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Rig-G negatively regulates SCF-E3 ligase activities by disrupting the assembly of COP9 signalosome complex

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ABSTRACT

We previously showed that Rig-G, an antiproliferative protein induced by interferon, can sequester CSN5 protein in the cytoplasm. Here, we report that Rig-G can destroy the functions of CSN5-containing COP9 signalosome (CSN), a highly conserved multiprotein complex implicated in protein deneddylation, deubiquitination, and phosphorylation. By damaging integrity and stability of the CSN complex, Rig-G can dramatically reduce the cellular content of CSN complex and inhibit its regulatory roles in assembly and activation of cullin-RING ubiquitin E3 ligases (CRL). Furthermore, Rig-G can cause excessive activation of CRL through inhibition of CSN-mediated deneddylation, largely decreasing protein levels of Cul1 and βTrCP, two important subunits of SCF (Skp1-Cul1-F-box protein)-E3 ligase. Rig-G can also attenuate the ability of CSN to recruit USP15 and impair CSN-associated deubiquitination. Increased autoubiquitination of βTrCP and concomitant accumulation of target substrates (such as IκBα) are observed in Rig-G-expressing cells. Taken together, our findings reveal for the first time the negative regulation of Rig-G on SCF-E3 ligase activities through disrupting CSN complex, not only contributing to further investigation on biological functions of Rig-G, but also leading to better understanding of the CSN complex as a potential target in tumor diagnosis and treatment.

1. Introduction

The retinoic acid-induced gene G (RIG-G) was first identified from an acute promyelocytic leukemia cell line NB4 treated with all-*trans* retinoic acid (ATRA) [1]. Subsequent studies indicated that RIG-G was an interferon (IFN)-inducible protein having antiproliferative activity in many cancer cells [2,3]. It could play a role in the inhibition of the cell cycle by enhancing p21 and p27 proteins. In addition, Rig-G could interact with CSN5 protein and alter its cellular distribution, indicating that Rig-G may have an inhibitory effect on the biological functions of CSN5 [3].

The CSN5 was initially found as a Jun activating binding protein, facilitating the transactivation of AP-1 transcription factor [4]. Further studies showed that CSN5 was also a component of COP9 signalosome (CSN) complex, which is composed of eight subunits (CSN1-8) with a total molecular mass of approximately 500 kDa [5,6]. The functions of CSN complex were tightly associated with its three major biological activities: deneddylation, deubiquitination, and phosphorylation [7,8]. It has been shown that CSN is directly involved in the cleavage of a ubiquitin-like modifier Nedd8 from the cullin subunit of the CRL (Cullin-RING ligase) complex [9]. CRL is a superfamily of ubiquitin E3 ligases characterised by a RING protein and a cullin-family member (Cul-1, 2, 3, 4A, 4B, 5 or 7) as an enzyme catalytic core [10,11]. Being the key mediators of post-translational regulation, CRLs are implicated in a lot of cellular processes. The turnover of Nedd8 linkage represents an important mechanism by which CRL activities can be regulated [12]. Theoretically, the attachment of Nedd8 to cullin protein (named neddylation) is required for an active CRL. However, continuous neddylation of cullin will lead to excessive activation of CRLs, thus causing the autoubiquitination of CRL subunits followed by destruction. The removal of Nedd8 from cullin subunit (named deneddylation) by the CSN complex can inactivate CRL, preventing the CRL subunits from self-degradation [7].

In addition to deneddylation, the role of CSN in the control of CRL activities is also reflected in its deubiquitination activity. It was found that the CSN complex could associate with Ubp12p, a deubiquitinase in yeast, antagonising the autoubiquitination of CRL subunits by removing the attached poly-ubiquitin chains in

Abbreviations: ATRA, all-*trans* retinoic acid; IFN, interferon; CAND1, cullin-associated and neddylation-dissociated protein 1; CRL, Cullin-RING ligase; CRL3, Cul3-containing E3 ligase; CSN, COP9 signalosome; Rig-G, retinoic acid-induced gene G; SCF, Skp1-Cul1-F-box protein; Ub, ubiquitin.

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the absence of substrates and relieving the instability of CRL subunits [13]. Accordingly, both CSN-dependent deneddylation and CSN-associated Ubp12p deubiquitination served as protective mechanisms to preserve CRL subunits from self-degradation and to regulate CRL activities [7,12].

