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Overexpression of SCYL1-BP1 stabilizes functional p53 by suppressing MDM2-mediated ubiquitination

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Abstract

Previously, we defined SCYL1-BP1 to be a substrate of Pirh2 that binds to MDM2. In the current study, we found that an increase in SCYL1-BP1 protein levels caused a parallel change in the amount of p53 protein due to the inhibition by SCYL-BP1 of MDM2-mediated p53 ubiquitination. SCYL1-BP1 was not able to alter the ubiquitination of p53 by human papillomavirus protein E6, indicating that the effect was specific for MDM2. Increases in the level of SCYL1-BP1 protein in cells led to the greater transcriptional activation of p21 and gadd45, reduced rate of cellular proliferation, increased levels of apoptosis and inhibition of tumorigenicity. Thus, we propose that SCYL1-BP1 is a novel regulator of the MDM2-p53 feedback loop and that it may be a potential tumor suppressor.

Keywords

SCYL1-BP1; p53; MDM2; tumorigenicity

Introduction

The tumor suppressor protein p53 is a transcription factor that acts through a number of pathways to protect cells from malignant transformation [11]. As a sequence-specific DNA-binding protein, upon activation, p53 triggers the transcription of its target genes through binding to specific DNA consensus sequences, such as those found in the genes *p21, gadd45,* and *PUMA* [20]. These p53 target genes subsequently regulate cell cycle progression, cell death, DNA repair and DNA replication to maintain genomic stability and prevent tumorigenesis. However, under normal conditions, the p53 protein is short-lived, primarily due to MDM2-mediated ubiquitination and degradation. MDM2, an E3 ubiquitin ligase, is also a transcriptional target of p53. MDM2 binds to the transactivation domain of p53 and inhibits p53-mediated transcription, shuttles p53 out of the nucleus and targets p53 for ubiquitin-mediated proteolysis [14]. Besides MDM2, p53 is also targeted by other ubiquitin ligases, including COP1 [7] Pirh2 [10] and E6AP [17], among others, although the regulation of these enzymes is less clear.

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SCYL1-BP1, which was originally cloned in our lab, was found to interact with both Pirh2 [21] and MDM2 [19]. Additionally, we found that SCYL1-BP1 was a substrate of Pirh2, but not MDM2. However, by binding to MDM2, SCYL1-BP1 induced MDM2 self-ubiquitination and thus a reduction in MDM2 protein levels. Because p53 is a common substrate of both Pirh2 and MDM2, we hypothesized that SCYL1-BP1 expression may affect p53 protein levels. In this paper, we demonstrate that SCYL1-BP1 stabilized the protein level of p53 *in vivo* and inhibited MDM2-dependent p53 ubiquitination. Moreover, we also identified a potential tumor-suppressive property of SCYL1-BP1.

Results

SCYL1-BP1 promoted p53 protein stabilization

Previously, we found that SCYL1-BP1 induced MDM2 self-ubiquitination and protein reduction [19]. Because MDM2 is a key E3 ubiquitin ligase of p53, we wanted to know whether or not SCYL1-BP1 was able to affect p53 protein level and/or cellular function. We first investigated the effect of SCYL1-BP1 on the steady-state levels of p53 in the cell line skhep1, which has endogenous wild-type p53 expression intact. The endogenous p53 protein level increased concomitantly with the increase in the protein level of SCYL1-BP1 (Fig. 1A). We also tested the endogenous p53 protein levels in both sk-hep1-pEGFPc and sk-hep1-pEGFP-SCYL1-BP1 stably transfected cells. The overexpression of SCYL1-BP1 resulted in a similar increase in the amount of p53 (Fig. 1B). Moreover, the upregulation of p53 by SCYL1-BP1 was attenuated when the cells were treated with the proteasome inhibitor MG132 (Fig. 1A, lane 1 and 6), suggesting that the regulation of p53 by SCYL1-BP1 was probably accomplished through an effect on protein turnover. To further examine this possibility, the half-life of p53 with or without SCYL1-BP1 overexpression was analyzed. The half-life of p53 was found to be extended by ectopically expressing SCYL1-BP1 (Fig. 1C,D). The data in Fig. 1F confirmed that upregulation of the p53 response was not correlated with an increased p53 mRNA level.

It is also possible that even a slight increase in SCYL1-BP1 expression could result in genotoxic stress, leading to p53 activation and stabilization. A hallmark of DNA damage is phosphorylation at the Ser15 residue of p53 [8]. To address this possibility, we assessed the Ser15 phosphorylation status of p53 as well as the total p53 levels. As shown in Fig. 1E, SCYL1-BP1 increased the level of total p53 significantly, but the level of p53 that was phosphorylated at Ser15 remained unaltered (Fig. 1E lane 1 and 2). In contrast, after treatment with the DNA-damaging agent cisplatin, similar increases in the total and Ser15-specific phosphorylation of p53 were noted in cells that were transfected with or without Myc-SCYL1-BP1 (Fig. 1E lane 3 and 4). These data indicate that SCYL1-BP1 increased p53 levels via a pathway that was most likely distinct from pathways that are utilized by cells undergoing genotoxic stress.

