Biochem. J. (2012) **441**, 227–236 (Printed in Great Britain) doi:10.1042/BJ20111500

Targeted polyubiquitylation of RASSF1C by the Mule and SCF$^{\beta\text{-TrCP}}$ ligases in response to DNA damage

Xin ZHOU*†‡, Ting-Ting LI*†‡, Xu FENG*†‡, Esther HSIANG§, Yue XIONG†‡‖, Kun-Liang GUAN*†¶ and Qun-Ying LEI*†‡[1]

*Key Laboratory of Molecular Medicine, Ministry of Education, Department of Biochemistry and Molecular Biology, Shanghai Medical College, Shanghai 200032, China, †Molecular and Cell Biology Lab, Institute of Biomedical Sciences, Fudan University, Shanghai 200032, China, ‡School of Life Sciences, Fudan University, Shanghai 200032, China, §Department of Human Evolutionary Biology, Harvard University, Cambridge, MA 02138, U.S.A., ‖Department of Biochemistry and Biophysics, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, NC 27599, U.S.A., and ¶Department of Pharmacology and Moores Cancer Center, University of California at San Diego, La Jolla, CA 92093-0815, U.S.A.

RASSF1A [Ras association (RalGDS/AF-6) domain family member 1A] and RASSF1C are two ubiquitously expressed isoforms of the *RASSF1* gene. The promoter of RASSF1A is frequently hypermethylated, resulting in inactivation in various human cancers. RASSF1A is implicated in the regulation of apoptosis, microtubule stability and cell cycle arrest. However, little is known about the regulation and function of RASSF1C. In the present study we show that exogenously expressed RASSF1C is a very unstable protein that is highly polyubiquitylated and degraded via the proteasome. Furthermore, RASSF1C degradation is enhanced when cells are exposed to stress signals, such as UV irradiation. Mule, a HECT (homologous with E6-associated protein C-terminus) family E3 ligase, but not SCF$^{\beta\text{-TrCP}}$ [where SCF is Skp1 (S-phase kinase-associated protein 1)/cullin/F-box and β-TrCP is β-bransducin repeat-containing protein] or CUL4 (cullin 4)-DDB1 (damage-specific DNA-binding protein 1), is the E3 ligase for RASSF1C under normal conditions, whereas both Mule and SCF$^{\beta\text{-TrCP}}$ target RASSF1C degradation in response to UV irradiation. GSK3 (glycogen synthase kinase 3) phosphorylates RASSF1C to promote RASSF1C degradation subsequently, which is negatively regulated by the PI3K (phosphoinositide 3-kinase)/Akt pathway. Thus the present study reveals a novel regulation of RASSF1C and the potentially important role of RASSF1C in DNA damage responses.

Key words: DNA damage, glycogen synthase kinase 3 (GSK3), phosphoinositide 3-kinase (PI3K), stability, Ras association (RalGDS/AF-6) domain family member 1C (RASSF1C), ubiquitylation.

INTRODUCTION

RASSF1 [Ras association (RalGDS/AF-6) domain family member 1] is located in chromosome 3p21.3, which frequently undergoes loss of heterozygosity in human tumours [1,2]. RASSF1A and RASSF1C, two major isoforms of the tumour suppressor RASSF1, are transcribed from two different promoters and are expressed in most normal human tissues [3,4]. RASSF1A and RASSF1C are identical in their C-terminal domains, containing a Ras-association domain, a SARAH (Sav/RASSF/Hpo) domain and an ATM (ataxia telangiectasia mutated) domain. RASSF1A has a 119 amino acid N-terminal region containing a cysteine-rich diacylglycerol-binding C1 domain, whereas RASSF1C has a 49 amino acid N-terminal region without a C1 domain [5]. The identities and differences in the amino acid sequences of RASSF1A and RASSF1C indicate they may have different physiological and pathological functions.

RASSF1A, but not RASSF1C, has been shown to block cell cycle progression and inhibit cyclin D1 accumulation [6], and regulate mitosis by inhibiting the APC (anaphase-promoting complex)–Cdc20 complex [7]. Both RASSF1A and RASSF1C are associated with microtubules, but only RASSF1A stabilizes microtubules [8,9]. In addition, RASSF1A has been implicated in the regulation of apoptosis [10,11], growth suppression [4] and DNA damage responses [12]. Compared with RASSF1A, the functions of RASSF1C are contradictory in different reports. Li et al. [13] showed that RASSF1C inhibited the proliferation of different cancer cell lines, such as LNCaP and KRC/Y; Vos et al. [14] reported that RASSF1C mediated Ras-dependent apoptosis in NIH 3T3 cells. However, RASSF1C has also been shown to promote osteoblast cell proliferation and breast cancer cell migration [15,16]. In contrast, Shivakumar et al. [6] showed that overexpression of RASSF1C did not affect proliferation of several different cell lines. Moreover, RASSF1C, but not RASSF1A, has been shown to release from the nucleus when DAXX (death-domain-associated protein) is degraded in response to UV irradiation [17]. Therefore the functions of RASSF1C remain largely unknown and are worth further investigation.

Hypermethylation of the RASSF1A promoter is a very common event in various human cancers, including small cell lung cancer, non-small cell lung cancer, breast cancer, bladder cancer, primary nasopharyngeal cancer and primary renal cell carcinoma [3,4,18–22]. Interestingly, no detection of hypermethylation of the RASSF1C promoter has been reported, despite RASSF1C mRNA having been detected in many human cancers and cancer cell lines via Northern blot or qPCR (quantitative PCR). Why cancer cells need to epigenetically inactivate RASSF1A but not RASSF1C is an open question in this field. One possibility is

Abbreviations used: CHX, cycloheximide; CK1, casein kinase 1; CUL, cullin; DAXX, death-domain-associated protein; DDB1, damage-specific DNA-binding protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK3, glycogen synthase kinase 3; HA, haemagglutinin; HECT, homologous with E6-associated protein C-terminus; HEK-293T, human embryonic kidney-293 cells expressing the large T-antigen of SV40 (simian virus 40); LC-MS/MS, liquid chromatography-tandem MS; PI3K, phosphoinositide 3-kinase; qPCR, quantitative PCR; RASSF, Ras association (RalGDS/AF-6) domain family; RNAi, RNA interference; SBP, streptavidin-binding peptide; SCF, Skp1/cullin/F-box; Skp, S-phase kinase-associated protein; siRNA, small interfering RNA; TAZ, transcriptional co-activator with PDZ-binding motif; β-TrCP, β-transducin repeat-containing protein.

[1] To whom correspondence should be addressed (email qlei@fudan.edu.cn).

that RASSF1A and RASSF1C might have very different functions. Indeed, Reeves et al. [16] proposed RASSF1C might be an oncogene. The other possible reason is that RASSF1C might be mainly regulated by post-translational events, which leads to low activity or decreased protein levels in tumorigenesis. Due to a commercial anti-RASSF1C antibody which can detect endogenous RASSF1C expression being unavailable, most studies on RASSF1C regulation have been limited to the transcriptional level.

In the present study, we showed that RASSF1C is a very unstable protein that responds to different stress signals such as UV damage. Furthermore, we define Mule, but not $SCF^{\beta\text{-TrCP}}$ [where SCF is Skp1 (S-phase kinase-associated protein 1)/cullin/F-box and β-TrCP is β-transducin repeat-containing protein] or CUL4 (cullin 4)-DDB1 (damage-specific DNA-binding protein 1), as a *bona fide* RASSF1C E3 ligase under normal conditions, whereas both Mule and $SCF^{\beta\text{-TrCP}}$ are RASSF1C E3 ligases in response to UV irradiation. Moreover, RASSF1C phosphorylation by GSK3 (glycogen synthase kinase 3) promotes the interaction between RASSF1C and the E3 ligase Mule, thus leading to RASSF1C polyubiquitylation and degradation. The present study reveals a novel mechanism of RASSF1C regulation and implies a potentially important role of RASSF1C in DNA damage responses.

EXPERIMENTAL

Plasmid construction

Full-length cDNAs of RASSF1C were cloned into the pCDNA3-HA (haemagglutinin), pRK7-N-FLAG or pQCXIH vector using standard protocols. Ubiqutin, β-TrCP and GSK3β were cloned into the pCDNA3-HA vector.

Cell culture and transfection

HEK-293T [human embryonic kidney-293 cells expressing the large T-antigen of SV40 (simian virus 40)] and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10 % fetal bovine serum (HyClone), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco). U2OS cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10 % fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cell transfection was performed using the calcium phosphate method or Lipofectamine™ 2000 (Invitrogen). Cells were harvested at 30 h post-transfection for protein analysis.

To establish stable RASSF1C-expressing cell pools, pQCXIH-SBP (streptavidin-binding peptide)-FLAG-RASSF1C retroviruses were generated and used to infect HEK-293T, HeLa and U2OS cells, and stable pools were selected with 50 mg/ml hygromycin B (Amresco) for 5 days.

SBP purification of RASSF1C protein complexes

Ten 15 cm plates of HEK-293T SBP-FLAG-RASSF1C stable cells were pretreated with MG132 for 6 h before harvest, and then lysed in 40 ml of 0.5 % Nonidet P40 buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl and 0.5 % Nonidet P40) containing 10 mM NaF, 1 mM Na_3VO_4 and protease inhibitor mixture (Roche) for 30 min. SBP-FLAG-RASSF1C in the supernatant was precipitated for 3 h with 100 μl streptavidin resin (GE HealthCare, 17-5113-01), which was then washed three times with 0.5 % Nonidet P40 buffer followed by three washes with 50 mM NH_4HCO_3. The precipitated protein was digested with trypsin as described previously [45]. The supernatant was collected, dried and dissolved in 10 % acetonitrile and 0.8 % formic acid solution. The peptides were analysed by LC-MS/MS (liquid chromatography-tandem MS).

Immunoprecipitation and Western blotting analysis

Cells were lysed in 0.5 % Nonidet P40 buffer containing protease and phosphatase inhibitors. Cell debris was removed by centrifugation and the lysates were incubated for 3 h at 4 °C with anti-FLAG M2 agarose (Sigma). The immunoprecipitates were washed four times with 0.5 % Nonidet P40 buffer and then analysed by Western blot. Antibodies against FLAG (A00170, GenScript or A2220, Sigma), HA (F7) (SC7392, Santa Cruz Biotechnology), DAXX (4533, Cell Signaling Technology), β-catenin (9562, Cell Signaling Technology) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; SC32233, Santa Cruz Biotechnology) were purchased commercially. Anti-RASSF1C antibody was raised in rabbits using a synthetic peptide (CSQEDSDSE) as an antigen by Shanghai Genomics.

