

# SCCRO (DCUN1D1) Promotes Nuclear Translocation and Assembly of the Neddylation E3 Complex<sup>\*[5]</sup>

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Guochang Huang, Andrew J. Kaufman<sup>1</sup>, Y. Ramanathan<sup>2</sup>, and Bhuvanesh Singh<sup>3</sup>

From the Department of Surgery, Laboratory of Epithelial Cancer Biology, Memorial Sloan-Kettering Cancer Center, New York, New York 10065

SCCRO/DCUN1D1/DCN1 (squamous cell carcinoma-related oncogene/defective in cullin neddylation 1 domain containing 1/defective in cullin neddylation) serves as an accessory E3 in neddylation by binding to cullin and Ubc12 to allow efficient transfer of Nedd8. In this work we show that SCCRO has broader, pleiotropic effects that are essential for cullin neddylation *in vivo*. Reduced primary nuclear localization of Cul1 accompanying decreased neddylation and proliferation in *SCCRO*<sup>-/-</sup> mouse embryonic fibroblasts led us to investigate whether compartmentalization plays a regulatory role. Decreased nuclear localization, neddylation, and defective proliferation in *SCCRO*<sup>-/-</sup> mouse embryonic fibroblasts were rescued by transgenic expression of *SCCRO*. Expression of reciprocal *SCCRO* and Cul1-binding mutants confirmed the requirement for *SCCRO* in nuclear translocation and neddylation of cullins *in vivo*. Nuclear translocation of Cul1 by tagging with a nuclear localization sequence allowed neddylation independent of *SCCRO*, but at a lower level. We found that in the nucleus, *SCCRO* enhances recruitment of Ubc12 to Cul1 to promote neddylation. These findings suggest that *SCCRO* has an essential role in neddylation *in vivo* involving nuclear localization of neddylation components and recruitment and proper positioning of Ubc12.

The cullin family of proteins anchor Cullin RING finger-type E3 ubiquitination complexes (CRL)<sup>4</sup> that regulate the degradation and activity of proteins involved in a wide range of cellular processes (1, 2). Several reports have shown that the activity of CRL complexes is primarily regulated by neddylation, a process

mechanistically analogous to ubiquitination, where the cullin family of proteins are covalently modified by ubiquitin (Ub)-like protein Nedd8 (3–11). All cullin proteins in humans (Cul1, Cul2, Cul3, Cul4a, Cul4b, Cul5, and PARC) are subject to neddylation, with the exception of Cul7 (12, 13). Lethality resulting from knocking out core components in all organisms studied (except budding yeast) emphasizes the indispensable role of neddylation in normal cellular function (14–20).

Like ubiquitination, neddylation involves a sequential, tripartite enzymatic cascade (21, 22). The vertebrate enzymes for neddylation are the heterodimeric complex APP-BP1/Uba3 (E1) and Ubc12 or Ube2f (E2) (21, 23–25). The presence of enzymatic activity in the RING domain of Roc1 combined with its requirement and sufficiency to promote Nedd8 conjugation *in vitro* supports a role for Roc1 as the E3 for neddylation (26). Recent work identified a novel protein *SCCRO*/DCUN1D1/DCN1 (squamous cell carcinoma-related oncogene/defective in cullin neddylation 1, domain containing 1/defective in cullin neddylation) that binds to components of the E3 complex for neddylation (Ubc12 and Cullin-Roc1) and increases neddylation efficiency *in vitro* (27–30). Biochemical studies and structural modeling suggest that DCN1 functions as an E3 promoting neddylation by reducing nonspecific Rub1 (Nedd8 in mammals) discharge and directing the active site of Ubc12 toward the acceptor lysine in Cdc53 (Cul1 in mammals) (31). These observations suggest that *SCCRO* is a nonessential component of the neddylation E3 complex that increases efficiency of the reaction. In contrast to *in vitro* observations, *in vivo* studies suggest that *SCCRO* has an essential role in neddylation, as knocking out *SCCRO* orthologs DCN1/Dcn1p in *Caenorhabditis elegans* and *Saccharomyces cerevisiae* results in reduced Cul3 neddylation and lethality (30). Our work shows that although *SCCRO*<sup>-/-</sup> mice are viable, neddylation is reduced, and these mice have several developmental defects including male specific infertility, runting, and high rates of perinatal mortality.<sup>5</sup> In this work we show that in addition to assembling the neddylation E3 complex, *SCCRO* facilitates the subcellular localization of neddylation components, which is required for cullin neddylation *in vivo*. These findings help to explain the differential requirement for *SCCRO* in *in vitro* and *in vivo* models.

## EXPERIMENTAL PROCEDURES

**Plasmids**—Human *SCCRO* and its mutants were cloned into pCMV-HA vector (Clontech) by standard PCR methodology

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<sup>1</sup> Present address: Dept. of Cardiothoracic Surgery, Mount Sinai School of Medicine, New York, NY 10029.

<sup>2</sup> To whom correspondence may be addressed: Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York NY 10065. Fax: 212-717-3302; E-mail: ramanaty@mskcc.org.

