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RESEARCH ARTICLE

The serine protease inhibitor serpinB2 binds and stabilizes p21 in senescent cells

Hsi-Hsien Hsieh[1], Ying-Chieh Chen[1], Jing-Ru Jhan[1] and Jing-Jer Lin[1,2,*]

ABSTRACT

SerpinB2 is a serine protease inhibitor 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 levels are increased in senescent cells and serpinB2 is thus considered a senescence biomarker. In this study, by mimicking the elevated levels of serpinB2 in senescent cells, 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 affect senescence. We also showed that serpinB2 is a direct downstream target of p53 that is activated by the DNA damage response pathway. Significantly, serpinB2 bound to and stabilized p21 to mediate senescence in a proteasome-independent manner, indicating that serpinB2 has a direct role in senescence. Thus, this study reveals a unique mechanism by which serpinB2 maintains senescence through stabilization of p21 protein levels.

KEY WORDS: Senescence, serpinB2, p21, Proteasome

INTRODUCTION

Normal human diploid fibroblasts have a finite proliferative capacity in the laboratory. After ∼60–80 population doublings, they enter the terminally nondividing 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 occur at a lower saturation density than nonsenescent cells. 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, senescent cells also have alterations in gene expression, microRNA expression and protein secretion (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 has been shown that 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).

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Senescence is executed mainly by a DNA damage response pathway, mediated by either of two important cell cycle inhibitors: p21 (also known as CDKN1A, p21Cip1, Waf1 or SDI1) or p16 (also known as CDKN2A or p16INK4a) (Brown et al., 1997; McConnell et al., 1998). Both pathways can establish and maintain growth arrest by maintaining retinoblastoma protein (Rb, also known as RB1) in a hypophosphorylated and active state. In normal human fibroblasts, the signal that induces senescence by telomere erosion is primarily driven by the tumor suppressor protein p53 (also known as TP53) (d'Adda di Fagagna, 2008). p53 is a transcriptional regulator activated by DNA damage. 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), is 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 distribution (Kruithof et al., 1995). The intracellular role of serpinB2 is poorly understood. Here, we identify a unique role of serpinB2 in senescence, in which it forms a complex with p21 to stabilize it and maintain senescence.

RESULTS

SerpinB2 induces cellular senescence in normal human fibroblasts

Both serpinB2 mRNA (Chen et al., 2004; Zhang et al., 2003) and protein (Kumar et al., 1992; West et al., 1996) levels have been 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 that serpinB2 expression induced growth arrest in these cells (Fig. 1B). This change was also accompanied by decreased bromodeoxyuridine (BrdU) incorporation (Fig. 1C).

Subsequently, the senescent phenotypes were analyzed. The serpinB2-expressing cells showed increased levels of SA-β-gal, a well-recognized biomarker for senescence (Fig. 1D). Senescent

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cells also display a signature heterochromatin foci formation (Narita et al., 2003) that can be identified by elevated H3K9me3 (a senescence-associated heterochromatin marker) (Fig. 1E). Moreover, senescent cells upregulate enzymes that degrade the extracellular matrix, and secrete immune modulators and inflammatory cytokines to reinforce senescence in an autocrine and paracrine manner (Sharpless and Sherr, 2015). The senescence-associated secretory phenotype (SASP) is reported to heighten inflammation through recruitment of inflammatory cells, and has detrimental effects on the tissue microenvironment through alteration of tissue composition and architecture (Sharpless and Sherr, 2015). In this study, the cellular senescence induced by serpinB2 also induced several signature SASP components, including IL6, IL8, CXCR2, WNT2 and IL1B (Fig. 1F). The results suggested that elevating serpinB2 levels is sufficient to induce a secretory phenotype that is similar to senescence.

To gain insight into the molecular basis of serpinB2-mediated growth arrest and senescence in normal fibroblasts, the expression

