Serine protease inhibitor SerpinB2 binds and stabilizes p21 in senescent cells

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Key words: senescence, SerpinB2, p21, proteasome

JCS Advance Online Article. Posted on 9 August 2017

Summary Statement

SerpinB2 is known as an inhibitor of uPA and tPA. Here we found SerpinB2 is a downstream target of p53 and has a role in senescence through stabilizing p21 level.

Abstract

SerpinB2 is a serine protease inhibitor that is also known as plasminogen activator inhibitor type 2 (PAI-2). It has been well documented that SerpinB2 is an inhibitor of urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA). Interestingly, SerpinB2 level is increased in senescent cells and is considered a senescence biomarker. Through mimicking the elevated level of SerpinB2 in senescent cells, this study showed the proliferating human fibroblasts were induced into senescence. Senescence induced by SerpinB2 did not relate to its extracellular function as inhibition of SerpinB2 secretion, exogenous introduced SerpinB2, or a SerpinB2 mutant that failed to bind to its extracellular target uPA, did not have an effect on senescence. This study also showed SerpinB2 is a direct downstream target of p53 that is activated through the DNA damage response pathway. Significantly, SerpinB2 bound to and stabilized p21 to mediate senescence in a proteasome-independent manner, indicating SerpinB2 has a direct role in senescence. Thus, this study reveals a unique mechanism that SerpinB2 maintains senescence through stabilization of the p21 protein level.

Introduction

Normal human diploid fibroblasts have a finite proliferative capacity in the laboratory. After ~60-80 population doublings (PDs), they enter the terminally non-dividing state termed replicative senescence or cellular senescence (Hayflick, 1965; Hayflick and Moorhead, 1961). Senescent cells cease proliferation and cannot initiate DNA synthesis despite adequate growth conditions. Senescent cells are generally larger, less motile, and decreased saturation density. They can be identified by increased expression of senescence-associated β-galactosidase (SA-β-gal) (Dimri et al., 1995). In addition to this specific microscopic phenotype, alteration of gene expression, microRNA expression, and protein secretion are also characterized in senescent cells (Acosta et al., 2008; Campisi and d'Adda di Fagagna, 2007; Chen et al., 2004; Grillari and Grillari-Voglauer, 2010; Kuilman et al., 2008; Kuilman and Peeper, 2009; Zhang et al., 2003). It is now clear cellular senescence is caused by telomere shortenings after repeated cycles of cell divisions (Allsopp et al., 1995; Bodnar et al., 1998). Senescence can also be induced by different cellular stressors and is considered a state of cell cycle arrest in response to various types of stresses (Campisi and d'Adda di Fagagna, 2007).

Senescence is executed mainly by a DNA damage response pathway. It is mediated through either of the two important cell cycle inhibitors, p21 (also termed CDKN1a, p21Cip1, Waf1 or SDI1) or p16 (also termed CDKN2a or p16INK4a) (Brown et al., 1997; McConnell et al., 1998). Both pathways can establish and maintain the growth arrest through maintaining retinoblastoma protein (RB) in a hypophosphorylated and active state. In normal human fibroblasts, the signal that induces senescence by telomere erosion is primarily driven through the tumor suppressor protein p53 (d'Adda di Fagagna, 2008). p53 is a transcriptional regulator activated by DNA damages. It mediates cell growth arrest through the transactivation of various cell cycle regulatory target genes (Zuckerman et al., 2009). p21 is among many growth inhibitory genes induced by p53. p21 binds to and inhibits the kinase

activity of the cyclin-dependent kinases (CDKs) to arrest cells at specific stages of the cell cycle (Abbas and Dutta, 2009).

The level of SerpinB2, also known as plasminogen activator inhibitor type 2 (PAI-2), was known to be elevated in senescent human skin fibroblasts (Chen et al., 2004; Kumar et al., 1992; West et al., 1996; Zhang et al., 2003). SerpinB2 is a member of the clade B serine protease inhibitor subgroup of the serpin superfamily (Law et al., 2006). A notable function of SerpinB2 is related to its role as an inhibitor of urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA) (Kruithof et al., 1986; Thorsen et al., 1988). It acts as a suicide substrate to form a covalent SerpinB2-uPA or SerpinB2-tPA complex (Kruithof et al., 1995). However, SerpinB2 lacks a classical secretory signal peptide and is considered an intracellular serpin. It is inefficiently secreted with the majority of the protein retained intracellularly, having nucleocytoplasmic distributions (Kruithof et al., 1995). The intracellular role of SerpinB2 is poorly understood. Here, we identify a unique role of SerpinB2 in senescence where it forms a complex with p21 to stabilize it and maintain senescence.

Results

SerpinB2 induces cellular senescence in normal human fibroblasts

Both the mRNA (Chen et al., 2004; Zhang et al., 2003) and protein (Kumar et al., 1992; West et al., 1996) levels of SerpinB2 were shown to be elevated in senescent fibroblasts. To test the role of SerpinB2 in cellular senescence, SerpinB2 was ectopically expressed in the proliferating (young) normal human diploid fibroblast line IMR90 to mimic the expression level of SerpinB2 in senescent cells (Fig. 1A). The growth of young cells was inhibited when SerpinB2 was expressed, suggesting SerpinB2 expression induced growth arrest in these cells (Fig. 1B). It is also accompanied by decreased BrdU incorporation (Fig. 1C).

The senescent phenotypes were analyzed next. The SerpinB2-expressing cells showed increased levels of SA-β-gal activities, a well-recognized biomarker for senescence (Fig. 1D). The senescent cells also harbor a signature heterochromatin foci formation (Narita et al., 2003) that can be marked by elevated H3K9me3 (a senescence-associated heterochromatin marker; Fig. 1E). Moreover, the senescent cells upregulate enzymes that degrade the extracellular matrix, and secret immune modulators and inflammatory cytokines to reinforce senescence in an autocrine and paracrine manner. The senescence-associated secretory phenotype (SASP) was reported to heighten inflammation through recruitment of inflammatory cells, and has detrimental effects on the tissue microenvironment by alterations of tissue composition and architecture (Sharpless and Sherr, 2015). The cellular senescence induced by SerpinB2 also induced several signature SASP components, including IL6, IL8, CXCR2, WNT2, and IL1b (Fig. 1F). The results suggest an elevated level of SerpinB2 is sufficient to induce a secretory phenotype similar to senescence.

To gain insight into the molecular basis of SerpinB2-mediated growth arrest and senescence in normal fibroblasts, the expression pattern of several key cell cycle regulators involved in senescence were evaluated using immunoblotting analysis. Expressing SerpinB2 increased p53 and p21 expressions and inhibited Rb protein expression and phosphorylation (Fig. 1G). Although p16 has been implicated as key mediators in senescence, its level is not significantly altered in IMR90 cells. These results suggest the p53/p21/Rb senescent pathway was activated in response to SerpinB2 overexpression in normal cells.

To test if SerpinB2 is required for senescence, the SerpinB2 level was reduced by RNA interference using shRNA (Fig. 1H) and then analyzed for the SA-β-gal activities (Fig. 1I). The analysis showed reduced SerpinB2 greatly decreased the number of senescent cells, suggesting SerpinB2 is required for senescence formation. It is well documented that telomere shortening is the main cause of replicative senescence. To show that the observed

SerpinB2 function was not due to its effect on telomere length, SerpinB2 were expressed in a telomerase-positive human normal fibroblast BJ-hTERT (Fig. S1) and lung cancer cell H1299 (Fig. S2). The results showed that SerpinB2 induced senescence in both BJ-hTERT and H1299 cells, suggesting that SerpinB2 did not induce senescence through affecting telomere length or telomerase activity.

Senescence induced by SerpinB2 is not related to its extracellular function

Since SerpinB2 is well documented for its binding and inhibiting uPA and tPA (Kruithof et al., 1986; Thorsen et al., 1988), the role of extracellular SerpinB2 in senescence was investigated. The senescence phenotype was first determined in cells treated with Brefeldin A to inhibit the secretion of SerpinB2 (Fig. 2A). The growth inhibitory effect of SerpinB2 did not appear to be affected by Brefeldin A. The result suggested inhibition of SerpinB2 secretion did not affect growth inhibition.

