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FBXL5-mediated degradation of single-stranded DNA-binding protein hSSB1 controls DNA damage response

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ABSTRACT

Human single-strand (ss) DNA binding proteins 1 (hSSB1) has been shown to participate in DNA damage response and maintenance of genome stability by regulating the initiation of ATM-dependent signaling. ATM phosphorylates hSSB1 and prevents hSSB1 from ubiquitin-proteasome-mediated degradation. However, the E3 ligase that targets hSSB1 for destruction is still unknown. Here, we report that hSSB1 is the *bona fide* substrate for an Fbxl5-containing SCF (Skp1-Cul1-F box) E3 ligase. Fbxl5 interacts with and targets hSSB1 for ubiquitination and degradation, which could be prevented by ATM-mediated hSSB1 T117 phosphorylation. Furthermore, cells overexpression of Fbxl5 abrogated the cellular response to DSBs, including activation of ATM and phosphorylation of ATM targets and exhibited increased radiosensitivity, chemosensitivity and defective checkpoint activation after genotoxic stress stimuli. Moreover, the protein levels of hSSB1 and Fbxl5 showed an inverse correlation in lung cancer cells lines and clinical lung cancer samples. Therefore, Fbxl5 may negatively modulate hSSB1 to regulate DNA damage response, implicating Fbxl5 as a novel, promising therapeutic target for lung cancers.

INTRODUCTION

DNA double-strand breaks (DSBs) could be induced by environmental exposure to ionizing radiation (IR), ultraviolet light and genotoxic agents as well as endogenous factors including replication fork collapse and oxidative stress (1). To counteract DNA damage, repair mechanisms specific for DSBs have evolved. Eukaryotic cells utilize two primary mechanisms to repair DNA DSBs: non-homologous end joining and homologous recombination (HR). HR is the major pathway for DSB repair (2). To initiate HR, DNA is resected and then bound by RPA, eukaryotic single-strand DNA (ssDNA)-binding protein (SSB), to facilitate Rad51 nucleofilament formation and strand invasion (3). RPA has three subunits (RPA70, RPA32 and RPA14) and plays essential roles in cell-cycle regulation and DNA replication and repair (4–6).

Recently, a novel SSB protein hSSB1 was recently identified as a key player in the cellular response to DNA damage (7). HSSB1 exists as a member of a heterotrimeric complex called Sensor of Single-Stranded DNA complex 1 (SOSS1), together with SOSSA(INTS3) and SOSSC(C9orf80) (8–11). Cells deficient in hSSB1 exhibit increased radiosensitivity, defective checkpoint activation and genomic instability, suggesting a role for hSSB1 in HR-mediated repair (7). HSSB1 is a short-lived protein and rapidly accumulated in the cell in response to DNA damage. Phosphorylation of hSSB1 at T117 by ataxia telangiectasia mutated (ATM) kinase prevents its degradation by the proteasome (7). However, the E3 ligase which targets hSSB1 is still unknown.

The Skp1-Cul1-F-box-protein (SCF) ubiquitin ligase is one of the most characterized E3 ligase complexes. Extensive structure studies reveal a well-conserved architecture for the multi-subunits of SCF complexes, in which the divergent F-box proteins dictating substrate specificity (12,13). The mammalian genome contains about 70 F-box proteins which are further classified into three subfamilies: Fbxws

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that contain WD-40 repeats; Fbxls contain leucine-rich repeats (LRRs); Fbxos that lack either WD-40 repeats or LRRs (14). Several F-box proteins have been reported to be involved in DNA damage response and play essential roles in the maintenance of genome stability (15).

In this study, we screened an F-box protein-targeted siRNA library to identify novel E3 ligase that is responsible for the ubiquitin-proteasome-degradation of hSSB1. We identified the F-box protein, Fbxl5, as the targeting subunit of a SCF E3 complex that ubiquitinates and targets hSSB1 for destruction.

MATERIALS AND METHODS

Cell culture and tissue samples

A549, NCI-H23 and NCI-H460 cells were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were culture in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. Paired lung cancer tissues and adjacent non-tumor lung tissues were collected from routine therapeutic surgery at our department. All samples were obtained with informed consent and approved by the institutional review board of Shanghai Chest Hospital.

Subcutaneous tumor model

Four weeks old male immune-deficient nude mice (BALB/c-nu) were purchased from Shanghai Slac Laboratory Animal Co., Ltd., bred at the facility of laboratory animals, Shanghai Jiao Tong University and housed in micro-isolator individually ventilated cages with water and food. All experimental procedures were carried out according to the regulations and internal biosafety and bioethics guidelines of Shanghai Jiao Tong University and the institutional review board of Shanghai Chest Hospital. Mice were divided into two groups of eight mice each. Each mouse was simultaneously injected subcutaneously with 5×10^6 of A549 cells transfected with Fbxl5 or vector control. Mice were monitored daily and all formed subcutaneous tumors. The tumor size was measured by with vernier caliper weekly, and calculated according to the formula as follow: $V = (1/2)\ ab2$, where V = tumor volume; a = the largest diameter of tumor; b = the most trails of tumor. At the sixth weekend after planting, all nude mice were euthanized, and xenograft tumors were weighted.

Antibodies and reagents

Immunoblotting was performed using antibodies as indicated: anti-Cullin1 (D-5, sc-17775, Santa Cruz), anti-Skp1 (H-6, sc-5281, Santa Cruz), anti-Chk1 (2G11D5, sc-56288, Santa Cruz), anti-GAPDH (G-9, sc-365062, Santa Cruz), anti-beta-TcrP (4394, Cell Signaling), anti-hSSB1 (ab85752, Abcam), anti-FBXL5 (ab68069, Abcam), anti-FBXO6 (C-20, sc-323856, Santa Cruz), anti-Skp2 (H-435, sc-7164, Santa Cruz), anti-Phospho-ATM(Ser1981) (D25E5, Cell Signaling), anti-ATM(D2E2, Cell Signaling), anti-Flag M2 (F1804, Sigma), anti-HA (3724, Cell Signaling), anti-HA (2365, Cell Signaling). FLAG M2 Affinity Gel (A2220) was purchased from Sigma. MLN4924 was purchased from Medkoo. Caffeine, etoposide and cycloheximide (CHX) were purchased from Sigma.