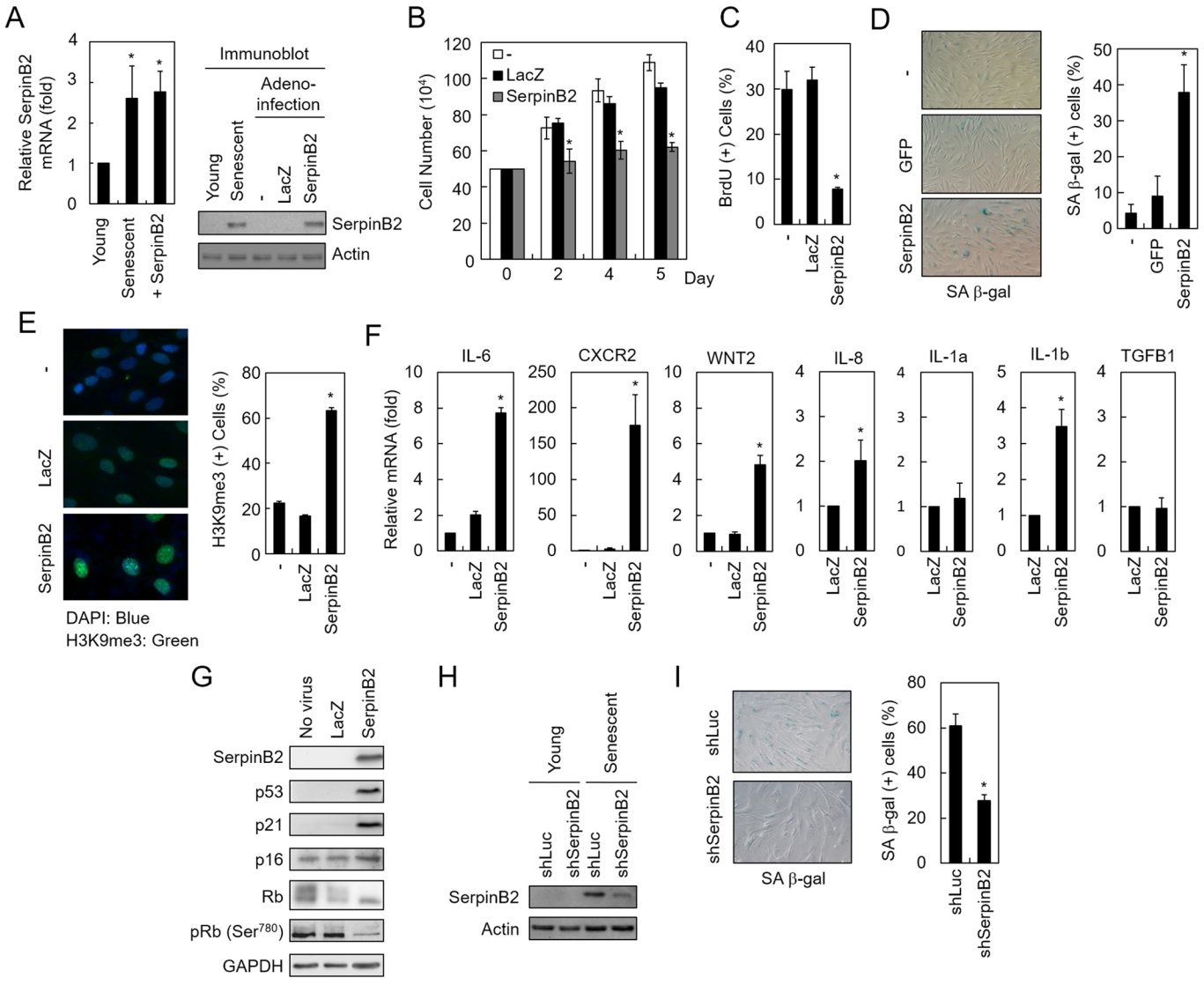

Fig. 1. SerpinB2 induces senescence in normal human fibroblasts. (A) IMR90 cells were collected at passages 16 (young) and 33 (senescent). Young IMR90 cells were transduced with adenoviruses carrying *SERPINB2* or *lacZ* and then cultured at 37°C for 3 days. Total mRNA and cellular proteins were prepared and analyzed for serpinB2 mRNA and protein levels using quantitative real-time PCR (left) and immunoblot (right) analysis, respectively. Data are mean±s.e.m. from three to four experiments. (B) IMR90 cells (~5×10^5) were transduced with adenovirus carrying *SERPINB2* or *lacZ*. The cells were then counted at the indicated times. Data are mean±s.e.m. from three experiments. (C) The virus-transduced cells were cultured in medium containing BrdU for 8 h. After labeling, the cells were fixed and stained with anti-BrdU antibody, and the percentage of BrdU-positive cells was determined. (D) SerpinB2- or GFP-transduced cells were stained for SA-β-gal (left), and the percentage of SA-β-gal-positive cells was quantified (right). Data are mean±s.e.m. from three independent experiments. (E) Mock control, *lacZ*- or *SERPINB2*-expressing IMR90 cells were stained with DAPI (blue) and anti-H3K9me3 antibody (green) (left), and the percentage of H3K9me3 foci-positive cells was determined (right). Data are mean±s.e.m. from three independent experiments. (F) Increased SASP in response to serpinB2 expression. IMR90 cells were transduced with adenovirus carrying *SERPINB2* or *lacZ*. The cells were lysed and their mRNA analyzed by quantitative real-time PCR. The level of mRNA in *lacZ*-treated cells was set as 1. (G) IMR90 cells were transduced with adenoviruses carrying *lacZ* or *SERPINB2*. Total cell extracts were prepared 4 days after transduction and then analyzed by immunoblotting 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. SerpinB2 levels were analyzed by immunoblotting using anti-serpinB2 antibodies. (I) The senescence phenotype of serpinB2 knockdown cells was analyzed by SA-β-gal analysis. shLuc and shSerpinB2 cells were stained for SA-β-gal (left) and the percentage of SA-β-gal-positive cells was determined (right). *P<0.05.

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pattern of several key cell cycle regulators involved in senescence was evaluated using immunoblotting. Expression of serpinB2 increased p53 and p21 expression, and inhibited Rb protein expression and phosphorylation (Fig. 1G). Although p16 has been implicated as a key mediator of senescence, its level was not significantly altered in serpinB2-expressing IMR90 cells. These results suggest that the p53–p21–Rb senescence pathway was activated in response to serpinB2 overexpression in normal cells.

To test whether serpinB2 is required for senescence, serpinB2 levels were reduced by RNA interference using shRNA (Fig. 1H) and then analyzed for SA-β-gal (Fig. 1I). The analysis showed that reducing serpinB2 levels greatly decreased the number of senescent cells, suggesting that serpinB2 is required for senescence formation. It is well documented that telomere shortening is the main cause of replicative senescence (Allsopp et al., 1995; Bodnar et al., 1998). To show that the observed serpinB2 function was not caused by its effect on telomere length, serpinB2 was expressed in telomerase-positive human normal fibroblast (BJ-hTERT) (Fig. S1) and lung cancer (H1299) (Fig. S2) cells. The results showed that serpinB2 induced senescence in both BJ-hTERT and H1299 cells, suggesting that serpinB2 did not induce senescence by affecting telomere length or telomerase activity.

Senescence induced by serpinB2 is not related to its extracellular function

Because serpinB2 is known to bind and inhibit 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 evaluated 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, suggesting that inhibition of serpinB2 secretion did not affect growth inhibition.

The effect of exogenous serpinB2 on senescence was then evaluated. SerpinB2 is not usually detectable in human plasma, except during pregnancy, when its 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, serpinB2 levels 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 that mimicked the patho- or physiological concentrations of serpinB2 (0.5 µg/ml and 1.5 µg/ml). Under the tested conditions, the addition of serpinB2 did not cause apparent cell growth or senescence in IMR90 cells (Fig. 2C,D). 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 was still functional (Fig. 2E). Of note, the serpinB2 protein level in the culture medium of senescent IMR90 cells was <30 ng/ml.

SerpinB2 contains a reactive site near the carboxyl terminus that acts as a pseudosubstrate for its cognate proteinase. It was reported that Arg380 is the reaction site of serpinB2 where its mutation (R380A) disrupts uPA binding activity (Dickinson et al., 1995; Fish and Kruithof, 2006). To test whether 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 that the R380A mutation did not affect senescence mediated by serpinB2 (Fig. 2G,H), suggesting that interaction between serpinB2 and uPA is not required for serpinB2-mediated senescence. Taken together, these results suggest that senescence induced by serpinB2 is not caused by its extracellular function.

SerpinB2 is a direct downstream target of p53

The mechanism of serpinB2 expression in senescent cells was next investigated. Because DNA damage response induced by telomere erosion is the causal factor for cellular senescence, the dependence of p53 on serpinB2 expression was determined. p21 is a direct downstream target of p53 and its level can be induced by the DNA damage agent doxorubicin in a p53-dependent manner (Fig. 3A, right). Both the mRNA and protein levels of serpinB2 were induced by doxorubicin (Fig. 3A,B). Similar to observed for p21, the extent to which serpinB2 levels were elevated was 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 by the *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 in the presence of the DNA damage-inducing agent doxorubicin. Upon treatment with doxorubicin, the expression of SEAP from the *SERPINB2* promoter was increased (Fig. 3D). By comparison, the human cytomegalovirus (CMV) promoter did not respond to doxorubicin treatment. Sequence analysis identified seven p53-binding consensus sequences near the transcriptional start site of *SERPINB2* (Fig. 3D). To show that 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). p53 also bound to the *P21* promoter in senescent cells.

It was also of interest to note that p53 levels were activated by serpinB2 expression (Fig. 1G). The results suggested that serpinB2 might have a positive regulatory effect on p53, but the mechanism by which serpinB2 could regulate p53 levels is not clear. It is possible that serpinB2 might stabilize p53 levels in senescent cells because 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 a 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 have been 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 expression was efficiently induced by these stresses (Fig. 3E). The results suggest that serpinB2 is a direct downstream target of p53 and can be induced by various types of stress.

p21 is required for serpinB2-mediated senescence

The requirement for p53 and p21 as part of serpinB2-mediated senescence was subsequently investigated. IMR90 cells were first transduced with shRNAs targeting p53 or p21, and then serpinB2 was introduced. As shown in Fig. 4A, the level of p53 was decreased upon shRNA treatment. In these p53 knockdown cells, the cell numbers decreased and percentage of SA-β-gal-positive cells increased (Fig. 4B,C). By contrast, following knockdown of p21 expression, the percentage of SA-β-gal-positive cells significantly

