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RAP80 interacts with the SUMO-conjugating enzyme UBC9 and is a novel target for sumoylation

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Abstract

RAP80, a nuclear protein with two functional ubiquitin interaction motifs (UIMs) at its N-terminus, plays a critical role in the regulation of estrogen receptor alpha and DNA damage response signaling. A yeast two-hybrid screen identified the SUMO-conjugating enzyme UBC9 as a protein interacting with RAP80. The interaction of RAP80 with UBC9 was confirmed by co-immunoprecipitation and GST pull-down analyses. The region between a.a. 122–204 was critical for the interaction of RAP80 with UBC9. In addition, we demonstrate that RAP80 is a target for SUMO-1 modification in intact cells. Expression of UBC9 enhanced RAP80 mono-sumoylation and also induced multisumoylation of RAP80. In addition to SUMO-1, RAP80 was efficiently conjugated to SUMO-3 but was only a weak substrate for SUMO-2 conjugation. These findings suggest that sumoylation plays a role in the regulation of RAP80 functions.

Introduction

Post-translational modifications are important in controlling the conformation, function, and activity of proteins and, as such, play a critical role in the regulation of many cellular processes. Ubiquitination and sumoylation represent a unique type of post-translational modification, in which ubiquitin or ubiquitin-like proteins are covalently conjugated to lysine residues in target proteins [1–6]. Polyubiquitination plays a key role in guiding the proteolytic degradation of proteins by the ubiquitin-proteasome system while monoubiquitination has been implicated in several processes, including the regulation of endocytosis, DNA-repair, and transcription [1; 2;7].

Members of the small ubiquitin-related modifier (SUMO) family are structurally related to ubiquitin [2–5]. SUMO-1 shows ~50% amino acid sequence identity to SUMO-2 and SUMO-3 while SUMO-2 and SUMO-3 exhibit ~95% amino acid sequence identity to each other. The pathway of sumoylation is analogous to that of ubiquitination, but involves a different set of enzymes. SUMO is activated by an E1-activating enzyme consisting of an AOS1/UBA2 heterodimer, transferred to the E2-conjugating enzyme, UBC9, and is subsequently (with the aid of various E3 ligases) attached to the ε-amino group of specific lysines in target proteins [2–5]. Sumoylation can be reversed by SUMO-cleaving enzymes. In some cases, UBC9 can catalyze sumoylation without the help of E3 ligases, but its efficiency and specificity is increased by E3 ligases. Sumoylation is emerging as a critical modifier of the conformation and activity of many, mostly nuclear, proteins with functions in many cellular processes,

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including transcriptional regulation, DNA damage repair, histone modification, and apoptosis [6;8–11].

Receptor-associated protein 80 (RAP80) (HUGO nomenclature: ubiquitin-interaction motif containing 1 or UIMC1) is a nuclear protein identified in our laboratory [12;13]. It contains two putative Cys-X_2-Cys-X_{11}-His-X_3-Cys zinc finger-like motifs at its carboxyl-terminus and two ubiquitin-interacting motifs (UIMs) near its amino-terminus. Recently, we demonstrated these UIMs are able to bind (poly)ubiquitin [12]. In addition, we reported that RAP80 can interact with the estrogen receptor α (ERα) in an agonist-dependent manner and regulate its transcriptional activity. Moreover, RAP80 interacts with the tumor suppressor BRCA1 and is essential for the translocation of BRCA1 to DNA damage foci after irradiation [14–17]. The UIMs play a critical role in these activities of RAP80 [12;17].

To obtain further insights into the functions of RAP80, we performed a yeast two-hybrid screen to identify potential RAP80 interacting proteins. In this study, we report that the SUMO E2-conjugating enzyme UBC9 was identified as a novel RAP80 binding partner. The interaction was confirmed by co-immunoprecipitation and GST pull-down assays. In addition, we demonstrate that RAP80 is a target of sumoylation in intact cells and that UBC9 overexpression promotes multi-sumoylation of RAP80. RAP80 sumoylation was independent of its UIMs. Ionizing radiation (IR) increases RAP80 ubiquitination but not sumoylation. Both ubiquitination and sumoylation may play an important role in regulating the activity and function of RAP80.

Materials and methods

Plasmids

pLXIN-3×FLAG-RAP80, pLXIN-3×FLAG-RAP80ΔUIM, ΔC582, ΔC504, ΔC404, ΔC304, ΔC204, and ΔC122 were described previously[12;13]. Details on other RAP80, UBC9, SUMO-1, 2, and 3 plasmids used in this study are provided in Supplementary data.