DNA-damaging stimuli

U2OS RASSF1C stable cells were cultured to approximately 70–80 % confluence in 35-mm-diameter dishes and were irradiated with 40 mJ/cm^2 UVC delivered via a HL-2000 HybriLinker with a 254 nm wavelength (Upvon), followed by the indicated recovery time before harvest to analyse RASSF1C protein levels or cellular localization.

Cytoplasmic and nuclear extracts

One 10-cm-diameter plate of U2OS stable cells were lysed in 1 ml Harvest buffer (10 mM Hepes, pH 7.9, 50 mM NaCl, 0.5 M sucrose, 0.1 mM EDTA, 0.5 % Triton X-100 and freshly added protease and phosphatase inhibitors) at 4 °C for 10 min, and then centrifuged at 500 ***g*** for 10 min to pellet the nucleus. The pellet was then washed three times with washing buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and freshly added protease and phosphatase inhibitors). The supernatant was subjected to 17 000 ***g*** centrifugation for another 10 min to remove any nuclear contamination and transferred to a new tube. Both the pellet and supernatant were boiled seperately in SDS sample buffer.

RNAi (RNA interference)

RNAi-mediated down-regulation of Mule was performed by either transfecting Mule siRNA (small interfering RNA) or control siRNA into HEK-293T or U2OS RASSF1C stable cells following the manufacturer's instructions for Lipofectamine™ RNAiMAX reagent (904203, Invitrogen). The knockdown effects were assessed 48 h after induction of RNAi. The siRNA sequences were as follows: siMule#1, 5′-GAGUUUGGAGUUUGUGAAGTT-3′; siMule#2, 5′-AAUUGCUAUGUCUCUGGGACA-3′; siControl, 5′-AAUUGCCAUGUAUCUGGGACA-3′; and siDDB1, 5′-CCUGUUGAUUGCCAAAAAC-3′.

RNA isolation and real-time PCR

Total cellular RNA was isolated from cultured cells using TRIzol® reagent following the manufacturer's instructions (Invitrogen). RNA was reverse transcribed with oligo(dT) primers and real-time PCR was conducted with gene-specific primers in the

presence of SYBR Premix Ex Taq (TaKaRa). qPCR was performed in triplicate, using GAPDH as a housekeeping control. Relative fold changes in the expression of Mule in control and Mule-knockdown cells were determined using the $\Delta\Delta C_t$ method.

The primer sequences used were: RASSF1C forward, 5′-GCTACTGCAGCCAAGAGGAC-3′; RASSF1C reverse, 5′-AGGTGTCTCCCACTCCACAG-3′; Mule forward, 5′-ACAACCTCGAGCAGCAGCGG-3′; Mule reverse, 5′-TTGTTAGCCCGGCGCGTGTC-3′; GAPDH forward, 5′-GATGACATCAAGAAGGTGGTGAAG-3′; and GAPDH reverse, 5′-TCCTTGGAGGCCATGTGGGCCAT-3′.

RESULTS

RASSF1C protein decreases in response to DNA damage

A previous study showed that DAXX underwent polyubiquitylation and degradation to promote RASSF1C release from the nucleus in response to DNA damage in HeLa cells [17]. This triggered us to test the effect of DNA damage on RASSF1C regulation. Surprisingly, we found both cytosolic and nuclear RASSF1C protein levels reduced significantly when U2OS cells were treated with UV irradiation (Figure 1A). The protein levels of RASSF1C decreased in a time-course-dependent manner in response to UV irradiation (Figures 1B and 1C). Interestingly, we found the levels of RASSF1C reduced by half in 15 min, which is much faster than DAXX or RASSF1A (Figure 1B). These results indicated that the nuclear RASSF1C reduction may not be due to release into the cytosol after DAXX degradation, but mediated by an unknown mechanism. Although we did not observe the translocation of RASSF1C in response to UV irradiation, we cannot rule out the different results of the present study compared with previous work [17] due to the different cell lines or the dose of UV used.

We also analysed RASSF1C protein levels when cells were exposed to a variety of stresses. Interestingly, treatment with doxorubicin, osmotic stress or serum starvation also reduced the protein levels of RASSF1C in a time-course-dependent manner (Figure 1D, upper panel, and Supplementary Figures S1A and S1B at http://www.BiochemJ.org/bj/441/bj4410227add.htm).

The possible reasons for UV-induced decrease of RASSF1C may be due to the transcription or protein stability of RASSF1C. To this end, we employed qPCR and found that the mRNA levels of RASSF1C changed little in comparison with the dramatic down-regulation of protein levels (Figures 1C and 1D, lower panels). Thus the decrease of RASSF1C induced by UV irradiation and doxorubicin might not due to transcriptional regulation.

RASSF1C stability is regulated by the 26S proteasome

These results promoted us to test whether the proteasome is involved in RASSF1C protein degradation. We treated HEK-293T and HeLa stable cells with the protein synthesis inhibitor CHX (cycloheximide) and measured the half-life of RASSF1C. Remarkably, we found that RASSF1C is a very unstable protein with a half-life of approximately 15 min, indicating the functional importance of this protein (Figure 1E and Supplementary Figure S2A at http://www.BiochemJ.org/bj/441/bj4410227add.htm). Treatment of the two stable cells with the proteasome inhibitor MG132 also increased the steady-state level of RASSF1C by 5- or 16-fold in HEK-293T or HeLa cells respectively (Figure 1F and Supplementary Figure S2B). Furthermore, we determined the polyubiquitylation of RASSF1C *in vivo*. Prior to the

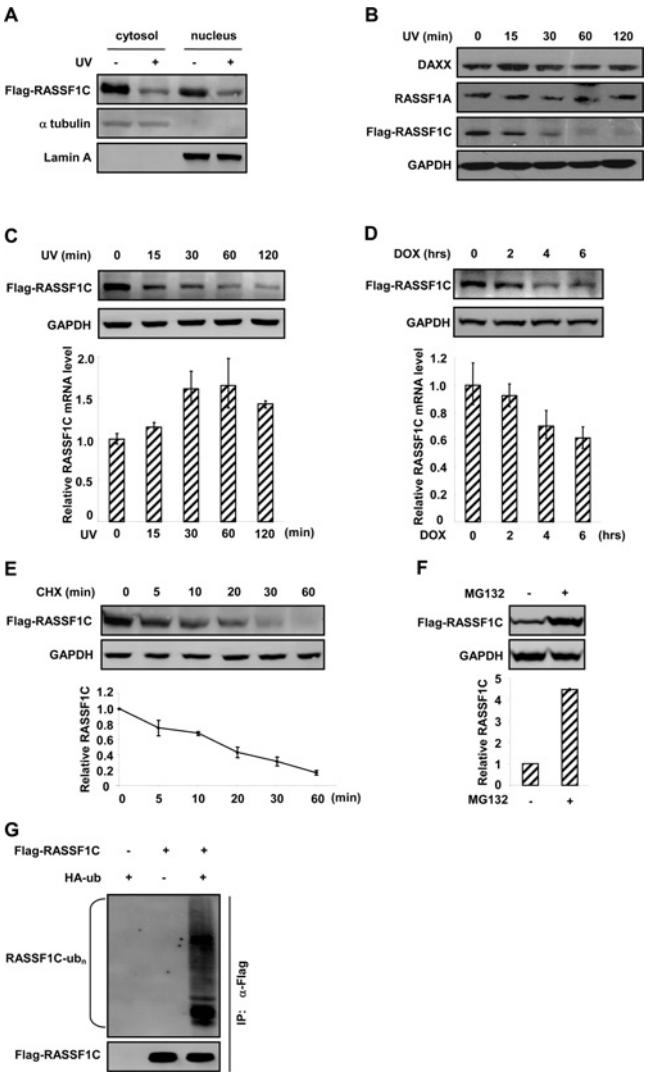

Figure 1 RASSF1C is a very unstable protein and decreases in response to DNA damage

(**A**) Both cytosolic and nuclear RASSF1C decrease after exposure to UV irradiation. After exposure to UV irradiation (40 mJ/cm^2) and recovery for 1 h, U2OS stable cells were subjected to cytosolic and nuclear fraction extraction as described in the Experimental section, and RASSF1C protein levels were determined by Western blot. Tubulin and lamin A were used as cytoplasmic and nuclear markers respectively. (**B**) UV-irradiation-induced RASSF1C reduction is faster than the decrease in DAXX. U2OS stable cells were treated with UV irradiation (40 mJ/cm^2) and recovered for the indicated times. DAXX, RASSF1A and RASSF1C protein levels were detected by Western blot. GAPDH was used as a loading control. (**C** and **D**) UV irradiation or doxorubicin reduces the protein levels of RASSF1C in a time-course-dependent manner. U2OS stable cells were treated with UV irradiation (40 mJ/cm^2) and recovered or treated with doxorubicin (10 μM) for the indicated times. Top panel, cell lysates were analysed by Western blot using specific antibodies. Bottom panels, relative mRNA levels of RASSF1C were determined by qPCR in the same experiment. (**E**) RASSF1C is an unstable protein. Top panels, HEK-293T stable cells were treated with CHX (20 μg/ml) for the indicated times and analysed by Western blot. Bottom panel, the amount of RASSF1C was quantified by densitometry and normalized to GAPDH. The ratio between RASSF1C and GAPDH at the zero time point was arbitrarily set to 1. (**F**) MG132 causes the accumulation of RASSF1C protein in HEK-293T stable cells. Cells were treated with either the solvent DMSO or 10 μM MG132. Top panel, cell lysates were analysed by Western blot. Bottom panelm relative RASSF1C levels were normalized to GAPDH and quantified by densitometry. (**G**) RASSF1C is highly polyubiquitylated. FLAG–RASSF1C was immunoprecipitated (IP) and polyubiquitylation of the precipitated RASSF1C (RASSF1C-ub$_n$) was determined by Western blot for the co-transfected HA–ubiquitin (HA-ub).

