Biochemical and Biophysical Research Communications 461 (2015) 624–629

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Both Rbx1 and Rbx2 exhibit a functional role in the HIV-1 Vif-Cullin5 E3 ligase complex *in vitro*

Xiaodan Wang [a, c, 1], Xiaoying Wang [a, 1], Weiran Wang [a], Jingyao Zhang [a], Jiawen Wang [a], Chu Wang [a], Mingyu Lv [a], Tao Zuo [a], Donglai Liu [a], Haihong Zhang [a], Jiaxin Wu [a], Bin Yu [a], Wei Kong [a, b], Hui Wu [a, b, *], Xianghui Yu [a, b, *]

[a] National Engineering Laboratory for AIDS Vaccine, College of Life Science, Jilin University, Changchun, Jilin Province, People's Republic of China
[b] Key Laboratory for Molecular Enzymology and Engineering of the Ministry of Education, College of Life Science, Jilin University, Changchun, Jilin Province, People's Republic of China
[c] Institute of Immunology, The First Bethune Hospital Academy of Translational Medicine, Jilin University, ChangChun, People's Republic of China

ARTICLE INFO

Article history:
Received 17 March 2015
Available online 23 April 2015

Keywords:
HIV-1
Protein degradation
RING box proteins
Ubiquitin-protein ligases
Vif

ABSTRACT

Rbx1 and Rbx2 are essential components of Cullin-RING E3 Ligases. Vif is generally believed to preferentially recruit the Cul5-Rbx2 module to induce proteasomal degradation of antiretroviral enzyme APOBEC3G, although some investigators have found that the Cul5-Rbx1 module is recruited. Here, to investigate the function of the two Rbx proteins in the Vif-Cul5 complex, we analyzed the performance of Cul5-Rbx1/Cul5-Rbx2 module in the activity of Vif E3 ligase and evaluated the interactions between Rbx1/Rbx2 and Cul5. We found that either Rbx1 or Rbx2 could promote ubiquitination of APOBE3G (A3G) *in vitro*. We also found that both Rbx1 and Rbx2 could bind Cul5 in cells and Rbx2 could dose-dependently inhibit the interaction of Rbx1 with Cul5. Furthermore, only the decrease of endogenous Rbx2 but not Rbx1 could impair the Vif-induced A3G degradation in cells. These findings indicate that Rbx1 and Rbx2 can both activate Cul5-Vif E3 ligase *in vitro*, but they may undergo a more delicate selection mechanism *in vivo*.

1. Introduction

The ubiquitin (Ub)-proteasome pathway is the most predominant mechanism for cellular protein catabolism in mammals. Ubiquitination of the target protein involves a three-step enzymatic reaction catalyzed by an E1 Ub-activating enzyme, an E2 Ub-conjugating enzyme and an E3 Ub protein ligase [1]. E3 ligases play a critical role in ubiquitination by recognizing substrates with specificity, recruiting Ub-loaded E2 and inducing the Ub transfer from the E2 to the target substrates [2]. Cullin (Cul)-RING E3 Ligases (CRLs), also known as Skp1–Cul–F-box proteins (SCF), comprise the largest E3 family containing Cul–Rbx modules [3]. The catalytic RING components consist of two members, Rbx1/ROC1/Hrt1 and Rbx2/ROC2/SAG [3].

Both Rbx1 and Rbx2 contain a conserved RING-H2 finger domain in their carboxy-terminus which is essential for their ligase activity [4,5]. Rbx1 expression is constitutive, whereas Rbx2 expression is inducible by stress, such as redox agents and hypoxia [6]. CRLs are activated by covalent attachment of the Ub-like protein NEDD8 to a C-terminal lysine residue of Cul proteins [7]. Like the Ub pathway, the NEDD8 cascade involves E1, E2 and E3 [8]. Rbx1 and Rbx2 were reported to function as E3s for the NEDD8 pathway and specifically regulate NEDD8ylation of Cul1–4 and Cul5, respectively [9]. Another crucial function of Rbx proteins is that they are able to directly bind E2s thioesterified with Ub (E2~Ub) and promote transfer of Ub from E2~Ub to the substrate [10].

HIV-1 Vif counteracts host antiviral factor APOBEC3 proteins by taking advantage of a host CRL. It serves as an adapter bridging APOBEC3s with the Cul5-CBF-beta-Elongin B/C-Rbx E3 ligase and