SCYL1-BP1 stabilized the protein level of p53 by inhibiting MDM2-mediated ubiquitination

The primary pathway of p53 turnover is through ubiquitination by MDM2 and subsequent proteasomal degradation. Because our previous data suggested that SCYL1-BP1 might interfere with this pathway, we examined the effect of MDM2 on the ability of SCYL1-BP1 to modulate p53 levels. We expressed constant levels of exotic p53 in embryonic fibroblasts from *p53/MDM2* double knockout mice (2KO-MEFs) for this investigation. The results showed that SCYL1-BP1 had a much more pronounced effect on p53 levels in the 2KOMEFs cells expressing MDM2 (Fig. 2A). Similarly, the obviously diminished ubiquitination of p53 caused by SCYL1-BP1 was detected only in the presence of MDM2 (Fig. 2B, lane 3 and 4). To expand on this data, we assessed the effect of SCYL1-BP1 on p53 ubiquitination in sk-hep1 cells (p53 wild-type) that were treated with MG132. The data

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show that the ubiquitinated forms of p53 were impaired in SCYL1-BP1-expressing cells compared with control cells, although the total levels of endogenous p53 were comparable (Fig. 2C).

It was also possible that the expression of SCYL1-BP1 could have altered the entire ubiquitination pathway in the cells. To exclude this possibility, we employed HeLa cells, in which p53 is dominantly ubiquitinated and degraded by E6AP, but not MDM2 [17]. As shown in Fig. 2D, SCYL1-BP1 overexpression failed to stabilize p53 in HeLa cells, indicating that the activation of p53 by SCYL1-BP1 was specifically occurring through the MDM2 pathway, rather than by affecting the whole ubiquitination process. The same ubiquitination pattern of p53 in HeLa cells occurred irrespective of the expression of SCYL1-BP1 (Fig. 2E), which was in agreement with our previous data.

SCYL1-BP1 affected the transcriptional activity of p53

As is well known, p53 is an important transcription factor that is present in very low amounts in normal cells. The activation of p53 results in the transactivation of many target genes that regulate corresponding biological processes. Thus, we also assessed the transcriptional activation ability of p53 under conditions of SCYL1-BP1 overexpression using *p21* and *gadd45* luciferase reporters that contain p53-responsive elements.

By transfecting 2KO-MEF cells with the plasmids cocktails that are illustrated in Fig. 3A and B, we found that SCYL1-BP1 had no significant effect on reporter activity without MDM2. However, in the presence of MDM2, although the reporter activity was decreased significantly by MDM2, SCYL1-BP1 was able to rescue the reduction in p21 and gadd45 transcription. The results show that SCYL1-BP1 stabilized functional p53 and promoted its transcriptional activity in an MDM2-dependent manner.

SCYL1-BP1 promoted apoptosis, inhibited cell growth and prevented tumorigenicity

Given its potential connection to p53, we tested whether SCYL1-BP1 may be a tumor suppressor in cell lines having wild-type p53. First, we established sk-hep1 cell lines stably expressing either GFP or GFP-SCYL1-BP1. We then measured the cellular proliferation rate and found that exogenous SCYL1-BP1 dramatically inhibited the proliferation of the sk-hep1 cells (Fig. 4A). Next, we examined whether SCYL1-BP1 induced apoptosis in the sk-hep1 cells. Flow cytometry was used to compare cells with different genotypes under normal conditions or the following types of genotoxic stress: Act.D or 5-FU treatment. Under these conditions, cells expressing GFPc-SCYL1-BP1 were more apoptotic than those expressing GFP (Fig. 4B). Furthermore, we tested the tumor-suppressive ability of SCYL1-BP1 in nude mice. We used another p53 wild-type cell line, BEL7402, to generate BEL7402-pEGFPc, BEL7402-pEGFP-mSCYL1-BP1 and BEL7402-pEGFP-hSCYL1-BP1 stable transfectants, and injected each of BEL7402 transfectants into six male BALB/c nude mice. Tumor growth was monitored for 21 days, at which time the mice were sacrificed and explants were established from the harvested tumors. As shown in Fig. 4C, all of SCYL1-BP1-expressing clones showed a drastic reduction in tumorigenicity. These results emphasize the important role of SCYL1-BP1 in the p53 pathway and suggest that SCYL1-BP1 may be a novel target for future tumor therapies.

Discussion

We previously used SCYL1 (also known as NTKL) as bait in a yeast two-hybrid assay to discover binding partners. We identified a protein named SCYL1-BP1, which is soluble, highly conserved and widely expressed in many tissues.[6] However, the specific function of SCYL1-BP1 remained unknown. In this study, we demonstrated that SCYL1-BP1 slowed

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down the cellular proliferation rate, induced apoptosis, and inhibited tumorigenicity. As for how exactly SCYL1-BP1 exercises these functions, we have shown that SCYL1-BP1 elevated the level of p53 protein by impairing p53 ubiquitination and degradation. Several E3 ubiquitin ligases for p53 regulation have been reported, including MDM2 [9], COP1 [7], E6AP [16] and Pirh2 [10]. Our experiments demonstrated that the ability of SCYL1-BP1 to activate p53 is MDM2-dependent. Parallel tests, including E6AP in HeLa cells, effectively exclude the possibility of a general inhibition by SCYL1-BP1 of p53 ubiquitination and degradation, and established a specific role for SCYL1-BP1 in the MDM2-p53 pathway.