Yeast two-hybrid screening

The Gal4 yeast two-hybrid system was purchased from BD Biosciences. Library screening was conducted according to the manufacturer's instructions and details on it are provided in Supplementary data.

GST pull-down assay

The methods of purifying GST or GST-UBC9 fusion proteins and their binding to [^{35}S]-methionine-labeled RAP80 were previously described [12].

Co-immunoprecipitation assay

HeLa or HEK293T cells were transiently transfected with pLXIN-3×FLAG-RAP80 (full-length or mutants) and pCMV-Myc-UBC9 using Fugene 6 transfection reagent (Roche, Indianapolis, IN). Forty-eight h after transfection, cells were processed as previously described [17].

Sumoylation analysis

HEK293T cells were transiently transfected with pLXIN-3×FLAG-RAP80 (full-length or mutants), pCMV-HA-SUMO-1, and pCMV-Myc-UBC9 as indicated. Forty-eight h later, cells were harvested and lysed for 1 h in modified RIPA buffer (50 mM Tris/HCl, pH 7.8, 150 mM NaCl, 5 mM EDTA, 15 mM $MgCl_2$, 0.5% NP-40, 0.3% Triton X-100, 0.5% sodium deoxycholate, 1 mM dithiothreitol, 10 mM N-ethylmaleimide, and 0.1% SDS) containing

protease inhibitor cocktails. The cell lysates were centrifuged for 10 min at 14,000 × g and 4 °C. The supernatants were incubated overnight with anti-HA antibody (Roche) and protein-G agarose (Sigma) to pull down sumoylated proteins. The agarose was then washed three times with RIPA buffer. The bound proteins were solubilized in sample buffer and analyzed by Western blot analysis using anti-FLAG M2 antibody.

Results

Identification of UBC9 as a RAP80-interacting protein

To identify proteins that interact with RAP80 or modulate RAP80 function, a yeast two-hybrid screen was performed using full-length RAP80 as bait and an E17 mouse embryo library as prey. From the 1.5×10^6 cDNA clones screened, several positive clones were isolated. One of the positive clones encoded the full-length region of the SUMO-conjugating enzyme UBC9.

The interaction between RAP80 and UBC9 was validated by co-immunoprecipitation analysis. Myc-UBC9 and FLAG-RAP80 were transiently expressed in HeLa cells and cell lysates incubated with anti-FLAG M2 affinity resin to isolate FLAG-RAP80 protein complexes. FLAG-RAP80 protein complexes were then examined by Western blot analysis. As shown in Fig. 1A, UBC9 was co-immunoprecipitated with FLAG-RAP80 in a specific manner. These data indicate that RAP80 and UBC9 interact with each other consistent with results of the yeast two-hybrid analysis.

We next examined the interaction of RAP80 with UBC9 by *in vitro* pull-down analysis using GST-UBC9 fusion protein and *in vitro* translated, [^{35}S]-methionine labeled RAP80. GST-UBC9 protein complexes were isolated with glutathione-Sepharose beads and analyzed by SDS-PAGE. As shown in Fig. 1B, GST-UBC9 fusion protein effectively pulled down RAP80 (Fig. 1B). Very little RAP80 was bound to GST. These observations suggest that RAP80 interacts directly with UBC9.

Mapping the RAP80 region required for the interaction with UBC9

RAP80 contains two UIMs and two putative zinc fingers. The UIMs are critical for RAP80 function [12;17]. To determine which region of RAP80 was required for its interaction with UBC9, a series of RAP80 deletion mutants (Fig. 2A) were examined by co-immunoprecipitation analysis for their ability to bind UBC9. The results demonstrated that the UIMs of RAP80 were not required for its interaction with UBC9 (Fig. 2B). Carboxyl-terminal deletions up to Ser^{204} had little effect on the ability of RAP80 to pull-down UBC9; however, RAP80ΔC122 was unable to interact with UBC9 (Fig. 2C). These data indicated that the region between Arg^{122} and Ser^{204} is crucial for the interaction of RAP80 with UBC9 and that the 2 zinc finger-like motifs located at the carboxyl-terminus are not required for binding.