* Corresponding authors. National Engineering Laboratory for AIDS Vaccine, College of Life Science, Jilin University, Changchun, Jilin Province 130012, People' s Republic of China. Fax: +86 0431 85167674.
E-mail addresses: topwuhui@jlu.edu.cn (H. Wu), xianghui@jlu.edu.cn (X. Yu).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2015.04.077
0006-291X/

induces their proteasomal degradation [11,12]. In the initial report on this Vif-Cul5 E3 complex in 2003, Rbx1 was found to be recruited to form the Cul5-Rbx module [11]. Other researchers also supported the notion that Cul5 can pair with Rbx1 [13–17]. However, more recent studies have proposed that Rbx2 preferentially and exclusively interacts with Cul5 [9,18–21]. In this work, to evaluate which Rbx protein is more likely required in Vif function, we analyzed the performance of Cul5-Rbx1 and Cul5-Rbx2 module in the activity of Vif E3 ligase and found that Rbx1 and Rbx2 can both activate Cullin5-Vif E3 ligase *in vitro* but only the deficiency of Rbx2 could induce an obvious suppression of the Vif-mediated depletion of A3G in cells. We also investigated the interactions between Cul5 and Rbx1/Rbx2 in detail. In addition, we discussed the possible explanations about interaction of Cul5 and Rbx1 which seems to be redundant. Our results demonstrated that Rbx1 and Rbx2 can bind and activate Vif-Cul5 E3 ligase equivalently *in vitro*. However, the function of Cul5 in cells preferentially depends on the presence of Rbx2, indicating that the selection of RING domain proteins by Cul5 may undergo a more delicate mechanism *in vivo*.

2. Materials and methods

2.1. Plasmids

The polycistronic expression plasmid pST39/pET3aTr (a generous gift from Song Tan, Pennsylvania State University, University Park, PA) was used to produce the plasmids pST39-RBX1-HisCullin5, pST39-RBX2-HisCullin5 and pST39-CBF-beta-EloB-EloC-HisVif for co-expression of protein complexes in *Escherichia coli*. In these plasmids, the coding sequences of the following proteins were subcloned into the indicated restriction sites: RBX (RBX1/RBX2), *Xba*I and *Bam*HI; HisCullin5, *Bsp*EI and *Mlu*I; CBF-β, *Xba*I and *Bam*HI; EloB, *Eco*RI and *Hind*III; EloC, *Sac*I and *Kpn*I; and HisVif, *Bsp*EI and *Mlu*I [22]. VR-RBX1, VR-RBX2 and VR-Cullin5 were generated by subcloning RBX1, RBX2 and Cullin5, respectively, into the VR1012 vector at the *Pst*I and *Bam*HI restriction sites. VR-Cul5-Myc, VR-RBX1-HA, VR-RBX2-HA, VR-RBX1 I44A-HA, VR-RBX2 I52A-HA, VR-Vif-HA, VR-Vif-Myc and pcDNA3.1-A3G-HA (PC-A3G-HA) have been previously described [9,11]. The infectious molecular clone HXB2ΔVif was obtained from the National Institutes of Health AIDS Research and Reference Reagents Program (NIH-ARRRP), Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID) in Germantown, MD.

2.2. Cells, antibodies and transfections

HEK293T (CRL-11268) cells and HEK293 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2. Antibodies used for Western blotting in this study were as follows: anti-Myc mouse monoclonal antibody (mAb, Millipore, Billerica, MA), anti-HA mouse mAb (Covance, Emeryville, CA), anti-tubulin mouse mAb (Covance), anti-Cul5 mAb (Santa Cruz Biotechnology, Santa Cruz, CA), anti-ROC1/RBX1 antibody (Abcam, Cambridge, MA), anti-RBX2 (Santa Cruz Biotechnology), anti-EloB rabbit polyclonal antibody (pAb, Abcam), anti-EloC mouse mAb (BD Transduction Lab, San Jose, CA), anti-CBF-β mouse mAb (Santa Cruz Biotechnology), anti-Vif antibody (NIH-ARRRP) anti-APOBEC3G antibody (Abcam), anti-NEDD8 antibody (Bioworld, Dublin, OH, USA), anti-biotin antibody (Invitrogen, Carlsbad, CA), Alkaline phosphatase (AP)-conjugated goat anti-rabbit and goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA, USA) Transient plasmid transfections into HEK293T cells were performed by using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen).

2.3. RNA interference (RNAi)

RBX2 shRNA (target sequence: TAAACTGCACTTTATTTGT) and the Negative Control shRNA constructs were obtained from Shanghai GenePharma Co., Ltd (Shanghai, China). 1.2 μg shNC or shRBX2 expressing plasmids were transfected into 0.8×10^6 HEK293 cells. The monoclonal cells were selected with G418 (450 ug/ml) and re-cultured repeatedly for two weeks to establish the Rbx2 shRNA stably-transfected cell line. The following double-stranded siRNAs targeted to human RBX1 were also purchased from Shanghai GenePharma: RBX1-siRNA1 (siRBX1-1), CAAGAGGACUGUGUUGUGGTT; RBX1-siRNA2 (siRBX1-2), GCAGGAACCACAUUAUGGATT and RBX1-siRNA3 (siRBX1-3), GGACAACAGAGAGUGGGAATT. The AllStars Negative Control siRNA was purchased from Qiagen (siNC, Hilden, Germany). Rbx1 siRNAs were transfected into shRbx2 or shNC stable HEK293 cells by using HiPerfect transfection reagent (Qiagen) at a final concentration of 20 nM according to the manufacturer's instructions. The efficiency of siRNA silencing of Rbx1 was evaluated by immunoblotting 2 days after transfection.

