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The Oncogene PIM1 Contributes to Cellular Senescence by Phosphorylating Staphylococcal Nuclease Domain-Containing Protein 1 (SND1)

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The oncogene PIM1, encoding a constitutively active serine/threonine protein kinase, is involved in the regulation of cell proliferation, survival, differentiation, and apoptosis. There is a growing body of literature on the role of PIM1-mediated cellular senescence, but the precise mechanism remains unclear.

Material/Methods: Silver staining and LC–MS/MS analysis were performed to investigate the protein interacting with PIM1. Immunofluorescence, Co-IP, and Western blot assay were used to assess the interaction of PIM1 and SND1. EdU incorporation and CCK8 assay were used to detect cell proliferation and immunohistochemistry was used to detect the level of the indicated protein.

Results: We found that PIM1 can bind directly and phosphorylate SND1. In addition, decreased expression of SND1 leads to the upregulation of SASP. SND1 is involved in cellular senescence induced by PIM1.

Conclusions: We investigated the role of PIM1 in oncogene-induced normal cellular senescence. Our results promote further understanding of the mechanisms underlying OIS and suggest potential applications for preventing tumorigenesis.

MeSH Keywords: Cell Aging • Phosphorylation • Proto-Oncogene Proteins c-pim-1

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Background

Cellular senescence, which is generated by a variety of stimuli, including DNA damage, oncogene activation, telomere shortening, constitutively activated oncogene expression, and cleavage of chromatin, is a stress response that accompanies stable exit from the cell cycle [1]. Cellular senescence can be divided into 2 main types: replicative senescence and stress-induced premature senescence (SIPS); the former depends on telomere erosion or dysfunction, while the latter is telomere-independent [2]. Oncogene-induced senescence (OIS) is also a kind of cellular senescence. In addition, long-term research showed that OIS can act as a tumor-suppressor mechanism [3]. Therefore, the identification of a new OIS mechanism can further our knowledge of senescence regulation and provide new insights for cancer treatment strategies.

The oncogene PIM1 is a member of the evolutionarily conserved serine/threonine kinase family, which is a type of calcium/calmodulin-regulated kinase (CAMK) that contains a characteristic kinase domain, the so-called ATP anchor and active site. Pim2 and Pim3, which are 2 other kinase family members, have high sequence homology to Pim1 [4,5]. Subsequent studies have shown that Pim1 plays an important role in cell proliferation, differentiation, and apoptosis, and is related to tumor development. New strategies for exploring PIM1 as a cancer therapeutic target are emerging [6]. Previous reports published by our laboratory and others have indicated that PIM1 is critical in regulating cellular senescence; it can promote the senescence-associated heterochromatin (SAHF) by phosphorylating HP1, and increases the genomic DNA instability of senescent cells through targeting protein UHRF1, suggesting that PIM1 can regulate various phenotypes of senescent cells. Additionally, previous studies found that overexpressing PIM1 can result in cellular senescence rather than enhancing growth [7]. However, we are still unclear of the specific relevant molecular mechanisms.

Staphylococcal nuclease domain-containing protein 1 (SND1), a multifunctional protein also known as p100, is highly conserved in organisms. SND1 has 4 staphylococcal nuclease (SN) domains, one Tudor domain, and an incomplete SN domain [8]. SND1 is a transcriptional coactivator that is associated with EBNA-2, STAT6, and STAT5, and it facilitates their transcriptional activity [9]. In addition, SND1 is also involved in cell cycle, DNA damage repair, proliferation, and apoptosis in eukaryotic cells [10]. It was also reported that SND1 has an important role during tumorigenesis and development. For instance, SND1 is increased in most cancer cells and promotes cancer cell viability and tumorigenicity in hepatocytes [11]. However, the role of SND1 in aging remains unclear. In this study, we demonstrated that PIM1 promotes cellular senescence by phosphorylating SND1, revealing a new explanation of the occurrence of cellular senescence.

Material and Methods

Cell culture

2BS cells and HEK293T cells were obtained from the National Institute of Biological Products of China and were maintained as described elsewhere [12]. Briefly, 2BS and HEK293T cells were cultured in RPMI 1640 medium (GIBCO) and DMEM (Dulbecco's modified Eagle's medium) (GIBCO), respectively. All the culture media were supplemented with 10% FBS (fetal bovine serum) (GIBCO) and 100 U/ml penicillin and 100 μg/ml streptomycin, and the cells were then cultured with 5% CO_2 at 37°C in a humidified incubator. MG132 (Sigma) was dissolved in DMSO and added into the medium at a concentration of 20 mM.