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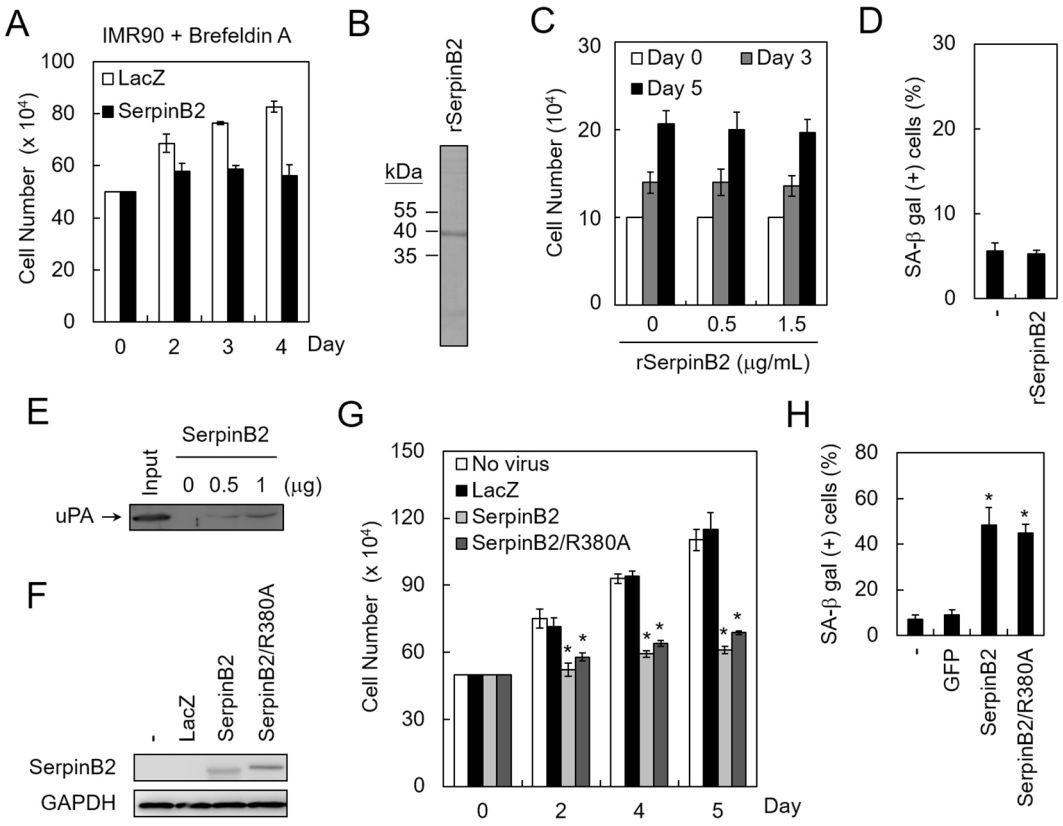

Fig. 2. Extracellular serpinB2 does not induce senescence in IMR90 cells. (A) IMR90 cells were transduced with adenoviruses carrying *SERPINB2* or *lacZ*, and then cultured in the presence of 75 ng/ml Brefeldin A. The cells were counted on the indicated days. (B) 6×His-tagged recombinant serpinB2 was expressed and isolated from *E. coli*. Then, 1 µg isolated serpinB2 was separated on 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 cells were counted on days 0, 3 and 5. (D) SA-β-gal analysis in 1.5 µg/ml recombinant serpinB2-treated IMR90 cells. (E) 6×His-tagged serpinB2 (0, 0.5 or 1 µg) was incubated with 100 µg cell extracts isolated from A549 cells at 4°C for 1 h, and then loaded onto Ni-NTA agarose beads. The beads were eluted with 500 mM imidazole and then analyzed by 10% SDS-PAGE. Immunoblot 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. (G) The cells were counted on the indicated days. (H) SA-β-gal analysis in the IMR90 cells.

decreased (Fig. 4D–F), indicating that reducing p21 expression can restore serpinB2-mediated senescence. Thus, p21 is required for serpinB2-mediated senescence in normal cells.

In an independent experiment, an isogenic colorectal cancer cell line, HCT116, and its p53 and p21 knockouts, were evaluated (Bunz et al., 1998). Upon serpinB2 overexpression, the HCT116 cells ceased to proliferate (Fig. S3). Proliferation was recovered in HCT116 cells carrying $p21^{-/-}$, but not the $p53^{-/-}$ 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 the $p21^{-/-}$ mutation, indicating that serpinB2-mediated senescence is p21 dependent. These results indicated that p21 is required for senescence mediated by serpinB2 in both normal and cancer cells.

Proteasome-independent stabilization of p21 by serpinB2

The mechanism of p21 involvement in serpinB2-mediated senescence was then analyzed. Although transcriptional regulation is considered the initial control point for p21 expression, post-transcriptional and post-translational regulation also play a critical role in p21 expression and activity (Jung et al., 2010). The accumulation of p21 could be caused by elevated p21 expression and/or reduced p21 degradation. We found that expressing serpinB2 caused an increase in p21 expression (Fig. S4), likely to be caused by p53 activation (Fig. 1F). The elevated p21 levels had subsided after ∼3 days. As senescence induced by serpinB2 can last for at least 30 days, a transient elevation in p21 expression is not sufficient to maintain senescence. Thus, an additional mechanism is involved for serpinB2 to maintain p21 levels.

SerpinB2 appeared to directly impact p21 levels, 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 that 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 (data not shown), indicating that p21 is indeed co-immunoprecipitated by serpinB2. Thus, although the nature of the p21 band shift is not clear, serpinB2 preferentially interacted with the mobility-shifted p21. The effect of serpinB2 on the stability of p21 protein was then analyzed. p21 protein is short living; it has a half-life <30 min (Fig. 5B). SerpinB2 greatly extended the half-life of p21 to ∼160 min. To determine whether the proteasome-dependent pathway is involved in stabilization of p21, the proteasome inhibitor MG132 was introduced. As shown in