Although in recent years significant progress has been made in determining the structure and functions of CSN, research regarding the activity regulation of the CSN complex remains limited. Rig-G was previously found to act as a negative regulator of monomeric CSN5 functions by preventing CSN5 from entering the nucleus [3]. Since the CSN5 protein could exert its functions not only in monomers independently of CSN complex, but also as a major component of CSN holocomplex, we wondered whether Rig-G had some effects on the CSN holocomplex, and whether Rig-G could regulate CRL activities through the CSN holocomplex. In this study, we intensively detail the potential role of Rig-G in CSN-dependent regulation of CRL activities and outline its possible mechanism of action.

2. Materials and methods

2.1. Cell culture and reagents

The Rig-G-inducible cell line U937T-RIG-G and its control cell line U937T-pTRE were established in our previous work [3]. The cells were maintained in RPMI medium 1640 supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine and 2 μg/mL doxycycline. The 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine. All cells were incubated at 37 °C with 5% CO_2. MG132 (Merck, Whitehouse Station, NJ) and cycloheximide (Sigma, St. Louis, MO) were, respectively dissolved in DMSO and ethanol as stock solution at 10 mmol/L. Doxycycline (Sigma) was dissolved in PBS as a stock solution at 1 mg/mL. Protease inhibitors PMSF (AMRESCO, Solon, OH) and cocktail (Roche, Switzerland) were, respectively dissolved in isopropanol and PBS as stock solution at 100 mmol/L and 50×. All stock solutions were stored at −20 °C.

2.2. Antibodies

The generation of rabbit polyclonal anti-Rig-G sera was described previously [3]. Other primary antibodies used in this study were as follows: CSN3 and CSN5 (GeneTex, San Antonio, TX); CSN8 (Proteintech, Chicago, IL); C2 (ALEXIS, London, UK); Cul1 (Invitrogen, Grand Island, NY); Cul3 (BD, Franklin Lakes, NJ); USP15, CAND1, Lamin B, and ubiquitin (Santa Cruz, CA); Flag and β-actin (Sigma); IκBα, IKKβ, and βTrCP (Cell Signaling, Danvers, MA).

2.3. Nondenaturing polyacrylamide gel electrophoresis (Native-PAGE)

2–4 × 10^7 of cells were lysed in the hypotonic buffer [20 mmol/L Hepes (pH 7.4), 1.5 mmol/L MgCl2, 1% NP40, 1 mmol/L PMSF, and 1X cocktail] on ice for 1 h. Protein content of the supernatants was determined by BCA Protein Assay Kit (Thermo, Rockford, IL). 20 μg of total proteins were then mixed with an equal volume of 2× nondenaturing loading buffer [100 mmol/L Tris (pH 6.8), 0.2% bromophenol blue, 20% glycerol] and loaded onto a native discontinuous gradient gel without SDS. The gel was prepared with two separating gels (12% and 6%) and a stacking gel (3%). The electrophoresis was performed at 15 mA for 0.5 h followed by 10 mA for 4 h. The membrane transfer and the immunoblotting analysis were as same as those described for the ordinary Western blot test [3].

2.4. Gel filtration chromatography

The total protein extracts were prepared from 1 × 10^8 of cells in the aforementioned hypotonic buffer. The proteins (approximately 3 mg) were loaded onto a Superdex 200 10/300 GL column (GE Healthcare, Pittsburgh, PA) and eluted with Hepes buffer (20 mmol/L Hepes, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, and 1 mmol/L PMSF) at a sample flow rate of 0.5 mL/min. Thirteen fractions were collected and resolved on a 10% SDS–PAGE, then transferred onto nitrocellulose membrane and analysed by Western blot. The proteins given in HMW Calibration Kits (GE Healthcare) were used as molecular weight standards.

2.5. Plasmids and transfection

The Rig-G expression plasmid was constructed as previously described [3]. The pCMV4-Flag-CSN5 plasmid was kindly provided by Naumann [14]. These two plasmids were cotransfected into 293T cells by SuperFect (Qiagen, Hilden, Germany) according to the manufacturer's procedure.

2.6. Coimmuoprecipitation

Protein extracts were prepared in the buffer containing 150 mmol/L NaCl, 50 mmol/L Tris–HCl (pH 8.0), and 0.5% NP40, then mixed with protein A-agarose (Santa Cruz) and the indicated antibodies at 4 °C overnight with rotation. The precipitated proteins were then eluted by boiling beads in SDS-loading buffer [4% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.2% bromophenol blue, 100 mmol/L Tris (pH 6.8)] and analysed by Western blot.

