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UHRF2, another E3 ubiquitin ligase for p53

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ABSTRACT

UHRF2, ubiquitin-like with PHD and ring finger domains 2, is a nuclear E3 ubiquitin ligase, which is involved in cell cycle and epigenetic regulation. UHRF2 interacts with multiple cell cycle proteins, including cyclins (A2, B1, D1, and E1), CDK2, and p[21], moreover, UHRF2 could ubiquitinate cyclin D1 and cyclin E1. Also, UHRF2 has been shown to be implicated in epigenetic regulation by associating with DNMTs, G9a, HDAC1, H3K9me2/3 and hemi-methylated DNA. We found that UHRF2 associates with tumor suppressor protein p53, and p53 is ubiquitinated by UHRF2 in vivo and in vitro. Given that both UHRF2 and p53 are involved in cell cycle regulation, this study may suggest a novel signaling pathway on cell proliferation.

1. Introduction

p53 is a crucial tumor suppressor that could be activated by a plethora of cellular stress signals. Activation of p53 would cause multiple outcomes, including cell cycle arrest, apoptosis, senescence, and autophagy [1–3]. In response to DNA damage, p53 levels increase and directly results in the transcription of p21 (CIP1/WAF1) [4], a tight p53-controlled target that binds to and inhibits phosphorylation activity of cyclin D/CDK4/6 and cyclin E/CDK2 complexes on pRb. As long as pRb remains hypophosphorylated, cells stay in G1 phase [5–8].

UHRF2 is a multi-domain E3 ubiquitin ligase, which comprises an ubiquitin-like (UBL) domain, a tudor domain, a PHD finger domain, a SRA/YDG domain and a RING finger domain [9,10]. Previous researches reported that UHRF2 induces G1 arrest by interacting with inactivated CDK2-cyclin E complex [11]; UHRF2 associates with multiple cell cycle proteins, which includes cyclins (A2, B1, D1, and E1) and pRb; moreover, UHRF2 could ubiquitinate cyclin D1, E1, and PCNA [12,13].These results suggest UHRF2 would play a very important role in cell cycle regulation.

Ubiquitination is an important mechanism in cellular p53 levels regulation. In unstressed cells, p53 levels are constantly hold in check by its negative regulator MDM2, an E3 ubiquitin ligase which itself is a product of p53 that binds to and targets p53 for ubiquitin-mediated degradation [14]. Several other E3 ligases have also been proposed for p53, including human papilloma virus E6-associated cellular protein E6AP, Pirh2, COP1, ARF-BP1, WWP1, E4F1, and Ubc13. As polyubiquitination labels substrate proteins for degradation, monoubiquitination of target proteins often connects to several degradation-independent processes, including endocytosis and transcriptional regulation. It is reported that Mdm2-mediated ubiquitination of p53 could inhibit p21 gene expression by interfering with DNA binding activity of p53. On the contrary, ubiquitination of p53 by E4F1 reversely enhances transcription of p21. Additionally, there are evidences that monoubiquitination of p53 relates to its nuclear export [15–19].

Here, we describe that UHRF2 associates with p53 in vivo and in vitro, and UHRF2 serves as another E3 ubiquitin ligase for p53.

2. Materials and methods

2.1. Plasmid construction

Coding sequences for pGST-UHRF2 and all the domain-based GST fusion proteins expression constructs (pGST-UBL, pGST-Tudor, pGST-PHD, pGST-SRA/YDG, pGST-RING) were amplified from pCMV-3xFLAG-UHRF2 by standard PCR method. Subsequently, PCR amplicons were cloned into pGEX-4T-1 (GE Healthcare). pcDNA3-HA-p53 was a kind gift from Professor Jaewhan Song of Yonsei University, Korea.