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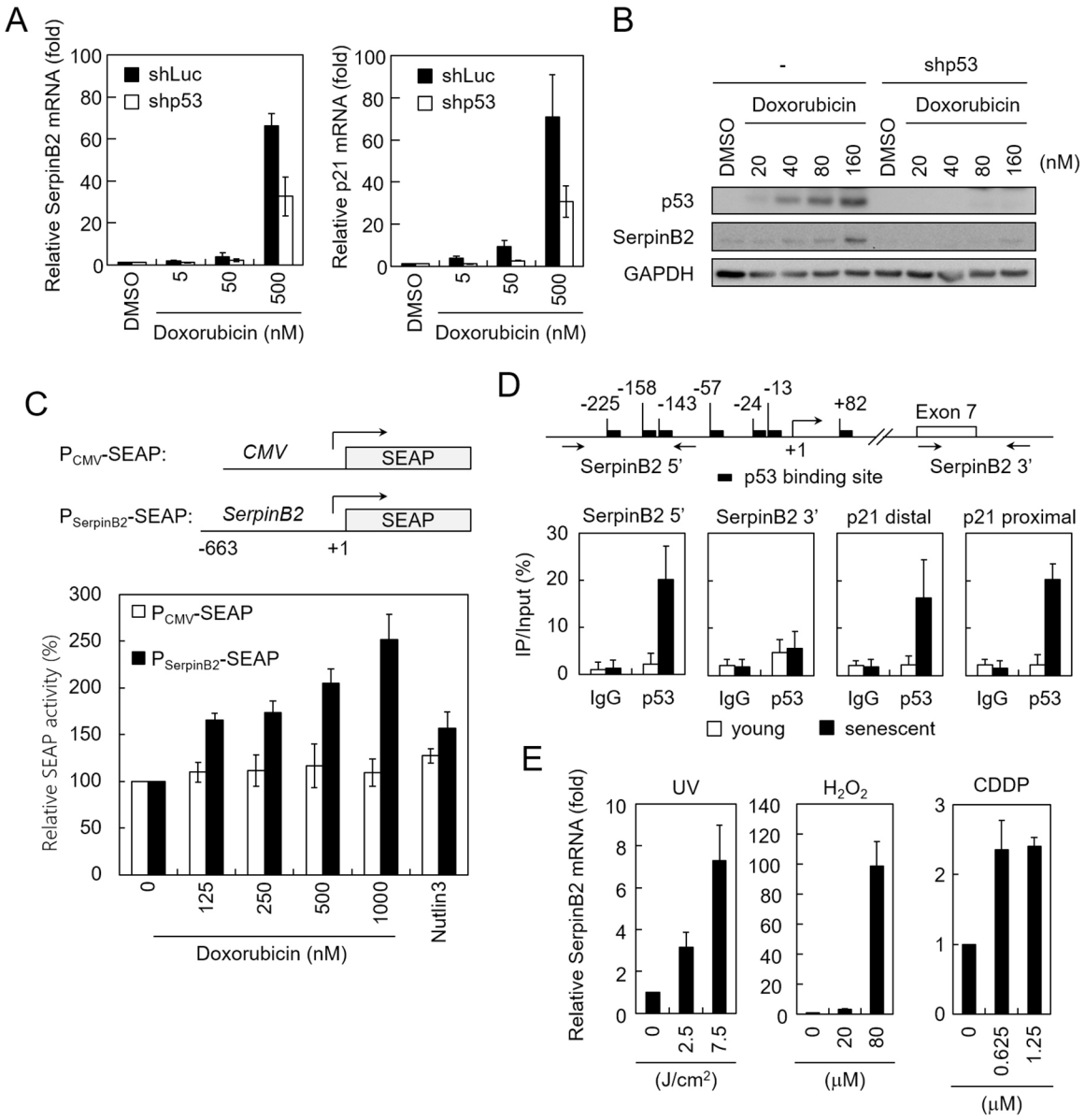

Fig. 3. SerpinB2 is a direct downstream target of p53. (A) IMR90 cells were treated with the indicated concentration of doxorubicin. Total mRNAs were prepared from treated cells and *SERPINB2* mRNA levels were determined by quantitative real-time PCR. *P21* mRNA levels were also determined. p53 knockdown was achieved using lentiviruses carrying shLuc or shp53. Data are mean±s.e.m. from three experiments. (B) p53 and serpinB2 levels were determined using immunoblotting. (C) P_{CMV}-SEAP and $P_{serpinB2}$-SEAP reporter plasmids were transfected into IMR90 cells and analyzed for basal expression activities without doxorubicin treatment. The relative phosphatase activities of CMV- and serpinB2-driven expression in the presence of the indicated amounts of doxorubicin are presented, using the basal levels as 100%. Cells treated with the Mdm2 antagonist Nutlin3 were used as a control. (D) The 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 of the *SERPINB2* promoter region showing the locations of predicted p53 binding sites and primer sets used for ChIP (top). 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 real-time PCR. Data are mean±s.e.m. from three experiments.

Fig. 5C, MG132-treated cells showed higher p21 accumulation in both control and serpinB2-expressing cells. The results suggest that serpinB2 might stabilize p21 through a pathway that is independent of proteasome. Indeed, MG132 treatment 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). These results suggest that serpinB2 has a general role in binding and stabilizing p21 in maintenance of senescence. It is also interesting to note that p53 levels were slightly reduced in cells with reduced serpinB2 levels, suggesting a positive effect of serpinB2 on p53.

DISCUSSION

SerpinB2 is known to increase in senescent cells (Chen et al., 2004; Kumar et al., 1992; West et al., 1996; Zhang et al., 2003), and is considered a senescent marker with no known function related to senescence. This study showed that 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 levels

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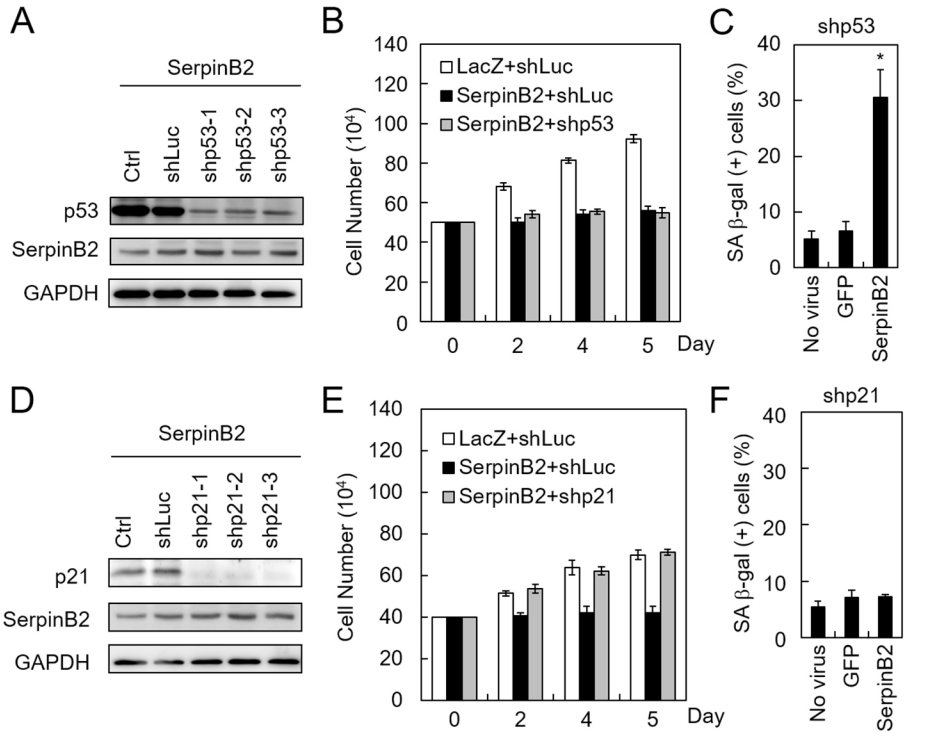

Fig. 4. p21 is required for serpinB2-mediated senescence. (A) IMR90 cells were infected with three different shRNAs against p53 (shp53-1/2/3) and then cultured at 37°C for 2 days. The cells were then infected with adenoviruses carrying serpinB2 and cultured at 37°C for another 3 days. Cell extracts were prepared and analyzed by immunoblotting using antibodies against p53, serpinB2 or GAPDH. (B) serpinB2-expressing cells harboring shRNAs against p53 or shLuc were prepared as described above. The cells were counted at the indicated times. (C) In a parallel experiment, the percentage of SA-β-gal-positive cells was determined. Data are mean±s.e.m. from three independent experiments. *P<0.05. (D,E) IMR90 cells were infected with three shRNAs against p21 (shp21-1/2/3) and serpinB2. The cells were then analyzed as above and the percentage of SA-β-gal-positive cells was quantified.

