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CRL4B^{RBBP7} targets HUWE1 for ubiquitination and proteasomal degradation

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ABSTRACT

The E3 ubiquitin ligase HUWE1/Mule/ARF-BP1 plays an important role in diverse biological processes including DNA damage repair and apoptosis. Our previous study has shown that in response to DNA damage HUWE1 was downregulated in CUL4B-mediated ubiquitination and subsequent proteasomal degradation, and CUL4B-mediated regulation of HUWE1 was important for cell survival upon DNA damage. CUL4B is a core component of the CUL4B Ring ligase complexes containing ROC1, DDB1 and a DDB1-Cullin Associated Factors (DCAFs), the latter of which are DDB1-binding WD40 adaptors critical for substrate recognition and recruitment. However, the identity of DCAF in CRL4B that mediates degradation of HUWE1 remains elusive. Here we report that RBBP7 is the DCAF in the CRL4B complex bridging the DDB1-CUL4B-ROC1 to HUWE1. Loading of HUWE1 to the E3 ubiquitin ligase complex resulted in its polyubiquitination, and consequently its proteasome mediated degradation. Overexpression of RBBP7 promoted HUWE1 protein degradation, while depletion of RBBP7 stabilized HUWE1, and hence accelerated the degradation of MCL-1 and BRCA1, two substrates of HUWE1 that are critical in apoptosis and DNA damage repair. Taken together, these data reveal CRL4B^{RBBP7} is the E3 ligase responsible for the proteasomal degradation of HUWE1, and further provide a potential strategy for cancer therapy by targeting HUWE1 and the CUL4B E3 ligase complex.

1. Introduction

HUWE1 (also known as Mule, ARF-BP1, E3 Histone, UREB1, HECTH9 and LASU1) is a HECT E3 ubiquitin ligase involved in various cellular processes, such as DNA replication [1], apoptosis [2], DNA damage repair [3–8], stem cells development and maintenance [9,10], and transcriptional regulation [11–15]. Among them, apoptosis and DNA damage repair seem to be the main function of HUWE1. MCL-1, a member of the BCL-2 anti apoptosis family, is currently being confirmed to be the most important substrate of HUWE1. It has been shown that HUWE1-regulated ubiquitination and proteasomal degradation of MCL-1 are required to induce apoptosis in response to DNA damage [2]. Other apoptosis related proteins, such as Mfn2 and RASSF1C were also found to be substrates of HUWE1 [16,17]. HUWE1 targets these proteins to ubiquitination and proteasomal degradation, thereby promoting apoptosis process. At the same time, HUWE1 is a p53 protein ubiquitin E3 ligase [15], and can regulate the p53 dependent apoptotic pathway by targeting p53 for degradation. Recently, we discovered that BRCA1 can be regulated by HUWE1 through the ubiquitin-proteasomal system (UPS) [8]. Overall, these findings highlight a critical role of HUWE1 in the mediation of apoptosis and DNA damage repair.

CUL4-based complexes (CRL4) encompass CUL4A/B-DDB1-ROC1, where DDB1 bridges the association between CUL4A/B and substrate receptors. Substrate receptors for CRL4 complexes normally contain a linker protein termed DDB1 and CUL4-associated factors (DCAF) which contains specific WD40 repeats [18]. Recently, multiple WD40 proteins were found to interact with DDB1 and serve as the substrate-recognition subunits of the CUL4-DDB1 ubiquitin ligase complex [19–23]. One of these WD40 domain-containing proteins, Retinoblastoma binding protein P46 (RBBP7), also known as RbAp46, is an integral subunit in HDAC complex [24,25], ATP-dependent nucleosome-remodeling factor (NURF) complexes(22), and EZH2/EED methylase complexes. In

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addition, studies revealed that RBBP7 acts as a tumor suppressor which inhibits the malignant phenotype of various cancers types, including breast cancer [26], lung cancer and bladder cancer [27]. CRL4 complexes are involved in a wide variety of cellular processes, including DNA repair, DNA replication and chromatin remodeling [5].

In this study, using a GST pull-down approach, we have identified HUWE1 and DDB1 as two major binding partners of RBBP7. Our data show that the $CRL4^{RBBP7}$controls the stability of HUWE1, which in turn regulates MCL-1 and BRCA1 protein levels. In addition, HUWE1 is recruited to the E3 ligase through RBBP7, resulting in its poly-ubiquitination and degradation. This study sheds light on the mechanism through which HUWE1 is regulated and has significance for cancer treatment.

2. Materials and methods

2.1. Plasmids

In this study pEYFP-RBBP7 was first constructed by PCR-amplification of the coding sequence of *RBBP7* from Hela cDNA and subcloned into pEYFP plasmid, using primers BglII-Rbbp7F (5′GGAAGATCTATGGCGAGTAAAGAGATGTTT3′) and Kpn I-Rbbp7R (5′CGGGGTACCTTAAGATCCTTGTCCCTCCAG3′). pcDNA3.1-Flag-HA-RBBP7 was generated by PCR using primers XbaI-Rbbp7-1 (5′GCTCTAGAATGGCGAGT AAAGAGATGTTT3′) and Kpn I- Rbbp7R and subcloned into pcDNA3.1-Flag-HA/Hygro in between the XbaI and KpnI cloning sites; For construction of pGEX-6P-3-RBBP7, plasmid pEYFP-RBBP7 was cleaved by BamH1 and BglIIenzymes, and the released ORF of *Rbbp7* was subcloned into pGEX-6P-3. pcDNA3-ARF-BP1-V5-His was provided by Professor Wei Gu (Columbia University), and pFast-Bac-Mule was provided by Professor Xiaodong Wang (UT Southwestern Medical Center). pFast-Bac1-GST-Huwe1-1-2500 was constructed by introducing a Glutathione S-transferase (GST)-tag fused HUWE1 DNA fragment encoding amino acids 1–2500 of HUWE1 protein.

