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USP13 regulates cell senescence through mediating MDM2 stability

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Abstract

Aims: Lung aging results in altered lung function, reduced lung remodeling and regenerative capacity, and increased susceptibility to acute and chronic lung diseases. The molecular and physiological underlying mechanisms of lung aging remain unclear. Mounting evidence suggests that deubiquitinating enzymes (DUBs) play a critical role in tissue aging and diseases through regulation of cellular signaling pathways. Here we investigate the role of Ubiquitin-Specific Protease 13 (USP13) in cell senescence and lung aging and its underlying mechanisms.

Main methods: Protein levels of USP13 and MDM2 in lung tissues from aged and young mice were compared. Gene silencing and overexpression of USP13 in human cell lines were performed. MDM2 levels were examined by Quantitative Real-Time PCR and Western blotting analysis. The cell senescence levels of human cells were checked by the β-galactosidase staining.

Key findings: Lung tissues from aged mice showed higher levels of USP13 compared to younger mice. We found a negative correlation between USP13 and MDM2 expression in lung tissues of aged mice. The increased protein levels of MDM2 were detected in lung tissues of USP13 deficient mice. Furthermore, overexpression of USP13 promoted cell senescence. Knockdown of USP13 increased MDM2 levels in lung cells, while overexpression of USP13 reduced it. The degradation of MDM2 caused by USP13 was prevented by the proteasome inhibitor MG132. Furthermore, we showed that USP13 targeted and reduced K63-linked polyubiquitination of MDM2. These results demonstrate that USP13 is involved in the aging signaling pathway in lungs through regulation of MDM2.

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Declaration of competing interest

The authors declare no competing interest.

CRediT authorship contribution statement

J.H.: conceptualization, investigation, data analysis, writing, review, editing. B.B.: investigation, data analysis. S.T.: investigation, data analysis, editing. J.Z.: funding acquisition, editing. Y.Z.: funding acquisition, conceptualization, editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2023.122044.

Keywords

Cell senescence; USP13; MDM2; Deubiquitination; Lung aging

1. Introduction

Studies have thoroughly shown that lung aging is an extremely important factor related to morbidity and mortality and promotes the malignant development of various diseases [1]. As the aging population and aging-related diseases both increase, it is crucial to study the physiological and cellular effects of aging on the lungs. Aging of the lungs leads to decreases in their remodeling and regenerative capacity, as well as an increased susceptibility to acute and chronic lung diseases [1].

A common hallmark of aging is the increased accumulation of damaged, dysfunctional proteins, indicating that protein degradation plays an important role in cellular quality control with increasing of age [2]. As E3 ubiquitin ligase and deubiquitinating enzymes (DUBs) are involved in the degradation of proteins by the proteasome or lysosome; therefore the enzymes that regulate protein stability play a pivotal role in aging. Mounting evidence suggests that MDM2 is an important E3 ubiquitin ligase related to aging and cell senescence. Depletion of MDM2 causes skin aging phenotypes in mice, which includes thinning of the epidermis, reduced wound healing, and progressive loss of fur [3]. MDM2 not only acts as an oncogene, but also regulates the normal physiological functions of cells in a p53-dependent and independent manner [4,5]. Researchers discovered an MDM2 mutation leading to dysregulation of p53 activity linked to premature aging [6]. This dysregulation of the MDM2/p53 axis leads to increase in p53 level and stability [6]. This finding suggests that mutations of the MDM2 gene or dysfunctional MDM2 activity may be a driver of aging [6,7]. Similarly, mutations in MDM4, a homolog of MDM2, also leads to shortened telomeres in both human patients and mice [8] along with tissue aging.

Previous studies have revealed that the p53/p21Cip1 (p21), p16INK4a (p16)/RB1, and PTEN/p27 pathways are three key senescence signaling pathways [9]. As MDM2 regulates protein expression levels of cellular senescence markers p53 [10,11], p21 [4,12–14], p16 [15], and RB1 [16], MDM2 can be called a master regulator of aging [7]. In particular, MDM2 can promote the degradation of p21 [4] and RB1 [16]. Additionally, MDM2 has also been found to determine respiratory progenitor cell number and lung size [17]. These findings suggest that the expression level of MDM2 may be involved in the process of lung aging.

DUBs can remove ubiquitin from proteins, edit ubiquitin chains, and process ubiquitin precursors. Ubiquitin-specific protease 13 (USP13), a member of the USP superfamily of DUBs, is implicated in many diseases. The roles of USP13 have been widely reported. USP13 is associated with regulation of the cell cycle, DNA damage repair, cell differentiation, autophagy, infection, inflammation, and cancers. USP13 is involved in the regulation of signal proteins in cells, such as PTEN [18], STING [19], and SIGIRR [20]. A recent study showed that inhibition of USP13 reduced autophagy and ROS accumulation, and attenuated pyroptosis. However, these effects were greatly blocked by

USP13 overexpression which causes increases in autophagy as well as ferroptosis [21]. Recent studies also have reported that USP13 is upregulated in Alzheimer's disease (AD) and Parkinson's disease (PD) [22]. As both AD and PD are related to aging, especially neuronal senescence, these results suggest that USP13 expression levels are associated with tissue aging.

In this study, we compared lung tissues from both young and aged mice and found that USP13 protein levels are significantly upregulated in aged mice while MDM2 protein expression was significantly downregulated. Increase in USP13 promoted cell senescence. We also checked the protein levels of MDM2 in both USP13 knockout and wild type mice, and found that MDM2 protein was upregulated in USP13-knockout mouse lung tissues. This study is the first to report that USP13 is involved in aging by deubiquitinating MDM2.

2. Materials and methods

2.1. Animals

Mice were maintained and handled in all studies according to the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of The Ohio State University. C57BL/6 wild type (WT) mice were purchased from the Jackson Laboratory (Sacramento, CA, USA). The USP13 knockout USP13KO (USP13−/−) mice were generated by the CRISPR/Cas9 system [20]. Age-matched mice were used in all animal experiments.

2.2. Cell lines and cell culture

Human lung microvascular endothelial cells (HLMVEC) were cultured in endothelial basal medium-2 (EBM-2) Basal Medium (Lonza) supplemented by EGM-2 MV Microvascular Endothelial Cell Growth Medium SingleQuots supplements (Lonza). Human primary pulmonary artery endothelial cells (HPAEC) were cultured in EBM-2 Basal Medium supplemented with the EGM-2 SingleQuot Kit (Lonza). Human vascular smooth muscle cells (HVSMC) were cultured in Smooth Muscle Cell Basal Medium (PromoCell, C-22262) supplemented by Growth Medium Supplement-Mix (PromoCell, C-39267). A549 and HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % Fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). All cells were cultured in a 5 % CO2 incubator at 37 °C.