RNA interference

The SMARTpool siRNA library targeting all known human F box genes was purchased from Dharmacon. For siRNA screening cells were transfected with 150 nM siRNA with the DharmaFECT 1 reagent. Expression levels of Fbxl5 and actin were analyzed by western blot. The Lentiviral Human FBXL5 shRNA was purchased from Thermo scientific and the target sequences for short hairpin RNA (sh-RNA)-expressing plasmids were 5′-AAACCCAGGGCTGCCTTGGAAAAG-3′ and 5′-AAACCCAGGGCTGCCTTGGAAAAG-3′.

Plasmids

FBXL5, hSSB1, Cullin1 and SKP1 were amplified from A549 cells by polymerase chain reaction and cloned into the pBabe retroviral vector or pcDNA3.1 vector. Beta-TrcP and His-ubiquitin plasmid were previously described (16,17). HSSB1 mutants were generated using QuickChange Site-Directed Mutagenesis Kit (Stratagene). All cDNAs were completely sequenced.

Immunoprecipitation (IP)

The IP procedure was described previously (16). Briefly, cells at ∼80% confluence were lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Nonidet P40, Roche complete ethylenediaminetetraacetic acid-free protease inhibitor cocktail) for 20 min at 4°C. Lysates were cleared using centrifugation (13 000 revolutions per minute, 10 min), and the resulting material subjected to IP with 50 μl of anti-FLAG M2 affinity resin (Sigma) overnight at 4°C with gentle inversion. Resin containing immune complexes was washed with ice cold lysis buffer four times followed by three Tris-buffered saline (TBS) washes. Proteins were eluted with 3 × Flag-peptide (Sigma) in TBS for 30 min.

Western blot

Cells were harvested and lysed with lysis buffer (62.5 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% w/v SDS, 10% glycerol). After centrifugation, proteins were quantified and separated by 10 sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to NC membrane. After blocking with 5% non-fat milk in phosphate buffered saline (PBS), membranes were immunoblotted with antibodies as indicated, followed by horseradish peroxidase-linked secondary antibodies (Cell Signaling). The signals were detected by SuperSignal West Pico Chemiluminescent Substrate kit according to manufacturer's instructions.

In vivo ubiquitination assays

Cells were lysed in a phosphate/guanidine buffer A (6 M guanidine-HCl, 10 mM Tris-HCl, pH 8.0, 50 mM Na2HPO4/NaH2PO4, 100 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol). The ubiquitinated proteins were precipitated with Ni-NTA-agarose (QIA-GEN, Chatsworth, CA, USA) overnight at 4°C. The beads were washed five times with buffer B (8 M urea, 100 mM NaCl, 50 mM Na2HPO4/NaH2PO4, pH 8.0). The bound proteins were eluted by boiling in SDS-PAGE sample buffer and analyzed by immunoblot.

In vitro ubiquitylation assays

Flag-hSSB1 was co-transfected with either HA-Fbxl5 or HA-Fbxl5(ΔF-box) into HEK293T cells. Thirty-six hours after transfection, cells were incubated with MG132 for 3 h before collection. Fbxl5(wild-type or mutant) was immunoprecipitated with anti-HA agarose beads. The beads were washed four times in lysis buffer and twice in ubiquitylation reaction buffer (10 mM TrisHCl, pH 7.5, 100 mM NaCl and 0.5 mM DTT). *In vitro* ubiquitylation assays were performed on immunoprecipitated beads with 0.1 μM E1 0.25 μM Ubch3, 0.25 μM Ubch5c, 1 μM ubiquitin aldehyde, 2.5 μg/μl ubiquitin and 5 mM ATP. Samples were incubated for 2 h at 30°C and analyzed by immunoblot.

Clonogenic assays

Sensitivity of A549 and NCI-H460 cells to irradiation was determined by clonogenic assay after the variable doses of radiation (0Gy, 2Gy, 4Gy, 6Gy, 8Gy) using a linear accelerator (Siemens, Germany). After incubation for 10–14 days, colonies formed were fixed with methanol and stained with 1% crystal violet. Only colonies consisting of more than 50 cells were counted. The data were fitted to linear-quadratic model using Sigmaplot software, where survival curves were generated and radiosensitivity parameters were calculated. Experiments were repeated three times.

Apoptosis assays

Annexin-V assay was carried out by Annexin V-FITC Apoptosis Detection Kit according to manufacturer's instruction (BD, San Diego, CA, USA). Briefly, 3×10^5 cells were collected, washed twice with cold PBS, resuspended in binding buffer and incubated with Annexin V-FITC at room temperature, followed by addition of propidium iodide for 5 min. Fluorescent intensities were determined on flow cytometry (Beckman Coulter, Miami, FL, USA).

Statistical analysis

Data are expressed as the mean ± SEM from at least three separate experiments. Differences between groups were analyzed using Student's *t*-test and chi-square test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Cullin1-based ubiquitin E3 ligase mediates the degradation of hSSB1

The stability of hSSB1 is regulated by the ubiquitin proteasome system, as hSSB1 was rapidly stabilized in the presence of MG132 in A549 and NCI-H460 cells (Figure 1A, Supplementary Figure S1). IR and anti-cancer drugs, which can induce DNA DSB, could significantly prevent the degradation of the basal level of hSSB1 in A549 cells (Figure 1B, Supplementary Figure S2). These observations raise the issue of which E3 ligase is required for the destruction of hSSB1. Because the Cullin-based ubiquitin E3 ligases are involved in the degradation of many key proteins during DNA damage response (18), we then asked whether one of the Cullin-based ubiquitin E3 ligases is required for the degradation of hSSB1. To test this possibility, A549 cells were treated with MLN4924, which is an investigational NEDD8-activating enzyme inhibitor (19,20). As depicted in Figure 1C, MLN4924 treatment significantly removed Cullin1 Neddnaytion and caused hSSB1 accumulation in a dose-dependent manner. To further test which Cullin is responsible for the degradation of hSSB1, four dominant negative (DN) Cullin members, including DN-Cullin1, DN-Cullin2, DN-Cullin3 and DN-Cullin4A, were expressed into A549 cells. As shown in Figure 1D, among those DN-Cullin members, only DN-Cullin1 could significantly prevent the degradation of hSSB1 (Figure 1D). Force expression of Cullin1 resulted in the decrease of hSSB1, which could be rescued by either MG132 or MLN4924 administration, indicating hSSB1 maybe a substrate of a SCF E3 ligase (Figure 1E). To this end, we asked whether HSSB1 interacts with the components of SCF complex. Endogenous hSSB1 was immunoprecipitated by hSSB1 antibody from A549 cell lysate and both Cullin1 and Skp1 were detected in the precipitated HSSB1 complex (Figure 1F). These results suggest that hSSB1 associates with the SCF E3 ligase complex *in vivo*.