2.2. Cell culture and transfection

HEK293 and COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfections were carried out using Effectene reagent (Qiagen, Valencia, CA, USA) or Lipofectamine 2000 (Invitrogen, CA, USA) according to manufacturer's protocols. After transfection, cells

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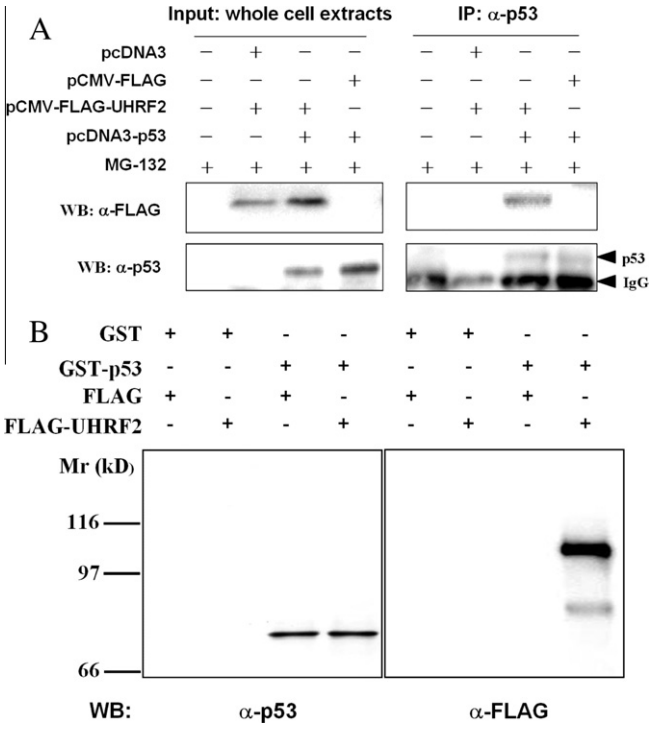

Fig. 1. UHRF2 interacts with p53 in vivo and in vitro: (A) UHRF2 interacts with p53 in vivo. HEK293 cells were co-transfected with pCMV-FLAG-UHRF2 and pcDNA3-p53 expression constructs; cell lysates were immunoprecipitated with Protein G agarose beads and anti-p53 antibody followed by Western blot analysis with anti-FLAG or anti-p53 antibodies. (B) UHRF2 interacts with p53 in vitro. Purified GST-UHRF2 was incubated with glutathione 4B-Sepharose immobilized GST-p53 or GST (control). Binding complex was subjected to Western blot assay, and blots were detected with anti-p53 or anti-FLAG antibodies.

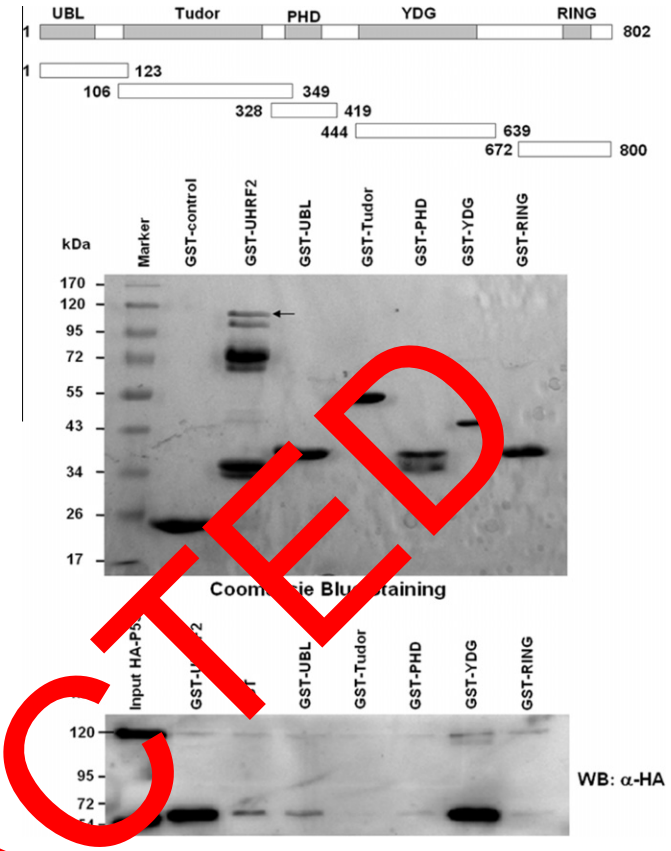

Fig. 2. UHRF2 interacts with p53 through its SRA/YDG domain: Domain-based GST fusion proteins were purified with glutathione 4B-Sepharose. Each GST fusion protein was incubated with equal amount of HA-p53 (transfected HEK293 cells extracts). Only GST-YDG (444–639) is associated with HA-p53 (bottom panel).

were further cultured for 48 h. MG-132 (10 μM) was added 12 h prior to harvest if necessary.