2.3. Western blotting

Proteins were extracted from human cells or mouse lung tissues using lysis buffer (Thermo Fisher Scientific, USA) containing protease and phosphatase inhibitors. The lysates were then sonicated and centrifuged at 12,000 rpm at 4 °C for 10 min. Protein quantification of the supernatant was performed using the a protein assay kit (Bio-Rad, USA). Equal amounts of cell lysate (20 μg) were loaded into SDS-PAGE gels and transferred to a nitrocellulose blotting membrane (0.2 μm, Amersham Protran, Germany). The membranes were incubated with 5 % nonfat milk in TBST buffer (25 mM Tris-HCl, 137 mM NaCl, and 0.1 % Tween 20) for 1 h for blocking. After blocking, the membranes were then incubated with primary antibodies overnight following by the incubation with secondary antibodies. Protein bands

were detected were using the Enhanced Chemiluminescence Detection Kit (Thermo Fisher Scientific, USA) and Azure C600 Imaging System (VWR, USA).

2.4. Co-immunoprecipitation (Co-IP)

Cell lysates containing an equal amount of protein (1 mg) were incubated with specific antibodies overnight at 4 °C. 50 μl protein A/G agarose beads were added to the mixture and incubated for another 2 h at 4 °C. The immunoprecipitated complexes were washed 3 times with cold PBS then combined with SDS sample buffer and boiled at 95 °C for 10 min. After centrifugation, the supernatant was collected and analyzed by immunoblotting with the indicated antibodies.

2.5. In vivo ubiquitination assay

Cells were harvested with PBS, followed by centrifugation at 1000 rpm for 5 min. Cell pellets were added 60–80 μl of 2 % SDS lysis buffer containing ubiquitin aldehyde and N-ethylmaleimide. Cell lysates were boiled at 100 °C for 10 min, followed by dilution with 600–800 μl of TBS. Equal quantities of protein (1 mg) from each sample was subjected to Co-IP.

2.6. Gene silencing and plasmid transfection

The cells were transfected with siRNA using PepMute reagent (SignaGen Laboratories, USA) after reaching 80–90 % confluence. The Genjet Plus reagent (SignaGen Laboratories, USA) was used for plasmid transfections for gene over-expression. The V5 tagged USP13 plasmid was generated in the laboratory previously. The Flag tagged USP13 plasmid was a gift from Dr. Ze'ev Ronai, Director of Sanford Burnham Prebys Medical Discovery Institute (La Jolla, CA, 92037 USA). Human MDM2 Gene ORF cDNA clone expression plasmid was purchased from Sino Biological US (Cat. No.: HG11206-NY). All siRNAs used in the experiments were purchased from Sigma.

2.7. Immunofluorescence (IF)

Cells were grown in glass-bottom dishes and maintained in appropriate medium. Cells were washed twice with cold PBS buffer and fixed with 4 % paraformaldehyde (PFA) for 20 min at room temperature (RT). Permeabilization of cells was performed by incubation in PBS containing 0.2 % Triton X-100 for 2 min at room temperature. The cells were incubated with the appropriate primary antibody at 4 °C overnight following washing and blocking. The cells were then incubated with Alexa-488 or 546-conjugated secondary antibody for 1 h at room temperature and mounted with mounting solution containing DAPI. Images were captured with a Nikon confocal microscope.

2.8. β-Galactosidase staining

The cells were incubated in glass bottom dishes, and fixed with 2 % paraformaldehyde. Cells were then stained with Cell Event™ Senescence Green Detection Kit (Catalog No.: C10850, Invitrogen, USA) for 2 h at 37 °C in line with the manufacturer's instructions. A confocal microscope was used for imaging using an Alexa Fluor™ 488/FITC filter set.

2.9. Quantitative real-time PCR

The total RNA from the cells was extracted using an RNA Extraction Kit (Invitrogen). RT-PCR was performed with SsoFast Eva Green Supermix (Bio-Rad) after cDNA was synthesized using the Supermix kit (Bio-Rad). MDM2 primers: forward 5′-TGGGCAGCTTGAAGCAGTTG-3′, reverse 5′-CAGGCTGCCATGTGACCTAAGA-3′; GAPDH primers: forward 5′-GCACCGTCAAGGCTGAGAAC-3′, reverse primer: 5′-TGGTGAAGACGCCAGTGGA-3′.

2.10. Quantification and statistical analysis

Data are presented as mean ± SD of triplicate samples from three independent experiments. Quantification of immunoblots was performed by ImageJ and normalized to β-actin. Quantification of the intensity of the images was also analyzed using ImageJ. Quantitative data were statistically analyzed using a two-tailed student's *t*-test. p values <0.05 were considered significant.

3. Results

3.1. The USP13 expression levels are correlated with lung aging as well as MDM2 protein expression

To understand the physiological function of USP13 in lung aging, we compared protein expression levels in lung tissue and cytokine interleukin-6 (IL-6) in bronchoalveolar lavage (BAL) from 8 week and 90-week-old wild type (WT) mice. The results of immnoblotting analysis indicate that the protein levels of USP13 were significantly elevated in the tissue lysates from 90-week-old mouse lungs compared to those from 8-week-old mouse lungs (Fig. 1A and B). USP13 levels were also increased in lungs from 45-week-old mice (Supplemental Fig. 1S). In contrast, the protein levels of MDM2 were significantly decreased in lung tissue lysates from 45- and 90-week-old mice compared to tissue lysates from 8-week-old mice (Fig. 1A, B, and Supplemental Fig. 1S). Additionally, enzyme-linked immunosorbent assay (ELISA) results indicate that IL-6 protein levels in BAL were significantly increased in aged mice (90 weeks) compared to that of young mice (8 weeks) (Fig. 1E). In order to further study the relationship between USP13 and MDM2, immunoblotting analysis was performed using lung tissue lysate from 12-week-old WT and USP13 knockout (USP13KO) mice. The results showed that the protein level of MDM2 in lung tissue from USP13KO mice was significantly higher than that of WT mice (Fig. 1C and D). In addition, ELISA results indicate that IL-6 levels in BAL were significantly decreased in the USP13 KO mice compared to that of WT mice (Fig. 1F).

3.2. Overexpression of USP13 leads to cellular senescence

In order to investigate the role of USP13 in cell senescence, HVSMC cells were transfected with Flag-tagged USP13 plasmid to over-express USP13 or treated with cobalt chloride ($CoCl_2$) as a positive control, followed by staining with a β-galactosidase kit (Fig. 2A). The results showed that most of the cells were positive to cell senescence marker after $CoCl_2$ treatment. USP13 overexpressed cells showed significant increased cell senescence marker levels (Fig. 2A and B). The results indicate that overexpression of USP13 promotes cell

senescence. In order to further confirm the conclusion, we investigated additional proteins related to cell senescence. HVSMC cells were transfected with USP13-V5 plasmid and harvested after 72 h for immunoblotting analysis. The levels of senescence-related markers p21, p27, and IL-6 were evaluated. IL-6 expression levels were significantly increased in USP13-overexpressed cells. However, the expression levels of p21 and p27 did not change (Fig. 2C and D).