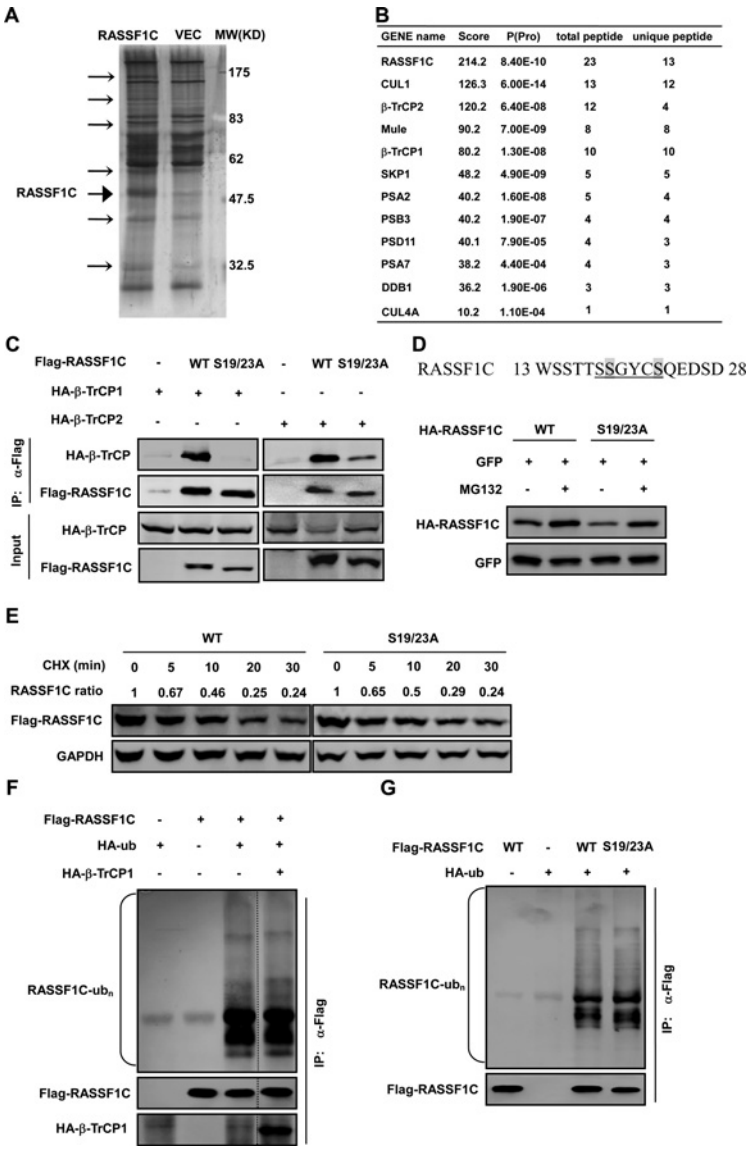

Figure 2 SCF$^{\beta\text{-TrCP}}$ and CUL4-DDB1 are not involved in RASSF1C degradation under normal conditions

(**A**) Silver staining of affinity-purified RASSF1C-containing protein complexes. Cell extracts that were prepared from HEK-293T cells stably expressing SBP-FLAG–RASSF1C or control cells were subjected to streptavidin affinity purification. Proteins bound to streptavidin-conjugated beads from SBP-FLAG–RASSF1C stable cells and control cells (VEC) were analysed by SDS/PAGE and visualized by silver staining. Arrows indicate RASSF1C and the specific protein bands that interact with it. Molecular mass in kDa is given on the right-hand side. (**B**) Proteins involved in the degradation of RASSF1C were identified by MS analysis. Affinity-purified proteins were identified by MS analysis and the detailed peptide sequences are summarized in Tables S1 and S2 (at http://www.BiochemJ.org/bj/441/bj4410227add.htm). (**C**) The mutant of the potential phosphorylation sites S19A/S23A (S19/23A) disrupts the interaction between RASSF1C and β-TrCP. HA–β-TrCP was co-transfected with RASSF1C. FLAG–RASSF1C was immunoprecipitated (IP) and the associated HA–β-TrCP was detected by Western blot with anti-HA antibody. The RASSF1C S19A/S23A mutant showed weak interaction with β-TrCP. (**D**) The binding-deficient mutant of RASSF1C remains an unstable protein. The sequences surrounding the phosphodegrons in the N-terminal of RASSF1C are shown. Potentially phosphorylated serine residues are shaded (top panel). HA-tagged wild-type (WT) or S19A/S23A RASSF1C was cotransfected with GFP (green fluorescent protein), which is referred as loading control. At 24 h after transfection, cells were pretreated with either the solvent DMSO or 10 μM MG132 for 6 h before harvest, followed by Western blot analysis. (**E**) S19A/S23A mutant does not affect the half-life of RASSF1C. HEK-293T stable cells were transfected with the wild-type or S19A/S23A mutant of RASSF1C, and then split into 5 different wells. At 24 h after transfection, cells were treated with CHX (20 μg/ml) for the indicated times. RASSF1C protein levels were determined by Western blot. The amount of RASSF1C was quantified by densitometry and normalized with GAPDH. (**F**) β-TrCP overexpression does not result in the increase of characteristic ladders. FLAG–RASSF1C, HA–ubiquitin (HA-ub) and HA–β-TrCP were cotransfected into cells as indicated. Cells were lysed in 1 % SDS buffer and subsequently subjected to immunoprecipitation and Western blot to detect the polyubiquitin ladders. (**G**) The S19A/S23A mutant doesn't affect RASSF1C polyubiquitylation. FLAG-tagged wild-type or S19A/S23A RASSF1C was cotransfected with HA–ubiquitin as indicated, FLAG–RASSF1C was immunoprecipitated and ubiquitylation of the precipitated RASSF1C was detected by Western blot for the co-transfected HA–ubiquitin.

analysis of RASSF1C polyubiquitylation, cells were treated with MG132 to enrich polyubiquitylated proteins. Co-expression of RASSF1C with ubiquitin resulted in the detection of characteristic incremental ladders, the indicative of polyubiquitylated species (Figure 1G). Collectively, our data suggests that RASSF1C protein stability is regulated by the proteasome and may respond to DNA damage.

SCF$^{\beta\text{-TrCP}}$ and CUL4-DDB1 are not involved in RASSF1C degradation under normal conditions

Proteasome-mediated degradation depends on polyubiquitylation of target proteins. A direct interaction between the E3 ubiquitin ligase and target protein indicates the selective polyubiquitylation of the target protein. To search for RASSF1C-interacting proteins,

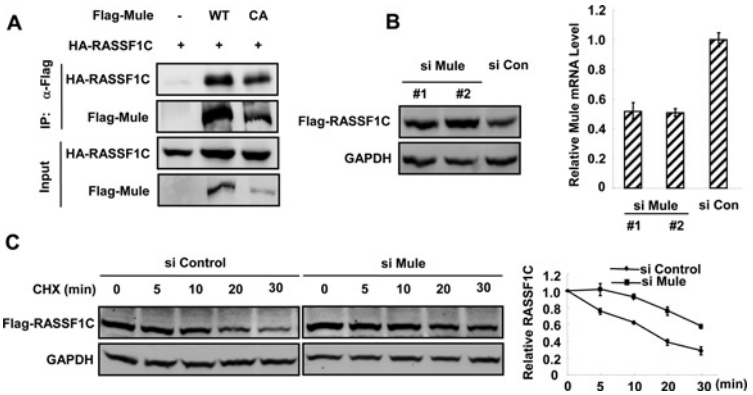

Figure 3 Mule promotes RASSF1C degradation under normal conditions

(**A**) Mule interacts with RASSF1C. HA–RASSF1C was co-transfected with FLAG–Mule wild-type (WT) or catalytically inactive mutant C4341A (CA). FLAG–Mule was immunoprecipitated (IP) and the associated HA–RASSF1C was determined by Western blot using an anti-HA antibody. The Mule C4341A mutant showed the same degree of interaction. (**B**) Knocking down Mule leads to accumulation of RASSF1C. Control (si Con) or two different Mule (si Mule) siRNA oligonucleotides were transfected into HEK-293T cells for Western blot analysis. Both of the Mule siRNAs, but not the control oligonucleotide, resulted in dramatic up-regulation of RASSF1C protein (left-hand panel). Mule knockdown efficiency was determined by qPCR with specific primers (right-hand panel). (**C**) Mule knockdown significantly increases RASSF1C stability. HEK-293T stable cells transfected with Mule siRNA or control oligonucleotides were treated with CHX (20 μg/ml) for the indicated times. Total protein lysates were subjected to Western blot analysis with the indicated antibodies (left-hand panels). The amount of RASSF1C was quantified by densitometry and normalized with GAPDH (right-hand panel).

we performed MS analysis of affinity-purified RASSF1C (Figure 2A). The candidate degradation-related RASSF1C-interacting proteins identified in this search are shown in Figure 2(B).

Interestingly, CUL1, Skp1, β-TrCP1 and β-TrCP2 were identified in a potential RASSF1C-interacting protein complex. These identified peptides are shown in Supplementary Table S1 (at http://www.BiochemJ.org/bj/441/bj4410227add.htm). CUL1, Skp1, β-TrCP1 and β-TrCP2, which belong to the SCF class of E3 ubiquitin ligase, have been implicated in the degradation of many growth-promoting proteins, such as IκB (inhibitory κB), β-catenin and Emi1 (early mitotic inhibitor 1) [23]. To confirm the interaction between RASSF1C and the $SCF^{\beta\text{-TrCP}}$ E3 ligase complex, we performed co-immunoprecipitation experiments and found that both β-TrCP1 and β-TrCP2 could be readily pulled down by RASSF1C (Figure 2C) as described previously [24]. β-TrCP can recognize the D/SpSGXXpS phosphodegron of target proteins, and phosphorylation of this phosphodegron generates the binding sites for $SCF^{\beta\text{-TrCP}}$ and subsequently leads to proteasome degradation. RASSF1C contains the SSGYCS degron, and we generated the S19A/S23A phosphodegron mutant to determine whether this mutant could disrupt the interaction. As expected, the S19A/S23A mutant dramatically decreased the interaction between RASSF1C and β-TrCP1 (Figure 2C, left-hand panel) or β-TrCP2 (Figure 2C, right-hand panel).

On the basis of this data, we believed that the S19A/S23A mutant should be resistant to proteasome-mediated degradation. To our surprise, MG132 treatment still led to obvious RASSF1C accumulation (Figure 2D) and the half-life of S19A/S23A mutant is the same as wild-type RASSF1C (Figure 2E). Furthermore, β-TrCP1 overexpression didn't result in an increase of characteristic ladders (Figure 2F). Moreover, the S19A/S23A mutant retained the same amount of polyubiquitin ladders (Figure 2G). Taken together, these results suggest that $SCF^{\beta\text{-TrCP}}$ is not the potential E3 ligase for RASSF1C degradation under normal conditions.