in senescent cells. Thus, serpinB2 has a critical role in senescence. SerpinB2 was also found to be elevated by different inducers in various cell types. For example, serpinB2 levels were shown to increase several hundred fold within hours of exposing hippocampal neurons to bicuculline, a $GABA_A$ receptor antagonist (Zhang et al., 2009). Elevated serpinB2 appears to promote the survival of cultured hippocampal neurons and protect against seizure-induced neuronal death by kainic acid. SerpinB2 was also shown to be upregulated in monocytes/macrophages following infection or stimulation with inflammatory mediators (Schroder et al., 2010). Moreover, a recent report showed that FOXO1 can enhance the expression of serpinB2 under high glucose conditions to inhibit keratinocyte migration. Further analysis showed that reduced keratinocyte migration can be rescued by reducing serpinB2 (Zhang et al., 2015). Together, these observations suggest that serpinB2 might be considered a common stress response protein. It can be induced to help cells accommodate stress. Consistent with this notion, a notable function of serpinB2 is related to its anti-apoptotic function. It was reported that serpinB2 maintains the survival of TNF-stimulated cells by interacting with transglutaminase (TGM2) to inactivate procaspase-3 (Delhase et al., 2012). A similar mechanism might be involved for serpinB2 to protect cells against apoptosis. The binding and stabilization of p21 by serpinB2 might stop unchecked cell proliferation, providing an opportunity for cells to adjust to various types of stress.

This study showed that the role of serpinB2 in senescence is mediated by an intracellular pathway through stabilization of the p21 protein. This result is consistent with the nucleocytoplasmic distribution of serpinB2 and its function as an intracellular serpin (Kruithof et al., 1995). This study also provided evidence that the function of serpinB2 in senescence is not likely to be mediated through inhibition of uPA and tPA (Kruithof et al., 1986; Thorsen et al., 1988). It is also interesting to note that plasminogen activator inhibitor type 1 (PAI-1) is a direct target of p53 (Kunz et al., 1995). PAI-1 is an extracellular serpin and known to inhibit the activity of secreted uPA by forming a stable complex. It has a direct role in senescence by inhibiting the phosphoinositide 3-kinase, PKB (Akt) protein and GSK3β signaling pathways both *in vitro* and *in vivo* (Eren et al., 2014; Kortlever et al., 2006). Although the serpins serpinB2 and PAI-1 share several similar functions, they contribute to senescence through mechanistically distinct pathways.

p21 binds CDKs and inhibits their activities, and needs to be efficiently removed from CDKs for cells to undergo 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 <30 min. Degradation of p21 is mediated mainly through ubiquitin-dependent proteasome pathways (Bendjennat et al., 2003; Bloom et al., 2003). In such pathways, proteolysis of p21 is mediated by E3 ubiquitin ligases, including SCF^{SKP2}, $CRL4^{CDT2}$, APC/C^{CDC20}, ZNF313 (also known as RNF114), RNF115 and BCA2 (Amador et al., 2007; Bornstein et al., 2003; Han et al., 2013; Wang et al., 2005, 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 lysines 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 proteasomes through binding the proteasomal C8 subunit. It is also reported that the MDM2 oncogene has a role in ubiquitin-independent degradation through promoting the binding of p21 to the C8 subunit (Jin et al., 2003; Zhang et al., 2004). It facilitates p21 degradation independent of ubiquitylation and the E3 ligase function of MDM2. This study showed that serpinB2 does not affect proteasome degradation of p21 in senescent cells. The mechanism by which serpinB2 stabilizes p21 is unclear. A simple explanation could be that the binding of serpinB2 prevents p21 from proteasome degradation. However, because serpinB2 is considered a protease inhibitor, it is also possible that the activity of a specific p21-degrading protease is inhibited by serpinB2. Alternatively, since this study showed that serpinB2 interacts with an unidentified version of modified p21 (Fig. 5A), it is possible that serpinB2 induces such a p21

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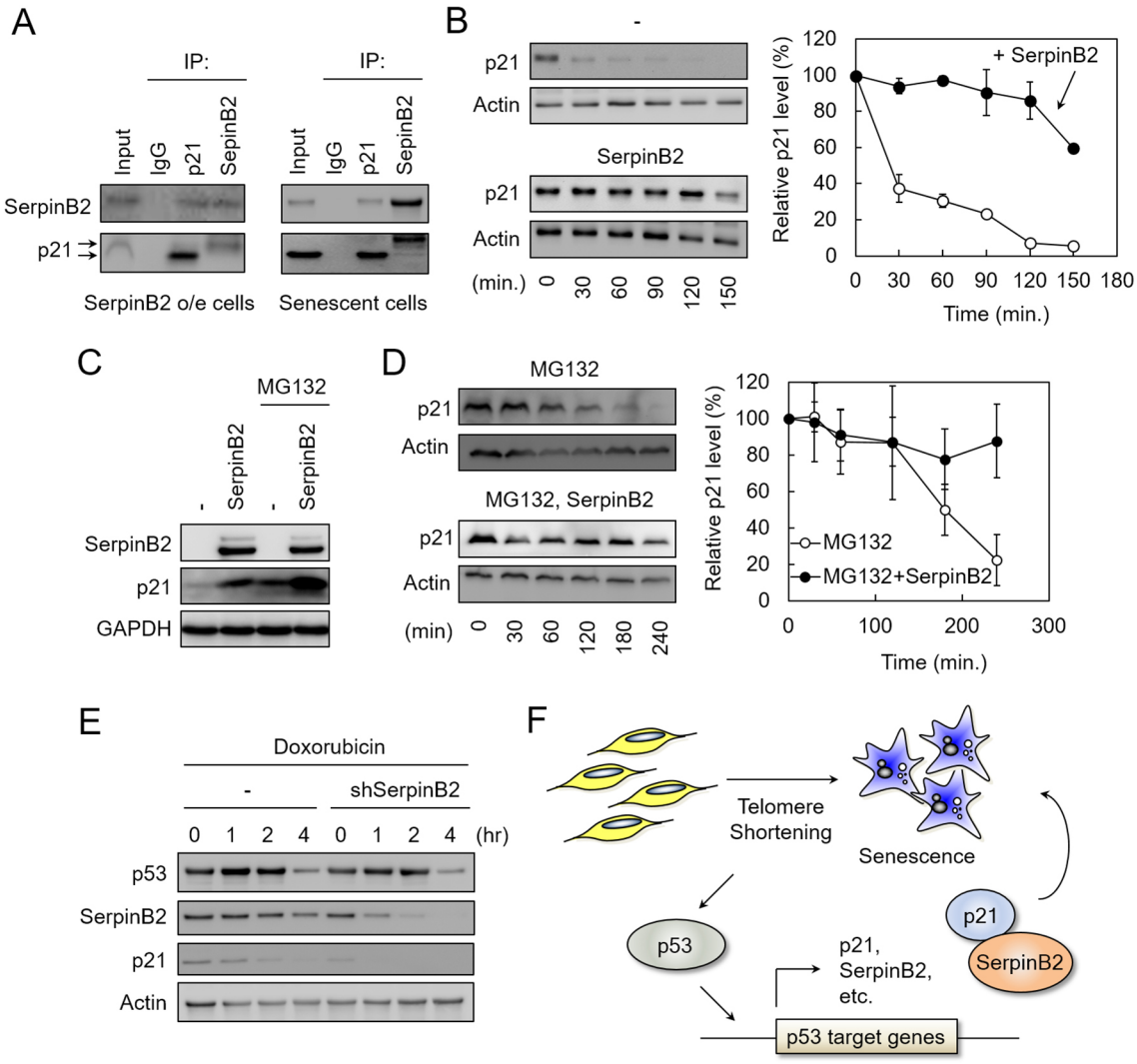