3.3. USP13 regulates the protein stability of MDM2

To investigate if USP13 controls the protein stability of MDM2, we analyzed MDM2 protein levels in various USP13-overexpressing cells. MDM2 protein levels were detected using Western blotting. The results showed that protein levels of endogenous MDM2 were decreased in USP13 over-expressing HVSMCs compared to that of empty vector-transfected cells (Fig. 3A and B, Supplemental Fig. S3). To further investigate the regulation mechanisms of USP13 on MDM2, A549 and HPAEC cell lines were co-transfected with HA-MDM2 and USP13-V5 plasmids. Cells were harvested and the MDM2 protein levels were examined using immunoblotting. The results showed that MDM2 levels were decreased in USP13-overexpressed cells compared to control (Fig. 3C and D, Supplemental Fig. S3). The results indicate that exogenous MDM2 levels were decreased by over-expression of USP13. To determine the mechanism by which USP13 regulates MDM2 expression, MDM2 protein turnover in cells was examined by inhibition of protein synthesis by cycloheximide (CHX). CHX administration led to a remarkable decrease in MDM2 in a time dependent manner (Figs. 3E and S3). We observed that the half-life of MDM2 is quite short, only about 15 min, suggesting that endogenous MDM2 undergoes fast degradation at the steady state. Additionally, the half-life of MDM2 protein was found to be significantly shorter after over-expression of USP13 compared with that observed in control (Fig. 3E–G, and Supplemental Fig. S4). When endogenous USP13 was knocked down by siRNA, significant increase of MDM2 protein was observed (Fig. 3H). Notably, the mRNA levels of MDM2 were not affected by knockdown of USP13 (Supplemental Fig. S5). In addition, we found that compared with the control group, when USP13 was knocked down, the phosphorylation of MDM2 was increased, and the expression of p16 was reduced (Fig. 3H, and Supplemental Fig. S6).

3.4. USP13 is associated with MDM2

To determine whether endogenous USP13 directly targets MDM2, we conducted co-immunoprecipitation (Co-IP) and co-immunofluorescence (Co-IF) staining. Cell lysates were immuno-precipitated with anti-MDM2 or anti-USP13 specific antibody; normal rabbit immunoglobulin G (IgG) was used for control IP's antibody. The Co-IP results demonstrate that USP13 is associated with MDM2 (Fig. 4A and B). To further confirm the interaction between USP13 and MDM2, Co-IP experiments after over-expression of exogenous genes were conducted. A549 cells were co-transfected with USP13 and MDM2 plasmids for overexpression. The results demonstrate that exogenously expressed USP13 and MDM2 proteins are associated each other (Fig. 4C and D). To investigate the co-localization between USP13 and MDM2, Co-IF staining was conducted to examine the sub-cellular locations of both proteins. As shown in Fig. 4E, co-localization of USP13 and MDM2 in the cells were identified by IF staining. USP13 was found to be co-localized with MDM2 in

the nucleus of HVSMC cells, and knockdown of USP13 lead to increase in MDM2 levels. These experimental results indicate that USP13 interacts with MDM2 in the nucleus.

3.5. USP13 negatively regulates MDM2 through K63-linked ubiquitination

To elucidate the mechanisms of regulation of MDM2 protein by USP13, we examined in which by pathway USP13 reduces MDM2 levels. USP13-V5 transfected HVSMC cells were treated with MG132, carbobenzoxy-valyl-alanyl-aspartyl-[*O*-methyl]-fluoromethylketone (ZVAD), or caspase-2 inhibitor (CSPS2-Inhi). MG132 is an inhibitor of the proteasome, whereas ZVAD is a pan-caspase inhibitor. MDM2 protein levels were decreased in cells overexpressing USP13. However, MG132 prevented the loss of MDM2 compared to the control group and both the ZVAD- and CSPS2-Inhi-treated groups (Fig. 5A and B), suggesting MDM2 is degraded in the proteasome. Considering the direct association between USP13 and MDM2 and the role of USP13 in the degradation of MDM2, we examined if USP13 deubiquitinates MDM2. HVSMC cells were transfected with control siRNA or siUSP13. MDM2 was found to be ubiquitinated in cells from the control group, likely through autoubiquitination. The ubiquitination of MDM2 was significantly increased in siUSP13-transfected cells (Fig. 5C). The results indicate that USP13 deubiquitinates MDM2. We also used *in vitro* experiments to examine the deubiquitination of MDM2 by USP13 using a purified human recombinant protein USP13 (rhUSP13) and MDM2 autoubiquitination kit. The results showed that the autoubiquitination of MDM2 was significantly reduced when USP13 protein was presented in the reaction solution compared with the control group and the BSA group (Fig. 5D). The results indicate that USP13 deubiquitinates MDM2. Thus, USP13 appears to be a deubiquitinase of MDM2. K63-linked ubiquitination has been shown to stabilize MDM2 [23,24]. Next, we examined USP13 regulates K63-linked ubiquitination of MDM2. The cells were transfected with siUSP13 (or control siRNA), followed by IP with anti-MDM2 antibody after collecting and lysing the cells. Knockdown of USP13 resulted in an increase in K63-linked ubiquitination of MDM2 (Fig. 5E), not K48-linked ubiquitination (Supplemental Fig. S7). These results indicate that USP13 removes the K63-linked ubiquitination of MDM2.

3.6. Overexpression of MDM2 rescues the effect of USP13 on cellular senescence

In order to study if the overexpression of MDM2 can rescue the cell senescence caused by USP13, HVSMC cells were co-transfected with Flag-tagged USP13 and HA-tagged MDM2 plasmids. The cells were stained with a β-galactosidase kit (Fig. 6A). The results showed that over-expression of USP13 induced cell senescence. However, the effect was attenuated by co-overexpression of MDM2. This result suggests that over-expression of MDM2 may rescue cellular senescence caused by over-expression of USP13. Next, we downregulated MDM2 with siRNA in HVSMC cells and stained with the β-galactosidase kit to examine senescence of cells in the absence of MDM2. Cell senescence was significantly increased when MDM2 was knocked down (Fig. 6B). Western blotting analysis showed that knocking down MDM2 increased the expression levels of cell senescence marker protein p21, but did not alter the levels of USP13 (Fig. 6C and D). These results are consistent with previous studies showing that inhibition of MDM2 resulted in an increase in senescence marker protein p21 [12–14]. Taken together, this data indicates that over-expression of USP13 causes the reduction and inhibition of MDM2 and thus promotes the senescence of cells.