2.4. Co-immunoprecipitation assay

Two days after transfection, HEK293T cells were harvested, washed twice with cold PBS and lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100) supplemented with Complete protease inhibitor cocktail tablets (EDTA Free, Roche, Mannheim, Germany) at 4 °C for 1 h. The cell lysates were centrifuged at 10,000× *g* for 30 min and then incubated with anti-HA Ab-conjugated agarose beads (Roche) at 4 °C for 3 h. For Myc-tag immunoprecipitation, the pre-clarified cell lysates were mixed and incubated with anti-Myc (Millipore) for 3 h, followed by incubation with Protein G-agarose (Roche) for an additional 3 h at 4 °C. Subsequently, the beads were washed three times with washing buffer (20 mM Tris, pH = 7.5, 100 mM NaCl and 0.05% Tween-20), boiled in SDS sample buffer and then subjected to SDS-PAGE and analyzed by Western blotting.

2.5. NEDD8 conjugation assay

Rbx1/Rbx2-Cul5 proteins (2 μM) were conjugated with NEDD8 at 37 °C for 1 h in the reaction buffer containing 250 nM E1 (APPBP1/Uba3), 5 μM E2 (UbcH12 and UBE2F), 25 μM NEDD8, 1 mM Mg-ATP, 2 mM ATP, according to the manufacturer's recommendations (Boston Biochem, Cambridge, MA). After the NEDD8 reaction was completed, one-sixth of the mixture was subjected to the ubiquitination assay.

2.6. Ubiquitination assay

Ubiquitination assays were performed at 37 °C for 1 h. The reaction system contained: E1, 200 nM; E2 (UbcH5b), 4 μM; Biotinylated Ubiquitin (Bt-Ub), 1 μM; Mg-ATP, 10 mM; Rbx1/Rbx2-Cul5 (NEDD8 modified), 0.625 μM; His Vif-CBF-beta-EloB-EloC, 0.938 μM; and substrate A3G, 1 μM (provided by NIH-ARRRP), according to the instructions of the Ubiquitinylation Kit (Enzo, Farmingdale, NY). Finally, the ubiquitination reactions were quenched by the addition of 2 x SDS loading buffer (Enzo).

3. Results and discussion

3.1. Both Rbx1 and Rbx2 can play a functional role in the reconstituted Vif E3 ligase in vitro

To investigate the function of the two Rbx proteins in the Vif-Cul5 complex, we reconstituted an active Vif E3 complex by taking advantage of the pST39 polycistronic expression system to co-express the components of the His-Cul5-Rbx1, His-Cul5-Rbx2 or His-Vif-CBF-beta-EloB-EloC complex [22,23]. The His-Vif-CBF-beta-EloB-EloC protein complex was obtained with high purity (Fig. 1A), and both Rbx1 and Rbx2 could be co-purified with Cul5 *in vitro* (Fig. 1B, C).

To evaluate which Cul5-Rbx module is more likely required in Vif function, we combined the His-Vif-CBF-beta-EloB-EloC complex with the His-Cul5-Rbx1 or His-Cul5-Rbx1 complex and compared the abilities of the two combinations to induce A3G ubiquitination. Because NEDDylation is essential for Cul5 to mediate A3G ubiquitination, we modified Cul5 with NEDD8 before mixing the Cul5-Rbx modules with the His-Vif-CBF-beta-EloB-EloC complex. As Rbx2 RING has been reported to be inherently specific for UBE2F [9], we also supplemented the kit with His6-UBE2F to the same amount as UBE2M from the kit. The NEDD8-modified Cul5-Rbx modules were then examined for the ability to induce ubiquitin chain formation on the A3G substrate. The A3G substrate was mixed with the Ubiquitination Kit components and Neddylated His-Cul5-Rbx1, His-Cul5-Rbx2 complex or both in the absence or presence of His-Vif-CBF-beta-EloB-EloC (VCBC) complex, Ub and E1-E2. The results showed that both the Cul5-Rbx1 and Cul5-Rbx2 module could promote A3G polyubiquitination *in vitro*, and this promotion depended on the presence of the VCBC complex (Fig. 1D).

3.2. Rbx2 suppresses the interaction between Rbx1 and Cul5 in HEK293T cells

Rbx1 was reported to be able to interact with all human Cul proteins in the yeast two hybrid system [24]. When the Vif-Cul5 E3 complex was identified for the first time in 2003 [11], Rbx1 was generally believed to be recruited into the Cul5-Rbx module [13–16]. However, Rbx2 but not Rbx1 has increasingly been shown to interact with Cul5 to mediate E2 binding [9,18,20,21,25]. Given the fact that the Cul5-Rbx2 module is preferentially utilized by most Cul5-containing E3 complexes, we further questioned why an interaction between Cul5 and Rbx1 would still be detectable and whether it is redundant [11,13–16,24].