2.3. GST pull-down

GST fusion proteins were purified using glutathione–Sepharose 4B (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. Purified GST fusion proteins were then incubated with equal amount of HA-p53 (transfected HEK293 cells extracts) or purified FLAG-UHRF2 (anti-FLAG M2 affinity gel) for 2 h at 4 °C with constant shaking. Glutathione-Sepharose 4B beads were washed with ice-cold phosphate-buffered saline (pH 7.4) for 3 times. Bound proteins were eluted by elution buffer (50 mM Tris–Cl, 10 mM reduced glutathione, pH 8.0); eluted proteins were subjected to Western blot.

2.4. Antibodies

Antibodies used here included anti-FLAG monoclonal antibody (Sigma), anti-ubiquitin monoclonal antibody (Zymed, San Francisco, CA, USA), anti-Myc monoclonal antibody (Santa Cruz, CA, USA), anti-p53 monoclonal antibody (Santa Cruz, CA, USA) and anti-HA monoclonal antibody (Beyotime, Haimen, China).

2.5. Immunoprecipitation and Western blot

For immunoprecipitation, cells were collected and lysed in NP-40-based lysis buffer for 30 min, followed by centrifugation at 12,000×*g* for 15 min. Protein concentration were determined by bicinchoninic acid (BCA) assay (Beyotime, Haimen, China). Respectively, indicated antibodies and protein G agarose slurry (sc-2001, Santa Cruz, USA) were added into cell lysates and incubated for 2 h at 4 °C. Precipitates were washed for three times using ice-cold lysis buffer; bound proteins were eluted with sample buffer (0.35 M Tris–Cl, pH 6.8; 10.28% SDS, w/v; 36% glycerol, v/v; 20% 2-mercaptoethanol, v/v; 0.012% bromphenol blue, w/v;). Western blot was performed according to standard protocols.

FLAG-tagged proteins were precipitated with anti-FLAG M2 affinity gel (sigma) in accordance with manufacturer's recommendation. The gel was then washed three times with washing buffer (50 mM Tris–Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40) and washed once with phosphate-buffer saline; FLAG-tagged proteins were eluted by 3xFLAG peptide solution (150 ng/μl); then eluted proteins were used for immunoblotting or other downstream purposes.

2.6. In vitro ubiquitination

GST-UHRF2 and GST-p53 were purified using glutathione–Sepharose 4B resin. Complete reaction system was established consisting of reaction buffer (25 mM HEPES pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.05% Triton X-100, 0.5 mM dithiothreitol, 3 mM ATP, 10 μg FLAG-tagged ubiquitin [St. Louis, MO, USA]), 200 ng of E1, 200 ng of E2 (UbcH5a, Boston Biochem, Cambridge, MA, USA), 500 ng or 1 μg of GST-UHRF2, and 1 μg of GST-p53, in a final volume of 50 μl. Ubiquitination reactions were carried out at 37 °C for 60 min. When the reaction is completed, anti-FLAG M2 affinity gel was used to purify FLAG-Ub tagged protein.

