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Chromatin remodeling factor BAZ1A regulates cellular senescence in both cancer and normal cells

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ABSTRACT

Aims: Cellular senescence is a well-known cancer prevention mechanism, inducing cancer cells to senescence can enhance cancer immunotherapy. However, how cellular senescence is regulated is not fully understood. Dynamic chromatin changes have been discovered during cellular senescence, while the causality remains elusive. *BAZ1A*, a gene coding the accessory subunit of ATP-dependent chromatin remodeling complex, showed decreased expression in multiple cellular senescence models. We aim to investigate the functional role of *BAZ1A* in regulating senescence in cancer and normal cells.

Materials and methods: Knockdown of *BAZ1A* was performed via lentivirus mediated short hairpin RNA (shRNA) in various cancer cell lines (A549 and U2OS) and normal cells (HUVEC, NIH3T3 and MEF). A series of senescence-associated phenotypes were quantified by CCK-8 assay, SA-β-Gal staining and EdU incorporation assay, etc.

Key findings: Knockdown (KD) of *BAZ1A* induced series of senescence-associated phenotypes in both cancer and normal cells. *BAZ1A*-KD caused the upregulated expression of SMAD3, which in turn activated the transcription of p21 coding gene *CDKN1A* and resulted in senescence-associated phenotypes in human cancer cells (A549 and U2OS).

Significance: Our results revealed chromatin remodeling modulator BAZ1A acting as a novel regulator of cellular senescence in both normal and cancer cells, indicating a new target for potential cancer treatment.

1. Introduction

Cellular senescence is an important anticancer mechanism that inhibits proliferation of damaged or premalignant cells [1,2]. A recent study demonstrated that induction of cancer cells to enter into senescent state can activate the immune cells such as natural killer cells to clear tumor cells in a KRAS-mutant lung cancer mouse model [3]. Thus, discovery of new genes that induce senescence of cancer cells could help to unclose potential novel targets for cancer immune therapy. It is well known that multiple stresses can induce cellular senescence [4–6]. For example, telomere shortening can cause replicative senescence. In addition, γ-irradiation induced DNA damage can lead to premature senescence. Overexpression of oncogene serves as another source to induce senescence, termed as oncogene-induced senescence (OIS). Senescent cells are characterized by several features including increased activity of senescence-associated β-galactosidase (SA-β-Gal) [7], cell cycle arrest, reduced proliferation rate and increased secretion of pro-inflammatory cytokines which is termed as senescence-associated secretory phenotype (SASP) [8]. Two major pathways, p53-p21 signaling cascade and p16-Rb signaling cascade, are widely accepted to be involved in cellular senescence [9,10]. At the molecular level, both transcriptional and post-transcriptional regulation can influence senescence-associated phenotypes, such as transcription factor (TF)-mediated transcriptional regulation, microRNA-mediated post-transcriptional regulation [11,12] and RNA binding protein (RBP)-regulated mRNA translation efficiency [13,14], all of which can affect cellular senescence and individual aging.

Epigenetics is the study of heritable changes in gene expression not due to alternations in the DNA sequence, and has emerged as an important mechanistic insight into genetic and environmental risk factors for human diseases [15]. These gene expression alternations typically arise owing to DNA methylation, histone modification and chromatin remodeling [16,17]. Notably, increasing evidences support that epigenetic factors, including enzymes modifying DNA and histones or

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remodeling chromatin, hold important functions in diverse biological processes [18,19]. While gene expression affected by factors modulating DNA methylation and histone modifications are intensively studied in various biological processes [20,21], knowledge regarding chromatin remodeling factors contributing to gene expression is limited, especially in cellular senescence, a biological process known to be one of the key tumor suppression mechanisms [22]. Chromatin remodeling is a dynamic and regulated process modulating DNA accessibility to various regulatory proteins, and then influence transcription of genes nearby [23]. Transcription generally requires ATP-dependent chromatin remodelers to alter histone-DNA interaction and mobilize, evict, or replace nucleosomes [24]. Among the most conserved chromatin remodeling families, Imitation switch (ISWI) is a member of the SWI2/SNF2 superfamily of ATP-dependent chromatin remodelers, which regulate transcription and maintain chromatin structure [25]. ISWI chromatin remodelers pair one of two ATPase subunits, SMARCA5 (SNF2H) or SMARCA1 (SNF2L), with one of the six regulatory subunits: BAZ1A (ACF1), BAZ1B (WSTF), BAZ2A (TIP5), BPTF, CECR2 or RSF1, some of which have been implicated to affect gene transcriptional regulation, chromosome organization and DNA replication [25,26]. Recently, several studies highlighted that mice depleted of components of this complex displayed abnormalities including impaired mice growth and cell proliferation rate, cognitive defects, neurodevelopmental deficits, and even compromised viability [27–31].

BAZ1A (bromodomain adjacent to zinc finger domain, 1A), also known as ACF1, is an accessory, non-catalytic subunit of ACF (the ATP-dependent chromatin assembly factor) [32]. BAZ1A is thought to enhance nucleosome sliding efficiency and regulate template specificity of the ATPase subunit SNF2H [33,34]. BAZ1A has been reported to modulate the ATPase activity of ACF complex and participate in gene transcription, DNA damage checkpoint and double-strand break repair [35–37]. BAZ1A can also function in neurodevelopment [38] and spermatogenesis through regulating expression of associated genes [37]. Intriguingly, knockdown of SMARCA5 (SNF2H), a catalytic subunit of ACF which always functions incorporation with BAZ1A, leads to slower cell proliferation and migration rate in breast cancer cells [39]. Nevertheless, whether BAZ1A functions in cell proliferation and even cellular senescence remains unclear.

Based on the findings that dynamic changes of histone modifications and DNA methylation occur during cellular senescence in mammals [40,41], we wonder whether other epigenetic factors, especially chromatin remodeling factors could play a functional role in mammal cellular senescence. In this study, we are excited to discover the chromatin remodeling factor BAZ1A as a novel factor regulating senescence-associated phenotypes in cancer and normal cells. It adds another layer of gene expression regulation for cellular senescence and may also have implication in cancer prevention.