<sup>3</sup> To whom correspondence may be addressed: Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, NY 10065. Fax: 212-717-3302; E-mail: singhb@mskcc.org.

<sup>4</sup> The abbreviations used are: CRL, Cullin RING ligases; DCUN1D1, defective in cullin neddylation 1, domain containing 1; DCN1, defective in cullin neddylation; E1, ubiquitin (or Nedd8)-activating enzyme; E2, ubiquitin (or Nedd8) carrier protein; E3, ubiquitin (or Nedd8)-protein isopeptide ligase; MEF, mouse embryonic fibroblast; NES, nuclear export sequence; NLS, nuclear localization signal; *SCCRO*, squamous cell carcinoma-related oncogene; Ub, ubiquitin.

<sup>5</sup> A. J. Kaufman, G. Huang, R. Ryan, L. Hury, G. Hunnicut, Y. Romin, P. Morris, K. Manora, Y. Ramanathan, and B. Singh, unpublished data.

## Translocation and Assembly of Neddylation E3 by SCCRO

(27). Double HA-tagged human *Roc1* cDNA as well as *Cul1* and its mutants were either obtained from a commercial source (Addgene) or developed using GeneTailor Site-directed Mutagenesis System (Invitrogen) and sequence verified. Nuclear export sequence (NES) (32) and nuclear localization signal (NLS) (33) tags were coded by 5'-GACCTCCAAAAGAAGC-TGGAGGAGCTGGAGCTGGACGAG-3' and 5'-GATCCAAAAGAAGAGAAAGGTAGATCCAAAGAGAAAGAA-GAAGTA-3', respectively, and were cloned into the N-terminal region of *SCCRO* or *Cul1*.

**Cell Culture, Growth Assay, Transfection, Retrovirus Infection, and Fractionation**—Primary mouse embryonic fibroblasts (MEFs) were harvested from littermate wild-type, heterozygous, or *SCCRO*<sup>-/-</sup> embryos at age 12 days after coitus. MEF and human U2OS were cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (and containing 55  $\mu$ M 2-mercaptoethanol for MEF) at 37 °C in 5% CO<sub>2</sub>. For proliferation assays, 0.2 million MEFs were seeded, cells were harvested daily, and cell numbers were counted, in triplicate. To introduce the *SCCRO* or *SCCRO* mutant into MEFs, we used the pBABE retroviral system. Transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Cytoplasmic and nuclear fractions were prepared using established protocol with modifications. In brief, cells were harvested and resuspended in buffer A, pH 7.9 (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM DTT) for 15 min on ice. Nonidet P-40 was then added to 0.6%. After a quick vortex, nuclei were separated from cytoplasm by centrifugation at 10,000  $\times$  *g* for 30 s. Nuclei were further lysed with an equal volume of radioimmune precipitation assay buffer.

**UV Irradiation**—MEFs were irradiated at 300 millijoules using GS Gene Linker (Bio-Rad). Cells were then incubated for another hour before harvest for immunoblotting or fixation for immunofluorescence.

**Antibodies and Immunoprecipitation**—The following antibodies were used in this study: anti-Cul1 (Zymed Laboratories), anti-Cul3 (BD Biosciences), anti-Roc1 (Abcam), anti-Ub (P4D1) (Santa Cruz Biotechnology), anti-HA (Covance), anti-Myc (Santa Cruz Biotechnology), anti-FLAG (Sigma), anti-tubulin (Calbiochem), and anti-P300/CBP-associated factor (Santa Cruz Biotechnology). Anti-*SCCRO* monoclonal antibody was produced and utilized as described previously (27). Immunoprecipitations were performed essentially as described earlier (34). In brief, cells were lysed using mammalian cell lysis buffer (Cell Signaling). Lysate was incubated with 20  $\mu$ l of anti-HA-conjugated agarose beads (Abcam) by gentle rocking at 4 °C overnight. The beads were washed three times with lysis buffer and once with PBS. Bound proteins were eluted by the addition of 2 $\times$  Laemmli buffer, resolved on SDS-polyacrylamide gels, and analyzed by immunoblotting.

**In Vivo and In Vitro Neddylation Assay**—For *in vivo* neddylation, cell lysates were directly subjected to immunoblotting for cullin(s). *In vitro* neddylation was performed essentially as described earlier (27). The source of Cul-Roc1 substrate was either endogenous or transfected cullins. The reaction mixture contained 2  $\mu$ M recombinant Nedd8, 10 nM E1, and 4 mM ATP.