RAP80 is a novel target of sumoylation

Since UBC9 functions as an E2 SUMO conjugating enzyme, we determined whether RAP80 was a target of sumoylation. To investigate this, HEK293T cells were transfected with pLXIN-3×FLAG-RAP80, pCMV-HA-SUMO-1 in the presence or absence of pCMV-Myc-UBC9. Forty-eight hours later cell extracts were prepared and sumoylated proteins immunoprecipitated with an anti-HA antibody. Analysis of immunoprecipitated proteins by Western blot analysis with an anti-FLAG antibody showed that RAP80 was sumoylated. SUMO-1 has a calculated mass of 11.5 kD and migrates in SDS-PAGE at about 17 kD while RAP80 migrates at 105 kD [12;18]. Sumoylated RAP80 migrated at about 120 kD suggesting that it is mono-sumoylated (Fig. 3A). Often sumoylated RAP80 appeared as 2–3 bands migrating closely together what might represent monosumoylation of different RAP80 molecules at two distinct sites resulting in slightly different electrophoretic mobilities.

Co-expression with Myc-UBC9 greatly enhanced mono-sumoylation of RAP80. Moreover, several additional, higher molecular weight RAP80 species were observed suggesting that multiple lysines in RAP80 were sumoylated (Fig. 3A). These results suggested that UBC9 expression leads to multi-sumoylation of RAP80. Recent studies have reported that proteins can be poly-sumoylated *in vitro* [19;20]. To determine whether the higher molecular weight, sumoylated RAP80 species represents multi-monosumoylation or poly-sumoylation, we examined sumoylation of RAP80 by SUMO-1(KO) in which all lysines were mutated. SUMO-1(KO) cannot form a poly-SUMO-1 chain but can still mono-sumoylate proteins. As shown in Supplementary Fig. 1, RAP80 is still multi-sumoylated when co-expressed with UBC9 and SUMO-1(KO) supporting the conclusion that RAP80 is multi-monosumoylated on distinct lysines.

To determine whether RAP80 could be covalently modified by SUMO-2 and SUMO-3, HEK293T cells were transfected with pLXIN-3×FLAG-RAP80 and expression vectors encoding HA-tagged SUMO-1, -2, or -3 and 48 h later the level of sumoylated RAP80 analyzed. As shown in Fig. 3B, RAP80 was efficiently conjugated to SUMO-1 and -3 but was only a weak substrate for SUMO-2 conjugation.

IR induces translocation of RAP80 to DNA damage foci [14–17]. We, therefore, examined whether IR had an effect on RAP80 sumoylation. As shown in Fig. 3C, IR did not influence the extent of RAP80 sumoylation. In contrast to sumoylation, IR enhanced RAP80 ubiquitination.

The amino-terminus of RAP80 is sumoylated

Previously, we reported that the UIMs of RAP80 can bind (poly)ubiquitin and are of critical importance for several RAP80 functions [12;17]. We therefore examined whether the UIMs had a role in the sumoylation of RAP80. As shown in Supplementary Fig. 2, as wild type RAP80, FLAG-RAP80ΔUIM was efficiently mono-sumoylated. Deletion of the UIMs also did not affect the multi-sumoylation of RAP80 induced by exogenous UCB9 expression. These results suggest that the UIMs are not required for RAP80 sumoylation.

To obtain greater insight into the regions within RAP80 that are targeted for sumoylation, the effect of a series of carboxyl-terminal truncations on the sumoylation of RAP80 was examined. As was often observed for full-length RAP80, sumoylated RAP80ΔC582 appeared as doublet what might be due to mono-sumoylation of different RAP80 molecules. RAP80ΔC122, the shortest deletion mutant tested, was still sumoylated (Fig. 4A). These observations indicated that the amino-terminus of RAP80 contains lysine(s) that are targeted for sumoylation.

As shown in Fig. 4A, the amino terminus of RAP80 contains 17 lysine residues, some of which are part of a nuclear localization signal [13]. Sequence analysis for potential sumoylation consensus motifs (http://www.abgent.com/doc/sumoplot and http://bioinformatics.lcd-ustc.org/sumosp/prediction.php) revealed that this region does not contain any high probability sumoylation sites but has five low probability sumoylation sites at K9, K19, K31, K52, and K61. Because K52 received the highest score, we first examined the effect of the K52A single mutation on RAP80 sumoylation. The result in Fig. 4B shows that this mutation did not affect RAP80 sumoylation. Next, we examined the effects of the K9A, K19A, K31A, and K61A single mutations and the K19,52A double mutation on the sumoylation of RAP80ΔC122. None of these mutations affected sumoylation (Fig. 4C). Lastly, we generated a RAP80ΔC122(5K/A) mutant in which all 5 potential sites were mutated. Fig. 4C shows that RAP80ΔC122(5K/A) was still sumoylated.

