Neoplasia • Vol. 8, No. 12, December 2006, pp. 1042–1054 1042

www.neoplasia.com

SAG/ROC–SCF$^{\beta\text{-TrCP}}$ E3 Ubiquitin Ligase Promotes Pro–Caspase-3 Degradation as a Mechanism of Apoptosis Protection[1]

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Abstract

Skp1–cullin–F-box protein (SCF) is a multicomponent E3 ubiquitin (Ub) ligase that ubiquitinates a number of important biologic molecules such as p27, β-catenin, and IκB for proteasomal degradation, thus regulating cell proliferation and survival. One SCF component, *SAG*/ROC2/Rbx2/Hrt2, a RING finger protein, was first identified as a redox-inducible protein, which, when overexpressed, inhibited apoptosis both *in vitro* and *in vivo*. We report here that sensitive to apoptosis gene (*SAG*), as well as its family member ROC1/Rbx1, bound to the proinactive form of caspase-3 (pro–caspase-3). Binding was likely mediated through F-box protein, β-transducin repeat-containing protein (β-TrCP), which binds to the first 38 amino acids of pro–caspase-3. Importantly, β-TrCP1 expression significantly shortened the protein half-life of pro–caspase-3, whereas expression of a dominant-negative β-TrCP1 mutant with the F-box domain deleted extended it. An *in vitro* ubiquitination assay showed that *SAG*/ROC–SCF$^{\beta\text{-TrCP}}$ promoted ubiquitination of pro–caspase-3. Furthermore, endogenous levels of pro–caspase-3 were decreased by overexpression of *SAG*/ROC–SCF$^{\beta\text{-TrCP}}$ E3 Ub ligases, but increased on siRNA silencing of *SAG*, regulator of cullin-1 (ROC1), or β-TrCPs, leading to increased apoptosis by etoposide and TNF-related apoptosis-inducing ligand through increased activation of caspase-3. Thus, pro–caspase-3 appears to be a substrate of *SAG*/ROC–SCF$^{\beta\text{-TrCP}}$ E3 Ub ligase, which protects cells from apoptosis through increased apoptosis threshold by reducing the basal level of pro–caspase-3. *Neoplasia* (2006) **8,** 1042–1054

Keywords: *SAG*, SCF E3 ligase, apoptosis, caspase-3, protein ubiquitination.

Introduction

Apoptosis is a genetically programmed process of cell death required for maintaining homeostasis under physiological conditions and for responding to various internal and external stimuli [1]. Cells committed to apoptosis are characterized by membrane blebbing, cytoplasmic shrinkage, nuclear chromatin condensation, and DNA fragmentation [2]. Among many molecular players that regulate apoptosis, caspases, a family of cysteine proteases, play a critical and essential role. Caspases exist in cells in an inactive prozymogen form [proinactive form of caspase-3 (pro–caspases)]. Two major pathways in apoptosis-associated caspase activation have been defined in mammalian cells: the extrinsic death receptor pathway, which is triggered by members of the death receptor superfamily, and the intrinsic mitochondrial pathway, which is activated in response to extracellular cues and internal insults, such as DNA damage [3]. Both pathways converge at caspase-3, whose activation, through proteolytic cleavage by other initiator caspases, leads to the cleavage of many cellular protein substrates and to committed apoptotic cell death [4,5]. Thus, caspase-3 is a key effector caspase [4].

It has been recently shown that caspase-3 is subjected to ubiquitination, followed by 26*S* proteasome–mediated degradation. Oxidative stress induced caspase-3 polyubiquitination [6], whereas proteasome inhibitors induced accumulation of caspase-3 subunits [7] and enhanced apoptosis induced by overexpression of pro–caspase-3 [8]. Furthermore, XIAP, a RING finger antiapoptosis protein, was found to bind to rev-caspase-3 [9], an artificially active caspase-3 generated by rearranging small subunits (p12) to precede the large subunit (p20), with capability for autocatalytic processing to active form

Abbreviations: β-TrCP, β-transducin repeat-containing protein; ROC1, regulator of cullin-1; TRAIL, TNF-related apoptosis-inducing ligand; SCF, Skp1–cullin–F-box protein; *SAG*, sensitive to apoptosis gene; Ub, ubiquitin

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[1]This work was supported largely by a grant from the Stuart and Barbara Padnos Endowed Research Fund of the University of Michigan Comprehensive Cancer Center and by National Institutes of Health (NIH) grant R01-CA111554 to Y.S., and partially by NIH/National Cancer Institute (NCI) grants CA93614, CA095441, and CA 079721 to H.L. Jayme R. Gallegos was supported by an NIH/NCI training grant on the molecular basis of skin pathobiology (1-T32-CA106195).
Received 15 August 2006; Revised 28 September 2006; Accepted 10 October 2006.

DOI 10.1593/neo.06568

[10]. After binding, intrinsic ubiquitin (Ub) ligase activity of XIAP promoted the ubiquitination and degradation of rev-caspase-3 and protected cells from apoptosis [9]. However, XIAP did not bind to the native inactive form of caspase-3, pro–caspase-3 [9]. Moreover, cIAP-2, another RING finger antiapoptosis protein with intrinsic E3 ligase activity, promoted *in vitro* monoubiquitination of caspase-3 [11]. Monoubiquitination or diubiquitination of caspase-3 subunits p17 and p12 was seen after proteasome inhibitor treatment of transfected cells [7]. Thus, it appears that caspase-3 is targeted by the Ub pathway for degradation. However, it is still largely unknown how the stability of native inactive form of caspase-3 (pro–caspase-3) is regulated and by which E3 Ub ligase.

Ubiquitination of a target protein involves a well-defined multistep reaction catalyzed by a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub ligase (E3). Skp1–cullin–F-box protein (SCF) complexes are the largest family of E3 Ub ligases that ubiquitinate a variety of regulatory proteins for 26*S* proteasome degradation. SCF complexes consist of Skp1, cullin/Cdc53, an F-box protein, and a RING finger protein *SAG*/ROC/Rbx/Hrt [12,13]. A recent crystal structure of the SCF complex revealed that cullin-1 acts as a scaffold protein, which, at its N-terminus, binds to the Skp1–F-box complex and, at its C-terminus, binds to ROC/Rbx that recruits E2 [14]. The substrate specificity of the SCF complex is determined by F proteins that bind to Skp1 and cullins through its F-box domain and to substrates through its WD or LRR domain [14,15]. The large number of F-box proteins found in the human genome [16] suggests that SCF E3 Ub ligases would play a major role in the regulation of a variety of biologic processes.

We have previously cloned and characterized a redox-inducible gene, sensitive to apoptosis gene (*SAG*) [17]. *SAG* was found to be essential in yeast growth [18], to promote cell growth under serum-starved conditions [19,20], and to inhibit reactive oxygen species (ROS)–induced apoptosis both in *in vitro* cell culture models [17] and in *in vivo* mouse brain and rat cardiomyocyte ischemia/reperfusion models [21,22]. The antiapoptotic function of *SAG* was mediated, at least in part, through its antioxidant activity by metal ion binding and free radical scavenging, which inhibited ROS-induced cytochrome *c* release and caspase activation [17,23]. Because *SAG* is the second family member of ROC1/Rbx1, which activates SCF E3 Ub ligase activity through neddylation of cullins and recruitment of E2 [24], we sought to explore a ligase-associated mechanism of *SAG* apoptosis inhibition. We report here that *SAG* and its family member ROC1 which also showed antiapoptotic activity [13], bound to pro–caspase-3 most likely through the F-box protein β-transducin repeat-containing protein (β-TrCP). *SAG*/ROC1–$SCF^{\beta\text{-TrCP}}$ E3 ligase promoted ubiquitination of pro–caspase-3 *in vitro* and induced degradation of either exogenously expressed pro–caspase-3 or endogenous pro–caspase-3. However, siRNA silencing of *SAG*, ROC1, or β-TrCP increased the endogenous levels of pro–caspase-3 and sensitized cancer cells to anticancer agents, etoposide, or TNF-related apoptosis-inducing ligand (TRAIL) through caspase-3 activation.

Materials and Methods

Cell Culture, DNA Transfection, and Drug Treatment

The human kidney cell line 293, the breast carcinoma line MCF7, and the cervical carcinoma line HeLa were grown in DMEM supplemented with 10% fetal bovine serum. Cells were subcultured into 100-mm dishes 16 to 24 hours before transfection to reach 90% confluency. DNA transfection was conducted using Lipofectamine 2000 reagent (InVitrogen, Carlsbad, CA) according to the manufacturer's instructions. For drug treatment, after 24 to 36 hours of transfection, cells were exposed to a proteasome inhibitor MG132 (10 μM) for 16 hours, to etoposide (25 μM) for 24 hours, or to TRAIL (50 ng/ml) for 8 hours. Cell viability was determined by trypan blue exclusion assay. For half-life determination, MCF7 cells were cotransfected with pro–caspase-3 and β-TrCP1 or β-TrCP2 or their F-box–deleted mutants, along with pcDNA3 vector control. Twenty-four hours posttransfection, cells were treated with cycloheximide (CHX; 10 μg/ml) and harvested at various time points, then subjected to Western blot analysis.