In most previous reports supporting the Cul5-Rbx1 module, it was identified in the context of Rbx1 over-expression [13–15]. Cul5 and Rbx1 were shown to interact so stably *in vitro* that they were able to be co-crystallized [17]. To determine whether Cul5 can interact with endogenous Rbx1, a vector expressing Cul5-Myc or empty vector VR1012 was transfected into HEK293T cells for analysis by anti-Myc immunoprecipitation. Endogenous Rbx1 could be efficiently co-immunoprecipitated with Cul5-Myc (Fig. 2A), consistent with a previous observation by Harada et al. [11,16]. We also attempted to examine the interaction between endogenous Rbx2 and Cul5-Myc, but no visible Rbx2 could be detected in the cell lysate and immunoprecipitated proteins, perhaps due to low extraction efficiency of endogenous Rbx2 by the RIPA lysis buffer and poor affinity of the Rbx2 antibody (data not shown).

To confirm whether over-expressed Rbx1 and Rbx2 could bind to Cul5 in cells, we transfected the HA-tagged Rbx1 or Rbx2 expression vectors separately or together into HEK293T cells in the absence or presence of Cul5-Myc expression vector. Both over-

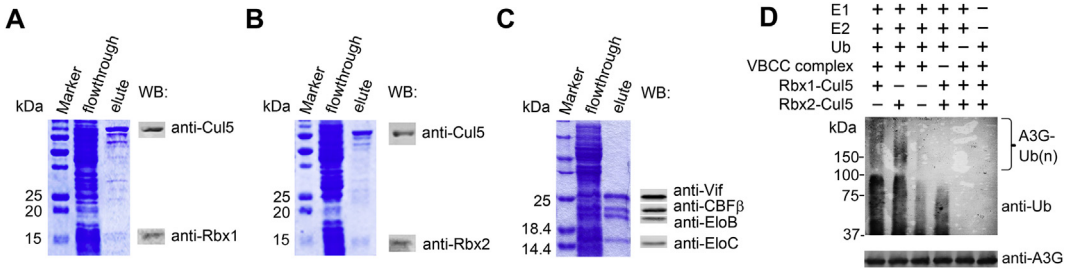

Fig. 1. Reconstitution of active Vif E3 ligase with Cul5-Rbx1 and Cul5-Rbx2 module *in vitro*. (A), (B) The cell lysate was applied onto a Ni-affinity column (GE Healthcare, Piscataway, NJ) and washed with wash buffer (25 mM Tris–HCl, 40 mM imidazole, 500 mM NaCl, pH = 8.0). HisCul5-RBX1/HisCul5-RBX2 complex was eluted with elution buffer (25 mM Tris–HCl, 80 mM imidazole, 500 mM NaCl, pH = 8.0). The purified proteins were subjected to SDS–PAGE and analyzed by Coomassie Brilliant Blue staining and Western blotting (WB). (C) His Vif-CBF-beta-EloB-EloC complex was purified and identified as described above. (D) NEDD8-modified Cul5-Rbx1/Cul5-Rbx2 was mixed with His Vif-CBF-beta-EloB-EloC at a ratio of 1:1.5 (mol:mol) and then subjected to the ubiquitination assay, followed by SDS–PAGE and immunoblot analysis with an anti-biotin antibody (anti-Ub) and anti-A3G antibody.

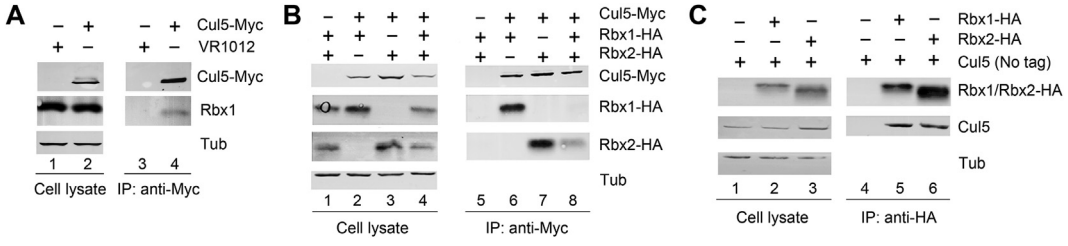

Fig. 2. Immunoprecipitation analysis of Rbx1/Rbx2 and Cullin5 interactions. (A) HEK293T cells (10^6) were transfected with 1 μg of VR-Cul5-Myc or VR1012. Cell lysates were immunoprecipitated with anti-Myc antibody, followed by SDS–PAGE and immunoblot analysis with anti-Myc, anti-Rbx1 and anti-tubulin antibodies. (B) HEK293T cells (10^6) were co-transfected with 1 μg of VR-Cul5-Myc or VR1012 and 1.5 μg of VR-RBX1-HA or 1.5 μg of VR-RBX2-HA or both of them. Cell lysates were immunoprecipitated with an anti-Myc antibody, followed by SDS–PAGE and immunoblot analysis with anti-Myc, anti-Rbx1, anti-Rbx2 and anti-tubulin antibodies. (C) HEK293T cells (10^6) were transfected with 1 μg VR-Cul5 and 1.5 μg VR-RBX1-HA or 1.5 μg VR-RBX2-HA. Cell lysates were immunoprecipitated with anti-HA antibody, followed by SDS–PAGE and immunoblot analysis with anti-Cul5, anti-HA and anti-tubulin antibodies.