Fbxl5 is required for the destruction of hSSB1

SCF complex is a family of multi-subunit ubiquitin E3 ligase, in which the F-box proteins determine the substrate specificity (13). We reasoned that loss of the F-box protein that targets hSSB1 for degradation would increase the basal level of hSSB1, as observed in Cullin1-depleted cells (Figure 2B). To identify potential F-box proteins that specifically recognize hSSB1 as a substrate, we transfected A549 cells with a validated SMARTpool siRNA library (Dharmacon) targeting all known human F-box genes. Immunoblot analysis of A549 cell extracts revealed 70–90% reduction in the level of several representative F-box proteins at 48 h after siRNA transfection (Figure 2B). After siRNA transfection, we found that depletion of fbxl5, but not other F-box proteins, including beta-TrcP1, Skp2 and Fbxo6, consistently increased the basal expression level of hSSB1 (Figure 2B and Supplementary Figure S3). An independent shRNA vector targeting Fbxl5 also increased the level of endogenous hSSB1 (Figure 2C). Moreover, force expressed Fbxl5 caused the decrease of endogenous hSSB1 in a dose-dependent manner (Figure 2D). Taken together,

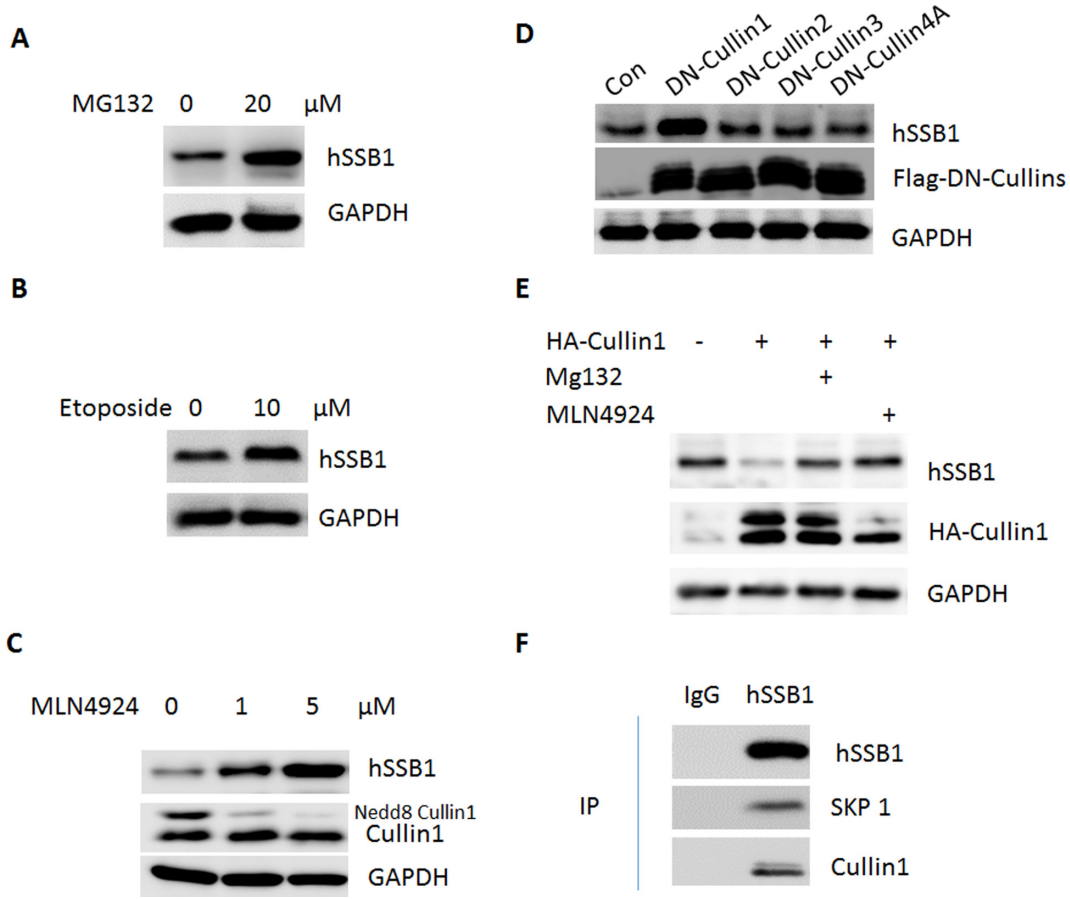

Figure 1. Cullin1-based ubiquitin E3 ligase mediates the degradation of hSSB1. (A) Western blot analysis of A549 cells treated with 20 μM MG132 for 8 h. (B) Western blot analysis of A549 cells treated with 10 μM Etopside for 24 h. (C) Western blot analysis of A549 cells treated with 0, 1, 5 μM MLN4924 for 6 h. (D) Western blot analysis of A549 cells transfected with DN-Cullin1, DN-Cullin2, DN-Cullin3 or DN-Cullin4A plasmid, respectively. (E) Western blot analysis of A549 cells transfected with control or HA-Cullin1plasimd for 24 h, 20 μM MG132 or 5 μM MLN4924 were added 6 h before harvested. (F) Endogenous HSSB1 was immunoprecipitated by HSSB1 antibody from A549 cell lysate. Immunoprecipitates was detected by western blot using indicated antibodies.

these data suggested that Fbxl5 is required for the destruction of hSSB1.

