SAG/RBX2 and RBX1 are two family members of RING components of Cullin-RING ligases (CRLs), required for their enzymatic activity. Previous studies showed that SAG prefers to bind with CUL5, as well as CUL1, whereas RBX1 binds exclusively to CULs1–4. Detailed biochemical difference between SAG and RBX1, and whether SAG mediates cross-talk between CRL5 and CRL1 are previously unknown. Here we report that the levels of SAG and β-TrCP1 are inversely correlated, and SAG-CUL5-β-TrCP1 forms a complex under physiological condition. SAG-CUL5, but not RBX1-CUL1, negatively modulates β-TrCP1 levels by shortening its protein half-life through promoting its ubiquitylation via atypical K11-linkage. Consistently, chemical inducers of SAG reduced β-TrCP1 level. Furthermore, SAG mainly binds to E2s UBCH10 and UBE2S known to mediate K11 linkage of ubiquitin, whereas RBX1 exclusively binds to E2s CDC34 and UICH5C, known to mediate K48 linkage of ubiquitin. Finally, silencing of either UBCH10 or UBE2S, but not UICH5C, caused accumulation of endogenous β-TrCP1, suggesting that β-TrCP1 is a physiological substrate of SAG-UBCH10C/UBE2S. Our study, for the first time, differentiates SAG and RBX1 biochemically via their respective binding to different E2s; and shows a negative cross-talk between CRL5 and CRL1 through SAG mediated ubiquitylation of β-TrCP1.

Protein ubiquitylation is a post-translational modification, that via modulating protein stability, activity, or localization regulates many cellular pathways including proinflammatory signaling, DNA damage response, and apoptosis. Protein ubiquitylation is catalyzed by an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase, that is responsible for substrate recognition, and catalyzes the transfer of ubiquitin from the E2 to the substrate. Multiple run of this cascade reaction results in polyubiquitylation of a substrate. Such ubiquitin chains can be connected through the N-terminus of ubiquitin or through one of its seven lysine residues, leading to assembly of diverse polyubiquitin chains with different topologies for distinct structures and functions. While K63-linked chains are mostly implicated in proinflammatory signaling, K48-linked polyubiquitin chains predominantly target proteins for proteasomal degradation. K11-linked chains have been less studied than K48 or K63 linkages, but they seem to serve as a degradation signal for APC/C substrates in the regulation of cell division.

The mechanisms of linkage specificity in polyUb chain synthesis by E2s and E3s are not well understood and remain an area of active investigation. Unlike HECT E3 ligases, which possess an active-site cysteine that receives Ub from a charged E2 (E2~Ub) and subsequently transfers it onto a substrate lysine, the RING ligases lack a catalytic cysteine and act instead by bringing the substrate lysine and catalytic cysteine of E2~Ub together in a conformation suitable for Ub transfer. Thus, E2s of RING ligases determine which polyUb linkage(s) will be synthetized. It has been established that CDC34 or UICH5C E2s couples with CRL1, also known as SCF (SKP1-Cullin1-F box protein) E3, to assemble the ubiquitin chain via the K48 linkage, whereas UBCH10/UBE2C and UBE2S couples with APC/C (Anaphase Promoting Complex/cyclosome) E3 to assemble the ubiquitin chain via the K11
negative mutant 23 (Fig. 2A), excluding the possibility of degradation via self-ubiquitylation. Combination of a novel substrate of CRL5. We, therefore, determined whether accumulation. Our study, therefore, revealed, for the first time, that there is a negative cross-talk between CRL1 and CRL5 through SAG-mediated ubiquitylation and degradation of β-TrCP1, and that CRL5 E3 can mediate and CRL5 through SAG-mediated ubiquitylation and degradation of β-TrCP1. SAG-CUL5 had no effect on the levels of FBXL3 and FBXL11 (Fig. 2A). We further determined which protein, its combination with CUL1 or CUL5 (Fig. S2A). Thus, SAG plays a critical role in negative regulation of β-TrCP1 level, whereas RBX1 had no effect regardless its combination with CUL1 or CUL5 (Fig. 2D). Moreover, β-TrCP1-SAG binding was significantly enhanced when protein degradation was inhibited by MG132 (Fig. 1D). Thus, β-TrCP1 forms a complex with SAG-CUL5 under non-stressed physiological conditions.

**SAG-CUL5, but not RBX1-CUL1 reduces the β-TrCP1 level via shortening its protein half-life.**

Given that SAG-CUL5 form active CRL5 E3 ubiquitin ligase, we tested our hypothesis that β-TrCP1 could be a novel substrate of CRL5. We, therefore, determined whether β-TrCP1 protein level is negatively regulated by SAG or CUL5, alone or in combination, as compared to RBX1 and CUL1 in combination. In a co-transfection experiment in 293 cells, transfection of neither SAG nor CUL5 alone had any significant effect on the levels of ectopically expressed β-TrCP1. Interestingly, when transfected in combination, SAG-CUL5, but not RBX1-CUL1, significantly reduced the levels of ectopically expressed β-TrCP1 as well as β-TrCP1ΔE, a ligase-dead dominant negative mutant (Fig. 2A), excluding the possibility of degradation via self-ubiquitylation. Combination of SAG-CUL5 had no effect on the levels of FBXL3 and FBXL11 (Fig. 2A). We further determined which protein, among SAG, RBX1 CUL5 and CUL1, is responsible for mediating β-TrCP1 reduction and found that SAG, in combination with either CUL5 or CUL1, is able to reduce β-TrCP1 level, whereas RBX1 had no effect regardless its combination with CUL1 or CUL5 (Fig. 2A). Thus, SAG plays a critical role in negative regulation of β-TrCP1.