We previously found that SCYL1-BP1 binds to MDM2 and promotes the self-ubiquitination of MDM2 [19]. The ability of SCYL1-BP1 to suppress MDM2 ubiquitination of p53, but promote ubiquitination of MDM2 itself, is interesting. In contrast to C23 [15] and other ribosomal proteins [18,3], it seems that SCYL1-BP1 selectively affects the ubiquitination of MDM2 target proteins. A similar phenomenon was also observed with regard to the effect of MTBP on p53-MDM2 loop [2]. MTBP promotes the ubiquitination of p53 but inhibits the ubiquitination of MDM2. However, MTBP binds to the region between amino acids 167 and 304, encompassing the central acidic region of MDM2 [1]. Moreover, NS [5] and C23 [15] also associate with this region of MDM2 and inhibit MDM2 E3 ligase activity on both itself and p53. All of these data suggests that the central acidic region of MDM2 plays an important, yet diverse, regulatory function on the ubiquitination activity of MDM2. A second p53 binding site has been identified in MDM2 (amino acid 211-361), including the acidic domain and the zinc finger domain [12]. The interaction between the central domain of MDM2 and the core domain of p53 is critical for the ubiquitination and degradation of p53 [12]. We propose that the exogenously overexpressed SCYL1-BP1 may have bound to the central acidic domain of MDM2 and occupied the second p53 binding site on MDM2, although p53 can also loosely bind to the N-terminus of MDM2 with its own N-terminus. Then, it would be more difficult for MDM2 to deliver the ubiquitin chains onto p53. In contrast, binding to SCYL1-BP1 may alter the conformation of MDM2, which in turn would facilitate the attachment of ubiquitin chains to its own lysines. As shown in Fig. 5, SCYL1-BP1, MDM2, p53 and the poly-ubiquitin chain may form a transient complex in the cells. We confirmed the binding *in vitro* (data not shown), but whether it is possible to form such a complex under natural physiological conditions is still a topic of research in our lab.

In conclusion, we found that SCYL1-BP1 stabilized p53 by inhibiting MDM2-mediated p53 ubiquitination and degradation and that this resulted in apoptosis, cell growth inhibition and tumor prevention.

MATERIALS AND METHODS

Expression plasmids

Human *SCYL1-BP1* cDNA [6] was derived from a human fetal liver cDNA library (Clontech) and cloned into the pCMV-Myc (Stratagene) and pEGFPc-1 (Clontech) vectors. The p53 expression plasmid was constructed by cloning the full-length human p53 sequence into the pcDNA3.0 vector (Invitrogen). His-Ub was generously provided by Dr. H. Lu [4].

Cell lines

Human sk-hep1, HepG2, H1299, HEK293 and BEL7402 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50-U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in a 5% CO2 humidified atmosphere. The p53 and MDM2 double knockout MEFs (2KO-MEFs) were a gift from Dr. G. Lozano [13].

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For the transient transfections, cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. To generate stable cell lines, sk-hep1 cells were seeded at 5×10^5 cells per 60-mm plate and transfected with 2 μg of GFP-SCYL1-BP1 or 1 μg of GFPc vectors. Post-transfection (18 h), the cells were replated at a density of 10^4 cells per 10-cm dish, in duplicates. GFP-expressing cells were selected in DMEM media containing 1200-μg/ml G418 (MERCK) for 21 days, with the drug-containing media replaced every week. Individual clones were subsequently isolated and expanded over a two to four week-long period.

Antibodies

The primary antibodies used for immunoprecipitation or western blotting were as follows: rabbit polyclonal anti-Myc antibody (Santa Cruz), mouse monoclonal anti-GFP (Santa Cruz), mouse monoclonal anti-p53 (DO-7, Abcam) and rabbit polyclonal anti-p53, phosphorylated on Ser15 (pS15p53, Cell Signaling Technology).

Luciferase Assays

Luciferase reporter constructs containing the p53 responsive sequence p21-Luc was a gift from Dr. Moshe Oren [9], while gadd45-Luc was from Dr. John A. Di Battista [8].

For luciferase assays, 2KO cells were seeded in 24-well plates 24 h before transfection. The cells were transfected with the plasmids using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions at 60% confluence (with 200 ng of reporter constructs). When the cells were ready, 50 ng of the p53 and 100 ng of the MDM2 plasmids were co-transfected. Total plasmid DNA per well was adjusted to an equal level by adding empty vector. The cells were harvested at 48 h post-transfection and prepared using the Dual Luciferase Assay System (Promega) following the manufacturer's protocol. The luciferase activity was measured in a Laser Capture Microdissection (LCM) System Veritas™ (Turner Biosystems). For each construct, the relative luciferase activity was defined as the mean value of the firefly luciferase/Renilla luciferase ratios that were obtained from at least four independent experiments.

Apoptosis

For apoptosis analysis, cells under normal conditions or treated with either 10-nM Actinomycin D (Act. D) or 20-μg/ml fluorouracil (5-FU) for 12 h prior to harvest were washed twice in phosphate-buffered saline (PBS), fixed with 4% PFA and permeabilized in 0.1% Triton X-100/PBS on ice for 10 min. The fixed cells were then washed and stained with a propidium iodide solution containing 50-μg/ml propidium iodide, 0.05% Triton X-100, 37-μg/ml EDTA, and 100-U/ml ribonuclease in PBS. After incubation for 45 min at 37° C, the DNA content was determined by quantitative flow cytometry using the standard optics of the FACScan flow cytometer (Becton-Dickinson FACStar). GFP-positive cells were gated and the percentage of apoptotic cells were quantitated from the sub-G1 events.

Cell growth rate analysis

Cell growth rate was determined by using the Cell Counting Kit-8 (CCK-8, Dojindo Laboratories) according to the manufacturer's instructions. Cells were cultured in a 96-well plate and every 24 h, four of the wells were treated with 10 μl of CCK-8 solution per well and incubated for 2 h at 37°C over 6 days. The amount of formazan dye generated by cellular dehydrogenase activity was then measured by absorbance at 450 nm with a microplate fluorometer.