The ROC1-CUL4-DDB1 E3 ligase is reported to regulate chromatin formation, cell cycle checkpoint, DNA replication and DNA damage responses [25]. As shown in Figure 2(B), CUL4A and DDB1 were also coimmunoprecipitated by RASSF1C. However, DDB1 knockdown also didn't lead to the accumulation of RASSF1C (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/441/bj4410227add.htm). Collectively, these results indicated that ROC1-CUL4-DDB1 is also not involved in the degradation of RASSF1C under normal conditions.

Mule promotes RASSF1C degradation under normal conditions

Given that $SCF^{\beta\text{-TrCP}}$ and CUL4-DDB1 are not the potential E3 ligases responsible for RASSF1C degradation, we tested another potential E3 ligase, Mule, which was identified in our SBP affinity purification (Supplementary Table S2 at http://www.BiochemJ.org/bj/441/bj4410227add.htm). Mule belongs to the HECT (homologous with E6-associated protein C-terminus) family and ubiquitylates multiple proteins such as Mcl-1 and p53 [26–29]. The strong interaction between Mule and RASSF1C can be easily detected. Similarly, the catalytically inactive Mule mutant C4341A also strongly interacted with RASSF1C (Figure 3A). As shown in Figure 3(B), two different Mule knockdown, but not control, oligonucleotides resulted in dramatic accumulation of RASSF1C. To prove that the Mule-knockdown-induced accumulation of RASSF1C protein was due to decreased degradation, we analysed the half-life of RASSF1C in Mule knockdown or control cells. We observed that Mule knockdown HEK-293T cells displayed higher RASSF1C protein levels (Figure 3B, left-hand panel) and the half-life of RASSF1C was prolonged from 15 min to longer than 30 min (Figure 3C), although the knockdown efficiency is only 50 % (Figure 3B, right-hand panel). Altogether, these results indicate that Mule promotes the proteasomal degradation of RASSF1C under normal conditions.

The PI3K (phosphoinositide 3-kinase)/Akt pathway inhibits GSK3 to upregulate RASSF1C protein levels

RASSF1A has been reported to undergo phosphorylation-induced degradation [30]. Given that RASSF1C is identical to RASSF1A except for 49 amino acids at the N-terminal, we hypothesized that phosphorylation regulation might also play a role in RASSF1C

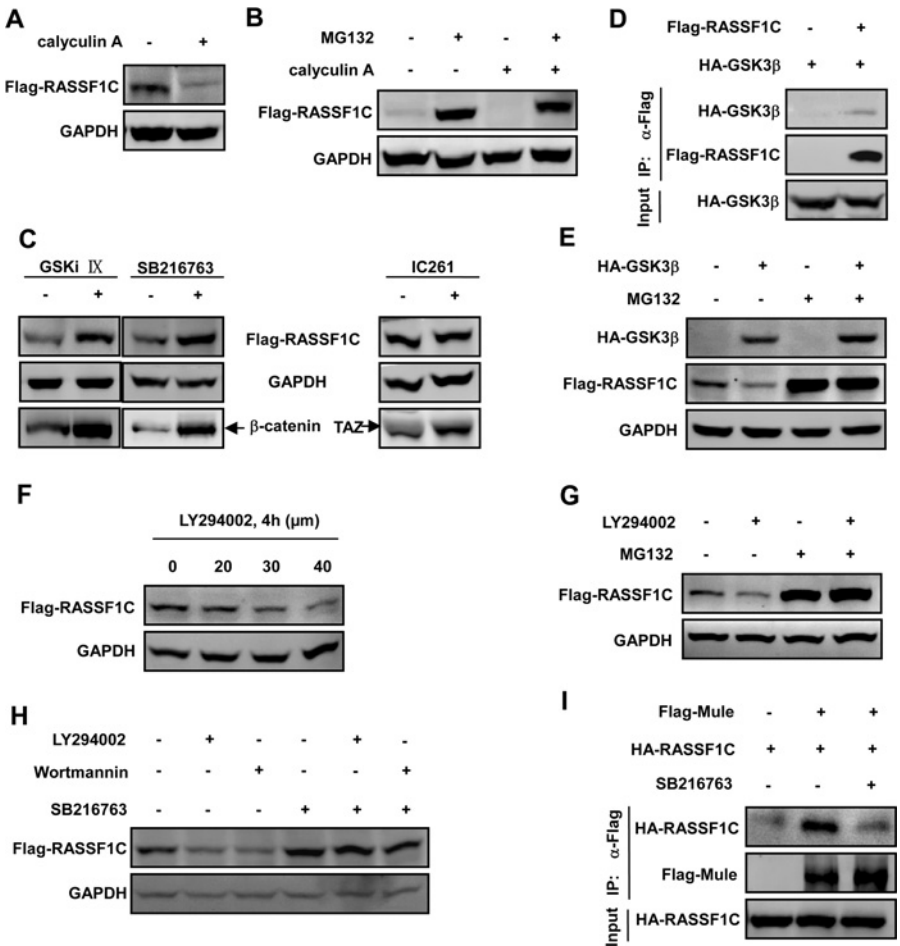

Figure 4 PI3K/Akt pathway inhibits GSK3 to upregulate RASSF1C protein levels

(**A**) Calyculin A decreases RASSF1C protein levels. HEK-293T stable cells were treated with the serine/threonine phosphatase inhibitor calyculin A (100 nM) for 30 min. RASSF1C protein levels were determined by Western blot along with the GAPDH control. (**B**) Calyculin A-induced RASSF1C degradation is blocked by MG132. HEK-293T stable cells were treated with or without Calyculin A or MG132 as indicated and RASSF1C protein levels were determined. (**C**) GSK3β inhibitors but not a CK1 inhibitor leads to accumulation of RASSF1C proteins. HEK-293T stable cells were treated with the GSK3 inhibitor SB216763 (20 μM) or GSKi IX (2 μM), or the CK1-specific inhibitor IC261 (10 μM) for 4 h and RASSF1C protein levels were determined. β-Catenin and TAZ served as positive controls respectively. (**D**) GSK3β binds to RASSF1C. HA–GSK3β was co-transfected with FLAG–RASSF1C and co-immunoprecipitation (IP) was performed to determine the interaction between RASSF1C and GSK3β. The associated HA–GSK3β was detected by Western blot. (**E**) GSK3β induces proteasome-dependent degradation of RASSF1C. HA–GSK3β was transfected into HEK-293T stable cells, followed by treatment with 10 μM MG132 for 6 h and then RASSF1C protein levels were determined. (**F**) Inhibition of PI3K reduces RASSF1C protein levels in a dose-dependent manner. HEK-293T stable cells were treated with the PI3K inhibitor LY294002 as indicated for 6 h and then cell lysates were analysed by Western blot. (**G**) Inhibition of PI3K induces proteasome-dependent RASSF1C degradation. HEK-293T stable cells were treated with or without the PI3K inhibitor LY294002 (40 μM) or MG132 (10 μM) for 6 h as indicated, and RASSF1C protein levels were determined. (**H**) GSK3 inhibitor blocks RASSF1C degradation induced by PI3K inhibition. HEK-293T stable cells were treated with or without the PI3K inhibitor LY294002 (50 μM), wortmannin (1 mM) or the GSK3 inhibitor SB216763 (20 μM) for 6 h as indicated. Cell lysates were analysed by Western blot. (**I**) The GSK3 inhibitor SB216763 disrupts the interaction between Mule and RASSF1C. HA–RASSF1C was co-transfected with FLAG–Mule. At 24 h post-transfection, cells were treated with or without SB216763 (20 μM). FLAG–Mule was immunoprecipitated (IP) and the associated HA–RASSF1C was detected by Western blot analysis.

degradation. To test whether phosphorylation is involved in RASSF1C degradation, we examined the effect of the phosphatase inhibitor calyculin A on RASSF1C protein levels. Interestingly, we found calyculin A treatment led to the decrease of RASSF1C and the shift of the RASSF1C protein band, indicating the regulation of the phosphorylation of RASSF1C (Figure 4A). Furthermore, MG132 significantly blocked calyculin A-induced RASSF1C degradation (Figure 4B).

Interestingly, we found that RASSF1C contains a potential motif recognized by GSK3 using a motif scan (http://scansite.mit.edu/motifscan_seq.phtml). In addition, RASSF1A has been shown to be phosphorylated by GSK3β [31]. These led us to examine if GSK3 is responsible for RASSF1C phosphorylation. As shown in Figure 4(C), two different GSK3 inhibitors, but not a CK1 (casein kinase 1) inhibitor, resulted in RASSF1C accumulation, with β-catenin and TAZ (transcriptional co-activator with PDZ-binding motif) serving as positive controls respectively. When we examined the interaction between RASSF1C and GSK3β, RASSF1C readily pulled down GSK3β (Figure 4D). Furthermore, GSK3β overexpression led to the decrease of RASSF1C. More importantly, this effect was totally blocked by MG132 (Figure 4E).

GSK3 activity is regulated by different cell signalling pathways, such as the Wnt signalling pathway, the PI3K/Akt pathway and the MAPK (mitogen-activated protein kinase) pathway. To figure out which pathway is involved in the regulation of RASSF1C stability through GSK3, we treated cells with the PI3K inhibitor LY294002, and found that LY294002 led to decreased RASSF1C protein levels in a dose-dependent manner as shown in Figure 4(F). MG132 totally blocked LY294002-induced RASSF1C degradation (Figure 4G). Moreover, the GSK3 inhibitor SB216763 totally blocked

RASSF1C degradation induced by LY294002 and wortmannin (Figure 4H). More importantly, the interaction between Mule and RASSF1C can be blocked by SB216763 (Figure 4I). Collectively, GSK3β is the *bona fide* RASSF1C kinase, and phosphorylation regulation promotes RASSF1C degradation by enhancing the interaction between the E3 ligase and target protein. It would be worth further study to identify GSK3β-specific phosphorylation site(s) in RASSF1C.