3. Results

3.1. Rig-G disrupts the assembly of CSN complex

By using Tet-off transfection system, we previously established a Rig-G-inducible U937 subline (U937T-RIG-G) in which Rig-G expression could be induced in the absence of doxycycline. To investigate the effects of Rig-G protein on CSN complex, we first evaluated whether the protein levels of endogenous CSN components changed in U937T-RIG-G and U937T-pTRE cells before and after doxycycline removal. With the antibodies against CSN3, CSN5, and CSN8, respectively, we showed by immunoblotting that these three CSN subunits exhibited no obvious protein level changes under the expression of Rig-G (Fig. 1A). We then performed a native (nondenaturing) PAGE to compare the CSN holocomplex in the cells with and without Rig-G induction. A band of approximately 450–550 kDa, corresponding to the intact CSN holocomplex, was revealed by each of the three aforementioned antibodies (Fig. 1B). Notably, this band was dramatically decreased when cells expressed Rig-G, suggesting that Rig-G may interfere with the stability of the CSN holocomplex.

To further confirm the effects of Rig-G on the assembly of the CSN holocomplex, we carried out gel filtration chromatography with the homogenates from U937T-RIG-G and U937T-pTRE cells after doxycycline withdrawal and analysed the expression of CSN subunits in the elution fractions (Fig. 1C). As a result, the subunits CSN3, CSN5, and CSN8 eluted mainly in the same fractions 3–5, in line with the molecular mass of the CSN holocomplex (450–550 kDa). In addition, the CSN5 protein also eluted in lower molecular mass fractions 10–11 (about 40 kDa), corresponding to its monomeric form as expected. Importantly, we found that Rig-G expression could result in a decreased level of the subunits CSN3, CSN5, and CSN8 in their association in the CSN holocomplex, and an increased expression of CSN5 monomers (Fig. 1C), further

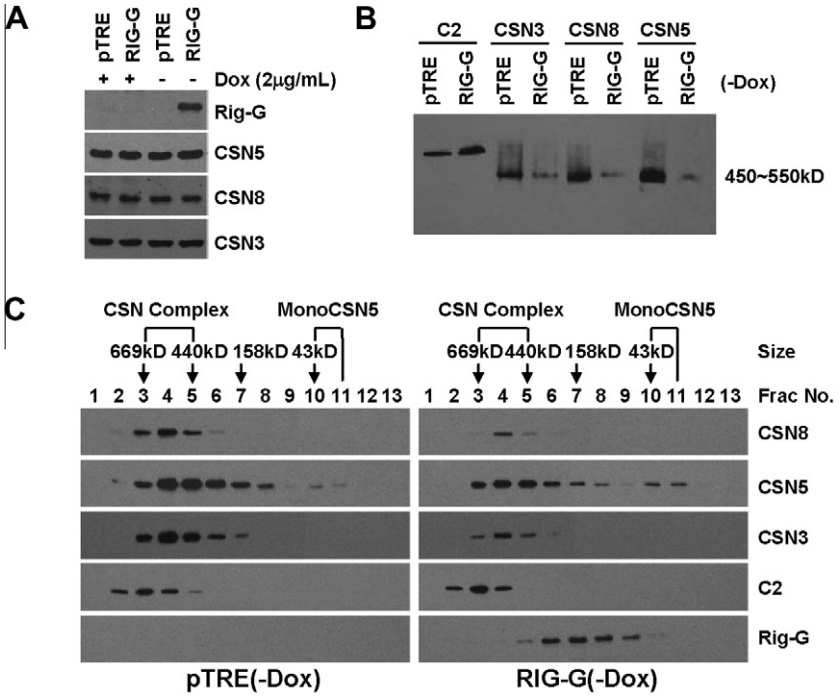

Fig. 1. Rig-G disrupts the assembly of CSN complex. (A) The sublines U937T-pTRE (pTRE) and U937T-RIG-G (RIG-G) were, respectively cultured with or without doxycycline (Dox). Effects of inducible Rig-G on CSN3, CSN5, and CSN8 protein levels were analysed by Western blot. (B) The lysates from pTRE and RIG-G cells without doxycycline were separated by a nondenaturing PAGE and analysed by immunoblotting using the indicated antibodies. C2 subunit of 20S proteasome was used as a loading control. (C) The protein extracts from pTRE and RIG-G cells without doxycycline were separated by gel filtration chromatography. Fractions 1–13 were collected and then analysed by Western blot with the indicated antibodies.

indicating that Rig-G could indeed reduce the entire content of CSN holocomplex in the cells through disruption of CSN assembly.