The effect of exogenous SerpinB2 on senescence is then evaluated. SerpinB2 is not usually detectable in human plasma, except during pregnancy where the concentration increases to 260 ng/mL at term (Kruithof et al., 1987). SerpinB2 can also be detected in other human bodily fluids, including gingival fluid, saliva, peritoneal fluid, and infectious pleural effusions (Croucher et al., 2008). For example, the SerpinB2 level in the dentogingival region of patients with Papillon–Lefevre syndrome (PLS) can reach 1.38 μg/mL (Ullbro et al., 2004). Here, recombinant SerpinB2 was expressed and isolated from *E. coli* (Fig. 2B). The isolated recombinant protein was used to treat IMR90 cells at concentrations (0.5 and 1.5 μg/mL) that mimicked the patho- or physiological concentrations of SerpinB2. Under the tested conditions, the addition of SerpinB2 did not cause apparent cell growth or senescence in IMR90 cells (Fig. 2C-2D). To validate the function of the recombinant SerpinB2, an *in vitro* co-pulldown analysis was conducted. The recombinant SerpinB2 was capable of pulling down uPA in a concentration dependent manner, suggesting that the isolated SerpinB2

protein is still functional (Fig. 2E). Of note, the SerpinB2 protein level in the culture medium of senescent IMR90 cells is <30 ng/mL.

SerpinB2 contains a reactive site near the carboxyl terminus that acts as a pseudosubstrate for its cognate proteinase. It was reported the Arg380 is the reaction site of SerpinB2 where its mutation (R380A) disrupted the uPA binding activity (Dickinson et al., 1995; Fish and Kruithof, 2006). To test if binding to uPA is required for SerpinB2-mediated senescence, the R380A mutant was generated and introduced into IMR90 cells (Fig. 2F). Both cell growth and SA-β-gal analyses showed the R380A mutation did not affect the senescence mediated by SerpinB2 (Fig. 2G-2H), suggesting interaction between SerpinB2 and uPA is not required for senescence mediated by SerpinB2. Together, these results suggest senescence induced by SerpinB2 is not due to its extracellular function.

SerpinB2 is a direct downstream target of p53

The mechanism of SerpinB2 expression in senescent cells is investigated next. Since DNA damage response induced by telomere erosion is the causal factor for cellular senescence, the dependence of p53 on SerpinB2 expression is determined. p21 is a direct downstream target of p53 and its level can be induced by DNA damage agent doxorubicin in a p53 dependent manner (Figure 3A, right panel). Both the mRNA and protein levels of SerpinB2 were induced by doxorubicin (Figure 3A and 3B). Similar to that observed in p21, the elevated SerpinB2 levels can be reduced when p53 was decreased using shRNA. Thus, SerpinB2 expression is upregulated by p53 during senescence. To show that the expression of SerpinB2 is mediated through activating SerpinB2 promoter, the SerpinB2 promoter was also fused to a secreted alkaline phosphatase (SEAP) gene for reporter analysis (Fig. 3C). The expression of SEAP was used as the criterion for the measurement of SerpinB2 expression efficiency in the presence of DNA damage-inducing agent doxorubicin. Upon treatment with doxorubicin, the expressions of SEAP from the SerpinB2 promoter were increased (Fig. 3D). As a comparison, the expression efficiency of CMV promoter did not respond to doxorubicin

treatments. Sequence analysis identified seven p53-binding consensus sequences near the transcriptional start site of SerpinB2 (Fig. 3D). To show p53 is bound to the promoter region of SerpinB2, chromatin immunoprecipitation analyses were conducted. The SerpinB2 promoter was effectively precipitated by p53 in senescent cells, but not in young cells (Fig. 3D). As a comparison, p53 also bound to the p21 promoter in senescent cells.

It is also interesting to note that the p53 level is activated by SerpinB2 expression (Fig. 1G). The results suggested SerpinB2 might have a positive regulatory role for p53. The mechanism of how SerpinB2 regulates p53 level is not clear. It is possible that SerpinB2 might stabilize p53 level in senescent cells since SerpinB2 was reported to prevent degradation of p53 in endothelial cells (Boncela et al., 2011). Thus, p53 and SerpinB2 might form a positive feedback loop to maintain cells in senescent state.

The expression of SerpinB2 was also tested under various stress conditions that induce p53, including UV irradiation, hydrogen peroxide exposure, and Cisplatin CDDP. All these stress conditions were shown to activate p53 and induce senescence in human normal fibroblasts (Chen and Ames, 1994; Chen et al., 1998; Debacq-Chainiaux et al., 2005; Zhao et al., 2004). Under similar treatment conditions, SerpinB2 expressions were efficiently induced by these stresses (Fig. 3E). The results suggest SerpinB2 is a direct downstream target of p53 and can be induced by various types of stresses.

p21 is required for SerpinB2-mediated senescence

The requirement for p53 and p21 as part of the SerpinB2-mediated senescence was investigated. The IMR90 cells were first transduced with shRNAs targeting p53 or p21 and then introduced with SerpinB2. As shown in Fig. 4A, the level of p53 was decreased upon shRNA treatments. In these p53 knockdown cells, the cell numbers decreased and the SA-β-gal-positive cells increased (Fig. 4B-4C). On the contrary, the growth of SerpinB2-expressing cells increased and the numbers of SA-β-gal-positive cells significantly decreased upon knocking down p21 expression (Fig. 4D-4F), indicating reduced p21

expression could restore SerpinB2-mediated senescence. Thus, p21 is required for SerpinB2-mediated senescence in normal cells.

In an independent experiment, isogenic colorectal cancer cell lines, HCT116 and its p53 and p21 knock-outs, were applied in this study (Bunz et al., 1998). Upon SerpinB2 overexpression, the HCT116 cells ceased to proliferate (Fig. S3). The proliferation was recovered in HCT116 cells carrying $p21^{-/-}$, but not $p53^{-/-}$ mutation. The senescent phenotype was also analyzed (Fig. S3). Elevated senescent cells were observed in HCT116 cells expressing SerpinB2. The elevated senescent cells were not observed in HCT116 cells carrying $p21^{-/-}$ mutation, indicating SerpinB2-mediated senescence is p21-dependent. Together, our results indicated p21 is required for senescence mediated by SerpinB2 in both normal and cancer cells.

Proteasome-independent stabilization of p21 by SerpinB2.

The mechanism of how p21 is involved in SerpinB2-mediated senescence was then analyzed. Although transcriptional regulation is considered the initial control point for p21 expression, post-transcriptional and post-translational regulations also play a critical role in p21 expression and activity. The accumulation of p21 could be caused by elevated p21 expression and/or reduced p21 degradation. We found expressing SerpinB2 caused a boost in p21 expression (Fig. S4). The observed p21 elevation is likely due to p53 activation (Fig. 1F). The elevated p21 level subsided at ~3 days. Since senescence induced by SerpinB2 could last for as many as 30 days, a transient elevation of p21 expression is not sufficient to maintain senescence. Thus, additional mechanism is involved for SerpinB2 to maintain p21 level.

SerpinB2 appeared to directly impact the p21 level as decreased SerpinB2 also caused a reduced level of p21 (Fig. S5). The interaction between p21 and SerpinB2 was first tested. Co-immunoprecipitation experiments showed SerpinB2 formed a complex with p21 in cells that were either overexpressing SerpinB2 or senescent (Fig. 5A). Interestingly, p21 immunoprecipitated by SerpinB2 appeared to have slow mobility. To show that the

mobility-shifted bands were indeed p21, the shifted bands were excised and subjected to analysis by mass spectrometry. The mass analysis readily detected p21 peptides, indicating that p21 is indeed co-immunoprecipitated by SerpinB2. Thus, although the nature of p21 band shift is not clear, SerpinB2 preferentially interacted with the mobility-shifted p21. The role of SerpinB2 on the stability of p21 protein was then analyzed. p21 protein is short lived, it has a half-life less than 30 min (Fig. 5B). SerpinB2 greatly extended the half-life of p21 to ~160 min. To determine if the proteasome-dependent pathway is involved in stabilization of p21, proteasome inhibitor MG132 was introduced in this study. As shown in Fig. 5C, MG132-treated cells showed higher p21 accumulations in both cells. The results suggest SerpinB2 might stabilize p21 through a pathway that is independent of proteasome. Indeed, MG132 treatments further extended the half-life of p21 to >240 min in SerpinB2 expressing cells (Fig. 5D). The observed phenomenon is not limited to ectopically expressed SerpinB2 because the p21 level was also stabilized by SerpinB2 in doxorubicin-treated cells (Fig. 5E). The results suggest SerpinB2 has a general role in binding and stabilizing p21 in maintaining senescence. It is also interesting to note that the p53 level was slightly reduced in cells with reduced SerpinB2 level, suggesting a positive role of SerpinB2 on p53.