Fig. 5. Proteasome-independent stabilization of p21 by serpinB2. (A) serpinB2 interacts with p21. Co-immunoprecipitation experiments were conducted in serpinB2-expressing (left) or senescent (right) IMR90 cells using IgG, anti-p21 or anti-serpinB2 antibodies. (B) serpinB2-transduced IMR90 cells were incubated with cycloheximide, and p21 levels were analyzed at the indicated times (left) and quantified (right). Data are mean±s.e.m. from three independent experiments. (C) serpinB2-transduced IMR90 cells were incubated with MG132 for 1 h and p21 levels determined using immunoblotting. (D) serpinB2-transduced IMR90 cells were incubated with MG132 for 1 h and then treated with cycloheximide. p21 levels were analyzed at the indicated times using immunoblotting (left) and quantified (right). Data are mean±s.e.m. from three independent experiments. (E) IMR90 cells were transduced with shserpinB2 and then incubated with 500 nM doxorubicin. p53, serpinB2 and p21 levels were determined using immunoblotting. (F) Schematic of serpinB2 involvement in senescence.

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) cell line was purchased from the Food Industry Research and Development Institute (Hsinchu City, Taiwan) and maintained in minimum essential medium (MEM, Gibco Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS). Both proliferation rate and SA-β-gal activity were used to evaluate the senescent status of the IMR90 cells. The IMR90 cells could be cultured for an additional ∼30 passages before showing signs of senescence. In this study, cells with <30 passages were considered 'young' cells. The percentage of SA-β-gal-positive cells was <10%. Cells with >30 passages were considered 'senescent' cells; they had >30% SA-β-gal-positive cells. The human immortalized foreskin fibroblast BJ-hTERT, AD293, 293T and human lung adenocarcinoma A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco Thermo Fisher Scientific) with 10% FBS. The colorectal cancer HCT116 and isogenic $p53^{-/-}$ and $p21^{-/-}$ cells were maintained in McCoy's 5A medium (Sigma-Aldrich) with 10% FBS. The H1299 cells were maintained in RPMI Medium 1640 (Gibco Thermo Fisher Scientific) containing 10% FBS.

Growth curves

The proliferative capacity of cells was monitored by seeding 5×10^5 cells in a 30-mm dish containing 10% FBS. Adenovirus carrying *lacZ* or *SERPINB2* was added the subsequent day. Cell numbers were determined by digesting the cells with trypsin, staining with 0.2% Trypan Blue and counting using a hemocytometer.

SA-β-gal 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 min 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 h. For each sample, ≥250 cells were counted in randomly chosen fields.

Immunofluorescence assay

The cells were grown on slides and fixed with 4% formaldehyde at room temperature for 10 min. 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 (NA61, Calbiochem, 1:400) or anti-H3K9me3 (07-442, EMD Millipore, 1:400) 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 h. DAPI staining was conducted using mounting solution (Prolong Gold antifade reagent with DAPI), and the cells were visualized using fluorescence or confocal microscopy.

Immunoblotting

Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl) 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 to nitrocellulose membranes and probed with anti-serpinB2 (polyclonal rabbit antibodies generated from recombinant serpinB2), anti-p53 (OP43, Calbiochem), anti-p21 (2947, Cell Signaling Technology), anti-p16 (NA29, Calbiochem), anti-Rb (OP66, Calbiochem), anti-phospho-Rb (Ser780) (9307, Cell Signaling Technology), 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 PCR

The total RNA was extracted using TriPure Isolation Reagent (Roche). First-strand cDNA was synthesized from 0.5 µg total RNA with a RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Real-time PCR was then performed using SYBR Green Master Mix (Roche) and a StepOne Real-Time PCR System (Applied Biosystems). The primer pairs for p21 were 5′-TCAGAGGAGGTGAGAGAGCGG-3′ and 5′-CGCAT-GGGTTCTGACGGACA-3′. Primer pairs for GAPDH were 5′-GAAGG-TGAAGGTCGGAGTCAA-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} reached 0.4, and 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added. The cells were grown at 25°C for another 16 h before harvest by centrifugation. Cells were resuspended in 10 ml 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 9000 ***g*** for 20 min at 4°C to obtain total cell free extracts. Then, 0.5 ml Ni-NTA-agarose (ABT) was added to the total cell free extracts and incubated at 4°C for 16 h. The resin was serially washed with sonication buffer containing 20 mM and 30 mM imidazole, and eluted with 2 ml sonication buffer containing 200 mM imidazole and 20% glycerol. Purified protein was aliquoted and frozen in a dry-ice and ethanol bath.

Chromatin immunoprecipitation

IMR90 cells were incubated with 0.75% formaldehyde for 10 min. 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 1 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 min with shaking. The supernatants were incubated with RNase A (0.5 mg/ml) at 65°C for 4 h and purified using a 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′-TGCAGAGG-ATGGATTGTTCA-3′), p21 distal promoter (5′-CCTATGCTGCCTGCT-TCCCAGGAA-3′ and 5′-TAGCCACCAGCCTCTTCTATGCCAG-3′) (Ishikawa et al., 2007); serpinB2 promoter region (5′-TCTTGAAACTG-GGGCTGACA-3′ and 5′-CCTCTGTCTTTTGATCTGTGTCC-3′), serpinB2 C-terminal region (5′-ATGGTCCTGGTGAATGCTGT-3′ and 5′-TAGCAAGTCTATAATGAGCGGTCT-3′).

Reporter analysis

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, 5×10^5 cells were transfected with P_{CMV}-SEAP and $P_{serpinB2}$-SEAP, respectively, and 1×10^4 of each of the transfected cell types were grown in 96-well plates and incubated at 37°C for 24 h. Varying amounts of doxoribicin were added, and the cells were incubated for another 24 h. Culture media were collected and heated at 65°C for 10 min to inactivate heat-labile phosphatases. An equal amount of SEAP buffer (2M diethanolamine, 1 mM $MgCl_2$ and 20 mM L-homoarginine) was added to the medium, and *p*-nitrophenylphosphate was added to a final concentration of 12 mM. Absorptions at 405 nm were measured, and the rate of absorption increase was determined.

Statistical analysis

Student's *t*-test was applied to determine the statistical differences between the means of two groups. $P<0.05$ was considered significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: H.-H.H., Y.-C.C., J.-R.J., J.-J.L.; Methodology: H.-H.H., Y.-C.C., J.-R.J., J.-J.L.; Validation: H.-H.H., J.-R.J.; Formal analysis: H.-H.H.; Investigation: H.-H.H., Y.-C.C., J.-R.J.; Resources: H.-H.H., Y.-C.C., J.-J.L.; Data curation: H.-H. H., Y.-C.C.; Writing - original draft: H.-H.H., J.-J.L.; Writing - review & editing: H.-H.H., J.-J.L.; Supervision: J.-J.L.; Project administration: J.-J.L.; Funding acquisition: J.-J.L.