3.2. Rig-G damages the stability of SCF-E3 ligase

CRL1 is a type of canonical CRL-E3 ligase with Cul1 protein as a scaffold. It is also designated as an SCF (Skp1-Cul1-F-box protein)-E3 ligase in which the Cul1 subunit is combined with the adaptor Skp1, an F-box-containing substrate receptor, as well as with a RING protein [10,11]. Since CSN complex plays a crucial role in the regulation of CRL activities, we should question the effects of Rig-G on CRL. Two important subunits of SCF-E3 ligase, Cul1 and βTrCP, were examined in U937T-RIG-G and U937T-pTRE cells with or without doxycycline. We observed a significant reduction in levels for both the Cul1 and βTrCP proteins upon Rig-G expression (Fig. 2A). Moreover, such down-regulation could be practically recovered by the proteasome inhibitor MG132 (Fig. 2B), indicating that the decrease of Cul1 and βTrCP in Rig-G-expressing cells should be due to a proteasome-dependent post-translational regulation. By using the protein synthesis inhibitor cycloheximide, we further compared the turnover of Cul1 protein in the cells with and without Rig-G expression. About 50% of Cul1 degraded in Rig-G-expressing cells after 4 h treatment with cycloheximide, while nearly 90% of Cul1 was still present at the same time point in control cells (Fig. 2C and D), emphasising that Rig-G could greatly accelerate degradation of the Cul1 protein and damage the stability of SCF-E3 ligase.

It has been reported that the substrate receptor βTrCP can be specifically recruited into the activated/neddylated SCF-E3 ligases responsible for ubiquitination and degradation of IκBα protein [15], which generally binds to transcription factor NF-κB and retains them inactive in the cytosol. Here, we found that IκBα accumulated in Rig-G-expressing cells (Fig. 2E); moreover, this accumulation of IκBα was associated with decreased ubiquitination (Fig. 2F). The evidence that Rig-G to some extent could impair the ubiquitination of IκBα strongly supports the findings that Rig-G may have an inhibitory effect on $SCF^{\beta TrCP}$-mediated IκBα proteolysis.

3.3. Rig-G cannot destabilise Cul3 protein

To study whether Rig-G had similar effects on other CRLs except SCF-E3 ligase, we examined an immunoblot of Cul3 in Rig-G-expressing cells. No marked change in Cul3 protein level was observed before and after Rig-G expression. In addition, we found that the expression of IKKβ, one substrate of the Cul3-containing E3 ligase (CRL3) Cul3-Keap1-Rbx1 [16], was also not affected by Rig-G (Fig. 3A). We then used a subcellular fractionation assay to analyse Cul1 and Cul3 proteins, respectively, in cytoplasmic and nuclear fractions. As a result, we noted that in Rig-G-expressing cells the ratio of neddylated to unneddylated Cul3 was markedly decreased in the nucleus (Fig. 3B and C). These findings indicate that, while Rig-G had little effect on the stability of the Cul3 subunit, the Nedd8 modification of Cul3 protein was still influenced by Rig-G. More recently, it has been shown that CAND1 (cullin-associated and neddylation-dissociated protein 1), a cullin-interacting protein, could protect unneddylated Cul3 but not Cul1 from proteasomal degradation in Drosophila [17]. Interestingly, we found that CAND1 could be significantly up-regulated in U937T-RIG-G cells when Rig-G was induced (Fig. 3D). Whether CAND1 played a role in fine-tuning CRL3 activity in our case remained to be explored.