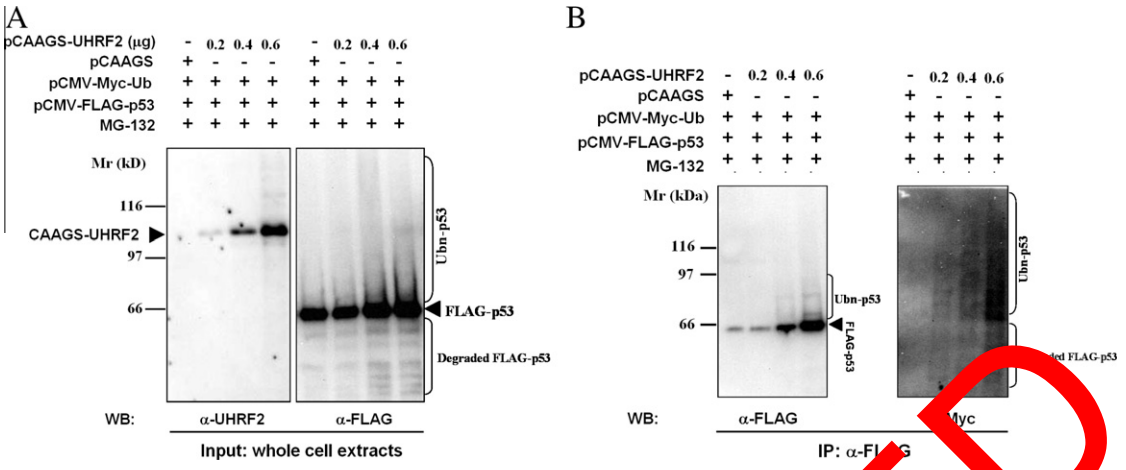

Fig. 3. UHRF2 could ubiquitinate p53 in vivo: (A) Increasing amount of pCAAGS-UHRF2 was co-transfected with pCMV-Myc-Ub and pCMV-FLAG-p53 in the presence of proteasome inhibitor MG-132 (5 μM) in HEK293 cells. Whole cell extracts were analyzed by immunoblotting using anti-UHRF2 or anti-FLAG antibodies. (B) FLAG-p53 was precipitated by anti-FLAG M2 affinity gel, and precipitated proteins were subjected to Western blot analysis using anti-FLAG or anti-Myc antibodies.

3. Results

3.1. UHRF2 could interact with p53 in vivo and in vitro

By carrying out co-immunoprecipitation assay, we found interaction between UHRF2 and p53 (Fig. 1A). Co-immunoprecipitation of p53 by UHRF2 indicates UHRF2 and p53 could, at least, form a supramolecular complex in vivo. To further verify the association of p53 and UHRF2, we performed GST pull-down assay. FLAG-UHRF2 was purified from COS-7 cells and incubated with purified GST-p53 or GST control. As demonstrated in Fig. 1B, FLAG-UHRF2 could directly bind to p53 in vitro.

3.2. UHRF2 interacts with p53 through its SRA/YDG domain

UHRF2 consists of 5 distinct domains: an ubiquitin like (UBL) domain, a Tudor domain, a PHD finger domain, a SRA/YDG domain, and a RING finger domain [9,10]. Among these domains, SRA/YDG domain, exclusively found in mammal and only found in UHRF2 and its family member UHRF1 (ICBP90) [20,21], could bind to methylated CpG DNA. Tudor and PHD domains are able to interact with H3K9, and they are required for UHRF2's pericentric heterochromatin localization [21,22]. RING finger domain, notably, is a landmark of E3 ubiquitin ligase. In order to pinpoint the exact domains required for binding of UHRF2 to p53, we generated 5 domain-based GST fusion proteins expression constructs (Fig. 2). GST pull-down results (Fig. 2) showed only the SRA/YDG domain is required for the association of UHRF2 with p53.

3.3. UHRF2 ubiquitinates p53 in vivo

Previous studies pointed out that UHRF2 is capable of ubiquitinating PCNP (a PEST-containing protein) and cyclins [12,13]. We then investigated if direct association of UHRF2 with p53 would lead to ubiquitination of p53. As shown in Fig. 3, intensifying signal of ubiquitin-conjugated p53 was detected (Fig. 3B), which indicated ubiqutination of p53 by UHRF2. Interestingly, degraded p53 was also observed (Fig. 3B), which may suggest that UHRF2 ubiquitinates p53 and promotes its degradation.

3.4. UHRF2 mediates ubiquitination of p53 in vitro

To further investigate ubiquitination of p53 by UHRF2, we carried out in vitro ubiquitination assay. As shown in Fig. 4, increasing

