression and NAD + level in YMSCs and AMSCs. Compared with YMSCs, NAMPT expression and NAD + level were robustly decreased in AMSCs, indicating that downregulation of NAMPT–NAD biosynthesis is closely associated with MSC senescence (Fig. 4A and B). Subsequently, we treated YMSCs with TRAF3IP2-lentivirus and revealed that overexpression of TRAF3IP2 enhanced SA-β-gal activity along with an increase in the expression of p21and p16, and reduced NAD + level and NAMPT expression in YMSCs (Fig. 4C–E). Notably, these effects were largely reversed by P7C3 treatment, the NAMPT activator (Fig. 4C–E), indicating that TRAF3IP2 induced MSC senescence by downregulating NAMPT–NAD biosynthesis. Furthermore, TRAF3IP2-siRNA treatment ameliorated the senescence of AMSCs, reduced the expression of p21 and p16 and increased NAD + level and NAMPT expression (Fig. 4F–H). Importantly, the alleviation of senescence of AMSCs by TRAF3IP2-siRNA treatment was partially abrogated by FK866, the NAMPT inhibitor (Fig. 4F–H). Collectively, these results revealed that TRAF3IP2 regulates MSC senescence via mediation of NAMPT–NAD biosynthesis.

3.5. TRAF3IP2 regulates NAMPT–NAD biosynthesis via the AMPK signaling pathway

Previous studies have demonstrated that the AMPK signaling pathway plays a critical role in regulating NAMPT-NAD biosynthesis [33,34]. We explored whether TRAF3IP2 regulates NAMPT-NAD biosynthesis via the AMPK signaling pathway. First, we found that the protein level of p-AMPK was greatly reduced in AMSCs compared with YMSCs (Fig. 5A). Overexpression of TRAF3IP2 dramatically reduced the expression of p-AMPK and NAMPT and level of NAD+ in YMSCs (Fig. 5B and C). These effects were partially reversed by the AMPK activator, AICAR (Fig. 5B and C). Furthermore, TRAF3IP2-siRNA treatment significantly upregulated the expression of p-AMPK and NAMPT and the level of NAD+ in AMSCs (Fig. 5D and E). Nevertheless treatment with Compound C, an AMPK inhibitor, partially inhibited upregulation of p-AMPK and NAMPT and the level of NAD+ in TRAF3IP2-siRNA-treated AMSCs (Fig. 5D and E). These results suggest that TRAF3IP2 regulates NAMPT-NAD biosynthesis by mediating the AMPK signaling pathway in MSCs.

3.6. TRAF3IP2 mediates the replicative senescence of MSCs

The above results highlight that TRAF3IP2 contributed to the physiological senescence of MSCs. We determined whether TRAF3IP2 also regulates the replicative senescence of MSCs by examining its effects on YMSCs at P3, P6 and P12. With an increase in passage number, YMSCs exhibited increased SA-β-gal-positive cells (Fig. 6A), and elevated TRAF3IP2, p16 and p21 expression but decreased p-AMPK, NAMPT expression (Fig. 6B) and level of NAD+ (Fig. 6C). Next, we used TRAF3IP2-siRNA to treat the P12 MSCs. Compared with the untreated group, TRAF3IP2-siRNA treatment greatly decreased β-gal + cells, TRAF3IP2, p16, and p21 expression but enhanced p-AMPK and NAMPT expression and level of NAD+ (Fig. 6D–F). In addition, TRAF3IP2-siRNA treatment significantly downregulated the secretion of IL-6 and TNF-α and upregulated the secretion of IL-10 (Fig. 6G). Nevertheless these effects were largely abrogated by Compound C or FK866 (Fig. 6D–G). These data indicate that TRAF3IP2 regulated the replicative senescence of MSCs via regulation of NAMPT–NAD biosynthesis by the AMPK signaling pathway.

4. Discussion

In the current study, we report several novel findings. First, the level of TRAF3IP2 was significantly increased in AMSCs compared with YMSCs. Second, TRAF3IP2 mediated MSC senescence via regulation of NAMPT-NAD + biosynthesis. Third, the AMPK signaling pathway is involved in TRAF3IP2-regulated NAMPT-NAD + biosynthesis. Finally, TRAF3IP2 mediated the replicative senescence of MSCs during longterm culture *in vitro*. This study highlights TRAF3IP2 as a promising target for rejuvenating AMSCs to enhance their therapeutic efficacy.

