

The Ubiquitin-associated (UBA) Domain of SCCRO/DCUN1D1 Protein Serves as a Feedback Regulator of Biochemical and Oncogenic Activity^{*[5]}

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Background: SCCRO/DCUN1D1 is commonly amplified in squamous cell carcinomas. Although the SCCRO PONY domain is required for its function, the contribution of its UBA domain is undefined.

Results: Binding of polyubiquitin chains to UBA domain inhibits SCCRO-promoted neddylation and oncogenic activity.

Conclusion: UBA domain serves as a sensor and regulator of SCCRO.

Significance: Targeting the UBA domain may inhibit SCCRO function in human cancers.

Amplification of squamous cell carcinoma-related oncogene (*SCCRO*) activates its function as an oncogene in a wide range of human cancers. The oncogenic activity of *SCCRO* requires its potentiating neddylation domain, which regulates its E3 activity for neddylation. The contribution of the N-terminal ubiquitin-associated (UBA) domain to *SCCRO* function remains to be defined. We found that the UBA domain of *SCCRO* preferentially binds to polyubiquitin chains in a linkage-independent manner. Binding of polyubiquitin chains to the UBA domain inhibits the neddylation activity of *SCCRO* *in vivo* by inhibiting *SCCRO*-promoted nuclear translocation of neddylation components and results in a corresponding decrease in cullin-RING-ligase-promoted ubiquitination. The results of colony formation and xenograft assays showed a mutation in the UBA domain of *SCCRO* that reduces binding to polyubiquitin chains, significantly enhancing its oncogenic activity. Analysis of 47 lung and head and neck squamous cell carcinomas identified a case with a frameshift mutation in *SCCRO* that putatively codes for a protein that lacks a UBA domain. Analysis of data from The Cancer Genome Atlas showed that recurrent mutations cluster in the UBA domains of *SCCRO*, lose the ability to bind to polyubiquitinated proteins, and have increased neddylation and transformation activities. Combined, these data suggest that the UBA domain functions as a negative regulator of *SCCRO* function. Mutations in the UBA domain lead to loss of inhibitory control, which results in increased biochemical and oncogenic activity. The clustering of mutations in the UBA domain of *SCCRO* suggests that mutations may be a mechanism of oncogene activation in human cancers.

Posttranslational modification by ubiquitin regulates the activity of proteins involved in a wide range of essential cellular processes. Although ubiquitin modifications primarily target proteins for degradation by the 26 S proteasome, they can also signal for nonproteolytic effects on protein function. The pleiotropic effects of the ubiquitin signal are encoded for by variations in length and linkage of the ubiquitin chain. In general, Lys⁴⁸-linked polyubiquitin chains signal for protein degradation, whereas monoubiquitin and non-Lys⁴⁸-linked polyubiquitin chains signal for nonproteolytic effects (Lys⁶, Lys¹¹, Lys²⁷, Lys²⁹, Lys³³, and Lys⁶³) (1–5). However, the same ubiquitin linkage can have varying consequences: for example, Lys¹¹-linked chains can promote degradation of the targeted proteins by either the proteasome or the endoplasmic reticulum as well as promote endocytosis and nondegradative activities that lead to NF- κ B activation (6). The diversity of effects imparted by the ubiquitin signal are mediated by motifs that bind to ubiquitin and/or ubiquitin chains and transduce their effects, analogous to the role played by binding of phosphorylated proteins to phospho-binding domains (7–10). Collectively known as ubiquitin-binding domains (UBDs),² these motifs comprise more than 20 different families. The ubiquitin-associated (UBA) domain, the prototypic and most common type of UBD, is composed of a motif of 40 residues that are arranged in three tightly packed α -helices that share structural homology with coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE)-type UBDs (9, 11–14). It is estimated that more than 79 proteins contain UBA domains. The majority of UBA domains recognize a canonical Ile⁴⁴/Val⁷⁰-binding patch on ubiquitin and preferentially bind to polyubiquitin chains (15, 16). However, the affinities of individual UBA domains vary consider-

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[5] This article contains supplemental Table 1.

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² The abbreviations used are: UBD, ubiquitin-binding domain; UBA, ubiquitin-associated; *SCCRO*, squamous cell carcinoma-related oncogene; *DCUN1D1*, defective in cullin neddylation 1 domain-containing 1; PONY, potentiating neddylation domain; TCGA, The Cancer Genome Atlas; CRL, cullin-RING-ligase; CUE, coupling of ubiquitin conjugation to endoplasmic reticulum degradation; NLS, nuclear localization signal; Ub, ubiquitin.

ably, binding to ubiquitin chains of different lengths and linkages (10). UBA domains are thought to limit ubiquitin chain elongation and to help with localization of the bound ubiquitinated proteins to the 26 S proteasome for degradation. Recent studies show that UBA domains can also transduce their effects for a variety of other consequences, including modulation of protein interactions and subcellular localization as well as non-proteasomal turnover of the ubiquitinated proteins and/or the UBA domain-containing proteins (9, 17, 18).

The presence of a UBA domain in SCCRO (also known as DCUN1D1), a protein that functions as an E3 in neddylation (conjugation of the ubiquitin-like protein NEDD8), raises several intriguing possibilities about its function. In contrast to ubiquitination, only a limited number of proteins are subject to neddylation with the cullin protein family (CUL1, CUL2, CUL3, CUL4, CUL5, and CUL7) being the best characterized targets (19). Cullins serve as the scaffold for assembly of cullin-RING-ligase (CRL)-type E3 ligases, the most common type of ubiquitination E3s (20, 21). Neddylation of cullins promotes assembly of the CRL complex and optimizes its conformation to allow efficient transfer of ubiquitin from the E2 to the substrate protein. In its role in promoting neddylation, SCCRO activity regulates levels of ubiquitination activity in cells: an increase in SCCRO activity increases levels of ubiquitinated proteins by promoting CRL activity, whereas a decrease in SCCRO activity has the opposite effect (22–27).

So what is the role of the UBA domain in SCCRO? The high degree of conservation and the existence of splice forms of the SCCRO orthologue DCN1 in lower organisms and SCCRO paralogues in higher organisms that lack a UBA domain suggest that, although it is not required, the UBA domain plays an important role in the function of SCCRO. *In vitro* studies confirm that the UBA domain is not required for the neddylation activity of SCCRO (26). Our finding that SCCRO regulates neddylation *in vivo* by promoting nuclear translocation of the neddylation components (27) combined with recent reports that the UBA domain promotes monoubiquitination of SCCRO leading to its nuclear export (28) suggests that the UBA domain may regulate the neddylation activity of SCCRO *in vivo*. In this study, we show that binding of polyubiquitin chains to the UBA domain inhibits the activity of SCCRO, a core protein in the ubiquitin-proteasome pathway. In this role, the UBA domain serves as a feedback regulator of the *in vivo* neddylation and oncogenic activity of SCCRO. Moreover, clustering of mutations in the UBA domain of SCCRO that mitigate binding to polyubiquitinated proteins suggests that it is a mechanism for oncogene activation. Furthermore, the presence of mutations in the UBA domain of many other proteins suggests that this may be an underappreciated mechanism of oncogene activation in human cancers. Given the conserved binding characteristics of the UBA domain, targeting it may have therapeutic benefit in the treatment of human cancer.

EXPERIMENTAL PROCEDURES

Alignment and Sequence Analyses—Database and BLAST searches were performed at the National Center for Biotechnology Information. Sequence alignments were performed using the ClustalW program.

Reagents—All mutants were generated by PCR mutagenesis and verified by sequencing. Proteins were expressed as glutathione *S*-transferase (GST) fusions in *Escherichia coli* using the pGEX4T3 vector. The proteins were purified using glutathione-Sepharose 4B beads (Amersham Biosciences) followed by thrombin cleavage where required. CUL1-ROC1 was expressed and purified from *E. coli* as described previously (29). APPBP1/UBA3, UBC12, NEDD8, and all ubiquitin chains were obtained from a commercial source (Boston Biochem, Cambridge, MA). The proteasome inhibitor Z-Leu-Leu-Leu-al (here referred to as MG132) was purchased from Sigma-Aldrich. The following antibodies were used in this study: anti-CUL1 (Zymed Laboratories Inc., South San Francisco, CA); anti-CUL3 (BD Biosciences); anti-UBC12 (Rockland Immunochemicals, Gilbertsville, PA); anti-NEDD8 (Invitrogen); anti-CAND1 (BD Biosciences); anti- α -tubulin (Calbiochem); anti-HA, anti-His₆, and anti-ROC1 (Abcam, Cambridge, MA); and anti-MYC and anti-ubiquitin (Santa Cruz Biotechnology, Dallas, TX). Anti-SCCRO (rabbit polyclonal) antibody was produced and used as described previously (30). Anti-SCCRO monoclonal antibody was raised against an N-terminal region of the protein in *SCCRO*^{-/-} mice. This antibody was found to be highly sensitive and specific (data not shown). All cell lines used in this study were obtained from the American Type Culture Collection (Manassas, VA) and maintained as recommended.

cDNAs for mammalian transfection were cloned into the pBAGE-puro vector (Addgene, Cambridge, MA) or pUSE-amp (Clontech). NIH 3T3 cells were doubly exposed to virus in the presence of 7 μ g/ml Polybrene (Sigma-Aldrich) for 24 h each. Infected cells were selected for by use of puromycin (Sigma-Aldrich). pUSE-amp transfection was performed using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's specifications.