Western Blot Analysis and Immunoprecipitation (IP)

Cell pellets were lysed in a Triton lysis buffer (20 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton, 5 mM EGTA, and 5 mM EDTA) with a freshly added protease inhibitor tablet for 1 hour on ice, followed by a 30-minute centrifugation. Supernatants were measured for protein concentration using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) and subjected to Western blot analysis as described [17] using antibodies to FLAG tag, HA tag, β-actin (Sigma, St. Louis, MO), caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA), and Myc tag (Bethyl Laboratories, Montgomery, TX). For IP, cell lysates were precleaned with protein A/G beads, then immunoprecipitated with bead-conjugated goat anti–caspase-3 antibody (Santa Cruz Biotechnology), anti-FLAG antibody, or anti-HA antibody, with preimmuno IgG as negative control. Immunoprecipitates were washed with Triton lysis buffer three to four times and subjected to Western blot analysis.

Construction of Pro–Caspase-3 Mutants

The Myc tag pro–caspase-3 and its four deletion mutants were derived by polymerase chain reaction (PCR) using wild-type pro–caspase-3 as template [25]. Primer sequences for wild type are Casp3-05 (5′-GGATCCGCCACCATGGAGAACACTGAAAAC-3′) and Casp3-06 (5′-GGTACCGTGATAAAAATAGAGTTC-3′); for *mutant-1* (*MT1*; p20, subunit, AA 1–175) are Casp3-05 and Casp3-08 (5′-GGTACCGTCTGTCTCAATGCCACAG-3′); for *mutant-2* (*MT2*; p12, small subunit, AA 175–277) are Casp3-07 (5′-GGATCCGCCACCATGAGTGGTGTTGATGATGAC-3′) and Casp3-06; for *mutant-3* (*MT3*; AA 39–277) are Casp3-09 (5′-GGATCCGCCACCATGGATTATCCTGAGATGGGTTTATG-3′) and Casp3-06; and for *mutant-4* (*MT4*; dNdC, AA 39–175) are Casp3-09 and Casp3-08. PCR products were first subcloned into TA vector, then into pcDNA3.1/Myc-His vector, followed by sequencing confirmation. The resulting constructs would express pro–caspase-3, or its deletion mutants with Myc epitope and His tag at the C-terminus.

Preparation of ROC1–SCF$^{\beta\text{-TRCP1}}$ Complex

Hi-5 cells were maintained in HfQ SFX insect cell medium (Perbio, Belgium) with 1× penicillin–streptomycin (Cellgro, Herndon, VA) at 25°C. Baculoviral expression constructs of ROC-1, HA-Cul1, HIS-Skp1, and FLAG-β-TRCP1 were titered for optimal expression in Hi-5 cells. Hi-5 cells were grown to approximately 50% confluence on 100-mm plates before infection. The constructs were infected separately for maximum expression. After 4 days, the cells were then harvested and centrifuged at 3000 rpm for 10 minutes at 4°C. The medium was removed, and the cells were washed once in 20 ml of PBS and centrifuged a second time. The pellets were lysed by vigorous pipetting and repetitive freeze–thaw in nickel lysis buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, and 10 mM imidazole) with phenylmethylsulfonyl fluoride and pepstatin A. Lysates were spun at high speed for 10 minutes to remove debris, and the supernatants from each construct were combined. The combined lysis was rotated at 4°C for 2 hours, and then 1 ml of clean 50% Ni-NTA agarose bead slurry (Qiagen, Valencia, CA) was added. The bead was rotated for an additional 2 hours. The lysate was then centrifuged at 6000 rpm for 3 minutes, and the supernatant was removed. Bead pellet was resuspended in Ni wash buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, and 20 mM imidazole) and loaded onto an elution column where it was washed with 1 ml of Ni wash buffer for six times. Proteins were then eluted in 200 μl of a Ni elution buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, and 250 mM imidazole) for six fractions. The fractions were pooled into two groups according to concentration and dialyzed in 4 l of Buffer C-100 (20 mM Tris–Cl pH 7.5, 100 mM KCl, 0.2 mM EDTA, and 20% glycerol) for at least 2 hours. The fractions were run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and checked by Western blot analysis using polyclonal anti-ROC1 (Neomarkers, Fremont, CA), monoclonal 12CA5 anti-HA (generated in Lu Laboratory), monoclonal anti-FLAG (Sigma), and monoclonal anti-HIS (Covance, Princeton, NJ) antibodies to detect separate complex components.

Preparation of SAG–*SCF$^{\beta\text{-TRCP1}}$ Complex*

The *SAG*–SCF$^{\beta\text{-TRCP1}}$ complex was prepared as above, except for the *SAG*, which was purified separately before complex reconstitution. GST-*SAG* was grown in LB broth to log phase and induced using 0.4 mM IPTG (Fisher, Chino, CA). Bacteria were harvested after 6 hours at 37°C, centrifuged at 35,000 for 20 minutes, and lysed in 20 ml of bacterial lysis buffer (1× PBS, 10% glycerol, and 0.1% NP-40) by French press. The lysate was spun at high speed for 10 minutes to remove cell debris and then incubated for 5 minutes at room temperature with 500 μl of GSH slurry. After binding, GST-*SAG* beads were washed thrice in GST wash buffer (1× PBS, 10% glycerol, and 500 mM NaCl) and eluted using 5 mM reduced glutathione (Sigma). The fractions were dialyzed using the same conditions as above, and the protein was examined for concentration and purity using SDS-PAGE followed by Coomassie staining. The fraction with the highest purity was combined with insect lysates obtained by the same procedure as above. After the steps noted above, purified complex fractions were rebound to GSH beads, washed twice in lysis buffer (50 mM Tris pH 7.5, 5 mM EDTA, 150 mM NaCl, and 0.5% NP-40), and diluted to 50% slurry. *In vitro* ubiquitination was preformed on the same day to maintain complex activity.

Expression and Purification of FLAG-Tagged Pro–Caspase-3

Pro–caspase-3 substrate was prepared from an IPTG-inducible bacterial construct following a similar induction and preparation protocol as noted above for GST-*SAG*. After French press and centrifugation, 40 μl of 50% monoclonal FLAG M2-conjugated affinity agarose bead slurry (Sigma) was added to the clarified lysate and incubated at 4°C for 4 hours. The beads were washed twice in 1 ml of lysis buffer (50 mM Tris–Cl pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.5% NP-40) and twice in 1 ml of SNNTE lysis buffer (50 mM Tris–Cl pH 7.4, 500 mM NaCl, 5 mM EDTA, 5% sucrose, and 1% NP-40). They were then eluted using 6×30-μl fractions of 0.1 μg/μl FLAG peptide. Their concentration was then determined by pro–caspase-3 Western blot analysis, and samples of similar concentration were dialyzed in 4 l of Buffer C-100 for 2 hours at 4°C. Five microliters of these final fractions was used per sample for the assays.

In Vitro *Ubiquitination*

SAG/ROC1–SCF$^{\beta\text{-TRCP1}}$–mediated *in vitro* ubiquitination assay was performed as described previously [26]. Pro–caspase-3 *in vitro* ubiquitination assay was carried out in 40 μl of a reaction mixture containing 50 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 2 mM NaF, 3 μM okadaic acid, 400 ng of Uba-1 (Boston Biochem, Cambridge, MA), 400 ng of UbcH3 (Boston Biochem), 1000 ng of HA Ub, 5 mM ATP (Amersham Biosciences, Piscataway, NJ), 1.5 mM ATPγ (Fisher ICN, Chino, CA), and 20 μl of purified ROC1–SCF$^{\beta\text{-TRCP1}}$ complex or 8 μl of *SAG*–SCF$^{\beta\text{-TRCP1}}$ bead slurry. Five microliters of purified FLAG-tagged pro–caspase-3 was used in each reaction. The mixture was incubated at 37°C for 1.5 hours and analyzed afterward by SDS-PAGE followed by Western blot analysis. Ubiquitinated pro–caspase-3 was detected by immunoblot using monoclonal anti/pro–caspase-3 antibody (BD Transduction Laboratory, San Jose, CA).

siRNA Silencing of SAG*, ROC1, or β-TrCP*

Plasmid-based hairpin RNA were used to silence *SAG*, ROC1, or β-TrCPs. The pU6pro vector (a kind gift from Dr. David L. Turner, University of Michigan, Ann Arbor, MI) was used [27] as detailed previously (http://sitemaker.umich.edu/dlturner.vectors). Two sets of siRNA template oligonucleotides were synthesized (InVitrogen) to silence *SAG*. For psiSAG-1, the sequences are iSAG-1-1 (5′-*TTTG*AAGGCTTCTTAGCGCAGTTGTTCAGAGCCAAGAGGCTCTGAACAACTGCGCTAAGAAGCCTTTTTT-3′) and iSAG-1-2 (5′-*CTAG*AAAAAGGCTTCTTAGCGCAGTTGTTCAAGAGCCTCTTGGCTCTGAACAACTGCGCTAAGAAGCCTT-3′). For psi *SAG*-2, the sequences are iSAG-2-1