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Tumorigenicity Assay

The tumorigenicity assays were performed using BALB/c nude mice that were purchased from the Shanghai Laboratory Animal Center of China. The control (BEL7402-pEGFPc), BEL7402-pEGFPc-mSCYL1-BP1 and BEL7402-pEGFPc-hSCYL1-BP1 cells were injected subcutaneously into the flanks of the mice (10^6 cells/injection site, six mice/cell line). After 3 weeks, photographs were taken and tumors were harvested and individually weighed after the mice were anesthetized. Data are presented as tumor weight (mean ± SD).

Acknowledgments

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Abbreviations

MDM2	mouse double minute gene number 2
SCYL1-BP1	SCY1-like 1 binding protein 1

References

1. Boyd MT, Vlatkovic N, Haines DS. A novel cellular protein (MTBP) binds to MDM2 and induces a G1 arrest that is suppressed by MDM2. J. Biol. Chem. 2000; 275:31883–31890. [PubMed: 10906133]
2. Brady M, Vlatkovic N, Boyd MT. Regulation of p53 and MDM2 activity by MTBP. Mol. Cell Biol. 2005; 25:545–553. [PubMed: 15632057]
3. Chen D, Zhang Z, Li M, Wang W, Li Y. Ribosomal protein S7 as a novel modulator of p53-MDM2 interaction: binding to MDM2, stabilization of p53 protein, and activation of p53 function. Oncogene. 2007; 26:5029–5037. [PubMed: 17310983]
4. Dai MS, Lu H. Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5. J. Biol. Chem. 2004; 279:44475–44482. [PubMed: 15308643]
5. Dai MS, Sun X, Lu H. Aberrant expression of Nucleostemin activates p53 and induces cell cycle arrest via inhibition of MDM2. Mol. Cell Biol. 2008; 28:4365–4376. [PubMed: 18426907]
6. Di Y, Li J, Fang J, Xu Z, He X, et al. Cloning and characterization of a novel gene which encodes a protein interacting with the mitosis-associated kinase-like protein NTKL. J. Hum. Genet. 2003; 48:315–321. [PubMed: 12783284]
7. Dornan D, Wertz I, Shimizu H, Arnott D, Frantz GD, et al. The ubiquitin ligase COP1 is a critical negative regulator of p53. Nature. 2004; 429:86–92. [PubMed: 15103385]
8. Faour WH, He QW, Mancini A, Jovanovic D, Antoniou J, et al. Prostaglandin E2 stimulates p53 transactivational activity through specific Serine 15 phosphorylation in human synovial fibroblasts. J. Biol. Chem. 2006; 281:19849–19860. [PubMed: 16714289]
9. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. Nature. 1997; 387:296–299. [PubMed: 9153395]
10. Leng R, Lin Y, Ma W, Wu H, Lemmers B, et al. Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. Cell. 2003; 112:779–791. [PubMed: 12654245]
11. Levine AJ, Momand J, Finlay CA. The p53 tumor suppressor gene. Nature. 1991; 351:453–456. [PubMed: 2046748]
12. Ma JH, Martin JD, Zhang H, Auger KR, Ho TF, et al. Second p53 binding site in the central domain of MDM2 is essential for p53 ubiquitination. Biochem J. 2006; 45:9238–9245.

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13. Montes LR, Wagner DS, Lozano G. Rescue of early embryonic lethality in MDM2-deficient mice by deletion of p53. Nature. 1995; 378:203–206. [PubMed: 7477326]

14. Prives C. Signaling to p53: breaking the MDM2-p53 circuit. Cell. 1998; 95:5–8. [PubMed: 9778240]

15. Saxena A, Rorie CJ, Dimitrova D, Daniely Y, Borowiec JA. Nucleolin inhibits Hdm2 by multiple pathways leading to p53 stabilization. Oncogene. 2006; 25:7274–7288. [PubMed: 16751805]

16. Scheffner M, Huibregtse JM, Vierstra RD, Howley PM. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell. 1993; 75:495–505. [PubMed: 8221889]

17. Talis AL, Huibregtse JM, Howley PM. The Role of E6AP in the Regulation of p53 Protein Levels in Human Papillomavirus (HPV)-positive and HPV-negative Cells. J Biol Chem. 1998; 273:6439–6445. [PubMed: 9497376]

18. Weber JD, Kuo ML, Bothner B, DiGiammarino EL, Kriwacki RW. Cooperative signals governing ARF-MDM2 interaction and nucleolar localization of the complex. Mol. Cell Biol. 2000; 20:2517–2528. [PubMed: 10713175]

19. Yan J, Zhang D, Di Y, Shi H, Rao H, Huo K. A newly identified Pirh2 substrate SCYL1-BP1 can bind to MDM2 and accelerate MDM2 self-ubiquitination. FEBS Letters. 2010; 584:3275–3278. [PubMed: 20598683]

20. Yu J, Zhang L, Hwang PM, Rago C, Kinzler KW, et al. Identification and classification of p53-regulated genes. P Natl Acad Sci. 1999; 96:14517–14522.

21. Zhang L, Li J, Wang C, Ma Y, Huo K. A new human gene hNTKL-BP1 interacts with hPIRH2. Biochem Bioph Res Co. 2005; 330:293–297.