Fbxl5 interacts with hSSB1

F-box protein-mediated degradation of its substrates requires the physical interaction between F-box protein and its substrates. We then examined the interaction between Fbxl5 and hSSB1 using tagged proteins exogenously expressed in 293T cells. Both Fbxl5 and beta-TrcP1 were able to pull down the intact SCF complex. However, immunoprecipitates of Fbxl5, but not beta-TrcP1 contained Flag-tagged hSSB1 (Figure 3A), suggesting the specificity of the interaction between Fbxl5 and hSSB1. Furthermore, endogenous Fbxl5 was co-immunoprecipitated with hSSB1 in extracts from non-transfected cells (Figure 3B). Moreover, GST-hSSB1 but not GST tag alone captured HA-tagged Fbxl5 purified from 293T cell extract (Figure 3C). To identify the interaction interfaces on hSSB1 with Fbxl5, we further utilized the *in vitro* GST pull-down assay and found that the 101–158 AA of hSSB1 was required for the binding to Fbxl5. Taken together, these data indicated that Fbxl5 interacted with hSSB1 by recognizing its 101–158 AA domain.

Fbxl5 regulates hSSB1 ubiquitination and degradation

The interaction between Fbxl5 and hSSB1 suggests a direct role of Fbxl5 in the regulation of hSSB1 ubiquitination. To test this possibility, 293T cells were transfected with His-ubiquitin, Flag-hSSB1 with or without HA-Fbxl5. Lysate were incubated with Flag M2 beads to immunoprecipitate Flag-hSSB1. The immunoprecipitates were then detected with anti-His antibody. Alternatively, cells after transfection were lysed in 6M guanidine-HCl and nickel column pull-down were performed. As depicted in Figure 4A, both assays indicated Fbxl5 could significantly enhance hSSB1 ubiquitination. To test whether Fbxl5 could ubiquitinate hSSB1 *in vitro*, 293T cells were transfected with Flag-hSSB1, HA-Fbxl5 or HA-Fbxl5(ΔF-box). After immunopurification with anti-HA resin, *in vitro* ubiquitylation of hSSB1 was performed in the presence of E1, E2s and ubiquitin. Our data showed that immunopurified wild-type Fbxl5, but not Fbxl5(ΔF-box), promoted the *in vitro* ubiquitylation of hSSB1 (Figure 4B). Indeed, silencing the

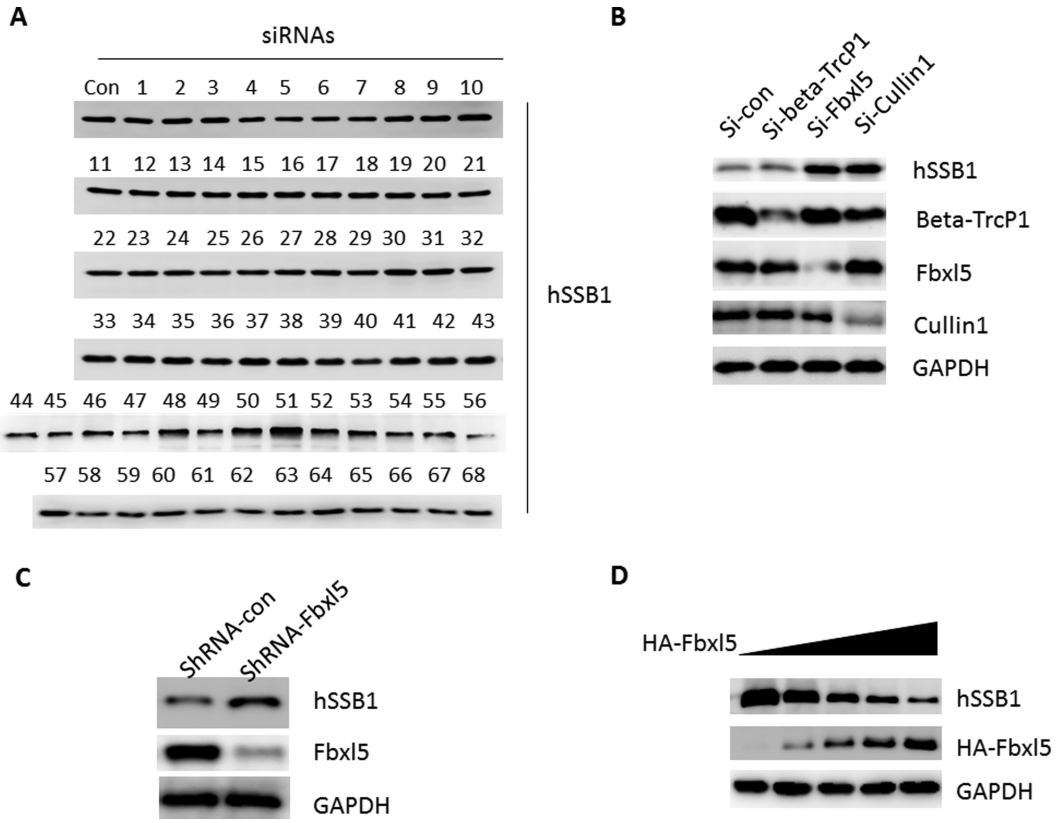

Figure 2. Fbxl5 is required for the destruction of hSSB1. (A) A549 cells were transfected with a validated SMARTpoolTM siRNA library (Dharmacon) targeting all known human F-box genes. Cells were collected and cell lysate was analysis with western blot assay with hSSB1 antibody. (B) A549 cells were transfected with siRNAs for 48 h, targeting Beta-trcp1, Fbxl5 or Cullin1, respectively. The whole cell lysate was detected by western blot using indicated antibodies. (C) A549 cells were transfected with shRNAs targeting Fbxl5 for 36 h. The whole cell lysate was detected by western blot using indicated antibodies. (D) A549 cells were transfected with increase dose of Fbxl5 plasmids for 36 h. The whole cell lysate was detected by western blot using indicated antibodies.

expression of Fbxl5 enhanced the half-life of hSSB1 (Figure 4C), suggesting that Fbxl5-induced hSSB1 ubiquitination led to hSSB1 degradation. Finally, using endogenous immunoprecipitatation assay, we found that hSSb1 ubiquitination was decreased when Fbxl5 was silenced or upon DNA damage drug treatment (Figure 4D). Taken together, our data suggested DNA damage could prevent Fbxl5-induced hSSB1 ubiquitination and degradation.