(5′-*TTTG*AACAAGAGGACTGTGTTGTGGTCTGGCAAAGAGCCAGACCACAACACAGTCCTCTTGTTTTT-3′) and iSAG-2-2 (5′-*CTAG*AAAAACAAGAGGACTGTGTTGTGGTCTGGCTCTTGCCAGACCACAACACAGTCCTCTTGTT-3′). For psiROC1, the sequences are iROC1-1 (5′-*TTTG*AAGACTTCTTCCATCAAGCTTCAAGAAGAAGCTTGATGGAAGAAGTCTTTTT-3′) and iROC1-2 (5′-*CTAG*AAAAAGACTTCTTCCATCAAGCTTCTCTTTGAAGCTTGATGGAAGAAGTCTT-3′). For psi-β-TrCPs (silencing both β-TrCP1 and β-TrCP2), the sequences are iβ-TrCP01 (5′-*TTTG*AAGTGGAATTTGTGGAACATCTTTCAAGAGAAGATGTTCCACAAATTCCACTTTTT-3′) and iβ-TrCP02 (5′-*CTAG*AAAAAGTGGAATTTGTGGAAACATCTTCTCTTGAAGATGTTCCACAAATTCCACTT-3′). The control psiCont sequences are iCont01 (5′-*TTTG*ATTGTATGCGATCGCAGACTTCAAGAGAAGTCTGCGATCGCATACAATTTTT-3′) and iCont02 (5′-*CTAG*AAAAATTGTATGCGATCGCAGACTTCTCTTTGAAGTCTGCGATCGCATACAAT-3′). The cohesive ends of restriction enzymes *Bbs*I (TTTG) and *Xba*I (CTAG) are italicized. Annealed synthetic primers were ligated into the pU6pro vector predigested with *Bbs*I and *Xba*I. Recombinant plasmids of psiSAG-1, psiSAG-2, psi-β-TrCP, and psiCont were confirmed by DNA sequencing. The plasmids were transfected into HeLa cells using Lipofectamine 2000 reagent (InVitrogen), and silencing of endogenous *SAG*, ROC1, or β-TrCP1 was determined using antibodies against *SAG*, ROC1, or β-TrCP1 (Zymed, Carlsbad, CA), respectively.

Caspase-3 Activity Assay

Caspase-3 activity was measured by a fluorogenic caspase-3 assay using Ac-DEVD-AFC (Biomol, Plymouth meeting, PA) as substrate, as described previously [28]. Briefly, HeLa cells were seeded in 24-well plate 1 day before Lipofectamine transfection of siRNA plasmids targeting *SAG* and ROC1, along with a control plasmid with random sequence. Thirty-six hours posttransfection, cells were treated with either etoposide (25 μM) for 24 hours or with TRAIL (50 ng/ml) for 8 hours. Cells were then lysed in a lysis buffer (1.67 mM Hepes pH 7.4, 7 mM KCl, 0.83 mM $MgCl_2$, 0.11 mM EGTA, 0.11 mM EDTA, and 0.57% CHAPS) for 30 minutes, followed by centrifugation at 14,000 rpm for 15 minutes. Supernatants were collected and incubated with a caspase-3 activity assay buffer (120 mM Hepes pH 7.4, 630 mM sucrose, 0.25% CHAPS, 10 mM DTT, and 50 μM Ac-DEVD-AFC fluorescent substrate). The plates were read 2 hours after the addition of Ac-DEVD-AFC substrate at 400 nm (excitation) and 505 nm (emission) in a TECAN Ultra fluorescence reader (TECAN, Rancho Santa, CA).

Results

SAG *or ROC1 Bound to Endogenous Pro–Caspase-3*

Although it has been known that the active form of caspase-3 is ubiquitinated by XIAP E3 Ub ligase and that inactive pro–caspase-3 appears to be degraded through the ubiquitination and 26*S* proteasome pathway [6–9], it is completely unknown which E3 Ub ligase mediates the ubiquitination of the inactive prozymogen form of caspase-3 (pro–caspase-3). In an attempt to elucidate the E3 Ub ligase–associated mechanism by which *SAG* protects cells from apoptosis [17,21,22,29], we sought to determine whether pro–caspase-3 (32 kDa) is a substrate of *SAG*–SCF E3 Ub ligase. We first measured whether *SAG* physically bound to endogenous pro–caspase-3. FLAG-tagged *SAG* was transiently transfected into 293 cells. Endogenous pro–caspase-3 was immunoprecipitated with goat anti–caspase-3 antibody or with goat IgG as control, followed by Western blot analysis using anti-FLAG antibody to determine whether transfected *SAG* was coprecipitated with endogenous pro–caspase-3. Indeed, as shown in Figure 1*A*, FLAG-tagged *SAG* (15 kDa) was detected in *SAG*-transfected—but not control vector–transfected—cells in immunoprecipitates by pro–caspase-3 antibody, but not by control IgG. We next determined whether the *SAG* family member ROC1 would also bind to endogenous pro–caspase-3. HA-tagged ROC1 was transiently transfected into 293 cells. Again, endogenous pro–caspase-3 was immunoprecipitated by anti–caspase-3 antibody or control IgG, followed by Western blot analysis using anti-HA antibody to detect ROC1, if two proteins bind together. As shown in Figure 1*B*, ROC1 can be detected in immunoprecipitates pulled down by anti–caspase-3 antibody, but not by control IgG. Expression of both *SAG* and ROC1 was detected by direct Western blot analysis shown at the *bottom panels* (Figure 1, *A* and *B*). Thus, it appears that both *SAG* and its family member ROC1 bound to pro–caspase-3 *in vivo*.

F-box Protein β-TrCP1 or β-TrCP2 Bound to Pro–Caspase-3 at Its N-terminus

Based on the published crystal structure of the SCF complex [14], the binding of *SAG*/ROC to protein substrates such as pro–caspase-3 has to be mediated by an F-box protein. We therefore determined which F-box protein would mediate *SAG*/pro–caspase-3 binding. Because, like *SAG*, β-TrCP was also involved in the survival of cancer cells [30], it is a putative candidate. Before confirming β-TrCP/pro–caspase-3 binding, we first determined whether *SAG* bound to β-TrCPs. It has been well-established that ROC1 is included in the $SCF^{\beta\text{-}TrCP}$ complex [31,32], and we know that *SAG* bound to cullin-1 [18], which acts as a scaffold protein to bridge *SAG* and Skp1–F-box protein [14]. HA-tagged β-TrCP2 was cotransfected with FLAG-tagged *SAG* into 293 cells. *SAG* was pulled down with anti-FLAG antibody, along with preimmune IgG control, and β-TrCP2 was detected by IB using anti-HA antibody in immunoprecipitates, pulled down by FLAG (*SAG*), but not by control IgG (Figure 1*C*), indicating *in vivo SAG*–β-TrCP2 binding. Similarly, HA-tagged *SAG* was cotransfected into 293 cells with FLAG-tagged β-TrCP1, and *SAG* was detected in β-TrCP immunoprecipitates by anti-FLAG antibody but not in control IgG immunoprecipitates, (Figure 1*D*), again indicating *in vivo* binding of *SAG*–β-TrCP1. Expressions of cotransfected *SAG*, ROC, or β-TrCPs were shown at the two *bottom panels* by direct Western blot analysis.