In Vitro Neddylation—Cullin-ROC1 substrate for *in vitro* neddylation reactions was derived from either HeLa cell lysates or bacteria (see above). For reactions using HeLa-derived cullin-ROC1 complexes, 50 μ g of lysate (CUL1 concentration, ~20 fmol) was added to reactions containing 10 nM APPBP1/UBA3, 4 nM UBC12, and 0.9 μ M NEDD8 in neddylation buffer (50 mM Tris-HCl (pH 7.5), 55 mM NaCl, and 5 mM MgCl₂). Reactions were incubated at 30 °C and stopped with the addition of 6 \times Laemmli buffer. Reactions with bacterially derived substrate were performed at 30 °C in neddylation buffer containing 27 nM CUL1-ROC1, 4 mM ATP, 10 nM APPBP1/UBA3, 20 nM UBC12, and 0.9 μ M NEDD8. Proteins were resolved by SDS-PAGE and subjected to Western blot analysis. For quantification, Western blots were imaged using NIH ImageJ software.

GST Pulldown Assay and Immunoprecipitation—GST-tagged proteins were bound to glutathione-Sepharose beads by gentle rotation at 4 °C for 45 min. The beads were washed five times with EBC buffer (50 mM Tris-HCl (pH 7.5), 2.5 mM MgCl₂, 150 mM NaCl, and 0.5% Nonidet P-40) at 20 \times bead volume. The beads were incubated with HeLa cell lysate or purified proteins as indicated at 4 °C for 1 h followed by five washes with EBC buffer at 20 \times bead volume. Bound proteins were eluted by the addition of 6 \times Laemmli buffer, resolved by SDS-PAGE, and analyzed by Western blotting. GST-tagged

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proteins were visualized by use of Coomassie R-250 staining (Sigma-Aldrich). Immunoprecipitations were performed essentially as described previously (30). In brief, 1 mg of whole-cell lysate was incubated with antibody bound to agarose beads by gentle rocking at 4 °C for 2 h. The wash and detection were the same as described above for the GST pulldown assay.

Immunofluorescence—Rhodamine-conjugated anti-MYC and FITC-conjugated anti-HA antibodies were obtained from a commercial source (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells transfected with plasmid(s) were seeded in 6-well plates with cover glass. Forty-eight hours after transfection, cells were washed with PBS and fixed in 4% formaldehyde for 10 min. The fixed cells were permeabilized with 0.5% Triton X-100 for 5 min, incubated in blocking buffer (PBS containing 10% FCS) for 30 min, and stained overnight at 4 °C with fluorochrome-conjugated antibodies. The cells were washed three times with PBS, counterstained with DAPI, covered with ProLong Gold antifade reagent (Invitrogen), and examined with a Leica inverted confocal microscope fitted with appropriate fluorescence filters.

Colony Formation and Xenograft Assays—A soft agar assay was performed essentially as described previously (30). Xenograft assays were performed as described previously and in accordance with Institutional Animal Care and Use Committee guidelines. In brief, NIH 3T3 cells (5×10^6) were implanted into the flanks of 8-week-old female BALB/c severe combined immunodeficiency (SCID) mice (Taconic, Hudson, NY). Tumor size was measured every 3 days by a single observer and analyzed using Microsoft Excel and GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). According to institutional guidelines, mice were sacrificed when tumor burden impeded normal feeding and movement.

UBA Mutation Analysis—Sixty-four human UBA domain-containing proteins from Simple Modular Architecture Research Tool (SMART) and 15 UBA domain-containing proteins from the literature were analyzed. Of these proteins, 28 were included in the TCGA data set. These 28 proteins were assessed for the presence and location of mutations against 29 cancer types by use of the cBioPortal for Cancer Genomics.

Statistical Analysis—Paired *t* tests were used to compare the means \pm S.D. of soft agar colony formation counts by use of SPSS 19 software (IBM, Armonk, NY). Survival curves were generated using the Kaplan-Meier method and compared using the log rank test by use of GraphPad Prism. Disease-free survival was defined as the time from primary treatment of cancer to development of recurrence. Survival outcomes were censored for patients who died of intercurrent disease or who survived to the end of the study. A two-tailed *p* value ≤ 0.05 was considered to indicate statistical significance.

RESULTS

The UBA Domain of SCCRO Binds Polyubiquitin Chains in a Linkage-independent Manner—Results of sequence and structural analyses show that the UBA domain of SCCRO is composed of a three-helical bundle architecture that shares homology with several other UBA and CUE domains (26, 31). Hydrophobic residues that are critical for interactions with ubiquitin chains in structurally related domains are highly con-

served in the UBA domain of SCCRO (Fig. 1A). Whereas previous studies have shown that the UBA domain of the yeast orthologue of SCCRO (DCN1) binds to monoubiquitin, structurally related domains preferentially bind to polyubiquitin chains and have a low affinity for monoubiquitin (16, 22). To define the binding preferences, we used *in vitro* synthesized Lys⁴⁸-linked ubiquitin chains (Ub₂ and Ub₄) and assessed their binding to SCCRO by size exclusion chromatography. The results showed a preferential 1:1 co-elution of the Ub₄-SCCRO complex even in the presence of excess Ub or Ub₂ (Fig. 1B). To validate these binding preferences and precisely map the residues involved, GST-SCCRO and selected UBA and PONY domain mutants were used in a pulldown assay containing ubiquitin chains of varying lengths and linkages (Lys⁴⁸ or Lys⁶³). The results showed that SCCRO bound to both Lys⁴⁸- and Lys⁶³-linked Ub₄ or greater length chains with higher affinity than Ub or Ub₂ chains (Fig. 1C). Pulldown assays repeated using serial dilutions of equal concentrations of Lys⁴⁸- and Lys⁶³-linked polyubiquitin chains showed a higher affinity for binding to Lys⁶³-linked chains (Fig. 1D). Mutations in conserved hydrophobic residues in the UBA domain of SCCRO (L30A and A40E) reduced binding, whereas mutations of nonconserved residues in the UBA domain or of residues in the PONY domain did not affect binding to polyubiquitin chains (Fig. 1C). Immunoprecipitation for MYC in lysates from U2OS cells expressing MYC-tagged SCCRO or SCCRO mutants followed by Western blot analysis for ubiquitin showed that SCCRO binds to polyubiquitinated proteins *in vivo* and that this interaction requires conserved hydrophobic residues (Phe¹⁸, Leu³⁰, Ala⁴⁰, and Phe⁴⁵) but not other residues in the UBA domain (Fig. 1E). Mutations in two (SCCRO^{F18K/L30A} and SCCRO^{L30A/A40E}) or three conserved residues (SCCRO^{F18K/L30A/A40E}) in the UBA domain of SCCRO resulted in complete loss of binding to polyubiquitinated proteins (Fig. 1F). These findings confirm the requirement of the UBA domain in binding to polyubiquitinated proteins. Previous studies have suggested that UBA domain-containing proteins can be subclassified on the basis of the length and linkage of the ubiquitin chains to which they bind (32). Our results show that the UBA domain of SCCRO is best included in the class 4 subgroup, although it does not bind to monoubiquitin.

The UBA Domain of SCCRO Binds to Polyubiquitinated CRL Substrates—To determine whether SCCRO binds to proteins that are ubiquitinated by CRL complexes activated by neddylation, we performed tandem affinity purification to assess binding to known CUL1- and CUL3-anchored CRL substrates. GST-SCCRO pulldowns on whole-cell lysate from HeLa cells pretreated with MG132 were performed to enrich for SCCRO-binding proteins. After thrombin cleavage to release these proteins from the glutathione beads, immunoprecipitation was performed using anti-ubiquitin antibody to enrich for ubiquitinated proteins. Immunoblotting showed that SCCRO bound to polyubiquitinated Aurora B and RHOA, established substrates of CUL3-anchored CRL complexes, but not to cyclin E or p27, substrates of CUL1-anchored CRL complexes (Fig. 2A). However, as there is a limited amount of ubiquitinated Aurora B and RHOA in cellular lysates, extended exposure of the Western blot was required to detect binding. To verify these results, we