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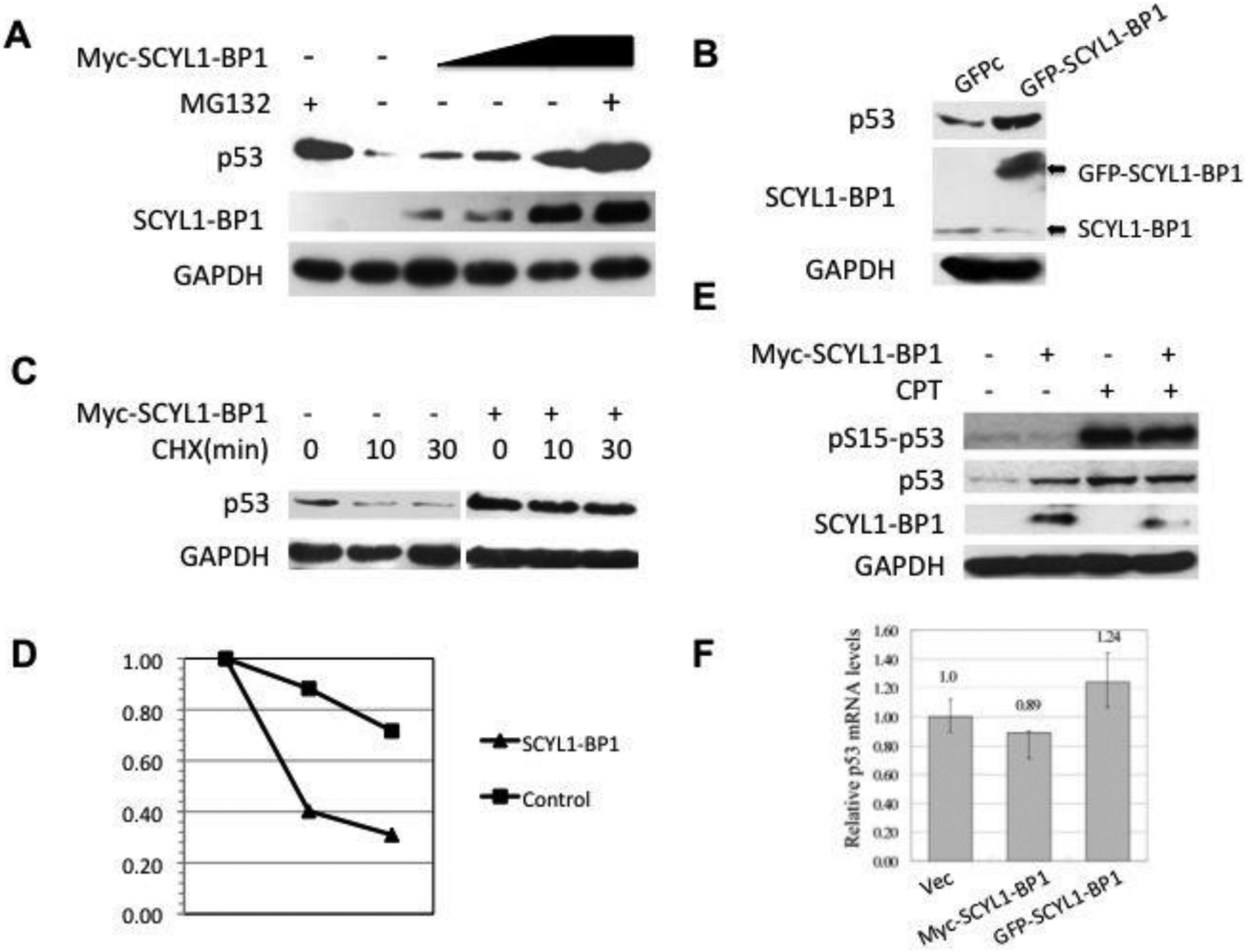

Fig 1.

SCYL1-BP1 stabilized p53 protein expression. (A) The overexpression of SCYL1-BP1 induced an increase in the p53 protein level *in vivo*. sk-hep1 cells were transiently transfected with increasing levels of a SCYL1-BP1-expressing plasmid. When necessary, the cells were treated with 30-μM MG132 for 6 h before harvest. The expression of p53, GAPDH and Myc-SCYL1-BP1 were assayed by western blot. (B) sk-hep1 cells were stably transfected with GFP-SCYL1-BP1 or GFPc (empty vector) plasmids, as indicated. Protein lysates were analyzed by western blotting for p53, endogenous SCYL1-BP1, GFP tagged SCYL1-BP1 and GAPDH. (C) SCYL1-BP1 increased the half-life of p53. Sk-hep1 cells were transfected with or without Myc-SCYL1-BP1. At 48 h post-transfection, the cells were treated with 50-μg/ml cycloheximide (CHX) for the indicated times. Lysates were prepared and analyzed by western blotting for p53 and the GAPDH loading control. (D) The p53 expression levels following cycloheximide treatment in cells expressing either Myc-SCYL1-BP1 (solid squares) or Myc-GFP alone (triangles), adjusted for the levels of loading control GAPDH, were plotted. (E) sk-hep1 cells were transfected with or without Myc-SCYL1-BP1 expression vector. As indicated, cells were treated with 2-mM CPT for 2 h to induce DNA damage. The lysates were analyzed for the phosphorylation status of Ser15 of p53 (pS15-p53), total p53 (p53), Myc-SCYL1-BP1 and GAPDH. (F) SCYL1-BP1 did not affect the p53 mRNA level. The sk-hep1 cells were transfected with SCYL1-BP1-expression plasmids as indicated. The relative expression of *p53* was measured by quantitative real-time PCR,

and then normalized to expression of *gapdh*. The values represented fold increase or decrease relative to untreated sk-hep1 cells.