DNA damage promotes the polyubiquitylation and degradation of RASSF1C

We have demonstrated that RASSF1C is a very unstable protein and can be polyubiquitylated by Mule, which promotes its proteasomal degradation. Notably, a variety of stresses, including UV irradiation, doxorubicin, osmotic stress and serum starvation can induce a decrease in RASSF1C protein levels (Figures 1C and 1D, and Supplementary Figures S1A and S1B). Thus we examined the effect of the proteasome inhibitor MG132 on stress-induced RASSF1C protein decrease. Interestingly, MG132 totally blocked DNA damage, osmotic stress and serum-starvation-triggered RASSF1C protein decrease (Figure 5A, and Supplementary Figure S4 at http://www.BiochemJ.org/bj/441/bj4410227add.htm), indicating that DNA damage promotes RASSF1C degradation via the proteasome. As expected, UV irradiation and doxorubicin promoted the polyubiquitylation of RASSF1C (Figure 5B). Moreover, RASSF1C is mainly degraded in the nucleus, as we noticed that MG132 can totally block UV-induced degradation in the nucleus (Figure 5C). In addition, the interaction between RASSF1C and Mule was enhanced when cells were exposed to UV irradiation (Figure 5D, top panel). Furthermore, RASSF1C protein levels were halved in 15 min in response to UV irradiation (as shown in Figures 1B and 5E), which was significantly delayed to 60 min when U2OS cells were transfected with Mule siRNA compared with control oligos (Figure 5E and Supplementary Figure S5 at http://www.BiochemJ.org/bj/441/bj4410227add.htm). At the same time, we found that the S19A/S23A mutant, which disrupts the interaction between β-TrCP and RASSF1C, has a comparable effect with Mule knockdown under UV irradiation conditions (Figure 5F). Similarly, we found that UV irradiation significantly increases the interaction between β-TrCP and RASSF1C (Figure 5D, bottom panel). However, DDB1 knockdown did not lead to resistance to UV-induced RASSF1C degradation (Supplementary Figure S3). Moreover, we found that the GSK3 inhibitor SB216763 can also block UV-induced RASSF1C degradation (Figure 5G). Collectively, DNA damage promotes GSK3 phosphorylation of RASSF1C and subsequent polyubiquitylation and degradation via Mule and $SCF^{\beta\text{-TrCP}}$, but not via CUL4A-DDB1 E3 ligase.

DISCUSSION

The *RASSF1* gene was first identified in a yeast two-hybrid screen for partners of the human DNA excision repair protein XPA (xeroderma pigmentosum A) [4]. Until now, numerous studies have demonstrated that RASSF1A plays a very important role in cancer development by regulating cell cycle arrest, microtubule stability and apoptosis [6,7,9–11,30]. However, the regulation and cellular function of RASSF1C were rarely reported, although several studies have debated it is a tumour suppressor or oncogene [6,13–16]. Besides the unavailable endogenous anti-RASSF1C antibody, the lack of systemic study on RASSF1C is certainly a reason for the absence of this information.

Therefore we addressed this question by generating an anti-RASSF1C antibody and using biochemical purification coupled with LC-MS/MS technology. Unfortunately, we produced an anti-RASSF1C antibody which can detect ectopically expressed but not endogenous RASSF1C protein (see Supplementary Figure S6 at http://www.BiochemJ.org/bj/441/bj4410227add.htm). Interestingly, RASSF1C affinity purification identified three different E3 ligase complexes with high scores, including $SCF^{\beta\text{-TrCP}}$, composed of CUL1, Skp1, β-TrCP1 and β-TrCP2, ROC1-CUL4-DDB1 E3 ligase and a HECT-domain-containing E3 ligase, Mule. This strongly suggests RASSF1C might be a very unstable protein and potentially regulated by polyubiquitylation and proteasome degradation. Consistently, we found that the half-life of RASSF1C is approximately 15 min and that the protein is mainly degraded via the proteasome. Notably, RASSF1C polyubiquitylation is regulated by stress signals including UV damage, doxorubicin treatment, osmotic stress and serum starvation. This is the first report that RASSF1C protein levels are regulated via post-translational modification.

Via mutant analysis and knockdown experiments, we identified Mule, but not DDB1 or $SCF^{\beta\text{-TrCP}}$, as the RASSF1C *bona fide* E3 ligase responsible for the degradation of RASSF1C under normal culture conditions. Interestingly, UV-damage-induced RASSF1C degradation can be partially blocked by Mule knockdown, indicating that other potential E3 ligases might also be involved in this process. To our surprise, the S19A/S23A mutant, which disrupts the interaction between β-TrCP and RASSF1C, but not DDB1, has a comparable effect with Mule under UV irradiation conditions. Taken together, these results suggest that Mule controls the polyubiquitylation and degradation of RASSF1C under normal conditions, whereas both Mule and $SCF^{\beta\text{-TrCP}}$ are responsible for RASSF1C proteasome degradation in response to UV irradiation (Figure 6).

UV light results in various DNA lesions, including oxidative lesions and DNA single-strand breaks [32]. It is worth noting that the SCF complex and Mule function as the other two common E3 ligases responsible for protein degradation in response to UV irradiation besides CUL4-DDB1, and the target protein degradation is usually controlled by more than one E3 ligase. Notably, UV irradiation target proteins such as p21 and Mcl-1 share three common features: (i) UV irradiation induces the degradation of both proteins; (ii) GSK3 phosphorylation leads to polyubiquitylation and degradation; and (iii) UV-induced degradation is via two different E3 ligase complexes, p21–SCF^{Skp2} and $CUL4^{Cdt2}$, Mcl-1–$SCF^{\beta\text{-TrCP}}$ and Mule respectively [29,33–37]. In the present study, we show another example that RASSF1C degradation in response to UV irradiation is controlled by Mule, and $SCF^{\beta\text{-TrCP}}$ and GSK phosphorylation. On the other hand, one can speculate the potential role of RASSF1C in DNA repair, cell cycle control and anti-apoptosis from this analogy with other target proteins such as p21 and Mcl-1.

As well as its roles in destabilizing growth-stimulating proteins such as cyclin D, cyclin E and β-catenin by phosphorylation [38–42], GSK3-regulated proteolysis also plays important roles in DNA damage responses. Lee et al. [36] reported that low-dose UV irradiation can increase GSK3 activity, and promote the phosphorylation of p21 for rapid degradation via proteasome. Another example is that GSK3 phosporylates Mcl-1 in response to UV irradiation, and was subsequently polyubiquitylated and degraded by $SCF^{\beta\text{-TrCP}}$, but not Mule [34]. Therefore phosphorylation of RASSF1C by GSK3 may provide a molecular mechanism for RASSF1C destabilization in response to UV irradiation. Consistent with this model, UV-irradiation-induced RASSF1C destabilization is significantly blocked by the GSK3 inhibitor SB216763. It is worth noting that RASSF1A is

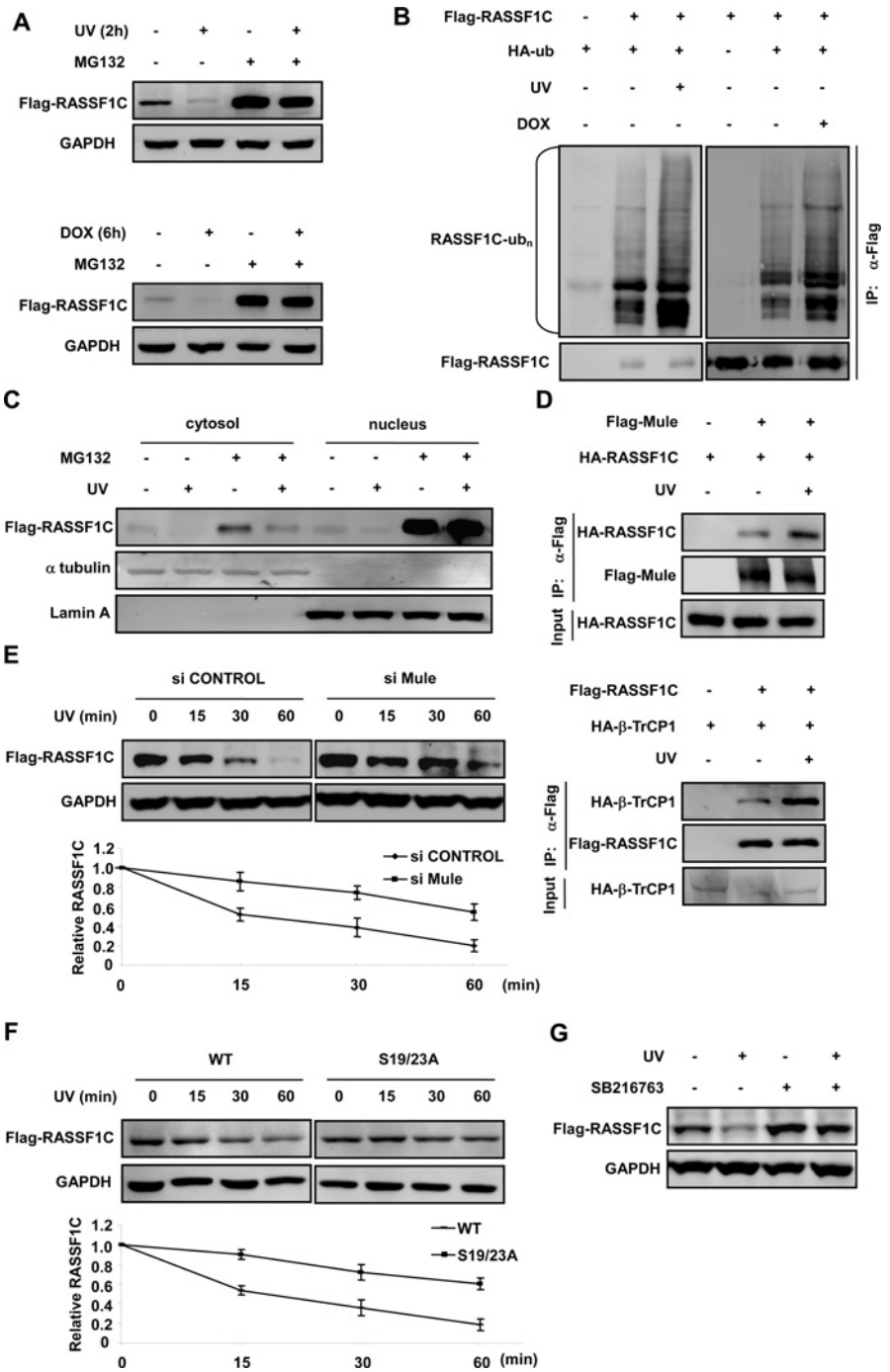

Figure 5 DNA damage promotes the degradation of RASSF1C via Mule and SCF$^{\beta\text{-TrCP}}$