ATM-mediated hSSB1 T117 phosphorylation prevents Fbxl5-induced hSSB1 destruction

It has been reported that ATM plays a role in preventing hSSB1 degradation by inducing hSSB1 T117 phosphorylation (7). Indeed, DNA damage-induced hSSB1 stabilization is absent in $ATM^{-/-}$ cells or cells treated with caffeine (Figure 5A, Supplementary Figure S4). Moreover, we performed Co-IP experiments of hSSB1 and Fblx5 with or without DNA damage and with or without ATM kinase activity, and found that the interaction between hSSB1 and Fbxl5 was weakening with DNA damage and ATM kinase deficiency could reverse this phenomenon (Figure 5A). Silencing the expression of Fbxl5 in $ATM^{-/-}$ cells caused an obvious increase of the basal hSSB1 expression (Figure 5C), conformed a role of Fbxl5 in the regulation of hSSB1 degradation. Unlike hSSB1 WT, the protein level of hSSB1 T117A was not increased in cells treated with etoposide (Figure 5D). To investigate how ATM prevents Fbxl5-induced hSSB1 destruction, Flag-hSSB1 WT, Flag-hSSB1 T117A or hSSB1 phosphorylation mimic T117E was co-expressed in 293T cells. As depicted in Figure 5E, Fbxl5 interacted with both Flag-hSSB1 WT and Flag-hSSB1 T117A efficiently but not with hSSB1 T117E. Moreover, Fbxl5 could significantly promote hSSB1WT but not hSSB1 T117E ubiquitination (Figure 5F). It was most likely that ATM-induced hSSB1 T117 phosphorylation caused a conformation change which prevented the interaction between Fbxl5 and hSSB1.

Overexpression of Fbxl5 sensitized lung cancer cells to genotoxic stress

Fbxl5 regulated the abundance of hSSB1, leading us to ask whether Fbxl5 plays a role in DNA damage response. To test this possibility, we evaluated the cell viability on A549 and NCI-H23 cells stable expression of exogenous Fbxl5 or control vector and treated with or without several DNA-damaging agents. A549 cells overexpression of Fbxl5 exhibited hypersensitivity to IR (Figure 6A). Moreover, when compared with control cells, A549 cells overexpressing of Fbxl5 showed increased apoptosis treated with etoposide (Figure 6B) and defective checkpoint activation (Figure 6C)

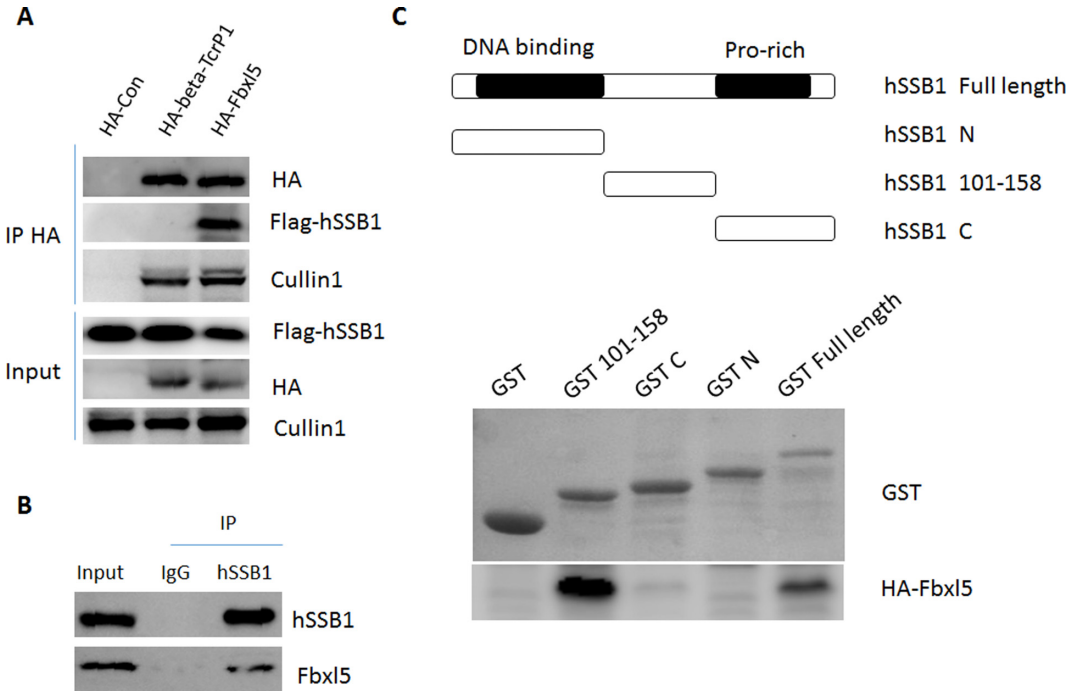

Figure 3. Fbxl5 interacts with hSSB1. (A) 293T cells were co-transfected with indicated plasmids for 48 h. The whole lysate were immunoprecipitated with HA antibody. Immunoprecipitates and the input were detected by western blot using indicated antibodies. (B) Endogenous HSSB1 was immunoprecipitated by HSSB1 antibody from A549 cell lysate. Immunoprecipitates was detected by western blot using indicated antibodies. (C) Bacterial produced GST or various GST-hSSB1 proteins were incubated with HA-Fbxl5 protein purified from 293T cells. Immunoprecipitates was detected by western blot using indicated antibodies.

when compared with mock transfected cells, including decreased activation of ATM and phosphorylation of ATM targets after etoposide treatment, suggesting that overexpression of Fbxl5 could sensitize lung cancer cells to genotoxic stress via decreased cellular response to DSBs. In consistent with these results, silencing the expression of Fbxl5 in NCI-H23 cells prevented etoposide-induced apoptosis and promoted ATM activation (Supplementary Figure S5).

To further demonstrate its function, we tested if forced expression of Fbxl5 promotes the ability of A549 cells to form xenograft tumors in nude mice. We injected $\sim 5 \times 10^6$ stable A549 cells subcutaneously into 4 weeks old BALB/c nude mice. The tumors were measured weekly for 6 consecutive weeks, and each tumor was individually weighed after the mice were euthanized. As a result, the tumor volume and weight were increased in Fbxl5-overexpressed tumors compared to control tumors (Figure 6D and E), suggesting Fbxl5 could promote tumor growth *in vivo*.