expressed Rbx1-HA and Rbx2-HA could be co-immunoprecipitated with Cul5-Myc. Interestingly, when the Rbx1 and Rbx2 vectors were co-transfected in equal amounts, Rbx2 was preferentially immunoprecipitated (Fig. 2B). To exclude the influence of the HA tag on the Cul5-Rbx interactions, we repeated this co-immunoprecipitation assay with Cul5-Myc and untagged Rbx1 or Rbx2 expression vectors, and the same result was obtained (data not shown). To rule out the possibility of non-specific binding of Rbx1 and Rbx2 with the Myc tag, the HA-tagged Rbx1 or Rbx2 or empty expression vector was also co-transfected into HEK293T cells together with an untagged Cul5 expression vector. Coincidentally, Cul5 could be co-immunoprecipitated with either Rbx1-HA or Rbx2-HA (Fig. 2C), indicating that both over-expressed Rbx1 and Rbx2 could interact with Cul5 with high specificity.

We observed that Rbx2 could impair the interaction between Rbx1 and Cul5 (Fig. 2B). In order to check the specificity of this impairment, the Rbx1-HA expression vector was co-transfected together with an increasing amount of the Rbx2-HA expression vector in the presence of a plasmid expressing Cul5-Myc. The results showed that Rbx2 could induce a dose-dependent depressive effect on the interaction between Rbx1 and Cul5 (Fig. 3A). To check whether Rbx1 could competitively inhibit the interaction between Rbx2 and Cul5, we performed the reverse titration experiment by keeping RBX2 constant while titrating RBX1. The increasing doses of RBX1 could not efficiently impair the Rbx2-Cul5 interaction as Rbx2 did the Rbx1-Cul5 interaction, supporting the presumption that Rbx2 was preferentially recruited by Cul5 (Fig. 3B).

In light of all the evidence outlined above, we can speculate on three scenarios in which the Cul5-Rbx module becomes functional. First, the Cul5-Rbx1 and Cul5-Rbx2 modules may be present simultaneously in the cell but are specifically selected by different E3 complexes. Alternatively, the abundance of the stress-inducible Rbx2 may be regulated, consequently inhibiting the formation of a constitutive Cul5-Rbx1 module which can function in all E3 complexes. Finally, some yet undiscovered mechanisms may facilitate the formation of the Cul5-Rbx2 but not the Cul5-Rbx1 module *in vivo*.

3.3. Down-regulation of endogenous Rbx2 but not Rbx1 impairs Vif-induced A3G degradation

To assess the scenarios proposed above in cells, we examined the effects of Rbx1/Rbx2 deficiency on the function of the Vif-Cul5 E3 ligase by constructing Rbx1/Rbx2 mutants defective for E2 binding or by knocking down endogenous expression of Rbx1 or Rbx2.

According to previous reports, a single point mutation resulting in an isoleucine-to-alanine (I-to-A) change at the E2-interacting surface could decrease the E2 affinity of RING proteins [9,26]. Here, we constructed mutants Rbx1 I44A-HA and Rbx2 I52A-HA, both containing the corresponding critical I-to-A mutation. In order to test which Rbx protein would have a greater influence on Vif-Cul5 E3 activity, we co-transfected PC-A3G-HA and VR-Vif-cmyc or VR1012 in the presence of wild-type VR-Rbx1-HA/VR-Rbx2-HA, the corresponding mutant VR-Rbx1 I44A-HA/VR-Rbx2 I52A-HA or VR1012. However, neither Rbx1 I44A-HA nor Rbx2 I52A-HA could obviously disrupt the level of Vif-mediated A3G degradation afforded by wild-type Rbx1-HA and Rbx2-HA, respectively, even co-expression of both Rbx1 and Rbx2 mutants could not influenced the degradation (Fig. 4A). We speculated that the Rbx1 and Rbx2 mutants may cause greater effects in NEDDylation than in the ubiquitination process, as the former is necessary but not always