Although MSCs are the most widely used stem cells in animal studies and clinical trials, there is substantial evidence that their senescence restricts their application [35–37]. MSCs isolated from senescent mice display increased cell cycle arrest, reactive oxygen stress and immune system dysfunction compared with those isolated from control mice [38]. Compared with YMSCs, transplantation of human AMSCs confers significantly weaker cardioprotective effects for myocardial infarction with reduced cell survival and angiogenic capacity in the presence of ischemia [10]. Moreover, transplantation of aged human umbilical cord-derived MSCs exhibit decreased protective effects against Alzheimer's disease [39]. Although the molecular mechanisms that underlie MSC senescence are not fully understood, this is consistent with the view that genetic alterations to MSCs contribute to their senescence. It has been reported that downregulation of Apelin-accelerated MSC senescence and replenishment of Apelin in AMSCs robustly rejuvenated senescent cells and enhanced their cardioprotective effects [40]. The expression of neuron-derived neurotrophic factor (NDNF) was

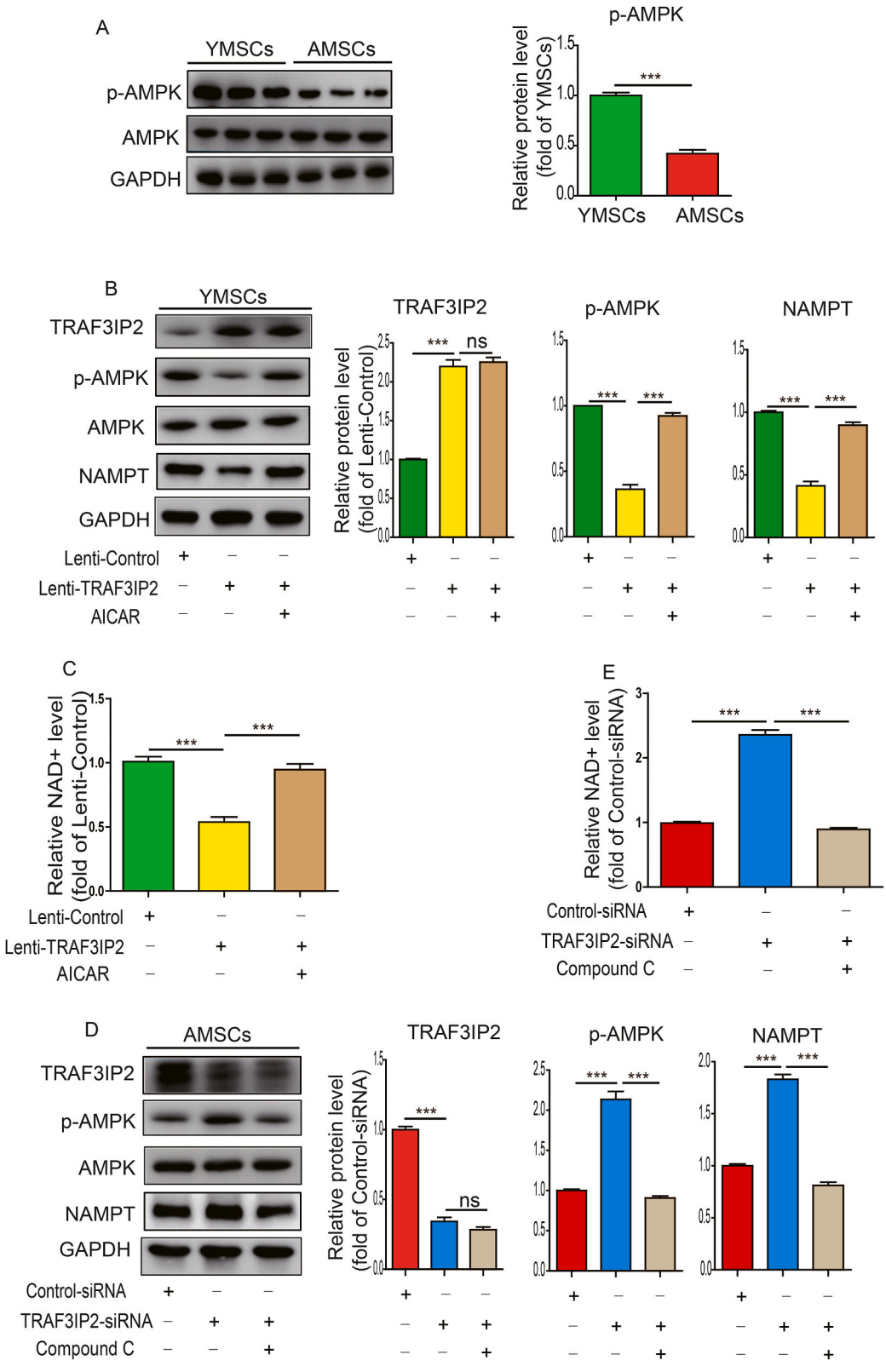

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Fig. 5. TRAF3IP2 regulates NAMPT–NAD biosynthesis via the AMPK signaling pathway. (A) Western blotting and quantitative analysis of p-AMPK and AMPK protein level in YMSCs and AMSCs. (B) Western blotting and quantitative analysis of p-AMPK, AMPK and NAMPT protein level in Control-lentivirus, TRAF3IP2-lentivirus or TRAF3IP2-lentivirus + AICAR-treated YMSCs. (C) Quantitative analysis of the intracellular NAD + concentration in Control-lentivirus, TRAF3IP2-lentivirus or TRAF3IP2-lentivirus + AICAR-treated YMSCs. (D) Western blotting and quantitative analysis of p-AMPK, AMPK and NAMPT protein level in Control-siRNA, TRAF3IP2-siRNA or TRAF3IP2-siRNA + Compound C-treated AMSCs. (E) Quantitative analysis