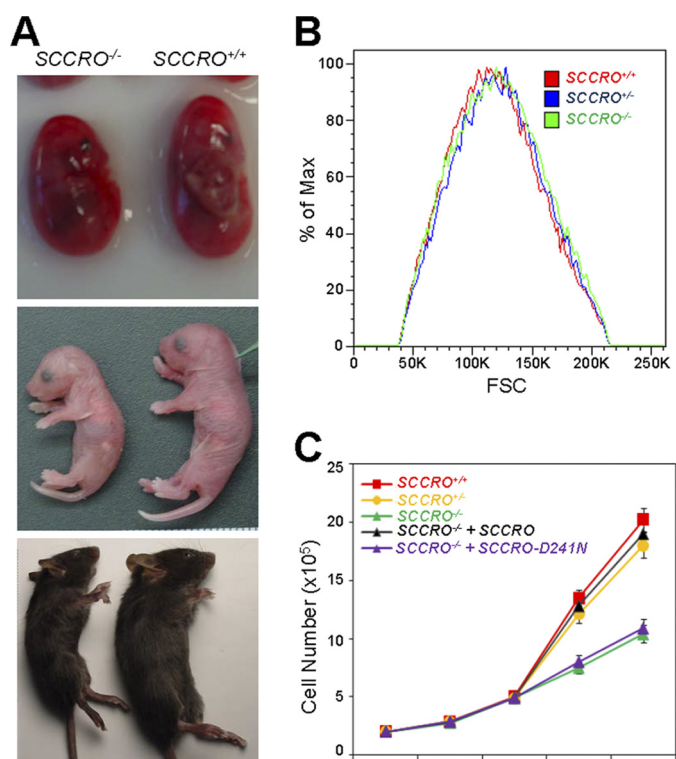
**Immunofluorescence**—Cy3-conjugated anti-HA, anti-Myc, and anti-FLAG and FITC-conjugated anti-HA antibodies were obtained from a commercial source (Jackson ImmunoResearch Laboratories). Cells transfected with plasmid(s) were seeded in 6-well plates with cover glass. Twenty four hours after transfection, cells were washed (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% FBS) for 30 min and stained overnight at 4 °C with fluorochrome-conjugated antibodies. The cells were washed three times with PBS, counterstained with 4',6-diamidino-2-phenylindole (DAPI), covered by ProLong Gold Antifade Reagent (Invitrogen), and examined with a Leica inverted confocal microscope fitted with appropriate fluorescence filters. For Cul1 staining, FITC-conjugated goat anti-rabbit secondary antibody was used. To calculate the percentage of cells with nuclear or nonnuclear localization, a minimum of 200 cells were counted for each experiment. All experiments have been repeated at least three times. Fisher's exact test was used to compare results from localization studies, and a *p* < 0.05 was considered significant.

**Analysis of Mutant Mice**—Spleen, lymph nodes, and other organs were dissected free from littermates. Tissues were minced into dissociated cells in equal volume of media. The cell numbers were then counted, and cell sizes were determined by forward scatter analysis using flow cytometry.

## RESULTS

**SCCRO Has Essential Function in Vivo**—Given the discrepancy in the function of *SCCRO* *in vivo* and *in vitro*, we first aimed to develop a model to define the requirement of *SCCRO* *in vivo* and assess mechanisms involved. Because body size changes can be attributed to either a decrease in cell size, cell number or both, runting in *SCCRO*<sup>-/-</sup> mice provides an attractive model to investigate the *in vivo* requirement and functions of *SCCRO* (Fig. 1A) (35). We first confirmed that the smaller size of *SCCRO*<sup>-/-</sup> mice is independent of gender or genetic background.<sup>5</sup> In addition, no obvious organ or hormonal defects that could explain the reduced body size were identifiable.<sup>5</sup> We assessed cell size and number in several organs from littermate *SCCRO*<sup>-/-</sup> and *SCCRO*<sup>+/+</sup> mice, showing no alterations in cell size in any organs tested (data not shown). As fewer cells were present in spleen and lymphatic tissues of *SCCRO*<sup>-/-</sup> mice, we focused on defects in proliferation. We isolated primary MEFs from day 12 *SCCRO*<sup>+/+</sup>, *SCCRO*<sup>+/-</sup>, and *SCCRO*<sup>-/-</sup> littermate embryos. No detectable morphological differences were observed in MEFs of different genotypes. Forward scatter analysis using flow cytometry also revealed no significant differences in the size of these MEFs (Fig. 1B). However, a decrease in proliferation was seen in *SCCRO*<sup>-/-</sup> MEFs, confirming that the loss of *SCCRO* has physiological consequences (Fig. 1C).

**In Vivo Functions of SCCRO Involve Its Neddylation Activity**—To determine whether reduced proliferation of *SCCRO*<sup>-/-</sup> MEF is related to defective neddylation, we assessed levels of neddylated cullins. Despite similar levels of neddylation components (APP-BP1, Cul1, Ubc12, and Nedd8) in *SCCRO*<sup>-/-</sup> and *SCCRO*<sup>+/+</sup> MEFs, there was a decrease in the basal level of