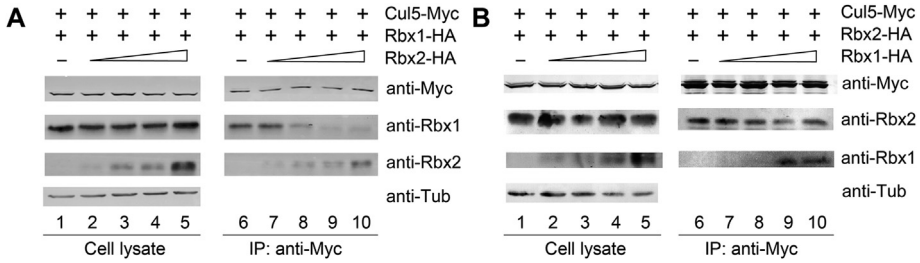

Fig. 3. Rbx2 suppresses the Rbx1-Cullin5 interaction in a dose-dependent manner. (A) HEK293T cells (2.5×10^6) were co-transfected with 1 μg VR-Cul5-Myc, 3 μg VR-HA-RBX1 and increasing amounts of VR-RBX2-HA (250 ng, 500 ng, 1 μg and 2 μg) or VR1012. Cell lysates were immunoprecipitated with an anti-Myc antibody, followed by SDS–PAGE and immunoblot analysis with anti-Myc, anti-Rbx1, anti-Rbx2 and anti-tubulin antibodies. (B) HEK293T cells (2.5×10^6) were co-transfected with 1 μg VR-Cul5-Myc, 2 μg VR-HA-RBX2 and increasing amounts of VR-RBX1-HA (250 ng, 500 ng, 1.5 μg and 3 μg) or VR1012. Cell lysates were immunoprecipitated with an anti-Myc antibody.

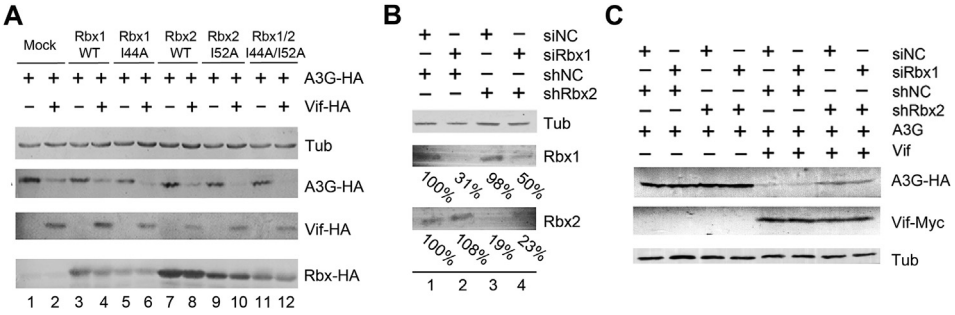

Fig. 4. The effects of Rbx1/Rbx2 deficiency on the function of the Vif-Cul5 E3 ligase. (A) HEK293T cells (10^6) were co-transfected with 100 ng PC-A3G-HA and 500 ng VR-Vif-HA or VR1012 in the presence of 0.75 μg VR-Rbx1-HA/VR-Rbx2-HA or corresponding mutant VR-Rbx1 I44A-HA/VR-Rbx2 I52A-HA or both. After 48 h of transfection, cells were harvested and examined for A3G expression. (B) The shRbx2 or shNC stably transfected HEK293 cells were seeded in 6-well plates at 0.8×10^6 cells/well and simultaneouly transfected with indicated siRNAs at a final concentration of 20 nM. The cells were harvested at 48 h post-transfection, and Rbx1/Rbx2 protein expression was analyzed by Western blot. Mock, cells treated with the transfection reagent only; siNC, cells transfected with negative control siRNA; shNC, the shNC stably transfected cells. Band intensities were quantified by using the Bandscan software and normalized to tubulin. (C) The shRbx2 or shNC stable HEK293 cells (cultured in 25 cm^2 flasks to about 80% confluency) were transfected with 100 ng of PC-A3G-HA, 500 ng of VR-Vif-Myc or VR1012 in the absence or presence of Rbx1 siRNAs. After 48 h of transfection, cells were examined for A3G expression.

positively related to the latter. For example, over-expression of Rbx1 was found to enhance the NEDD8 modification of Cul5, but it disturbed the ability of the Vif-Cul5 E3 complex to neutralize the antiviral activity of A3G [11].

To explore the effects of the two endogenous Rbx proteins on the function of Vif E3 ligase, we knocked down endogenous Rbx1 and Rbx2 by RNAi. However, we failed to obtain a high efficiency of knockdown by Rbx2-specific small interfering RNA (siRNAs) (data not shown). To improve the performance of Rbx2 knockdown, we established cell lines stably transfected with Rbx2-specific shRNA (shRbx2) or negative control shRNA (shNC). For Rbx1 down-regulation, we designed three Rbx1-specific siRNAs and mixed them to enhance the knockdown activity. The results showed that siRbx1 caused an approximately 70% decrease in Rbx1 protein in shNC stably transfected cells and a 50% decrease in shRbx2 stably transfected cells, while shRbx2 caused an approximately 80% decrease in Rbx2 protein whether or not siRbx1 was present (Fig. 4B).