Discussion

The SerpinB2 level is known to increase in senescent cells (Chen et al., 2004; Kumar et al., 1992; West et al., 1996; Zhang et al., 2003). It is considered a senescent marker with no known function related to senescence. This study showed SerpinB2 is a direct target of p53 induced in senescent cells through a DNA damage response pathway (Fig. 5F). SerpinB2 then binds and stabilizes p21 level in senescent cells. Thus, SerpinB2 has a critical role in senescence. In addition to senescence, SerpinB2 level was also found to be elevated by different inducers in various cell types. For example, SerpinB2 level was found to increase several hundred fold within hours of exposing hippocampal neurons to bicuculline, a $GABA_A$ receptor antagonist (Zhang et al., 2009). The elevated SerpinB2 appears to promote survival of cultured hippocampal neurons and protect against seizure-induced neuronal death by kainic acid. SerpinB2 was also shown to be up-regulated in monocyte/macrophages following infection or stimulation with inflammatory mediators (Schroder et al., 2010). Moreover, a recent report showed FOXO1 can enhance the expression of SerpinB2 under high glucose condition to inhibit keratinocyte migration. Further analysis showed the reduced keratinocyte migration can be rescued by reducing SerpinB2 (Zhang et al., 2015). Together these observations suggest SerpinB2 might be considered a common stress response protein in response to stresses. It can be induced to help cells accommodate stresses. Consistent with this notion, a notable function of SerpinB2 is related to its anti-apoptotic function. It was reported SerpinB2 maintains survival of TNF-stimulated cells from apoptosis through interacting with transglutaminase (TGM2) to inactivate procaspase-3 (Delhase et al., 2012). A similar mechanism might be involved for SerpinB2 to protect cells from apoptosis. The binding and stabilization of p21 by SerpinB2 might stop cells from un-checked proliferation, providing an opportunity for cells to adjust to various types of stresses.

This study showed the role of SerpinB2 in senescence is mediated through an intracellular pathway through stabilization of the p21 protein. The result is consistent with the

nucleocytoplasmic distributions of SerpinB2 and its function as an intracellular serpin (Kruithof et al., 1995). This study also provided evidence the function of SerpinB2 in senescence is not likely to be mediated through its role as an inhibitor of urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA) (Kruithof et al., 1986; Thorsen et al., 1988). It is also interesting to note the plasminogen activator inhibitor type 1 (PAI-1) is a direct target of p53 (Kunz et al., 1995). PAI-1 is an extracellular serpin and is known to inhibit the activity of the secreted protease uPA (urokinase plasminogen activator) by forming a stable complex. It has a direct role in senescence through inhibiting the PI(3)K, PKB, and GSK3β signaling pathway both in *vitro* and *in vivo* (Eren et al., 2014; Kortlever et al., 2006). Although these two serpins, SerpinB2 and PAI-1, share several similar functions, they contribute to senescence through mechanistically distinct pathways.

p21 binds and inhibits CDK activities, and it needs to be efficiently removed from the CDKs for the cells to facilitate proper cell cycle progression (Brown et al., 1997; El-Deiry et al., 1993; Harper et al., 1993; Stein et al., 1999; Xiong et al., 1993). Removal of p21 from CDKs is generally achieved by rapid degradation of p21. Indeed, p21 is a relatively short-lived protein, it has a half-life of ~30 min. Degradation of p21 is mediated mainly through ubiquitin dependent proteasome pathways (Bendjennat et al., 2003; Bloom et al., 2003). In this pathway, proteolysis of p21 is mediated by E3 ubiquitin ligases, including SCFSKP2, CRL4CDT2, APC/C^{CDC20}, ZNF313, and RNF115/BCA2 (Amador et al., 2007; Bornstein et al., 2003; Han et al., 2013; Wang et al., 2005; Wang et al., 2013). p21 can also be targeted directly to the proteasome for degradation without ubiquitylation. This is evident from the analysis of a p21 mutant with all of the lysines having been mutated to arginines. This p21 mutant protein degraded at the same rate as p21 *in vivo* (Sheaff et al., 2000). p21 is targeted directly to proteasome through binding to the proteasomal C8 subunit. It is also reported the MDM2 oncogene has a role in ubiquitin independent degradation through promoting the binding of p21 with the C8 subunit (Jin et al., 2003; Zhang et al., 2004). It

facilitates p21 degradation independent of ubiquitination and the E3 ligase function of MDM2. This study showed SerpinB2 does not affect proteasome degradation of p21 in senescent cells. The mechanism of how SerpinB2 stabilizes p21 is unclear. A simple explanation would be binding of SerpinB2 prevents p21 from proteasome degradation. However, since SerpinB2 is considered a protease inhibitor, it is also possible the activity of a specific p21-degrading protease is inhibited by SerpinB2. Alternatively, since this study showed SerpinB2 interacts with an unidentified version of modified p21 (Fig. 5A), it is possible SerpinB2 induces such a p21 modification to stabilize it. Nevertheless, this study reveals a unique mechanism to maintain p21 stability in senescent cells.

Materials and Methods

Cell lines and cell culture The human normal lung fibroblast IMR90 was purchased from the Food Industry Research and Development Institute (Taiwan) and maintained in minimum essential medium (MEM, Gibco Thermo Fisher Scientific Inc.) containing 10% fetal bovine serum (FBS). Both proliferation rate and senescence-associated β-galactosidase activity (SA-β-gal) were used to evaluate the senescent status of IMR90. The IMR90 cells could be cultured for additional ~30 passages before showing sign of senescence. In this study, cells with passages less than 30 were considered "young" cells. The percentage of SA-β-gal positive cells were less than 10%. Cells with passage numbers passed 30 were considered "senescent" cells. They have over 30% SA-β-gal positive cells. The human immortalized foreskin fibroblast BJ-hTERT, AD293, 293T, and human lung adenocarcinoma A549 were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco Thermo Fisher Scientific Inc.) with 10% FBS. The colorectal cancer HCT116 and its isogenic $p53^{-/-}$ and $p21^{-/-}$ cells were maintained in McCoy's 5A medium (Sigma-Aldrich Co.) with 10% FBS. The H1299 was maintained in RPMI Medium 1640 (Gibco Thermo Fisher Scientific Inc.) containing 10% FBS.

Growth Curves The proliferative capacity of cells was monitored by seeding $5x10^5$ cells into a 30-mm dish containing 10% fetal bovine serum. Adenovirus carrying LacZ or SerpinB2 was added the next day. Cell numbers were determined by digesting the cells with trypsin, stained with 0.2% trypan blue, and counted using a hemocytometer.

SA-β-galactosidase staining Cells were washed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and fixed for 5 minutes in 2% formaldehyde and 0.4% glutaraldehyde. The fixed cells were then incubated with staining solution (40 mM citrate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$ and sodium phosphate, pH 6.0) containing 1 mg/mL 5-bromo-4-chloro-3-indolyl -D-galactoside (X-Gal) at 37˚C for 16-24 hours. At least 250 cells were counted in randomly chosen fields for each sample.

Immunofluorescence assay The cells were grown on slides and fixed with 4% formaldehyde at room temperature for 10 minutes. The fixed cells were then permeabilized with blocking buffer (Tris-buffered saline with 1% bovine serum albumin and 0.1% Triton X-100). Primary antibody, anti-BrdU (cat #NA61, Calbiochem), or anti-H3K9me3 (07-442, EMD Millipore) antibody was added and incubated at 4˚C overnight. The cells were then washed and incubated with secondary antibody, FITC-conjugated anti-rabbit (Jackson ImmunoResearch) or Rhodamine-conjugated anti-mouse (Jackson ImmunoResearch) antibody for 1 hour. DAPI staining was conducted using the mounting solution (Prolong® Gold antifade reagent with DAPI). The cells were visualized using fluorescence or confocal microscopy.

Immunoblotting analysis Cells were lysed in RIPA buffer (Tris pH 7.4 50 mM, NP-40 1%, Na-deoxycholate 0.5%, SDS 0.1%, NaCl 150 mM) containing 1 mM PMSF and protease inhibitor cocktail (539134, Calbiochem) and the proteins were separated by 8%, 10%, 12% or 15% SDS-PAGE. The proteins were transferred onto nitrocellulose membranes and probed with anti-SerpinB2 (polyclonal rabbit antibodies generated from recombinant SerpinB2),

anti-p53 (OP43, Calbiochem), anti-p21 (#2947, Cell Signaling), anti-p16 (NA29, Calbiochem), anti-Rb (OP66, Calbiochem), anti-phospho-Rb (Ser780) (9307, Cell Signaling), anti-GAPDH (10494-1-AP, Proteintech), or anti-Actin antibodies (MAB1501, Merck Millipore). Bound antibodies were visualized by chemiluminescence using an ECL kit (Amersham Biosciences).

Quantitative real-time polymerase chain reaction The total amount of RNA was extracted by TriPure Isolation Reagent (Roche). First-strand cDNA was synthesized from 0.5 μg of total RNA with RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). Real-time PCR was then performed using SYBR Green Master Mix (Roche) and StepOne Real-Time PCR System (Applied Biosystems). The primer pairs for p21 are 5'-TCAGAGGAGGTGAGAGAGCGG-3' and 5'-CGCATGGGTTCTGACGGACA-3'. Primer pairs for GAPDH are 5'-GAAGGTGAAGGTCGGAGTCAA-3' and 5'-CGTTCTCAGCCTTGACGGT-3'.