4. Discussion

Here, we identify USP13 as a regulator of aging and cellular senescence. Aged mice showed higher levels of USP13 in lung tissues compared with young mice. Additionally, over-expression of USP13 in human vascular smooth muscle cells promotes cellular senescence. We found that USP13 regulates the stability of MDM2. Over-expression of USP13 in human cell lines leads to degradation of MDM2, indicating that cellular MDM2 levels are controlled by a dynamic balance between ubiquitination and deubiquitination. USP13 appears to function as a deubiquitinating enzyme for MDM2, as it removes K63-linked ubiquitin chains directly from MDM2. USP13 regulates the stability of MDM2, mediating its downstream proteins to participate in the regulation of cell senescence.

MDM2, known as a regulator of p53 and p21, is an oncogenic factor; however, the role of MDM2 in regulation of normal cellular physiological processes, especially for aging, remains poorly understood. Aging and cancer are major topics in the medical research field, and they are closely correlated. According to reports, most cancer patients in the United States are >70 years old [25]. MDM2 plays a pivotal role not only in cancer but also in tissue aging. Previous studies have shown that inhibiting MDM2 leads to early aging, while increased MDM2 attenuates cellular senescence in fibroblasts [26]. Furthermore, loss of MDM2 leads to p53-mediated senescence in epidermal stem cells and early skin aging [3]. In this study, we found that knockdown of MDM2 increases the level of p21 expression in cells and promotes cellular senescence. The results are highly consistent with previous reports that MDM2 plays an important role in cellular senescence [3,4,6,7,26].

Previous studies have shown that MDM2 itself is capable of autoubiquitination and is also degraded in the proteasome. For example, FKBP25 promotes the autoubiquitination of MDM2 to promote its proteasomal degradation [27]. Our data showed that as a deubiquitinating enzyme, USP13 regulates the protein level of MDM2 by removing K63-linked ubiquitination, thereby participating in the regulation of cell senescence and lung aging. In this study, we found that knockdown of USP13 increased the K63-linked ubiquitination of MDM2, which consequently promoted its protein stability. This finding is consistent with previously reports showing that K63-linked ubiquitination protects protein from degradation [23,24,28]. Previously reports showing that K63-type polyubiquitination by NEDD4–1 [24] and MARCH7 [23] competes with other types of polyubiquitination on MDM2, as a result, stabilizes MDM2 in cells. Protein ubiquitination does not necessarily result in the degradation of its target protein. Target proteins are modified with different types of ubiquitin chains in order to play different roles in different processes in the cells. Among them, K11-, K48-, and K63-linked ubiquitination are the most abundant ubiquitin chain types. K11- and K48-linked ubiquitination chains are generally thought to be involved in proteasomal degradation. However, K63-linked ubiquitination is mainly involved in the regulation of signal transduction, regulation of protein localization and interaction, regulation of transcription, modification of substrate activity, DNA damage repair, endocytosis, and regulation of cell cycle progression [29]. Tissue aging is a process characterized by a gradual loss of its functionality; it is associated with oxidative stress. Accumulation of damage by reactive oxygen species (ROS) due to aging impairs various functions of tissues and increases the incidence of age-related diseases. K63-linked

ubiquitination rapidly accumulates under the conditions of oxidative stress, and it seems to be a modulator of the oxidative stress response and aging [30]. A previous study indicated that p21 could promote the lung senescence and might influence chronic obstructive pulmonary diseases (COPD) progression [31]. However, the K63-ubiquitin of p21 was decreased could inhibit the senescence of lung cells and the lung aging procession [31]. Increase of K63-mediated ubiquitination results in reduction of proteasome-mediated protein degradation, and subsequently promotes protein aggregation and accumulation [23,24,32]. In this study, we showed that USP13 removes K63-linked ubiquitin chains from MDM2, but not K48-linked ubiquitin chains. Further studies are needed to determine the molecular mechanisms by which K63-linkd ubiquitination of MDM2 regulates MDM2 stability. It is possible that increased phosphorylation of MDM2 by downregulation of USP13 regulates MDM2 degradation.

A previous study indicated that USP13 affects p53 by deubiquitinating USP10 [33]. The study indicated that the expression levels of USP13 indirectly affect the protein expression of p53. However, MDM2 was not mentioned in this study. Previous studies have shown that knockdown of USP13 reduces PTEN, and over-expression of USP13 results in higher levels of PTEN [18]. Meanwhile, another study has shown that PTEN restricts MDM2 to the cytoplasm and promotes MDM2 degradation [34]. The study indicated that over-expression of PTEN reduces levels of MDM2 [34]. Based on the above studies, these results clearly indicated that the over-expression of USP13 indirectly resulted in the reduction of protein expression of MDM2. These results are consistent with our findings showing that USP13 regulates MDM2 protein levels. To the best of our knowledge, this study is the first report on the mechanism by which USP13 regulates MDM2.

In this study, we also found that overexpression of USP13 caused the reduction of p16, which is consistent with the conclusion that the reduction of p16 causes cell senescence [35]. This finding further supports the conclusion that USP13 promotes cellular senescence. The mechanism of how USP13 regulates the expression level of p16 needs to be further explored in the future.