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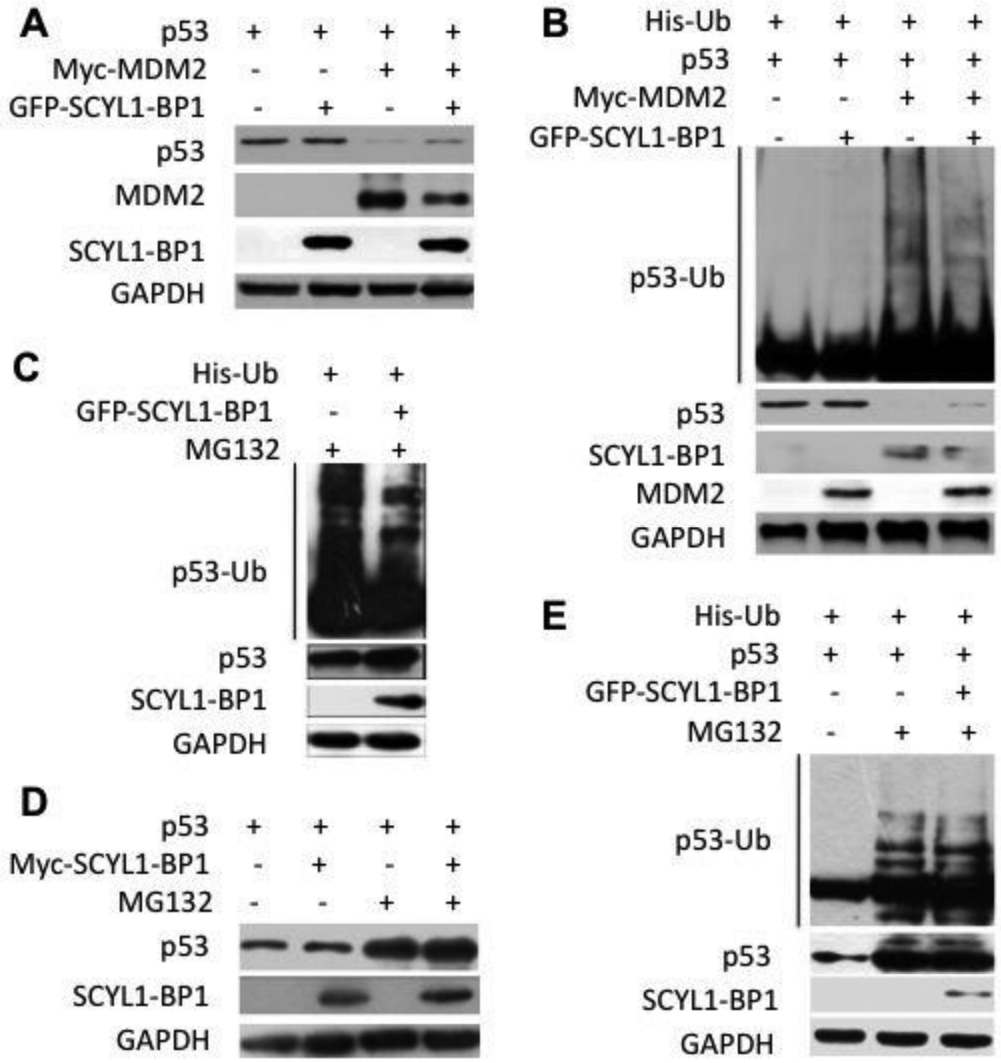

Fig. 2.

SCYL1-BP1 inhibited MDM2-dependent degradation of p53. (A) The stabilization of p53 by SCYL1-BP1 was dependant on MDM2. 2KO-MEFs cells were transfected with plasmids expressing p53, Myc-MDM2 or GFP-SCYL1-BP1, as indicated. The protein level of p53 was analyzed by western blotting. (B) SCYL1-BP1 impaired MDM2-mediated p53 ubiquitination *in vivo*. 2KO-MEFs cells were co-transfected with the plasmids indicated and then were cultured in the presence of MG132. The ubiquitinated conjugates purified by Ni-NTA-agarose beads were analyzed using anti-p53 antibodies. The cell lysates (10% input) were analyzed by western blotting using the indicated antibodies. (C) SCYL1-BP1 suppresses p53 ubiquitination in sk-hep1 cells. The sk-hep1 cells were transfected with His-Ub and Myc-SCYL1-BP1 plasmids. The His-tagged (ubiquitinated) species were then immunoprobed for the presence of p53. Total lysates were probed for p53 (total), Myc-SCYL1-BP1 and GAPDH. (D) SCYL1-BP1 had no effect on p53 protein level in HeLa cells. HeLa cells were transfected with p53 (lane 3 and 4) and Myc-SCYL1-BP1 plasmids as indicated. Total lysates were probed for SCYL1-BP1, p53 and GAPDH. (E) SCYL1-BP1 overexpression did not inhibit p53 ubiquitination in HeLa cells. HeLa cells were transfected with p53, His-Ub and Myc-SCYL1-BP1 plasmids. The His-tagged (ubiquitinated) species were isolated and then immunoprobed for p53. Total lysates were analyzed for SCYL1-BP1,

total p53 and GAPDH. The same total DNA amount was kept constant by transfection of empty vector in every individual experiment.