**FIGURE 1. SCCRO enhances cell proliferation by promoting cullin neddylation.** *A*, representative  $SCCRO^{+/+}$  and  $SCCRO^{-/-}$  mice at embryo (top), birth (middle), and weaning (bottom) stages. *B*, cell size of  $SCCRO^{+/+}$ ,  $SCCRO^{+/-}$ , and  $SCCRO^{-/-}$  MEFs determined by forward scatter analysis (FSC), showing no differences. *C*, cell proliferation assay showing reduced proliferation in  $SCCRO^{-/-}$  relative to  $SCCRO^{+/+}$  and  $SCCRO^{+/-}$  MEFs. Retroviral delivery of SCCRO but not  $SCCRO-D241N$  rescued the proliferation defect in  $SCCRO^{-/-}$  MEFs.

neddylated Cul1 in  $SCCRO^{-/-}$  MEF lysates as detected by immunoblotting (Fig. 2*A*, lanes 1 and 2, and supplemental Fig. 1). This difference was even more pronounced when neddylation was activated by supplementing the lysates with E1 (APP-BP1/Uba3), Nedd8, and ATP prior to immunoblotting (Fig. 2*B*). Moreover, global ubiquitination was also reduced in  $SCCRO^{-/-}$  MEFs, suggesting that reduced cullin neddylation has functional consequences (Fig. 2*B*).

To determine whether the defect in cell proliferation is due to a loss of SCCRO-augmented neddylation, we expressed SCCRO in  $SCCRO^{-/-}$  MEFs by retroviral infection and assessed effects on neddylation and proliferation. We expressed  $SCCRO-D241N$ , a mutant previously shown to lose neddylation promoting activity, as a control (27). Immunoblotting analysis showed that the levels of SCCRO in  $SCCRO^{-/-}$  MEFs after retroviral infection were equal to that in  $SCCRO^{+/+}$  MEF (Fig. 2*C*). Expression of SCCRO but not  $SCCRO-D241N$  rescued both defective proliferation and Cul1 neddylation in  $SCCRO^{-/-}$  MEF to wild-type levels (Figs. 1*C* and 2*C*). These findings suggest an association between the neddylation activity of SCCRO and its function *in vivo*.

**SCCRO Promotes Nuclear Translocation and Neddylation of Cul1**—The difference in requirement for SCCRO *in vitro* and *in vivo* may be explained by several factors, including post-translational modifications, compartmentalization, and/or requirement for other proteins/factors in the reaction. Work from

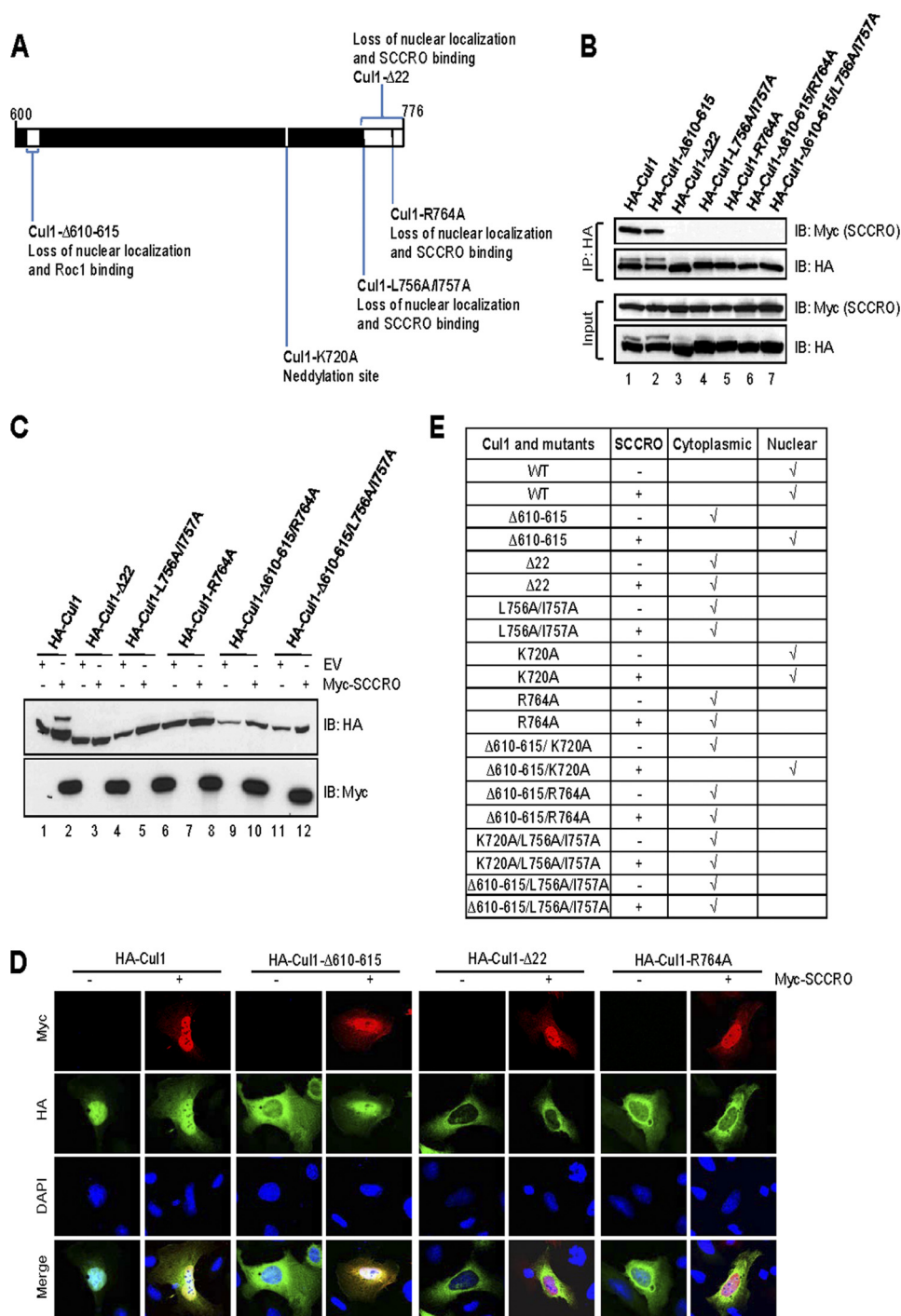
Furukawa *et al.* (36) suggests that nuclear translocation may be required for cullin neddylation. Accordingly, we compared the subcellular distribution of Cul1 in  $SCCRO^{-/-}$  and  $SCCRO^{+/+}$  MEFs. Interestingly, Cul1 was primarily nuclear in a significantly higher proportion of  $SCCRO^{+/+}$  (174/200; 87%) compared with  $SCCRO^{-/-}$  (50/200; 25%) MEFs (Fig. 2*D*). Transgenic expression of SCCRO, but not  $SCCRO-D241N$ , in  $SCCRO^{-/-}$  MEFs rescued the nuclear localization of Cul1 (Fig. 2*D*). To confirm that these observations are of physiological significance, we assessed cullin localization in response to neddylation-promoting stimuli. Among the various conditions tested, we found that UV irradiation resulted in the greatest increase in Cul1 neddylation and nuclear localization in  $SCCRO^{+/+}$  MEFs (Fig. 2, *A* and *D*). In contrast, no change in Cul1 neddylation or nuclear localization was observed in  $SCCRO^{-/-}$  MEFs upon UV exposure (Fig. 2, *A* and *D*). Combined, these observations suggest that SCCRO plays an important role in nuclear translocation of Cul1, which may explain differences in its requirement for neddylation *in vivo* and *in vitro*.