To determine whether RAP80ΔC122 was multi-sumoylated when Myc-UBC9 was co-expressed, HEK293T cells were transfected with pLXIN-3×FLAG-RAP80ΔC122, pCMV-

HA-SUMO-1, and pCMV-Myc-UBC9. Forty-eight h later cells were collected and sumoylation of RAP80ΔC122 analyzed. The data show that UBC9 effectively promoted multi-sumoylation of RAP80ΔC122 (Supplementary Fig. 3).

Discussion

Previously we reported that RAP80 interacts with ERα in an agonist-dependent manner and regulates its stability and transcriptional activity [12]. Recently, we demonstrated that RAP80 interacts with the tumor suppressor BRCA1 and has a critical role in DNA damage repair response [17]. The UIMs which bind (poly)ubiquitin, play a critical role in these activities of RAP80. In this study, we identified using a yeast two-hybrid screen UBC9 as a novel interacting partner of RAP80. This interaction was confirmed by immunoprecipitation and *in vitro* GST-pulldown analysis.

Our study also demonstrates that RAP80 is a novel target of sumoylation. RAP80 was effectively conjugated to SUMO-1 and SUMO-3 and to a much lesser extent to SUMO-2. This is in agreement with other studies showing that proteins can exhibit different specificities for SUMO-1–3 conjugation [21]. Based on the size of the sumoylated RAP80, it was concluded that RAP80 is mostly monosumoylated. Deletion analysis indicated that the amino-terminus (aa 1–122) of RAP80 can be modified by sumoylation. Although many proteins are sumoylated at the ΨKXD/E consensus sequence, several studies have reported that proteins can be sumoylated at sites that do not conform to the ΨKXD/E consensus sequence. For example, PML and Smad4 are sumoylated at lysines in AKCP and VKYC, respectively, while Mdm2 and CREB are sumoylated and do not contain a ΨKXD/E sequence [4;22;23]. How these sites are recognized has not yet been established. Although the amino-terminus contains 17 lysine residues none of them are part of a high-probability ΨKXD/E sumoylation motif. Five low-probability motifs were identified; however, mutation of all five sites did not have any effect on the sumoylation of RAP80 suggesting that another lysine can be a target for sumoylation. Sumoylation of many proteins involves binding of UBC9 to the consensus ΨKXD/E sumoylation motif; however, in the case of other proteins, including the transcription factor AP2, the co-repressor N-COR, and Sovar, UBC9 interacts with a region distant to the sumoylation site [24–26]. The amino-terminus is sumoylated but does not bind UBC9. Possibly, this sumoylation does not require a direct interaction between RAP80 and UBC9, and sumoylation is mediated by E3 ligases. Deletion analysis showed that the region of RAP80 between Arg^{122} and Ser^{204} is important for the interaction of RAP80 with UBC9; however, this region does not contain a high probability ΨKXD/E sumoylation motif. The binding of UBC9 to this region may involve different interaction motif and be implicated in a different activity of RAP80. Since RAP80 is part of a larger protein complex, it may recruit UBC9 to the complex thereby promoting the sumoylation of other proteins in the complex.

Sumoylation has been reported to be a critical modifier of the conformation and activity of many, mostly nuclear, proteins with functions in a variety of cellular processes [3;6;8–11]. For example, sumoylation plays an important role in the regulation of the NF-κB signaling pathway. After sumoylation NEMO, the regulatory subunit of IκB kinase, is translocated from the cytoplasm to the nucleus [27]. IκBα sumoylation targets the same lysines as ubiquitination thereby stabilizing the protein by preventing its ubiquitination and destruction by the proteasome [28]. Recently, we reported that RAP80 interacts with the tumor suppressor BRCA1 and translocates with BRCA1 to DNA damage foci after irradiation suggesting a role for RAP80 in DNA-damage repair signaling [17]. The UIMs play a critical role in the transfer of RAP80 to DNA damage foci, and as a consequence also in the transfer of BRCA1. Interestingly, ubiquitination and sumoylation have multiple functions in DNA damage repair signaling [6;8;9] and a number of proteins involved in DNA repair are subject to sumoylation. For example, Lys^{164} in proliferating cell nuclear antigen (PCNA) is a target for ubiquitination

as well as sumoylation [29]. By competing with ubiquitin for attachment to this lysine SUMO can block ubiquitination and inhibit damage-induced DNA repair. Rad51 and Rad52, which play a key role in homologous recombination, interact with UBC9 and are targets of sumoylation [30;31]. The DNA repair factor XPC becomes sumoylated after irradiation [32]. Sumoylation of DNA repair proteins can regulate their stability, protein-protein interactions, localization, and activity. Sumoylation did not affect the stability of RAP80 or its nuclear localization (Yan, J., unpublished observations). We hypothesize that this post-translational modification plays an important role in regulating the activity of RAP80 and its function in DNA damage response and possibly ERα signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GST, glutathione sulfotransferase; UIM, ubiquitin interaction motif; RAP80, receptor associated protein 80; ERα, estrogen receptor α; IR, ionizing radiation.