of the intracellular NAD + concentration in Control-siRNA, TRAF3IP2-siRNA or TRAF3IP2-siRNA + Compound C-treated AMSCs. All data were obtained from at least three independent experiments and each error bar represents the mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$. The raw data of Fig. 5A, B, D is presented in Supplementary Fig. 5.

decreased in AMSCs compared with YMSCs and over-expression of NDNF in AMSCs inhibited their senescence and apoptosis [8]. Therefore, identifying the key regulator that governs MSC senescence is vital. Recently, TRAF3IP2 has been identified as a critical mediator of cell proliferation [41,42]. It has been documented that chronic intermittent hypoxia stimulated human aortic smooth muscle cell proliferation via upregulation of TRAF3IP2 expression and the effect was partially reversed by knockdown of TRAF3IP2 [43]. In contrast, GATA4 inhibited the proliferation of human fibroblasts via activation of TRAF3IP2 [18]. This discrepancy may be because TRAF3IP2 regulates cell proliferation in a cell type-specific fashion. Consistent with a previous report [18], AMSCs exhibited a higher expression level of TRAF3IP2 along with a decreased proliferative ability compared with YMSCs. Furthermore, overexpression of TRAF3IP2 accelerated MSC senescence as evidenced by increased SA-β-gal activity and DNA damage and decreased proliferative ability. In contrast, knockdown of TRAF3IP2 in AMSCs ameliorated their senescence. More importantly, we found that expression of TRAF3IP2 was greatly increased in late passage MSCs and knockdown of TRAF3IP2 ameliorated MSC senescence, indicating that TRAF3IP2 also participated in regulation of the replicative senescence of MSCs. Based on the above results, we conclude that TRAF3IP2 mediates both the physiological and replicative senescence of MSCs and is an important regulator of MSC senescence. Nevertheless the molecular mechanisms that underlie this process remain unclear.