We next determined the effect of transfected SAG/CUL5 or RBX1/CUL1 on endogenous levels of β-TrCP1 in two lung cancer cell lines, A427 and A549 harboring relatively high levels of β-TrCP1 (Fig. S1A). Transfection of either SAG or CUL5 had moderate, if any, effect, but the combination of SAG-CUL5, not of RBX1-CUL1 remarkably reduced the levels of β-TrCP1 without affecting FBXL3 and FBXL11 (Fig. 2B and C). Again, SAG in combination with either CUL1 or CUL5 caused completely elimination β-TrCP1, whereas RBX1 had a minimal effect, if any, regardless its combination with CUL1 or CUL5 (Fig. 2D).

We then used loss-of-function approaches via either genetic siRNA knockdown or pharmacological small molecule inhibitor to further determine the role of SAG or RBX1 in combinations of CUL1 or CUL5 for β-TrCP1 targeting. Knockdown of SAG, CUL5, RBX1 or CUL1 alone or in combination was performed in H358 cells, which expressed a relatively low level of β-TrCP1 (Fig. S1A). A significant accumulation of endogenous β-TrCP1, but not FBXL3 or FBXL11, was observed only when both SAG and CUL5 were silenced simultaneously (Fig. 2E), indicating that β-TrCP1 is selectively degraded by SAG-CUL5, but not by RBX1-CUL1. We then used MLN4924, a small molecule inhibitor of NAE (NEDD8-Activating Enzyme), which indirectly inhibits the entire CRL E3 ligases by blocking cullin neddylation, and found a dose-dependent accumulation of β-TrCP1 in all three lung cancer cell lines tested (Figs 2F and S2B,C), further supporting the notion that β-TrCP1 is a novel substrate of CRLs. A dose-dependent accumulation of p27, serving as a positive control, was also observed (Fig. 2F and S2B,C).
Given SAG-CUL5 is a typical E3 ubiquitin ligase, we, therefore, determined whether SAG-CUL5, but not RBX1-CUL1 would shorten the protein half-life of β-TrCP1. Indeed, in a co-transfection experiment, SAG-CUL5, but not RBX1-CUL1, shortened the protein half-life of transfected HA-β-TrCP1 (Fig. S2D). SAG-CUL5 also shortened the protein half-life of HA-β-TrCP1ΔF (Fig. S2E). Likewise, the protein half-life of endogenous β-TrCP1 was also significantly shortened upon SAG transfection into A427 cells which expressed high β-TrCP1 but low SAG (Fig. 2G). Consistently, siRNA knockdown of SAG caused the accumulation of basal level β-TrCP1.
Figure 2. SAG negatively regulates β-TrCP1 protein level. (A–D) SAG negatively regulates β-TrCP1 protein level: Various cell lines, as indicated were transfected with indicated plasmids alone or in combination. Whole cell extracts were analyzed by immunoblotting with indicated antibodies. FLAG-1: CUL1 or CUL5; FLAG-2, SAG or RBX1. β-TrCP1 accumulation upon silencing of SAG and CUL5: H358 cells were transfected with siRNAs targeting SAG, RBX1, CUL1 or CUL5. Whole cell extracts were analyzed by immunoblotting with indicated antibodies. Accumulation of β-TrCP1 and p27: A427 cells were treated with indicated concentrations of MLN4924 for 24 hrs, followed by immunoblotting using indicated Abs. (G and H) SAG manipulates protein half-life of β-TrCP1: A427 or H358 cells were either transfected with FLAG-SAG (G) or si-SAG (H) for 12 hrs. Cells were then cultured in fresh medium containing CHX and incubated for indicated time periods before being harvested for immuno blotting with indicated Abs. SAG induction reduces β-TrCP1 level: A549 cells were treated with CoCl2 (250 μM) or TPA (20 ng/mL) for indicate periods of time, followed by immunoblotting analysis using indicated Abs.
SAG-CUL5, but not RBX1-CUL1 promotes the ubiquitylation of β-TrCP1 via K11 linkage. To further confirm that β-TrCP1 is indeed a new substrate of SAG-CUL5 E3 ligase, we determined whether β-TrCP1 is subjected to ubiquitylation by SAG-CUL5.