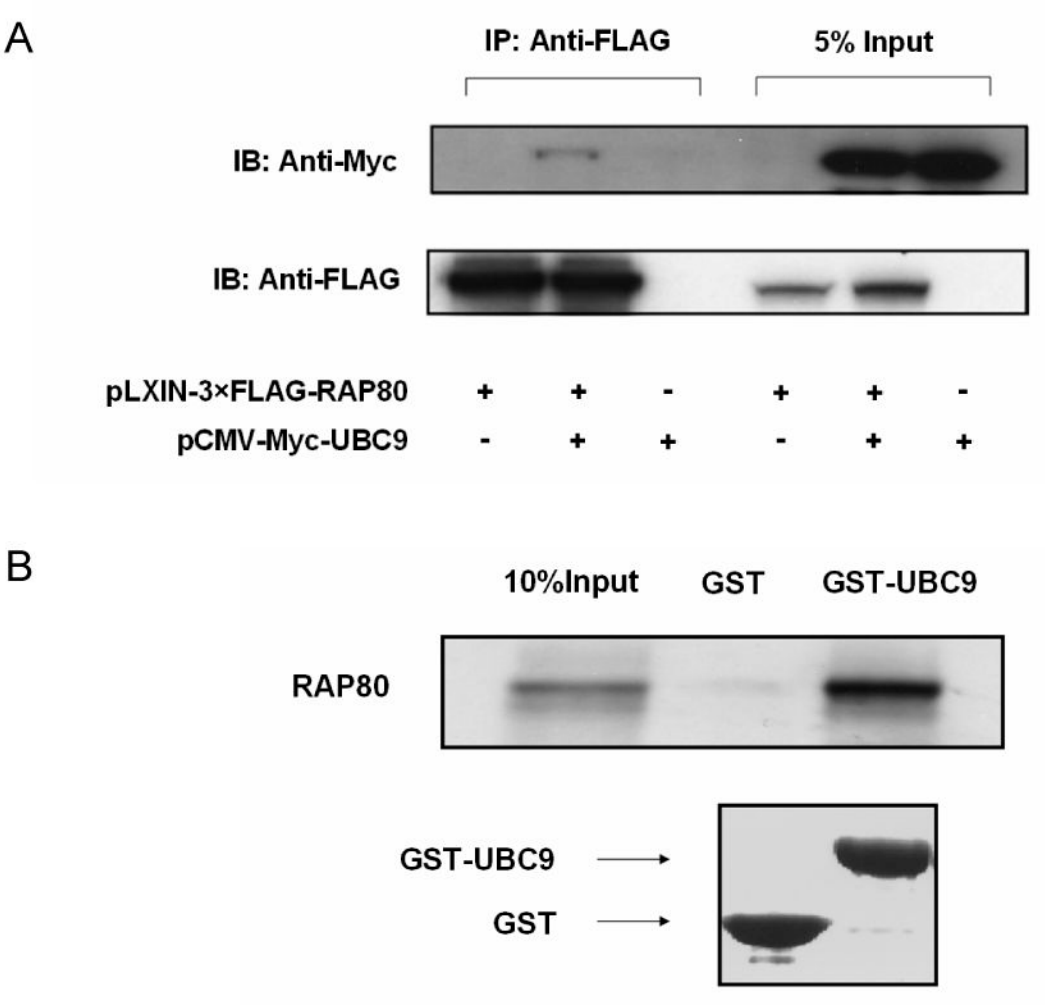

Fig 1.

RAP80 interacts with UBC9. A. HeLa cells were co-transfected with pCMV-Myc-UBC9 and pLXIN-3×FLAG-RAP80 expression plasmids as indicated. Cells were harvested 48 h later and cell lysates prepared. Five percent of each cell lysate was used directly for Western blot analysis while the remaining was incubated with anti-FLAG M2 affinity resin to isolate FLAG-RAP80 protein complexes. Protein complexes were then examined by Western blot analysis using anti-Myc and anti-FLAG antibodies. B. GST and GST-UBC9 fusion protein were bound to glutathione-Sepharose 4B beads and then incubated with [^{35}S]-methionine-labeled RAP80. After 1.5 h incubation, beads were washed extensively and bound proteins solubilized. Radiolabeled proteins were analyzed by SDS-PAGE and visualized by autoradiography. The first lane showed 10% of the input of radiolabeled RAP80. GST and GST-RAP80 proteins are shown in the lower panel.