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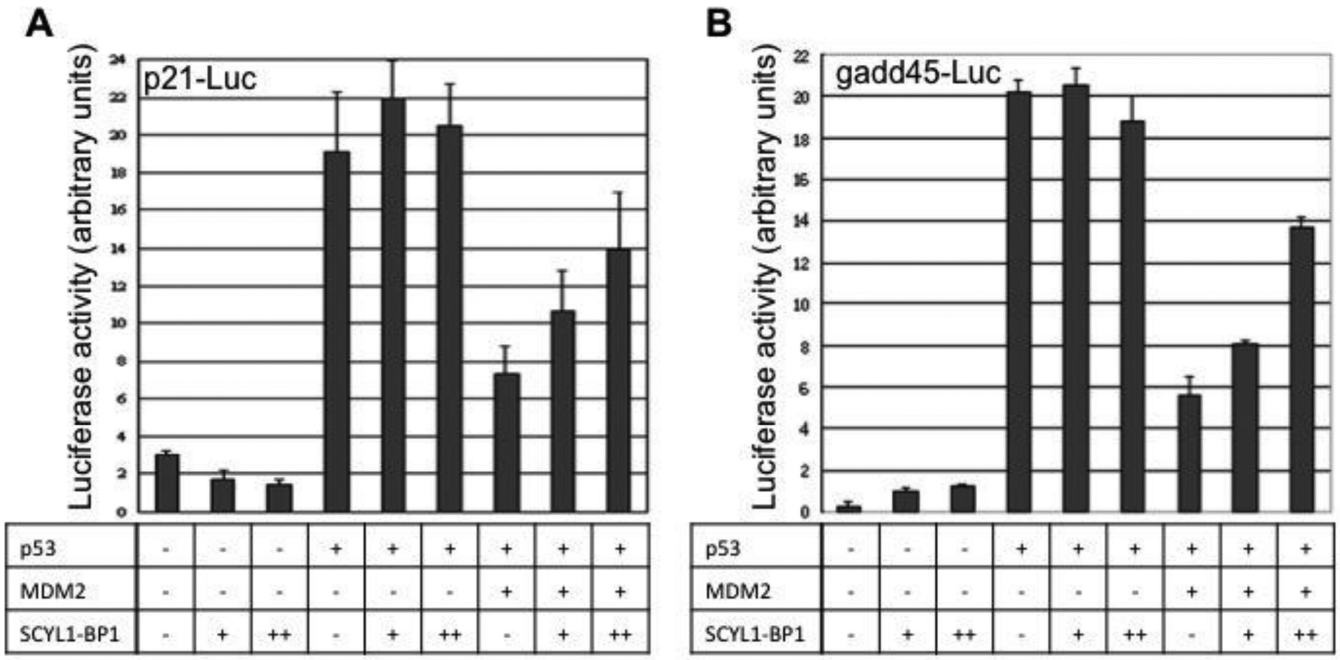

Fig. 3.

The effect of SCYL1-BP1 on the transcriptional activity of p53 was dependent on MDM2. 2KO-MEFs cells were transfected with a p53 responsive luciferase reporter derived from (A) p21 or (B) gadd45, and plasmids expressing human p53, MDM2 and Myc-BP1 (+ or ++ indicate that a low or high dose was given, respectively), as indicated, and luciferase activity was assayed. The results were normalized to Renilla luciferase activity, and represent the mean ± SD of three independent experiments.

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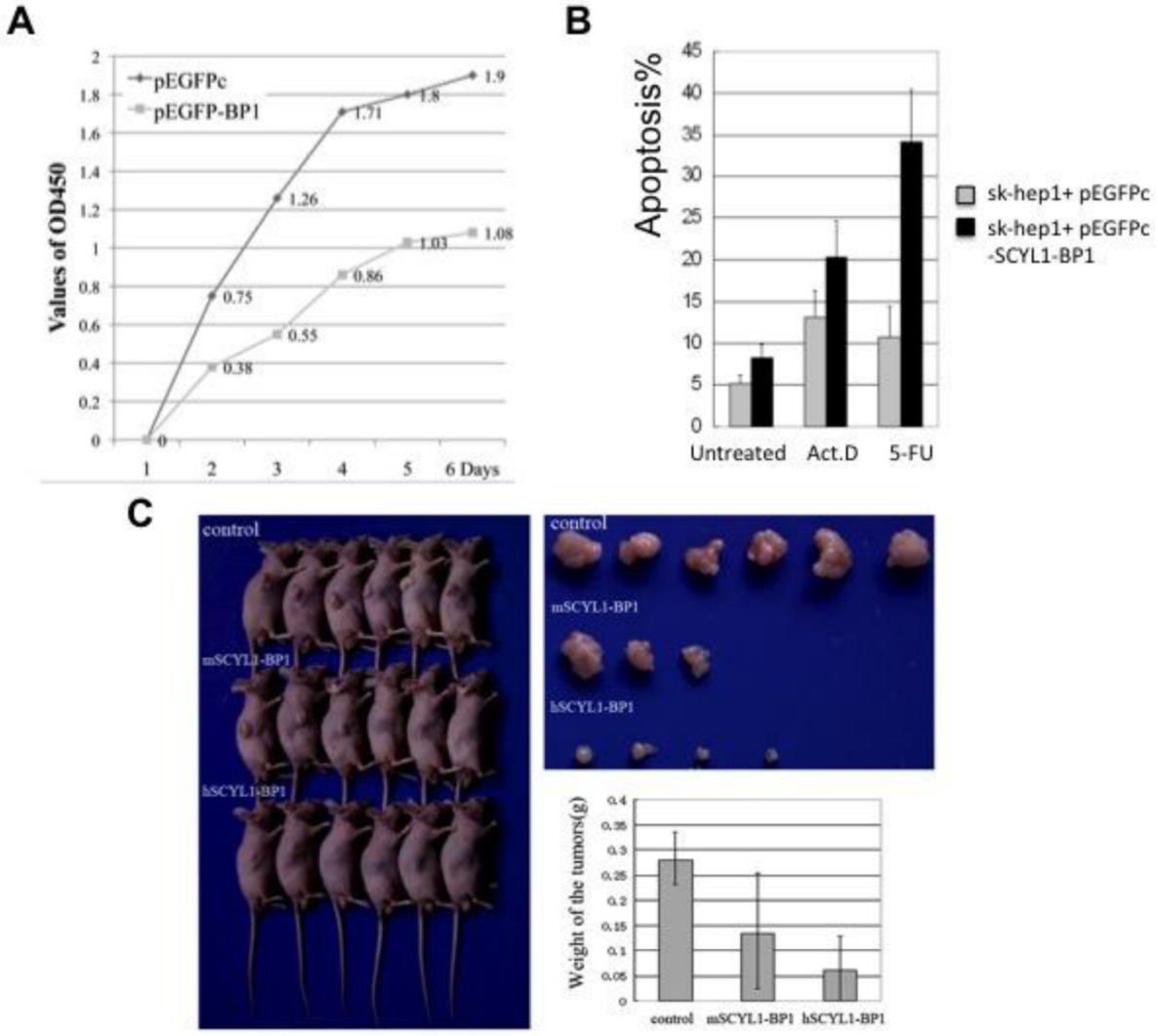

Fig. 4.