**SCCRO-mediated Nuclear Transport Is Required for Cul1 Neddylation *In Vivo***—To define the cause for the differential requirement for SCCRO *in vivo* and *in vitro*, we elected to use U2OS, an established cell line that is more amenable to experimental manipulations than MEFs. For *in vivo* neddylation, U2OS cells were transfected with HA-Ubc12 and HA-SCCRO or HA-SCCRO-D241N and lysates subjected to immunoblotting for Cul1 or Cul3. For *in vitro* neddylation, reactions were activated by the addition of Nedd8, E1, and ATP to the lysates prior to immunoblotting. Consistent with prior findings, expression of Ubc12 alone was sufficient to promote cullin neddylation *in vitro* with co-expression of SCCRO enhancing the reaction (Fig. 3*A*, compare lanes 4 and 5 with 6). In contrast, whereas SCCRO promoted Cul1 and Cul3 neddylation *in vivo*, expression of Ubc12 alone had no effect (Fig. 3*A*, lanes 4 and 5). Co-expression of SCCRO with Ubc12 synergistically enhanced Cul1 neddylation (Fig. 3*A*, lane 6). Given that Ubc12 is primarily nuclear, these findings suggest that SCCRO-promoted nuclear translocation may be required for neddylation of cullins *in vivo*.

To assess the requirement of SCCRO-promoted nuclear translocation of Cul1 for neddylation, we developed a SCCRO construct with a canonical NES to block its nuclear translocation. Co-transfection of HA-Cul1 with HA-SCCRO or HA-NES-SCCRO in U2OS cells followed by immunoblotting for HA showed that SCCRO but not NES-SCCRO promotes Cul1 neddylation (Fig. 3*B*; compare lanes 2 and 3). Taken together, these findings suggest that SCCRO enhances cullin neddylation by promoting its nuclear translocation.

**Binding Is Required for SCCRO-promoted Nuclear Translocation and Neddylation of Cul1**—Structural modeling shows that the C terminus of SCCRO binds to the Cul1 C terminus in the terminal helix and the loop between the C-terminal two  $\beta$  strands (37). Consistent with this, we found that deletion or mutations in selected conserved residues within the C terminus of Cul1 resulted in loss of binding based on immunoprecipitation assays (Fig. 4, *A* and *B*). To determine the requirement for binding to SCCRO for nuclear localization and neddylation, we expressed loss of binding mutants in U2OS