Although the potential mechanisms have not been fully understood, NAD + has been documented as a critical regulator of cellular senescence. The intracellular concentration of NAD + is greatly reduced in multiple organs during aging [44] and NAD + supplementation can extend life [45]. Amounts of NAD + have been shown to be remarkably decreased in fresh muscle stem cells isolated from aged mice compared with those isolated from young mice [46]. More importantly, NAD + repletion rejuvenated muscle stem cells in aged mice and improved their function. To the best of our knowledge, intracellular NAD + level is predominately regulated by NAMPT, a rate-limiting enzyme of NAD + biosynthesis. Expression of NAMPT was significantly decreased in senescent fibroblasts induced by H_2O_2 and NAMPT inhibitor FK866 also prompted fibroblast senescence via regulation of NAD+ [47]. Consistent with previous study [24], we also found that the concentration of NAD+ and the expression of NAMPT were reduced in AMSCs compared with YMSCs. More importantly, knockdown of TRAF3IP2 rejuvenated AMSCs by elevating NAMPT-NAD + biosynthesis, effects that were partially abrogated by an NAMPT inhibitor, suggesting that TRAF3IP2 mediates MSC senescence via regulation of NAMPT- NAD + biosynthesis. Furthermore, we demonstrated that knockdown of TRAF3IP2 rescued the replicative senescence of MSCs at a later passage. AMPK, a major energy sensor maintaining energy homeostasis, plays an essential role in regulating a variety of cellular processes including proliferation, senescence and apoptosis [48,49]. There is mounting evidence that AMPK activation can enhance NAD + expression by elevating NAMPT [50,51]. Similarly, we found that expression of p-AMPK along with that of NAMPT and NAD+ was greatly decreased in AMSCs compared with YMSCs. It has been reported that exercise or calorie restriction enhances NAMPT level in skeletal muscle by activating AMPK [52]. Given the relationship between AMPK and NAMPT-NAD biosynthesis, it is crucial to determine whether TRAF3IP2 regulation of NAMPT-NAD + biosynthesis in MSCs is via regulation of the AMPK pathway. In the current study, we showed that overexpression of TRAF3IP2 significantly downregulated the expression of NAMPT and NAD+ in YMSCs via inhibition of AMPK activation. These effects were greatly abrogated by the AMPK activator, AICAR, suggesting that TRAF3IP2 regulates NAMPT-NAD + biosynthesis in MSCs by mediating the AMPK pathway.

Several experimental limitations in the current study need to be highlighted. First, in addition to NAMPT-NAD + biosynthesis, whether TRAF3IP2 also regulates MSC senescence by mediating mitochondrial dysfunction, telomere shortening or autophagy impairment warrants investigation. Second, although we found that TRAF3IP2 mediated senescence of BM-derived MSCs, whether TRAF3IP2 also affects the cellular senescence of MSCs derived from other tissues such as adipose and umbilical cord requires further investigation. Third, although multiple pathways have been reported to regulate NAMPT-NAD + biosynthesis, the potential pathway underlying TRAF3IP2 regulation of NAMPT-NAD + biosynthesis remains to be elucidated in addition to the AMPK pathway.

5. Conclusion

In summary, our study comprehensively shows that TRAF3IP2 induced MSC senescence via downregulation of NAMPT-mediated NAD biosynthesis by inhibiting the AMPK pathway. These findings reveal a novel mechanism underlying MSC senescence and highlight TRAF3IP2 as a potential novel target that contributes to the rejuvenation of AMSCs.