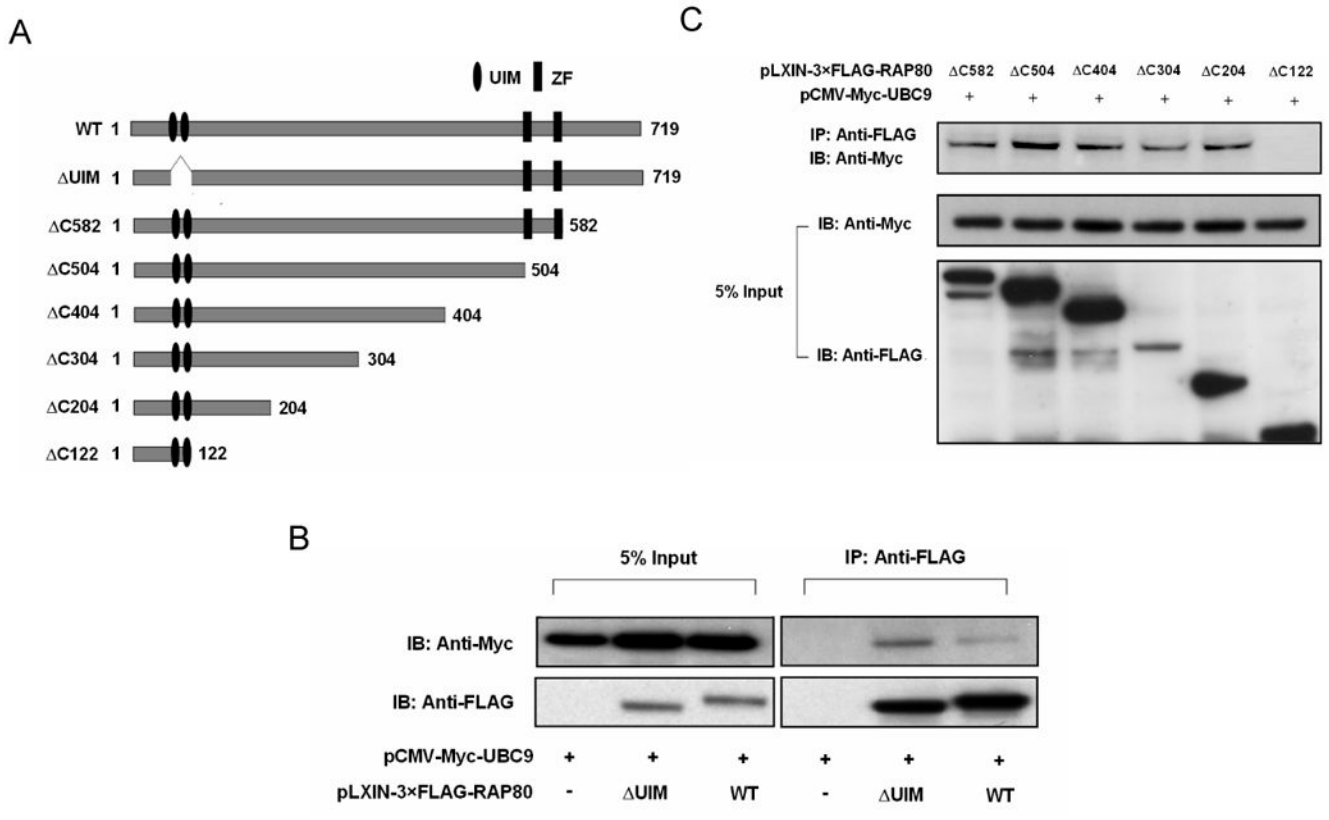

Fig 2.

Mapping of the UBC9 interacting region in RAP80. A. Schematic view of the different RAP80 deletion mutants. UIM and ZF indicate the two ubiquitin-interacting motifs and putative zinc fingers, respectively. B and C. Effect of different mutation on the interaction of RAP80 with UBC9. HeLa cells were co-transfected with pCMV-Myc-UBC9 and pLXIN-3×FLAG-RAP80 or pLXIN-3×FLAG-RAP80 mutants shown in A. After 48 h incubation cells were collected and processed as described in the legend to Fig. 1A.