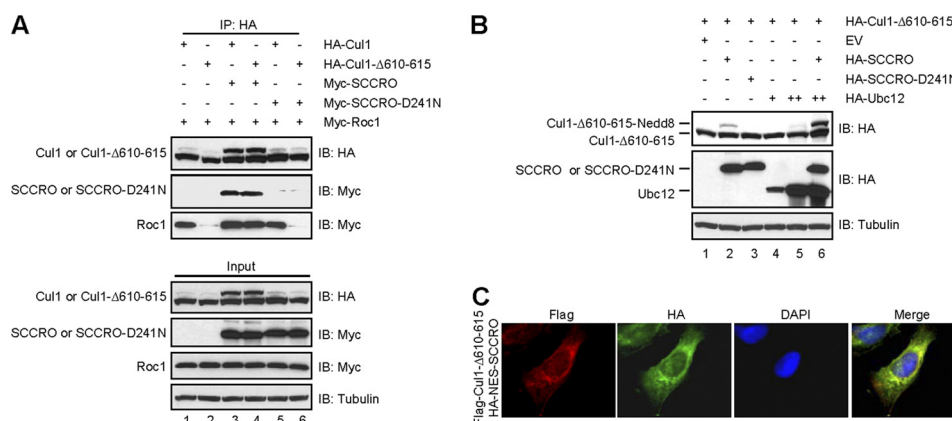


**FIGURE 4. Binding to SCCRO is required for neddylaton and nuclear localization of Cul1.** *A*, schematic representation of the C terminus of Cul1 showing cullin mutants used in these experiments. *B*, immunoblot on lysates from U2OS cells transfected with Myc-SCCRO and HA-Cul1 or selected mutants probed with the indicated antibodies following HA immunoprecipitation. SCCRO binds to HA-Cul1 and HA-Cul1-Δ610–615 (lanes 1 and 2) but not C-terminal Cul1 mutants (lane 3–7). *C*, immunoblots for Cul1 on lysates from U2OS cells co-transfected with Cul1 or its mutants with or without SCCRO showing mutants that lose SCCRO binding cannot be neddylated. *D*, immunofluorescence using Cy3-conjugated anti-Myc antibody and FITC-conjugated anti-HA antibody on U2OS cells transfected with HA-Cul1 or indicated HA-tagged Cul1 mutants with or without Myc-SCCRO. HA-Cul1 (first lane) was primarily nuclear (~74%) whereas Cul1 mutants (third, fifth, and seventh lanes) were primarily cytoplasmic in the absence of SCCRO expression. Co-expression of SCCRO increased the proportion of HA-Cul1 and rescued the localization defect of Cul1-Δ610–615 with primary nuclear localization. *E*, U2OS cells were transfected with Cul1 or its mutants with or without SCCRO. The results of Cul1 localization monitored by immunofluorescence by counting 200 cells for each transfection is tabulated. Nuclear localization by SCCRO requires the Cul1 C-terminal sequence.

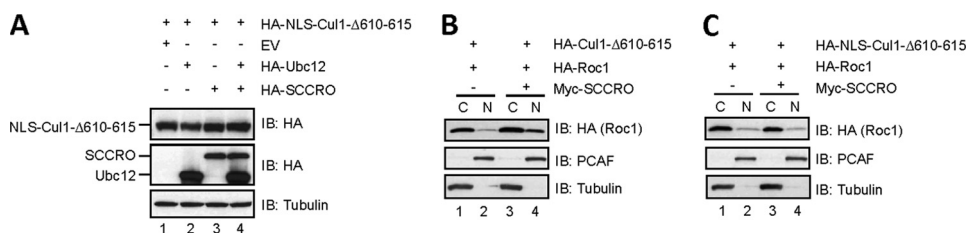
resulted in nuclear translocation of HA-Cul1-Δ610–615 (Fig. 4D). Validating a primary role for SCCRO in compartmentalization of cullins, the Cul1-Δ610–615/R764A double mutant, that loses both Roc1 and SCCRO binding, could not be translocated to the nucleus under any conditions tested (Fig. 4E).

Interestingly, defective neddylaton of Cul1-Δ610–615 was also salvaged by co-expression of SCCRO but not SCCRO-D241N (Fig. 5B, lanes 2 and 3). In contrast, expression of NES-SCCRO was unable to promote nuclear translocation of Cul1-Δ610–615 (Fig. 5C). Because Roc1 is required for cullin

## Translocation and Assembly of Neddyltion E3 by SCCRO



**FIGURE 5. SCCRO rescues nuclear localization and neddylation of Cul1-Δ610-615.** *A*, immunoblot on lysates from U2OS cells transfected with the indicated constructs and probed with anti-Myc or anti-HA antibodies following HA immunoprecipitation showing increased interaction between Roc1 and HA-Cul1-Δ610-615 with SCCRO co-expression (compare lanes 4 with 2 and 6). *B*, immunoblot on lysates from U2OS cells transfected with the indicated constructs probed with anti-HA antibodies showing rescue of HA-Cul1-Δ610-615 neddylation defect by SCCRO (top panel, lanes 2 and 6). *C*, immunofluorescence on U2OS cells co-transfected with FLAG-Cul1-Δ610-615 and HA-NES-SCCRO using Cy3-conjugated anti-FLAG antibody and FITC-conjugated HA antibody showing nuclear exclusion of SCCRO and loss of nuclear translocation of Cul1-Δ610-615.