2. Results

2.1. BAZ1A is downregulated during cellular senescence in human and mouse

Dynamic expression variance of *BAZ1A* was first examined at mRNA level in multiple cellular senescence models. We adopted the replicative senescence system of mouse embryo fibroblasts (MEFs), which exhibited typical senescence markers including increased expression of cyclin-dependent kinase inhibitor p21 (coded by *CDKN1A*), p16 (coded by *CDKN2A*) and decreased expression of cell proliferation marker *MKI67* (Supplemental Fig. 1A). We further validated the senescent state of passage 6 (P6) of MEFs compared to passage 3 (P3) via SA-β-Gal staining and calculating the percentage of positive stained cells (Supplemental Fig. 1B–C). Decreased expression of Baz1a in senescent MEFs was observed at both mRNA (Fig. 1A) and protein levels (Supplemental Fig. 2). Notably, the decreased expression of *Baz1a* at mRNA level was not specific to mouse cells, similar expression trend was observed in replicative senescent human umbilical vein endothelial cells (HUVECs) (Fig. 1B). Moreover, decreased *BAZ1A* mRNA was also found in multiple human replicative cellular senescence systems based on analysis of public RNA-seq datasets [42], including human foreskin fibroblasts (HFF), WI-38 and MRC-5 (Fig. 1C), whose senescent states were also featured by the expression of various senescence-associated markers (Supplemental Figs. 3–5). The above observations combined to indicate that *BAZ1A* exhibits down-regulated expression in multiple cellular senescence models.

2.2. Knockdown of BAZ1A induces cellular senescence

To examine whether down-regulation of *BAZ1A* contributes to cellular senescence, we stably knocked down (KD) *BAZ1A* by lentivirus mediated short hairpin RNA (shRNA) in three human cell lines (A549, a lung adenocarcinoma cell line; U2OS, Human Bone Osteosarcoma Epithelial Cells; HUVECs) and two mouse cell lines (NIH3T3, a murine embryonic fibroblast cell line; MEFs). The success of stable knockdown was validated at both mRNA and protein levels (Fig. 2A and Supplemental Figs. 6A, 7A, 8A and 9A). CCK-8 assay showed *BAZ1A*-KD cells had decreased proliferation rate compared to control cells (Fig. 2B and Supplemental Figs. 6B, 7B and 8B). In addition, cell cycle was arrested at G1 phase in *BAZ1A*-KD cells (Fig. 2C and Supplemental Figs. 6C, 7C and 8C). Noteworthy, all of the five *BAZ1A*-KD cell lines showed increased percentage of positive SA-β-Gal stained cells (Fig. 2D–E and Supplemental Figs. 6D–E, 7D–E, 8D–E and 9B–C). Moreover, reduced EdU incorporation rate was also observed in *BAZ1A*-KD cells (Fig. 2F and Supplemental Fig. 6F), reflecting decreased level of DNA synthesis, which is also a well-known molecular phenotype of senescent cells [43]. Furthermore, cellular senescence induced by *BAZ1A* knockdown was further proved by other molecular senescence-associated markers including increased expression of cyclin-dependent kinase inhibitor p21 (*CDKN1A*), decreased expression of cell cycle related Cyclin B2 (*CCNB2*) or cell cycle related Cyclin D1 (*CCND1*) and cellular proliferation marker MKI67 (Fig. 2G and Supplemental Fig. 6G). Besides, increased expression of CDKN1A in *BAZ1A*-KD cells was also validated by western blot (Fig. 2H and Supplemental Fig. 6H). These results demonstrated that BAZ1A deficiency can induce senescence-associated phenotypes in both normal and cancer cells.

2.3. Biological pathways involved in BAZ1A-KD induced senescence

To uncover the downstream regulatory pathways involved in *BAZ1A*-KD induced senescence, we performed RNA-seq profiling on both *BAZ1A*-KD A549 cells and control cells. Based on the previous report that BAZ1A acts as a repressive transcription factor [44], we speculated that the direct target genes should exhibit upregulated expression upon *BAZ1A* knockdown. We thus focused on upregulated genes shared by two batches of *BAZ1A*-KD cells transfected with two different shRNAs (sh1 and sh2), which resulted in 323 upregulated genes. These up-regulated genes were enriched in four pathways related to senescence, including p53 signaling pathway, FoxO signaling pathway, TGF-beta signaling pathway and Cell cycle (Fig. 3A). These genes were also enriched in five biological processes associated with senescence based on Gene ontology (GO), including GO terms such as DNA damage response, cell cycle arrest, response to hydrogen peroxide, unbiquitin-dependent SMAD protein catabolic process and intrinsic apoptotic signaling pathway in response to DNA damage (Fig. 3B). Interestingly, we noticed that the response to hydrogen peroxide pathway enriched genes with upregulated expression, suggesting changed cellular redox metabolism in *BAZ1A*-KD cells. To verify whether cellular superoxide level changed upon *BAZ1A* knockdown and eventually induced cellular senescence, we performed Dihydroethidium (DHE) assay to detect the intracellular superoxide level and found *BAZ1A*-KD indeed increased cellular superoxide level (Fig. 3C–D and Supplemental Figs. 10 and 11), which could induce DNA damage response and may