Inverse relationship between Fbxl5 and hSSB1 expression in lung cancer

To gain further insights into the regulation of hSSB1 by Fbxl5, we examined the expression of hSSB1 by Fbxl5 proteins in human lung cancer cell lines. HSSB1 and Fbxl5 proteins were variably expressed in these cell lines. Nonetheless, many of these cell lines exhibited a notable inverse relationship between the expression levels of hSSB1 by Fbxl5 (Figure 7A). There is no correlation between the levels of hSSB1 and Fbxl5 mRNA transcripts (data not shown), which is consistent with the idea that a post-transcriptional mechanism underlies the inverse relationship between these two proteins. We further examined the level of Fbxl5 protein in clinical lung cancer samples. Focusing on paired lung cancer and adjacent normal tissues, none of normal tissues expressed higher levels of Fbxl5 than cancers (Figure 7B). These data suggest that Fbxl5-mediated hSSB1 degradation may contribute to lung cancer development.

DISCUSSION

FBXL5 plays a critical role in the maintenance of cellular iron homeostasis by targeting IRP1 and IRP2 for ubiquitination and degradation in an iron-dependent manner (21,22). Global inactivation of the Fbxl5 gene results in embryonic lethality, with growth defects readily apparent prior to day E9 despite normal placentation, gastrulation and cardiovascular development (23). Loss of FBXL5 in mice induced apoptosis as a result of unrestrained IRP activity, and deletion of Irp2 in Fbxl5$^{-/-}$ mice prevented their embryonic death, suggesting that FBXL5 plays a pivotal role in the maintenance of appropriate concentrations of intracellular iron and that it is essential for embryonic development (23). However, whether Fbxl5 also participates in other biology process remains largely unexplored.

In the present study, our data reveal a novel role Fbxl5 in DNA damage response. By using unbiased siRNA library screen, we identify that Fbxl5 controls the stability of hSSB1. Our biochemical data reveal that hSSB1 is a novel substrate of Fbxl5. Fbxl5 interacts and targets hSSB1 for ubiquitination and degradation.