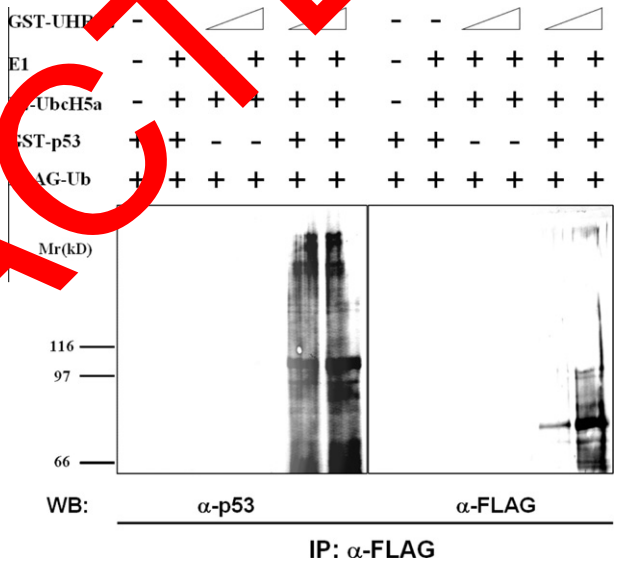

Fig. 4. UHRF2 could ubiquitinate p53 in vitro: In vitro ubiquitination reaction was established and performed as described in Section 2 with indicated combination of GST-UHRF2, E1, E2 (UbcH5a) and GST-p53. After completion of the reaction, proteins conjugated with FLAG-ubiquitin were purified with anti-FLAG affinity Gel followed by Western blot assay with indicated anti-p53 or anti-FLAG antibodies.

amount of ubiquitin-conjugated p53 could be detected by anti-p53 or anti-FLAG antibodies. However, we did not observe any self-ubiquitination bands of UHRF2 [13] (right panel of Fig. 4). Possibly, this may be the result of the conformation change caused by the GST tag, which may hinder necessary interaction required for self-ubiquitination, that was added to the N-terminus of UHRF2.

4. Discussion

4.1. UHRF2 as a central regulator in cell cycle

UHRF2 has been shown to play important roles in cell cycle regulation, epigenetic modulation, and nuclear Protein Quality Control machinery in mammalian cells [22–24]. UHRF2 could interact with multiple cell cycle proteins, including cyclins (A2, B1, D1, and E1), pRb, p53, and inactivated Cdk2–cyclin E complex, and it mediates ubiquitination of cyclin D1/E1 and PEST-containing nuclear protein

(PCNP) [11–13]. In classic p53-dependent cell cycle arrest, p53 could be stabilized in response to DNA damage by posttranslational modification. Increased p53 levels then directly lead to the transcription of p21 (CIP1/WAF1) [4], a cyclin-dependent kinase inhibitor that inhibits the phosphorylation activity of cyclin D/CDK4/6 and cyclin E/CDK2 complexes on pRb. As long as pRb is hypophosphorylated, pRb binds to and inhibits transcription activity of E2F-DP (E2 promoter-binding–protein-dimerization partners), which consequently stalls cells in G1 phase [5–8]. Although precise function of UHRF2 in cell cycle regulation is unclear, we could infer that UHRF2 is an important modulator in cell cycle control. Our data showed that UHRF2 could ubiquitinate p53, and possibly, p53 is polyubiquitinated (Fig. 3B, Fig. 4). If p53 is polyubiquitinated by UHRF2, then very likely, p53 is targeted for degradation. However, as shown in Fig. 3, although equal amount of p53 was transfected, ubiquitinated p53 levels increased as amount of UHRF2 increased. In accordance with previous evidences which reported that over-expressing of UHRF2 leads to G1 arrest [11], we tend to believe, by covalently conjugates ubiquitin to p53, UHRF2 may stabilize p53 and cause cell arrest in a p53-dependent manner. However, whether UHRF2 induced cell cycle arrest is dependent on p53, or reversely, whether p53-dependent G1 arrest is dependent on E3 ubiquitin ligase activity of UHRF2 on p53 needs to be tested out. Our study, which identified UHRF2 as an E3 ubiquitin ligase for p53, may contribute to another way of p53 regulation. At the same time, our data may suggest a novel signaling pathway on cell proliferation.

4.2. UHRF2 in epigenetic modulation

Recent researches demonstrated that UHRF2 has an opposite expression pattern with its family member UHRF1, and they do not functionally overlap [21]. Also, UHRF2 is associated with several important epigenetic modifiers, including histone deacetylase 1 (HDAC1), DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b), and histone methyltransferase G9a [24]. Moreover, UHRF2 specifically recognizes H3K9 methylation pattern (H3K9me2/3), and interaction of UHRF2 with this heterochromatin mark is required for its pericentric heterochromatin (PH) localization. It is reported that Tudor domain could bind to H3K9me2/3, and SRA/YDG domain could bind to hemimethylated DNA [10,20,21,24]. Our data suggest that UHRF2 associates with p53 through its SRA/YDG domain, but what does this association suggest needs more relevant researches. Similar to the study of UHRF2 on cell cycle regulation, although important associations with other candidate partners have been profoundly reported, how UHRF2 functions in these events remains elusive.

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