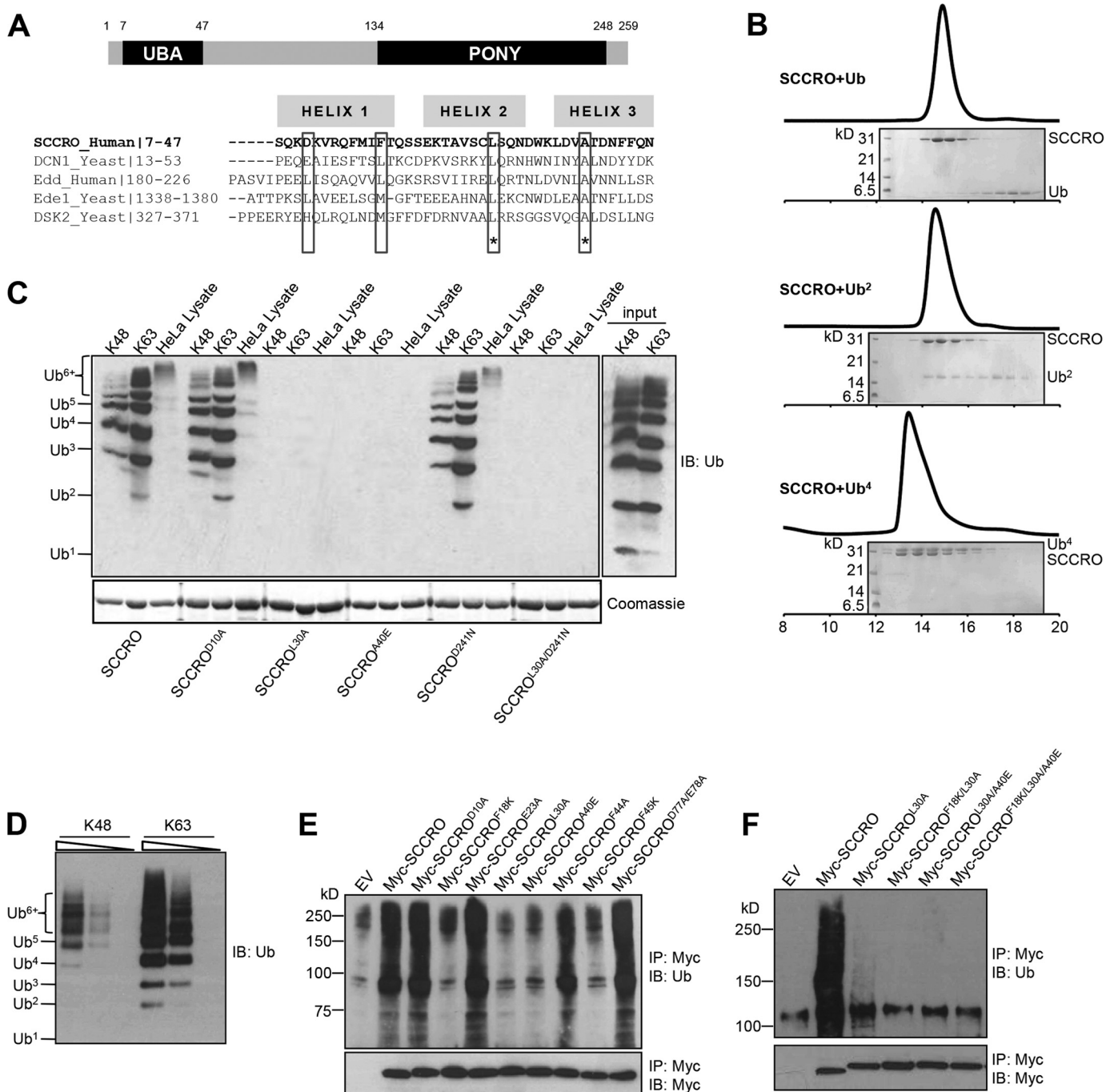


FIGURE 1. The UBA domain of SCCRO binds polyubiquitin chains in a linkage-independent manner. *A*, schematic depiction of the domain structure of SCCRO (*top panel*) and sequence analysis of the UBA domain of SCCRO with several other UBA and CUE domains (*bottom panel*). Highlighted are conserved hydrophobic residues critical for binding to ubiquitinated proteins. *B*, SCCRO preferentially binds Ub₄ over Ub or Ub₂. Lys⁴⁸-linked ubiquitin chains (Ub₂ and Ub₄) were synthesized *in vitro*. Pictured is a size exclusion chromatogram showing that SCCRO was co-eluted with Ub₄ but not with Ub or Ub₂. *C*, pull-down assay using GST-SCCRO and its mutants on Lys⁴⁸ or Lys⁶³ ubiquitin chains or HeLa cell lysates followed by Western blot analysis for ubiquitin showing that SCCRO binds to the ubiquitin chain in a linkage-independent manner. *D*, pull-down assay using GST-SCCRO on serially diluted Lys⁴⁸ or Lys⁶³ ubiquitin chains showing that SCCRO has preferential binding toward Lys⁶³-linked ubiquitin chains. *E* and *F*, immunoblot (*IB*) analysis of lysates from U2OS cells transfected with MYC-SCCRO and selected mutants probed with antibody against ubiquitinated proteins following immunoprecipitation (*IP*) for MYC showing that conserved hydrophobic residues in the UBA domain of SCCRO are critical for binding to ubiquitin chains. *EV*, empty vector.

repeated binding experiments on HeLa cells transfected with Aurora B or RHOA. Transfected cells were treated with MG132 and subjected to tandem affinity purification following the approach described above. As an additional control, we also included pull-downs with GST-SCCRO^{L30A} on the same lysates. We found clear binding of polyubiquitinated Aurora B and

RHOA to GST-SCCRO but not to GST or GST-SCCRO^{L30A} (Fig. 2*B*). Combined, these findings suggest that SCCRO binds to CUL3-anchored CRL targets.

The UBA Domain Does Not Affect Neddylaton Activity of SCCRO in Vitro—To determine whether the UBA domain modulates the neddylation activity of SCCRO, we performed

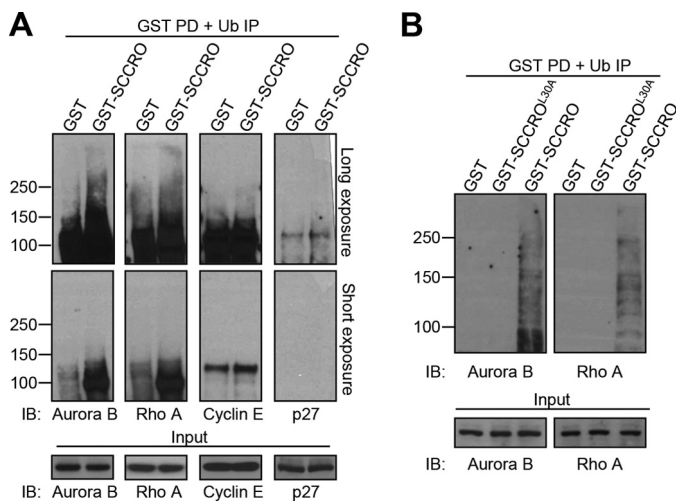


FIGURE 2. The UBA domain of SCCRO binds to polyubiquitinated CRL substrates. A, GST-SCCRO pull-down products from HeLa cell lysate pretreated with MG132 were released by thrombin cleavage. SCCRO and SCCRO-interacting proteins were further immunoprecipitated with antibody against polyubiquitinated proteins and analyzed by Western blotting with antibodies against Aurora B and RHOA (CUL3 substrates) and cyclin E and p27 (CUL1 substrates), which showed that SCCRO selectively bound to polyubiquitinated Aurora B and RHOA. B, experiments were performed as described for A except that HeLa cells were transfected with Aurora B or RHOA before MG132 treatment. Note that GST-SCCRO, but not GST or GST-SCCRO^{L30A}, bound to polyubiquitinated Aurora B and RHOA. IP, immunoprecipitation; IB, immunoblotting; PD, pull-down.

structure-function studies to investigate its effect in *in vitro* assays. *In vitro* reactions contained recombinant NEDD8, APPBP1/UBA3 (E1), UBC12 (E2), ATP, and whole-cell lysate from HeLa cells (as a source of CUL-ROC1 substrate) in the presence of varying concentrations of SCCRO or selected SCCRO mutants. As expected, the addition of SCCRO increased cullin neddylation in a dose-dependent manner. Deletion or mutation of the UBA domain (SCCRO^{ΔN} or SCCRO^{L30A}) did not alter the neddylation-promoting activity of SCCRO, whereas deletion or mutation of the PONY domain (SCCRO^{ΔC} or SCCRO^{D241N}) abrogated its activity (Fig. 3A) (23). Moreover, the addition of a 20-fold molar excess of Lys⁴⁸-linked Ub₄ did not affect the efficiency with which SCCRO and SCCRO^{L30A} increased levels of neddylated CUL1 or CUL3 (Fig. 3B). These findings are consistent with those reported previously for DCN1 in *Caenorhabditis elegans* and yeast (26), confirming that binding of ubiquitin chains to the UBA domain does not affect the neddylation activity of SCCRO *in vitro*.

The UBA Domain Affects Neddylation Activity of SCCRO *In Vivo*—Given our recent findings showing differential requirements for SCCRO in neddylation *in vitro* and *in vivo* (27), we next assessed the requirement of the UBA domain for the neddylation activity of SCCRO *in vivo*. To determine whether these effects were caused by binding of polyubiquitinated proteins to the UBA domain, HeLa cells were treated with sublethal doses of proteasome inhibitor (MG132), which resulted in accumulation of polyubiquitinated proteins relative to DMSO-treated controls. Whereas transfection of either SCCRO or SCCRO^{L30A} increased levels of neddylated CUL1 and CUL3 in DMSO-treated cells, levels of neddylated CUL1 and CUL3 increased only in SCCRO^{L30A}-transfected HeLa cells treated with MG132 (Fig. 4A).

To validate these findings, we expressed MYC-tagged SCCRO, SCCRO^{L30A}, SCCRO^{Δ210–259}, a neddylation-deficient mutant that retains binding to polyubiquitinated proteins, or SCCRO^{L30A/Δ210–259}, a neddylation-deficient mutant with reduced binding to polyubiquitinated proteins in U2OS cells, and confirmed effects of UBA mutations on binding to polyubiquitinated proteins by immunoprecipitation and Western blotting for Ub (Fig. 4B). Immunoprecipitation using anti-HA antibody on lysates co-transfected with HA-SCCRO and MYC-SCCRO^{Δ210–259} or MYC-SCCRO^{L30A/Δ210–259} followed by Western blotting for Ub showed reduced binding of polyubiquitinated proteins to HA-SCCRO when it was co-transfected with MYC-SCCRO^{Δ210–259} but not with MYC-SCCRO^{L30A/Δ210–259} (Fig. 4C, compare lanes 1–3) even though HA-SCCRO was expressed at equal levels (Fig. 4C, see IB: HA). This is likely due to sequestration of polyubiquitinated proteins by MYC-SCCRO^{Δ210–259}, reducing the pool available to bind HA-SCCRO. Co-expression of MYC-SCCRO with MYC-SCCRO^{Δ210–259} increased the levels of neddylated CUL3 relative to cells transfected with MYC-SCCRO alone (Fig. 4D, lanes 1 and 2). Co-transfection of either MYC-SCCRO^{Δ210–259} or MYC-SCCRO^{L30A/Δ210–259} did not affect levels neddylated CUL3 in MYC-SCCRO^{L30A}-transfected U2OS cells (Fig. 4D, lanes 4–6). Combined, our observations suggest that binding of polyubiquitinated proteins to the UBA domain of SCCRO inhibits its neddylation activity *in vivo*. These findings suggest that the UBA domain may serve as a sensor and inhibitor of the neddylation activity of SCCRO in response to increased levels of polyubiquitinated proteins.