Purification of recombinant SerpinB2 proteins The full-length SerpinB2 cDNA was amplified by PCR and cloned into pET6H plasmid (Lin et al., 2001) to generate plasmid pET6H-SerpinB2. To purify 6-His tagged SerpinB2 protein, a 500 mL culture of *E. coli* BL21 (DE3) harboring pET6H-SerpinB2 was grown at 37˚C until the OD_{600} reaches 0.4 and induced with the addition of 1 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside). The cells were grown at 25˚C for another 16 hours before harvest by centrifugation. Cells were resuspended in 10 ml of sonication buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.1 mM PMSF) and sonicated to release the cell contents. The sonicated cells were centrifuged at 9,000 *g* for 20 min at 4°C to obtain total cell free extracts. 0.5 ml of Ni-NTA-agarose (ABT) was added to the total cell free extracts and incubated at 4°C for 16 hours. The resin was serially washed with sonication buffer containing 20 mM and 30 mM imidazole and eluted with 2 ml of sonication buffer containing 200 mM imidazole and 20% glycerol. Purified protein was aliquoted and frozen by the dry ice-ethanol bath.

Chromatin immunoprecipitation IMR90 cells were incubated with 0.75% formaldehyde for 10 minutes. The cross-linking reactions were then terminated by adding 125 mM glycine. The treated cells were lysed with FA lysis buffer (100 mM HEPES, 300 mM NaCl, 2 mM EDTA, 2% Triton X-100, 0.2% sodium deoxycholate, 0.1% SDS) and the lysates were sonicated. Supernatants containing chromatins were incubated with anti-p53 antibodies for one hour, protein-G beads overnight, and then washed three times with low-salt buffer containing 20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 0.1% SDS, followed by washing with high-salt buffer containing 20 mM Tris-HCl, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 0.1% SDS. The beads were resuspended in elution buffer containing 100 mM $NaHCO_3$ and 1% SDS and then incubated at 30˚C for 15 minutes with shaking. The supernatants were incubated with RNase A (0.5 mg/mL) at 65˚C for 4 hours and purified by PCR clean kit (Geneaid). The eluates were then analyzed by quantitative real-time PCR. The primer pairs used were: p21 proximal promoter (5'-GAGGTCAGCTGCGTTAGAGG-3' and 5'-TGCAGAGGATGGATTGTTCA-3'), p21 distal promoter (5'-CCTATGCTGCCTGCTTCCCAGGAA-3' and 5'-TAGCCACCAGCCTCTTCTATGCCAG-3') (Ishikawa et al., 2007); SerpinB2 promoter region (5'-TCTTGAAACTGGGGCTGACA-3' and 5'-CCTCTGTCTTTTGATCTGTGTCC-3'), SerpinB2 C-terminal region (5'-ATGGTCCTGGTGAATGCTGT-3' and 5'-TAGCAAGTCTATAATGAGCGGTCT-3').

Reporter analysis Secreted alkaline phosphatase (SEAP) was used as the reporter system to monitor the transcriptional activity of SerpinB2. The SerpinB2 promoter ranging from -663 to +1 relative to the transcription starting site was PCR-amplified from IMR90 genomic DNA and cloned upstream to a SEAP reporter gene to generate $P_{SerpinB2}$-SEAP. The CMV promoter was also subcloned as a control, P_{CMV}-SEAP. The resulting mutations were verified by DNA sequencing of the plasmids. In the reporter assays, $5x10^5$ cells were transfected with P_{CMV}-SEAP and $P_{SerpinB2}$-SEAP, respectively, and $1x10^4$ each of the transfected cells were

grown in 96-well plates and incubated at 37°C for 24 hours. Varying amounts of doxoribicin were added, and cells were incubated for another 24 hours. Culture media were collected and heated at 65°C for 10 min to inactivate heat-labile phosphatases. An equal amount of SEAP buffer (2 M diethanolamine, 1 mM $MgCl_2$, and 20 mM L-homoarginine) was added to the media, and *p*-nitrophenylphosphate was added to a final concentration of 12 mM. Absorptions at 405 nm were taken, and the rate of absorption increase was determined.

Statistical analysis Student's T test was applied to assess whether the means of two groups are statistically different from each other. We consider $p<0.05$ as significant.

Acknowledgements

We thank Dr. Shu-Chun Teng for critical comments on the manuscript.

Competing interests

No competing interests declared.

Funding

This work was supported by the National Taiwan University Aim for Top University Project under grant 104R8955-2 and the Ministry of Science and Technology under grants MOST104-2311-B-002-028-MY3 and MOST104-2321-B-002-036.

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Figures

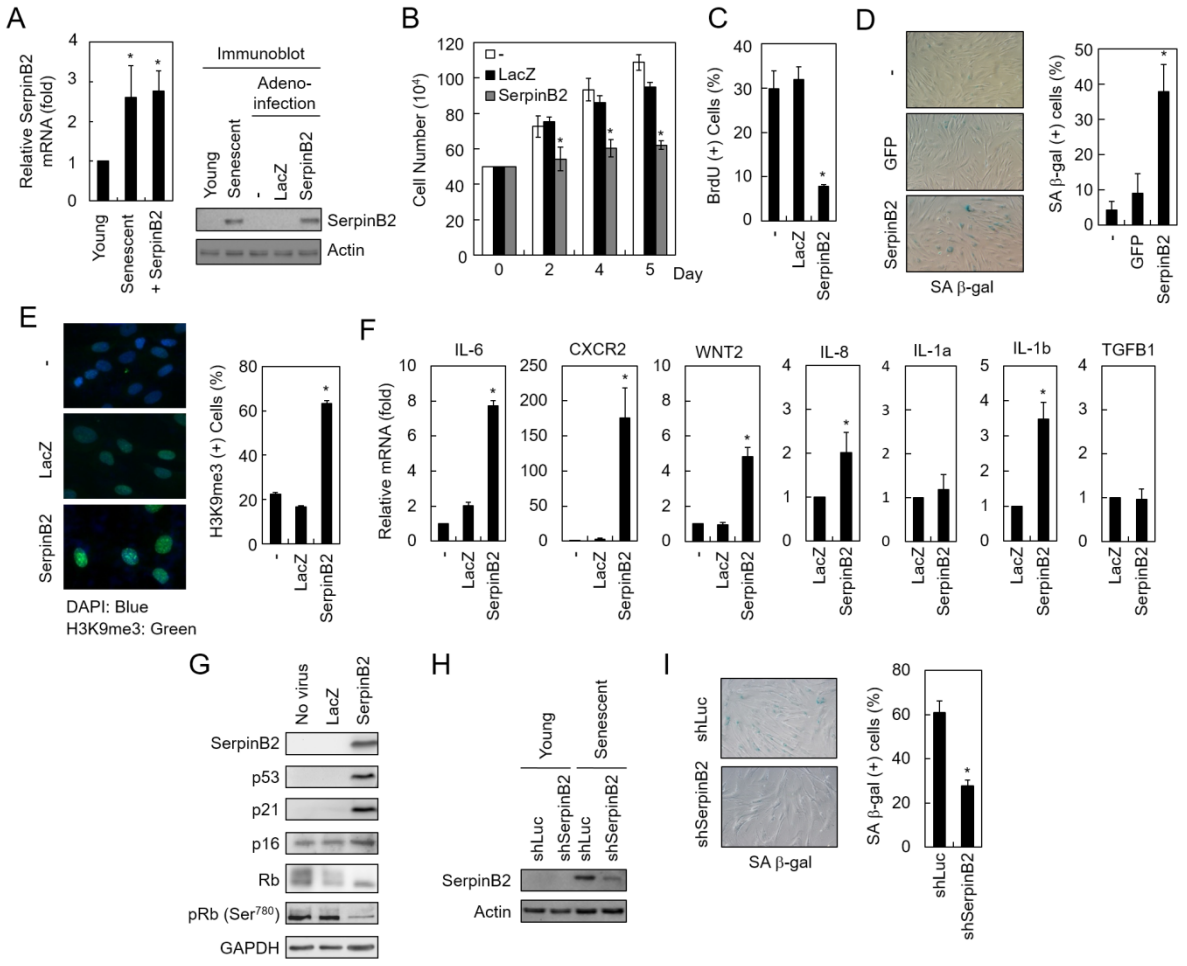

Figure 1. SerpinB2 induces senescence in normal human fibroblasts.

