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A Newly Identified Pirh2 substrate SCYL1-BP1 can bind to MDM2 and accelerate MDM2 self-ubiquitination

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Abstract

The SCYL1-BP1 protein was identified as an interacting partner of E3 ligase Pirh2 and MDM2 by yeast two-hybrid screening. Further investigation suggested there are two interactions involved in different mechanisms. SCYL1-BP1 can be ubiquitinated and degraded by Pirh2 but not by MDM2, which suggests that SCYL1-BP1 can be regulated by Pirh2. On the other hand, while SCYL1-BP1 binds to ubiquitin E3 ligase MDM2, it promotes MDM2 self-ubiquitination and results in a reduction of MDM2 protein level.

Keywords

SCYL1-BP1; MDM2; Pirh2; protein degradation; ubiquitination

1. Introduction

Murine double minute 2 (MDM2) is involved in cell growth and differentiation through its interaction with other cellular proteins. The MDM2 protein level is increased in a significant number of human tumors such as soft tissue sarcomas, osteosarcomas and breast tumors, underscoring its pivotal involvement in the development of human cancer [3]. MDM2 belongs to the family of RING Finger ubiquitin ligases [11], and the principal function of MDM2 is to mediate the ubiquitination and proteasome-dependent degradation of the p53 tumor suppressor protein and other growth regulatory proteins [9]. Accordingly, the ligase activity of MDM2 can be abolished by mutation of either one of eight cysteine and histidine residues involved in zinc coordination within the RING finger domain. Besides p53, MDM2 also ubiquitinates Numb [19], β-arrestin binding protein [17], histone acetyltransferase Tip60 [13] as well as MDM2 itself.

SCYL1-BP1, as we previously reported [6], is a highly conserved, widely expressed protein that binds to a p53-induced protein, Pirh2 [20]. Like MDM2, Pirh2 is also an E3 enzyme involved in an auto-regulatory feedback loop with p53. Then we indentified the binding between SCYL1-BP1 and MDM2, which forced us to ask questions like: Is SCYL1-BP1 the substrate of Pirh2, MDM2 or both? Can SCYL1-BP1 reversely affect Pirh2 or MDM2? And

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we found out SCYL1-BP1 was only a substrate of Pirh2, however could not be ubiquitinated or degraded by MDM2. Interestingly, by interaction with MDM2, SCYL1-BP1 promoted MDM2 self-ubiquitination and induced MDM2 protein degradation.

2. Materials and methods

2.1. Plasmids

Human SCYL1-BP1 cDNA [6] was derived from human fetal liver cDNA library (Clontech) and cloned into pCMV-Myc(Stratagene), pEGFPc-1(Clontech) and pGEX-5x-1 (Pharmacia Biotech)vectors respectively. The Myc-tagged full length and depletion mutants of human MDM2 were generated using PCR and were cloned into the pCMV-Myc vectors. The primers used were P1, P2 for the full length of MDM2; P1 and P3 for the MDM2ΔC1; P1 and P4 for the MDM2ΔC2; P5: and P2 for the MDM2ΔN1; P6 and P2 for the MDM2ΔN2; P7 and P2 for the MDM2ΔN3; P6 and P4 for MDM2-155-321 truncated mutation. Myc-Pirh2 expression plasmid was cloned by inserting full-length of Pirh2 cDNA into the pCMV-Myc vector.

2.2. Cell culture and transfection

The study employed sk-hep1 (p53 wt), HepG2 (p53 wt), Hep3B (p53 null), H1299 (p53 null) and HEK293 cells. DNA transfection was carried out using Lipofectamine2000 according to the manufacturer’s instruction. p53 and MDM2 double knockout MEFs (2KO-MEFs) cells were gifts from Dr. G. Lozano [15].

2.3. 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), rabbit polyclonal anti-p53 phosphorylated on Ser15 (pS15p53, Cell Signaling Technology) and mouse monoclonal anti-MDM2 (1B10, Novocatra). The anti-SCYL1-BP1 antiserum was generated by using purified His-tagged full-length SCYL1-BP1 protein as an antigen.