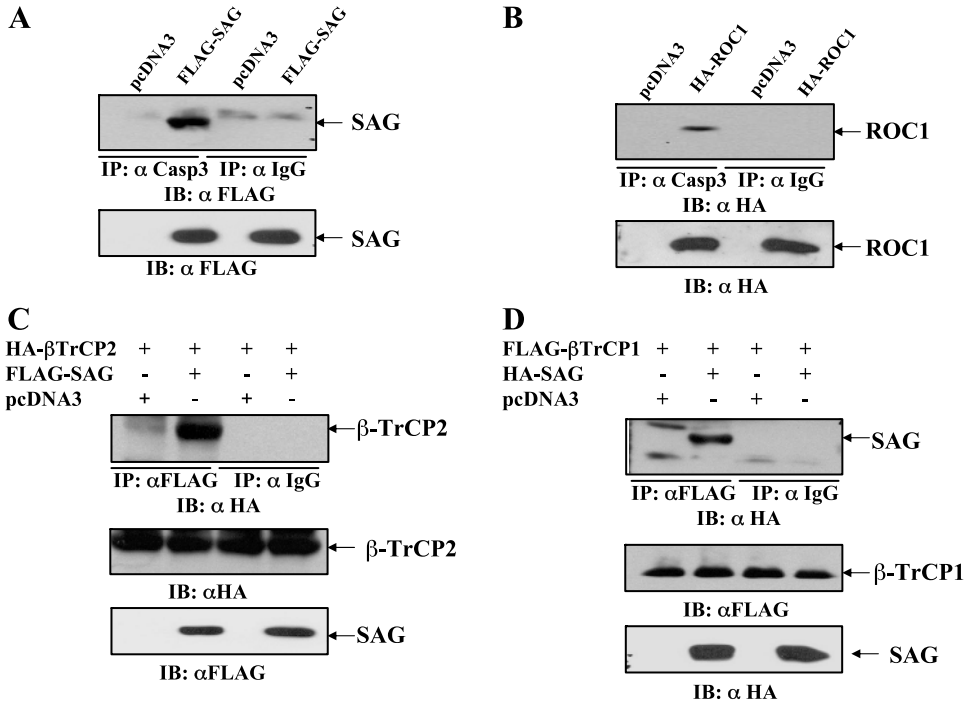

Figure 1. SAG *or ROC1 bound to endogenous pro–caspase-3 in 293 cells. Human 293 cells were transiently transfected with pcDNA3 vector control, FLAG-tagged* SAG *(A), or HA-tagged ROC1 (B), using Lipofectamine 2000. Cell lysates were prepared after transfection and subjected to IP with anti–caspase-3 antibody or control IgG, as indicated after preclearing with protein A/G-plus agarose beads, followed by immunoblotting using anti-FLAG antibody or anti-HA antibody, respectively. The bottom panel shows direct immunoblotting using whole-cell extract with anti-FLAG or anti-HA antibody, respectively, to show that transfected* SAG *or ROC1 was expressed in 293 cells. (C)* SAG–β*-TrCP2 binding. Human 293 cells were transiently cotransfected with HA-*β*-TrCP2 and FLAG-*SAG *or pcDNA3 vector control. After transfection, cell lysates were prepared and subjected to IP using anti-FLAG antibody or control IgG, as indicated after preclearing with protein A/G-plus agarose beads, followed by immunoblotting with anti-HA antibody (top). Expression of transfected* β*-TrCP2 (middle) and* SAG *(bottom) was detected by direct immunoblotting of whole-cell extract. (D)* SAG–β*-TrCP1 binding. Human 293 cells were cotransfected with FLAG-*β*-TrCP1 and HA-*SAG *or pcDNA3 vector control. Cell lysates were prepared and subjected to IP using anti-FLAG antibody or control IgG, as indicated after preclearing with protein A/G-plus agarose beads, followed by immunoblotting using anti-HA antibody (top). Expression of transfected* β*-TrCP1 (middle) and* SAG *(bottom) was detected by direct immunoblotting of whole-cell extract.*

Having confirmed *SAG*–Cul1–β-TrCP binding, we determined whether β-TrCP1 or β-TrCP2 directly bound to pro–caspase-3. HA-tagged β-TrCP2 was transfected into 293 cells and was detected in pro–caspase-3 immunoprecipitates, pulled down by anti–caspase-3 antibody, but not by preimmune IgG (Figure 2*A*). The *bottom panel* showed that HA-β-TrCP2 was expressed at an equal level in both transfections. Similarly, FLAG-tagged β-TrCP1 or its F-box deletion mutant β-*TrCP1ΔF* was transfected into 293 cells and was again detected in immunoprecipitates, pulled down by anti–caspase-3 antibody (Figure 2*B*). Expressions of β-TrCP1 and β-*TrCP1ΔF* were shown at the *bottom panel*. All these experiments indicated that, indeed, β-TrCP1 or β-TrCP2 bound to pro–caspase-3 *in vivo* and that the binding of *SAG*/pro–caspase-3 is most likely mediated through F-box protein β-TrCP1 or β-TrCP2.

It has been well established that β-TrCP binds to its substrate through its WD40 repeats at the C-terminus [33–35]. We therefore mapped the region in caspase-3 to which β-TrCP binds. Wild-type pro–caspase-3 and its four deletion mutants with C-terminal Myc tag were derived (Figure 2*C*): *MT1*, p20, the large subunit (AA 1–175); *MT2*, p12, the small unit (AA 176–277); *MT3*, dN (AA 39–277); and *MT4*, dNdC (AA 39–175). All four mutants, along with wild-type pro–caspase-3 (WT), were transiently transfected into 293 cells, and their expressions were detected by Western blot analysis with Myc-tag antibody. The levels of expression from high to low were WT, *MT1*, and *MT4*, in that order. No expression of *MT2* and *MT3* was detectable (data not shown). We therefore focused our attention on the potential binding of β-TrCP to *MT1* and *MT4*. The 293 cells were transiently cotransfected with HA-β-TrCP2 in a combination of WT, *MT1*, or *MT4*, respectively, and then subjected to direct Western blot analysis. As shown in Figure 2*D*, HA-β-TrCP2 was expressed equally well among all four transfections (80 μg of cellular protein was loaded from each transfection). However, the expression varied among WT and its mutants. As shown in Figure 2*E*, WT was expressed at the highest level (20 μg of cellular protein loaded), followed by p20 (60 μg of protein loaded) and dNdC (100 μg of protein loaded). Based on these different levels of expression, the amount of total cellular protein used for IP was 200 μg for WT, 600 μg for p20/*MT1* or pcDNA3 vector control, and 2.5 mg for dNdC/*MT4*. Following IP with HA antibody, along with preimmune IgG as negative control, potential β-TrCP2 binding to pro–caspase-3 was detected by Western blot analysis using anti-Myc-tag antibody. As shown in Figure 2*F*, a strong WT band was visualized when anti-HA antibody (*lane 2*), but not preimmuno-IgG (*lane 3*), was used for IP, indicating that WT strongly binds to β-TrCP2, consistent with the data shown in Figure 2*A*. A relatively weak

binding of p20/*MT1* to β-TrCP2 was detected (*lane 4*), consistent with its relatively low expression in comparison with WT. No β-TrCP2 binding was detected with pcDNA3-Myc vector control (*lane 1*), nor with dNdC mutant, even though 4-fold to 12-fold more input cellular proteins were used for IP to compensate for a lower expression (*lane 5*). The results suggested that the p12 subunit at the C-terminus is dispensable for β-TrCP2 binding, whereas the first 38 amino acids at the N-terminus are required for β-TrCP2 binding.

β-TrCP1 or β-TrCP2 Expression Shortened the Protein Half-Life of Pro–Caspase-3

We next determined whether β-TrCPs/pro–caspase-3 binding would lead to degradation of pro–caspase-3. For simplicity, we used MCF7 cells, a human breast cancer cell line that lacks endogenous pro–caspase-3 as a result of a 47-bp deletion in the exon 3 of the gene [36,37]. MCF7 cells were cotransfected with pro–caspase-3 and an increasing amount of β-TrCP1, along with the control plasmid pcDNA3. Cell lysates were prepared 38 hours posttransfection and subjected to Western blot analysis using anti–caspase-3 antibody. As shown in Figure 3*A*, pro–caspase-3 level decreased as the amount of β-TrCP1 increased, indicating a dose-dependent degradation of pro–caspase-3 by β-TrCP1.

Having established this, we next determined the potential shortening of the protein half-life of pro–caspase-3 by β-TrCPs. MCF7 cells were cotransfected with pro–caspase-3, along with β-TrCP1, its dominant-negative form *β-TrCP1ΔF*, or the empty vector control pcDNA3. Following 24 hours of transfection, cells were treated with CHX to block new protein synthesis. Samples were collected at various time points after CHX treatment and subjected to Western