3.4. Rig-G impairs the deubiquitination activity of CSN complex

Since the CSN complex can also recruit the ubiquitin-specific protease USP15 (homologue of Ubp12p in mammals) and possess deubiquitination abilities [18], we therefore examined whether Rig-G could exert an influence on USP15 recruitment by the CSN

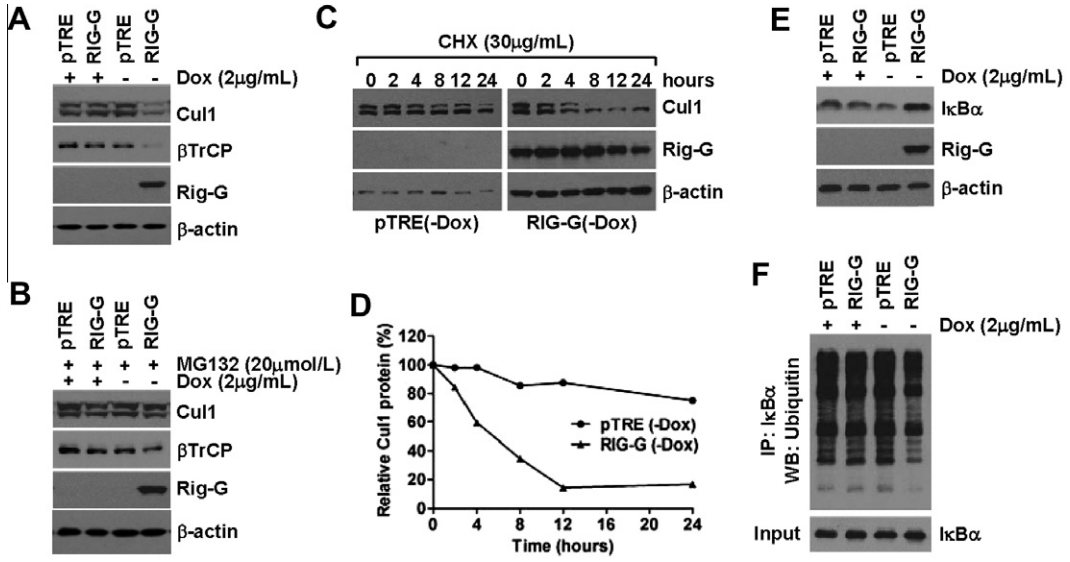

Fig. 2. Rig-G destabilises SCF-E3 ligase. (A) The pTRE and RIG-G cells were cultured with or without doxycycline (Dox). Effects of inducible Rig-G on Cul1 and βTrCP protein levels were analysed by Western blot. β-actin was used as a loading control. (B) Western blot analysis of Cul1 and βTrCP protein levels in above cells treated with 20 μmol/L proteasome inhibitor MG132 for 8 h. (C) In the absence of doxycycline, the pTRE and RIG-G cells were treated with 30 μg/mL cycloheximide (CHX) for the indicated lengths of time to analyse the effects of inducible Rig-G on Cul1 protein degradation rate. (D) Densitometric scanning of the results shown in (C) using Quantity One (Bio-Rad, Hercules, CA) software. The Cul1 values were normalised to β-actin content in each sample and plotted relative to the values of the cells untreated with cycloheximide (time 0). (E) Effects of inducible Rig-G on IκBα protein level were analysed by Western blot. (F) IκBα ubiquitination was analysed by coimmunoprecipitation. The indicated cells were incubated with 20 μmol/L MG132 for 4 h. Cell lysates (Input) were then immunoprecipitated (IP) with anti-IκBα antibody, followed by Western blot (WB) with anti-ubiquitin antibody.