5. Conclusions

In this study, we found that USP13 promotes cellular senescence. The study revealed for the first time the mechanism by which USP13 regulates lung aging and cellular senescence by deubiquitinating MDM2. Lung tissues from aged mice showed accumulation of USP13 compared to younger mice; meanwhile, the levels of MDM2 were increased in both USP13 knockout mouse lung tissues and USP13 knockdown human cells. USP13 interacts with MDM2 and removes K63-linked ubiquitin chains on MDM2. These experimental results proved evidence showing that USP13 is a regulator of lung aging and cellular senescence through the regulation of the protein stability of MDM2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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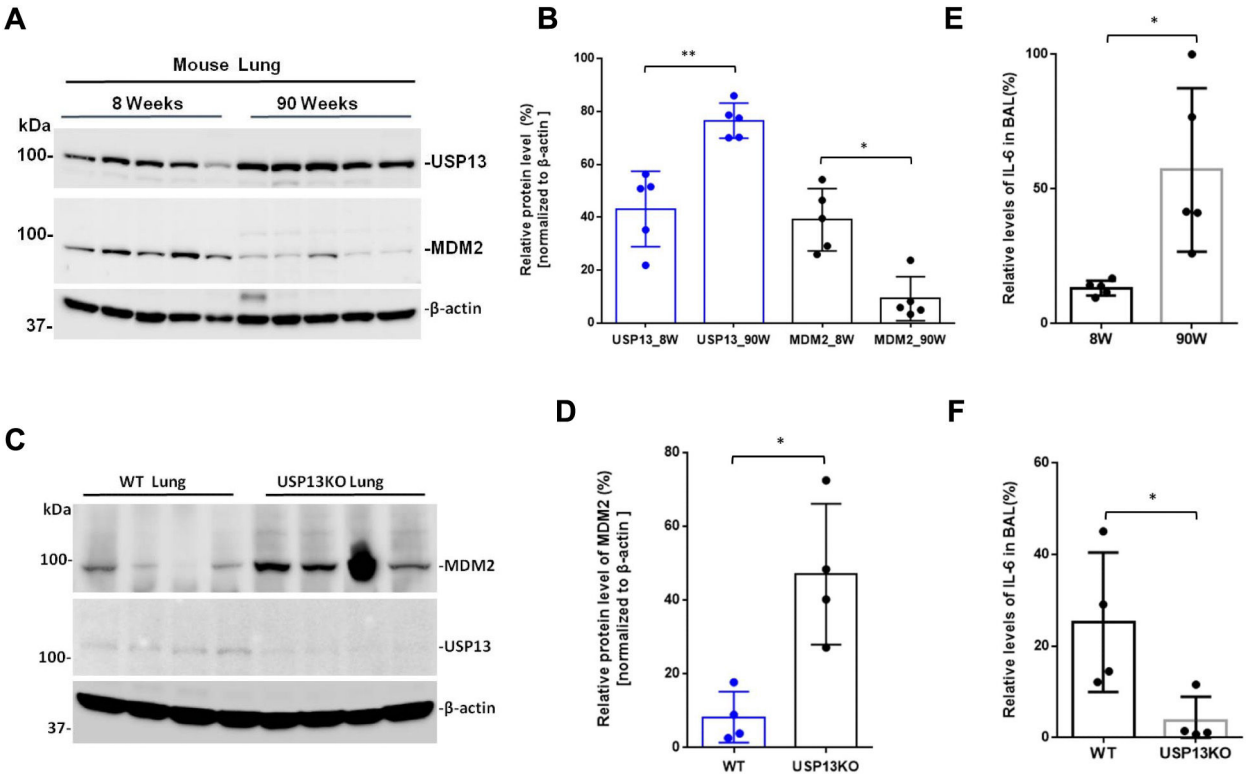

Fig. 1.

USP13 protein levels are associated with lung aging and MDM2 protein expression. Protein levels of USP13, MDM2, and β-actin in both 8 weeks (n = 5) and 90 weeks (n = 5) wild type (WT) mouse lung tissue lysates containing a constant amount of proteins (20 μg) were determined by Western blotting with specific antibodies (A). Lung lysates from 12-week-old wild type (WT) mice (n = 4) and USP13 knockout (KO) mice (n = 4, 12-week-old) containing total 20 μg proteins were subjected to Western blotting and detected by anti-USP13, MDM2, and β-actin specific antibodies (C). Blotting of β-actin served as loading control. The Dot plots shown in (B) and (D) are quantifications of (A) and (C) respectively. Protein levels were normalized to β-actin. Data are present as the means ± SD,** $p < 0.01$, * $p < 0.05$. (E) and (F): The relative levels of IL-6 in bronchoalveolar lavage solution (BAL) of mice (n = 5/each group) were determined by the enzyme-linked immunosorbent (ELISA) assay. Data are present as the means ± SD,* $p < 0.05$. Two-way parametric test and Mann Whitney test (for $n < 5$) were used.

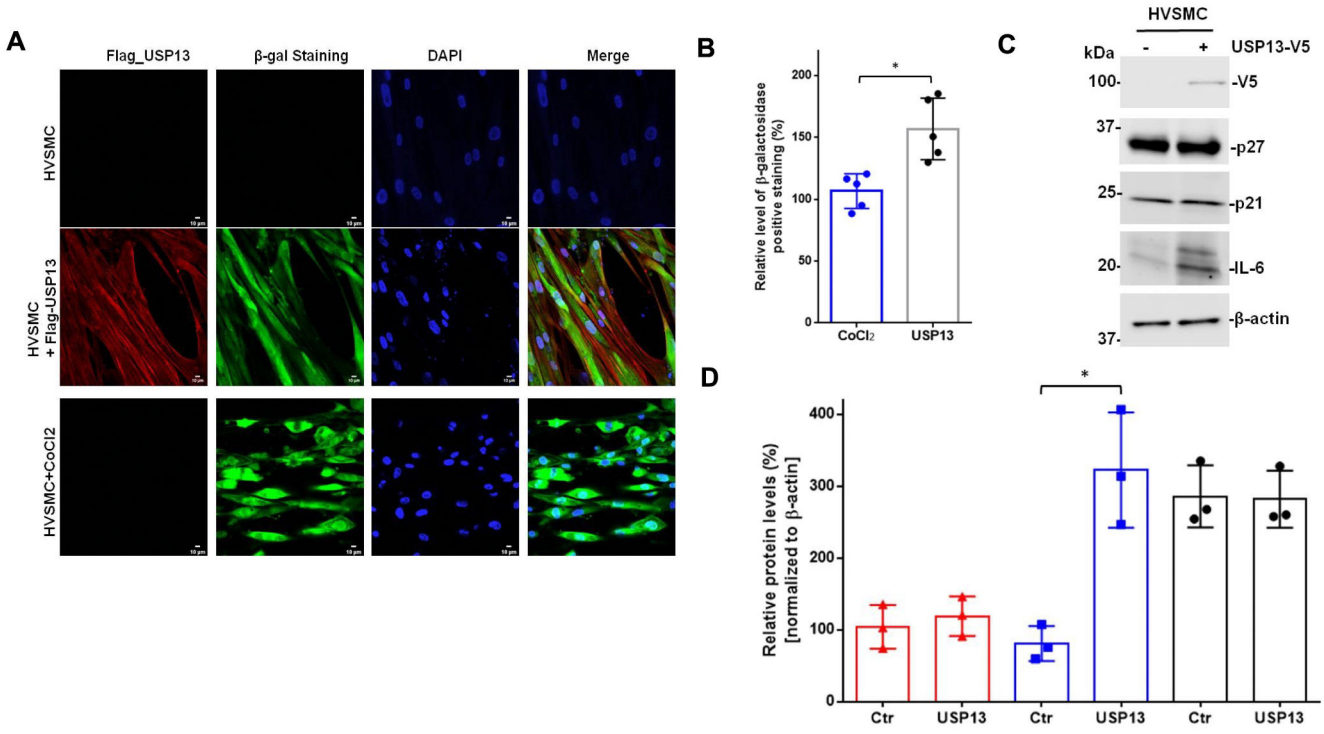

Fig. 2.