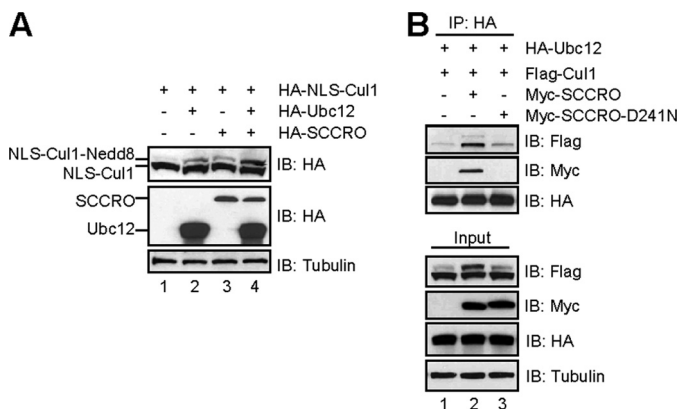


**FIGURE 6. NLS-Cul1-Δ610-615 cannot be neddylated *in vivo*.** *A*, immunoblot on lysates from U2OS cells transfected with the indicated constructs showing that neither HA-Ubc12 nor HA-SCCRO expression promotes neddylation of NLS-Cul1-Δ610-615. *B* and *C*, immunoblot on cytoplasmic (C) and nuclear (N) fractions of lysates from U2OS cells transfected with the indicated constructs probed with anti-HA antibody showing enrichment of Roc1 in the nuclear fraction of cells co-expressing SCCRO with HA-Cul1-Δ610-615 (*B*, top panel, lane 4) but not with HA-NLS-Cul1-Δ610-615 (*C*, top panel, lane 4). P300/CBP-associated factor (PCAF) and tubulin are nuclear and cytoplasmic loading controls, respectively.

neddylation, these findings suggest that SCCRO may help to recruit Roc1 to Cul1-Δ610-615 in the cytoplasm prior to nuclear translocation (36). To investigate this possibility, we performed immunoblots after HA immunoprecipitation of lysates from U2OS cells co-transfected with Myc-Roc1 and HA-Cul1-Δ610-615 with Myc-SCCRO or Myc-SCCRO-D241N. Binding between Cul1-Δ610-615 and Roc1 was enriched with Myc-SCCRO co-expression but not Myc-SCCRO-D241N (Fig. 5A, lane 4). To validate these findings, we tagged Cul1-Δ610-615 with nuclear localization sequence (NLS-Cul1-Δ610-615) thereby targeting it to the nucleus independent of SCCRO. Co-expression of SCCRO could not rescue neddylation of NLS-Cul1-Δ610-615, suggesting that SCCRO recruits Roc1 to Cul1-Δ610-615 prior to nuclear transport (Fig. 6A). Fractionation of the transfected cells followed by immunoblot analysis showed that the nuclear fraction of Roc1 increased with co-expression of Cul1-Δ610-615 with SCCRO (Fig. 6B, lane 4), but not in cells coexpressing NLS-Cul1-Δ610-615 (Fig. 6C, lane 4). Combined, these findings suggest that binding between SCCRO and Cul1 is required for both nuclear transport and neddylation *in vivo*.

**SCCRO Promotes Recruitment of Ubc12 to the Neddylation E3 Complex in the Nucleus**—The role of SCCRO in nuclear localization of Cul1 does not explain its effect on neddylation in *in vitro* reactions. To determine whether nuclear transport is the only contribution of SCCRO in augmenting neddylation, we developed a system to monitor neddylation independent of

Cul1 nuclear localization. Transfection of U2OS cells with Cul1 tagged with nuclear localization sequence (NLS-Cul1) followed by immunofluorescence confirmed its nuclear localization (supplemental Fig. 2). Co-transfection of Ubc12 was sufficient to promote neddylation of NLS-Cul1 but not wild-type Cul1. Expression of SCCRO also enhanced neddylation of NLS-Cul1. Co-expression of SCCRO with Ubc12 synergistically enhanced neddylation of NLS-Cul1 *in vivo* (Fig. 7A, lane 4). These findings suggest that in addition to subcellular localization, SCCRO promotes neddylation by other mechanisms *in vivo*. Scott *et al.* showed that SCCRO restricts the otherwise flexible Roc1-Ubc12~Nedd8 and orients the Ubc12 active site toward the Cul1 acceptor Lys (31). This increases the efficiency of Nedd8 transfer to Cul1. However, its effect on neddylation of NLS-Cul1 *in vivo* appears more pronounced than that observed in *in vitro* reactions where molar excess of SCCRO is required to see any activity. Accordingly, in addition to proper positioning to facilitate Nedd8 transfer, we questioned whether SCCRO also promotes assembly of the neddylation E3 complex by enhancing recruitment of E2 *in vivo*. Immunoprecipitation of lysates for HA-Ubc12 showed an increase in Cul1 binding when SCCRO was co-expressed (Fig. 7B). We have shown previously that SCCRO preferentially binds to Ubc12~Nedd8 thioester, suggesting that recruitment of Ubc12 by SCCRO constitutes a functional complex. Combined, these observations suggest that in addition to Cul1 nuclear localization, SCCRO enhances ned-