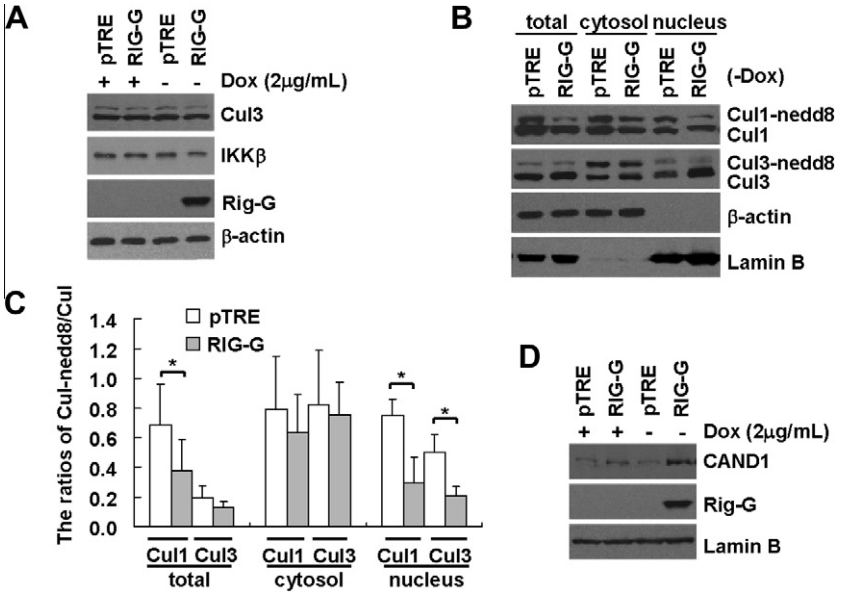

Fig. 3. Rig-G cannot destabilise CRL3. (A) The pTRE and RIG-G cells were cultured with or without doxycycline (Dox). Effects of inducible Rig-G on Cul3 and IKKβ protein levels were analysed by Western blot. (B) Western blot analysis of Cul1 and Cul3 protein levels in subcellular fractions from pTRE and RIG-G cells without doxycycline. β-actin and Lamin B were used as the internal controls for the cytosol and nucleus, respectively. (C) Quantification of the ratio of neddylated to unneddylated Cul1 and Cul3 shown in (B). The error bars indicate the standard deviations for the three independent experiments. Asterisks indicate significance by *t*-test (*$p < 0.05$). (D) Effects of inducible Rig-G on CAND1 protein levels were analysed by Western blot.

complex. The interaction between the CSN complex and USP15 was analysed by coimmunoprecipitation experiments in 293T cells transfected with Rig-G expression plasmid and empty vector, respectively. To enhance CSN complex assembly, these 293T cells were also co-transfected with Flag-tagged CSN5. As a result, we observed that less USP15 protein was combined with Flag-CSN5 in Rig-G-transfected cells than in control cells, despite the same amount of Flag-CSN5 having been precipitated (Fig. 4A), implying that Rig-G could impair the capacity of the CSN complex to recruit USP15.

We then detected the ubiquitination of βTrCP, the substrate receptor of $SCF^{\beta TrCP}$-E3 ligase, in the cells with or without Rig-G expression. As would be expected, Rig-G largely enhanced the autoubiquitination of βTrCP (Fig. 4B), further confirming the previous results that Rig-G could significantly reduce the βTrCP level by destabilising the CSN complex (Fig. 2A).

Taken together, CSN-mediated deubiquitination and deneddylation can synergistically prevent the subunits of $SCF^{\beta TrCP}$-E3 ligase from self-degradation. Rig-G can interfere with the protective effect