A cell-based in vivo ubiquitylation assay was performed where ubiquitylated proteins were captured with nickel nitrilotriacetic acid (Ni-NTA) affinity chromatography from 293 cells transfected with FLAG-RBX1-CUL1 or FLAG-SAG-CUL5, or the vector control, along with His-ubiquitin and HA-β-TrCP1, followed by detection of ubiquitylated β-TrCP1 using anti-β-TrCP1 antibody (for both exogenous and endogenous proteins). Indeed, SAG-CUL5, but not RBX1-CUL1, promoted substantial polyubiquitylation of β-TrCP1 as well as β-TrCP1ΔF (Fig. 3A). The same result was observed when H1299 cells were used for transfection (Figure S3A). Thus, SAG-CUL5 promotes β-TrCP1 ubiquitylation.

We then examined the type of ubiquitin chain linkage for ubiquitylated β-TrCP1, using various ubiquitin mutants with a single K → R substitution. We found that polyubiquitylation chain of β-TrCP1 or β-TrCP1ΔF, catalyzed by SAG-CUL5, can be formed when ubiquitin used was wild type, K48R or K63R mutant, but not K11R mutant (Figs 3B and S3B), suggesting that it is likely through a K11 linkage. We further confirmed this finding by using another set of ubiquitin mutants in which all seven lysine (K) residues were mutated to arginine (R), except one indicated K residue remaining as wild type. Consistent with above observation, polyubiquitylation chain of β-TrCP1 or β-TrCP1ΔF can be formed when ubiquitin used was wild type or K11 mutant, but not other K48 or K63 mutant (Figs 3C and S3C). Moreover, we co-transfected SAG-CUL1, followed by in vivo ubiquitylation assay, to determine whether the K11 linkage is SAG specific. Indeed, SAG-CUL1 can also promote polyubiquitylation of β-TrCP1 and β-TrCP1ΔF when wild type or K11 ubiquitin mutant, but not other mutants, was used (Fig. 3D and S3D). Likewise, SAG, in combination with either CUL1 or CUL5, promotes polyubiquitylation of β-TrCP1 or β-TrCP1ΔF when wild type or K48R, but not K11R ubiquitin was used (Fig. 3E). These results clearly demonstrated that SAG, but not CUL1 or CUL5, plays the key role in promoting the K11 linkage.

Our previous27 and recent28 studies showed that p27 and Erbin are substrates of SAG E3 ligase. Here we determined the type of ubiquitin chain linkage catalyzed by different combination of SAG/RBX1 with CUL1/CUL5, and found that in both ERBIN and p27 cases, SAG promotes the ubiquitylation through the K11 linkage, whereas RBX1 promotes ubiquitylation via the K48 linkage, in a manner independent of cullins (Fig. 3F and S3E). Taken together, these in vivo ubiquitylation assays clearly demonstrated that SAG and RBX1, two RING components of CRL E3, determine the ubiquitin chain linkage via K11 or K48, respectively.

SAG and RBX1 complex with different E2 enzymes to facilitate the formation of K11 or K48-linked ubiquitylation chain. It has been recently reported that UBCH10 (also known as UBE2C) and UBE2S are E2 conjugating enzymes, specifically for K11 linkage catalyzed by APC/C E3 ligase12,29. We, therefore, determined potential binding of SAG with UBCH10 or UBE2S, as well as RBX1 with UBCH5C or CDC34, two known E2s for K48 linkage30,31. Indeed, we found that ectopically expressed FLAG-SAG readily pulled down endogenous UBE2S and UBCH10 as well as trace amount of UBCH5C, but not all CDC34, whereas ectopically expressed FLAG-RBX1 pulled down endogenous K48 E2 UBCH5C and CDC34, exclusively (Figs 4A and S4A). The endogenous binding of these E2–E3 pairs was further confirmed in two lung cancer cell lines under physiological unstressed conditions in a pull-down assay using antibody against either SAG or RBX1 (Figs 4B and S4B), or antibodies against each of four E2s (Figs 4C and S4C). Interestingly, two K11 E2s can bind to each other, whereas two K48 E2s bind to each other, but there is essentially no cross-binding among these E2s, except a weak binding between UBE2S and UBCH5C, likely mediated via SAG (Figs 4C and S4C). Binding of SAG to both K11 and K48 E2s may suggest that SAG could mediate polyubiquitylation via K48 linkage in a subset of substrates, in addition to K11 linkage. Alternatively, SAG may mediate a mixed K48 and K11 linkage.

Finally, we used in vitro ubiquitylation assay to determine polyubiquitylation of β-TrCP1, catalyzed by different combinations of E2/E3. We found that polyubiquitylation of β-TrCP1 was found only when UBE2S and UBCH10 were in combination with either SAG-CUL5 (Fig. 4D) or SAG-CUL1 (Fig. 4E). Neither RBX1-CUL1 nor RBX1-CUL5 was able to promote β-TrCP1 polyubiquitylation, regardless which pair of E2s was used (Fig. 4D and E). In the case when ERBIN was used as the substrate, both K11 E2s (UBE2S/UBCH10) in combination with SAG-CUL5 E3, and K48 E2s (CDC34/UBCH5C) in combination of RBX1-CUL1 E3, promoted its polyubiquitylation (Fig. 4D). Taken together, our data indicate that through selective binding to K11 E2s UBE2S and UBCH10, SAG promotes poly-ubiquitylation of β-TrCP1 as well as other known substrates, Erbin and p27 via K11 linkage, whereas RBX1 binds exclusively to K48 E2s CDC34 and UBEH5C to promote the polyubiquitylation of p27 and Erbin via K48 linkage.