Silver staining and mass spectrometry

Cellular extracts of 2BS cells transfected with Flag-PIM1 were collected 48 h later. Anti-FLAG M2 affinity gel (Sigma) was used to prepare the anti-FLAG immune affinity columns following the manufacturer's instructions. The FLAG protein complex was eluted using FLAG peptide (0.2 mg/ml, Sigma). Fractions of the bed volume were collected and resolved on SDS-PAGE and silver stained; bands were excised and subjected to LC-MS/MS sequencing and data analysis. Silver staining was performed using the Pierce silver stain kit (Thermo) following the manufacturer's instructions and then subjected to LC–MS/MS analysis [13].

Western blot analysis and SA-β-gal

Total cell lysates from different experiments were obtained by lysing the cells in RIPA buffer (Wanlei Biotechnology, Beijing, China). The BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to quantify protein concentrations according to the manufacturer's protocols. We used 10% SDS-PAGE to resolve 30 ug proteins and then transferred them to PVDF membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, the membranes were incubated with primary antibodies at 4°C for 12 h, followed by incubation with a horseradish peroxidase-conjugated goat-anti-rabbit or goat-anti-mouse secondary antibody (Cell Signaling Technology, CA, USA) at room temperature for 1 h. Specific bands were visualized using enhanced chemiluminescence detection (Thermo Fisher Scientific, Inc.). The signal intensity was determined with ImageJ software version 1.48. The antibodies used were: PIM1 (3247, Cell Signaling, 1: 2000), SND1 (ab65078, Abcam, 1: 1500), Phosphoserine (ab9332, Abcam, 1: 3000), and β-actin (PM053, MBL, 1: 5000). SA-β-gal activity was assessed as described previously [12].

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GST pull-down and *in vitro* kinase assay

In cells, GST and GST-SND1 or HA-PIM1 or HA-K67M were purified with glutathione-sepharose 4B beads (GE Healthcare, Little Chalfont, UK). We added the same amounts of GST or GST-SND1 with HA-PIM1 into BC100 buffer at 4°C overnight. To remove unbound protein, the compound was centrifuged at 600 g for 5 min and repeated 2 times. Then, Western blot analysis was carried out to analyze the protein through the use of anti-HA and anti-GST. To evaluate the phosphorylation of SND1 by PIM1 *in vitro*, purified SND1 with PIM1 (purchased from Abcam) or purified K67M were co-incubated in kinase buffer (Cell Signaling) with 10 mM ATP for 30 min at 30°C. The p-Ser antibody was subjected to Western blot analysis.

Co-immunoprecipitation

For immunoprecipitation, specific antibodies (2–3 ug, anti-PIM1 or anti-SND1) were used to incubate the 500 ug proteins at 4°C for 12 h with constant rotation. Then, 60 ul 50% protein A/G agarose beads were added and continued for 2 h. Beads were washed 5 times using the lysis buffer. 2×SDS-PAGE loading buffer was used to resuspend the precipitated proteins from the beads. We used 10% SDS-PAGE gels to resolve the resultant materials from immunoprecipitation or cell lysates and then transferred it onto the acetate cellulose membranes. For Western blotting analysis, membranes were incubated with the appropriate antibodies overnight at 4°C, followed by incubation with the specific secondary antibody. Western blotting Luminal reagent (Santa Cruz Biotechnology) was used to visualize the immunoreactive bands according to the manufacturer's protocols.

EdU incorporation assay and CCK8 assay

EdU incorporation assay and CCK8 assay were performed as described previously [14]. EdU staining was performed using the Click-iTEdU imaging kit (Invitrogen) according to the manufacturer's instructions. For CCK8 assay, 10 ul CCK8 was added to each well and the plates were incubated for 4 h at 37°C. The optical density (OD) at a wavelength of 450 nm was determined using a microplate reader (Hitachi, Tokyo, Japan). The OD values reflect the relative number of viable cells.

Immunohistochemistry (IHC)

Immunohistochemistry (IHC) was performed by the streptavidin-peroxidase method (Zymed Laboratories, Inc., San Francisco, CA, USA). The antibodies used were SND1 (ab65078, Abcam, 1: 50), PIM1 (ab200889, Abcam, 1: 50), and CDKN2A/p16INK4a (ab189034, Abcam, 1: 50). Immunostaining was carried out using a peroxidase-conjugated goat anti-rabbit secondary antibody according to the manufacturer's instructions. Immunostaining was assessed and examined independently by 2 observers (LS Zhu and R Ding).