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Supplementary information

Supplementary information available online at
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References

Abbas, T. and Dutta, A. (2009). p21 in cancer: intricate networks and multiple activities. *Nat. Rev. Cancer* **9**, 400-414.

Acosta, J. C., O'Loghlen, A., Banito, A., Guijarro, M. V., Augert, A., Raguz, S., Fumagalli, M., Da Costa, M., Brown, C., Popov, N. et al. (2008). Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell* **133**, 1006-1018.

Allsopp, R. C., Chang, E., Kashefi-Aazam, M., Rogaev, E. I., Piatyszek, M. A., Shay, J. W. and Harley, C. B. (1995). Telomere shortening is associated with cell division *in vitro* and *in vivo*. *Exp. Cell Res.* **220**, 194-200.

Amador, V., Ge, S., Santamaría, P. G., Guardavaccaro, D. and Pagano, M. (2007). APC/C(Cdc20) controls the ubiquitin-mediated degradation of p21 in prometaphase. *Mol. Cell* **27**, 462-473.

Bendjennat, M., Boulaire, J., Jascur, T., Brickner, H., Barbier, V., Sarasin, A., Fotedar, A. and Fotedar, R. (2003). UV irradiation triggers ubiquitin-dependent degradation of $p21^{WAF1}$ to promote DNA repair. *Cell* **114**, 599-610.

Bloom, J., Amador, V., Bartolini, F., DeMartino, G. and Pagano, M. (2003). Proteasome-mediated degradation of p21 via N-terminal ubiquitinylation. *Cell* **115**, 71-82.

Bodnar, A. G., Ouellette, M., Frolkis, M., Holt, S. E., Chiu, C.-P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S. and Wright, W. E. (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**, 349-352.

Boncela, J., Przygodzka, P., Papiewska-Pajak, I., Wyroba, E. and Cierniewski, C. S. (2011). Association of plasminogen activator inhibitor type 2 (PAI-2) with proteasome within endothelial cells activated with inflammatory stimuli. *J. Biol. Chem.* **286**, 43164-43171.

Bornstein, G., Bloom, J., Sitry-Shevah, D., Nakayama, K., Pagano, M. and Hershko, A. (2003). Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase. *J. Biol. Chem.* **278**, 25752-25757.

Brown, J. P., Wei, W. and Sedivy, J. M. (1997). Bypass of senescence after disruption of $p21^{CIP1/WAF1}$ gene in normal diploid human fibroblasts. *Science* **277**, 831-834.

Bunz, F., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W. and Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G_2 arrest after DNA damage. *Science* **282**, 1497-1501.

Campisi, J. and d'Adda di Fagagna, F. (2007). Cellular senescence: when bad things happen to good cells. *Nat. Rev. Mol. Cell Biol.* **8**, 729-740.

Chen, Q. and Ames, B. N. (1994). Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells. *Proc. Natl. Acad. Sci. USA* **91**, 4130-4134.

Chen, Q. M., Bartholomew, J. C., Campisi, J., Acosta, M., Reagan, J. D. and Ames, B. N. (1998). Molecular analysis of H_2O_2-induced senescent-like growth arrest in normal human fibroblasts: p53 and Rb control G_1 arrest but not cell replication. *Biochem. J.* **332**, 43-50.

Chen, H.-L., Lu, C.-Y., Hsu, Y.-H. and Lin, J.-J. (2004). Chromosome positional effects of gene expressions after cellular senescence. *Biochem. Biophys. Res. Commun.* **313**, 576-586.

Croucher, D. R., Saunders, D. N., Lobov, S. and Ranson, M. (2008). Revisiting the biological roles of PAI2 (SERPINB2) in cancer. *Nat. Rev. Cancer* **8**, 535-545.

d'Adda di Fagagna, F. (2008). Living on a break: cellular senescence as a DNA-damage response. *Nat. Rev. Cancer* **8**, 512-522.

Debacq-Chainiaux, F., Borlon, C., Pascal, T., Royer, V., Eliaers, F., Ninane, N., Carrard, G., Friguet, B., de Longueville, F., Boffe, S. et al. (2005). Repeated exposure of human skin fibroblasts to UVB at subcytotoxic level triggers premature senescence through the TGF-β1 signaling pathway. *J. Cell Sci.* **118**, 743-758.

Delhase, M., Kim, S.-Y., Lee, H., Naiki-Ito, A., Chen, Y., Ahn, E.-R., Murata, K., Kim, S.-J., Lautsch, N., Kobayashi, K. S. et al. (2012). TANK-binding kinase 1 (TBK1) controls cell survival through PAI-2/serpinB2 and transglutaminase 2. *Proc. Natl. Acad. Sci. USA* **109**, E177-E186.

Dickinson, J. L., Bates, E. J., Ferrante, A. and Antalis, T. M. (1995). Plasminogen activator inhibitor type 2 inhibits tumor necrosis factor α-induced apoptosis. *J. Biol. Chem.* **270**, 27894-27904.

Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O. et al. (1995). A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc. Natl. Acad. Sci. USA* **92**, 9363-9367.

El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W. and Vogelstein B. (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell* **7**, 817-825.

Eren, M., Boe, A. E., Murphy, S. B., Place, A. T., Nagpal, V., Morales-Nebreda, L., Urich, D., Quaggin, S. E., Budinger, G. R. S., Mutlu, G. M. et al. (2014). PAI-1–regulated extracellular proteolysis governs senescence and survival in Klotho mice. *Proc. Natl. Acad. Sci. USA* **111**, 7090-7095.

Fish, R. J. and Kruithof, E. K. (2006). Evidence for serpinB2-independent protection from TNF-α-induced apoptosis. *Exp. Cell Res.* **312**, 350-361.

Grillari, J. and Grillari-Voglauer, R. (2010). Novel modulators of senescence, aging, and longevity: small non-coding RNAs enter the stage. *Exp. Gerontol.* **45**, 302-311.

Han, J., Kim, Y.-L., Lee, K.-W., Her, N.-G., Ha, T.-K., Yoon, S., Jeong, S.-I., Lee, J.-H., Kang, M.-J., Lee, M.-G. et al. (2013). ZNF313 is a novel cell cycle activator with an E3 ligase activity inhibiting cellular senescence by destabilizing $p21^{WAF1}$. *Cell Death Differ.* **20**, 1055-1067.

Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. and Elledge, S. J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75**, 805-816.

Hayflick, L. (1965). The limited *in vitro* lifetime of human diploid cell strains. *Exp. Cell Res.* **37**, 614-636.

Hayflick, L. and Moorhead, P. S. (1961). The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**, 585-621.

Ishikawa, K., Ishii, H., Murakumo, Y., Mimori, K., Kobayashi, M., Yamamoto, K.-I., Mori, M., Nishino, H., Furukawa, Y. and Ichimura, K. (2007). Rad9 modulates the $P21^{WAF1}$ pathway by direct association with p53. *BMC Mol. Biol.* **8**, 37.

Jin, Y., Lee, H., Zeng, S. X., Dai, M.-S. and Lu, H. (2003). MDM2 promotes $p21^{waf1/cip1}$ proteasomal turnover independently of ubiquitylation. *EMBO J.* **22**, 6365-6377.