ShNC or shRbx2 stably transfected HEK293 cells were transfected with PC-A3G-HA and VR-Vif-Myc or VR1012 in the absence or presence of Rbx1 siRNAs. We found that only Rbx2 knockdown could moderately impair Vif-induced A3G degradation (Fig. 4C), whereas Rbx1 knockdown caused no restoration of A3G protein levels (Fig. 4C), indicating that Rbx2 was preferred over Rbx1 by Vif E3 ligase in cells. When this experiment was repeated in the context of HXB2ΔVif transfection, similar results were obtained (data not shown), implying that other HIV proteins do not influence the preference of the Cul5-Rbx2 module by Vif. These results may rule out the second scenario proposed above, because Rbx1 was not shown to adequately compensate for the function of Rbx2 when the abundance of the latter protein was down-regulated.

In summary, the performance of the Cul5-Rbx1 and Cul5-Rbx2 modules in Vif E3 ligase activity and interactions between Cul5 and Rbx1/Rbx2 were analyzed. We demonstrated that both the Cul5-Rbx1 and Cul5-Rbx2 modules could reconstitute an active Vif E3 ligase together with the Vif-CBF-beta-EloB-EloC complex *in vitro*. We also found that both Rbx1 and Rbx2 could interact with Cul5 when over-expressed in HEK293T cells, and Rbx2 could dose-dependently inhibit the interaction between Rbx1 and Cul5. Furthermore, we observed that only knockdown of Rbx2 but not Rbx1 could cause a decline of Vif-Cul5 E3 ligase activity, indicating that the Vif-Cul5 E3 complex may be more likely to utilize the Cul5-Rbx2 module but not the Cul5-Rbx1 module. We propose two possible explanations for the selection of Cul5-Rbx module: (1) Both Cul5-Rbx1 and Cul5-Rbx2 modules may exist simultaneously in the cell, each exclusively recruited by different E3 complexes by yet undiscovered selective mechanisms. (2) The assembly of the Cul5-Rbx2 module but not the Cul5-Rbx1 module may be assisted by unknown mechanisms *in vivo*. Although additional investigations are necessary to clarify the seemingly redundant interaction of Cul5 and Rbx1, this study has further expanded our understanding of Cul5-containing E3 ligases.

Conflict of interest

None declared.

Acknowledgments

The authors would like to thank the NIH-ARRRP, Division of AIDS, NIAID for generosity in providing the A3G protein and Vif antibody. We are grateful to Xiao-Fang Yu (Johns Hopkins University, Baltimore, MD) for the gift of Vif and A3G expression plasmids, Song Tan (Pennsylvania State University, University Park, PA) for the pST39/pET3aTr vectors and Phuong Thi Sarkis for editorial assistance. This work was supported by Key Projects in the National Science & Technology Pillar Program in the Twelfth Five-year Plan Period (Grant No. 2013ZX10004608003) and the National Natural Science Foundation of China (Grant No. 31270807).

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.077.