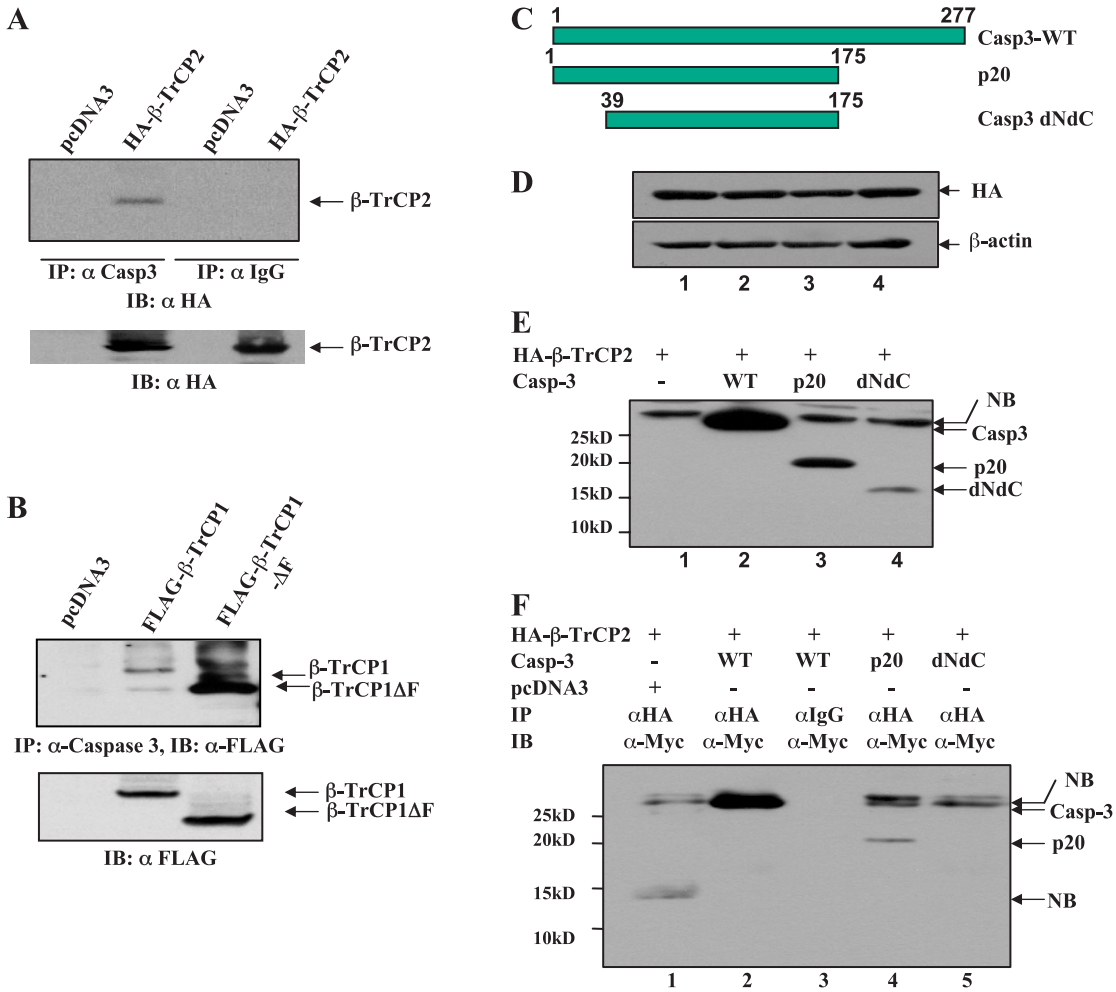

Figure 2. *β-TrCPs bound to pro–caspase-3 require prodomain. (A) β-TrCP2 bound to endogenous pro–caspase-3. Human 293 cells were transiently transfected with HA-β-TrCP2 or pcDNA3 vector control. Cell lysates were prepared and subjected to IP using anti–caspase-3 antibody or preimmune IgG, as indicated after preclearing with protein A/G-plus agarose beads, followed by immunoblotting using anti-HA antibody (top panel). A direct immunoblotting analysis of whole-cell extract was performed using anti-HA antibody to show β-TrCP2 expression (bottom). (B) β-TrCP1 bound to endogenous pro–caspase-3. Human 293 cells were transiently transfected with pcDNA3 vector control (lane 1), FLAG-β-TrCP1 (lane 2), and FLAG-β-TrCP1ΔF (lane 3). Cell lysates were prepared and subjected to IP using anti–caspase-3 antibody followed by Western blot analysis with anti-FLAG antibody (top), or whole-cell extracts were directly subjected to Western blot analysis to show expression of FLAG-β-TrCP1 and FLAG-β-TrCP1ΔF (bottom). Requirement of prodomain for β-TrCP-pro–caspase-3 binding. (C) Bar graphic presentation of pro–caspase-3 mutants. (D–F) Human 293 cells were cotransiently transfected with HA-β-TrCP2 in combination with WT pro–caspase-3 or its deletion mutants, along with the vector control. Thirty-eight hours posttransfection, cells were harvested and subjected to Western blot analysis (D and E) using indicated antibodies or IP, followed by Western blot analysis using myc antibody (F). NB, nonspecific band.*

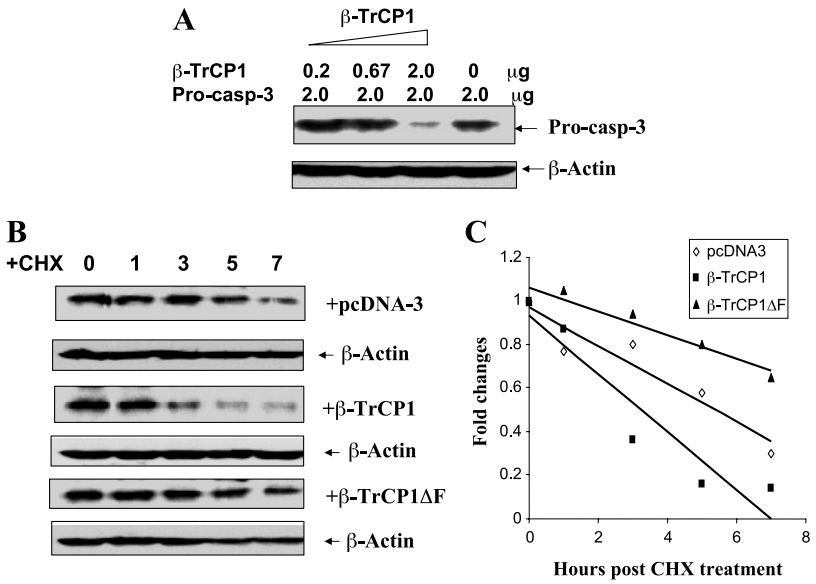

Figure 3. *β-TrCPs shortened the protein half-life of pro–caspase-3. (A) β-TrCP1 induced a dose-dependent reduction of pro–caspase-3. Pro–caspase-3–null MCF7 cells were cotransfected with FLAG-pro–caspase-3 and an increasing amount of β-TrCP1. Thirty-eight hours posttransfection, cell lysates were prepared and subjected to direct Western blot analysis using anti–caspase-3 antibody, as well as anti-actin antibody as loading control. (B and C) β-TrCP1 shortened—but its dominant-negative mutant extended—the pro–caspase-3 half-life. MCF7 cells were transiently cotransfected with FLAG-pro–caspase-3, along with pcDNA3 vector control, β-TrCP1, or* β-TrCP1ΔF*, as indicated. Twenty-four hours posttransfection, cells were treated with CHX (10 μg/ml) for indicated time periods up to 7 hours and harvested. Cell lysates were prepared and subjected to Western blot analysis with anti-FLAG antibody or anti–β-actin antibody as loading control. Densitometric quantification of pro–caspase-3 levels was shown in (C), with value at 0 hour after CHX treatment arbitrarily set to 1, on which the fold change was calculated.*

blot analysis to determine the pro–caspase-3 half-life. As shown in Figure 3*B*, with densitometric qualification data presented in Figure 3*C*, the protein half-life for transfected pro–caspase-3 in MCF7 cells is about 5.5 hours. Cotransfection with β-TrCP1 shortened the half-life to 3 hours, whereas cotransfection with β-*TrCP1ΔF* extended the half-life to > 7 hours. Similar results were observed when β-TrCP2 or β-TrCP2ΔF was cotransfected with pro–caspase-3 (data not shown). The results suggested that β-TrCP1 or β-TrCP2 bound to pro–caspase-3 and shortened its half-life likely by promoting its degradation. In contrast, β-*TrCP1ΔF* or β-TrCP2ΔF that bound to pro–caspase-3 as well (Figure 2*B*), but not to other components of SCF ligase [38], acted in a dominant-negative manner to block pro–caspase-3 degradation, thus extending its half-life.

SAG/*ROC–SCF*$^{\beta\text{-}TrCP}$ *E3 Ligase Promoted Pro–Caspase-3 Ubiquitination* In Vitro

We next established an *in vitro* ubiquitination assay to determine whether *SAG*/ROC–SCF E3 Ub ligase would promote ubiquitination of pro–caspase-3. We first purified ROC1–SCF or *SAG*–SCF complexes containing HA-Cul1, His-Skp1, and FLAG-β-TrCP1, plus either ROC1 or GST-*SAG*, as described in Materials and Methods section. These complexes were used as E3 in an *in vitro* ubiquitination reaction with purified FLAG-pro–caspase-3 as substrate. As shown in Figure 4*A*, several slow-migrating bands ranging from 54 to 100 kDa, reflecting ubiquitinated pro–caspase-3, were detected only when the reaction mixture contained Ub, E1, E2, ROC1–SCF, and pro–caspase-3 (*lane 4*). Omission of E1, E2, E3, and Ub (*lane 1*); E3 and Ub (*lane 2*); E1 and E2 (*lane 3*); or pro–caspase-3 (*lane 5*) all failed to detect it. Likewise, *SAG*–SCF E3 Ub ligase also induced ubiquitination of pro–caspase-3 in the presence of Ub, E1, and E2 (*left lane*), which is not detected in the absence of pro–caspase-3 (*right lane*), which serves as negative control. It is worth noting that pro–caspase-3 ubiquitination by *SAG*–SCF was not as pronounced as that by ROC1–SCF. This may reflect a stoichiometric difference during purification because ROC1–SCF was expressed and copurified from baculovirus as a complex, whereas in the *SAG*–SCF complex, GST-*SAG* was prepared from bacterial source and mixed with other SCF components prepared from baculovirus. Nevertheless, the results indicated that, indeed, pro–caspase-3 could be ubiquitinated by *SAG*/ROC–SCF E3 Ub ligase using components purified *in vitro*.