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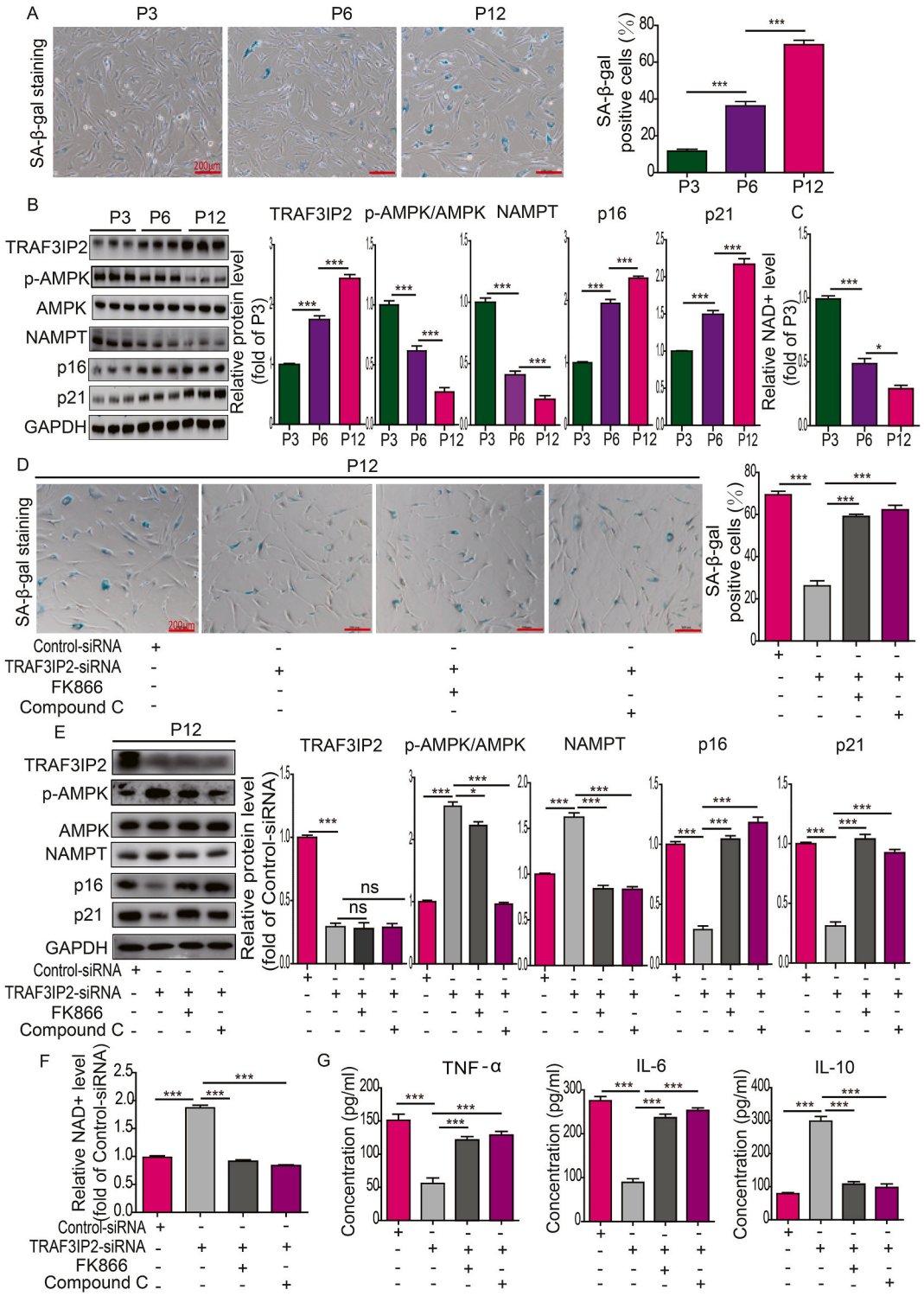

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Fig. 6. TRAF3IP2 mediates the replicative senescence of MSCs. (A) Representative images and quantitative analysis of SA-β-gal staining in P3, P6 and P12 of YMSCs. (B) Western blotting and quantitative analysis of p-AMPK, AMPK and NAMPT protein level in P3, P6 and P12 of YMSCs. (C) Quantitative analysis of the intracellular NAD + concentration in P3, P6 and P12 of YMSCs. (D) Representative images and quantitative analysis of SA-β-gal staining in P12 of YMSCs with Control-siRNA, TRAF3IP2-siRNA, TRAF3IP2-siRNA + Compound C or TRAF3IP2-siRNA + FMK866 treatment. (E) Western blotting and quantitative analysis of p-AMPK, AMPK and NAMPT protein level in P12 of YMSCs with Control-siRNA, TRAF3IP2-siRNA, TRAF3IP2-siRNA + Compound C or TRAF3IP2-siRNA + FMK866 treatment. (F) Quantitative analysis of the intracellular NAD + concentration in P12 of YMSCswith Control-siRNA, TRAF3IP2-siRNA, TRAF3IP2-siRNA + Compound C or TRAF3IP2-siRNA + FMK866 treatment. (G) Concentration of TNF-α, IL-6 and IL-10 in CdM isolated from P12 of YMSCs with Control-siRNA, TRAF3IP2-siRNA, TRAF3IP2-siRNA + Compound C or TRAF3IP2-siRNA + FMK866 treatment. All data were obtained from at least three independent experiments and each error bar represents the mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$. The raw data of Fig. 6B, E is presented in Supplementary Fig. 6.

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Author contribution statement

Yuelin Zhang, Xin Li and Weifeng Li: Conceived and designed the experiments.Xiaoran Huang, Baojuan Liu and Yaowen Liang: Performed the experiments; analyzed and interpreted the data.Cong Mai, Ying Shen, Xinran Huang and Jiaqi Chen: Analyzed and interpreted the data.Xiaoting Liang and Bei Hu: Contributed reagents, materials, analysis tools or data.Xiaoran Huang, Baojuan Liu, Yaowen Liang, Yuelin Zhang, Xin Li and Weifeng Li: Wrote the paper.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e19505.