(**A**) MG132 blocks UV irradiation or doxorubicin (DOX)-induced RASSF1C decrease. U2OS stable cells were treated with or without UV irradiation or doxorubicin, combined with or without MG132 as indicated. RASSF1C protein levels were determined by Western blot analysis. (**B**) UV irradiation and doxorubicin treatment promote RASSF1C polyubiquitylation. FLAG-tagged RASSF1C was cotransfected with HA–ubiquitin (HA-ub) as indicated, and 24 h after transfection, cells were treated with or without UV irradiation (40 mJ/cm^2, recovery for 2 h) or doxorubicin (10 μM, 6 h). FLAG–RASSF1C was immunoprecipitated and polyubiquitylation of the precipitated RASSF1C was determined by Western blot for the co-transfected HA–ubiquitin. (**C**) RASSF1C is primarily degraded in the nucleus. U2OS stable cells were exposed to UV irradiation with or without MG132. Cytosolic and nuclear fractions were prepared as described in the Experimental section, and then subjected to Western blot analysis. Tubulin and lamin A were used as markers for the cytoplasm and nucleus respectively. (**D**) UV irradiation promotes the interaction between Mule (top panel) or β-TrCP (bottom panel) and RASSF1C. RASSF1C was co-transfected with Mule or β-TrCP into U2OS cells as indicated. At 24 h after transfection, cells were treated with or without UV irradiation (40 mJ/cm^2, recovery for 2 h). FLAG–Mule or FLAG–RASSF1C was immunoprecipitated (IP) and the associated HA–RASSF1C or HA–β-TrCP was analysed by Western blot respectively. (**E**) Mule knockdown blocks UV-irradiation-induced RASSF1C degradation. U2OS stable cells transfected with Mule siRNA or control oligonucleotides were treated with UV irradiation (40 mJ/cm^2) and recovered for the indicated times. Total protein lysates were subjected to Western blot with the indicated antibodies (top panels). The RASSF1C protein levels were quantified by densitometry and normalized to GAPDH (bottom panel). (**F**) The S19A/S23A (S19/23A) mutant dramatically disrupts UV-induced degradation of RASSF1C. U2OS cells were transfected with wild-type (WT) or S19A/S23A mutant of RASSF1C, and then split into four different wells. At 24 h after transfection, cells were treated with UV irradiation (40 mJ/cm^2) and recovered for the indicated times. RASSF1C protein levels were determined by Western blot analysis. The amounts of RASSF1C were quantified by densitometry and normalized to GAPDH. (**G**) UV-irradiation-induced RASSF1C degradation can be blocked by the GSK3 inhibitor SB216763. U2OS stable cells were treated with or without UV irradiation (40 mJ/cm^2, recovery for 2 h), coupled with or without SB216763 (20 μM) as indicated. RASSF1C protein levels were determined by Western blot.

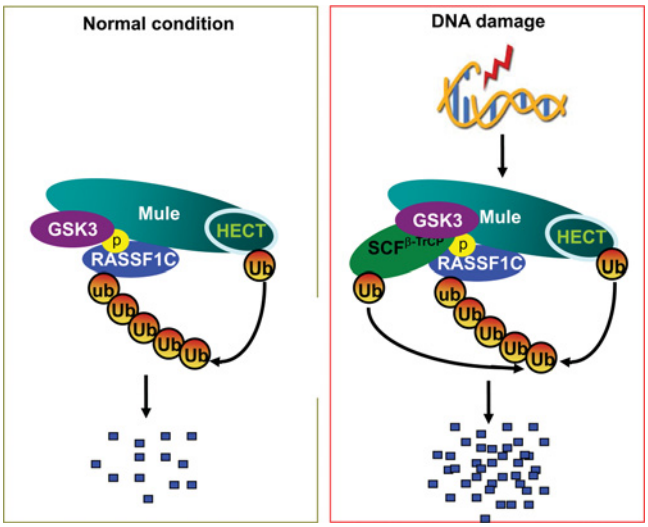

Figure 6 A model depicting the postulated Mule and SCF$^{\beta\text{-TrCP}}$-mediated degradation of RASSF1C in response to DNA damage

Mule, a HECT family E3 ligase, promotes RASSF1C degradation under normal conditions, whereas both Mule and SCF$^{\beta\text{-TrCP}}$ target RASSF1C degradation in response to UV irradiation. p, phosphorylation; ub, ubiquitin.

also regulated by GSK3β [31], and provides us the potential phosphorylation site(s) in RASSF1C.

Activation of the PI3K pathway is frequently observed in human cancers [43,44]. This can be achieved by an activating mutation in growth factor receptors and PI3K, or an inactivating mutation in PTEN (phosphatase and tensin homologue deleted on chromosome 10). Increased PI3K signalling results in constitutive Akt activation and GSK3 inhibition. Therefore, phosphorylation of RASSF1C by GSK3 may provide a molecular mechanism for RASSF1C stabilization by PI3K activation. When PI3K is activated, RASSF1C protein levels would accumulate, therefore contributing to the mitogenic activity of the PI3K pathway. Conversely, when PI3K signalling activity is low, RASSF1C protein levels would decrease, thus leading to apoptosis and growth inhibition. This hypothesis was verified when treatment of HeLa cells with serum starvation, which can inactive PI3K, led to the degradation of RASSF1C. Although no functional data was obtainable owing to the absence of an endogenous antibody, we propose that RASSF1C protein levels may contribute to tumorigenesis in cancer cells with dysregulated PI3K signalling.

In the present study, we demonstrated that RASSF1C is a very unstable protein and its stability is controlled by the Mule E3 ligase under normal conditions. After DNA damage, both Mule and SCF$^{\beta\text{-TrCP}}$ are involved in the control of RASSF1C proteasome degradation. The present study reveals a molecular mechanism of RASSF1C degradation by different E3 ligases in response to different signals and the regulation of RASSF1C stability by the PI3K/Akt/GSK3 pathway. We have provided new insights into the post-translational modification of RASSF1C and propose a functional importance in DNA damage responses and tumorigenesis in cancer cells with dysregulated PI3K signalling.

AUTHOR CONTRIBUTION

Xin Zhou designed the research, and Xin Zhou and Ting-Ting Li performed the experiments. Xu Feng and Esther Hsiang assisted with the experiments. Yue Xiong and Kun-Liang Guan analysed the data. Qun-Ying Lei devised the overall project, analysed the data and wrote the paper.

ACKNOWLEDGEMENTS

We thank the members of the Fudan Molecular and Cell Biology laboratory for discussions throughout this study. pCDNA4-FLAG-Mule (wild-type and C4341A mutant) plasmids were provided by Dr Qing Zhong (Department of Molecular & Cell Biology, University of California, Berkeley, CA, U.S.A.).

FUNDING

This work was supported by 973 [grant numbers 2009CB918401 and 2011CB910600], NCET [grant number 09-0315], NSFC (National Natural Science Foundation of China) [grant numbers 30871255 and 31071192] and NSFC-NIH (National Institutes of Health) [grant number 81110313]. This work was also supported by the 985 Program and Shanghai key project [grant number 09JC1402300]; 100 Talents Programme of Shanghai Health; the Shanghai Leading Academic Discipline Project [project number B110].

REFERENCES

1 Agathanggelou, A., Cooper, W. N. and Latif, F. (2005) Role of the Ras-association domain family 1 tumor suppressor gene in human cancers. Cancer Res. **65**, 3497–3508
2 Dammann, R., Schagdarsurengin, U., Seidel, C., Strunnikova, M., Rastetter, M., Baier, K. and Pfeifer, G. P. (2005) The tumor suppressor RASSF1A in human carcinogenesis: an update. Histol. Histopathol. **20**, 645–663
3 Burbee, D. G., Forgacs, E., Zochbauer-Muller, S., Shivakumar, L., Fong, K., Gao, B., Randle, D., Kondo, M., Virmani, A., Bader, S. et al. (2001) Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J. Natl. Cancer Inst. **93**, 691–699
4 Dammann, R., Li, C., Yoon, J. H., Chin, P. L., Bates, S. and Pfeifer, G. P. (2000) Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nat. Genet. **25**, 315–319
5 Richter, A. M., Pfeifer, G. P. and Dammann, R. H. (2009) The RASSF proteins in cancer; from epigenetic silencing to functional characterization. Biochim. Biophys. Acta **1796**, 114–128
6 Shivakumar, L., Minna, J., Sakamaki, T., Pestell, R. and White, M. A. (2002) The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. Mol. Cell. Biol. **22**, 4309–4318
7 Song, M. S., Song, S. J., Ayad, N. G., Chang, J. S., Lee, J. H., Hong, H. K., Lee, H., Choi, N., Kim, J., Kim, H. et al. (2004) The tumour suppressor RASSF1A regulates mitosis by inhibiting the APC–Cdc20 complex. Nat. Cell Biol. **6**, 129–137
8 Vos, M. D., Martinez, A., Elam, C., Dallol, A., Taylor, B. J., Latif, F. and Clark, G. J. (2004) A role for the RASSF1A tumor suppressor in the regulation of tubulin polymerization and genomic stability. Cancer Res. **64**, 4244–4250
9 Dallol, A., Agathanggelou, A., Fenton, S. L., Ahmed-Choudhury, J., Hesson, L., Vos, M. D., Clark, G. J., Downward, J., Maher, E. R. and Latif, F. (2004) RASSF1A interacts with microtubule-associated proteins and modulates microtubule dynamics. Cancer Res. **64**, 4112–4116
10 Baksh, S., Tommasi, S., Fenton, S., Yu, V. C., Martins, L. M., Pfeifer, G. P., Latif, F., Downward, J. and Neel, B. G. (2005) The tumor suppressor RASSF1A and MAP-1 link death receptor signaling to Bax conformational change and cell death. Mol. Cell **18**, 637–650
11 Matallanas, D., Romano, D., Yee, K., Meissl, K., Kucerova, L., Piazzolla, D., Baccarini, M., Vass, J. K., Kolch, W. and O'Neill, E. (2007) RASSF1A elicits apoptosis through an MST2 pathway directing proapoptotic transcription by the p73 tumor suppressor protein. Mol. Cell **27**, 962–975
12 Hamilton, G., Yee, K. S., Scrace, S. and O'Neill, E. (2009) ATM regulates a RASSF1A-dependent DNA damage response. Curr. Biol. **19**, 2020–2025
13 Li, J., Wang, F., Protopopov, A., Malyukova, A., Kashuba, V., Minna, J. D., Lerman, M. I., Klein, G. and Zabarovsky, E. (2004) Inactivation of RASSF1C during *in vivo* tumor growth identifies it as a tumor suppressor gene. Oncogene **23**, 5941–5949
14 Vos, M. D., Ellis, C. A., Bell, A., Birrer, M. J. and Clark, G. J. (2000) Ras uses the novel tumor suppressor RASSF1 as an effector to mediate apoptosis. J. Biol. Chem. **275**, 35669–35672
15 Amaar, Y. G., Baylink, D. J. and Mohan, S. (2005) Ras-association domain family 1 protein, RASSF1C, is an IGFBP-5 binding partner and a potential regulator of osteoblast cell proliferation. J. Bone Miner. Res. **20**, 1430–1439
16 Reeves, M. E., Baldwin, S. W., Baldwin, M. L., Chen, S. T., Moretz, J. M., Aragon, R. J., Li, X., Strong, D. D., Mohan, S. and Amaar, Y. G. (2010) Ras-association domain family 1C protein promotes breast cancer cell migration and attenuates apoptosis. BMC Cancer **10**, 562
17 Kitagawa, D., Kajiho, H., Negishi, T., Ura, S., Watanabe, T., Wada, T., Ichijo, H., Katada, T. and Nishina, H. (2006) Release of RASSF1C from the nucleus by Daxx degradation links DNA damage and SAPK/JNK activation. EMBO J. **25**, 3286–3297
18 Agathanggelou, A., Honorio, S., Macartney, D. P., Martinez, A., Dallol, A., Rader, J., Fullwood, P., Chauhan, A., Walker, R., Shaw, J. A. et al. (2001) Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumours. Oncogene **20**, 1509–1518