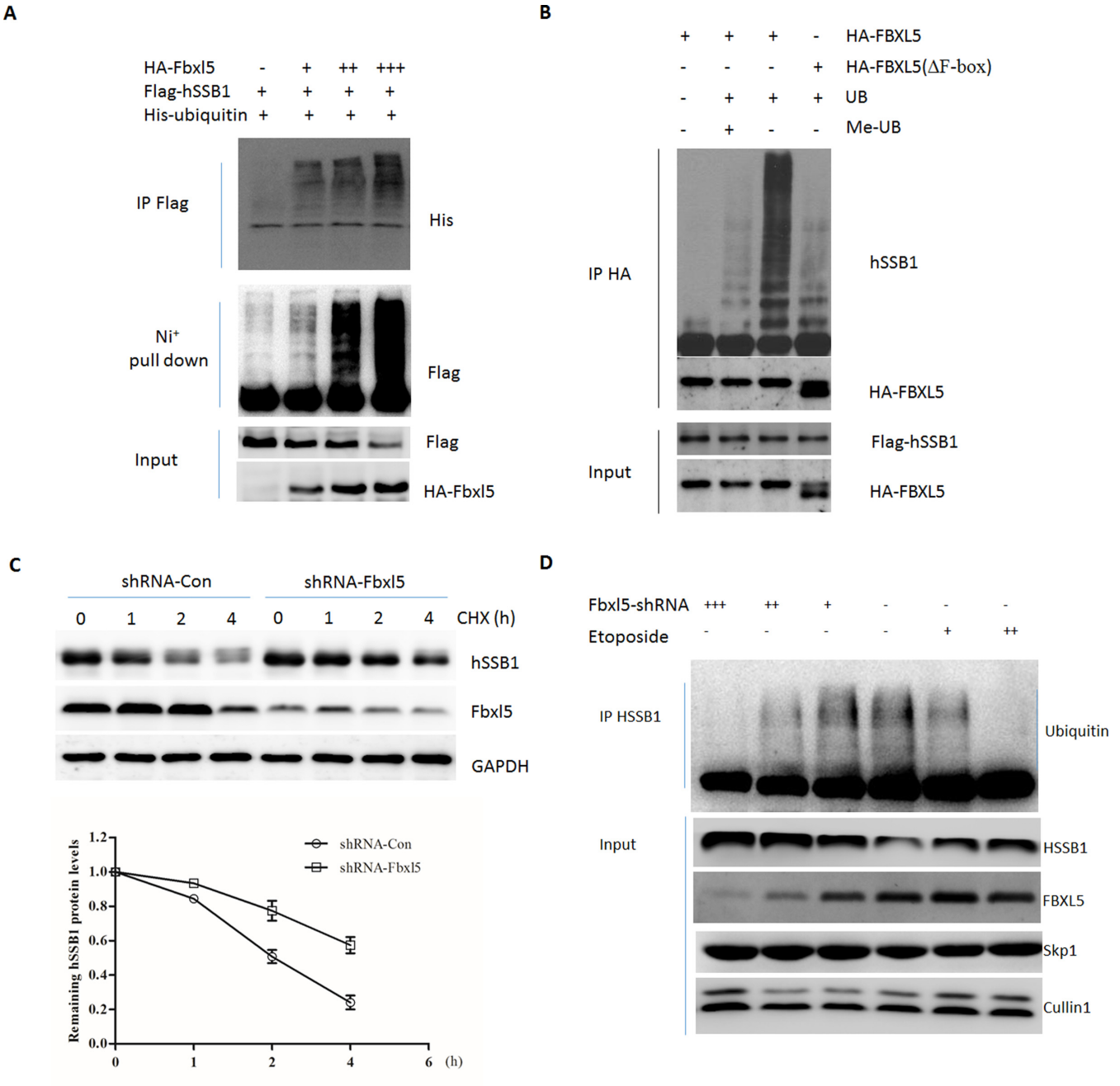

Figure 4. Fbxl5 regulates hSSB1 ubiquitination and degradation. (A) 293T cells were transfected with His-ubiquitin, Flag-hSSB1 with or without HA-Fbxl5 for 48h. Lysate were incubated with Flag M2 beads to immunoprecipitate Flag-hSSB1. Alternatively, cells after transfection were lysed in 6M guanidine-HCl and nickel column pull-down were performed. The immunoprecipitates and the input were then detected with indicated antibodies. (B) A549 cells were transfected with con-shRNA or shRNA targeting Fbxl5 for 40 h. 10 μM CHX was added for the indicated time. The whole cell lysate were detected by western blot using indicated antibodies. (C) 293T cells were transfected with Flag-hSSB1, HA-FBXL5 or HA-FBXL5(ΔF-box). After immunopurification with anti-HA resin, *in vitro* ubiquitylation of hSSB1 was performed in the presence of E1, E2s and ubiquitin (Ub). Where indicated, an excess of methylated ubiquitin(MeUb) was also added. Samples were analyzed by immunoblotting with the indicated antibodies. (D) A549 cells were transfected with con-shRNA or shRNA targeting Fbxl5 for 36 h with or without 10 μM etopside treatment. Cells were lysed and endogenous hSSB1 protein were enriched by hSSB1 antibody IP. The immunoprecipitates and the input were then detected with indicated antibodies.

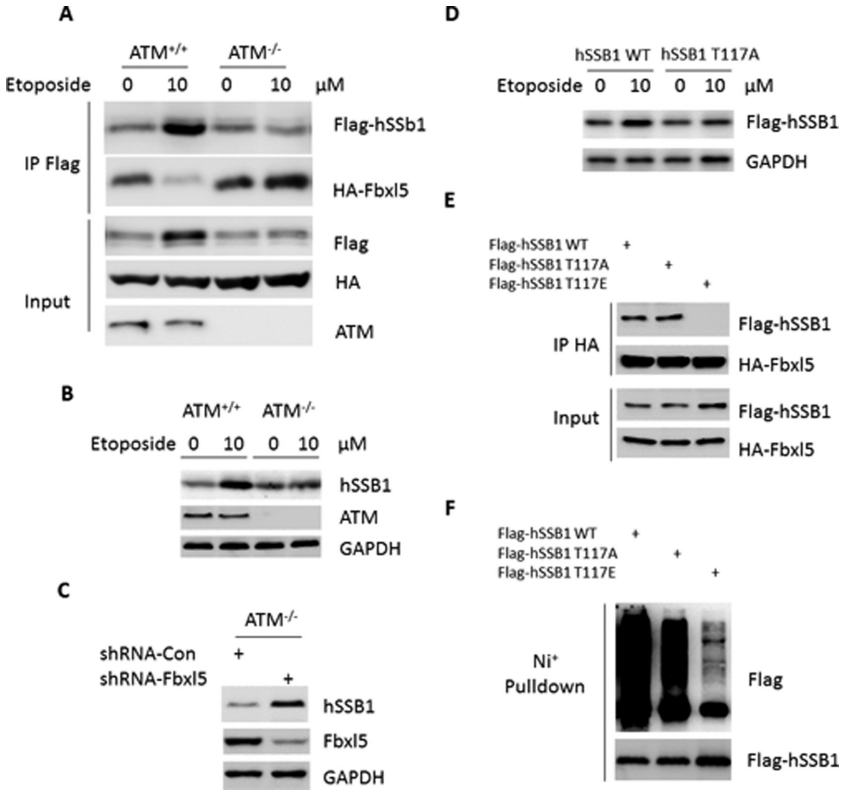

Figure 5. ATM-mediated hSSB1 T117 phosphorylation prevents Fbxl5-induced hSSB1 destruction. (A) GM00536 ($ATM^{+/+}$) and GM01526 ($ATM^{-/-}$) cells co-transfected with Flag-hSSB1 and HA-Fbxl5 were treated with or without 10 μM etopside for 24 h, lysate were incubated with Flag M2 beads to immunoprecipitate Flag-hSSB1. The immunoprecipitates and the input were detected by western blot using indicated antibodies. (B) GM00536 ($ATM^{+/+}$) and GM01526 ($ATM^{-/-}$) cells were treated with or without 10 μM etopside for 24 h, cell lysate were detected by western blot using indicated antibodies. (C) GM01526 ($ATM^{-/-}$) cells were transfected with con-shRNA or shRNA targeting Fbxl5 for 48 h. The whole cell lysate were detected by western blot using indicated antibodies. (D) A549 cells were transfected with hSSB1 WT or hSSB1 T117A for 24 h and treated with or without 10 μM etopside for 24 h. The whole cell lysate were detected by western blot using indicated antibodies. (E) 293T cells were co-transfected with HA-Fbxl5 with hSSB1 WT or mutants for 48h. The whole cell lysate was immunoprecipitated with HA antibody and followed by western blot with indicated antibodies. (F) 293T cells were co-transfected with His-Ubiquitin with hSSB1 WT or mutants for 48 h. Cells were lysed in 6M guanidine-HCl and nickel column pull-down were performed. The immunoprecipitates and the input were then detected with indicated antibodies.

Our studies provide new insights into mechanisms of DNA-damage signal transduction and reveal that Fbxl5 regulates cellular DNA damage response, including activation of ATM and phosphorylation of ATM targets. ATM kinase is a master regulator of the response to DNA DSBs, phosphorylating multiple targets to institute cell-cycle arrest and coordinate the repair of DNA damage (24). In response to genotoxic stress, ATM rapidly phosphorylates hSSB1 at T117 to prevent Fbxl5-hSSB1 interaction, leading to hSSB1 accumulation and efficient DNA DSBs repair. Dysregulation of DNA damage repair is associated with a predisposition to cancer and affects responses to DNA-damaging anti-cancer therapy (25). Although hSSB1 is an ATM target, hSSB1 function is required for activation of ATM kinase activity after DNA damage. HSSB1 has recently been shown to be required for the efficient recruitment of the MRN complex, bind directly with NBS1 and stimulate the endonuclease activity of the MRN complex to promote HR (26,27). Moreover, hSSB1 also could regulate cell-cycle progression and DNA damage checkpoints by modulating the stability of p53 and p21 in cancer cells (28,29). Cells deficient in hSSB1 exhibit increased radiosensitivity, defective checkpoint activation and genomic instability (7). We also observed that the protein levels of hSSB1 and Fbxl5 showed an inverse correlation in lung cancer cells lines and clinical lung cancer samples. In consistent with this, cells overexpression of Fbxl5 abrogates the cellular response to DSBs and exhibit increased radiosensitivity, chemosensitivity and defective checkpoint activation after genotoxic stress stimuli, leading to tumor formation *in vivo*. Thus, our results reveal a previous unknown positive feedback loop that enables the efficient activation of ATM and consequent phosphorylation of downstream proteins. Together, our data suggest that Fbxl5 may negatively modulate hSSB1 to regulate DNA damage response, implicating Fbxl5 as a novel, promising therapeutic target for lung cancer therapy.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