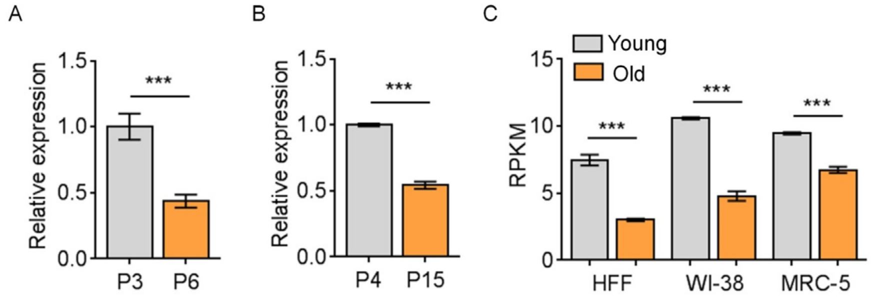

Fig. 1. Down-regulation of *BAZ1A* in multiple replicative senescence models. (A) The relative expression of *BAZ1A* was reduced in senescent MEFs (passage 6, P6) compared to younger MEFs (passage 3, P3), as quantified by qRT-PCR. (B) The down-regulation of *BAZ1A* was observed in senescent HUVECs (passage 15, P15) when compared to younger HUVECs (passage 4, P4), as quantified by qRT-PCR. *GAPDH* served as the internal control in both panel A and B. (C) Public RNA-seq datasets were analyzed to examine *BAZ1A* mRNA expression changes in three replicative senescence models of human fibroblasts, including human foreskin fibroblasts (HFF), WI-38 and MRC-5. *** indicates $p < 0.001$ of *t*-test with three qPCR reactions (for panel A and B) or three biological replicates (for panel C).

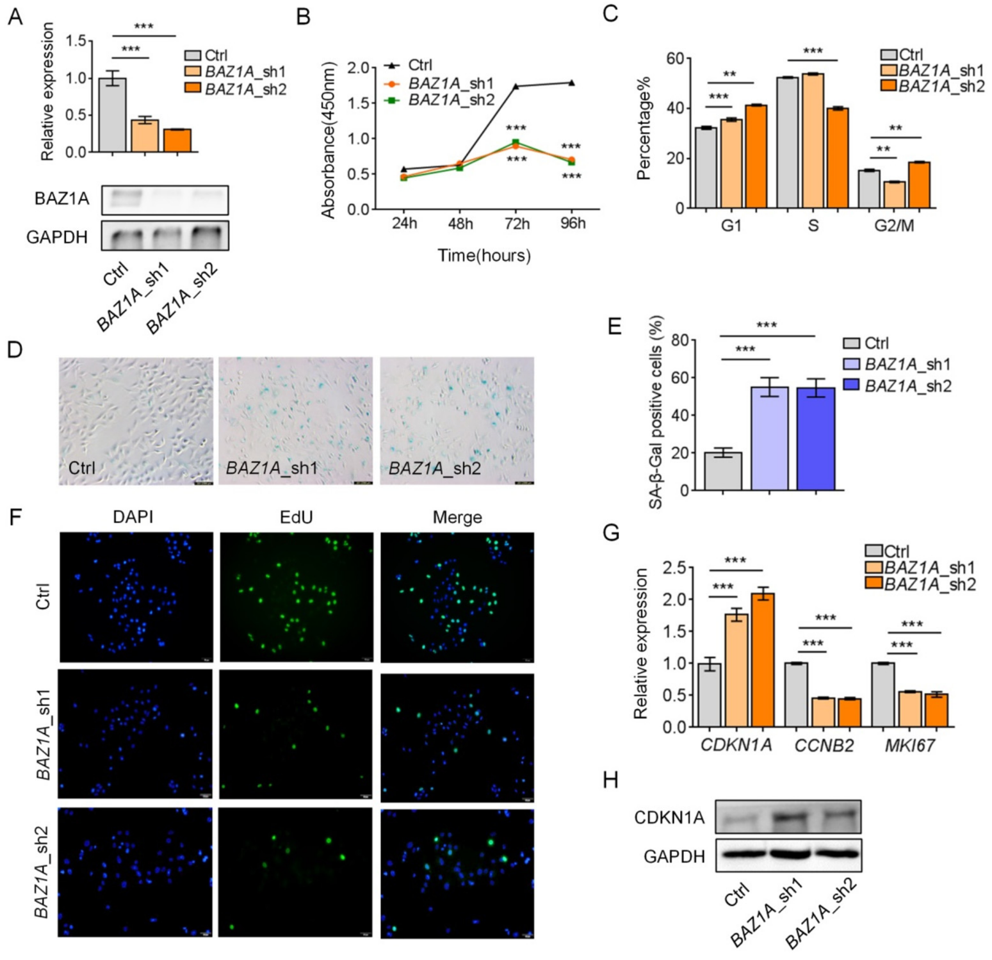

Fig. 2. Knockdown of *BAZ1A* induces senescence in human A549 cells. (A) qRT-PCR and Western blot to validate BAZ1A knockdown efficiency in human A549 cells transfected with two different shRNAs (sh1 and sh2). GAPDH served as the internal control. (B) Cell proliferation rate decreased in *BAZ1A*-knockdown A549 cells compared to control cells assayed by CCK-8 kit. (C) Cell cycle arrested in G1 phase in *BAZ1A*-knockdown A549 cells as detected by Fluorescence-activated cell sorting (FACS) analysis. (D, E) Representative images (D) and percentage of positive stained cells (E) of SA-β-Gal staining before and after *BAZ1A*-KD. (F) Decreased DNA replication rate in *BAZ1A*-KD A549 cells evaluated by EdU incorporation assay compared to control cells. Blue: DAPI stains nuclear DNA. Green: EdU labeled DNA, the newly synthesized DNA. (G) Cellular senescence-associated biomarkers including elevated *CDKN1A* (encoding p21) expression, decreased *CCNB2* and *MKI67* expression were detected by qRT-PCR after *BAZ1A*-KD in A549 cells. (H) Increased CDKN1A protein levels in *BAZ1A*-KD (sh1 and sh2) cells compared to control cells detected by Western blot. GAPDH served as the internal control. ** and *** represent $p < 0.01$ and $p < 0.001$, respectively, based on *t*-test with three qPCR reactions in panel A and G or four biological replicates in panel B and three biological replicates in panel C or three independent countings in panel E. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

eventually lead to senescence-associated phenotypes [45,46]. Together, these above results suggested that *BAZ1A*-KD induced cellular senescence can also be supported by related signaling pathways.