β-TrCP1 is mainly accumulated in U2OS-shUb-Ub (K11R) cells. To further demonstrate whether β-TrCP1 is degraded solely or mainly via K11 linkage at the cellular level, we detected β-TrCP1 expression in U2OS cells where endogenous ubiquitin was deleted and replaced with K11R, K48R and K63R mutants, respectively, using a tetracycline-inducible system25. We have validated this cellular system recently and found that it
Figure 3. SAG-CUL5, but not RBX1-CUL1, promotes the ubiquitylation of β-TrCP1 via K11 linkage. SAG-CUL5 promotes polyubiquitylation of β-TrCP1 and β-TrCP1ΔF in vivo: 293 T cells were transfected with indicated plasmids, lysed under denatured condition by 6M guanidinium solution, followed by Ni-bead pull-down. Washed beads were boiled and subjected to immunoblotting, along with whole cell lysates, using indicated Abs. (B–F) SAG/CUL5 promotes poly-ubiquitylation of β-TrCP1 via K11 linkage: H1299 (B) or 293 T (C–F) cells were cotransfected with indicated plasmids alone or in combination, along with ubiquitin and various ubiquitin mutants. Whole cell extracts and Ni-NTA affinity purified fractions were analyzed by immunoblotting using indicated Abs.

worked efficiently with wild type ubiquitin knockdown and mutant ubiquitin replacement upon tetracycline treatment34. It is anticipated that upon exposure to tetracycline, a given substrate will be accumulated in cells.
Figure 4. SAG and RBX1 form the complex with different E2s and β-TrCP is a substrate of SAG-UBCH10/UBE2S for targeted ubiquitylation via K11 linkage. The 293T cells were transfected with FLAG-SAG or FLAG-RBX1, followed by IP using FLAG Ab or normal IgG control, and immunoblotting with indicated Abs. (B and C) Whole cell extracts (WCE) from A549 cells were subjected to immunoprecipitation and immunoblotting, or directly subjected to immunoblotting with indicated Abs. (D and E) The 293T cells were transfected with indicated plasmids alone or in combination, and subjected to in vitro ubiquitylation as described in M&M. The reaction mixture was then loaded onto PAGE gel for immunoblotting using anti-β-TrCP Ab. (F) U2OS-shUb-Ub (WT), -Ub (K11R), -Ub (K48R), and -Ub (K63R) cells were treated with or without tetracycline (1 mg/mL) for 4 days before cell lysates were prepared for immunoblotting with indicated Abs. (G) A427 cells were transfected with siRNAs targeting UBCH10, UBE2S, or UBCH5C, along with scrambled controls. Cells were harvested 48 hr later and subjected to immunoblotting using indicated Abs.
expressing a particular ubiquitin mutant, if that substrate is ubiquitylated via that particular linkage for degradation. Using this system, we found that β-TrCP1 was accumulated at the highest level in tetracycline-inducible K11R cells (Fig. 4F, lanes 3 vs. 4). Interestingly, a moderate β-TrCP1 accumulation was also found in tetracycline-inducible K48R cells (Fig. 4F, lanes 5 vs. 6), suggesting that it is likely that β-TrCP1 is subjected to ubiquitylation and degradation via both K11 and K48 linkage, although mainly via K11 linkage. Serving as the controls, NOXA is mainly ubiquitylated via K11 linkage for degradation, as we reported recently33, whereas p27 is solely ubiquitylated via K48 linkage for targeted degradation under this experimental conditions (Fig. 4F).

β-TrCP1 is a physiological substrate of SAG-UBCH10/UBE2S. We next determined whether two SAG-binding, K11-linked E2s indeed play a role in SAG-mediated ubiquitylation and degradation of β-TrCP1. We, therefore, individually silenced UBCH10 or UBE2S, as well as K48-linked E2 UBCH5C as a control, to determine whether endogenous β-TrCP1 would be accumulated and under which knockdown conditions. As shown in Fig. 4G, silencing either UBCH10 or UBE2S, but not UBCH5C caused β-TrCP1 accumulation, whereas silencing UBCH5C, but not UBCH10 nor UBE2S, caused accumulation of p27, a protein whose ubiquitylation is mediated mainly by K48 linkage, although under overexpressed condition, SAG-CUL5 did promote its ubiquitylation via K11 linkage in 293 cells (Fig. S3E). Taken together, β-TrCP1 appears to be a physiological substrate of SAG-UBCH10/UBE2S.