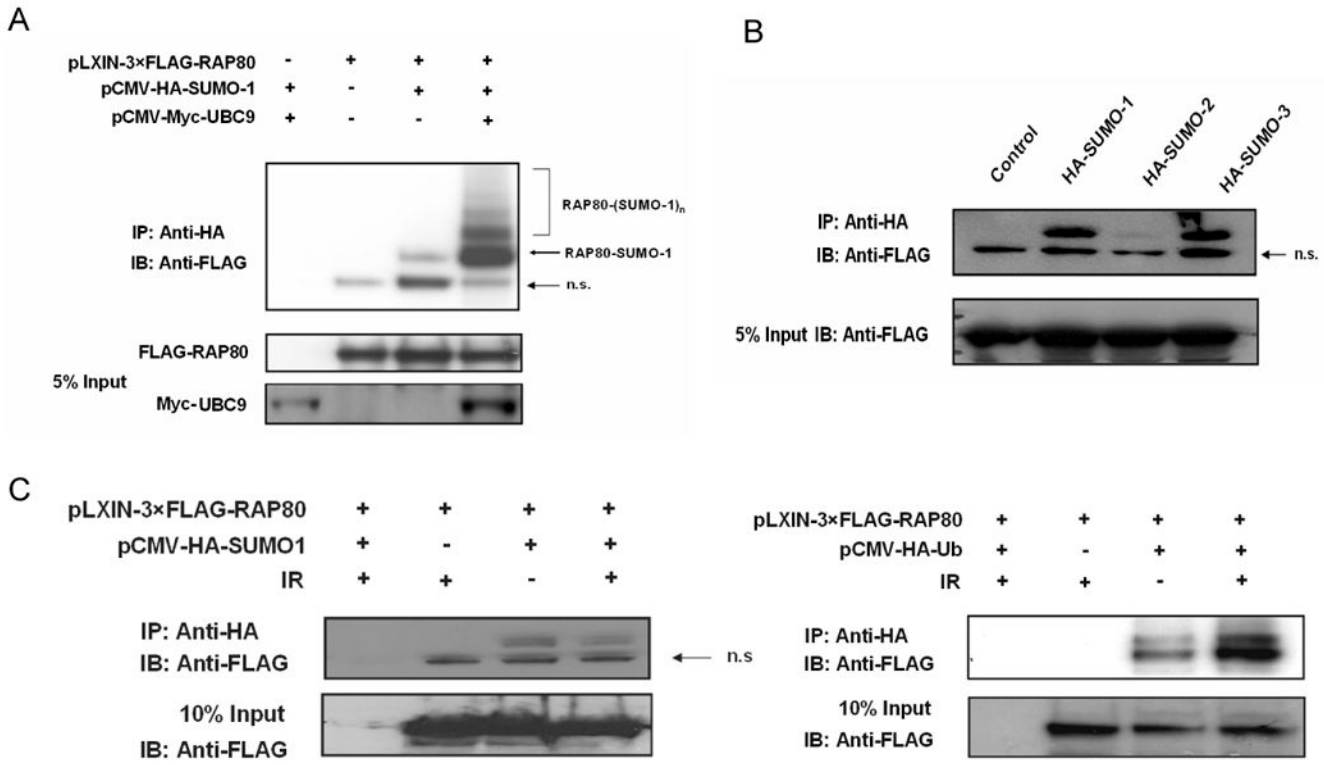

Fig 3.

RAP80 is a target for *in vivo* sumoylation. A. HEK293T cells were transfected with pLXIN-3×FLAG-RAP80, pCMV-HA-SUMO-1, and pCMV-Myc-UBC9 as indicated. Forty eight h later cell extracts were prepared and sumoylated proteins immunoprecipitated with an anti-HA antibody. Isolated proteins were subsequently examined by Western blot analysis with an anti-FLAG antibody. B. HEK293T cells were transfected with pLXIN-3×FLAG-RAP80, pCMV-HA-SUMO-1, pcDNA3-HA-SUMO-2 or pcDNA3-HA-SUMO-3 as indicated. Forty-eight hours later cell extracts were prepared and sumoylated proteins immunoprecipitated with anti-HA antibody. Proteins were then examined by Western blot analysis with an anti-FLAG antibody. C. HEK293T cells were transfected with pLXIN-3×FLAG-RAP80, pCMV-HA-SUMO-1, or pCMV-HA-Ub as indicated. Forty-eight hours later cells were irradiated (10 Gy) and collected after 3 hours incubation. Cells extracts were immunoprecipitated with anti-HA antibody. Proteins were then examined by Western blot analysis with an anti-FLAG antibody.