Immunofluorescence

Cells fixed with 4% formaldehyde were incubated at room temperature for 1 h with phosphate-buffered saline containing 0.1% triton X-100 and 2% bovine serum albumin, and then incubated with antibodies specific for SND1 (1: 500; Abcam) and PIM1 (1: 500; CST) overnight at 4°C. Cells were then washed with phosphate-buffered saline plus 0.1% triton X-100 and incubated with a second antibody of fluorophore conjugation. Cells were imaged under a microscope at 60×.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNA was extracted using Trizol reagent (Takara, Dalian, China) according to the manufacturer's protocol. We reverse-transcribed 1 μg total RNA to cDNA by M-MLV (Promega, Madison, WI, USA), and mRNA levels were assessed with RT-qPCR using SYBR Green (Takara, Dalian, China). The gene expression level was normalized to the endogenous GAPDH. The relative fold changes in the transcripts were calculated using the $2^{-\Delta\Delta Ct}$ method. The primers for used for RT-qPCR were:
SND1-Forward: 5'GTGAAGGAAGGGCTGGTC3',
SND1-Reverse: 5'TGGACCCAATCTACAACCC3';
IL-8-Forward: 5'TTTGAAGAGGGCTGAGAA 3',
IL-8-Reverse: 5'TTCTAGCAAACCCATTCA3';
CXCL1-Forward: 5'ACTGACCAGAAGGGAGGAG3',
CXCL1-Reverse: 5'GTGCAGGTAGAGTTAATCATAC3';
CXCL2-Forward: 5'ATGGCAAATCCAACTGAC3',
CXCL2-Reverse: 5'AACTAACTTGGGTTTGAC 3';
ANGPTL4-Forward: 5'CAGCCATTCCAACCTCAAC3',
ANGPTL4-Reverse: 5' TCGGGCAGAGCCAAGAGT3';
HGF-Forward: 5'TGGTGTCATTGTTCCTGGTC 3',
HGF-Reverse: 5'GTTGTATTGGTGGGTGCT3';
AREG-Forward: 5'AGCCATAGCTGCCTTTAT3',
AREG-Reverse: 5' AGGACCGACTCATCATTT3';
EREG-Forward: 5'CACCAGGTATCAAATCAA3',
EREG-Reverse: 5'GTACAAGTCATTACTCCC3';
GAPDH-Forward: 5'GCCCTCAACGACCACTTT3',
GAPDH-Reverse: 5'GGTCTACATGGCAACTGTGA3'.

Statistical analyses

Each experiment was performed in triplicate. The differences were analyzed using the 2-tailed *t* test. Multiple group comparisons were performed by 2-way analysis of variance (ANOVA). All analyses were performed using SPSS 19 for Windows (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5 for Windows (GraphPad Software, Inc., San Diego, CA, USA). $P \leq 0.05$ was considered statistically significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns, not significant).

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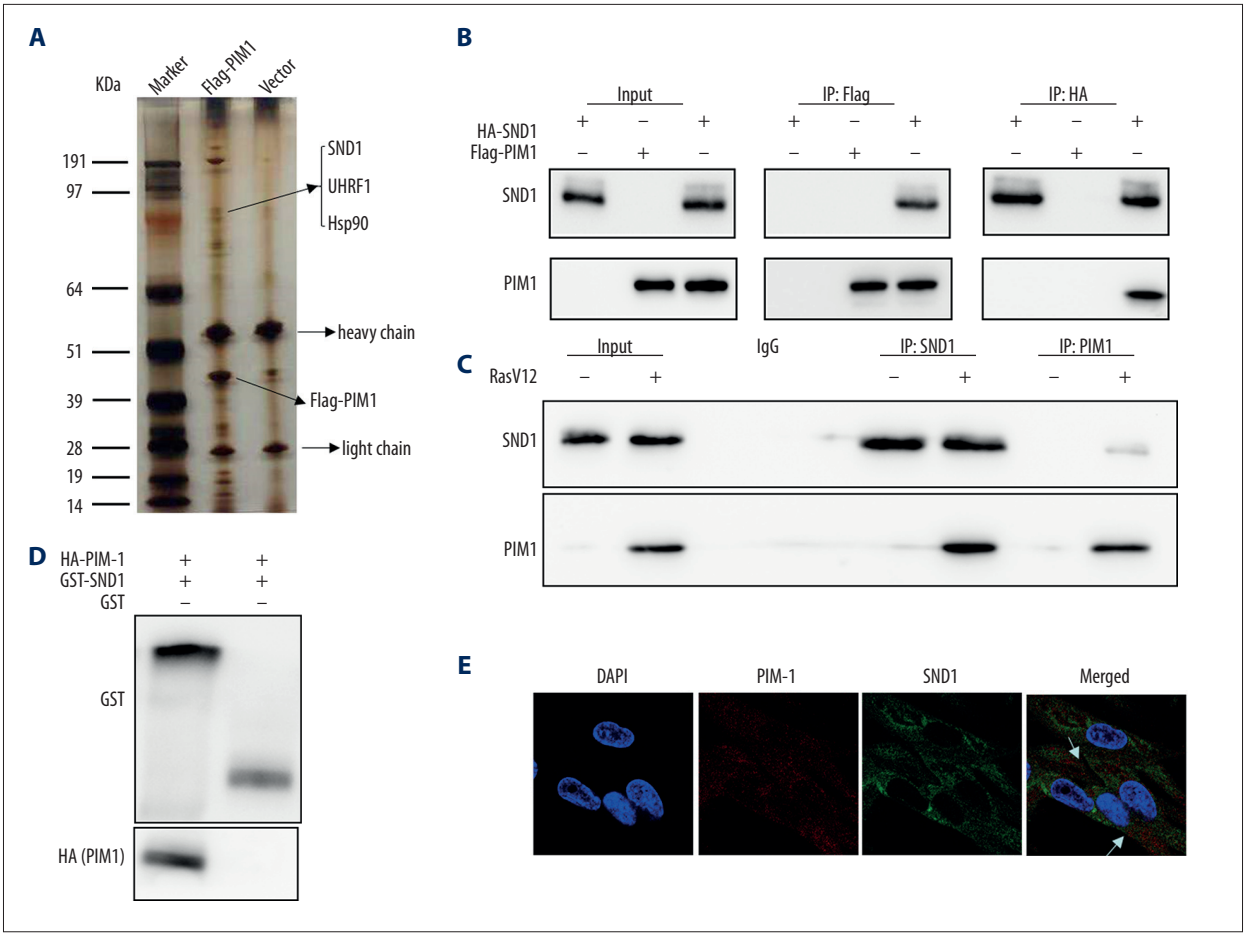