USP13 overexpression increases cell senescence. (A). HVSMC cells were transfected with Flag-tagged USP13 plasmid, and the positive control group cells were treated by Cobalt chloride ($CoCl_2$). The cells were immunostained with anti-Flag antibody followed by Alexa-546 conjugated anti-rabbit antibody. Senescence staining was performed using β-Galactosidase Staining Kit. The cell nucleus was stained with DAPI (blue). Red stands for Flag-USP13 and green stands for senescent cells. (B). The Dot plots shown here is quantifications of (A). Data are present as the means ±SEM,* $p < 0.05$. (C). The HVSMC cells were transfected with the V5-tagged USP13 plasmid. The cells were harvested, and protein levels of cell extract were detected by Western blotting with anti-V5, p27, p21, IL-6, and β-actin specific antibodies. (D). The Dot plots shown here is quantifications of (C). Red (▲) represents p21, blue (■) represents IL-6, and black (●) represents p27. The protein levels were normalized to β-actin. Data are present as the means ±SEM,* $p < 0.05$. Two-way parametric tests were used.

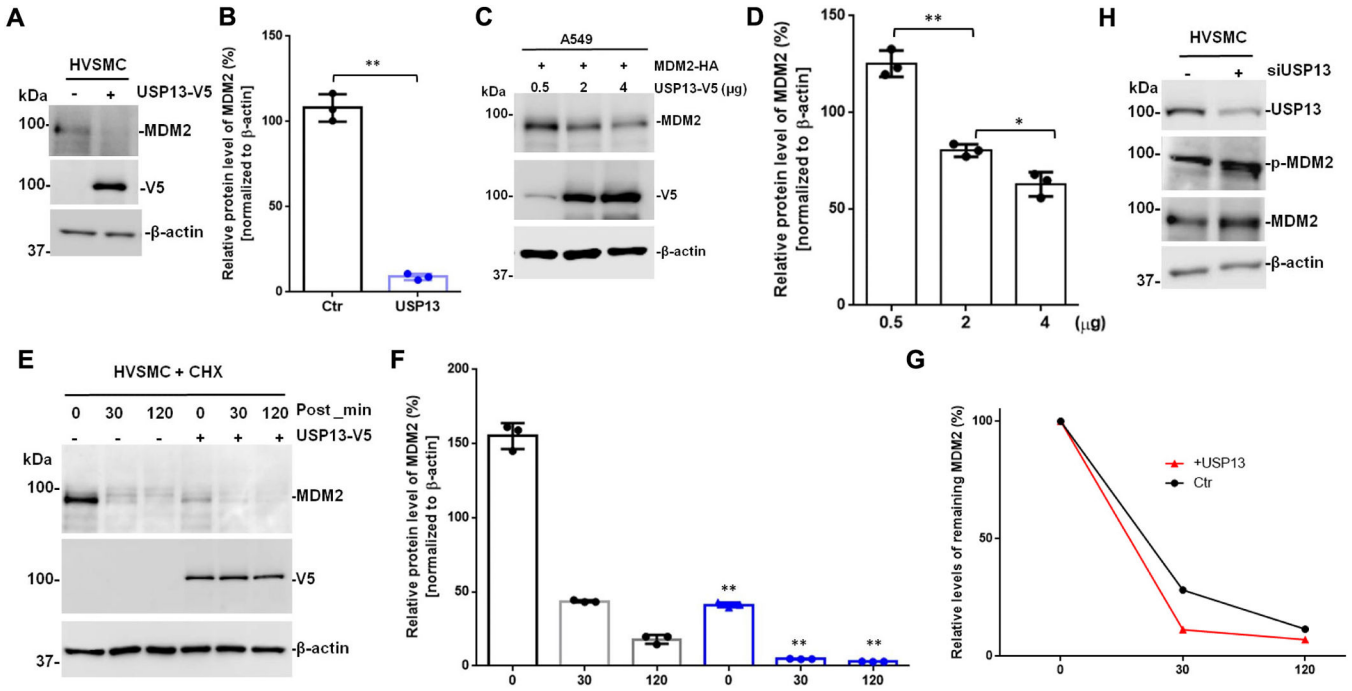

Fig. 3.

Overexpression of USP13 induces MDM2 degradation. The HVSMC cells were transfected with V5-tagged USP13 plasmid and the cells were collected (A). The A549 cells were co-transfected with the plasmids for expressing MDM2 (HA-MDM2, 1 μg) and USP13 (USP13-V5, 0.5, 2, and 4 μg) (C). The protein levels of cell extract were detected by Western blotting with anti-V5, MDM2, and β-actin specific antibodies. Western blot shown here is representative of 3 independent experiments. The blot of β-actin served as loading control. The Dot plots shown in (B) and (D) are quantifications of (A) and (C) respectively. The protein levels were normalized to β-actin. Data are present as the means ±SEM,** $p < 0.01$, * $p < 0.05$. (E). The HVSMC cells were transfected with V5 tagged USP13 plasmid (or control vector plasmid) and the cells were harvested after cycloheximide (CHX) treatment at indicated times. The protein levels of cell lysates were detected by Western blotting with anti-V5, MDM2, and β-actin specific antibodies. The shown in (F) and (G) are quantifications of (E). Data are present as the means ± SD,** $p < 0.01$. Two-way parametric tests were used. (H). The HVSMC cells were transfected with siRNA of USP13 (or control siRNA) and the cells were collected. The protein levels of cell extracts were identified by Western blotting with anti-USP13, p-MDM2, MDM2, and β-actin specific antibodies.