The UBA Domain Affects Compartmentalization Activity of SCCRO—To determine the mechanisms by which the UBA domain modulates the neddylation activity of SCCRO, we first investigated whether binding of polyubiquitinated proteins to the UBA domain affects interactions between SCCRO and other neddylation components (27). Lysates from HeLa cells transfected with HA-UBC12, HA-CUL1, and MYC-SCCRO or MYC-SCCRO^{L30A} were subjected to immunoprecipitation using anti-MYC antibody followed by Western blotting for neddylation components. We found no differences between the binding of MYC-SCCRO and MYC-SCCRO^{L30A} to neddylation components (HA-CUL1 or HA-UBC12) (Fig. 5A). Similar results were obtained from GST pull-down assays with no detectable differences in binding to neddylation E3 components between GST-SCCRO and GST-SCCRO^{L30A} in HeLa cell lysates treated with DMSO or MG132 (Fig. 5B).

Given the importance of nuclear localization to the function of SCCRO (27), we next investigated the effects of MG132 pretreatment on the subcellular localization of MYC-SCCRO or MYC-SCCRO^{L30A} in U2OS cells. Treatment of U2OS cells with MG132 resulted in increased cytoplasmic localization of MYC-SCCRO compared with treatment with DMSO cells (Fig. 5C). In contrast, treatment with MG132 had no effect on localization of MYC-SCCRO^{L30A}, which remained primarily nuclear. Next, we cotransfected U2OS cells with MYC-SCCRO or MYC-SCCRO^{L30A} and HA-CUL1^{Δ610–615}, a mutant that depends on SCCRO for nuclear translocation and neddylation *in vivo* (27). Both MYC-SCCRO and MYC-SCCRO^{L30A} promoted nuclear translocation of HA-CUL1^{Δ610–615} in U2OS cells treated with

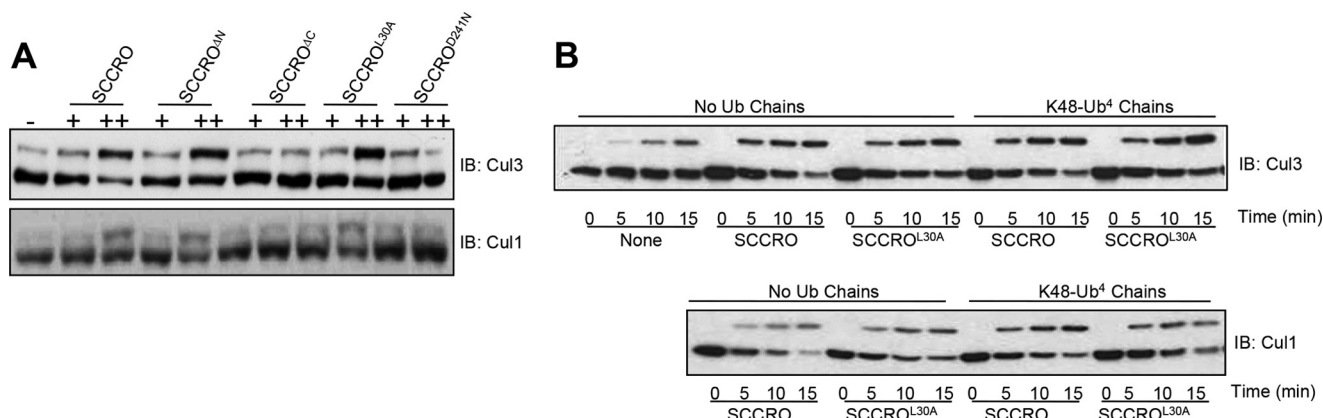


FIGURE 3. **The UBA domain of SCCRO does not affect neddylation activity *in vitro*.** *A*, *in vitro* neddylation reaction with concentration gradients of SCCRO or SCCRO mutants. Mutations in the PONY domain, but not the UBA domain, blocked the neddylation activity of SCCRO (ΔN , SCCRO with N-terminal 1–45 amino acids deleted; ΔC , SCCRO with C-terminal 151–259 amino acids deleted). *B*, *in vitro* neddylation reaction with purified recombinant SCCRO or SCCRO^{L30A} followed by Western blot analysis for CUL1 and CUL3 showing that the presence of a 20-fold molar excess of Lys⁴⁸-linked tetraubiquitin failed to inhibit the effect of SCCRO or SCCRO^{L30A} on neddylation. *IB*, immunoblotting.

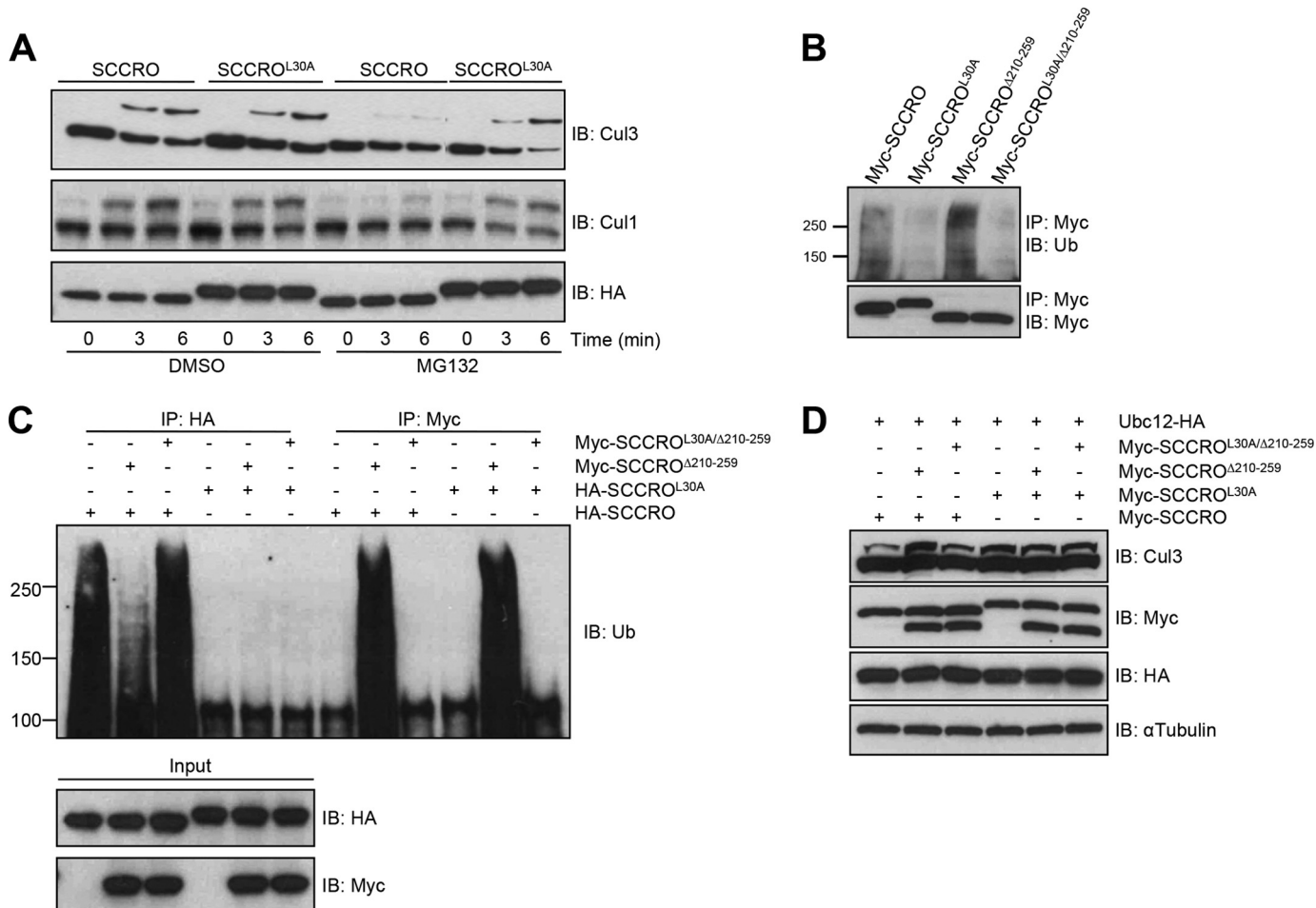
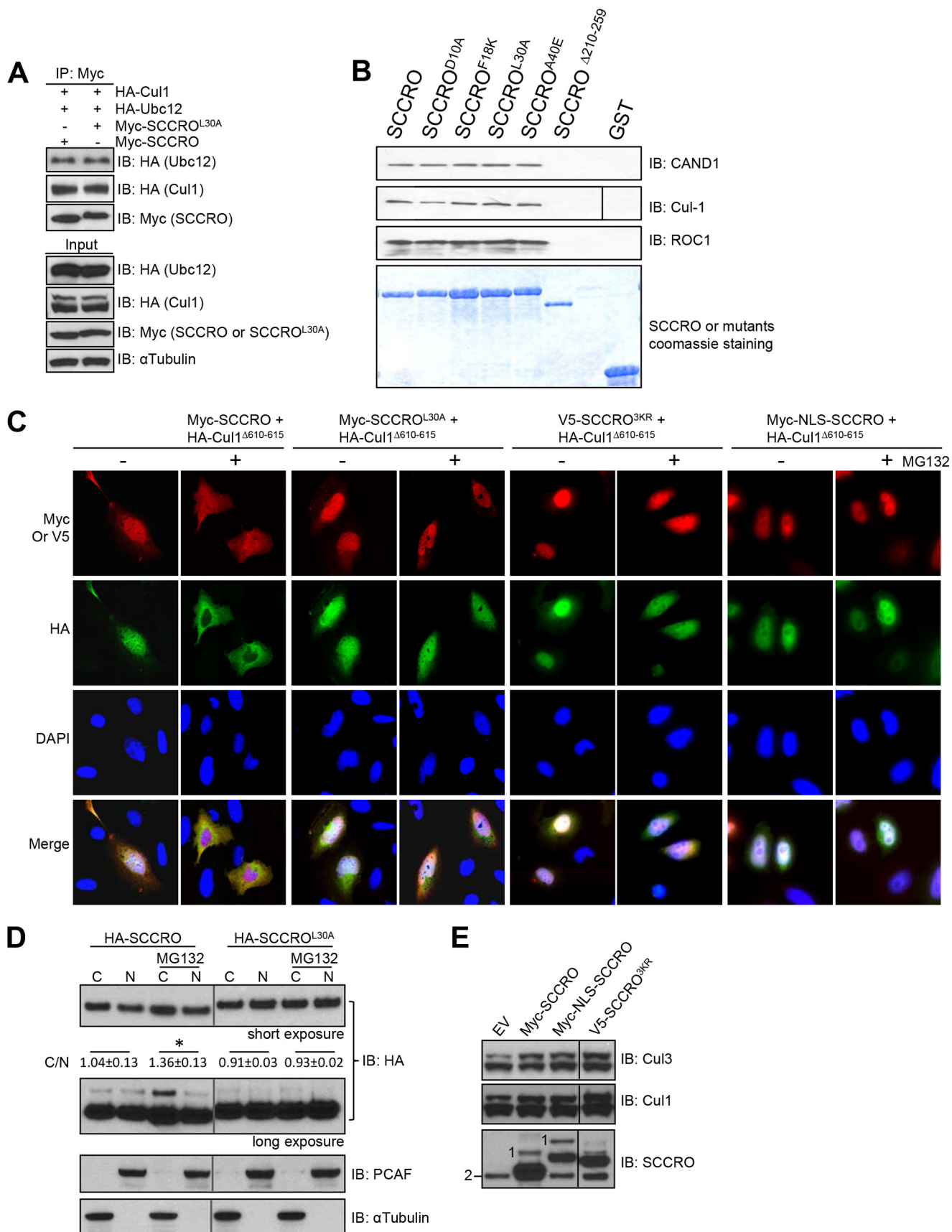