2.4. BAZ1A-KD increases SMAD3 expression to promote senescence

As narrated above, multiple signaling pathways may be involved in *BAZ1A*-KD induced senescence. Looking into these enriched pathways associated with senescence led us to an interesting finding. p53 signaling pathway contains p53-p21 signaling cascade, while TGF-beta signaling pathway has TGF-β-SMAD2/3-p21 axis, and ubiquitin-dependent SMAD catabolic process points to the key role of SMAD protein. Since p53-p21 is a classic signaling pathway functioning in senescence, we first look into the expression of p53 in *BAZ1A*-KD cells. However, we did not observe obvious expression changes at the protein level in both *BAZ1A*-KD A549 and U2OS cells (Supplemental Fig. 12). These clues combined together to imply that SMAD-p21 axis might be a signal transduction pathway to mediate *BAZ1A*-KD induced cellular senescence. BAZ1A has been reported to repress target gene's transcription when binding to its promoter region [44]. Consistent with this, we detected upregulated mRNA expression of *SMAD3* upon knockdown of *BAZ1A* (Fig. 4A and Supplemental Fig. 13A), while its paralog gene *SMAD2* did not display such trend (Supplemental Fig. 14). Besides, the protein level of SMAD3 was upregulated in *BAZ1A*-KD cells (Fig. 4B and Supplemental Fig. 13B). To validate whether BAZ1A directly regulates *SMAD3*, we performed Chromatin Immunoprecipitation coupled with PCR (ChIP-PCR) assay at the promoter region of *SMAD3* in A549 and U2OS cells. The results showed enriched signal of BAZ1A binding to the promoter region of *SMAD3* (Fig. 4C and Supplemental Fig. 13C) compared to non-specific IgG binding control, which was

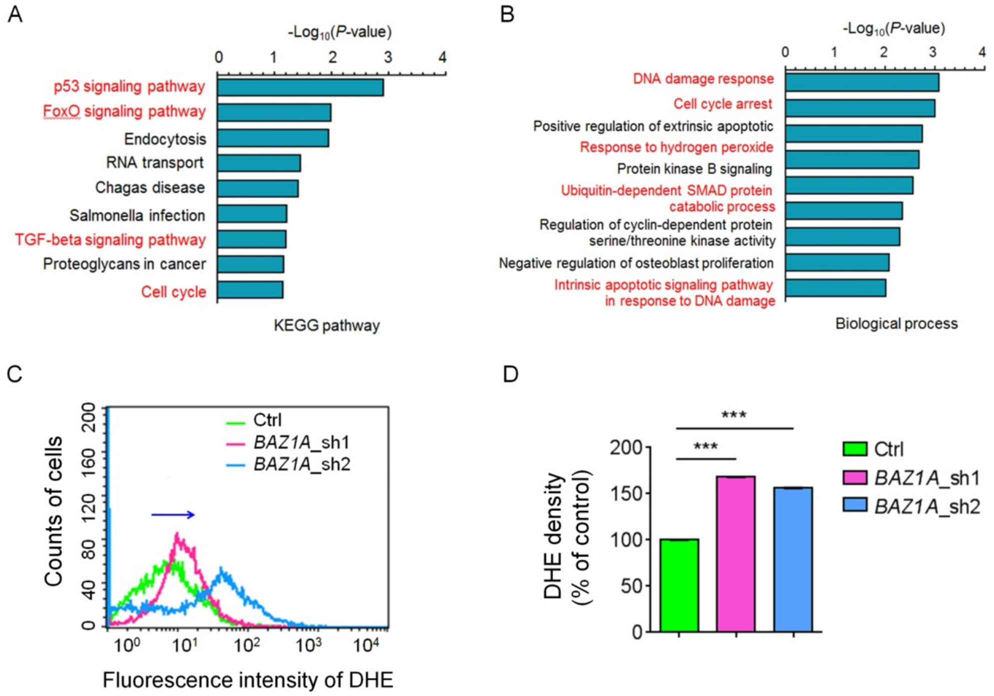

Fig. 3. Biological pathways involved in *BAZ1A*-KD induced senescence in A549 cells. (A, B) Functional enrichment analysis for upregulated genes in both *BAZ1A*-sh1-KD and *BAZ1A*-sh2-KD A549 cells based on KEGG pathway (A) and Gene ontology (GO) for biological processes (B). Red fonts indicate functional categories that were reported to be associated with senescence. (C) Cellular superoxide level in *BAZ1A*-KD (sh1 and sh2) cells and control cells assayed by Dihydroethidium (DHE) assay and Fluorescence-activated cell sorting (FACS). X axis represents the Fluorescence intensity while Y axis indicates cell counts. (D) Increased intracellular superoxide level in *BAZ1A*-KD A549 cells (two replicates, sh1 and sh2) compared to control cells. *** indicates $p < 0.001$ based on *t*-test with three technical replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

further validated by Chromatin Immunoprecipitation coupled with quantitative PCR (ChIP-qPCR) (Fig. 4D and Supplemental Fig. 13D). Several studies have demonstrated that SMAD3 activates the expression of p21 (*CDKN1A*) to suppress cell proliferation through binding to its promoter region [47,48]. Our data showed the upregulation of both SMAD3 and CDKN1A in *BAZ1A*-KD cells (Figs. 4A–B and 2G–H, Supplemental Figs. 13A–B and 6G–H), indicating upregulated SMAD3 activated the expression of CDKN1A and led to slower cell proliferation and ultimately cellular senescence [48–50]. Taken together, we discovered that BAZ1A-SMAD3-p21 was a new signal axis that could, at least in part, underlie *BAZ1A*-KD induced cellular senescence.