References

[1] Y. Han, J. Yang, J. Fang, et al., The secretion profile of mesenchymal stem cells and potential applications in treating human diseases, Signal Transduct. Targeted Ther. 7 (1) (2022) 92.
[2] N. Song, M. Scholtemeijer, K. Shah, Mesenchymal stem cell immunomodulation: mechanisms and therapeutic potential, Trends Pharmacol. Sci. 41 (9) (2020) 653–664.
[3] Y. Zhang, W. Zhu, H. He, et al., Macrophage migration inhibitory factor rejuvenates aged human mesenchymal stem cells and improves myocardial repair, Aging (Albany NY) 11 (24) (2019) 12641–12660.
[4] J. Dong, J. Liu, Y. Wen, et al., Down-regulation of lnc-CYP7A1-1 rejuvenates aged human mesenchymal stem cells to improve their efficacy for heart repair through SYNE1, Front. Cell Dev. Biol. 8 (2020), 600304.
[5] M. Al-Azab, M. Safi, E. Idiiatullina, F. Al-Shaebi, M.Y. Zaky, Aging of mesenchymal stem cell: machinery, markers, and strategies of fighting, Cell. Mol. Biol. Lett. 27 (1) (2022) 69.
[6] D. Cakouros, S. Gronthos, Epigenetic regulation of bone marrow stem cell aging: revealing epigenetic signatures associated with hematopoietic and mesenchymal stem cell aging, Aging Dis 10 (1) (2019) 174–189.
[7] Y. Hong, H. He, G. Jiang, et al., miR-155-5p inhibition rejuvenates aged mesenchymal stem cells and enhances cardioprotection following infarction, Aging Cell 19 (4) (2020), e13128.
[8] H.F. Song, S. He, S.H. Li, et al., Aged human multipotent mesenchymal stromal cells can Be rejuvenated by neuron-derived neurotrophic factor and improve heart function after injury, JACC Basic Transl Sci 2 (6) (2017) 702–716.
[9] D.Y. Zhang, T. Gao, R.J. Xu, et al., SIRT3 transfection of aged human bone marrow-derived mesenchymal stem cells improves cell therapy-mediated myocardial repair, Rejuvenation Res. 23 (6) (2020) 453–464.
[10] J. Dong, Z. Zhang, H. Huang, et al., miR-10a rejuvenates aged human mesenchymal stem cells and improves heart function after myocardial infarction through KLF4, Stem Cell Res. Ther. 9 (1) (2018) 151.
[11] X. Liu, D. Hu, Z. Zeng, et al., SRT1720 promotes survival of aged human mesenchymal stem cells via FAIM: a pharmacological strategy to improve stem cell-based therapy for rat myocardial infarction, Cell Death Dis. 8 (4) (2017), e2731.