SAG–*SCF*$^{\beta\text{-}TrCP}$ *E3 Ub Ligase Regulated the Endogenous Level of Pro–Caspase-3*

We have now shown that β-TrCP1 cotransfection shortened the protein half-life of pro–caspase-3 and that *SAG*–SCF$^{\beta\text{-}TrCP}$ E3 ubiquitin ligase promoted the ubiquitination of pro–caspase-3 *in vitro*. We next determined whether *SAG*/ROC–SCF$^{\beta\text{-}TrCP}$ E3 ubquitin ligase can decrease the steady-state levels of endogenous pro–caspase-3. The 293 cells were transiently cotransfected with plasmid expressing Ub, in combination with plasmids expressing pcDNA3 control, β-TrCP1, *SAG*, ROC1, *SAG* + β-TrCP1 + Cul1 + Skp1, or ROC1 + β-TrCP1 + Cul1 + Skp1, respectively. Cells were lysated after transfection and subjected to direct Western blot analysis using anti–caspase-3 antibody. As shown in Figure 5*A* (*top panel*), compared to the vector control (*lane 1*),

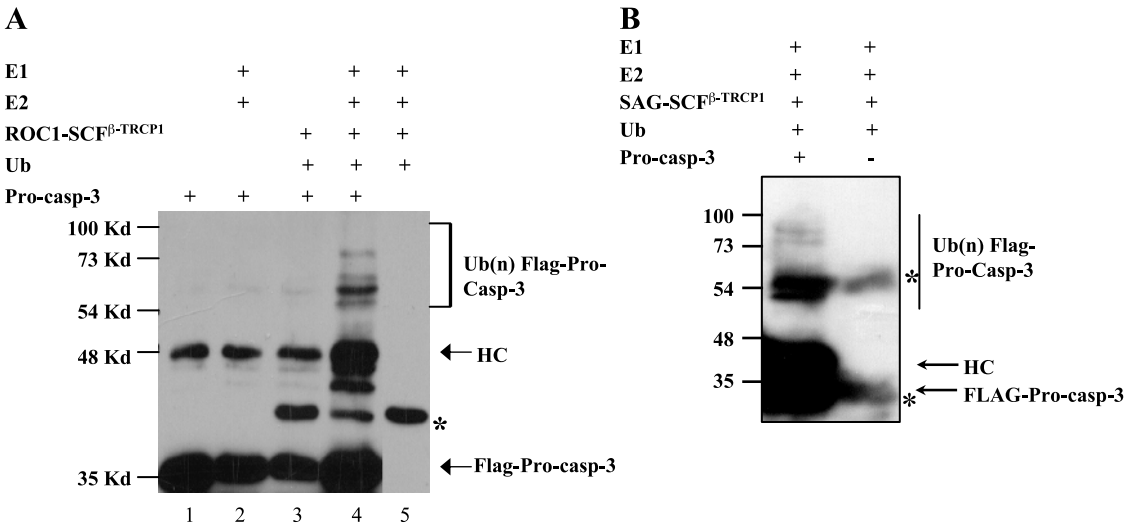

Figure 4. *Pro–caspase-3 ubiquitination by* SAG*/ROC–SCF E3 Ub ligase. (A) ROC1–SCF$^{\beta\text{-}TrCP}$ E3 ligase. Bacterially expressed and purified FLAG-tagged pro–caspase-3 was combined with Ub, Uba1 (E1), UbcH3 (E2), and E3, ROC1–SCF$^{\beta TrCP1}$, in which each component was individually expressed in baculovirus and copurified as a four-component complex, as described in Materials and Methods section. Omission of some components was carried out as indicated. Samples were incubated in the presence of ATP buffer for 1.5 hours at 37°C, run on a 10% to a 15% gradient SDS-PAGE, and blotted with anti-pro–caspase-3 antibody. *Nonspecific band. HC indicates the heavy chain of IgG. (B)* SAG*–SCF$^{\beta\text{-}TrCP}$. Bacterially purified FLAG-tagged pro–caspase-3 and GST-*SAG *were combined with Ub, Uba1 (E1), Ubc3 (E2), and SCF$^{\beta TrCP1}$, in which each component was individually expressed in baculovirus and copurified as a three-component complex, as described in Materials and Methods section. Omission of pro–caspase-3 on the right lane was indicated. Samples were incubated in the presence of ATP buffer for 1.5 hours at 37°C, run on a 10% SDS-PAGE, and blotted with anti/pro–caspase-3 antibody. HC, heavy chain from FLAG affinity purification. *Nonspecific signals.*

transfection with β-TrCP1 (*lane 2*), *SAG* (*lane 3*), and ROC1 (*lane 4*) alone did not significantly change the endogenous level of pro–caspase-3. However, when *SAG* or ROC1 was cotransfected with other components of SCF (*lanes 5* and *6*), endogenous pro–caspase-3 was significantly reduced to a nearly undetectable level, indicating a requirement of all SCF components in promoting the degradation of endogenous pro–caspase-3 in 293 cells. The lack of detection of caspase-3 subunits p20 and p12 (*lanes 5* and *6*) excluded the possibility that the decrease of pro–caspase-3 level is due to *SAG*/ROC–SCF–induced caspase-3 activation. Because β-TrCP1 is significantly stabilized in the presence of other SCF components (compare *lane 2 vs lanes 5* and *6*, *middle panel*), it raised a possibility that β-TrCP1 might be the rate-limiting component in *SAG*/ROC–SCF–mediated pro–caspase-3 degradation. In contrast, replacement of β-TrCP1 with its F-box deletion mutant β-TrCP1ΔF in the *SAG*–SCF complex completely abolished its pro–caspase-3–degrading activity (cf. *lanes 2* and *3 vs lane 1*, Figure 5*A*, *bottom panel*), suggesting that β-TrCP1 is the major—if not the only—F-box protein that mediated pro–caspase-3 degradation by *SAG*–SCF E3 Ub ligase, assuming that other F-box proteins are expressed in a sufficient amount in 293 cells. To further determine whether *SAG*–SCF E3–induced degradation of endogenous pro–caspase-3 is a proteasome-dependent process, *SAG*–SCF$^{\beta\text{-}TrCP}$–transfected cells were treated with MG132, a proteasome inhibitor (*lane 5*). Indeed, *SAG*–SCF$^{\beta\text{-}TrCP}$ ligase–induced reduction of endogenous pro–caspase-3 (Figure 5*A*, *bottom panel*, *lane 2 vs lane 1*) was completely blocked by MG132 (*lane 5 vs lane 4*, compared to *lane 2 vs lane 1*), indicating that pro–caspase-3 degradation is mediated by proteasomes.

Because overexpression of *SAG*/ROC1–SCF$^{\beta\text{-}TrCP}$ E3 ligase reduced the steady-state level of endogenous pro–caspase-3, we next determined whether siRNA silencing of *SAG*, ROC1, or β-TrCP could cause the accumulation of endogenous pro–caspase-3. HeLa cells were transiently transfected with plasmid-based hairpin RNA specifically targeting *SAG*, ROC, or β-TrCPs (both β-TrCP-1 and β-TrCP-2). The endogenous level of *SAG*, ROC1, or β-TrCP1 was measured by antibodies to each protein. As shown in Figure 5*B* (*bottom panel*), compared to control plasmid with random targeting sequence (*lane 1*), two psiSAG plasmids (*lane 2* for psiSAG-1 and *lane 3* for psiSAG-2) reduced endogenous *SAG* level up to five-fold. Likewise, psiROC1-1 (*lane 5*), but not control psiCont (*lane 4*), completely silenced endogenous ROC1 expression. As a result of *SAG* or ROC1 silencing, endogenous pro–caspase-3 level increased two-fold to four-fold (Figure 5*B*, *top panel*). Likewise, siRNA silencing of β-TrCP1 up to three-fold caused a two-fold accumulation of pro–caspase-3 (Figure 5*C*). In summary, when overexpressed, *SAG*/ROC1–SCF$^{\beta\text{-}TrCP}$ E3 ligase promoted a proteasome-dependent degradation of endogenous pro–caspase-3, whereas silencing of *SAG*, ROC1, or β-TrCPs induced its accumulation. Thus, it appears that pro–caspase-3 is a substrate of *SAG*–SCF$^{\beta\text{-}TrCP}$ E3 Ub ligase.