Figure 1. PIM1 interacts with SND1. (**A**) FLAG-PIM1 was overexpressed in 2BS cells, and then cellular proteins were collected for electrophoresis, silver staining, and mass spectrometric analysis to detect target proteins interacting with PIM1. (**B**) HA-SND1 and Flag-PIM1 plasmids were co-transfected into 293T cells. Co-immunoprecipitation and Western blot analysis were performed with corresponding antibodies. (**C**) In RasV12-induced senescent cells, co-immunoprecipitation was performed and precipitated complexes were subjected to Western blot analysis by using antibodies against PIM1 and SND1, respectively. (**D**) GST-SND1 and HA-PIM1, expressed from bacteria, were employed to perform GST pull-down experiments. (**E**) Co-localization of PIM1 and SND1 in the indicated 2BS cells by representative immunofluorescence staining. Data are presented as mean±SD. The experiments were repeated 3 times.

Results

PIM1 interacts with SND1

First, we assessed the specific mechanism by which PIM1 induced senescence through phosphorylating SND1. To determine whether there is direct interaction between PIM1 and SND1, we transiently expressed Flag-PIM1 into 2BS cells, which are frequently used for studying cellular senescence [15,16], and screened for target proteins that interact with PIM1 by immune-purification, silver staining, and mass spectrometry. The results showed that the group overexpressing PIM1 had a more pronounced band at approximately 90 kDa compared to the empty control group (Figure 1A). Mass spectrometry analysis showed that this band mainly contained 3 proteins (SND1, UHRF1, and HSP90). Among them, PIM1 has been reported to interact with UHRF1 and HSP90 [13,17]. Although the interaction between PIM1 and SND1 has also been reported [18], its role in cellular senescence is still unknown. Next, we transfected HA-SND1 and Flag-PIM1 plasmids into 293T cells, and then combined immunoprecipitation (Co-IP) and Western blot analysis to verify the physical binding of SND1 to PIM1 in cultured cells. The data revealed that Flag-PIM1 and HA-SND1 interact with each other (Figure 1B). To further confirm if endogenous SND1 was also associated with PIM1, we performed co-IP assay using RasV12-induced senescent 2BS cells with consistent upregulation of PIM1 expression [19, 20]. The positive SND1 signal was co-immunoprecipitated with PIM1, and PIM1 appeared in SND1 immunoprecipitations in reciprocal immunoprecipitations (Figure 1C). These data showed that