(A) IMR90 cells were collected at passages 16 (young) and 33 (senescent). Young IMR90 cells were transduced with adenoviruses carrying SerpinB2 or LacZ and then cultured at 37°C for 3 days. Total mRNA and cellular proteins were prepared and analyzed for SerpinB2 mRNA and proteins levels using quantitative RT-PCR (left) and immunoblot assays (right), respectively. Values show the average of 3 to 4 experiments. An *asterisk* indicates $p < 0.05$. (B) ~5x10^5 IMR90 cells were transduced with adenovirus carrying the SerpinB2 or LacZ. The cell numbers were then counted at the times indicated. Values show the average of three experiments. An *asterisk* indicates $p < 0.05$. (C) The virus-transduced cells were cultured in

medium containing BrdU for 8 hours. After labeling, the cells were fixed and stained with anti-BrdU antibody. The percentage of BrdUrd-positive cells is presented. (D) The SerpinB2 or GFP transduced cells were analyzed for SA-β-gal activity staining. Photographs of the X-gal-stained cells are shown (left). Quantification of the SA-β-gal positive-stained cells was conducted (right). Results were obtained from the average of three independent experiments. An asterisk indicates $p < 0.05$. (E) As above, the mock control or LacZ- or SerpinB2-expressed IMR90 cells were stained with DAPI (blue) and anti-H3K9me3 antibody (green). Images of the cells were presented. The Quantification of the H3K9me3 foci-positive cells was conducted. Results were obtained from the average of three independent experiments. An asterisk indicates $p < 0.05$. (F) Increased SASP by SerpinB2 expression. IMR90 cells were transduced with adenovirus carrying SerpinB2 or LacZ. The cells were lysed and the mRNA was analyzed by quantitative RT-PCR. The level of mRNA in LacZ-treated cells was used as 1. Asterisks indicate $p < 0.05$. (G) IMR90 cells were transduced with adenoviruses carrying LacZ or SerpinB2. Total cell extracts were prepared four days after transduction and then analyzed by immunoblotting assays using antibodies against SerpinB2, p53, p21, Rb, phosphor-Rb (Ser780), p16 or GAPDH. (H) shRNA expressing luciferase (shLuc) or SerpinB2 (shSerpinB2) was introduced into young or senescent IMR90 cells. The SerpinB2 levels were analyzed by immunoblots using anti-SerpinB2 antibodies. (I) As in (H), the senescence phenotype of SerpinB2 knock-down cells was analyzed by SA-β-gal analysis. An asterisk indicates $p < 0.05$.

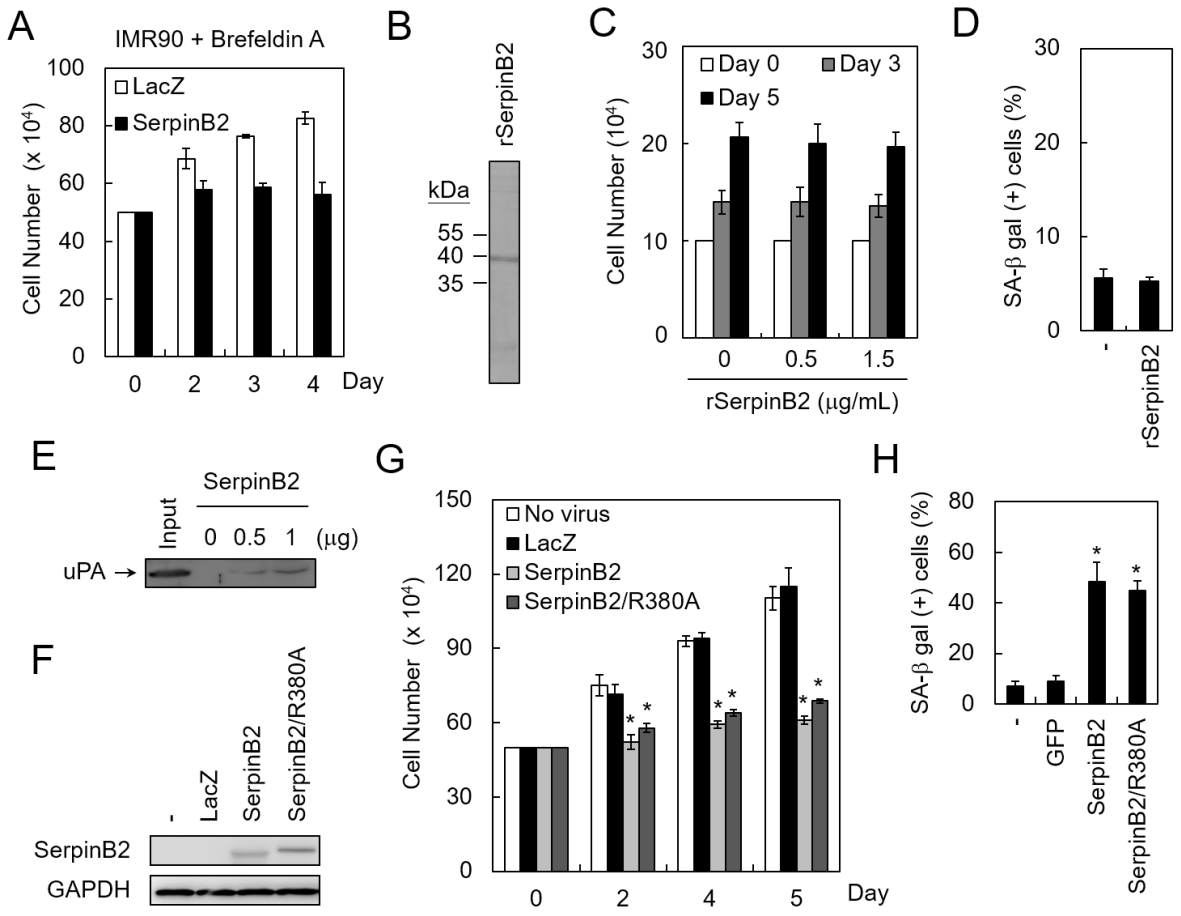

Figure 2. Extracellular SerpinB2 does not induce senescence in IMR90 cells. (A) IMR90 cells were transduced with adenoviruses carrying SerpinB2 or LacZ and then cultured in the presence of 75 ng/mL Brefeldin A. The cell numbers were determined at the indicated days. (B) The 6-His tagged recombinant SerpinB2 was expressed and isolated from *E. coli*. 1 μg of isolated SerpinB2 was separated by a 10% SDS-polyacrylamide gel and stained with coomassie blue. (C) IMR90 cells were treated with 0, 0.5, or 1.5 μg/mL recombinant SerpinB2 protein and the cell numbers were measured at days 0, 3, and 5. (D) SA-β-gal analyses were conducted in 1.5 μg/mL recombinant SerpinB2-treated IMR90 cells. (E) The 6-His tagged SerpinB2 (0, 0.5, or 1.5 μg) was incubated with 100 μg cell extracts isolated from A549 cells at 4°C for 1 hour and then loaded onto Ni-NTA agarose beads. The beads were eluted with 500 mM imidazole and then analyzed by 10% SDS-PAGE. Immunoblotting

analysis was conducted using anti-uPA antibody. (F) IMR90 cells were transduced with adenoviruses carrying LacZ, SerpinB2, or SerpinB2/R380A mutant. The cell extracts were prepared and analyzed for SerpinB2 expression using immunoblotting assays. (G) The cell numbers were measured at indicated days. (H) SA-β-gal analysis of IMR90 cells.