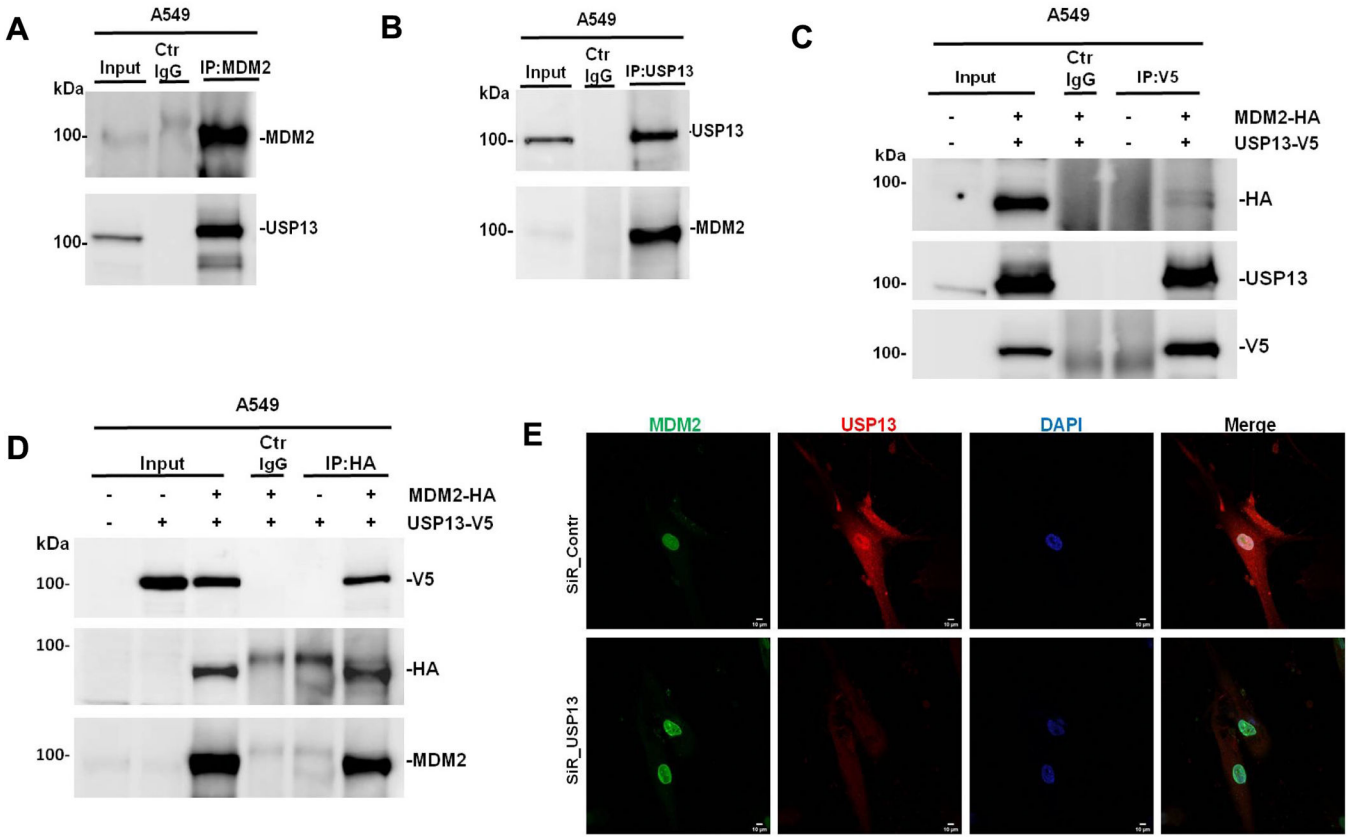

Fig. 4.

USP13 is associated with MDM2 in the nuclei. (A), (B). The cell lysates of A549 were immunoprecipitated (IP) with anti-MDM2 (or anti-USP13) antibody, and examined by Western blotting with anti-USP13 (or anti-MDM2) antibody. (C, D). The A549 cells were co-transfected with plasmids expressing HA-MDM2 or USP13-V5 as indicated. The cell lysates were immunoprecipitated with anti-V5 (or anti-HA) antibody, and the Co-immunoprecipitation (Co-IP) was examined by Western Blot with both anti-HA and anti-V5 antibodies. (E). Co-localization of USP13 and MDM2 was identified by Immunofluorescence Microscopy analysis. HVSMC cells were transfected with USP13 SiRNA (or control SiRNA) and the cells were immunostained with anti-USP13 and anti-MDM2 antibodies followed by Alexa-546 conjugated (red, USP13) and Alexa-488 conjugated (green, MDM2) antibodies. Scale bar is 10 μm.

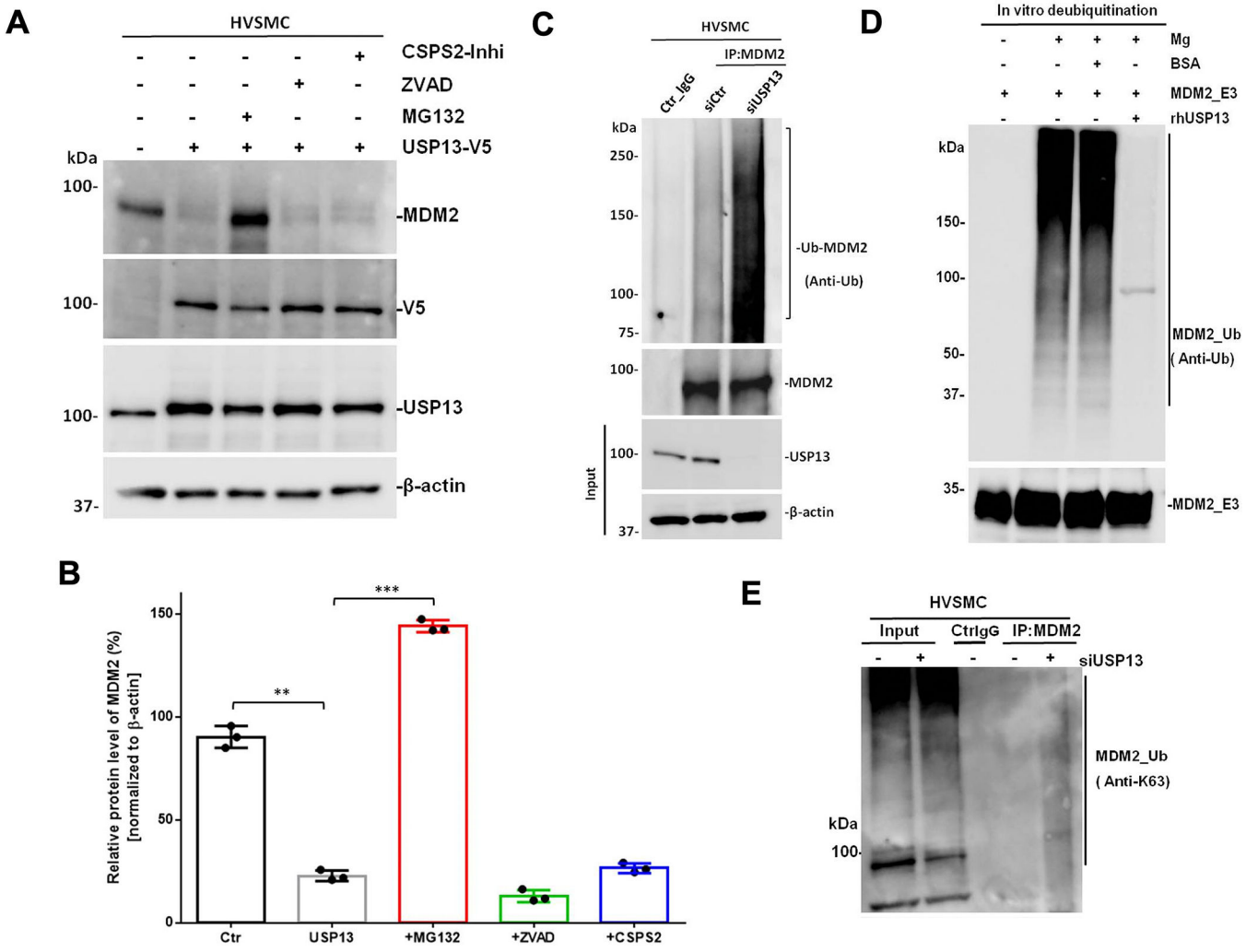

Fig. 5.