2.5. *SMARCA5 downregulates BAZ1A and induces cellular senescence*

As mentioned above, BAZ1A (also known as ACF1) and SMARCA5 (SNF2H) are the two subunits of the ACF chromatin-remodeler complex of ISWI family. Crosstalk existed between these two partners, for example, BAZ1A can modulate the remodeling properties of SMARCA5, while SMARCA5 can stabilize the stability of ACF1 protein [51]. To examine whether SMARCA5 can regulate the abundance of BAZ1A and

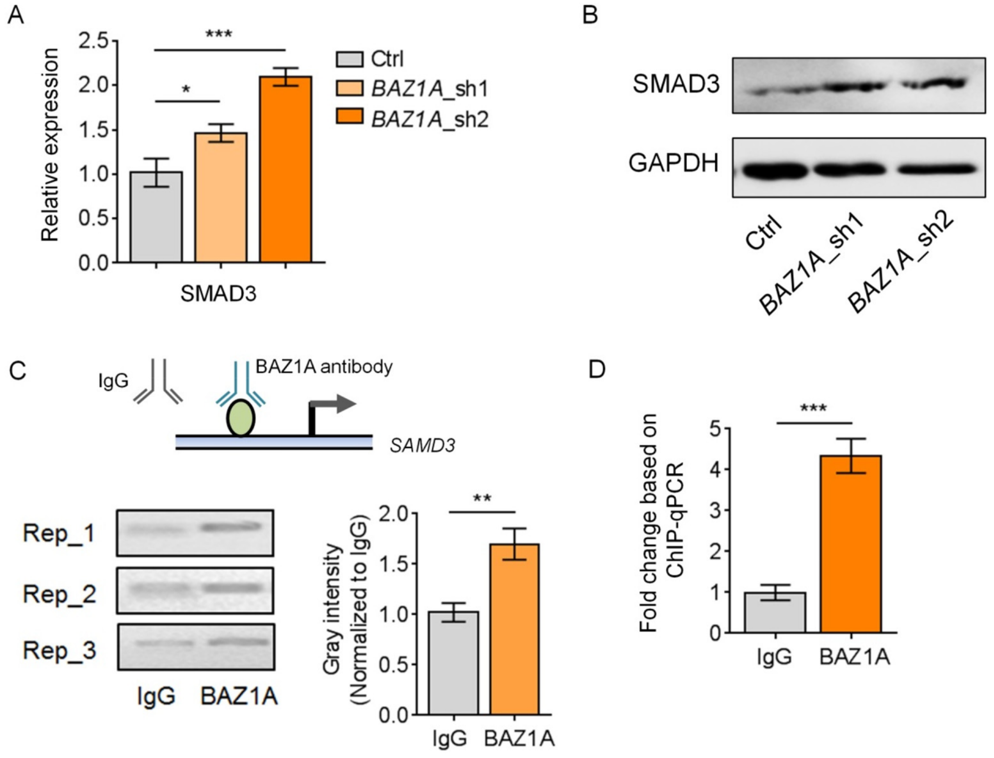

Fig. 4. Knockdown of *BAZ1A* directly upregulates SMAD3 in human A549 cells. (A) Upregulated expression of *SMAD3* at mRNA level in *BAZ1A*-KD cells with two replicates (sh1 and sh2) compared to control cells evaluated by qRT-PCR. *GAPDH* served as the internal control. (B) The protein abundance of SMAD3 was upregulated upon *BAZ1A*-KD detected by Western blot. GAPDH served as the internal control. (C) BAZ1A binding to the promoter region of *SMAD3* was validated by ChIP-PCR. IgG antibody served as non-specific binding control. The upper panel illustrates the binding location of BAZ1A on *SMAD3*. Down left panel represents agarose gel images of ChIP-PCR with three replications, while down right panel represents the gray intensity of ChIP-PCR gel images quantified by ImageJ software. (D) ChIP-qPCR to further validate the fold enrichment of BAZ1A bound to *SMAD3* promoter region. * and *** indicate $p < 0.05$ and $p < 0.001$, respectively, based on *t*-test with three qPCR reactions in panel A and D. ** in panel C represents $p < 0.01$ based on *t*-test with three technical replicates of ChIP-PCR.

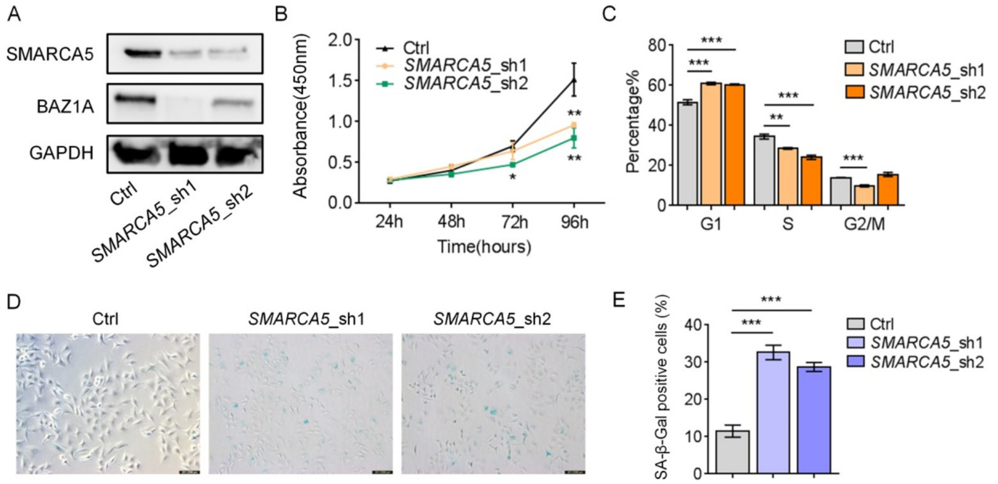

Fig. 5. SMARCA5 downregulates BAZ1A and induces cellular senescence in A549 cells. (A) Knockdown of *SMARCA5* reduced BAZ1A protein levels validated by Western blot. GAPDH served as the internal control. sh1 and sh2 denote two shRNAs for knocking down *SMARCA5*. (B) Cell proliferation rate was reduced in *SMARCA5*-knockdown A549 cells compared to control cells, as evaluated by CCK-8 assay. * and ** represent $p < 0.05$ and $p < 0.01$, respectively, based on *t*-test with four biological replicates. (C) Cell cycle arrested in G1 phase in *SMARCA5*-KD A549 cells when comparing to control cells, as detected by Fluorescence-activated cell sorting (FACS) analysis. ** and ** stand for $p < 0.01$ and $p < 0.001$, respectively, based on *t*-test with three technical replicates. (D, E) Representative images (D) and percentage of positive stained cells (E) of SA-β-Gal staining before and after knocking down of *SMARCA5* by two shRNAs. *** represents $p < 0.001$ based on *t*-test with three independent countings.