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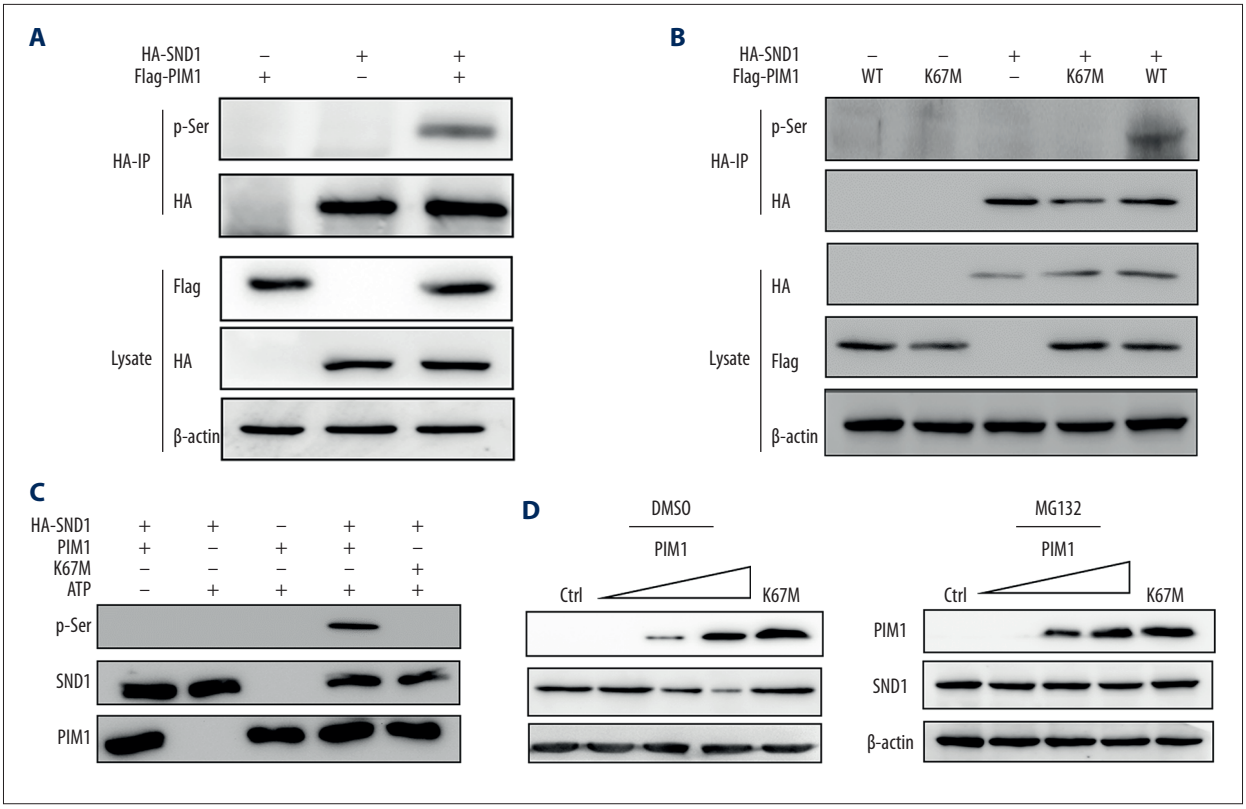

Figure 2. PIM1 phosphorylates SND1 to accelerate its degradation. (**A, B**) Specified plasmids were co-transfected into 293T cells, and the phosphorylation level of SND1 was detected by co-immunoprecipitation and Western blot analysis using the pan-p-Ser antibody. (**C**) HA-SND1 and K67M were purified from prokaryotic cells, and incubated in the presence of [γ-32p] ATP and recombinant PIM1 obtained from Abcam. (**D**) 2BS cells were infected with increasing amounts of PIM1 in the medium with added MG132 or not. Data are presented as mean±SD. The experiments were repeated 3 times.

both endogenous and exogenous SND1 could interact with PIM1, but whether this effect was direct or indirect was still unknown. Next, we co-incubated HA-PIM1 and the full-length of recombinant GST-SND1 to determine the nature of this interaction by performing a GST pull-down experiment. The results (Figure 1D) demonstrated that HA-PIM1 was specifically able to combine with full-length GST-SND1, but it was independent of free GST (Figure 1D). We also used immunofluorescence experiments to further assess the interaction between SND1 and PIM1, and the results showed that they were partially co-localized at the arrow mark (Figure 1E). In summary, this evidence confirmed our finding that the interaction between PIM1 and SND1 is direct.

PIM1 phosphorylates SND1 to accelerate its degradation

We further investigated whether SND1 could be phosphorylated by PIM1. First, we co-transfected Flag-PIM1 and HA-SND1 into 293T cells using HA beads to precipitate SND1. Then, we performed Western blot analysis and used p-Ser antibody to detect the phosphorylation level of the precipitated SND1 (Figure 2A). The results showed that there was significant phosphorylation of Ser in SND1, but not in the control group. Similarly, SND1 and wild-type Flag-PIM1 or Flag-K67M (a catalytically inactive mutant of PIM1) were co-transferred into 293T cells to again verify whether PIM1 can phosphorylate SND1. Western blot analysis confirmed that the phosphorylation level of SND1 was prominently increased by wild-type PIM1 compared to K67M (Figure 2B). To obtain further evidence that SND1 is a new phosphorylation substrate for PIM1 kinase, we performed an *in vitro* kinase assay. HA-SND1 and K67M were purified in prokaryotic cells in ATP-containing kinase assay buffer, and PIM1 was purchased from Abcam and then incubated together. As shown in Figure 2C, we observed that the Ser phosphorylation of SND1 could only be detected when HA-SND1 and Flag-PIM1 were simultaneously expressed under ATP-containing conditions, indicating that all 3 of the above were indispensable for the phosphorylation of SND1 (Figure 2C). Consistent with previous results, these results indicated that there was indeed phosphorylation of SND1 by PIM1. Nonetheless, the specific mechanism by which SND1 induces cellular senescence needs further elucidation. To answer this question, we added MG132 (a proteasome inhibitor that can reduce protein degradation) into 2BS cells infected