References

[1] A.M. Weissman, Themes and variations on ubiquitylation, Nat. Rev. Mol. Cell Biol. 2 (2001) 169–178.
[2] V. Nagy, I. Dikic, Ubiquitin ligase complexes: from substrate selectivity to conjugational specificity, Biol. Chem. 391 (2010) 163–169.
[3] A.R. Willems, M. Schwab, M. Tyers, A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin, Biochim. Biophys. Acta 1695 (2004) 133–170.
[4] M. Swaroop, M. Gosink, Y. Sun, SAG/ROC2/Rbx2/Hrt2, a component of SCF E3 ubiquitin ligase: genomic structure, a splicing variant, and two family pseudogenes, DNA Cell Biol. 20 (2001) 425–434.
[5] A. Chen, K. Wu, S.Y. Fuchs, P. Tan, C. Gomez, Z.Q. Pan, The conserved RING-H2 finger of ROC1 is required for ubiquitin ligation, J. Biol. Chem. 275 (2000) 15432–15439.
[6] D. Wei, Y. Sun, Small RING Finger proteins RBX1 and RBX2 of SCF E3 ubiquitin ligases: the role in cancer and as cancer targets, Genes Cancer 1 (2010) 700–707.
[7] Z.Q. Pan, A. Kentsis, D.C. Dias, K. Yamoah, K. Wu, Nedd8 on cullin: building an expressway to protein destruction, Oncogene 23 (2004) 1985–1997.
[8] L. Gong, E.T. Yeh, Identification of the activating and conjugating enzymes of the NEDD8 conjugation pathway, J. Biol. Chem. 274 (1999) 12036–12042.
[9] D.T. Huang, O. Ayrault, H.W. Hunt, A.M. Taherbhoy, D.M. Duda, D.C. Scott, L.A. Borg, G. Neale, P.J. Murray, M.F. Roussel, B.A. Schulman, E2-RING expansion of the NEDD8 cascade confers specificity to cullin modification, Mol. Cell 33 (2009) 483–495.
[10] R.J. Deshaies, C.A. Joazeiro, RING domain E3 ubiquitin ligases, Annu. Rev. Biochem. 78 (2009) 399–434.
[11] X. Yu, Y. Yu, B. Liu, K. Luo, W. Kong, P. Mao, X.F. Yu, Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex, Science 302 (2003) 1056–1060.
[12] B. Liu, P.T. Sarkis, K. Luo, Y. Yu, X.F. Yu, Regulation of Apobec3F and human immunodeficiency virus type 1 Vif by Vif-Cul5-ElonB/C E3 ubiquitin ligase, J. Virol. 79 (2005) 9579–9587.
[13] M.L. Heuze, F.C. Guibal, C.A. Banks, J.W. Conaway, R.C. Conaway, Y.E. Cayre, A. Benecke, P.G. Lutz, ASB2 is an Elongin BC-interacting protein that can assemble with Cullin 5 and Rbx1 to reconstitute an E3 ubiquitin ligase complex, J. Biol. Chem. 280 (2005) 5468–5474.
[14] T. Kamura, D. Burian, Q. Yan, S.L. Schmidt, W.S. Lane, E. Querido, P.E. Branton, A. Shilatifard, R.C. Conaway, J.W. Conaway, Muf1, a novel Elongin BC-interacting leucine-rich repeat protein that can assemble with Cul5 and Rbx1 to reconstitute a ubiquitin ligase, J. Biol. Chem. 276 (2001) 29748–29753.
[15] E. Querido, P. Blanchette, Q. Yan, T. Kamura, M. Morrison, D. Boivin, W.G. Kaelin, R.C. Conaway, J.W. Conaway, P.E. Branton, Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex, Genes Dev. 15 (2001) 3104–3117.
[16] J.N. Harada, A. Shevchenko, D.C. Pallas, A.J. Berk, Analysis of the adenovirus E1B-55K-anchored proteome reveals its link to ubiquitination machinery, J. Virol. 76 (2002) 9194–9206.
[17] D.M. Duda, L.A. Borg, D.C. Scott, H.W. Hunt, M. Hammel, B.A. Schulman, Structural insights into NEDD8 activation of cullin-RING ligases: conformational control of conjugation, Cell 134 (2008) 995–1006.
[18] N. Mahrour, W.B. Redwine, L. Florens, S.K. Swanson, S. Martin-Brown, W.D. Bradford, K. Staehling-Hampton, M.P. Washburn, R.C. Conaway, J.W. Conaway, Characterization of Cullin-box sequences that direct recruitment of Cul2-Rbx1 and Cul5-Rbx2 modules to Elongin BC-based ubiquitin ligases, J. Biol. Chem. 283 (2008) 8005–8013.
[19] T. Kamura, K. Maenaka, S. Kotoshiba, M. Matsumoto, D. Kohda, R.C. Conaway, J.W. Conaway, K.I. Nakayama, VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases, Genes Dev. 18 (2004) 3055–3065.
[20] S. Jager, D.Y. Kim, J.F. Hultquist, K. Shindo, R.S. LaRue, E. Kwon, M. Li, B.D. Anderson, L. Yen, D. Stanley, C. Mahon, J. Kane, K. Franks-Skiba, P. Cimermancic, A. Burlingame, A. Sali, C.S. Craik, R.S. Harris, J.D. Gross, N.J. Krogan, Vif hijacks CBF-beta to degrade APOBEC3G and promote HIV-1 infection, Nature 481 (2011) 371–375.

[21] W. Zhang, J. Du, S.L. Evans, Y. Yu, X.F. Yu, T-cell differentiation factor CBF-beta regulates HIV-1 Vif-mediated evasion of host restriction, Nature 481 (2011) 376–379.
[22] S. Tan, A modular polycistronic expression system for overexpressing protein complexes in Escherichia coli, Protein Expr. Purif. 21 (2001) 224–234.
[23] X. Wang, H. Zhang, M. Lv, T. Zuo, H. Wu, J. Wang, D. Liu, C. Wang, J. Zhang, X. Li, J. Wu, B. Yu, W. Kong, X. Yu, Interactions between HIV-1 Vif and human ElonginB-ElonginC are important for CBF-beta binding to Vif, Retrovirology 10 (2013) 94.
[24] T. Ohta, J.J. Michel, A.J. Schottelius, Y. Xiong, ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity, Mol. Cell 3 (1999) 535–541.
[25] J. Kohroki, T. Nishiyama, T. Nakamura, Y. Masuho, ASB proteins interact with Cullin5 and Rbx2 to form E3 ubiquitin ligase complexes, FEBS Lett. 579 (2005) 6796–6802.
[26] P.S. Brzovic, J.R. Keeffe, H. Nishikawa, K. Miyamoto, D. Fox 3rd, M. Fukuda, T. Ohta, R. Klevit, Binding and recognition in the assembly of an active BRCA1/BARD1 ubiquitin-ligase complex, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 5646–5651.