19 Dreijerink, K., Braga, E., Kuzmin, I., Geil, L., Duh, F. M., Angeloni, D., Zbar, B., Lerman, M. I., Stanbridge, E. J., Minna, J. D. et al. (2001) The candidate tumor suppressor gene, RASSF1A, from human chromosome 3p21.3 is involved in kidney tumorigenesis. Proc. Natl. Acad. Sci. U.S.A. **98**, 7504–7509
20 Lee, M. G., Kim, H. Y., Byun, D. S., Lee, S. J., Lee, C. H., Kim, J. I., Chang, S. G. and Chi, S. G. (2001) Frequent epigenetic inactivation of RASSF1A in human bladder carcinoma. Cancer Res. **61**, 6688–6692
21 Lo, K. W., Kwong, J., Hui, A. B., Chan, S. Y., To, K. F., Chan, A. S., Chow, L. S., Teo, P. M., Johnson, P. J. and Huang, D. P. (2001) High frequency of promoter hypermethylation of RASSF1A in nasopharyngeal carcinoma. Cancer Res. **61**, 3877–3881
22 Maruyama, R., Toyooka, S., Toyooka, K. O., Harada, K., Virmani, A. K., Zochbauer-Muller, S., Farinas, A. J., Vakar-Lopez, F., Minna, J. D., Sagalowsky, A. et al. (2001) Aberrant promoter methylation profile of bladder cancer and its relationship to clinicopathological features. Cancer Res. **61**, 8659–8663
23 Fuchs, S. Y., Spiegelman, V. S. and Kumar, K. G. (2004) The many faces of β-TrCP E3 ubiquitin ligases: reflections in the magic mirror of cancer. Oncogene **23**, 2028–2036
24 Estrabaud, E., Lassot, I., Blot, G., Le Rouzic, E., Tanchou, V., Quemeneur, E., Daviet, L., Margottin-Goguet, F. and Benarous, R. (2007) RASSF1C, an isoform of the tumor suppressor RASSF1A, promotes the accumulation of beta-catenin by interacting with βTrCP. Cancer Res. **67**, 1054–1061
25 Jackson, S. and Xiong, Y. (2009) CRL4s: the CUL4-RING E3 ubiquitin ligases. Trends Biochem. Sci. **34**, 562–570
26 Chen, D., Kon, N., Li, M., Zhang, W., Qin, J. and Gu, W. (2005) ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor. Cell **121**, 1071–1083
27 Shmueli, A. and Oren, M. (2005) Life, death, and ubiquitin: taming the mule. Cell **121**, 963–965
28 Warr, M. R., Acoca, S., Liu, Z., Germain, M., Watson, M., Blanchette, M., Wing, S. S. and Shore, G. C. (2005) BH3-ligand regulates access of MCL-1 to its E3 ligase. FEBS Lett. **579**, 5603–5608
29 Zhong, Q., Gao, W., Du, F. and Wang, X. (2005) Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. Cell **121**, 1085–1095
30 Song, M. S., Song, S. J., Kim, S. J., Nakayama, K., Nakayama, K. I. and Lim, D. S. (2008) Skp2 regulates the antiproliferative function of the tumor suppressor RASSF1A via ubiquitin-mediated degradation at the G1-S transition. Oncogene **27**, 3176–3185
31 Ghazaleh, H. A., Chow, R. S., Choo, S. L., Pham, D., Olesen, J. D., Wong, R. X., Onyskiw, C. and Baksh, S. (2010) 14-3-3 mediated regulation of the tumor suppressor protein, RASSF1A. Apoptosis **15**, 117–127
32 Roos, W. P. and Kaina, B. (2006) DNA damage-induced cell death by apoptosis. Trends Mol. Med. **12**, 440–450
33 Bendjennat, M., Boulaire, J., Jascur, T., Brickner, H., Barbier, V., Sarasin, A., Fotedar, A. and Fotedar, R. (2003) UV irradiation triggers ubiquitin-dependent degradation of p21(WAF1) to promote DNA repair. Cell **114**, 599–610
34 Ding, Q., He, X., Hsu, J. M., Xia, W., Chen, C. T., Li, L. Y., Lee, D. F., Liu, J. C., Zhong, Q., Wang, X. and Hung, M. C. (2007) Degradation of Mcl-1 by β-TrCP mediates glycogen synthase kinase 3-induced tumor suppression and chemosensitization. Mol. Cell. Biol. **27**, 4006–4017
35 Kim, Y., Starostina, N. G. and Kipreos, E. T. (2008) The CRL4Cdt2 ubiquitin ligase targets the degradation of p21Cip1 to control replication licensing. Genes Dev. **22**, 2507–2519
36 Lee, J. Y., Yu, S. J., Park, Y. G., Kim, J. and Sohn, J. (2007) Glycogen synthase kinase 3β phosphorylates p21WAF1/CIP1 for proteasomal degradation after UV irradiation. Mol. Cell. Biol. **27**, 3187–3198
37 Maurer, U., Charvet, C., Wagman, A. S., Dejardin, E. and Green, D. R. (2006) Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. Mol. Cell **21**, 749–760
38 Welcker, M., Singer, J., Loeb, K. R., Grim, J., Bloecher, A., Gurien-West, M., Clurman, B. E. and Roberts, J. M. (2003) Multisite phosphorylation by Cdk2 and GSK3 controls cyclin E degradation. Mol. Cell **12**, 381–392
39 Alt, J. R., Cleveland, J. L., Hannink, M. and Diehl, J. A. (2000) Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. Genes Dev. **14**, 3102–3114
40 Cohen, P. and Frame, S. (2001) The renaissance of GSK3. Nat. Rev. Mol. Cell Biol. **2**, 769–776
41 Hart, M. J., de los Santos, R., Albert, I. N., Rubinfeld, B. and Polakis, P. (1998) Downregulation of β-catenin by human Axin and its association with the APC tumor suppressor, β-catenin and GSK3β. Curr. Biol. **8**, 573–581
42 Diehl, J. A., Cheng, M., Roussel, M. F. and Sherr, C. J. (1998) Glycogen synthase kinase-3β regulates cyclin D1 proteolysis and subcellular localization. Genes Dev. **12**, 3499–3511
43 Dahia, P. L. (2000) PTEN, a unique tumor suppressor gene. Endocr.-Relat. Cancer **7**, 115–129
44 Maehama, T., Taylor, G. S. and Dixon, J. E. (2001) PTEN and myotubularin: novel phosphoinositide phosphatases. Annu. Rev. Biochem. **70**, 247–279
45 Rybak, J. N., Ettorre, A., Kaissling, B., Giavazzi, R., Neri, D. and Elia, G. (2005) *In vivo* protein biotinylation for identification of organ-specific antigens accessible from the vasculature. Nat. Meth. **2**, 291–298

Received 18 August 2011/10 September 2011; accepted 12 September 2011
Published as BJ Immediate Publication 12 September 2011, doi:10.1042/BJ20111500

Biochem. J. (2012) **441**, 227–236 (Printed in Great Britain) doi:10.1042/BJ20111500

online data

SUPPLEMENTARY ONLINE DATA

Targeted polyubiquitylation of RASSF1C by the Mule and SCF$^{\beta\text{-TrCP}}$ ligases in response to DNA damage

Xin ZHOU*†‡, Ting-Ting LI*†‡, Xu FENG*†‡, Esther HSIANG§, Yue XIONG†‡‖, Kun-Liang GUAN*†¶ and Qun-Ying LEI*†‡[1]

*Key Laboratory of Molecular Medicine, Ministry of Education, Department of Biochemistry and Molecular Biology, Shanghai Medical College, Shanghai 200032, China, †Molecular and Cell Biology Lab, Institute of Biomedical Sciences, Fudan University, Shanghai 200032, China, ‡School of Life Sciences, Fudan University, Shanghai 200032, China, §Department of Human Evolutionary Biology, Harvard University, Cambridge, MA 02138, U.S.A., ‖Department of Biochemistry and Biophysics, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, NC 27599, U.S.A., and ¶Department of Pharmacology and Moores Cancer Center, University of California at San Diego, La Jolla, CA 92093-0815, U.S.A.

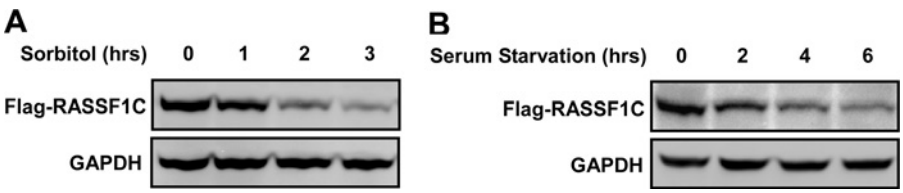

Figure S1 RASSF1C decreases in response to osmotic stress or serum starvation

Osmotic stress (**A**) or serum starvation (**B**) reduces the protein levels of RASSF1C in a time-course-dependent manner. Stable HeLa cells expressing RASSF1C were treated with sorbitol (0.5 M, **A**) or serum starvation (**B**) for indicated time. RASSF1C protein levels were analysed by Western blot. GAPDH was included as a loading control.