SAG-βTrCP1 interaction has a complicated effect on βTrCP1 substrates. Finally, we determined potential effect of SAG on the turnover of several known substrates of β-TrCP1. We focused on IκBα34, PHLPP135, and MCL136 after SAG manipulation for any changes in their protein half-lives. As shown in Fig. 5A and B, protein half-life of IκBα, like that of β-TrCP1, were shortened or extended upon SAG overexpression or silencing, respectively, suggesting that IκBα is likely a direct SAG substrate, as we previously shown19,37. In addition, although protein half-life of PHLPP1 had no change upon SAG overexpression, it is significantly extended upon SAG silencing, again suggesting that it is also likely a direct SAG substrate. In contrast, half-life of MCL1 had little change upon SAG overexpression, but was extended upon SAG silencing, suggesting that MCL1 is likely a direct substrate of β-TrCP1.
Discussion

In this study, we made two novel findings: (1) there is a cross-talk between two CRLs, namely CRL5 and CRL1/SCF E3s. Specifically, CRL5 can inhibit SCFβ-TRCP1 E3 ligase activity by promoting β-TRCP1 ubiquitylation via an atypical K11 linkage for subsequent degradation, mediated by SAG. (2) SAG preferentially forms the complex with K11 E2s (UBCH10/UBE2C and UBE2S) to promote the formation of atypical polyubiquitylation chain via K11 linkage, whereas RBX1 exclusively forms the complex with K48 E2s (e.g., CDC34 and UBCH5C) to promote the formation of polyubiquitylation chain via typical K48 linkage. It is SAG or RBX1, via binding to different E2s, determines the linkage specificity in a manner independent of cullins.

Several E3 ubiquitin ligases have been previously shown to be involved in β-TRCP1 degradation, although detailed characterizations were lacking. Examples include SMURF2[28] and SKP2[29]. Pursuit to our unexpected observations that the levels of SAG and β-TRCP1 were inversely correlated in mouse embryonic stem cells, in mouse pancreatic tissues and in multiple lung cancer lines, we reported here that β-TRCP1 is indeed a novel substrate of SAG-CUL5 or SAG-CUL1 E3s through UBC10/UBE2S-mediated, K11-linked polyubiquitylation with the following lines of supporting evidence: (a) β-TRCP1 and SAG bind to each other under physiological conditions; (b) β-TRCP1 levels are reduced or increased upon SAG-CUL5 overexpression or SAG silencing, respectively; (c) β-TRCP1 protein half-life is shortened by SAG-CUL5 overexpression, but extended by SAG knockdown; (d) β-TRCP1 is subjected to polyubiquitylation by SAG-CUL5 or SAG-CUL1; and (e) β-TRCP1 is accumulated upon expression of ubiquitin K11R mutant in U2-OS cells; and (f) β-TRCP1 is accumulated upon siRNA silencing of UBC10 or UBE2S. Thus, β-TRCP1 joins a growing list of SAG substrates[46]. Given that β-TRCP1 is a substrate-recognizing subunit of SCF/CRL1 that regulates a variety of biological processes by promoting ubiquitylation and degradation of many key signaling molecules[22,23], our study suggested that SAG-CRL5 can indirectly regulate these processes by counteracting the effect, established a negative cross-talk network among CRLs. It is worth noting that SAG is not the substrate-recognizing subunit of CRL5 E3 ligase, direct binding of SAG-CUL5 complex with β-TRCP1 is most likely mediated by one of SOCS (Suppressor of cytokine signaling) box-containing proteins (for reviews, see Refs 40 and 41). Future study is directed to identify and characterize such a SOCS protein.

Although our mouse germline knockout studies revealed that Rbx1 and Sag are not redundant and cannot compensate with each other during mouse embryonic development, as evidenced by embryonic lethality if either gene was disrupted[21,27], biochemical difference between two members of RING family of CRL E3 ligase was previously unknown. In the present study, we provided convincing experimental data, using both in vivo and in vitro ubiquitylation assays and various ubiquitin mutants, clearly showed that SAG, by coupling with E2s UBC10/UBE2C and UBE2S, promotes the formation of polyubiquitylation chain via K11 linkage, whereas RBX1, by coupling with E2 CDC34 or UBCH5C, catalyzes the formation of polyubiquitylation chain via K48 linkage. Moreover, we demonstrated that SAG-mediated polyubiquitylation of its substrates, such as β-TRCP1, p27 and Erbin via K11 linkage is E2-dependent and cullin-independent. We further demonstrated that β-TRCP1 is subjected to ubiquitylation and subsequent degradation by SAG-CUL1/5, but not by RBX1-CUL1, whereas p27 and Erbin can be ubiquitylated by either SAG-CUL1/5 or RBX-CUL1 via K11 or K48 linkage, respectively. This biochemical difference in selective E2 binding and substrate degradation can explain why Sag and Rbx1 are not functionally redundant in vivo, particularly during mouse embryogenesis[15,17].