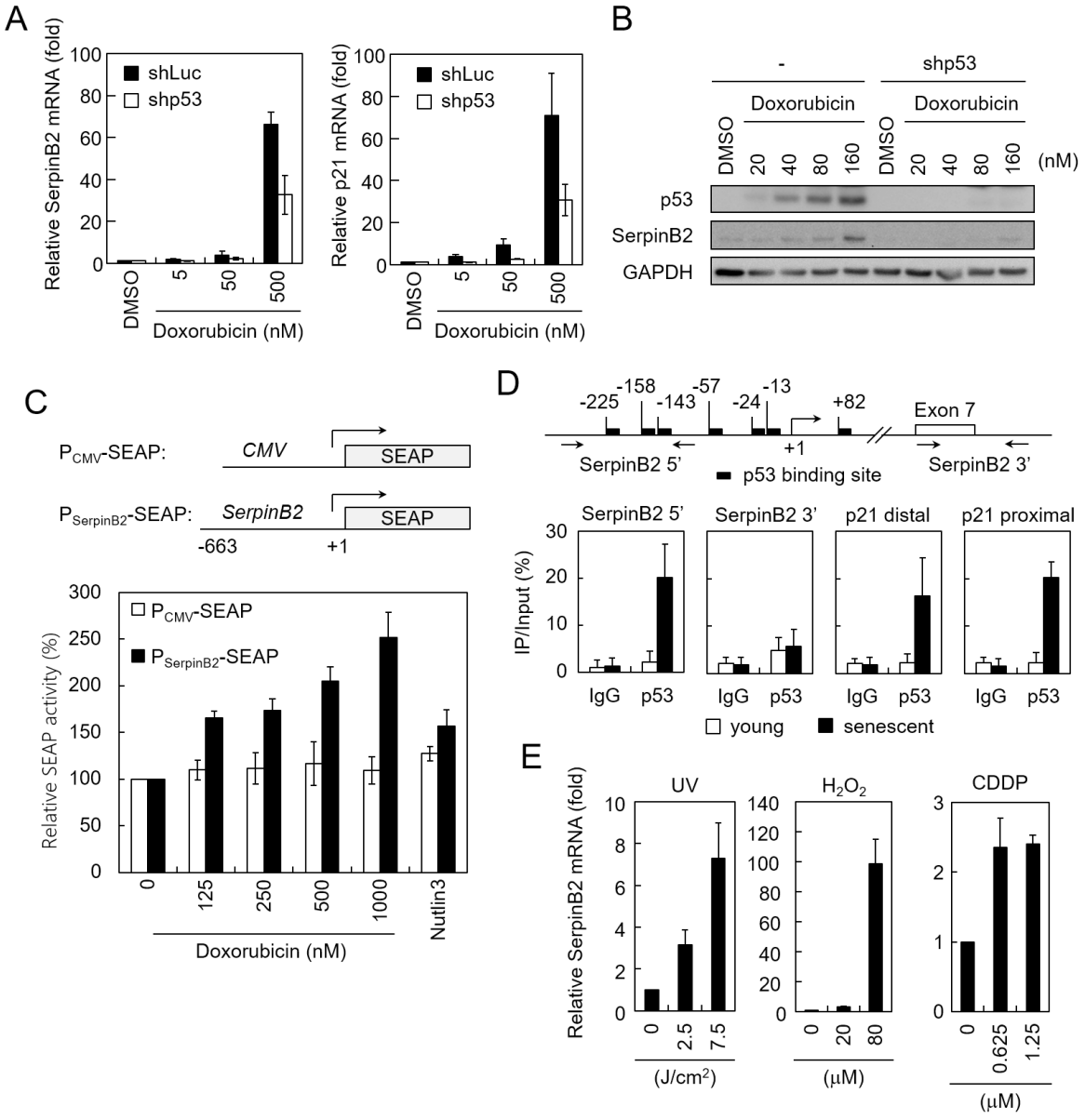

Figure 3. SerpinB2 is a direct downstream target of p53. (A) IMR90 cells were treated with the indicated concentration of doxorubicin. Total mRNAs were prepared from treated cells and SerpinB2 mRNA levels were determined by quantitative RT-PCR. The p21 mRNA levels were also determined. p53 knockdown experiments were achieved using lentiviruses carrying shLuc or shp53. Values show the average of three experiments. (B) As above, p53 and SerpinB2 levels were determined using immunoblotting assays. (C) The P_{CMV}-SEAP and $P_{SerpinB2}$-SEAP reporter plasmids were transfected into IMR90 cells and analyzed for their basal expression activities without doxorubicin treatment. The relative phosphatase activity

of CMV- and SerpinB2-driven expressions in the presence of indicated amounts of doxorubicin were presented using the basal levels as 100%. Addition of Mdm2 antagonist Nutlin3 was used as a control. (D) IMR90 cells were collected at passages 25 (young) and 41 (senescent) and cross-linked by formaldehyde. Chromatin immunoprecipitation (ChIP) analyses were conducted using antibody against p53. Schematic representation of the SerpinB2 promoter region showing the locations of predicted p53 binding sites and primers sets used for ChIP. ChIP was also conducted using primer sets located at the distal and proximal regions of the p21 promoter (Ishikawa et al., 2007). (E) IMR90 cells were treated with the indicated doses of UV, H_2O_2 or CDDP. The treated cells were collected and analyzed for SerpinB2 mRNA levels by quantitative RT-PCR. Values show the average of three experiments.

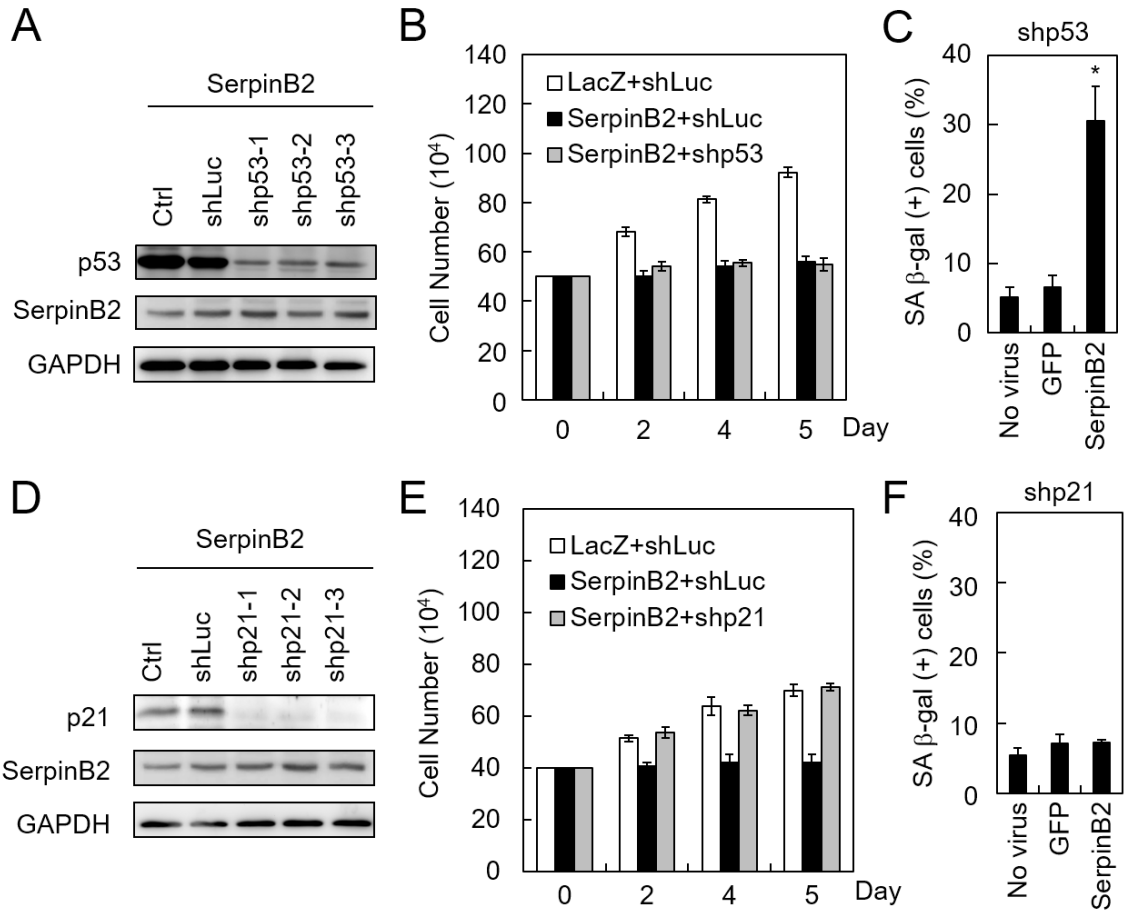

Figure 4. p21 is required for SerpinB2-mediated senescence. (A) IMR90 cells were infected with three different shRNAs against p53 (shp53-1 to -3) and then cultured at 37 °C for 2 days. The cells were then infected with adenoviruses carrying SerpinB2 and cultured at 37 °C for another 3 days. Cell extracts were prepared and analyzed by immunoblotting assays using antibodies against p53, SerpinB2 or GAPDH. (B) The SerpinB2-expressing cells harboring shRNAs against p53 or shLuc were prepared as described above. The cell numbers were determined at the indicated times. (C) In a parallel experiment, the SA-β-gal activities were examined and quantified. The results from the average of three independent experiments are presented. An *asterisk* indicates $p < 0.05$. (D-E) IMR90 cells were infected with three different shRNAs against p21 (shp21-1 to -3) and SerpinB2. The cells were then analyzed as above.