siRNA Silencing of SAG*, ROC1, or β-TrCPs Caused an Increased Activity of Caspase-3 on Activation and Sensitized Cancer Cells to Apoptosis Induced by Anticancer Agents*

We have previously shown that *SAG* inhibited apoptosis in several cancer cell lines, as well as in two ischemia/reperfusion models, both *in vitro* and *in vivo* [17,21,22]. Here we showed that *SAG*–SCF promoted pro–caspase-3

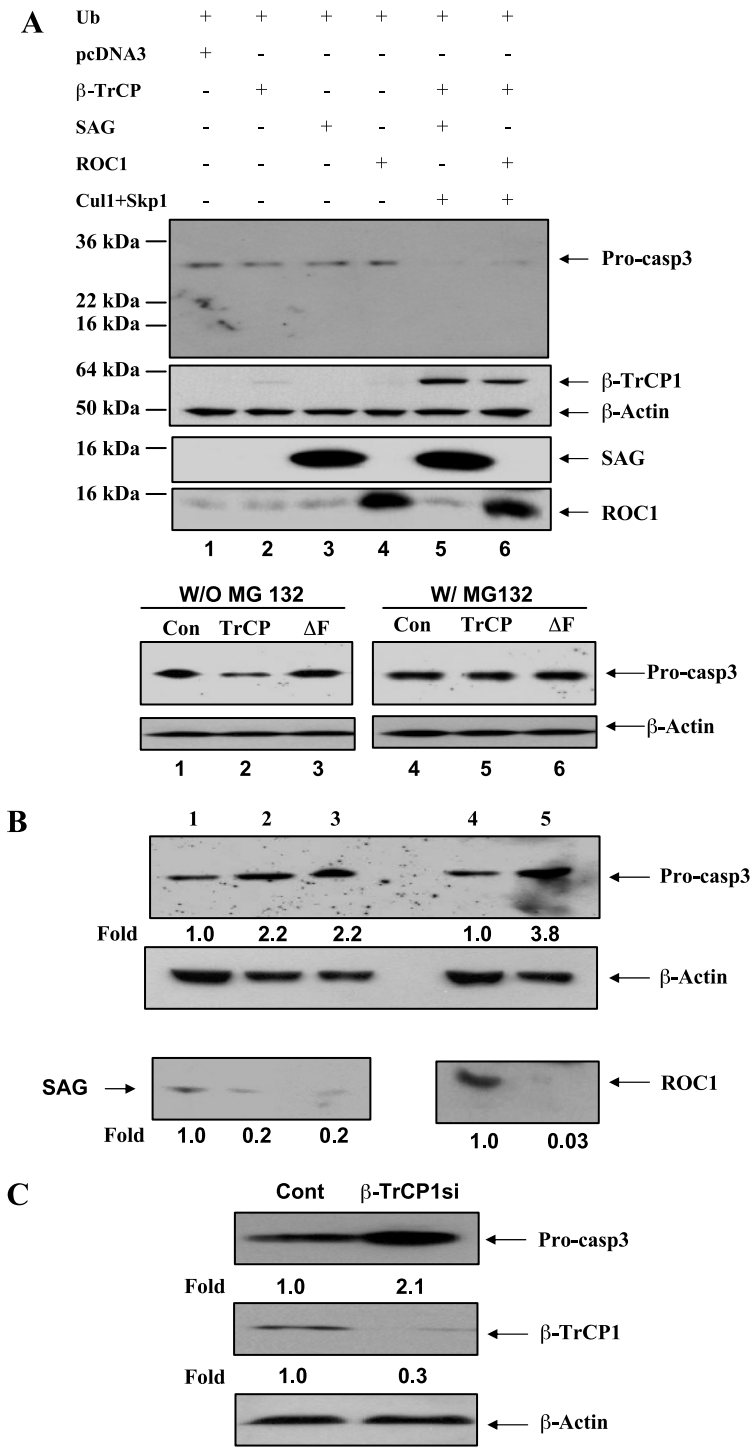

Figure 5. *Pro–caspase-3 is a substrate of* SAG*/ROC1–SCF*$^{\beta\text{-}TrCP}$ *E3 ligase. (A)* SAG*/ROC–SCF*$^{\beta\text{-}TrCP}$ *E3 Ub ligase promoted the degradation of endogenous pro–caspase-3. Top panel: Human 293 cells were transiently transfected with Ub, along with the pcDNA vector control (lane 1), FLAG-β-TrCP (lane 2), FLAG-*SAG *(lane 3), HA-ROC1 (lane 4), all four components of* SAG*–SCF E3 Ub ligase (*SAG*–SCF,* SAG *+ β-TrCP1 + Cul1 + Skp1, lane 5), or all four components of ROC1–SCF E3 ligase (ROC1–SCF; lane 6), respectively. Cell lysates were prepared 38 hours posttransfection and subjected to Western blot analysis with anti–caspase-3 antibody. The blots were stripped and reprobed with antibodies against FLAG tag for the expression of β-TrCP1 (panel 2),* SAG *(panel 3), HA tag for ROC1 expression (panel 4), and β-actin (panel 2) for loading control. Bottom panel: Proteasomal-dependent degradation of pro–caspase-3 by* SAG*–SCF*$^{\beta\text{-}TrCP1}$ *E3 ligase. Human 293 cells were transiently transfected with Ub, along with the pcDNA vector control (lanes 1 and 4),* SAG*–SCF*$^{\beta\text{-}TrCP1}$ *(lanes 2 and 5), or* SAG*–SCF*$^{\beta\text{-}TrCP1\Delta F}$ *(a substitution of wild-type β-TrCP1 with its F-box–deleted mutant; lanes 3 and 6). Twenty-four hours posttransfection, cells were either left untreated (left panel) or treated with 10 μM MG132 (right panel) for 16 hours. Cell lysates were then prepared and subjected to Western blot analysis, with antibodies against caspase-3 or actin as loading control. (B) siRNA silencing of* SAG *or ROC1 increased the endogenous level of pro–caspase-3. HeLa cells were transfected with plasmid-based hairpin RNA designed to silence* SAG *or ROC1: psiSAG-1 (lane 2) and psiSAG-2 (lane 3), as well as psiROC-1 (lane 5), along with control psiCont (lanes 1 and 4). Thirty-eight hours posttransfection, cell lysates were prepared and subjected to Western blot analysis using antibodies against caspase-3 (top panel), actin (middle panel) or* SAG *(lanes 1–3, bottom panel), or ROC1 (lanes 4 and 5, bottom panel). The value of densitometric quantification after normalization with β-actin for equal protein loading was shown on the bottom of each panel, with control number setting at 1, on which the fold change was calculated. (C) siRNA silencing of β-TrCP1 increased the endogenous level of pro–caspase-3. HeLa cells were transfected with plasmid-based hairpin RNA designed to silence β-TrCP1: β-TrCP1-si, along with control psiCont (Cont). Thirty-eight hours posttransfection, cell lysates were prepared and subjected to Western blot analysis using antibody against caspase-3 (top panel), β-TrCP1 (middle), or β-actin (bottom). The value of densitometric quantification after normalization with β-actin for equal protein loading was shown on the bottom of each panel, with control number setting at 1 on which the fold change was calculated.*

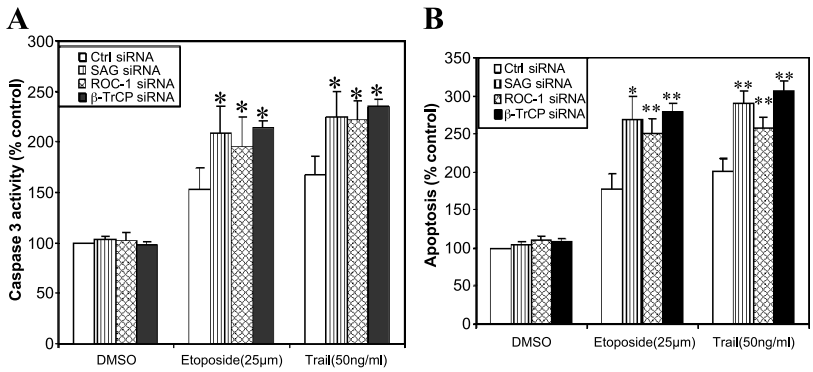

Figure 6. *Silencing of SCF components results in caspase-3 activation and apoptosis induction. (A) Silencing of* SAG*, ROC1, or β-TrCPs increased caspase-3 activity upon activation and (B) cell death induced by anticancer agents. HeLa cells were transfected with psiSAG-2, psiROC-1, or β-TrCP-si alone, along with psiCont plasmid. Thirty-six hours posttransfection, cells were treated with either DMSO or etoposide (25 μM) for 24 hours, or TRAIL (50 ng/ml) for 8 hours, followed by caspase-3 activity assay (A) or trypan blue staining assay for cell viability (B). The results were presented as percent control (mean ± SEM) from three independent experiments. Paired Student's t test was used to define statistically significant levels at *P < .05 or **P < .01.*