then influence senescence, we stably knocked down *SMARCA5* by lentivirus mediated short hairpin RNA in A549 and U2OS cells, and reduced BAZ1A protein levels were found in *SMARCA5*-KD cells (Fig. 5A and Supplemental Fig. 15A), which also exhibited senescence-associated phenotypes, including decreased proliferation rate (Fig. 5B and Supplemental Fig. 15B), cell cycle arrest at G1 phase (Fig. 5C and Supplemental Fig. 15C), and increased percentage of positive SA-β-Gal stained cells (Fig. 5D–E and Supplemental Fig. 15D–E). Interestingly, SMARCA5 was also found downregulated in senescent MEFs (Supplemental Fig. 16). These results suggested that in addition to BAZ1A, SMARCA5, the other subunit of ACF chromatin remodeling complex, can also regulate senescence-associated phenotypes. Further, *SMARCA5*-KD induced senescence was mediated, at least in part, through downregulated abundance of BAZ1A, in line with the stabilization function of SMARCA5 to BAZ1A.

3. Discussion

BAZ1A as one of the chromatin remodelers has emerged as an important regulator of many biological processes, including neurodevelopment, DNA damage recovery and spermatogenesis [35,37,52]. However, whether BAZ1A plays a crucial role in cellular senescence is completely unknown. Cellular senescence has been widely accepted as one of the key cancer prevention mechanisms [53,54]. In this study, we were surprised to find that *BAZ1A* is prevalently downregulated in multiple senescence models. Knockdown of *BAZ1A* led to senescence-associated phenotypes in both normal and cancer cells. Conversely, elevated *BAZ1A* expression was observed in various types of tumors compared to the matched normal tissues (Supplemental Fig. 17) based on analysis of TCGA public datasets available in the GEPIA website [55]. Further, knockdown of *BAZ1A* in cancer cell line A549 exhibited reduced colony formation ability (Supplemental Fig. 18). As A549 is a widely used lung cancer cell line for senescence-associated tumor immunotherapy study [56], we speculated that chromatin remodeler BAZ1A may be a novel regulator of cellular senescence and a novel target with potential implication in cancer treatment.

Several studies have reported that BAZ1A (or ACF1) is a multi-domain protein containing several conserved motifs for interaction with DNA, histones, and other members of the ACF complex [32,57]. Due to its ability of binding to DNA, BAZ1A acts partly as a transcription factor [58]. BAZ1A has been reported as a transcription repressor via organizing other transcription factors to VDR (Vitamin D3 Receptor) gene and represses its transcription under the condition lack of vitamin D3 [44]. In the present study, we discovered that BAZ1A bound to the promoter region of *SMAD3* and inhibited its transcription. Meanwhile, it has been demonstrated that SMAD3 activates *CDKN1A* (coding for p21) transcription [47,48,59], which is a key mediator of cell senescence [9]. In line with this, our results also showed p21 upregulation upon *BAZ1A* knockdown (Fig. 2G–H and Supplemental Fig. 6G–H), which implied that BAZ1A-SMAD3-p21 is a new signal axis in promoting cellular senescence in human cancer cell lines (A549 and U2OS) (Fig. 6). Since p53-p21 is a classic signaling pathway functions in senescence, we further noticed SMAD3 has been reported interacting with p53 and bound to target gene promoters to regulate gene expression [60,61]. Interestingly, *CDKN1A* did not show significant changes after knockdown of *BAZ1A* in two cancer cell lines with *TP53* genetic alterations (MDA-MB-231 with *TP53* point mutation and NCI-H1299 with

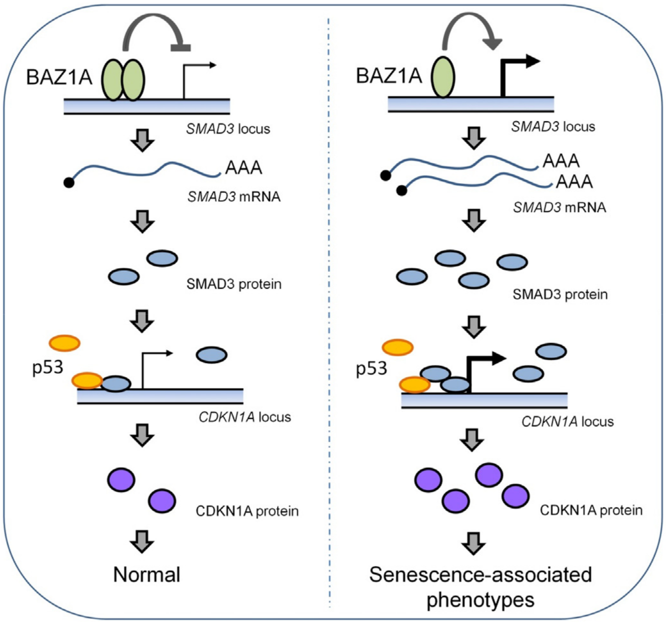

Fig. 6. Working model for BAZ1A-SMAD3-CDKN1A signal axis in regulating cellular senescence in A549 and U2OS cells. Chromatin remodeling factor BAZ1A exhibits downregulated expression in multiple cellular senescence models. BAZ1A binds to the promoter region of *SMAD3* to inhibit its transcription, thus the reduced BAZ1A level leads to increased expression of SMAD3, which may cooperate with p53 to regulate the expression of CDKN1A (coding for p21) [47,48,60,61]. SMAD3-induced CDKN1A upregulation ultimately promotes senescence-associated phenotypes.