2.4. In *vivo* ubiquitylation assays

Cells plated in 100mm plate were transfected with combinations plasmids as indicated in the figure legends. To inhibit proteasome-mediated protein degradation, the cells were treated with 30μM Proteasome inhibitor MG132 (Calbiochem) for 6h before harvest. Cells were harvested at 48h after transfection from each plate and separated into two aliquots: one was for immunoblotting and the other for ubiquitination assays. Cell pellets were lysed in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50mM Tris-HCl, pH 7.5, 1mM PMSF, 10μg/ml aprotinin, 5mg/ml leupeptin). The lysates were sonicated on ice and clarified through centrifugation followed by incubation with Ni-NTA beads at room temperature for 4h. The bound proteins were washed three times with RIPA buffer and eluted by boiling for 5 min in protein sample buffer (200mM imidazole, 0.15 M Tris-HCl (pH 6.7), 30% glycerol, 0.72M β-mercaptoethanol, and 5% SDS). The eluted proteins were analyzed by immunoblot with indicated antibodies.

2.5. Co-immunoprecipitation assay

For co-immunoprecipitation assay between exogenous SCYL1-BP1 and MDM2, HEK293 cells were co-transfected with pEGFP-SCYL1-BP1 and pCMV-Myc-MDM2 plasmids. Cell extracts were prepared with lysis buffer (50mM Tris-HCl (pH 7.5), 150mM NaCl, 0.1% NP-40, 5mM EDTA, 5mM EGTA, 15mM MgCl2, 60mM β-glycerophosphate, 0.1mM sodium orthovanadate, 0.1m M NaF, 0.1mM benzamide, 10mu;g/ml aprotinin, 10mu;g/ml

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leupeptin, 1mM PMSF), followed by incubation with anti-Myc antibodies. Precipitated proteins were analyzed by immunoblot with anti-GFP antibodies.

To detect the protein-protein interaction between endogenous SCYL1-BP1 and MDM2, sk-hep1 cells were lysed in the same lysis buffer as mentioned before. And the lysates were incubated with mouse IgG or anti-MDM2 antibodies, precipitated proteins were detected by SCYL1-BP1 anti-serum.

3. Results

3.1. SCYL1-BP1 binds to MDM2 both in *vivo* and in *vitro*

To examine the interaction between SCYL1-BP1 and MDM2, we co-transfected HEK293 cells with plasmids expressing Myc-tagged MDM2 and GFP-tagged SCYL1-BP1, and performed co-immunoprecipitation assay. As expected, MDM2 can be co-immunoprecipitated with SCYL1-BP1 (Fig. 1A). This interaction was further confirmed by co-immunoprecipitation of endogenous SCYL1-BP1 with endogenous MDM2 from sk-hep1 cell lysates (Fig. 1C). Co-immunoprecipitation of MDM2 and GFP-SCYL1-BP1 was also observed in a p53 deficient H1299 cells, indicating that their interaction was not mediated by p53 (Fig. 1B). Moreover, GST-pull down experiments using purified GST-SCYL1-BP1 and MDM2 protein demonstrated a direct interaction of SCYL1-BP1 with MDM2 in *vitro* (Fig, 1D).

After establishing the in *vivo* and in *vitro* interaction between MDM2 and SCYL1-BP1 we decided to define the minimal interaction domain on MDM2. A diagram illustrating the known structural motifs within MDM2 is shown in Fig. 2A. When full length MDM2 and a series of MDM2 deletion derivatives were used to assess the interaction with SCYL1-BP1, only the MDM2-N3 (322-491aa) truncation mutant failed to associate detectably with SCYL1-BP1 in the co-immunoprecipitation experiments (Fig. 2B). Thus, we concluded that the region of MDM2 necessary for binding to SCYL1-BP1 might reside on the amino acid residues 155-321 comprising the central acidic domain of MDM2. Then we made the clone (Myc-MDM2-155-321), tested the binding of this truncation mutant to GFP-SCYL1-BP1. The result showed that indeed SCYL1-BP1 directly bound to this central acidic resided region of MDM2 (Fig. 2C).