A key feature of ubiquitin is its ability to form polymers, in which individual moieties are linked via one of seven Lys residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 or Lys63 linkages) or the N terminus (linear linkage) in vivo and in vitro[4]. The K48, K63, and K11 linkages have consistently been reported as the most abundant forms of polyUb in yeast and mammalian cells by mass spectrometry methods[42,43]. The cellular roles of Lys48- and Lys63-linked polyubiquitin have been extensively studied. While Lys48-linked polyubiquitin serves as a targeting signal for proteasomal degradation[44], Lys63-linked ubiquitin chains are involved in cell signaling, initiate membrane trafficking events, and DNA damage response[45]. Although the cellular functions of Lys11-linked chains are less well understood, recent studies have, however, found the involvement of this linkage type in diverse cellular pathways, such as cell cycle[46], endocytosis[47], TNF signaling[48], and WNT signaling[49]. Nevertheless, the best studied case for Lys11 linkage is catalyzed by APC/C (Anaphase-Promoting Complex/Cyclosome) E3 ligase complex, leading to rapid degradation of substrates during cell cycle progression, particularly at the mitosis[49,50]. The APC/C can utilize ‘priming’ E2 enzymes such as UBE2C/UBCH10 or UBE2D to decorate substrates with mono-ubiquitin and short ubiquitin chains[45,51], and UBE2S then attaches ubiquitin to the already attached ubiquitin molecules, elongating polyubiquitin chains in a K11 linkage-specific manner[52]. In this study, we made a novel finding that SAG, on one hand, complexes with these two E2s, and on the other hand, binds to CUL5 or CUL1 to promote polyubiquitylation of β-TRCP1 via K11 for targeted degradation. Thus, it is likely that SAG-CRLs and APC/C would compete with each other for selective binding of K11 E2s in cell/tissue context- and stress-dependent manners to precisely regulate complicated biological processes. Our study, therefore, established yet another cross-talk between CRLs and APC/C, two largest E3 ubiquitin ligases co-existing competing for K11 E2s.

Finally, we examined potential effect of SAG manipulation on the turnovers of several known substrates of β-TRCP1. This appears to be a complicated analysis, given that fact that both SAG and β-TRCP1 are E3 ligases that could target the same substrates[16,53], while β-TRCP1 itself is yet a direct substrate of SAG. It is anticipated that the turnover of a given substrate should be accelerated or reduced upon SAG overexpression or depletion, respectively, if it is a direct SAG substrate, whereas the opposite would be true, if it is a direct substrate of β-TRCP1. In our experimental setting, we found IκBα and PHLPP1 are likely direct substrates of SAG, whereas MCL1 is more subjected to targeted degradation by β-TRCP1. Thus, the net biological outcome will be determined by whether the substrates are targeted by SAG directly or indirectly via β-TRCP1, as well as whether β-TRCP1 is directly targeted by SAG in a given cell line. The biological effect is, therefore, truly cell context dependent. Our previous studies as well as the studies from other groups have clearly demonstrated that SAG is a bona fide anti-apoptotic protein (for review, see ref. 16) through directly targeting its substrates, likely including β-TRCP1.
Materials and Methods

Cell culture. Human embryonic kidney HEK293T and various human lung cancer cells were purchased from American Type Culture Collection. Establishment of U2OS cells expressing shUb-Ub (WT), -Ub (K11R), -Ub (K48R), and -Ub (K63R) were described previously33. Mouse embryonic stem cells (AB2, wild type and AB1, Sag-null) were cultured as described39. HEK293, U2OS-shUb-Ub (WT), -Ub (K11R), -Ub (K48R), and -Ub (K63R) were grown in DMEM with 10% FBS; A427 and H358 were grown in RPMI-1640 with 10% FBS. All cell lines were tested and free of mycoplasma contamination.

Transfection. Transfections of plasmids or siRNAs (synthesized by Dharmacon, Lafayette, CO) were performed using the lipofectamine 2000 (Invitrogen), following the manufacturer’s instruction. The sequences of siRNAs targeting SAG, RBX1, CUL-1, CUL-5, UBC10, UBE2S, UBC5C, and ubiquitin (all from Boston Biochem), and above purified substrates and E3s. 