SCYL1-BP1 inhibited cell growth, promoted apoptosis and prevented tumorigenicity. (A) SCYL1-BP1 inhibited cellular proliferation as determined by the CCK-8 kit. The sk-hep1 cells that were stably transfected with pEGFPc or pEGFPc-SCYL1-BP1 were plated in 96-wells. The average values of the absorbance at 450nm derived from four independent experiments for each sample were shown in panel A. (B) SCYL1-BP1 promoted apoptosis in sk-hep1 cells. sk-hep1 cells were transfected with pEGFPc or pEGFPc-SCYL1-BP1 plasmids as indicated. Then, cells were treated with 10-nM Actinomycin D (Act. D) or 20-μg/ml 5-FU for 12 h to induce the genotoxic stress. Apoptosis was measured by flow cytometric analysis of propidium iodide staining. (C) SCYL1-BP1 suppressed tumor growth after transplantation into nude mice. BEL7402 cells were stably transfected pEGFPc, pEGFPc-mSCYL1-BP1 or pEGFPc-hSCYL1-BP1 plasmids. Stably transfected cells were then injected into nude mice. Three weeks later, photographs were taken (left panel). Tumors were removed and weighed. Results are shown as the mean ± SD of tumor weight (right panel).

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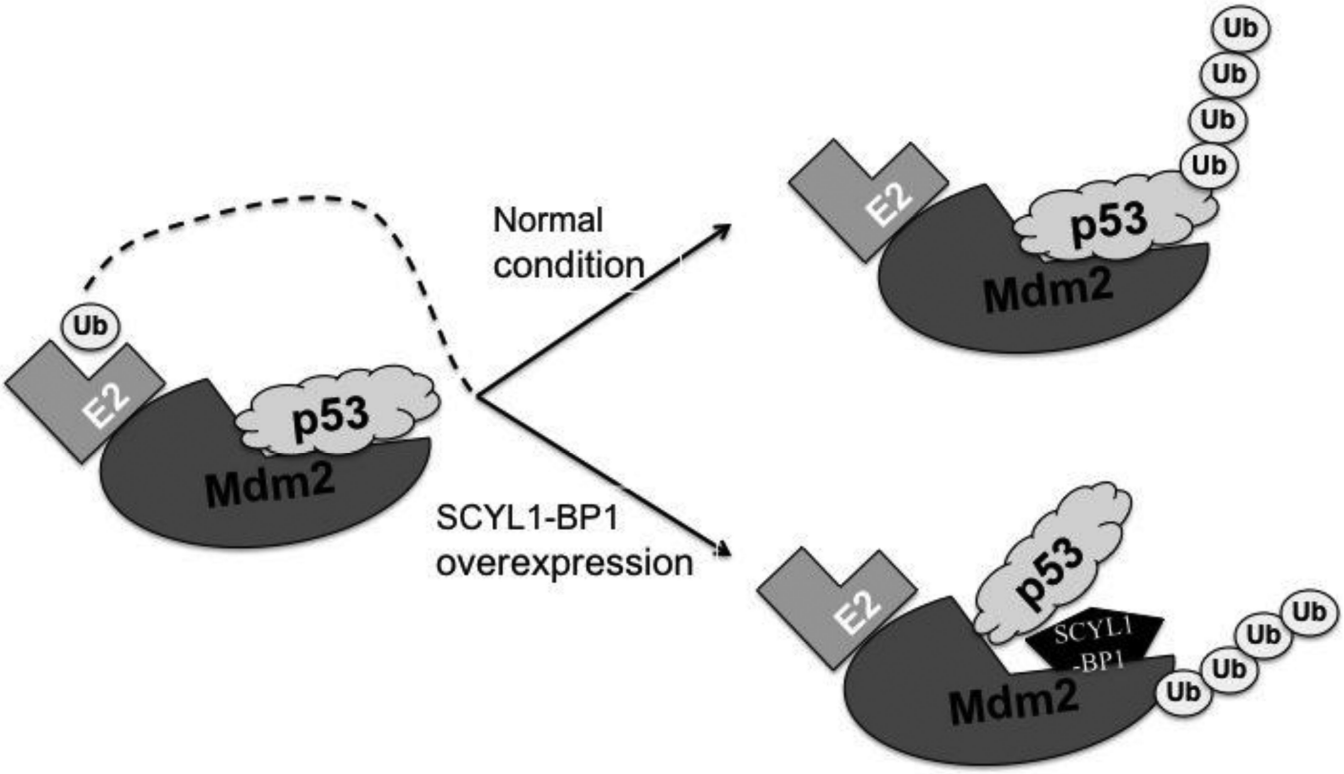

Fig. 5.

A schematic model illustrates the potential mechanism underlying the distinct roles of SCYL1-BP1 on MDM2 auto-ubiquitination and the MDM2-mediated ubiquitination of p53.