TP53 null mutation) (Supplemental Figs. 19F and 20F). These results further indicated that SMAD3 mediated *CDKN1A* upregulation required p53 in *BAZ1A*-KD cells, however, how they function cooperatively in *BAZ1A*-KD induced senescence reserves further study.

In addition, *BAZ1A*-KD induced senescence-associated phenotypes in MDA-MB-231 and NCI-H1299 cells bypass the way of increasing *CDKN1A* (Supplemental Figs. 19 and 20) also hints that BAZ1A-SMAD3-p21 might not be the only signal axis functioning in *BAZ1A*-KD induced senescence. Actually, some results did indicate that other signaling pathways might also underlie *BAZ1A*-KD induced senescence. For example, genes involved in response to hydrogen peroxide were also found differentially expressed in *BAZ1A*-KD cells (Fig. 3B), which was further proved by elevated cellular superoxide level evaluated by DHE assay in *BAZ1A*-KD cells (Fig. 3C–D and Supplemental Fig. 11). Recent studies revealed that accumulated superoxide can cause increased mitochondrial DNA mutations and respiratory chain damage, as well as cellular macromolecular compound injury, which ultimately led to cellular senescence [62,63]. Therefore, superoxide mediated pathway could be another mechanism to explain *BAZ1A*-KD induced senescence, which warrants further investigation. In addition, FoxO signaling pathway, a conserved pathway mediating both senescence and individual aging [64–66], was also enriched with differentially expressed genes upon *BAZ1A*-KD (Fig. 3A). Moreover, we looked into the published BAZ1A ChIP-seq results [58], and validated some reported target genes of BAZ1A through ChIP-qPCR in A549 cells, and found that BAZ1A enriched in the promoter region of *FOXK1* and *ZFP36L2* (Supplemental Fig. 21), which were responsible for cell proliferation and cell cycle control [67,68]. However, whether they contributed to *BAZ1A*-KD induced cellular senescence deserves further study.

In the present study, *SMARCA5* was found to influence the steady level of BAZ1A and contribute to senescence, which indicated the regulatory roles of factors affecting BAZ1A. Other factors such as transcription factors and microRNAs could also affect the expression of *BAZ1A* and contribute to senescence, which deserves further investigation. Besides, elevated *SMARCA5* expression was observed in various types of tumors (Supplemental Fig. 22), wherein *BAZ1A* also exhibited upregulated expression compared to the matched normal tissues based on TCGA public datasets available in the GEPIA website [55]. Moreover, SMARCA5 overexpressed in breast cancer and knockdown of *SMARCA5* reduced proliferation and invasion rate of breast cancer cells [39]. However, whether and how elevated SMARCA5 and BAZ1A contribute to cancer-related phenotypes deserve further study.

In conclusion, we revealed for the first time that downregulation of BAZ1A induced cellular senescence in both normal and cancer cells. Cellular senescence is considered to be an anti-cancer therapy and our results implied that *BAZ1A*-KD may have an anti-tumor effect, though further study is required to verify the therapeutic potential of targeting *BAZ1A*.

4. Conclusions

We found that BAZ1A was downregulated in senescent cells compared to young cells in both human and mouse. Knockdown of *BAZ1A* induced senescence-associated phenotypes in both normal and cancer cells. Multiple biological pathways, including p53 signaling pathway and TGF-β signaling pathway, were associated with *BAZ1A*-KD induced senescence. Moreover, we found knockdown of *BAZ1A* increased cellular superoxide levels, another aspect contributing to the senescence phenotype. Mechanistically, BAZ1A could bind to the promoter region of *SMAD3* and repress its expression. SMARCA5 could be one of the factors modulating BAZ1A expression and senescence-associated phenotypes. These collectively presented that BAZ1A was a novel regulator for cellular senescence and had potential implications for cancer prevention.

5. Materials and methods

5.1. Cell culture and transfection

293T, A549, U2OS, HUVEC, MDA-MB-231, NCI-H1299, NIH3T3 and MEFs were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibico) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C with 5% CO_2. Stable knockdown of *BAZ1A* in two human cell lines (A549, HUVEC) and two mouse cell lines (NIH3T3, MEFs) were performed utilizing two short hairpin RNAs (shRNAs) packaged in lentiviral vector and pLkO.1 served as control plasmid. The clone IDs of shRNAs were obtained from Sigma-Aldrich as follows: human_*BAZ1A*_sh1: TRCN0000034279; human_*BAZ1A*_sh2: TRCN0000034282; mouse_*BAZ1A*_sh1: TRCN0000244323; mosue_*BAZ1A*_sh2: TRCN0000241701. Lentiviral vectors were constructed according to established protocols from the Broad Institute RNAi Consortium. 293T cells of 90% confluence in 6-well plate were transfected with lipofectamine 2000 (Invitrogen). After culturing 24 h post transfection, the virus supernatant of 293T cells was harvested to infect A549, HUVEC, NIH3T3 and MEFs. The medium was replaced with fresh DMEM medium supplemented with puromycin (2 μg/mL) at 24 h post the infection. After one day selection, the survival cells were cultured for additional two days in the medium with puromycin (2 μg/mL). Then the cells were harvested for RNA and protein extraction to validate *BAZ1A* knockdown efficiency.