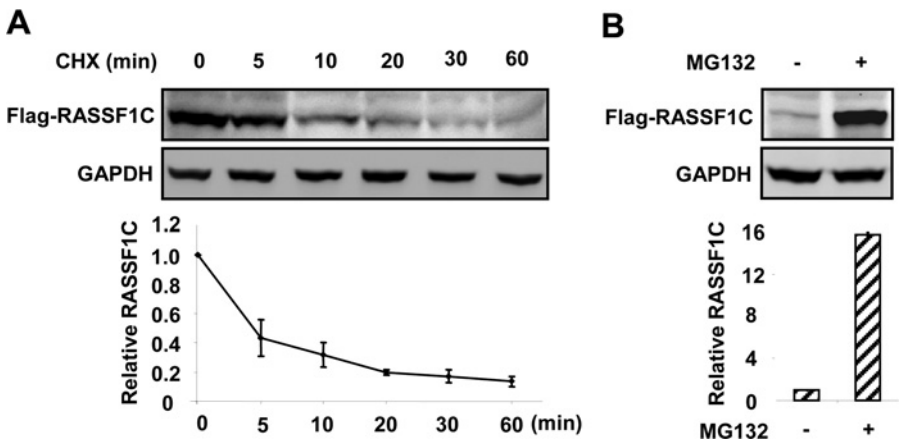

Figure S2 RASSF1C is a very unstable protein

(**A**) Stable HeLa cells expressing RASSF1C were treated with CHX (20 μg/ml) for the indicated times. RASSF1C protein levels were determined (top panel) and quantified by densitometry (bottom panel). (**B**) MG132 increases RASSF1C protein levels in stable HeLa cells expressing RASSF1C. Cells were treated with either DMSO or 10 μM MG132. Cell lysates were analysed by Western blot (top panel) and quantified by densitometry (bottom panel).

[1] To whom correspondence should be addressed (email qlei@fudan.edu.cn).

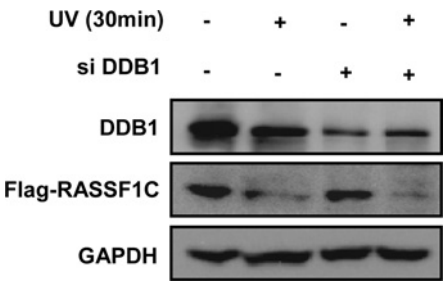

Figure S3 ROC1-CUL4-DDB1 is not involved in the degradation of RASSF1C either under normal conditions or during UV-induced DNA damage

Stable U2OS cells expressing RASSF1C were transfected with DDB1 siRNA (si DDB1) or control oligonucleotides were exposed to UV irradiation (40 mJ/cm^2) and recovered for 30 min. Cells were harvested and the protein levels of RASSF1C and DDB1 were determined by Western blot analysis.

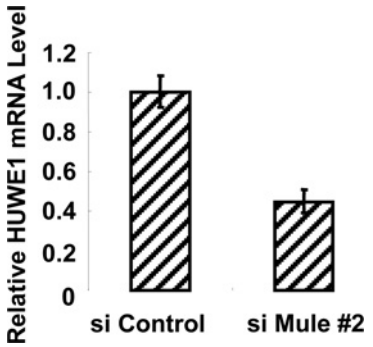

Figure S5 Mule knockdown efficiency analysis in U2OS cells

U2OS cells were transfected with Mule siRNA (si Mule) or control oligos (si Control). Mule knockdown efficiency was determined by qPCR with specific primers.

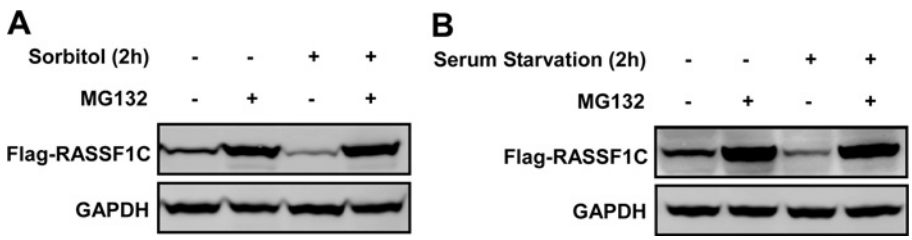

Figure S4 Osmotic stress or serum starvation promotes RASSF1C proteasomal degradation

MG132 blocks the decrease of RASSF1C protein levels induced by osmotic stress (**A**) or serum starvation (**B**). Stable HeLa cells expressing RASSF1C were treated with 0.5 M sorbitol (**A**) or serum starvation (**B**) and with or without MG132 as indicated. RASSF1C protein levels were analysed by Western blot.

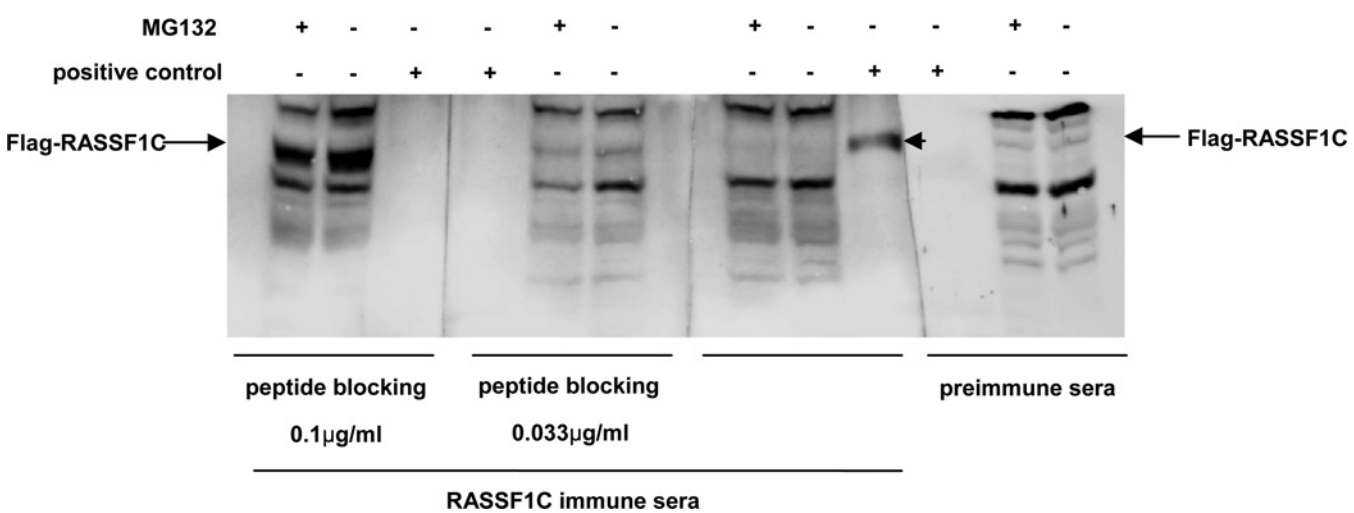

Figure S6 Characterization of the generated anti-RASSF1C antibody

HeLa cells were treated with or without MG132. Cell lysates were analyzed by Western blot with preimmune sera, immune sera or antigen peptide-blocked immune sera of RASSF1C as indicated. The positive control was immunoprecipitated from RASSF1C-transfected HEK-293T cells, and as a control monitoring peptide blocking efficiency and also a molecular mass indicator for endogenous RASSF1C.

Table S1 The peptide sequences of the $SCF^{\beta\text{-}TrCP}$ complex identified by RASSF1C affinity purification

The precipitated proteins from stable HEK-293T cells expressing SBP-FLAG–RASSF1C was digested with trypsin [1]. The supernatant was collected, dried and dissolved in 10 % (v/v) acetonitrile and 0.8 % formic acid solution. The peptides were analysed by LC-MS/MS.

Protein name	Peptide sequence
CUL1	-.IQDGLGELK.-
	R.VQVYLHESTQDELAR.K
	K.FYTQQWEDYR.F
	K.LLIQAAIVR.I
	K.DGEDLMDESVLK.F
	-.SLALVTWR.-
	Y.SLALVTWR.D
	M.SAFNNDAGFVAALDK.A
	K.GQTPGGAQFVGLELYK.R
	K.ESFESQFLADTER.F
	R.ESTEFLQQNPVTEYMK.K
	R.LISGVVQSYVELGLNEDDAFAK.G
	K.NPEEAELEDTLNQVMVVFK.Y
β-TrCP2	R.GWDQYLFK.N
	K.VWDLQAALDPR.A
	-.VWDLQAALDPR.-
	R.DFITALPEQGLDHIAEN.I
	R.VWDVNTGEVLNTL.I
	-.SIAVWDMASATDITLR.-
	R.VWDVNTGEVLNTLIHH.N
	R.VWDVNTGEVLNTLIH.H
	-.DFITALPEQGLDHIAENIL.-
	-.DFITALPEQGLDHIAENILS.-
	-.DFITALPEQGLDHIAENILSY.-
	R.DFITALPEQGLDHIAENILSY.L
β-TrCP1	R.LVVSGSSDNTIR.L
	R.AAVNVVDFDDK.Y
	-.DFITALPAR.-
	R.DFITALPAR.G
	R.AAVNVVDFDDKYIVSASGDR.T
	R.GWGQYLFK.N
	K.IIQDIETIESNWR.C
	-.LWDIECGACLR.-
	K.VWDLVAALDPR.A
SKP1	-.KDDPPPPEDDENKEK.-
	K.NDFTEEEEAQVR.K
	K.LQSSDGEIFEVDVEIAK.Q
	R.TDDIPVWDQEFLK.V
	-.VDQGTLFELIL.-

Table S2 The peptides sequences of Mule identified by RASSF1C affinity purification

The precipitated proteins from stable HEK-293T cells expressing SBP-FLAG–RASSF1C were digested with trypsin [1]. The supernatant was collected, dried and dissolved in 10 % (v/v) acetonitrile and 0.8 % formic acid solution. The peptides were analysed by LC-MS/MS.

Protein name	Peptide sequence
Mule	-.SHHAASTTTAPTPAARSTASA.-
	R.SDGSGESAQPPEDSSPPASSESSSTR.D
	K.GNDTPLALESTNTEK.E
	K.AIQDPAFSDGIR.H
	K.FLGDEQDQITFVTR.A
	R.LLSLISIALPENK.V
	R.ALAELFGLLVK.L
	K.QLAAFLEGFYEIIPK.R
	R.IPIPLMDYILNVMK.F

REFERENCE

1 Rybak, J. N., Ettorre, A., Kaissling, B., Giavazzi, R., Neri, D. and Elia, G. (2005) *In vivo* protein biotinylation for identification of organ-specific antigens accessible from the vasculature. Nat. Meth. **2**, 291–298

Received 18 August 2011/10 September 2011; accepted 12 September 2011
Published as BJ Immediate Publication 12 September 2011, doi:10.1042/BJ20111500