**FIGURE 7. SCCRO promotes recruitment of Ubc12 onto Cul1-Roc1 complex after nuclear translocation.** *A*, immunoblot on lysates from U2OS cells transfected with the indicated constructs showing an increase in neddylation of HA-NLS-Cul1 with co-expression of HA-Ubc12 or HA-SCCRO (lanes 2 and 3). A synergistic effect was seen with co-expression of both HA-Ubc12 and HA-SCCRO (lane 4). *B*, immunoblot on lysates from U2OS cells transfected with the indicated constructs following anti-HA immunoprecipitation showing an increase in interaction between Ubc12 and Cul1 with SCCRO co-expression (lane 2).

neddylation E3 activity *in vivo* by promoting recruitment of E2 to the neddylation complex.

## DISCUSSION

Inasmuch as mechanisms are highly conserved, conjugation of Ub and Ub-like protein also share factors regulating reaction dynamics (38, 39). The rate-limiting step in Ub and Ub-like protein conjugation is E3 activity. Regulation of E3 activity is most closely regulated for ubiquitination by CRL-type ligases and neddylation of cullins. In both reactions, the RING domain in Roc1 provides the enzymatic activity, and assembly of the complex serves as the rate-limiting step. Although the presence of Roc1 along with charged E2 is sufficient *in vitro*, other factors are involved in *in vivo* ligation activity (4, 26, 36, 40, 41). For CRL complexes, activation of E3 requires neddylation of the cullin *in vivo*, but can proceed without cullin neddylation *in vitro* (5, 41). Prior studies show that neddylation affects CRL activity by promoting assembly of the complex through recruitment and positioning of the E2 to allow Ub transfer and chain extension on the substrate protein (8, 42, 43). Combined, our findings that SCCRO recruits E2 and the work of Scott *et al.* showing that it promotes a favorable orientation for efficient Nedd8 transfer suggest that SCCRO affects the activity of neddylation E3 complexes as neddylation does for cullins in CRL complexes.

Although this defines the biochemical role of neddylation and SCCRO in the respective E3 activity, it fails to explain why neddylation and SCCRO are required *in vivo* but not *in vitro* for Ub and Nedd8 conjugation, respectively. Our laboratory, as well as others, have shown that neddylation of cullins can occur efficiently *in vitro* in the absence of SCCRO (27, 28). This would suggest that the role of SCCRO may not be essential. However, decreased neddylation combined with the detrimental effects of knocking out SCCRO in *in vivo* model organisms suggest that its function is required (30). Our findings help to explain this difference by showing that SCCRO promotes nuclear translocation of Cul1-Roc1 complexes *in vivo*. We found that Ubc12

exists almost exclusively in the nucleus (supplemental Fig. 3). Accordingly, for neddylation to occur, Ubc12 either has to be translocated to cytoplasm or Cullin-Roc1 complexes to the nucleus. The importance of nuclear transport is supported by the finding that NLS-Cul1, but not wild-type Cul1, can be neddylated by the expression of Ubc12 alone. However, even after nuclear localization of Cul1 by tagging with NLS, co-expression of SCCRO with Ubc12 synergistically enhances neddylation, at levels much higher than that seen *in vitro*. These observations may be explained by enhanced recruitment of Ubc12 to Cul1-Roc1 by SCCRO *in vivo*.

Our findings raise several possibilities and questions. As the SCCRO-Cul1-Roc1 complex is relatively large, it requires active transport through a nuclear pore. However, none of the proteins in the complex contains a canonical nuclear targeting sequence. Although it is possible that a cryptic nuclear targeting sequence may be present, it is also possible that other unidentified proteins/factors may be involved in nuclear translocation. Second, given the conservation in reaction dynamics, these findings raise the possibility that CRL activity may also be regulated by compartmentalization. Interestingly, in our preliminary studies we found that neddylation of cullins primarily exist in the nucleus, raising the possibility of ubiquitination occurring in the nucleus (supplemental Fig. 4). These findings need to be reconciled with reports suggesting SCCRO3 acts at the membrane to promote neddylation (44). Similar to work from Ma *et al.* (45), we found that SCCRO3 functions as a tumor suppressor in human cancers rather than an oncogene like other SCCRO family members.<sup>6</sup> Our results suggest that SCCRO3 binds to Cul1 *in vivo* but has no independent neddylation activity, suggesting that its function may be context-dependent *in vivo*. Finally, as SCCRO4/DCUN1D4 and SCCRO5/DCUN1D5 both have a functional NLS in their N terminus, their role in neddylation remains to be explained.

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