5.2. RNA extraction, cDNA synthesis, qRT-PCR and Western blot

Total RNA was extracted with TRIzol reagent (Sigma) according to the manufacturer's instruction. For cDNA synthesis, 1 μg total RNA was reversely transcribed into cDNA by FastQuant RT Kit (TIANGEN). Then quantitative PCR was performed using 2 × SYBR mix (KAPA) on Bio-Rad CFX manager machine. For Western blot, proteins were extracted with TRIzol Reagent (Sigma) according to the manufacturer's instructions, then resolved in 5% SDS solution. 20 μg total proteins were subjected to SDS-PAGE, then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). Membranes were incubated with anti-BAZ1A (Bethyl, Cat.no. A301-318A), anti-p21 (abcam, Cat.no. ab109199) and anti-SMAD3 (Sangon Biotech, Cat.no. D155234-0025) with 1:1000 dilution separately at room temperature for 2 h, then washed with TBST buffer. Subsequently, the membranes were incubated with corresponding secondary antibodies conjugated with horseradish peroxidase (HRP) for 1 h with 1:5000 dilution. Finally, protein levels were determined by ECL (Tanon) and visualized with enhanced chemiluminescence system (GE Healthcare Life Sciences).

5.3. Cell proliferation assay and EdU incorporation assay

Cell proliferation rate was assayed using Cell Counting Kit-8 (CCK-8) (DOJINDO, Japan) and DNA replication rate was detected by EdU incorporation assay (KGA331-100, KeyGen BioTECH). For CCK-8 assay, after selection by puromycin, the survival cells were trypsinized and diluted, then subcultured in a 96-well plate, with 1000 cells per well and four replicates for each time point. CCK-8 reagent (DOJINDO, Japan) was then added to each well every 24 h post seeding according to the manufacturer's protocol, lasting for four days. After each treatment, cells were incubated for 2 h at 37 °C, then the optical density (OD) at 450 nm and 630 nm were measured respectively for each well by microplate reader (Biorad). For EdU incorporation assay, cells were seeded in a 24-well plate and incubated with 10 μM EdU for 2 h. Changes in cell growth rate were then assayed following the kFluor488 Click-iT EdU image kit instruction. The images were captured by fluorescence microscope (Olympus).

5.4. Cell colony formation

After the cells were selected by puromycin, the survival cells were harvested and 200 cells were seeded into each well of 6-well plates and grown for 14 days. The growth medium was replaced every 3 days. At the end of the experiment, the cells were washed with PBS and then fixed with paraformaldehyde solution for 15 min at room temperature. After that cells were washed by PBS and then stained with 1% crystal violet solution. Cells colonies were viewed and photographed under an microscope (Leica).

5.5. Cell cycle analysis

Cells were seeded in the 6-well plate one day in advance at 60%–70% confluence. After 24 h incubation, cells were harvested then fixed with 70% cold ethanol and stained with Propidium Iodide (PI) for 10 min at room temperature. The fluorescence intensity of PI was analyzed with a FACS vantage SE flow cytometry (Becton Dickinson, USA) equipped with Cell Quest software (Becton Dickinson, USA).

5.6. SA-β-Gal activity assay

Cells were seeded in the 12-well plate one or two days before staining. Briefly, we adopted Sigma SA-β-Gal staining kit according to the manufacturer's instruction. After washing cells with PBS for twice, cells were fixed with the fixation buffer, followed by three washes with PBS and then incubated with SA-β-Gal staining solution at 37 °C separated from CO_2 overnight. Then images were captured under the microscope (Leica).

5.7. Determination and quantification of superoxide

Cells were seeded in the 6-well plate at 60% confluence. Two days later, cells were washed with PBS for three times and then treated with 5 μM Dihydroethidium (DHE) assay (Yeasen, Cat.no. 50102ES02) in PBS for 60 min at 37 °C in darkness. After 60 min, cells were washed with PBS for three times and then harvested with trypsin. For each sample, cells were resuspended in 300 μL PBS and to quantify the intracellular superoxide level, relative fluorescence intensities were determined by FACS vantage SE flow cytometry (Becton Dickinson, USA).

5.8. RNA-seq library construction and gene expression analysis

The dUTP-based strand-specific RNA-seq libraries were constructed following a previously described protocol [69] and sequenced using an Illumina HiSeq X TEN platform in a paired-end 2 × 150-bp manner. Processed raw data were aligned to the human genome (version hg38) using TopHat2 [70]. Differentially expressed genes between *BAZ1A*-KD and control cells were analyzed by Cuffdiff module of Cufflinks package with default parameter [71]. The raw RNA-seq data for this study can be accessed at the NCBI Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra/) with the accession number PRJNA542430.

5.9. GO and pathway enrichment analysis

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) [72] was used for both GO and pathway enrichment analysis on differentially expressed genes. We selected Biological Process (BP) for GO analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database for pathway analysis.

5.10. ChIP-PCR and ChIP-qPCR assay

After harvesting cells, we isolated the nuclei and then sheared chromatin using Bioruptor instrument (Bioruptor UCD-300). Either anti-BAZ1A antibody (Bethyl, Cat.no. A301-318A) or IgG (ABMART, B30011) was added to the sheared chromatin. The samples were incubated with the antibody for 2 h and then with protein G beads for another 2 h. After immunoprecipitation and purification of DNA, both PCR and qPCR was performed. PCR program: 2 μL of each sample was used per PCR reaction and the PCR was performed following 94 °C for 2 min; then 30 cycles of 98 °C for 10 s, 56 °C for 30 s and 68 °C for 30 s. qPCR program: DNA samples were heated for 5 min at 65 °C. 2 μL of each sample was used per qPCR reaction. Each sample was run in triplicate. The following mixture was prepared for per sample: 5.25 μL qPCR mix (SYBR Green, Vazyme); 0.375 μL forward and reverse primers mix (each at 10 μM); 2.375 μL ddH_2O. Each 2 μL from the DNA matrix samples was then added and the PCR was performed.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Author contributions

T.N. G.W. initiated, designed and supervised the research project. X.L. and D.D. performed the experiment. G.W. and X.L. performed the sequencing data analysis. X.L. drafted the manuscript while T.N. and G.W. revised it.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.05.023.

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