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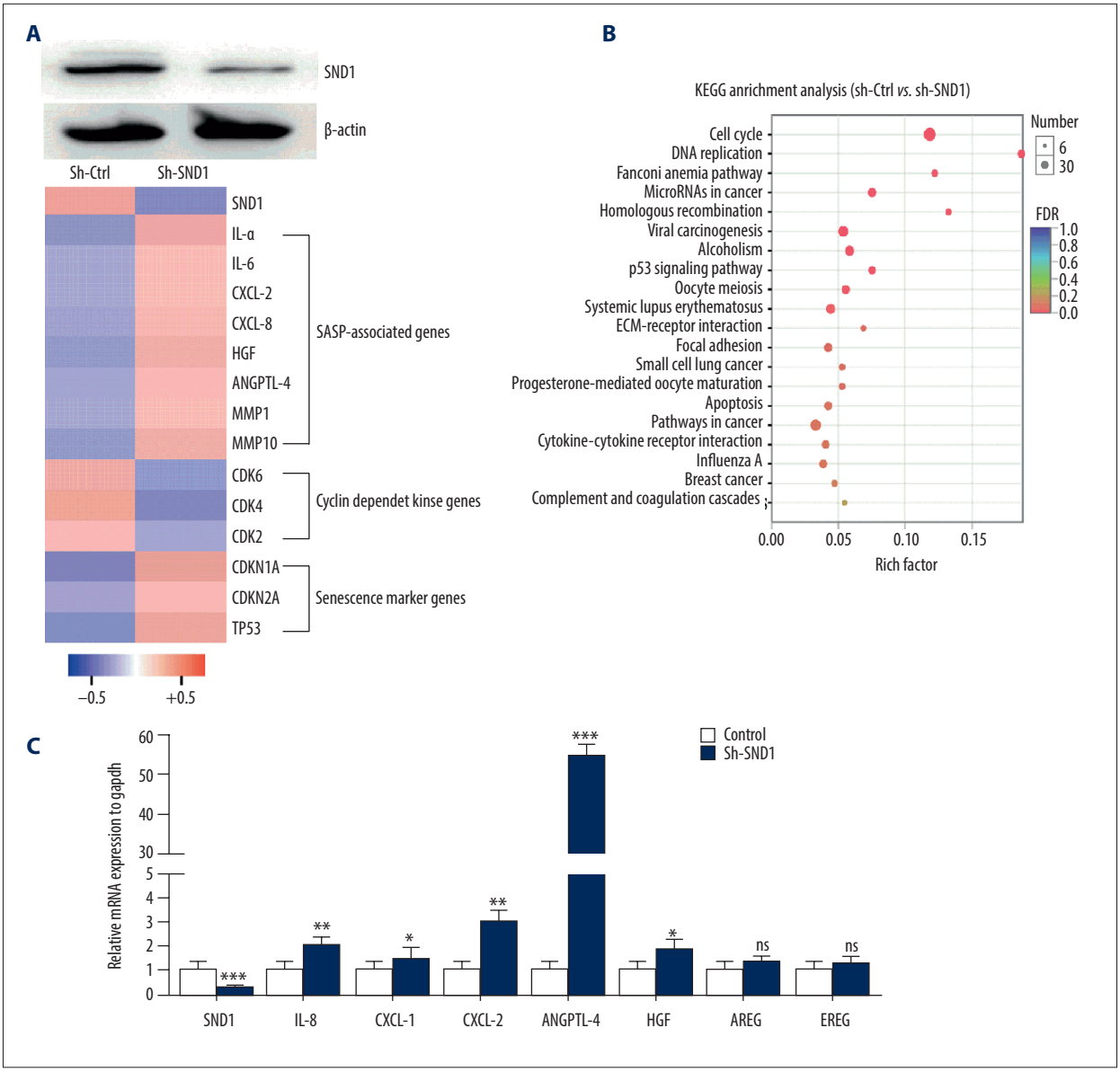

Figure 3. Decreased expression of SND1 results in the upregulation of SASP. (**A**) Heatmap of SASP-associated genes identified by RNA-seq using 2BS cells stably infected with lentivirus carrying SND1 short hairpin RNA (shRNA) or control shRNA. Typical immunoblot shows the knockdown of SND1 expression. (**B**) KEGG pathway analysis of genes differentially expressed between 2BS cells stably infected as in (**A**). (**C**) The expression of SASP-associated genes in 2BS cells infected with indicated vectors was measured by RT-PCR. Data are presented as mean±SD. The experiments were repeated 3 times. * p<0.05, ** p<0.01, *** p<0.001.

with PIM1 in advance. As illustrated in Figure 2D, the decrease in the expression level of SND1 was dependent on the increase in the expression of PIM1, but this was not observed in the group treated with MG132. At the same time, in the 2BS cells with the overexpression of K67M and MG132 had no effect on the protein level of SND1 (Figure 2D). These results indicated that PIM1 can promote the degradation of SND1. To sum up, we demonstrated that PIM1 can promote the degradation of SND1 through phosphorylation.