3.2. SCYL1-BP1 is a substrate of Pirh2 but not MDM2

Since SCYL1-BP1 interacts with both MDM2 and Pirh2, two well characterized RING-finger-domain E3s that can ubiquitinate and degrade p53 independently, it appears reasonable to suspect that SCYL1-BP1 may be the substrate of MDM2 and/or Pirh2. To test this idea, we co-transfected HEK293 cells with SCYL1-BP1 and Pirh2 or MDM2, and monitored the protein level of SCYL1-BP1. As shown in Fig. 3A, a fast degradation of SCYL1-BP1 was observed with the co-transfection of Pirh2, but not with the co-transfection of MDM2, suggesting that SCYL1-BP1 may be a substrate of Pirh2 E3 ligase in *vivo*. To confirm this premise, HEK293 cells were co-transfected with Myc-tagged pirh2, GFP-tagged SCYL1-BP1 and HA-tagged ubiquitin, after cells were lysed, the immunoprecipitates by anti-GFP antibody were blotted with anti-HA antibody. Consistent with the degradation data, SCYL1-BP1 was indeed ubiquitinated with co-transfection of Pirh2, but not MDM2 (Fig. 3B, 3C). Together, our results strongly suggest that Pirh2, but not MDM2, functions as the ubiquitin ligase of SCYL1-BP1 and mediates its degradation.

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3.3. SCYL1-BP1 promoted proteasome-dependant down-regulation and self-ubiquitination of MDM2

In the co-immunoprecipitation study of MDM2/SCYL1-BP1 interation, we unexpectedly encountered the striking observation that the protein level of MDM2 was significantly depleted when co-expressed with SCYL1-BP1 (Fig. 1B comparing lane 1 and 3). To confirm this result, MDM2 protein level was monitored in HepG2 cells co-transfected with MDM2 plasmid and incremental amount of SCYL1-BP1 plasmid. As expected, a decrease of MDM2 level was observed with the increase of SCYL1-BP1 expression (Fig. 4A). A similar phenotype was also observed in p53 deficient H1299 cells, indicating that this interplay between MDM2 and SCYL1-BP1 is p53-independent (Fig. 4B). Interestingly, this SCYL1-BP1-mediated reduction of MDM2 was partially rescued by treating the cells with proteasome inhibitor, MG132, suggesting that MDM2 is degraded through ubiquitin-proteasome pathway (Fig. 4C). Given the self-ubiquitination activity of MDM2, we carried out a cell-based ubiquitination assay to examine the effect of SCYL1-BP1 on MDM2 self-ubiquitination. As shown in Fig. 4D, co-transfection of SCYL1-BP1 led to a decrease of MDM2, and concomitantly stimulated the ubiquitination pattern of MDM2 in 2KO-MEFs cells. Therefore, we concluded that SCYL1-BP1 promoted MDM2 degradation through proteasome pathway by stimulating its self-ubiquitination.

4. Discussion

Previously we used SCYL1 (also known as NTKL) as the bait to screen for its binding partners and identified SCYL1-BP1, a soluble protein highly conserved and widely expressed in many tissues. SCYL1-BP1 was also reported to interact with Rad6 and suggested to be a golgin by Hennies et al. [10]. And we also observed some aberrant expressions of some key cancer marker genes in our Human Cancer cDNA Expression Array assay (data not shown). But what exact function SCLY1-BP1 executes in *vivo*, by what kind of mechanism and through which pathway are still under research in our lab.