[12] Z.I. Grunewald, F.I. Ramirez-Perez, M.L. Woodford, et al., TRAF3IP2 (TRAF3 interacting protein 2) mediates obesity-associated vascular insulin resistance and dysfunction in male mice, Hypertension 76 (4) (2020) 1319–1329.
[13] S. Sukhanov, Y. Higashi, T. Yoshida, et al., The SGLT2 inhibitor Empagliflozin attenuates interleukin-17A-induced human aortic smooth muscle cell proliferation and migration by targeting TRAF3IP2/ROS/NLRP3/Caspase-1-dependent IL-1β and IL-18 secretion, Cell. Signal. 77 (2021), 109825.
[14] E.U. Alt, P.M. Wörner, A. Pfnür, et al., Targeting TRAF3IP2, compared to Rab27, is more effective in suppressing the development and metastasis of breast cancer, Sci. Rep. 10 (1) (2020) 8834.
[15] S. Mummidi, N.A. Das, A.J. Carpenter, et al., RECK suppresses interleukin-17/TRAF3IP2-mediated MMP-13 activation and human aortic smooth muscle cell migration and proliferation, J. Cell. Physiol. 234 (12) (2019) 22242–22259.
[16] N.K. Somanna, M. Yariswamy, J.M. Garagliano, et al., Aldosterone-induced cardiomyocyte growth, and fibroblast migration and proliferation are mediated by TRAF3IP2, Cell. Signal. 27 (10) (2015) 1928–1938.
[17] Y. Song, L. Chen, Y. Li, et al., Knockdown of TRAF3IP2 suppresses the expression of VEGFA and the proliferation of keratinocytes and vascular endothelial cells, Heliyon 5 (5) (2019), e01642.
[18] C. Kang, Q. Xu, T.D. Martin, et al., The DNA damage response induces inflammation and senescence by inhibiting autophagy of GATA4, Science 349 (6255) (2015) aaa5612.
[19] T. Wang, F. Zhang, W. Peng, et al., Overexpression of NMNAT3 improves mitochondrial function and enhances antioxidative stress capacity of bone marrow mesenchymal stem cells via the NAD+-Sirt3 pathway, Biosci. Rep. 42 (1) (2022), BSR20211005.
[20] A. Nikiforov, V. Kulikova, M. Ziegler, The human NAD metabolome: functions, metabolism and compartmentalization, Crit. Rev. Biochem. Mol. Biol. 50 (4) (2015) 284–297.
[21] J. Wang, L. Liu, Z. Ding, et al., Exogenous NAD(+) postpones the D-gal-induced senescence of bone marrow-derived mesenchymal stem cells via Sirt1 signaling, Antioxidants 10 (2) (2021), 254.
[22] X. Lai, S. Huang, S. Lin, et al., Mesenchymal stromal cells attenuate alveolar type 2 cells senescence through regulating NAMPT-mediated NAD metabolism, Stem Cell Res. Ther. 13 (1) (2022) 12.
[23] E. Koltai, Z. Szabo, M. Atalay, et al., Exercise alters SIRT1, SIRT6, NAD and NAMPT levels in skeletal muscle of aged rats, Mech. Ageing Dev. 131 (1) (2010) 21–28.
[24] C. Ma, C. Pi, Y. Yang, et al., Nampt expression decreases age-related senescence in rat bone marrow mesenchymal stem cells by targeting Sirt1, PLoS One 12 (1) (2017), e0170930.
[25] Q. Lian, Y. Zhang, X. Liang, F. Gao, H.F. Tse, Directed differentiation of human-induced pluripotent stem cells to mesenchymal stem cells, Methods Mol. Biol. 1416 (2016) 289–298.
[26] R. Farahzadi, E. Fathi, S.A. Mesbah-Namin, N. Zarghami, Anti-aging protective effect of L-carnitine as clinical agent in regenerative medicine through increasing telomerase activity and change in the hTERT promoter CpG island methylation status of adipose tissue-derived mesenchymal stem cells, Tissue Cell 54 (2018) 105–113.
[27] E. Fathi, S. Vandghanooni, S. Montazersaheb, R. Farahzadi, Mesenchymal stem cells promote caspase-3 expression of SH-SY5Y neuroblastoma cells via reducing telomerase activity and telomere length, Iran J Basic Med Sci 24 (11) (2021) 1583–1589.
[28] Y. Li, J. Li, C. Zhao, et al., Hyperglycemia-reduced NAD(+) biosynthesis impairs corneal epithelial wound healing in diabetic mice, Metabolism 114 (2021), 154402.
[29] X. Liang, Y. Ding, Y. Zhang, et al., Activation of NRG1-ERBB4 signaling potentiates mesenchymal stem cell-mediated myocardial repairs following myocardial infarction, Cell Death Dis. 6 (5) (2015) e1765.
[30] Y. Zhang, X. Liang, S. Liao, et al., Potent paracrine effects of human induced pluripotent stem cell-derived mesenchymal stem cells attenuate doxorubicin-induced cardiomyopathy, Sci. Rep. 5 (2015), 11235.
[31] F.D. Khaidizar, Y. Bessho, Y. Nakahata, Nicotinamide phosphoribosyltransferase as a key molecule of the aging/senescence process, Int. J. Mol. Sci. 22 (7) (2021), 3709.