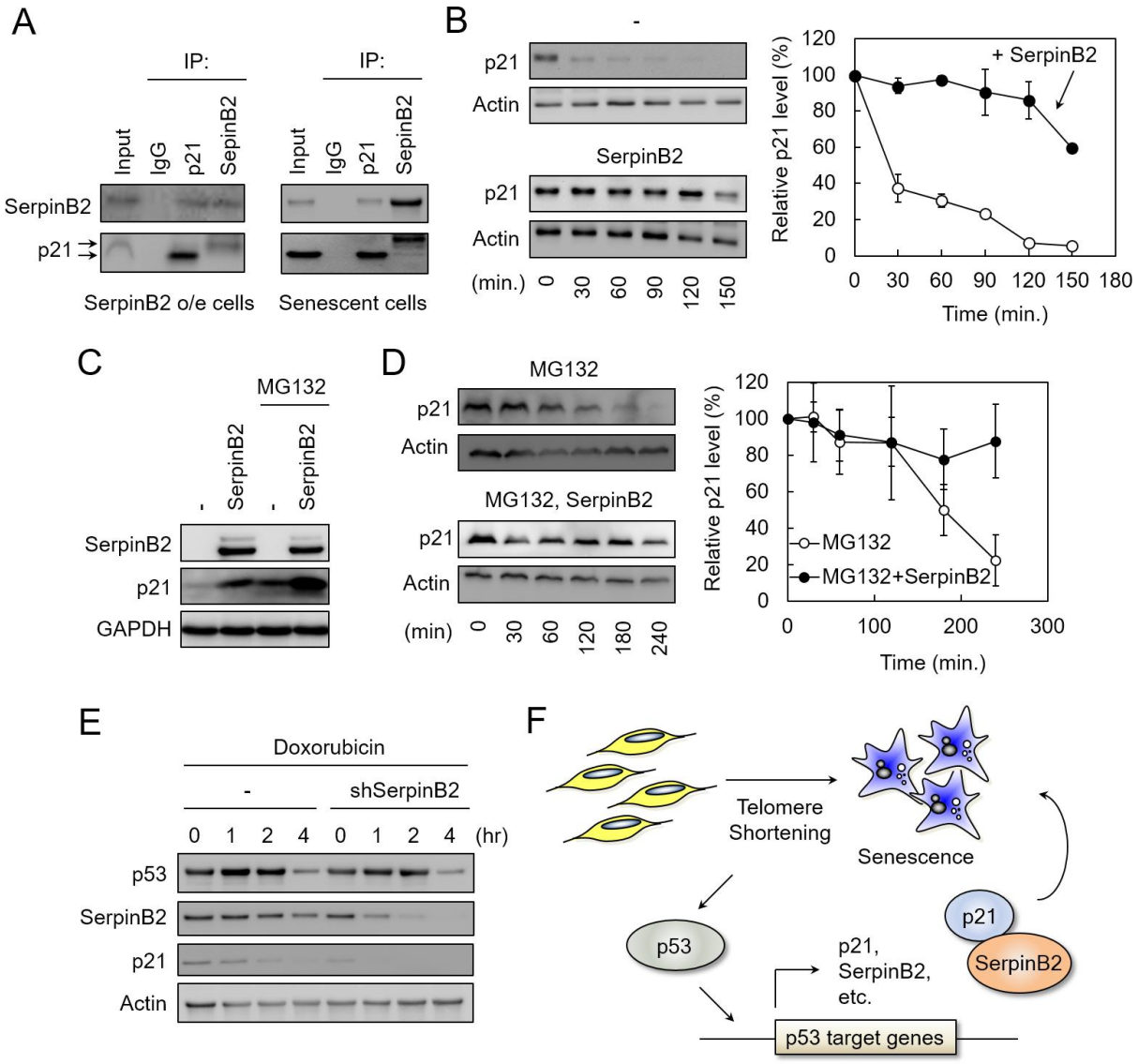

Figure 5. Proteasome-independent stabilization of p21 by SerpinB2. (A) SerpinB2 interacts with p21. Co-immunoprecipitation experiments were conducted in SerpinB2-expressing (right panel) or senescent (right panel) IMR90 cells using IgG, anti-p21, or anti-SerpinB2 antibodies. (B) SerpinB2-transduced IMR90 cells were incubated with cycloheximide and analyzed p21 levels at the indicated times. The right panel shows the quantification of the p21 levels. The results are the average of three independent experiments. (C) SerpinB2-transduced IMR90 cells were incubated with MG132 for 1 hour and then the p21 level was determined using immunoblotting assay. (D) SerpinB2-transduced IMR90 cells were incubated with MG132 for 1 hour and then treated with cycloheximide. The p21 levels were analyzed at the indicated times using immunoblotting assays. The right panel shows the

quantification of p21 levels. The results are the average of three independent experiments. (E) IMR90 cells were transduced with shSerpinB2 and then incubated with 500 nM doxorubicin. The p53, SerpinB2, and p21 levels were determined using immunoblotting assays. (F) Model for how SerpinB2 is involved in senescence.

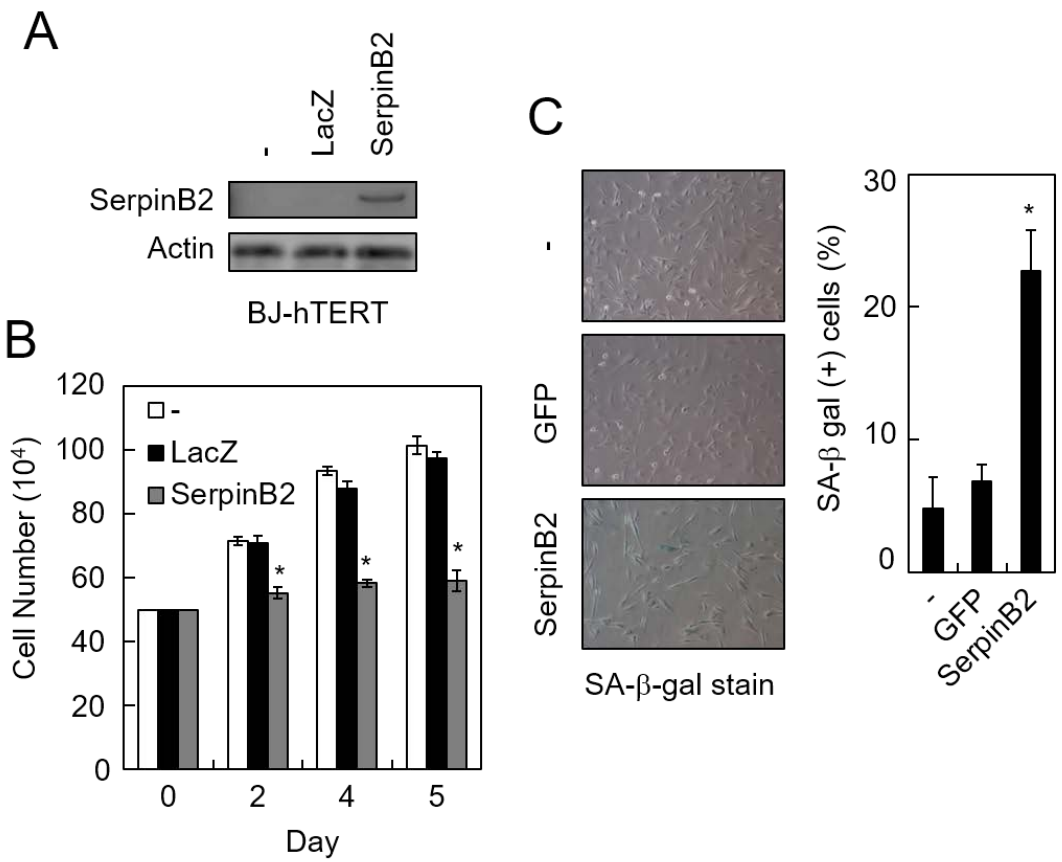

Figure S1. Telomerase cannot rescue SerpinB2-induced senescence. (A). Telomerase immortalized BJ-hTERT cells were transduced by either adenovirus carrying SerpinB2 or LacZ and cultured at 37°C for 3 days. The cell lysates were separated and analyzed by 10% SDS-PAGE followed by immunoblotting analysis using antibodies against SerpinB2 or actin. (B) ~$5x10^5$ cells were transduced with adenovirus carrying the SerpinB2 or LacZ. The cell numbers were then counted at the times indicated. Values show the average of three experiments. (C) The SerpinB2 or GFP transduced cells were analyzed for SA-β-gal activity staining. Photographs of the X-gal-stained cells are shown (left). Quantification of the SA-β-gal positive-stained cells was conducted (right). Results were obtained from the average of three independent experiments. An asterisk indicates $p < 0.05$.