Following up our previous studies, here we show that SCYL-BP1 can bind to two RING finger E3: Pirh2 [20] and MDM2. Unlike p53, which is a common ubiquitination substrate of both Pirh2 and MDM2, SCYL1-BP is only degraded and ubiquitinated by Pirh2. However, by binding to MDM2, SCYL1-BP1 promotes the self-ubiquitination and degradation of MDM2. Recently growing numbers of MDM2 binding proteins have been reported, including nucleolar proteins (e.g. B23 [12] and C23 [16]), ribosomal proteins (e.g. ARF [18], S7 [2], L5 [4], NS [5] etc.) and centrosomal protein (Lat2 [1]). Interestingly, C23 shares several similar properties with SCYL1-BP1, such as binding to the center acidic region of MDM2, possessing a coiled-coil domain and impairing MDM2 protein level. It was also reported that C23 interacted with p53 when cells were subjected to genotoxic stress [16]. However, in our experiments we failed to detect any stable association between SCYL1-BP1 and p53 (data not shown), suggesting that SCYL1-BP1 and p53 bind to MDM2 asynchronously.

The different response of SCYL1-BP1 to MDM2 and Pirh2 is interesting. In the paper by Duan et al, they listed several differences between Pirh2 and MDM2, and suggested that Pirh2 promotes ubiquitination of p53 in an MDM2-independent manner [8]. It was also reported that increasing level of Pirh2 could reduce the tumor suppression function of p53 in the lung tumor cells [7]. As it appears critical for the cells to determine when, where and using which ubiquitin E3 to degrade p53 under different circumstances, the research on SCYL1-BP1 may provide us further clues on this regulation.

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Abbreviations

Pirh2	p53-induced RING H2 protein
MDM2	mouse double minute gene number 2
SCYL1	SCY1-like 1
SCYL1-BP1	SCY1-like 1 binding protein 1

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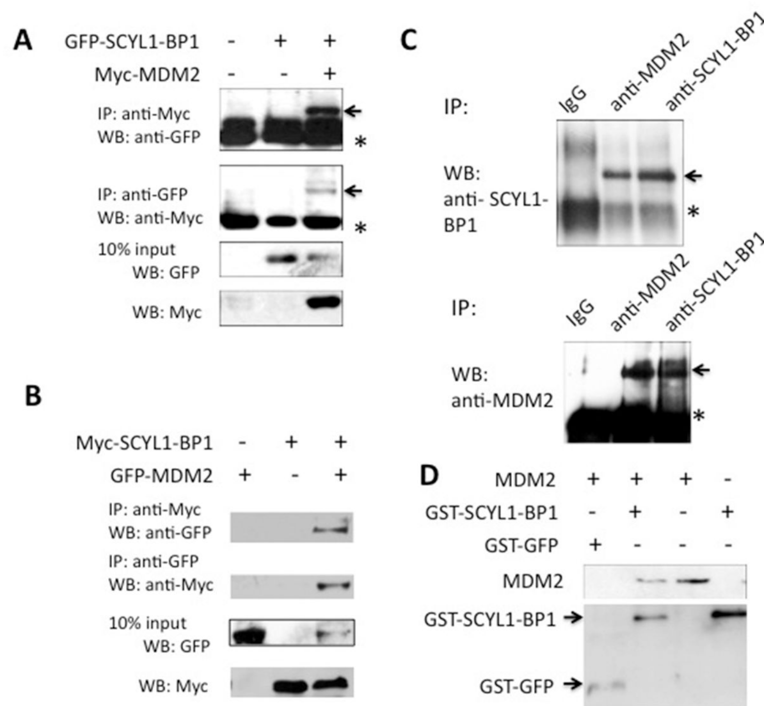

Fig. 1.