[32] C. Pi, Y. Yang, Y. Sun, et al., Nicotinamide phosphoribosyltransferase postpones rat bone marrow mesenchymal stem cell senescence by mediating NAD(+)-Sirt1 signaling, Aging (Albany NY) 11 (11) (2019) 3505–3522.
[33] M. Fulco, Y. Cen, P. Zhao, et al., Glucose restriction inhibits skeletal myoblast differentiation by activating SIRT1 through AMPK-mediated regulation of Nampt, Dev. Cell 14 (5) (2008) 661–673.
[34] H. Gong, H. Chen, P. Xiao, et al., miR-146a impedes the anti-aging effect of AMPK via NAMPT suppression and NAD(+)/SIRT inactivation, Signal Transduct. Targeted Ther. 7 (1) (2022) 66.
[35] J. Zupan, K. Strazar, R. Kocijan, et al., Age-related alterations and senescence of mesenchymal stromal cells: implications for regenerative treatments of bones and joints, Mech. Ageing Dev. 198 (2021), 111539.
[36] H. Chen, O. Liu, S. Chen, Y. Zhou, Aging and mesenchymal stem cells: therapeutic opportunities and challenges in the older group, Gerontology 68 (3) (2022) 339–352.
[37] J.A. Fafián-Labora, M. Morente-López, M.C. Arufe, Effect of aging on behaviour of mesenchymal stem cells, World J. Stem Cell. 11 (6) (2019) 337–346.
[38] M. Li, K. Guo, Y. Adachi, S. Ikehara, Immune dysfunction associated with abnormal bone marrow-derived mesenchymal stroma cells in senescence accelerated mice, Int. J. Mol. Sci. 17 (2) (2016), 183.
[39] S. Ma, X. Zhou, Y. Wang, et al., MG53 protein rejuvenates hUC-MSCs and facilitates their therapeutic effects in AD mice by activating Nrf2 signaling pathway, Redox Biol. 53 (2022), 102325.
[40] H. Zhang, C. Zhao, G. Jiang, et al., Apelin rejuvenates aged human mesenchymal stem cells by regulating autophagy and improves cardiac protection after infarction, Front. Cell Dev. Biol. 9 (2021), 628463.
[41] Q. Yan, B. Li, TRAF3IP2 regulated by FOXO4 affects fibroblast proliferation, migration and extracellular matrix deposition in keloid through the TGF-β1/Smad pathway, J. Cosmet. Dermatol. (2022) 5148–5155.
[42] S. Mummidi, N.A. Das, A.J. Carpenter, et al., Metformin inhibits aldosterone-induced cardiac fibroblast activation, migration and proliferation in vitro, and reverses aldosterone+salt-induced cardiac fibrosis in vivo, J. Mol. Cell. Cardiol. 98 (2016) 95–102.
[43] Y. Higashi, R. Dashek, J.J. Russell, R.S. Rector, B. Chandrasekar, Empagliflozin inhibits intermittent hypoxia-induced TRAF3IP2/NF-κB/HIF-1α/IL-6-dependent human aortic smooth muscle cell proliferation, Faseb. J. 36 (Suppl 1) (2022).
[44] J. Yoshino, K.F. Mills, M.J. Yoon, S. Imai, Nicotinamide mononucleotide, a key NAD(+) intermediate, treats the pathophysiology of diet- and age-induced diabetes in mice, Cell Metabol. 14 (4) (2011) 528–536.
[45] L. Mouchiroud, R.H. Houtkooper, N. Moullan, et al., The NAD(+)/Sirtuin pathway modulates longevity through activation of mitochondrial UPR and FOXO signaling, Cell 154 (2) (2013) 430–441.
[46] H. Zhang, D. Ryu, Y. Wu, et al., NAD⁺ repletion improves mitochondrial and stem cell function and enhances life span in mice, Science 352 (6292) (2016) 1436–1443.
[47] X. Han, H. Tai, X. Wang, et al., AMPK activation protects cells from oxidative stress-induced senescence via autophagic flux restoration and intracellular NAD(+) elevation, Aging Cell 15 (3) (2016) 416–427.
[48] X. Li, Y. Hong, H. He, et al., FGF21 mediates mesenchymal stem cell senescence via regulation of mitochondrial dynamics, Oxid. Med. Cell. Longev. (2019), 4915149, 2019.
[49] S. Tabibzadeh, Signaling pathways and effectors of aging, Front Biosci (Landmark Ed). 26 (1) (2021) 50–96.

[50] H.S. Zhang, X.Y. Chen, T.C. Wu, F.J. Zhang, Tanshinone II A inhibits tat-induced HIV-1 transactivation through redox-regulated AMPK/Nampt pathway, J. Cell. Physiol. 229 (9) (2014) 1193–1201.
[51] J.S. Kim, C.S. Yoon, D.R. Park, NAMPT regulates mitochondria biogenesis via NAD metabolism and calcium binding proteins during skeletal muscle contraction, J. Exerc. Nutrition Biochem. 18 (3) (2014) 259–266.
[52] C. Cantó, Z. Gerhart-Hines, J.N. Feige, et al., AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity, Nature 458 (7241) (2009) 1056–1060.