2.2. Cell culture, transfection and RNA interference

HEK293T, Hela H3 and Hela cells were cultured in DMEM containing10% FBS (Hyclone) and 100 μg/ml penicillin-streptomycin at 37 °C in a 5% CO_2 incubator. Cells were transient transfected with various plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol., *RBBP7* and the negative control siRNAs were synthesized from Invitrogen or Genepharma. The siRNAs targeting sequences were: siHUWE1, 5′-AAUUGCUAUGUCUCUGGGACA-3′; siRBBP7-1, 5′-GGACACACUGCUAAGAUUU-3′; siRBBP7-2, 5′-GGAUAAGACCGUAGCUUUA-3′. Cells were transfected with siRNAs using Lipofectamine RNAi MAX (Invitrogen) according to the manufacturer's protocol.

2.3. GST pull-down assay

For *in vitro* GST pull-down, GST and GST-RBBP7 immobilized on glutathione-agarose beads were incubated with Hela cell lysate at 4 °C for 4 h, respectively. The beads were washed, and the proteins were eluted by adding 5 × SDS loading buffer, boiled, and analyzed by Western blotting.

2.4. Cycloheximide (CHX) chase assay

293T and Hela cells were transfected with the indicated plasmids or siRNAs. 48 h after transfection, cells were treated with cycloheximide(100 μg/ml), harvested at indicated time points and then subjected to immunoblotting analysis.

2.5. Immunoprecipitation and western blotting

Cells were harvested and lysed in NETENG-400 buffer (50 mM Tris-HCl, pH 8.0, 400 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 10% Glycerol) with protease inhibitors (1 mM PMSF; protease inhibitor cocktail, Sigma) and protein phosphatase inhibitors, for 20 min on ice, and centrifuged at 13,000 rpm for 15 min. The lysate was diluted with 1.68 volume of the lysate of the NETENG-0 buffer without NaCl to obtain a final concentration of NaCl at 150 mM (50 mM Tris-HCl, pH 8.0,150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 10% Glycerol). The diluted lysate was incubated with the appropriate antibodies or anti-FLAG-M2 Agarose at 4 °C with rocking for 2 h. Protein-G beads was then added or not, and the incubation was continued for an additional 4 h. The proteins bound to the beads were washed three times with NETENG-150 buffer. The bound proteins were eluted by 100 mM Glycine, pH 2.5, neutralized by adding 1 M Tris-HCl, pH 8.5 (1/10 volume of elution buffer), followed by 3–15% SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. For western blotting, total cell lysate was obtained by using RIPA buffer (50 Mm Tris-HCl (pH 8.0), 0.1% SDS, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and protease inhibitor cocktail).

2.6. Ubiquitination assays

For *in vitro* ubiquitination assay, different combinations of E1 (100 ng, Boston Biochem), E2(100 ng, Boston Biochem) and E3 (CRL4B, GST-RBBP7) were mixed with 100 ng of recombinant GST-HW2500 substrate in a ubiquitin ligase reaction buffer (2 μg of HA-ubiquitin, 2 mM ATP, 5 mM $MgCl_2$, 2 mM DTT, 50 mM Tris-HCl, pH 7.4). Reactions were carried out for 90 min at 30 °C, terminated by boiling for 10 min in a SDS sample buffer followed by 3–8% SDS-PAGE gel, and blotted with anti-HA or anti-HUWE1 antibody.

2.7. Purification of protein

The purification details of GST-HUWE12500, GST-RBBP7 and Flag-HA-CUL4B complex proteins were available in Supplementary materials.

2.8. Statistical analysis

Data are presented as mean values ± S.E. Statistical significance was analyzed using GraphPad Prism 7 software, Western Blot gray value is calculated by Image J software. All experiments in which the significance levels are shown were performed for at least three times.

3. Results

3.1. RBBP7 links HUWE1 to DDB1-CUL4B-ROC1 E3 ligase complex

In a previous report, we showed that DNA damage-induced activation of CUL4B targets HUWE1 for proteasomal degradation and that the integrity of the DDB1-CUL4B-ROC1 ubiquitin ligase complex is indispensable for this process [28]. Additionally, CUL4B was reported to associate with the WD40-containing adaptor protein RBBP7 *in vivo* [29], which was further confirmed by our current study (Fig. 1A). More importantly, RBBP7 was found to interact with BRCA1, a substrate of HUWE1 [8,30,31]. Since RBBP7 and DDB1 have been shown to assemble with CUL4B and ROC1 to form an E3 ubiquitin ligase complex, we asked whether RBBP7 is an adaptor protein in the CRL4B complex responsible for the recognition and recruitment of HUWE1. Aim to this, we first examined interactions between RBBP7 and HUWE1 using

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