Interaction of SCYL1-BP1 and MDM2 in *vitro* and in *vivo*. (A) SCYL1-BP1 Bound to MDM2 in HEK293 cells. HEK293 cells were co-transfected with plasmids expressing GFP-SCYL1-BP1 and Myc-MDM2. Immunoprecipitates prepared by anti-Myc or anti-GFP antibody plus protein-G-agarose, then analysed by western blot using anti-GFP or anti-Myc antibodies as indicated, as well as the cell lysates (10% input). (B) SCYL1-BP1 interacted with MDM2 in the absence of p53. H1299 cells were co-transfected with plasmids expressing Myc-SCYL1-BP1 and GFP-MDM2. Lysates (10% input) and immunoprecipitates prepared by anti-Myc or anti-GFP antibodies with protein-G-agarose were analyzed by western blot using indicated antibodies. (C) Endogenous SCYL1-BP1 interacted with MDM2 in sk-hep1 cells. Lysates from sk-hep1 cells were split into two parts. One were immunoprecipitated with mouse immunoglobulin G (IgG), monoclonal anti-MDM2 antibody or anti-BP1 antiserum then detected by anti-SCYL1-BP1 antiserum; another one were immunoprecipitated by rabbit IgG, anti-BP1 antiserum, or monoclonal anti-MDM2 antibody, and then detected by anti-MDM2 antibodies. (D) SCYL1-BP1 bound to MDM2 in *vitro*. MDM2 protein (purchased from Bostonchem) was incubated with beads containing GST-GFP or GST-SCYL1-BP1. Following binding 3h in room temperature and extensive washing, the beads-bound material was analyzed by western-blot using anti-MDM2 and anti-GST antibodies. MDM2 alone and GST-SCYL1-BP1 alone were used as controls. (* Indicated unspecific band.)

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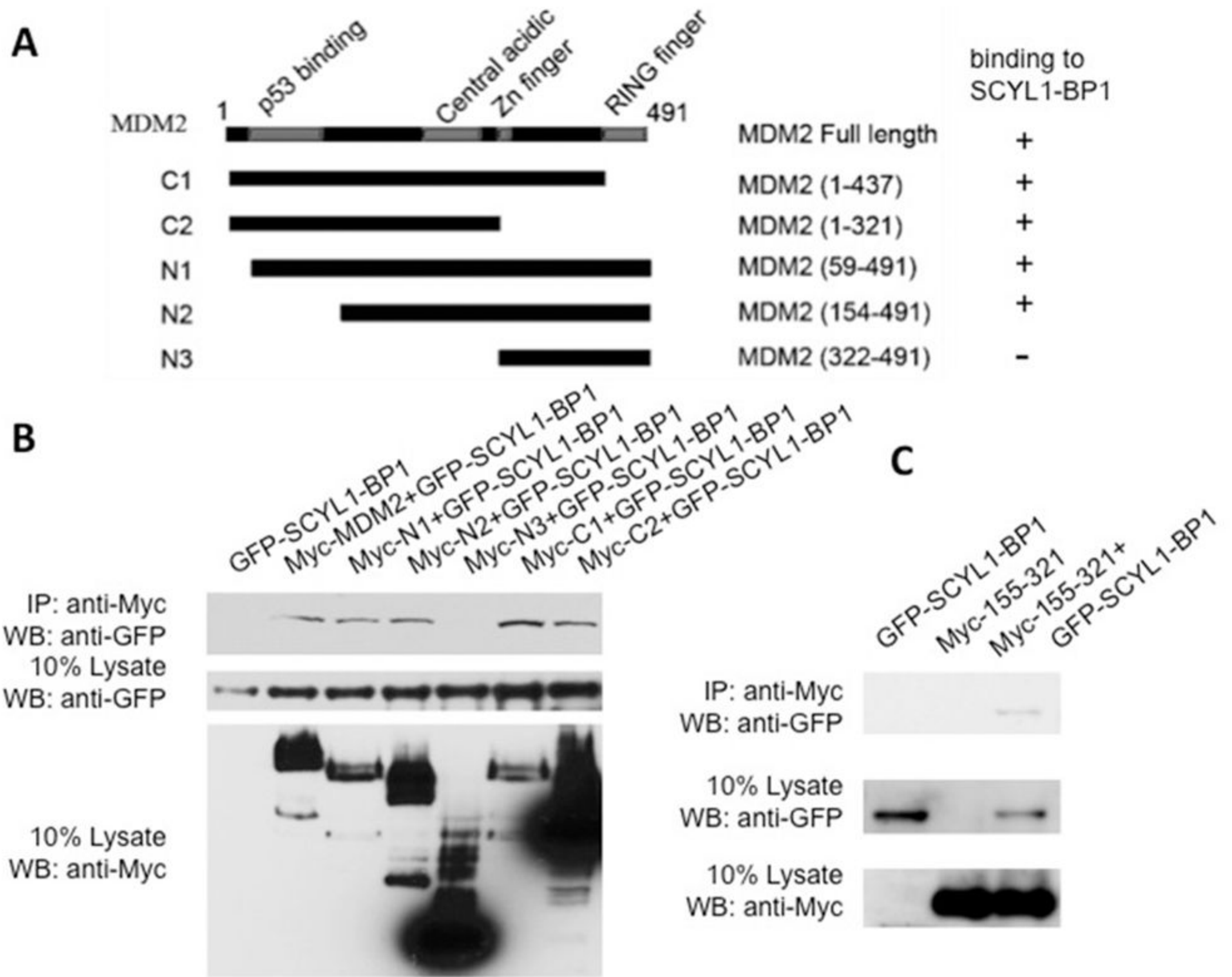