FUNDING

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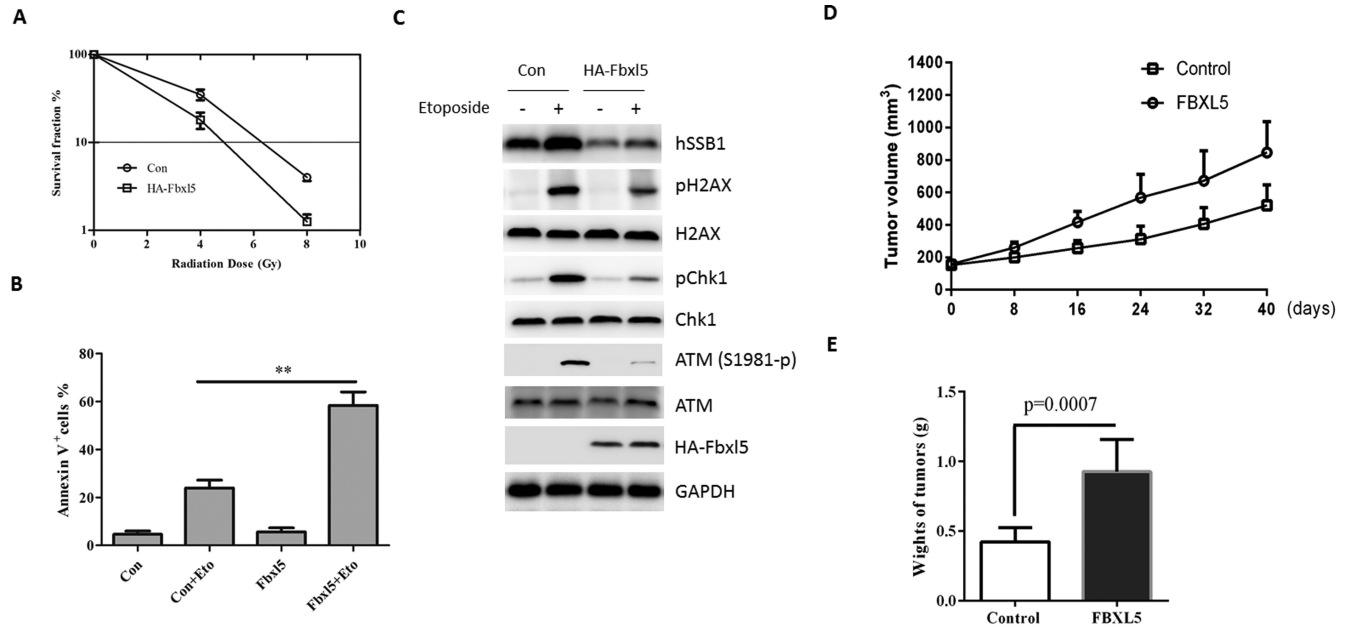

Figure 6. Overexpression of Fbxl5 sensitized lung cancer cells to genotoxic stress. (A) A549 cells stably transfected with control or HA-Fbxl5 were irradiated as indicated and clonogenic cell survival assays carried out. Data shown are the mean and SE from three independent experiments. (B) Control A549 cells and A549 cells stable expressing HA-Fbxl5 were treated with or without 10 μM etopside for 24 h and annexin-V+ cells% was determined on flow cytometry. (C) Control A549 cells and A549 cells stable expressing HA-Fbxl5 were treated with or without 10 μM etopside for 24 h. The whole cell lysate were detected by western blot using indicated antibodies. (D) Four weeks old male immune-deficient nude mice (BALB/c-nu) were simultaneously injected subcutaneously with 5×10^6 of A549 cells transfected with Fbxl5 or vector control. Mice were monitored daily and all formed subcutaneous tumors ($n = 6$–8 for each group) and followed up for tumorigenesis. The tumor size was measured by with vernier caliper weekly. Growth curve of tumor volumes were showen. (E) Tumor weights were taken 40 days after injection.

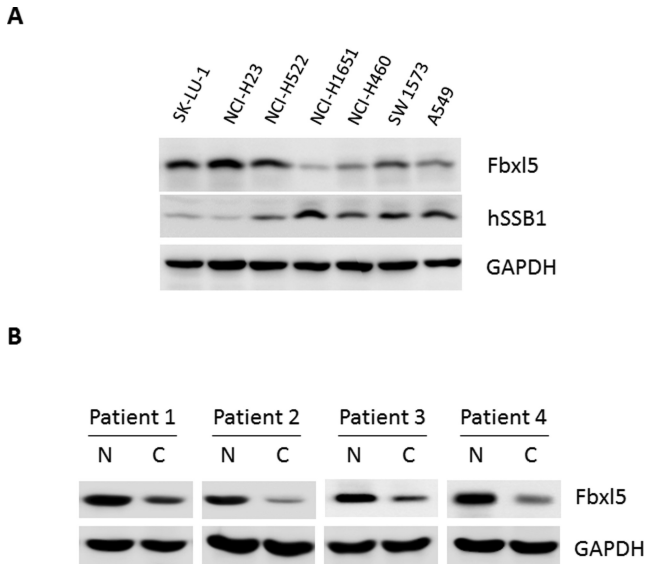

Figure 7. Inverse relationship between Fbxl5 and hSSB1 expression in lung cancer. (A) Asynchronously growing cells derived from non-small cell lung cancer were lysed and detected by western blot using indicated antibodies. (B) The protein levels of Fbxl5 and hSSB1 in four paired lung cancer samples were detected by western blot using indicated antibodies. N, adjacent normal tissue; C, cancer tissue.

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Conflict of interest statement. None declared.

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