References
1. Swatek, K. N. & Komander, D. Ubiquitin modifications. Cell research 26, 399–422, doi: 10.1038/cr.2016.39 (2016).
2. Zhou, W., Wei, W. & Sun, Y. Genetically engineered mouse models for functional studies of SKP1-CUL1-F-box-protein (SCF) E3 ubiquitin ligases. Cell Res 23, 599–619, doi: 10.1038/cr.2013.44 (2013).
3. Hershko, A. & Ciechanover, A. The ubiquitin system. Annual review of biochemistry 67, 425–479 (1998).
4. Hochstrasser, M. Origin and function of ubiquitin-like proteins. Annual review of biochemistry 78, 101807.093809 [pii] 10.1146/annurev.biochem.78.101807.093809 (2009).
5. Suryadhatu, R., Roesler, S. N., Yang, G. & Sarcievic, B. Mechanisms of generating polyubiquitin chains of different topology. Cells 3, 674–689, doi: 10.3390/cells3030674 (2014).
6. Ikeda, F. & Dikic, I. Atypical ubiquitin chains: new molecular signals. ‘Protein Modifications: Beyond the Usual Suspects’ review EMBO Rep 9, 536–542, doi: embo200893 [pii] 10.1038/embo.2008.93 (2008).
7. Komander, D. The emerging complexity of protein ubiquitination. Biochemical Society transactions 37, 937–953, doi: 10.1042/BS0307937 (2009).
8. Kirkpatrick, D. S. et al. Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology. Nature cell biology 8, 700–710, doi: 10.1038/nclb1436 (2006).
9. Jin, L., Williamson, A., Banerjee, S., Philipp, I. & Rape, M. Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex. Cell 133, 653–665, doi: 10.1016/j.cell.2008.04.012 (2008).
10. Deshaies, R. J. SCF and Cullin/Ring H2-based ubiquitin ligases. Annu Rev Cell Dev Biol 15, 435–467 (1999).
11. Meyer, H. J. & Rape, M. Enhanced protein degradation by branched ubiquitin chains. Cell 157, 910–921, doi: 10.1016/j.cell.2014.03.037 (2014).
12. Sun, Y., Tan, M., Duan, H. & Swaroop, M. SAG/RubX1/RubHr, a zinc RING finger gene family: molecular cloning, biochemical properties, and biological functions. Antioxid Redox Signal 3, 635–650 (2001).
13. Kamura, T. et al. VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases. Genes Dev 18, 3055–3065 (2004).
14. Cate, R. J. B. & Cooper, J. J. X. HOG-signaling lies at the crossroad of homeostasis and stress response. Trends Biochem Sci 28, 575–581 (2003).
15. Jin, L., Williamson, A., Banerjee, S., Philipp, I. & Rape, M. Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex. Cell 133, 653–665, doi: 10.1016/j.cell.2008.04.012 (2008).
16. Deshaies, R. J. SCF and Cullin/Ring H2-based ubiquitin ligases. Annu Rev Cell Dev Biol 15, 435–467 (1999).
17. Meyer, H. J. & Rape, M. Enhanced protein degradation by branched ubiquitin chains. Cell 157, 910–921, doi: 10.1016/j.cell.2014.03.037 (2014).
18. Sun, Yan, Tan, Master Degree, A., Duan, H., & Swaroop, M. SAG/RubX1/RubHr, a zinc RING finger gene family: molecular cloning, biochemical properties, and biological functions. Antioxid Redox Signal 3, 635–650 (2001).
19. Kamura, T. et al. VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases. Genes Dev 18, 3055–3065 (2004).
20. Tan, M. J. et al. SAG/RubX2/RubC E3 Ubiquitin Ligase Is Essential for Vascular and Neural Development by Targeting NF1 for Degradation. Dev Cell 21, 1062–1076, doi: 10.1016/j.devcel.2011.09.014 (2011).
16. Sun, Y. & Li, H. Functional characterization of SAG/RBX2/ROC2/RNF7, an antioxidant protein and an E3 ubiquitin ligase. *Protein Cell* 4, 103–116, doi: 10.1007/s13238-012-2105-7 (2013).

17. Tan, M., Davis, S. W., Saunders, T. L., Zhu, Y. & Sun, Y. RBX1/ROC1 disruption results in early embryonic lethality due to proliferative failure, partially rescued by simultaneous loss of p27. *Proceedings of the National Academy of Sciences of the United States of America* 106, 6203–6208, doi: 10.1073/pnas.0812425106 (2009).

18. Sawaarop, M. et al. Yeast homolog of human SAG/ROC2/Rbx2/Ret12 is essential for cell growth, but not for germination: chip profiling implicates its role in cell cycle regulation. *Oncogene* 19, 2855–2866, doi: 10.1038/sj.onc.1203635 (2000).

19. Tan, M. et al. Disruption of SAG/Rbx2/Roc2 induces radiosensitization by increasing ROS levels and blocking NF-kB activation in mouse embryonic stem cells. *Free Radic Biol Med* 49 976–983 (2010).

20. Frescas, D. & Pagano, M. Deregulated proteolysis by the F-box proteins SKP2 and beta-TRCP: tipping the scales of cancer. *Nat Rev Cancer* 8, 438–449, doi: nrc2396 [pii] 10.1038/nrc2396 (2008).

21. Huang, D. T. et al. E2-RING expansion of the NEDD8 cascade confers specificity to cullin modification. *Mol Cell* 33, 483–495, doi: S1097-2765(09)00308-0 [pii]. doi: 10.1016/j.molcel.2009.01.011 (2009).

22. Nakayama, K. I. & Nakayama, K. Ubiquitin ligases: cell cycle control and cancer. *Nat Rev Cancer* 6, 369–381 (2006).

23. Soucy, T. A. et al. An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. *Nature* 458, 732–736, doi: nature07884 [pii]. doi: 10.1038/nature07884 (2009).

24. Gu, Q., Tan, M. & Sun, Y. SAG/ROC2/Rbx2 is a novel activator protein-1 target that promotes c-Jun degradaion and inhibits 12-O-tetradecanoylphorbol-13-acetate-induced neoplastic transformation. *Cancer Res* 67, 3616–3625 (2007).

25. Tan, M. et al. SAG/ROC2/RBX2 is a HIF-1 target gene that promotes HIF-1alpha ubiquitination and degradation. *Oncogene* 27, 1401–1411 (2008).