Fig. 2.

SCYL1-BP1 bound to the central acidic domain of MDM2. (A) Region of MDM2 necessary for binding to SCYL1-BP1. Various MDM2 mutants were expressed in HEK293 cells. (+) and (–) indicate presence and absence, respectively, of binding. Top row is schematic structure of wild-type MDM2 (modified from Michael and Oren, 2003). (B) SCYL1-BP1 failed to bind to the MDM2 mutant lack of the central acidic region. GFP-SCYL1-BP1 alone (lane 1) or with Series of Myc-tagged MDM2 mutants were transfected into HEK293 cells. Cell lysates were immunoprecipitated with anti-Myc antibody, followed by WB with anti-GFP antibody. (C) SCYL1-BP1 bound directly to the 155-321aa region of MDM2. GFP-SCYL1-BP1 and myc-MDM2-155-321 expression plasmids were transfected into HEK293 cells as indicated. Cell lysates were immunoprecipitated with anti-Myc antibody, followed by WB with anti-GFP antibody.

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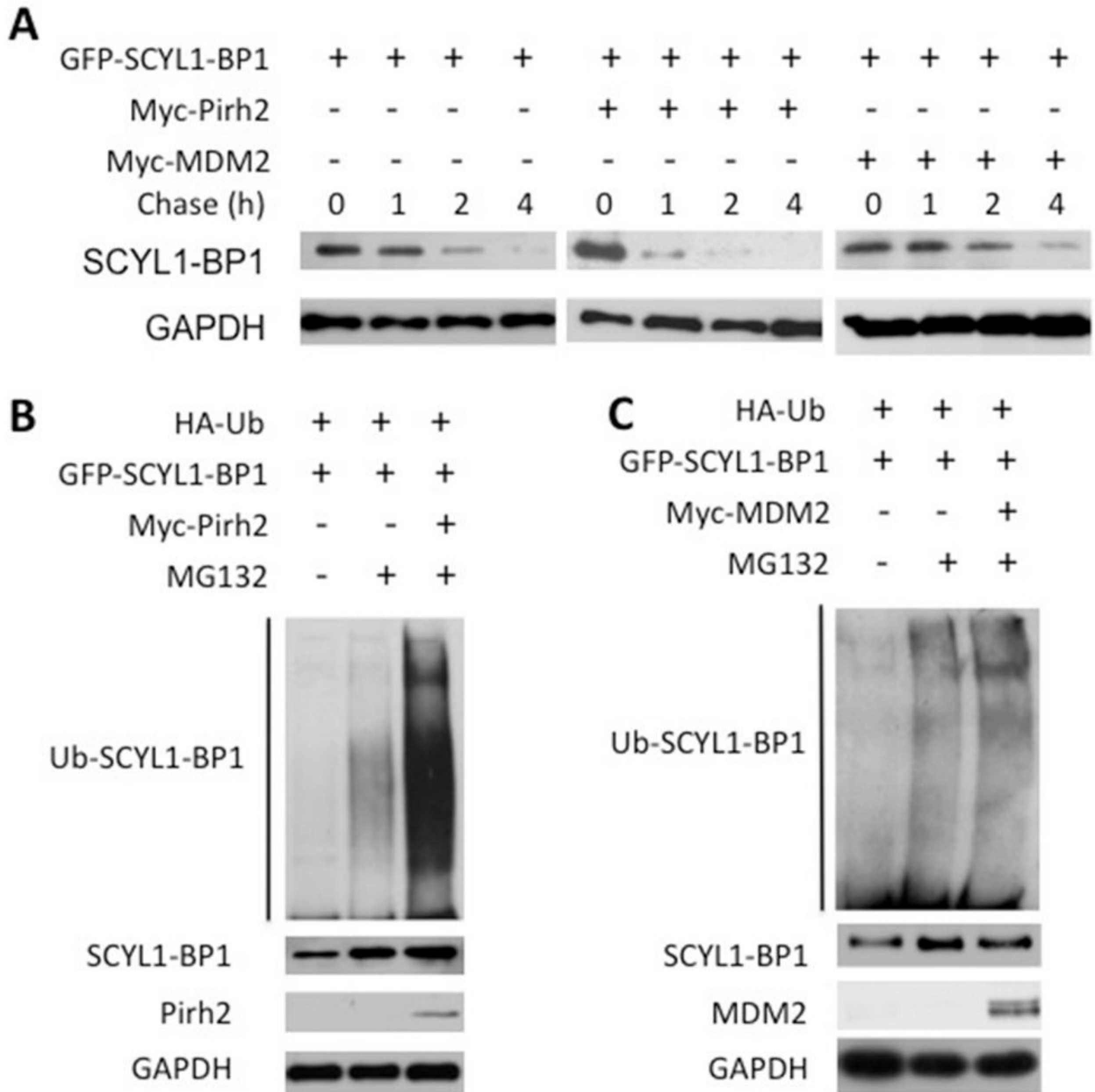