Decreased expression of SND1 results in the upregulation of SASP.

Previous studies have shown that the expression of SND1 is downregulated by PIM1. Based on the function of SND1 and the purpose of this study, we wanted to address the question of whether the downregulation of SND1 causes changes in downstream senescence-related target genes. To test this hypothesis, the short hairpin RNA (shRNA) was used to stably

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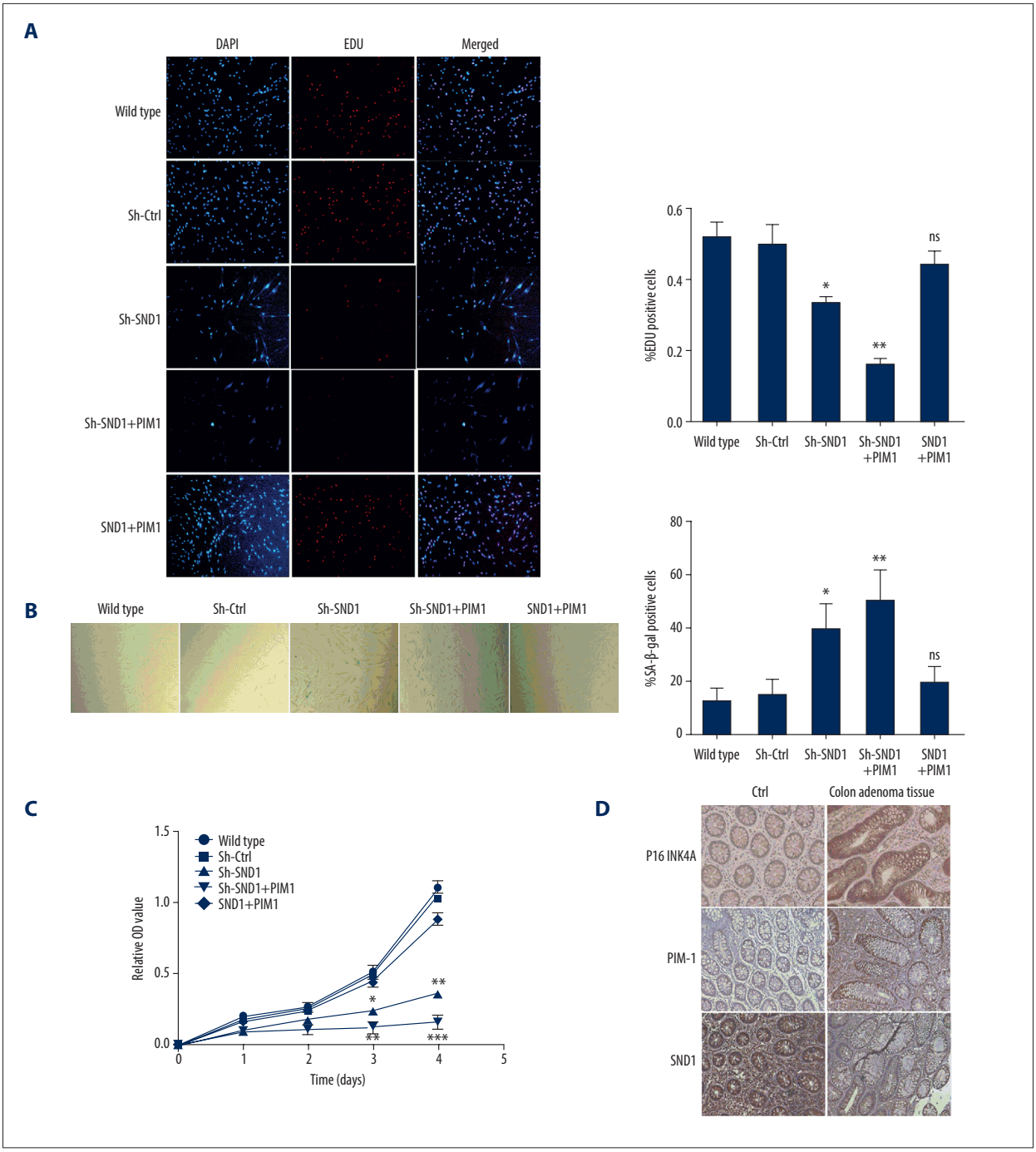