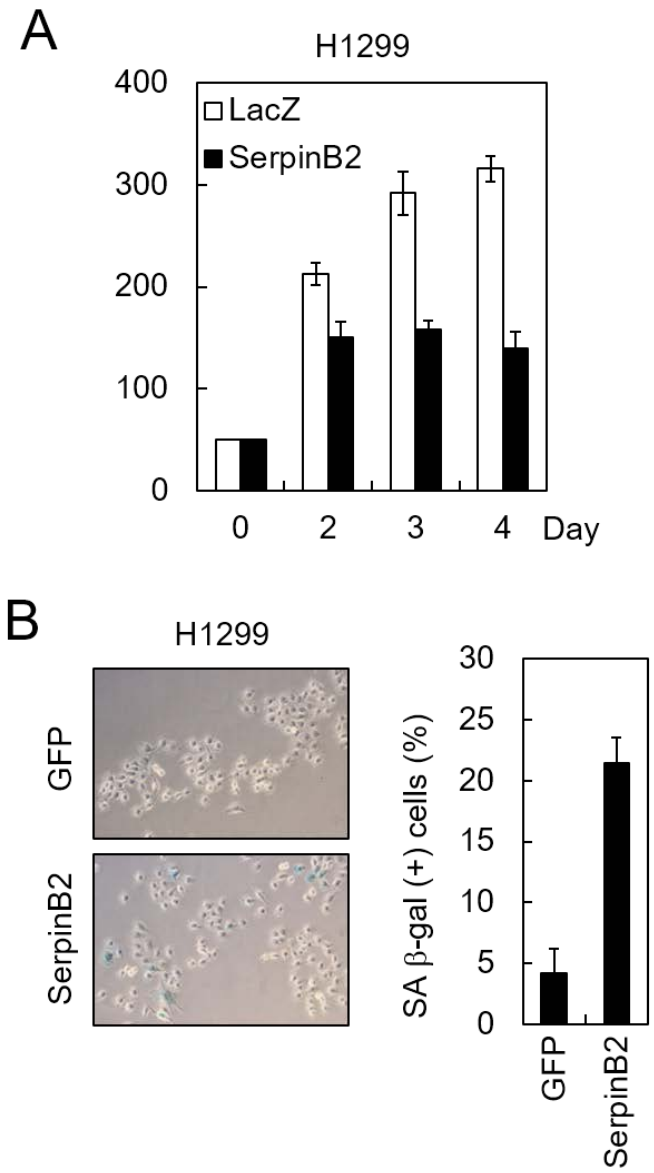

Figure S2. SerpinB2 induces senescence in H1299 cells. (A). ~5x10^5 lung cancer H1299 cells were transduced by either adenovirus carrying SerpinB2 or LacZ and cultured at 37^{o}C. The cell numbers were then counted at the times indicated. Values show the average of three experiments. (C) The SerpinB2 or GFP transduced cells were analyzed for SA-β-gal activity staining. Photographs of the X-gal-stained cells are shown (left). Quantification of the SA-β-gal positive-stained cells was conducted (right). Results were obtained from the average of three independent experiments. An asterisk indicates $p < 0.05$.

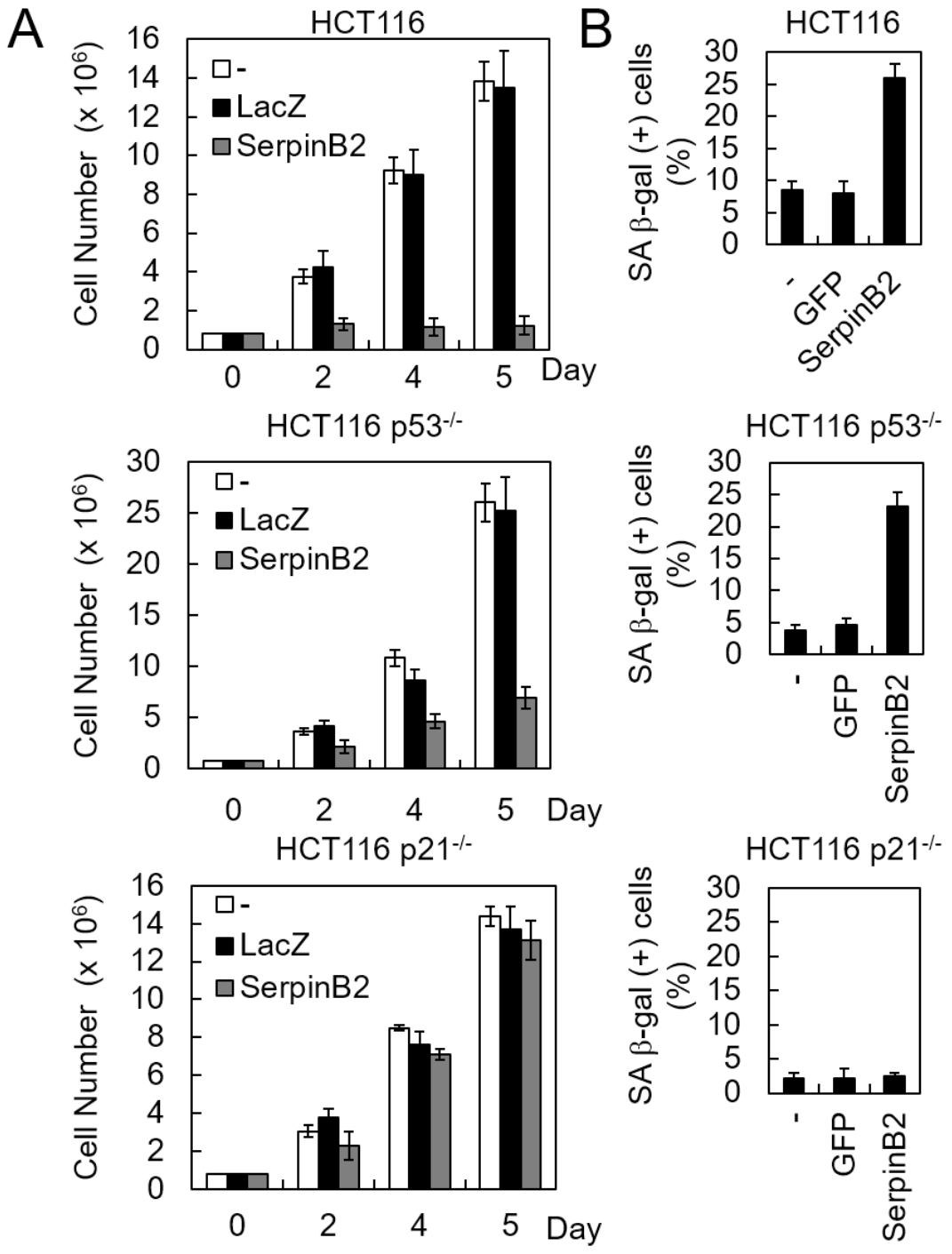

Figure S3 SerpinB2-mediated senescence requires p21 in HCT116. (A) HCT116 cells were transduced with adenovirus carrying LacZ or SerpinB2. The number of cells were calculated at indicated days. (B) The transduced cells were then analyzed for SA-β-gal activity staining. Quantification of the SA-β-gal positive cells were presented.

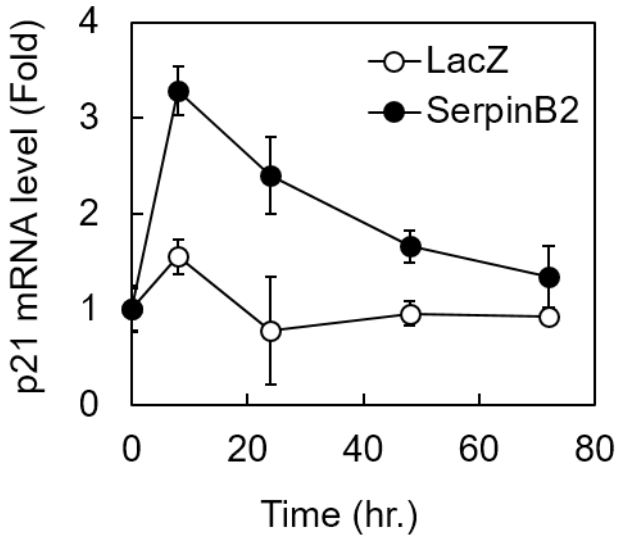

Figure S4. Expression of p21 mRNA in SerpinB2-expressing cells. IMR90 cells were transduced with adenoviruses carrying LacZ or SerpinB2. mRNA were collected and analyzed by qPCR at indicated times.

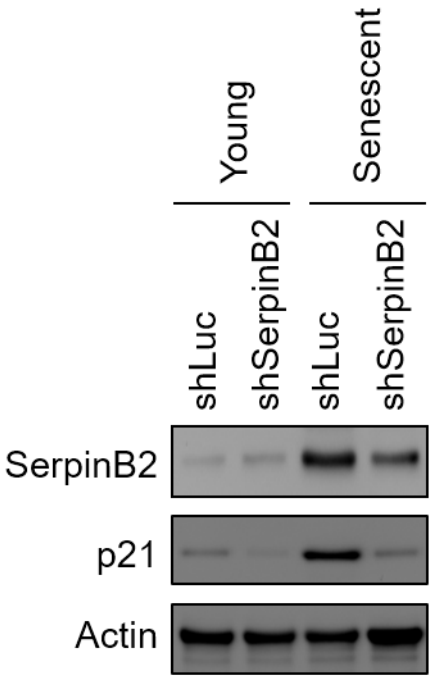

Figure S5. Reduction of p21 by knocking down SerpinB2. IMR90 cells were transduced with lentivirus carrying shLuc or shSerpinB2. Total cellular proteins were collected and analyzed by immunoblots using antibodies against SerpinB2, p21, or actin.