Fig. 3.

SCYL1-BP1 was a ubiquitination substrate of Pirh2 but not MDM2's substrate. (A) Only Pirh2 induced the degradation of SCYL1-BP1. GFP-SCYL1-BP1, Myc-Pirh2 and Myc-MDM2 plasmids were transfected into HEK293 cells as indicated. Before harvest, cells were treated with 50ug/ml CHX for 0,1,2,4 h. Then cell lysates were assayed for SCYL1-BP1 and GAPDH. (B) SCYL1-BP1 was ubiquitinated by Pirh2 but not MDM2.HEK293 cells were co-transfected with plasmids HA-ub, GFP-SCYL1-BP1 and Myc-Pirh2 or Myc-MDM2(C), cultured in the presence (+) or absence (–) of 30uM MG132 6h before harvest. The ubiquitinated conjugates purified by anti-GFP antibody combined with protein G agarose beads were analyzed by anti-HA antibodies. Cell lysates (10% input) were detected by western blot using the indicated antibodies.

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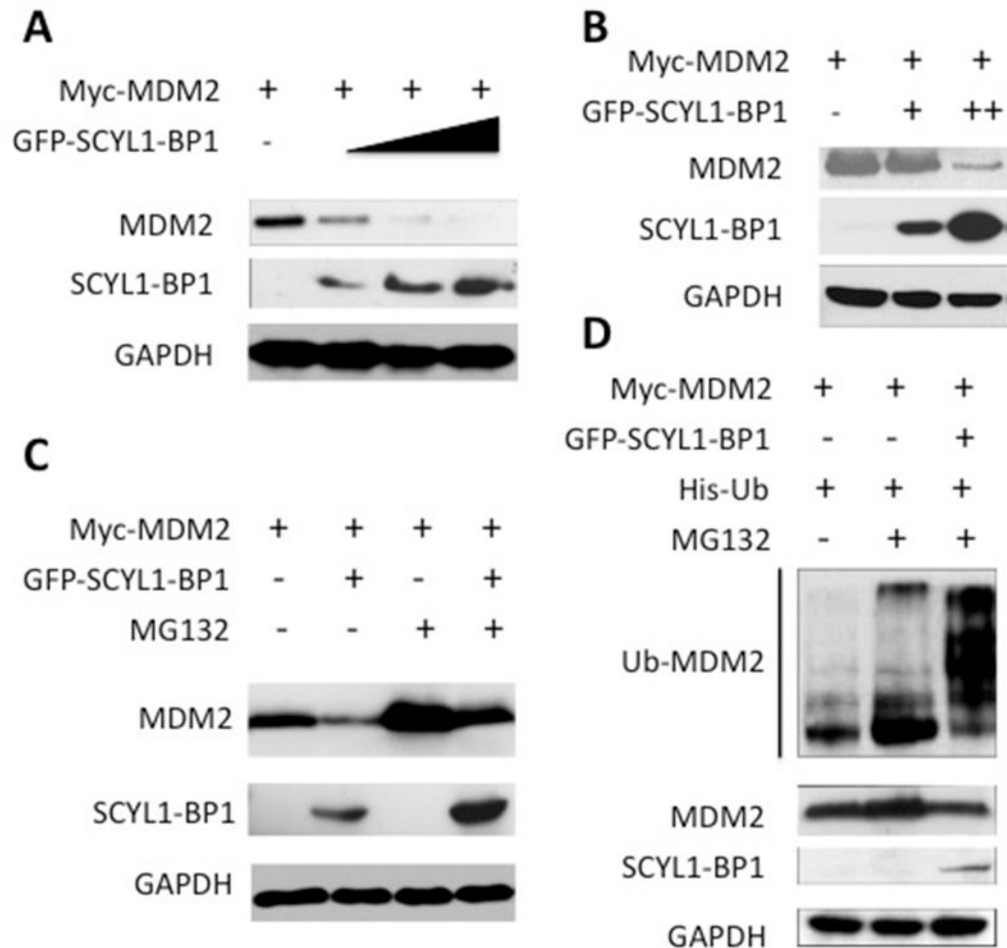

Fig. 4.

SCYL1-BP1 promoted mdm2 degradation by activating mdm2 auto-ubiquitination. (A) HepG2 (B) H1299 cells were transfected with increasing amount of GFP-SCYL1-BP1 plasmid, when required, cells were treated