26. He, H., Gu, Q., Zheng, M., Normolle, D. & Sun, Y. SAG/ROC2/Rbx2 E3 ligase promotes UVB-induced skin hyperplasia, but not skin tumors, by simultaneously targeting c-Jun/AP-1 and p27. *Carcinogenesis* 29, 858–865, doi: bgn021 [pii]. doi: 10.1093/carcin/bgn021 (2008).

27. Xie, C. M., Liu, J. & Gao, T. beta-TrCP-mediated ubiquitination and degradation of PHLPP1 are negatively regulated by Akt. *Mol Cell Biol* 27, 4096–4017, doi: 10.1128/MCB.06200-06 (2007).

28. Xie, C. M. et al. Neddylation E2 UBE2T is the E2 in the Fanconi anemia pathway and undergoes negative autoregulation. *Mol Cell* 6796–6802 (2005).

29. Li, H. Functional characterization of SAG/RBX2/ROC2/RNF7, an antioxidant protein and an E3 ubiquitin ligase. *Protein Cell* 4, 1107–1120, doi: 10.1007/s13238-010-0033-5 (2010).

30. Petroski, M. D. & Deshaies, R. J. Mechanism of lysine 48-linked ubiquitin-chain synthesis by the cullin-RING ubiquitin-ligase SCF complex. *Trends Biochem Sci* 36, 275–286, doi: S1097-2765(09)00376-5 [pii]. doi: 10.1016/j.tibs.2011.04.004 (2011).

31. Furukawa, M., Ohta, T. & Xiong, Y. Activation of UBC5 ubiquitin-conjugating enzyme by the RING finger of ROC1 and assembly of active ubiquitin ligases by all cullins. *J Biol Chem* 277, 15738–15765, doi: 10.1074/jbc.M108652200 (2002).

32. Xu, M., Skaug, B., Zeng, W. & Chen, Z. J. A ubiquitin replacement strategy in human cells reveals distinct mechanisms of IKK complex SCF-Cdc34. *Cell* 123, 1107–1112, doi: 10.1016/j.cell.2005.09.033 (2005).

33. Chen, Z. J. & Sun, L. J. Nonproteolytic functions of ubiquitin in cell signaling. *Mol Cell* 33, 275–286, doi: S1097-2765(09)00058-6 [pii]. doi: 10.1016/j.molcel.2009.01.014 (2009).

34. Kotliar, N. et al. Contribution of lysine 11-linked ubiquitination to MIR2-mediated major histocompatibility complex I internalization. *J Biol Chem* 225, 3531–35319, doi: 10.1074/jbc.M111.127673 (2010).

35. Dynek, J. N. et al. c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signaling. *EMBO J* 29, 4198–4209, doi: 10.1002/embo.2010.300.1010 (2011).

36. Hay-Koren, A., Caspi, M., Zilberberg, A. & Rosin-Arbesfeld, R. The EDD E3 ubiquitin ligase ubiquititates and up-regulates beta-catenin. *Molecular biology of the cell* 22, 399–411, doi: 10.1091/mbc.E10-05-0440 (2011).

37. Bremm, A. & Komander, D. Emerging roles for Lys11-linked polyubiquitin in cellular regulation. *Trends Biochem Sci* 36, 355–363, doi: 10.1016/j.tibs.2011.04.004 (2011).

38. Williamson, A. et al. Identification of a physiological E2 for the human anaphase-promoting complex. *Proc Natl Acad Sci USA* 106, 18213–18218, doi: 10.1073/pnas.0907877106 (2009).

39. Summers, M. K., Pan, B., Mukhyala, K. & Jackson, P. K. The unique N terminus of the UbcH10 E2 enzyme controls the threshold for APC activation and enhances checkpoint regulation of the APC. *Mol Cell* 31, 544–556, doi: 10.1016/j.molcel.2008.07.014 (2008).

40. Wiekuffe, K. E., Loven, S., Wemmer, D. E., Kuryiyan, J. & Raper, M. The mechanism of linkage-specific ubiquitin chain elongation by a single-subunit E2. *Cell* 144, 769–781, doi: 10.1016/j.cell.2011.01.035 (2011).

41. Sun, Y. E3 ubiquitin ligases as cancer targets and biomarkers. *Neoplasia* 8, 645–654 (2006).

42. Wagner, K. W. et al. Overexpression, genomic amplification and therapeutic potential of inhibiting the UbcH10 ubiquitin conjugase in human carcinomas of diverse anatomic origin. *Oncogene* 23, 6621–6629, doi: 10.1038/sj.onc.1207861 (2004).

43. Machida, Y. J., et al. UBES7 is the E2 in the Fanconi anemia pathway and undergoes negative autoregulation. *Mol Cell* 23, 589–596, doi: 10.1016/j.molcel.2006.06.024 (2006).

44. Zhou, W., Xu, J., Zhao, Y. & Sun, Y. SAG/RBX2 is a novel substrate of NEDD4-1 E3 ubiquitin ligase and mediates NEDD4-1 induced chemosensitization. *Oncotarget* 5, 6746–6755 (2014).
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Author Contributions
Y.S. designed and directed the studies. P.K., M.T., W.Z., and Q.Z. performed experiments and analyzed the data. Y.S. wrote the paper.

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