FIGURE 4. **Mutations in the UBA domain augment the neddylation activity of SCCRO *in vivo*.** *A*, neddylation reaction on HeLa cell lysates with and without pretreatment with MG132 showing that the level of neddylated CUL1 and CUL3 was reduced in cells transfected with HA-SCCRO but not with HA-SCCRO^{L30A} when pretreated with MG132. *B*, immunoblot (*IB*) analysis with antibody against polyubiquitinated proteins following immunoprecipitation (*IP*) for MYC on lysates from U2OS cells transfected with MYC-SCCRO mutants showing that SCCRO^{Δ210-259}, but not SCCRO^{L30A} or SCCRO^{L30A/Δ210-259}, retains binding to ubiquitinated proteins. *C*, immunoblot analysis with antibody against polyubiquitinated proteins following immunoprecipitation for either HA or MYC on lysates from U2OS cells transfected as indicated showing that SCCRO^{Δ210-259}, but not SCCRO^{L30A/Δ210-259}, competes with SCCRO for polyubiquitinated proteins. *D*, Western blot analysis of neddylated CUL3 in U2OS cells transfected as indicated showing that coexpression of SCCRO with SCCRO^{Δ210-259}, but not with SCCRO^{L30A/Δ210-259}, resulted in increased levels of neddylated CUL3 (lanes 1–3). In contrast, neither of the SCCRO mutants affected neddylation of CUL3 promoted by SCCRO^{L30A} (lanes 4–6).

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DMSO. However, treatment with MG132 blocked MYC-SCCRO- but not MYC-SCCRO^{L30A}-promoted nuclear translocation of HA-CUL1^{Δ610–615} (Fig. 5C). These observations suggest that the subcellular localization of SCCRO is regulated by binding of polyubiquitinated proteins to the UBA domain. Combined with our previous work showing the importance of SCCRO-promoted nuclear translocation of CRL complexes for the neddylation of cullins, these results provide an explanation for why the UBA domain of SCCRO differentially affects cullin neddylation *in vitro* and *in vivo*.

Binding of Polyubiquitinated Proteins to the UBA Domain Promotes Monoubiquitination and Nuclear Export of SCCRO—Previous studies have shown that the UBA domain is required for monoubiquitination of SCCRO, which promotes its nuclear export (28). We therefore sought to determine whether cytoplasmic localization of SCCRO in MG132-treated cells is due to increased monoubiquitination of SCCRO. We expressed HA-SCCRO or HA-SCCRO^{L30A} in U2OS cells. Whole-cell lysates from cells treated with either MG132 or DMSO were fractionated and immunoblotted for HA. HA-SCCRO, but not HA-SCCRO^{L30A}, was monoubiquitinated and was present only in the cytoplasmic fraction of MG132-treated cells (Fig. 5D). Consistent with previous findings (28), SCCRO^{L30A} was not monoubiquitinated under any conditions tested (Fig. 5D).

To determine whether monoubiquitination is required for nuclear export promoted by binding of polyubiquitinated proteins to the UBA domain of SCCRO, we cotransfected V5-SCCRO^{3KR} (a monoubiquitination-deficient mutant; Fig. 5E) and HA-CUL1^{Δ610–615} into U2OS cells. In contrast to cells expressing SCCRO, treatment with MG132 had no effect on localization of V5-SCCRO^{3KR}- or V5-SCCRO^{3KR}-promoted nuclear translocation of HA-CUL1^{Δ610–615} (Fig. 5C), suggesting that monoubiquitination is important for nuclear export. We next questioned whether the nuclear export of SCCRO is important for the inhibitory effects exerted by binding of polyubiquitinated proteins to the UBA of SCCRO. MYC-NLS-SCCRO and HA-CUL1^{Δ610–615} when cotransfected into U2OS cells did not translocate to the cytoplasm upon treatment with MG132 (Fig. 5C). Combined with the observed changes in localization and neddylation activity of SCCRO that resulted from treatment with MG132, these data suggest that binding of polyubiquitinated proteins to the UBA domain promotes monoubiquitination and nuclear export of SCCRO, thereby inhibiting its neddylation activity.

Mutation of the UBA Domain Increases Oncogenic Activity of SCCRO—We reported previously that overexpression of SCCRO increases cullin neddylation, which promotes malignant transformation *in vivo*. To determine whether the UBA domain affects the function of SCCRO *in vivo*, we assessed the effects of stable expression of HA-SCCRO, HA-SCCRO^{L30A}, or HA-SCCRO^{F44Y} on transformation of NIH 3T3 cells. Independent stable clones with relatively equal levels of transgene expression were selected for each construct. We first confirmed the functional effects of SCCRO and SCCRO UBA mutants. Levels of neddylated CUL1 or CUL3 were assessed by immunoblot analysis on lysates from at least two independent stable clones for each construct. We found that levels of neddylated CUL1 and CUL3 were higher in cells transfected with SCCRO^{L30A} than in cells transfected with SCCRO or SCCRO^{F44Y} (Fig. 6A, compare lanes 5, 6, and 7 with the other lanes). These observations suggest that the UBA domain modulate the neddylation activity of SCCRO in these cells. Next we assessed oncogenicity in stably transfected NIH 3T3 clones by soft agar assay, which showed that clones expressing SCCRO^{L30A} formed significantly more colonies than NIH 3T3 clones expressing SCCRO (Fig. 6, B and C). In contrast, no differences in colony formation were seen between clones expressing SCCRO^{F44Y} (which retains binding to polyubiquitinated proteins) and those expressing SCCRO. To confirm that the increase in anchorage-independent growth in UBA domain mutants depends on the neddylation activity of SCCRO, we investigated the transforming activity of the neddylation-dead mutant (SCCRO^{D241N}) and a double mutant in both the UBA domain and the PONY domain (SCCRO^{L30A/D241N}). Neither the SCCRO^{D241N} mutant nor the SCCRO^{L30A/D241N} double mutant was able to form colonies in soft agar, which suggests that the neddylation activity of SCCRO plays an essential role in its oncogenic activity (Fig. 6, B and C). To validate these findings, we performed *in vivo* xenograft assays in BALB/c nude mice using SCCRO- or SCCRO^{L30A}-transfected NIH 3T3 clones. We found that there was shortened latency and significantly higher growth for SCCRO^{L30A}-transfected than SCCRO-transfected NIH 3T3 cells (Fig. 6, D–F). Necropsy of mice and histopathologic analyses showed that tumor xenografts resulting from injection of SCCRO^{L30A}-expressing NIH 3T3 cells were poorly differentiated and metastasized to pelvic lymph nodes in all mice (data not shown). Conversely, tumor xenografts resulting from injection of SCCRO-expressing NIH 3T3