USP13 reverses K63-linked polyubiquitination of MDM2. (A). The HVSMC cells were transfected with USP13-V5 plasmid (or control vector plasmid), and then cells were treated with Caspase 2 inhibitor (CSPS2-Inhi), ZVAD, or MG132. The cell lysates were subjected to use for Western blotting analysis with anti-V5, MDM2, USP13, and β-actin specific antibodies. (B). The Dot plots shown here is quantifications of (A). Red represents cells were treated with MG132, Green represents cells were treated with ZVAD, and blue represents cells were treated with CSPS2-Inhi. The levels of proteins were normalized by β-actin. Data are present as the means ± SD,** $p < 0.01$, *** $p < 0.001$. Two-way parametric tests were used. (C). HVSMC cells were transfected with siUSP13 (or control siRNA), and after 72 h incubation the cells were harvested in RIPA buffer and IP was performed with anti-MDM2 antibodies. The ubiquitination of MDM2 were detected by anti-ubiquitin and anti-MDM2 antibodies. Western blotting analysis of cell lysates with anti-USP13 antibody was performed to confirm the expression levels of USP13. Western blotting of anti-β-actin was performed as a loading control. (D). *In vitro* deubiquitination assay was performed using the purified human recombinant protein USP13 (rhUSP13) and the MDM2 autoubiquitination kit. The reaction mixture solution containing E3 active center

of MDM2 (MDM2_E3), magnesium ions (Mg) and rhUSP13 was incubated at 37 °C for 1 h. Regarding the positive control, the reaction mixture did not contain magnesium ions (Mg), and as a negative control, BSA was used instead of rhUSP13. After the incubation, the reaction mixtures were analyzed by Western blotting with anti-ubiquitin and anti-MDM2 antibodies. (E). The HVSMC cells were transfected with siUSP13 (or control siRNA), and 72 h later the cells were harvested and IP was performed with anti-MDM2 antibody. The Western blotting analysis was performed with anti-K63 ubiquitin antibody. The normal IgG (CtrIgG) was serving as a control for IP.

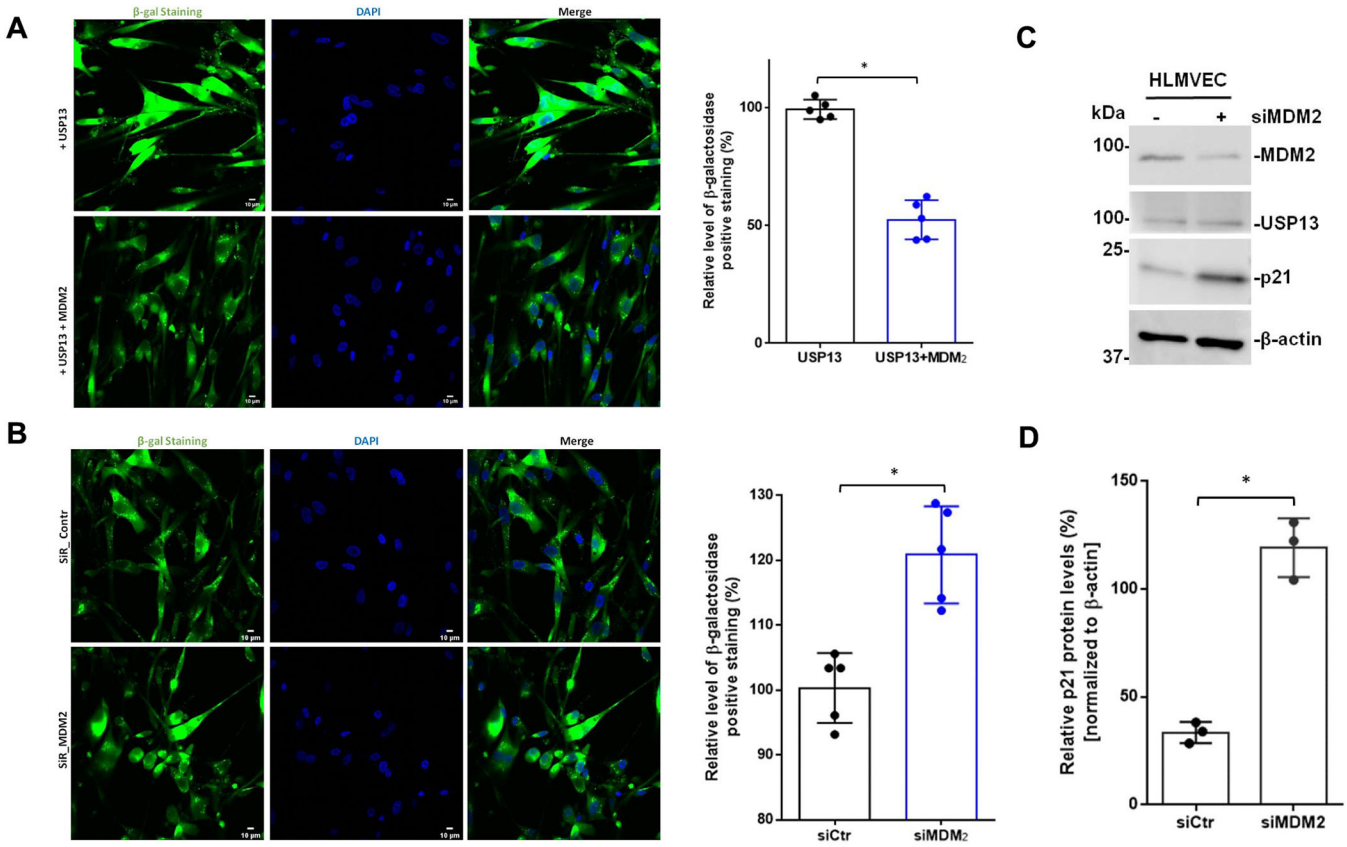

Fig. 6.

Overexpression of MDM2 attenuates the effect of USP13 on cell senescence. (A). HVSMC cells were co-transfected with Flag tagged USP13 and HA-tagged MDM2 plasmid. The control group cells were transfected with Flag tagged USP13 plasmid only. The cells were then stained with β-Galactosidase Staining Kit and the cell nucleus was stained with DAPI (blue). The green stands for senescent cells. (B). The HVSMC cells were transfected with siMDM2 (or control siRNA), and 48 h later the cells were then stained with β-Galactosidase Staining Kit and cell nucleus was stained with DAPI (blue). The green indicates for senescent cells. (C). HVSMC cells were transfected with siMDM2 (or control siRNA), and 72 h later the cells were harvested and Western Blotting was performed with anti-MDM2, anti-USP13, anti-p21 and anti-β-actin antibodies. The β-actin was serving as a loading control. (D). The Dot plots shown here is quantifications of (C). The protein levels of p21 were normalized to β-actin. Data are present as the means ± SD, * $p < 0.05$. Two-way parametric tests were used.