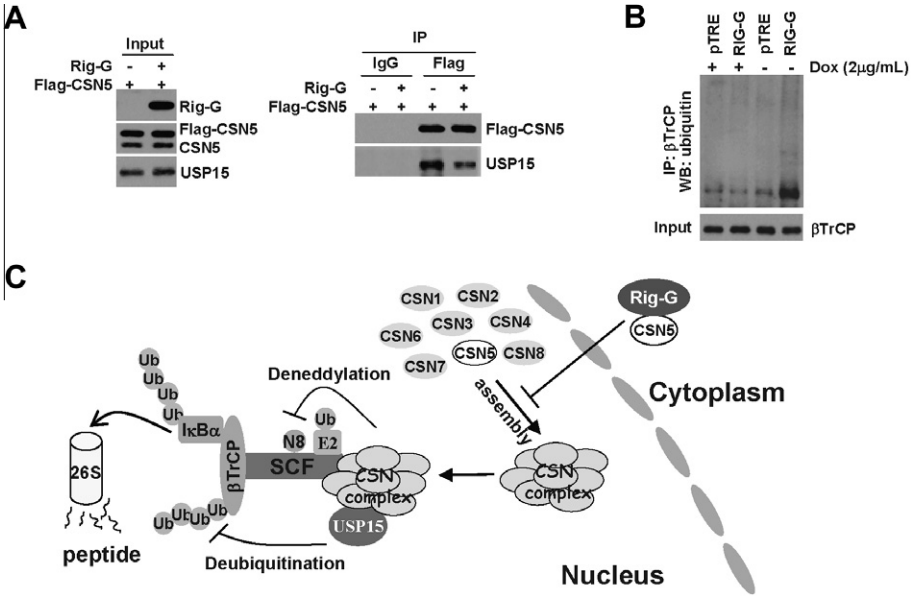

Fig. 4. Rig-G impairs the deubiquitination activity of the CSN complex. (A) The interaction of CSN5 and USP15 was analysed by coimmunoprecipitation. The 293T cells were cotransfected with Flag-CSN5 and RIG-G expression plasmids or empty vector. 48 h after transfection, the lysates (Input) were immunoprecipitated (IP) with anti-Flag antibody, followed by Western blot (WB) with anti-USP15 and anti-CSN5 antibodies. (B) βTrCP ubiquitination was analysed by coimmunoprecipitation. The indicated cells were treated with 20 μmol/L MG132 for 4 h. Cell lysates (Input) were then immunoprecipitated (IP) with anti-βTrCP antibody followed by Western blot (WB) with anti-ubiquitin antibody. (C) Schematic illustration of Rig-G regulation on $SCF^{\beta TrCP}$-E3 ligase through disassembling CSN complex. Ub: ubiquitin; N8: Nedd8; 26S: 26S proteasome; E2: ubiquitin-conjugating enzyme.

of the CSN complex on SCF-E3 ligase through sequestering CSN5 protein in the cytoplasm, thereby decreasing the cellular content of $SCF^{\beta TrCP}$-E3 ligase and resulting in the accumulation of substrate target (Fig. 4C).

4. Discussion

Currently, the best characterised biochemical function of CSN complex is its control over CRL-mediated protein ubiquitination and degradation. There are two counterbalancing effects of the CSN complex that should be considered when CRL activities are regulated. Although CSN can inhibit cullin neddylation, returning CRL activities to a basal level, CSN can more importantly also protect CRL subunits from auto-destruction and is actually necessary to the activation of CRLs [8,19,20]. Loss of CSN function will cause CRL instability due to its excessive activation [21–23].

Here, we showed that Rig-G could disrupt the integrity of CSN holocomplex, reducing the entire amount of CSN holocomplex in the cells. We found a drastic decrease in certain subunits of $SCF^{\beta TrCP}$-E3 ligase accompanied by accumulation of substrate target IκBα in Rig-G-inducible cells. But we failed to observe the increasing levels of neddylated cullins that should have manifested because of CSN down-regulation. On the contrary, the neddylated to unneddylated ratio of both Cul1 and Cul3 was markedly decreased by Rig-G vs. control (Fig. 3B and C). Our results again reflected the CSN paradoxes. Since hyperneddylated cullins were intrinsically unstable because of unusual activation of SCF-E3 ligases [19,24], we hypothesised that the existence of excessive neddylated cullins might be too instantaneous for detection. The overall decline of Cul1 protein and the decreased ratio of neddylated to unneddylated cullins in Rig-G-expressing cells fully indicated that the CSN complex was indeed essential for CRL-mediated protein degradation.

Meanwhile, we noted that the total amount of Cul3, unlike Cul1, was hardly affected by Rig-G, suggesting that the stability of CRL subunits was not solely controlled by the CSN complex. In this respect, several related arguments in the literature have attracted our attention. It was reported that in Arabidopsis CSN depletion resulted in a drastic decrease in Cul3 but a significant increase in Cul4, whereas Cul1 remained almost unchanged [25]. Cope et al. found that targeted silencing of CSN5 in human HEK293 cells resulted in enhanced neddylation of cullins 1–4, but the total levels of all four cullins were largely unaltered, except for a modest reduction in Cul2 [26]. All these findings emphasised the complexity of regulation of CRL stability and activity. Some unidentified mechanisms behind these phenotypes need to be elucidated. The result that Rig-G can upregulate CAND1 in U937 cells may give us a new insight into the regulation of CRLs stability by CAND1 in mammals [17].

It should be pointed out that Rig-G is almost undetectable in tumor cells, and usually certain anti-tumor drugs (such as IFN or ATRA) are required to induce its expression [1–3,27]. The results presented in this study were mainly obtained in an inducible cell line expressing Rig-G. Though the extent of over-expressed Rig-G in U937T-RIG-G cells could be comparable with the endogenous Rig-G levels achieved in IFN or ATRA-treated cells [3], further experiments should be carried out to precise the physiological role of Rig-G in the next studies.

In summary, we demonstrate here that Rig-G is able to negatively regulate SCF-E3 ligase activities by disrupting the stability of the CSN holocomplex as well as its pleiotropic functions. Our findings not only further reveal the involvement of Rig-G in the regulation of CRL activities, but also lead to a better understanding of the CSN complex as a potential target for therapeutic intervention in human diseases, particularly in tumorigenesis [28,29].

Acknowledgments

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