Jung, Y.-S., Qian, Y. and Chen, X. (2010). Examination of the expanding pathways for the regulation of p21 expression and activity. *Cell. Signal.* **22**, 1003-1012.

Kortlever, R. M., Higgins, P. J. and Bernards, R. (2006). Plasminogen activator inhibitor-1 is a critical downstream target of p53 in the induction of replicative senescence. *Nat. Cell Biol.* **8**, 878-884.

Kruithof, E. K. O., Vassalli, J.-D., Schleuning, W.-D., Mattaliano, R. J. and Bachmann, F. (1986). Purification and characterization of a plasminogen activator inhibitor from the histiocytic lymphoma cell line U-937. *J. Biol. Chem.* **261**, 11207-11213.

Kruithof, E. K. O., Tran-Thang, C., Gudinchet, A., Hauert, J., Nicoloso, G., Genton, C., Welti, H. and Bachmann, F. (1987). Fibrinolysis in pregnancy: a study of plasminogen activator inhibitors. *Blood* **69**, 460-466.

Kruithof, E. K. O., Baker, M. S. and Bunn, C. L. (1995). Biological and clinical aspects of plasminogen activator inhibitor type 2. *Blood* **86**, 4007-4024.

Kuilman, T. and Peeper, D. S. (2009). Senescence-messaging secretome: SMS-ing cellular stress. *Nat. Rev. Cancer* **9**, 81-94.

Kuilman, T., Michaloglou, C., Vredeveld, L. C. W., Douma, S., van Doorn, R., Desmet, C. J., Aarden, L. A., Mooi, W. J. and Peeper, D. S. (2008). Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell* **133**, 1019-1031.

Kumar, S., Millis, A. J. T. and Baglioni, C. (1992). Expression of interleukin 1-inducible genes and production of interleukin 1 by aging human fibroblasts. *Proc. Natl. Acad. Sci. USA* **89**, 4683-4687.

Kunz, C., Pebler, S., Otte, J. and von der Ahe, D. (1995). Differential regulation of plasminogen activator and inhibitor gene transcription by the tumor suppressor p53. *Nucleic Acids Res.* **23**, 3710-3717.

Law, R. H. P., Zhang, Q., McGowan, S., Buckle, A. M., Silverman, G. A., Wong, W., Rosado, C. J., Langendorf, C. G., Pike, R. N., Bird, P. I. et al. (2006). An overview of the serpin superfamily. *Genome Biol.* **7**, 216.

Lin, Y.-C., Hsu, C.-L., Shih, J.-W. and Lin, J.-J. (2001). Specific binding of single-stranded telomeric DNA by Cdc13p of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **276**, 24588-24593.

McConnell, B. B., Starborg, M., Brookes, S. and Peters, G. (1998). Inhibitors of cyclin-dependent kinases induce features of replicative senescence in early passage human diploid fibroblasts. *Curr. Biol.* **8**, 351-354.

Narita, M., Nuñez, S., Heard, E., Narita, M., Lin, A. W., Hearn, S. A., Spector, D. L., Hannon, G. J. and Lowe, S. W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* **113**, 703-716.

Schroder, W. A., Le, T. T. T., Major, L., Street, S., Gardner, J., Lambley, E., Markey, K., MacDonald, K. P., Fish, R. J., Thomas, R. et al. (2010). A physiological function of inflammation-associated SerpinB2 is regulation of adaptive immunity. *J. Immunol.* **184**, 2663-2670.

Sharpless, N. E. and Sherr, C. J. (2015). Forging a signature of *in vivo* senescence. *Nat. Rev. Cancer* **15**, 397-408.

Sheaff, R. J., Singer, J. D., Swanger, J., Smitherman, M., Roberts, J. M. and Clurman, B. E. (2000). Proteasomal turnover of $p21^{Cip1}$ does not require $p21^{Cip1}$ ubiquitination. *Mol. Cell* **5**, 403-410.

Stein, G. H., Drullinger, L. F., Soulard, A. and Dulić, V. (1999). Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. *Mol. Cell. Biol.* **19**, 2109-2117.

Thorsen, S., Philips, M., Selmer, J., Lecander, I. and Astedt, B. (1988). Kinetics of inhibition of tissue-type and urokinase-type plasminogen activator by plasminogen-activator inhibitor type 1 and type 2. *Eur. J. Biochem.* **175**, 33-39.

Ullbro, C., Kinnby, B., Lindberg, P. and Matsson, L. (2004). Tissue plasminogen activator (t-PA) and placental plasminogen activator inhibitor (PAI-2) in gingival crevicular fluid from patients with Papillon-Lefevre syndrome. *J. Clin. Periodontol.* **31**, 708-712.

Wang, W., Nacusi, L., Sheaff, R. J. and Liu, X. (2005). Ubiquitination of p21Cip1/WAF1 by SCFSkp2: substrate requirement and ubiquitination site selection. *Biochemistry* **44**, 14553-14564.

Wang, Z., Nie, Z., Chen, W., Zhou, Z., Kong, Q., Seth, A. K., Liu, R. and Chen, C. (2013). RNF115/BCA2 E3 ubiquitin ligase promotes breast cancer cell proliferation through targeting $p21^{Waf1/Cip1}$ for ubiquitin-mediated degradation. *Neoplasia* **15**, 1028-1035.

West, M. D., Shay, J. W., Wright, W. E. and Linskens, M. H. K. (1996). Altered expression of plasminogen activator and plasminogen activator inhibitor during cellular senescence. *Exp. Gerontol.* **31**, 175-193.

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Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R. and Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. *Nature* **366**, 701-704.

Zhang, H., Pan, K.-H. and Cohen, S. N. (2003). Senescence-specific gene expression fingerprints reveal cell-type-dependent physical clustering of up-regulated chromosomal loci. *Proc. Natl. Acad. Sci. USA* **100**, 3251-3256.

Zhang, Z., Wang, H., Li, M., Agrawal, S., Chen, X. and Zhang, R. (2004). MDM2 is a negative regulator of $p21^{WAF1/CIP1}$, independent of p53. *J. Biol. Chem.* **279**, 16000-16006.

Zhang, S.-J., Zou, M., Lu, L., Lau, D., Ditzel, D. A. W., Delucinge-Vivier, C., Aso, Y., Descombes, P. and Bading, H. (2009). Nuclear calcium signaling controls expression of a large gene pool: identification of a gene program for acquired neuroprotection induced by synaptic activity. *PLoS Genet.* **5**, e1000604.

Zhang, C., Ponugoti, B., Tian, C., Xu, F., Tarapore, R., Batres, A., Alsadun, S., Lim, J., Dong, G. and Graves, D. T. (2015). FOXO1 differentially regulates both normal and diabetic wound healing. *J. Cell Biol.* **209**, 289-303.

Zhao, W., Lin, Z. X. and Zhang, Z. Q. (2004). Cisplatin-induced premature senescence with concomitant reduction of gap junctions in human fibroblasts. *Cell Res.* **14**, 60-66.

Zuckerman, V., Wolyniec, K., Sionov, R. V., Haupt, S. and Haupt, Y. (2009). Tumour suppression by p53: the importance of apoptosis and cellular senescence. *J. Pathol.* **219**, 3-15.