FIGURE 5. Binding of ubiquitinated proteins affects SCCRO-promoted nuclear translocation of cullins. A, immunoblot (IB) analysis of lysates from U2OS cells transfected with the indicated constructs following immunoprecipitation (IP) for MYC showing no differences between the binding of SCCRO and SCCRO^{L30A} to neddylation components. B, Western blot analysis of the pull-down products of GST-SCCRO and SCCRO mutants from HeLa cell extracts probed with the indicated antibodies showing that SCCRO binds to CAND1, CUL1, and ROC1. The cullin-binding mutant SCCRO^{Δ210–259} loses its ability to bind to CAND1, CUL1, and ROC1; in contrast, UBA mutants do not. C, immunofluorescence analysis using rhodamine-conjugated anti-MYC or anti-V5 and FITC-conjugated anti-HA on U2OS cells transfected with MYC-SCCRO, MYC-SCCRO^{L30A}, V5-SCCRO^{3KR}, or MYC-NLS-SCCRO and HA-CUL1^{Δ610–615} in the presence or absence of MG132. Pretreatment with MG132 caused translocation of a significant proportion of MYC-SCCRO, but not MYC-SCCRO^{L30A}, V5-SCCRO^{3KR}, or MYC-NLS-SCCRO, from the nucleus to the cytoplasm (first row). In addition, pretreatment with MG132 blocked nuclear translocation of HA-CUL1^{Δ610–615} promoted by SCCRO but not by SCCRO^{L30A}, V5-SCCRO^{3KR}, or MYC-NLS-SCCRO (second row). D, fractionation analysis of U2OS cells transfected with HA-SCCRO showing increased levels of cytosolic SCCRO and monoubiquitinated SCCRO but not SCCRO^{L30A} after treatment with MG132. Results from densitometry measurement of Western blots from three independent experiments are shown (C, cytoplasm; N, nucleus; C/N stands for ratios of the levels of cytosolic SCCRO or SCCRO^{L30A} versus the levels of nuclear SCCRO or SCCRO^{L30A}). *, $p = 0.043$. E, Western blot analysis of neddylated CUL1 and CUL3 in U2OS cells transfected as indicated showing that expression of NLS-SCCRO or SCCRO^{3KR} resulted in increased levels of neddylated CUL1 and CUL3 to an extent similar to expression of SCCRO. Western blotting for SCCRO on the same lysates showed that although both MYC-SCCRO and MYC-NLS-SCCRO underwent monoubiquitination V5-SCCRO^{3KR} did not under identical conditions (1, monoubiquitinated SCCRO; 2, endogenous SCCRO). PCAF, p300/CBP-associated factor; EV, empty vector.

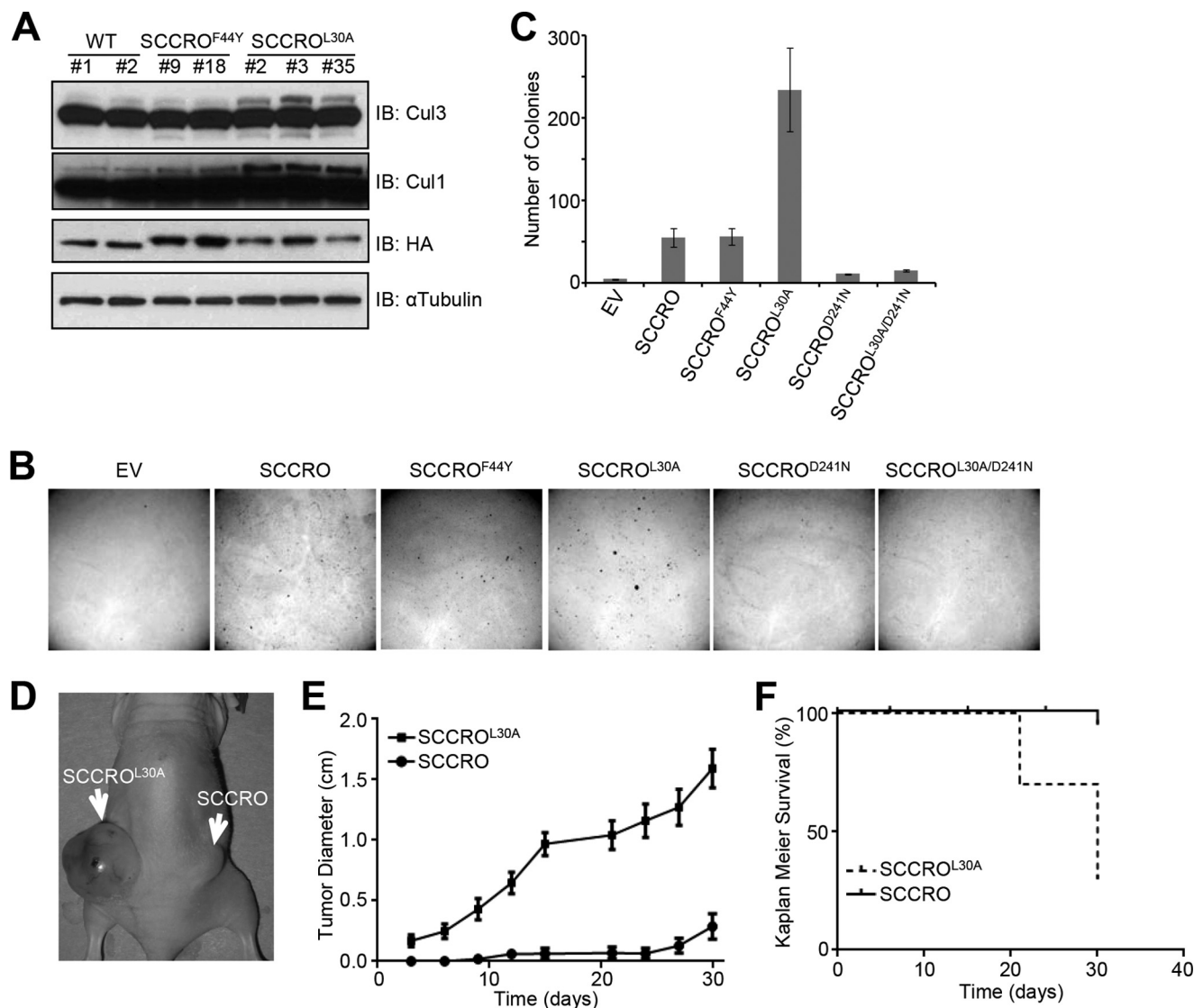


FIGURE 6. Disruption of ubiquitin binding increases transforming activity of SCCRO. *A*, Western blot analysis showing that levels of neddylated CUL1 and CUL3 are higher in NIH 3T3 cells transfected with HA-SCCRO^{L30A} than in those transfected with HA-SCCRO (WT) or HA-SCCRO^{F44Y}. *B*, representative results from soft agar assay showing that in NIH 3T3 cells stable expression of SCCRO^{L30A} resulted in increased colony formation compared with expression of SCCRO. Note that NIH 3T3 cells expressing PONY domain mutants (SCCRO^{D241N} and SCCRO^{L30A/D241N}) had a similar number of colonies as empty vector (EV) controls did. *C*, graph quantification of colonies for *B*. *D* and *E*, *in vivo* xenograft assays in nude mice with NIH 3T3 clones expressing SCCRO or SCCRO^{L30A} showing more aggressive tumor growth (*D*) with a larger tumor diameter (*E*) for SCCRO^{L30A} than for SCCRO. *F*, Kaplan-Meier survival curves showing that mice transplanted with NIH 3T3 clones expressing SCCRO^{L30A} had shorter survival than those transplanted with NIH 3T3 clones expressing SCCRO. *IB*, immunoblotting.

cells were more differentiated and rarely metastasized to lymph nodes. These results suggest that the oncogenic activity of SCCRO is modulated by its UBA domain. To determine the relevance of these findings to human tumors, we screened 47 lung and head and neck squamous cell carcinomas for mutations using the Sequenom method and identified one with a frameshift mutation that putatively produces a truncated SCCRO protein with an alternate start site downstream of the mutation and without a UBA domain (Fig. 7A). However, because of a lack of tumor tissue, we could not confirm the effect of this mutation on the production of SCCRO protein. We further screened available whole-genome sequencing results from TCGA projects for different human cancers. This analysis showed that, although they are rare, mutations in SCCRO cluster in the UBA domain (Fig. 7B). To assess whether TCGA-identified UBA domain mutants bind to ubiquitin

chains, we carried out an immunoprecipitation assay to assess their binding to ubiquitin chains of varying lengths and linkages (Lys⁴⁸ or Lys⁶³). In contrast to wild-type SCCRO, we found that none of the tumor-derived UBA mutants bound to ubiquitin chains (Fig. 7C). Moreover, immunoprecipitation for MYC in lysates from U2OS cells expressing MYC-tagged SCCRO or UBA mutants identified in TCGA followed by Western blot analysis for ubiquitin showed that all TCGA mutants lost the ability to bind to polyubiquitinated proteins *in vivo* (Fig. 7D).

To determine whether UBA mutants identified in the TCGA data set affect the biochemical and oncogenic functions of SCCRO, we assessed their effect on cullin neddylation and transformation. Whereas transfection of SCCRO, SCCRO^{R13H}, or SCCRO^{Q20A} increased levels of neddylated CUL1 and CUL3 in DMSO-treated HeLa cells, neddylated CUL1 and CUL3 only increased in SCCRO^{R13H}- or SCCRO^{Q20A}-transfected cells after