with 30 μM MG132 for 6 h before harvest. Cell lysates were assayed for MDM2, GFP-SCYL1-BP1 and GAPDH. (C) SCYL1-BP1 regulated MDM2 proteasomal degradation. H1299 cells were transfected with Myc-MDM2 and SCYL1-BP1 plasmids and then treated with or without MG132 as indicated. Cell lysates were assayed for MDM2, GFP-SCYL1-BP1 and GAPDH. (D) SCYL1-BP1 promotes MDM2 auto-ubiquitination in 2KO-MEFs cells. Lysates prepared from 2KO-MEFs cells transfected with the plasmids expressing His-Ub, Myc-MDM2 and GFP-SCYL1-BP1 plasmids were incubated with Ni-NTA-agarose. The precipitates were analyzed for ubiquitinated MDM2 using monoclonal anti-MDM2. 10% of total lysates were analyzed for MDM2, SCYL1-BP1 and GAPDH.

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Table 1

Primers used in this study

Name	Sequence (5′-3′)
P1	GGCCATACGGCCATGTGCAATACCAACATGTCTGTACC
P2	GGCCGAGGCGGCCCTAGGGGAAATAAGTTAGCACAATCAT
P3	GGCCGAGGCGGCCCTAAGGTTCAATGGCATTAAGGGGC
P4	GGCCGAGGCGGCCCTATCTGTTGCAATGTGATGGAAG
P5	GGCCATTACGGCCTGAAAGAGGTTCTTTTTTATCTTGGC
P6	GGCCGAGGCGGCCCTATCTGTTGCAATGTGATGGAAG
P7	GGCCATTACGGCCTGTTGGGCCCTTCGTGAGAATT