Figure 4. SND1 is involved in cell senescence induced by PIM1. (**A**) EdU incorporation assay of cellular proliferation. (**B**) Representative microscopic view of 2BS cells infected with the indicated vectors with SA-b-gal activity staining. (**C**) Cell viability of 2BS cells infected with the indicated vectors was measured by CCK8 assay. (**D**) Immunohistochemistry staining of PIM1 and SND1 in young and aging colon adenoma tissues. Data are presented as mean±SD. The experiments were repeated 3 times. * p<0.05, ** p<0.01, *** p<0.001.

knock down SND1 in 2BS cells, and RNA sequencing (RNA-seq) was performed (Supplementary Table 1 [Supplementary/raw data available from the corresponding author on request.]). As shown in Figure 3A, there were 2 bands that showed in many SASP-associated genes and senescence maker genes in sh-SND1 immunoblots. Meanwhile, KEGG analysis demonstrated the top 20 enriched pathways, including pathways that are highly relevant to aging, such as cell cycle and p53 signaling

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pathway (Figure 3B). Some papers have demonstrated that P53 and Rb are considered to be the "master regulators" of cellular senescence [21]. To study this further, we used RT-PCR to detect changes of SASP-related genes under different conditions (Figure 3C). The results showed that the expression of IL-8, CXCL1/2, ANGPTL-4, and HGF were elevated to varying degrees. Taken together, our data suggest that PIM1 can downregulate SND1, and decreased expression of SND1 can lead to the upregulation of SASP. As is well-known, senescence-associated secretory phenotype (SASP) is one of the main features of cellular senescence. Thus, it was also indirectly verified that SND1 indeed participates in PIM1-induced cellular senescence.

SND1 is involved in cellular senescence induced by PIM1

To obtain more direct evidence that SND1 affects cellular senescence in a PIM1-dependent manner, we performed functional experiments. Initially, we knocked down SND1 in 2BS cells to construct a Sh-SND1 stable cell line, and then used the EdU incorporation assay to detect cell proliferation. We found that SND1 silencing inhibits cellular proliferation. Then, we overexpressed PIM1 on the basis of SND1 silencing and found that this phenomenon is more obvious, and it was inhibited when SND1 and PIM1 were co-overexpressed in 2BS cells (Figure 4A). Similar trend was also observed in the CCK8 experiment (Figure 4C). Furthermore, the SA-β-gal activity staining assay revealed that, compared with knockdown of SND1 with or without PIM1-infected cells, overexpressing SND1 in 2BS cells infected with PIM1 decreased the percentage of stained cells (Figure 4B). Since human colon adenoma tissues are frequently used as an aging model due to their increased expression of $P16^{INK4A}$ [22,23], we used immunohistochemistry to measure the expression of PIM1 and SND1. We found significantly enhanced PIM1 expression in aging colon adenoma tissues compared to control tissues, and this was negatively correlated with SND1 expression (Figure 4D). These data indicate that the overexpression of SND1 effectively neutralizes PIM1-induced senescence, providing evidence that SND1 is associated with PIM1-induced cellular senescence.

Discussion

Proliferation inhibition is the main feature of cellular senescence. In addition, cellular senescence is increasingly regarded as a protective mechanism against carcinogenesis [24–26]. Therefore, exploring new mechanisms of senescence may provide new ideas for the treatment of cancer. The oncogene PIM1 is a serine/threonine kinase that has an important role in regulation of cellular senescence. It was reported that PIM1 can also regulate growth and transformation of many types of malignant cells [27], while the function of PIM1 in senescence has only recently been reported. In the present study, we found that the degradation of SND1 was increased after being phosphorylated by PIM1, which resulted in cellular senescence. This discovery may be a novel mechanism by which PIM1 drives cellular senescence.

Although we confirmed that SND1 is phosphorylated by PIM1, its specific phosphorylation site is unclear. Further studies are needed to identify specific phosphorylation sites to provide stronger evidence for the involvement of SND1 in PIM1-induced senescence. In addition, the relationship between UHRF1 and PIM1 was studied in our laboratory before, while UHRF1 is an E3 ligase and the degradation of SND1 is increased after the phosphorylation of PIM1, so we want to know whether there is any relationship between SND1 and UHRF1. Previous reports have shown that the p53 signaling pathway [28] and p16/Rb signaling pathway are 2 important molecular pathways in cellular senescence [29,30], although we also discovered that SND1 may be related to the regulation of PIM1-driven OIS, but its specific mechanism and signaling pathway require further elucidation.

Conclusions

We found that PIM1 promotes cellular senescence by phosphorylating SND1, which directly interacts with SND1. These findings expand our understanding of cellular senescence. The pleiotropic effects of PIM1- SND1 signaling suggest that it could be an effective target for senescence therapy.

Conflicts of interest

None.

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