degradation, as well as its accumulation on *SAG* silencing. We next determined whether accumulation of pro–caspase-3 by siRNA silencing of *SAG*, ROC1, or β-TrCPs leads to a higher caspase-3 activity on activation by exposure to its activator etoposide (mitochondrial pathway) or TRAIL (death receptor pathway) and whether *SAG*, ROC1, or β-TrCP silencing sensitizes cancer cells to these two apoptosis-inducing agents. HeLa cells were transiently transfected with psiSAG-2, psiROC1-1, or β-TrCP-si, which was shown to reduce endogenous *SAG*, ROC1, or β-TrCP1, respectively (Figure 5*B*). Thirty-six hours posttransfection, cells were treated with DMSO control, etoposide (25 μM), or TRAIL (50 ng/ml) to induce caspase-3 activation through the mitochondrial pathway or the death receptor pathway, respectively. Caspase-3 activity was measured and shown in Figure 6*A*. As expected, silencing of *SAG*, ROC1, or β-TrCPs did not change the basal activity of pro–caspase-3 (DMSO), whereas etoposide or TRAIL treatment caused a 50% increase of caspase-3 activity. Significantly, a combination of the silencing of *SAG*, ROC1, or β-TrCPs alone with etoposide or TRAIL treatment caused a > 100% increase in caspase-3 activity. Consistent with this, a two-fold induction of apoptosis by etoposide or TRAIL alone was further increased up to three-fold on the silencing of *SAG*, ROC1, or β-TrCPs, respectively (Figure 6*B*). These results demonstrated that the accumulation of pro–caspase-3 by the silencing of *SAG*, ROC1, or β-TrCPs, respectively, translated into a higher caspase-3 activity on activation and an increased apoptosis induction on exposure to anticancer agents.

Discussion

SCF E3 Ub ligase is one of the well-defined E3 Ub ligases in eukaryotes that mainly control cell cycle progression [12]. Recent work has implicated its role in apoptosis regulation as well. Through F-box protein Fbw7 or Cdc4, SCF E3 ligase promoted the ubiquitination and degradation of c-Jun, thus blocking apoptotic JNK signal pathway [39]. However, through F-box protein β-TrCP, SCF E3 ligase could protect cells from apoptosis through the ubiquitination and degradation of IκB [38,40], leading to activation of NFκB, an anti-apoptotic transcription factor that regulates cell survival and renders cancer cells resistant to chemotherapeutic agents [41,42]. Importantly, an elevated expression of β-TrCP1 was found in colon cancers, particularly in those with metastases, which was associated with the activation of both β-catenin and NFκB and may contribute to inhibition of apoptosis and tumor metastasis [43]. Furthermore, an increased level of β-TrCP1 was found in pancreatic carcinoma cells, which correlated with constitutive NFκB activation and chemoresistance [44]. However, targeting β-TrCP1 through siRNA silencing or overexpression of dominant-negative mutant suppressed the growth and survival of human breast cancer cells [30], consistent with our observation that siRNA silencing of β-TrCPs sensitized cancer cells to apoptosis induced by anticancer agents.

In this study, we demonstrated yet another potential mechanism by which *SAG*–$SCF^{\beta\text{-}TrCP}$ E3 Ub ligase protects cancer cells from apoptosis. Through the binding of *SAG*/ROC-β-TrCPs with pro–caspase-3, the protein half-life of pro–caspase-3 was shortened as a result of increased ubiquitination and degradation. Due to a key role played by caspase-3 in mediating apoptosis, the level of pro–caspase-3 in unstressed cells would determine the threshold of cellular sensitivity to apoptosis induced by agents that activate either the death receptor pathway or the mitochondrial apoptosis pathway. Indeed, it has been shown that pro–caspase-3–deficient mice had decreased apoptosis in the brain with premature lethality [45]. In cultured cells, reduction or inactivation of pro–caspase-3 dramatically reduced apoptosis in diverse settings, including resistance to apoptotic-inducing anticancer agents, whereas overexpression of pro–caspase-3 increased apoptosis at both basal and induced levels [8,46–49]. We further showed here that accumulation of pro–caspase-3 level can be achieved by siRNA silencing of *SAG*, ROC1, or β-TrCP, which led to increased caspase-3 activity and apoptosis induction on exposure to etoposide and TRAIL. Thus, it appears that the level of pro–caspase-3

contributes to cellular apoptosis threshold, and approaches that increase the cellular level of pro–caspase-3 would enhance caspase-3 activity on activation, leading to an increased sensitivity to apoptosis. We propose that anti-apoptosis activity of *SAG* could be mediated through the activation of $SCF^{\beta\text{-}TrCP}$ E3 ligase to promote the ubiquitination and degradation of pro–caspase-3, thus reducing the basal level of pro–caspase-3 to increase apoptosis threshold.

It has been shown recently that pro–caspase-3 level was decreased in some carcinomas of the prostate, colon, and breast [46,47,50–52], whereas levels of *SAG* or β-TrCP were increased in a subset of human colon, pancreatic, and lung cancers [20,32,43,44,53]. Future research is directed at identifying a subset of human cancer cells with an increased expression of *SAG*/β-TrCP and a decreased expression of pro–caspase-3 to determine whether targeting *SAG*/β-TrCP through siRNA or *SAG*–$SCF^{\beta\text{-}TrCP}$ E3 Ub ligase with a small molecule inhibitor such as pyrrolidine dithiocarbamate [54] would increase pro–caspase-3 level, induce apoptosis, and/or sensitize these cells to chemotherapy or radiotherapy. This would further validate *SAG*–$SCF^{\beta\text{-}TrCP}$ E3 ligase as a promising cancer target for novel anticancer drug discovery [55].

Earlier studies have demonstrated that substrates of $SCF^{\beta\text{-}TrCP}$ E3 Ub ligase contain a β-TrCP–consensus binding motif (destruction motif) D**S**Gxx**S** with both serine residues phosphorylated before β-TrCP binding [56–58]. Recent studies revealed some degeneracy in this motif requirement for β-TrCP binding. It has been shown that β-TrCP bound to ATF4 [33] and the NFκB1 precursor p105 [59] through a D**S**Gxxx**S** motif, to Cdc25A through a D**S**Gxxxx**S** motif [60], and to Wee1 that lacks a consensus motif, with two phosphate serine residues far apart from each other [61]. Most recently, β-TrCP has been shown to bind to a previously undescribed nonphosphorylated destruction motif (DDGϕXD, enriched in acidic residues) in Cdc25A and Cdc25B [62]. In the case of pro–caspase-3, we identified two putative β-TrCP–binding sites with some degeneracy: one is at ^{28}**DSG**I.**S** (one amino acid missing between residues G and S) and the second is at ^{175}**DSG**VD**D** (an S→D substitution at the second serine residue). Among all caspases examined at the corresponding regions [63], only pro–caspase-7 has a degeneracy site: ^{198}**DSG**PI**N**. However, pro–caspase-7 did not bind to β-TrCP (data not shown). We showed here that the first 38 amino acids are required for β-TrCP binding. The fact that the deletions of this sequence, which also completely deleted the first potential β-TrCP–binding site, abolished such binding suggested that the ^{28}**DSG**I.**S** motif might be important for binding. However, our further analysis using site-directed mutants indicated that β-TrCP/pro–caspase-3 binding requires neither degenerated D**S**Gxx**S** motif nor DDGϕXD motif because: 1) S→A mutations at both putative binding motifs did not abolish their binding; 2) S→D mutations that constitutively mimic serine phosphorylation did not enhance binding; and 3) D→A mutations at ^{28}D and ^{34}D, or at ^{175}D and ^{179,180,181}D (to abolish acidic residues) did not abolish their binding (unpublished data). Thus, due to this unconventional nature of β-TrCP/pro–caspase-3 binding, we cannot exclude the possibility that other cellular protein(s) may also be involved in the β-TrCP/pro–caspase-3 complex that would facilitate pro–caspase-3 degradation.

In summary, we identified pro–caspase-3 as a substrate for *SAG*/ROC–$SCF^{\beta\text{-}TrCP}$ E3 Ub ligase. It appears that the ligase-associated mechanism by which *SAG* protects cells from apoptosis is mediated, at least in part, by binding and ubiquitinating/degrading pro–caspase-3, leading to an increased apoptosis threshold and reduced apoptosis.

Acknowledgements

We would like to thank Yinon Ben-Neriah (Hebrew University Hadassah Medical School) for plasmids expressing β-TrCP1 and *β-TrCP1ΔF*; Zhen-qiang Pan (Mount Sinai School of Medicine) for plasmids expressing β-TrCP2 and β-TrCP2ΔF; Michele Pagano (New York University School of Medicine) for recombinant baculovirus expressing ROC–$SCF^{\beta\text{-}TrCP}$ E3 Ub ligase; and David Turner (University of Michigan) for the pU6pro vector.

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