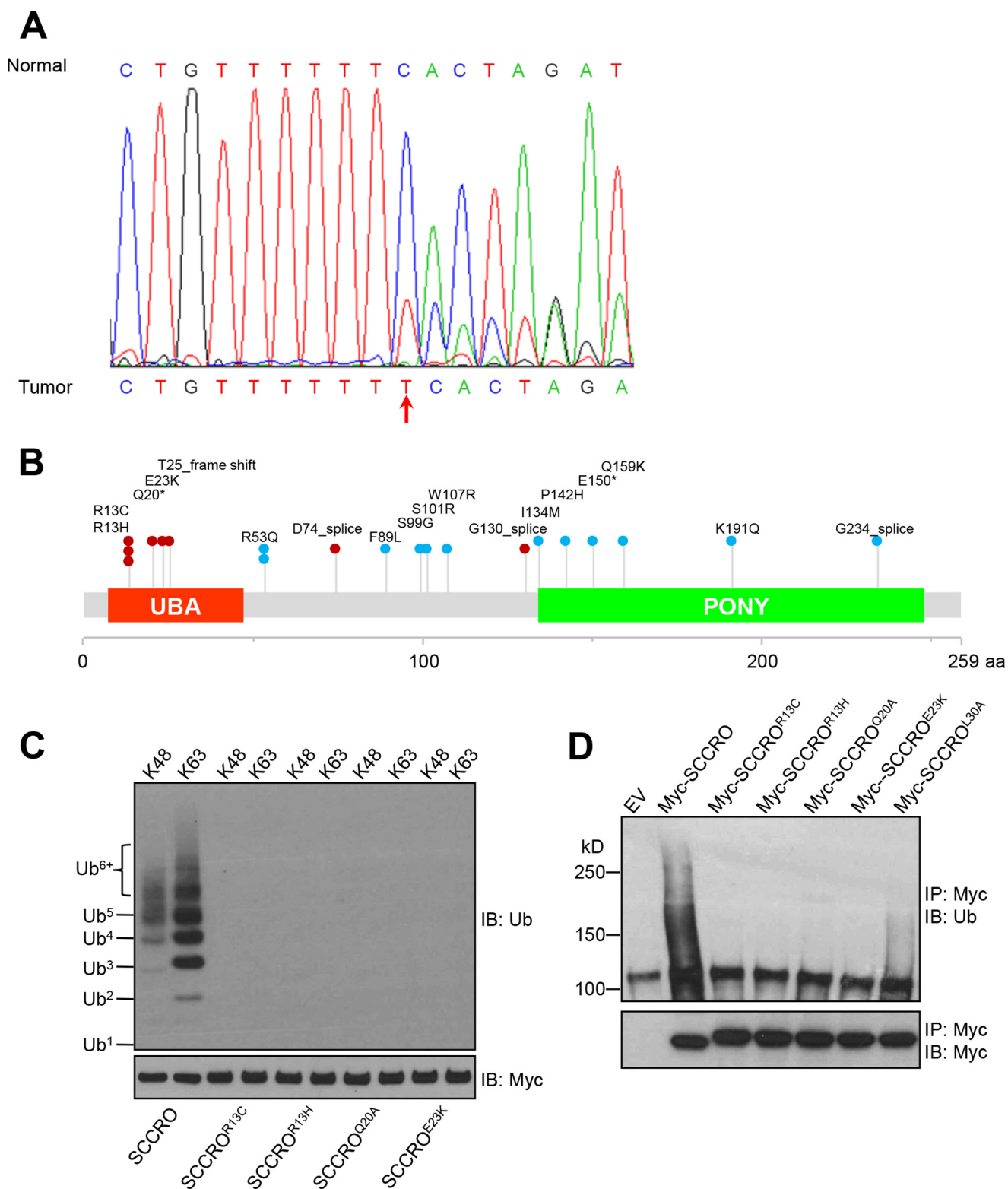


FIGURE 7. **Mutations in the UBA domain of SCCRO are present in human cancers.** *A*, frameshift mutation in the UBA domain identified in one case of 47 lung and head and neck squamous cell carcinomas assessed by use of the Sequenom method. A truncated SCCRO mutant without a UBA domain is predicted. *B*, mutations in SCCRO identified in TCGA projects. *Red dots* represent UBA domain point mutations or splice mutations putatively producing truncated SCCRO without a UBA domain; *light blue dots* represent non-UBA domain mutations. Each *dot* represents a separate cancer. The *asterisk* (*) indicates a nonsense mutation. *C*, immunoprecipitation (IP) assay using MYC-SCCRO and its TCGA UBA mutants on Lys⁴⁸- or Lys⁶³-linked ubiquitin chains followed by Western blot analysis for ubiquitin showing that TCGA-identified UBA domain mutants do not bind ubiquitin chains. *D*, immunoblot (IB) analysis of lysates from U2OS cells transfected with MYC-SCCRO or TCGA UBA mutants probed with antibody against ubiquitinated proteins following immunoprecipitation for MYC showing that all mutants lose the ability to bind to polyubiquitinated proteins. *EV*, empty vector.

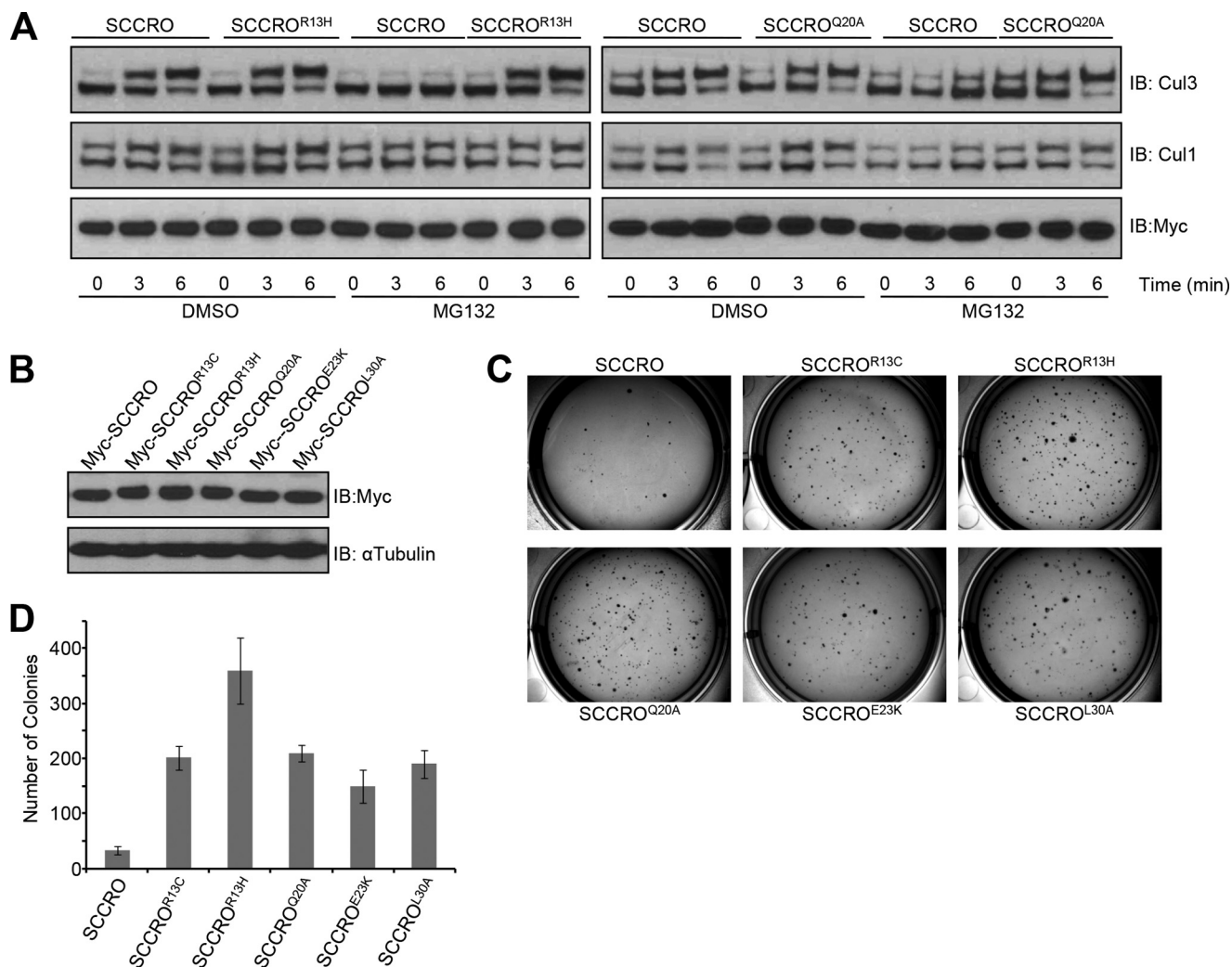


FIGURE 8. Tumor-derived UBA mutations increase transforming activity of SCCRO. *A*, TCGA-identified mutations in the UBA domain augment the neddylation activity of SCCRO *in vivo*. A neddylation reaction on lysates derived from HeLa cells with and without pretreatment with MG132 shows that the levels of neddylated CUL1 and CUL3 are reduced by pretreatment with MG132 in MYC-SCCRO-transfected but not MYC-SCCRO^{R13H}- or MYC-SCCRO^{Q20A}-transfected cells. Results were confirmed by densitometry measurement. *B*, Western blot analysis of lysates from NIH 3T3 cells stably transfected with the indicated SCCRO constructs and probed with anti-MYC antibody showing equivalent expression. *C*, representative results from a soft agar assay showing that stable expression of TCGA-identified UBA domain mutants in NIH 3T3 cells results in increased colony formation in soft agar relative to expression of SCCRO. *D*, graph showing the average number of colonies for each NIH 3T3 clone from *B*. *IB*, immunoblotting.

MG132 treatment (Fig. 8A). Next, to assess the transforming activities of tumor-derived SCCRO UBA mutants, we stably transfected NIH 3T3 cells and selected stable clones with similar transgene expression levels (Fig. 8B). These cells were subjected to soft agar assays, which showed significantly higher colony formation in NIH 3T3 cells expressing TCGA-identified SCCRO UBA mutants relative to those expressing wild-type SCCRO (Fig. 8, C and D). Combined, these findings suggest that the UBA domain of SCCRO modulates its neddylation activity and that this has functional relevance to tumorigenesis.