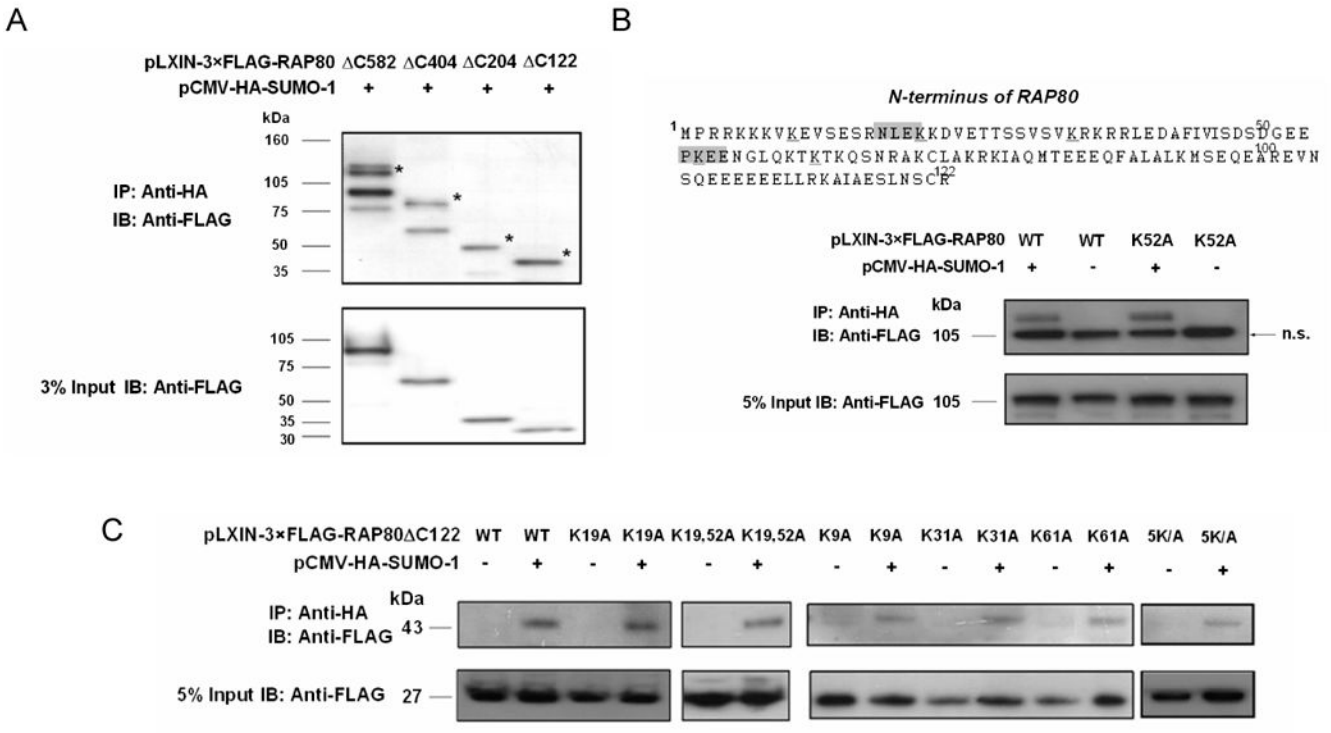

Fig 4.

RAP80 is sumoylated at its amino-terminus. A. HEK293T cells were transfected with pCMV-HA-SUMO-1 and a series of carboxyl-terminal deletion mutants of pLXIN-3×FLAG-RAP80. Forty-eight hours later sumoylated proteins were immunoprecipitated with an anti-HA antibody and examined by Western blot analysis with an anti-FLAG antibody. (*) indicates the migration of the sumoylated mutant RAP80 proteins. B. Effect of several mutations on RAP80 sumoylation. The amino acid sequence of the N-terminal region of RAP80 (a.a. 1–122) is shown; potential sumoylation sites are underlined. HEK293T cells were transfected with pCMV-HA-SUMO-1, wild type pLXIN-3×FLAG-RAP80 or its K52A mutant. The cells were collected 48 h later and processed as described under A. C. HEK293T cells were transfected with pCMV-HA-SUMO-1 and wild type pLXIN-3×FLAG-RAP80ΔC122 or RAP80Δ122 mutants as indicated. The cells were collected 48 h later and processed as described under A. 5K/A indicates the RAP80Δ122 (5K/A) mutant in which all five potential sites are mutated.