DISCUSSION

Through its role as an E3 in neddylation, SCCRO promotes CRL activity, which results in an increase in cellular levels of polyubiquitinated proteins (27). We found that binding of polyubiquitinated proteins to the N-terminal UBA domain of SCCRO inhibits its neddylation activity, suggesting the presence of a classical negative feedback loop that regulates CRL-

promoted ubiquitination activity (Fig. 9). In this model, the N-terminal UBA domain of SCCRO serves as a sensor of polyubiquitinated proteins as well as an effector by promoting the autoubiquitination of SCCRO. The effects of the UBA domain do not appear to be allosteric as the addition of polyubiquitin chains or mutation of the UBA domain had no effect on the neddylation function of SCCRO *in vitro*, whereas accumulation of polyubiquitinated proteins showed classical negative feedback dynamics *in vivo*. Interestingly, the requirement for SCCRO in neddylation was also different *in vitro* and *in vivo* with its essential effects *in vivo* involving compartmentalization of neddylation components. As UBC12, the E2 for neddylation, is primarily located in the nucleus, SCCRO-promoted nuclear translocation of cullin-ROC1 complexes is required for neddylation. Consistent with this, we found that the effect that binding of polyubiquitinated proteins to the UBA domain had on the function of SCCRO was imparted by cytoplasmic translocation of SCCRO: an increase in levels of polyubiquitinated

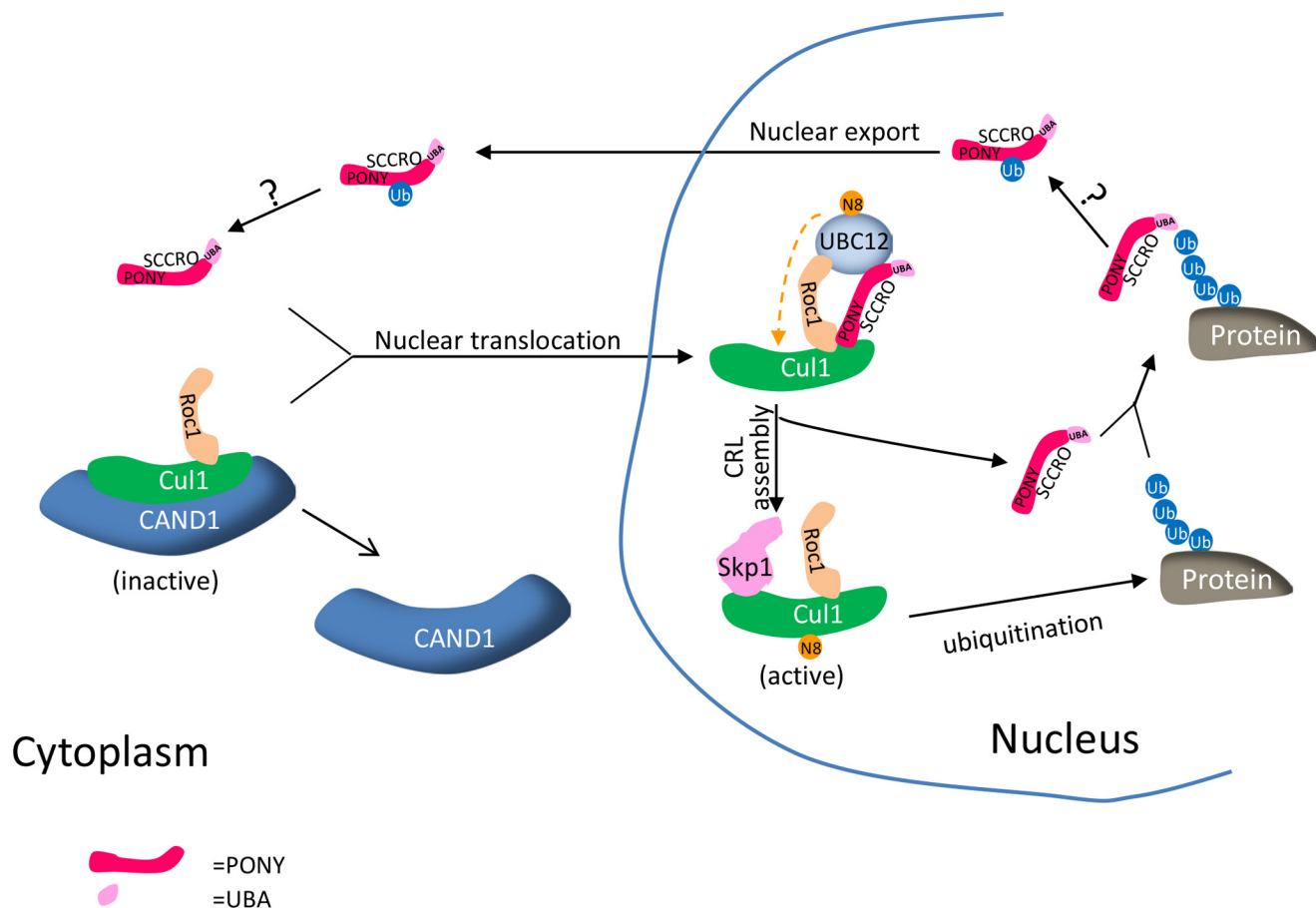


FIGURE 9. Proposed model of UBA domain-mediated regulation of SCCRO.

proteins increased cytoplasmic localization of SCCRO and inhibited nuclear localization of neddylation components, whereas mutation in the UBA domain abrogated these effects.

These findings raise obvious questions about how binding of polyubiquitinated proteins to the UBA domain affects the subcellular localization and function of SCCRO. Recent work by Wu *et al.* (28) shows that the UBA domain of SCCRO is required for its monoubiquitination and that monoubiquitination promotes nuclear export of SCCRO. Conversely, mutations in the UBA domain blocked monoubiquitination of SCCRO and consequently its nuclear export (Fig. 5, C and D) (28). These findings suggest that the binding of polyubiquitinated proteins to the UBA domain serves as a signal for the monoubiquitination of SCCRO. A limitation of this work is the reliance on SCCRO mutants to assess structure-function relationships. It remains possible that the generated mutants affect protein function in unanticipated ways. Mitigating this issue at least in part, we found that blocking the monoubiquitination of SCCRO by mutation of ubiquitination residues in SCCRO or restricting the nuclear export of SCCRO by NLS tag had identical effects on SCCRO localization and function as UBA domain mutants expressed in cells exposed to MG132.

UBD-coupled autoubiquitination has been reported for several other proteins, including STS1, STS2, EPS15, EPS15R, Vps27p/HRS, Vps9p, and RABEX-5 (9). Although the precise underlying mechanisms remain to be defined, a range of functional consequences are known to accompany UBD-coupled

autoubiquitination, including effects on inter- and intraprotein interactions and subcellular localization. Various UBDs have been implicated in coupled autoubiquitination, including ubiquitin-interacting motif, CUE, motif interacting with ubiquitin, and A20 zinc finger domains; however, this has not been reported previously for UBA domain-containing proteins. In the case of SCCRO, coupled autoubiquitination leads to its cytoplasmic translocation and functional inhibition *in vivo*. What remains to be defined is how monoubiquitinated SCCRO is translocated and sequestered in the cytoplasm. Given the relatively low affinity of the UBA domain in SCCRO for monoubiquitin, it is unlikely that intraprotein interaction is the cause of changes in the localization of SCCRO. These effects are also unlikely to be caused by changes in interactions with neddylation components as the presence of polyubiquitin chains and/or mutations in the UBA domain did not affect binding between SCCRO and CUL1 or UBC12. Given that SCCRO and other neddylation E3 components lack a canonical nuclear localization sequence, one possibility for nuclear export is that monoubiquitination of SCCRO inhibits interactions with NLS-containing protein partners involved in nuclear import.

In the broader context, the principal presence of the UBA domain in E2s and E3s (33) raises the question of whether UBA domains serve as general feedback regulators of the ubiquitination pathway. In a related model, autoubiquitination and ubiquitin binding of the UBD in RABEX-5 affect its ubiquitin ligase

The UBA Domain of SCCRO/DCUN1D1

activity (34). It remains to be determined whether UBA domains play similar roles in other proteins.

Although specific mechanisms require definition, it is quite clear that mutations in the UBA domain that inhibit interactions with ubiquitinated proteins increase the neddylation activity of SCCRO *in vivo*. We have shown previously that increased neddylation activity resulting from overexpression of SCCRO promotes oncogenesis *in vitro* and *in vivo* (30). In primary tumors, the oncogenic activity of SCCRO is promoted by amplification that is present in many types of cancers, including lung, ovarian, head and neck, and esophageal cancers (35–37). Interestingly, we found that transgenic expression of UBA domain mutants of SCCRO was more oncogenic than that of wild-type SCCRO. Moreover, we found that, although they are rare, somatic mutations cluster in the UBA domain of SCCRO. As the spontaneously occurring somatic UBA domain mutations lose the ability to bind to polyubiquitinated proteins and have increased neddylation and transforming activity, they represent an alternate mechanism for activation of the oncogenic activity of SCCRO in human cancers.

The role of the UBA domain in cancer pathogenesis has been well established. For example, germ line mutations in the UBA domain of SQSTM1 are associated with the development of Paget disease of bone. These mutations impair the ability of SQSTM1 to bind to ubiquitin chains, altering proteasome- and/or autophagy-based turnover of the bound ubiquitinated proteins (38–42). Although the specific polyubiquitinated proteins involved remain to be identified, the carcinogenic effects that result from loss of binding due to mutation in the UBA domain of SQSTM1 are achieved through activation of NF- κ B signaling (39, 40). Moreover, the identification of mutation clusters in the UBA domains of other proteins suggests that these domains may play a larger and more direct role in oncogenesis than appreciated previously (supplemental Table 1). In the broader context, functional domains typically have similar activities even when present in different proteins. As such, functionally related mutations in cancers may cluster in domains rather than in individual genes or gene families. Domain-based analysis of genome-wide sequencing data may identify novel targets for anticancer therapies. For example, the activity of an oncogene may be mitigated by agents that bind to or mimic the UBA domain of the oncoprotein. In one such instance, overexpression of the UBA domain of hFAF1 significantly promotes cell death by increasing the degradation of polyubiquitinated proteins (43).

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