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Sumoylation of the GTPase Ran by the RanBP2 SUMO E3 Ligase Complex*

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Volkan Sakin[‡1], **Sebastian M. Richter**[‡], **He-Hsuan Hsiao**[§], **Henning Urlaub**[§¶], **and Frauke Melchior**[‡2]

From the [‡]*Zentrum für Molekulare Biologie der Universität Heidelberg (ZMBH), DKFZ, ZMBH Alliance, Heidelberg, Germany,* [§]*Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany, and* [¶]*Department of Clinical Chemistry, University Medical Center, 37075 Göttingen, Germany*

Background: The GTPase Ran is the key regulator of nucleocytoplasmic transport.
Results: RanGDP is modified with SUMO1 by the E3 ligase RanBP2 and deSUMOylated by the isopeptidase SENP1.
Conclusion: Ran is subject to reversible sumoylation at nuclear pore complexes.
Significance: SUMOylation of Ran might be a novel way of regulating the directionality of nucleocytoplasmic transport for certain cargoes.

The SUMO E3 ligase complex RanBP2/RanGAP1*SUMO1/Ubc9 localizes at cytoplasmic nuclear pore complex (NPC) filaments and is a docking site in nucleocytoplasmic transport. RanBP2 has four Ran binding domains (RBDs), two of which flank RanBP2's E3 ligase region. We thus wondered whether the small GTPase Ran is a target for RanBP2-dependent sumoylation. Indeed, Ran is sumoylated both by a reconstituted and the endogenous RanBP2 complex in semi-permeabilized cells. Generic inhibition of SUMO isopeptidases or depletion of the SUMO isopeptidase SENP1 enhances sumoylation of Ran in semi-permeabilized cells. As Ran is typically associated with transport receptors, we tested the influence of Crm1, Imp β, Transportin, and NTF2 on Ran sumoylation. Surprisingly, all inhibited Ran sumoylation. Mapping Ran sumoylation sites revealed that transport receptors may simply block access of the E2-conjugating enzyme Ubc9, however the acceptor lysines are perfectly accessible in Ran/NTF2 complexes. Isothermal titration calorimetry revealed that NTF2 prevents sumoylation by reducing RanGDP's affinity to RanBP2's RBDs to undetectable levels. Taken together, our findings indicate that RanGDP and not RanGTP is the physiological target for the RanBP2 SUMO E3 ligase complex. Recognition requires interaction of Ran with RanBP2's RBDs, which is prevented by the transport factor NTF2.

Sumoylation, isopeptide bond formation between the carboxyl terminus of Small ubiquitin-related modifier (SUMO)[3] proteins and lysine residues in specific target proteins, is an essential protein modification that regulates hundreds of proteins in all eukaryotic cells (1, 2). Mammalian cells usually express three SUMO proteins, two of which are nearly identical (SUMO2 and 3). Both share 50% amino sequence identity with SUMO1. Sumoylation requires energy and an enzymatic cascade consisting of the SUMO-specific E1-activating enzyme Aos1/Uba2, the E2-conjugating enzyme Ubc9 and an E3 ligase. One of the few known SUMO E3 ligases is the vertebrate nuclear pore complex (NPC) protein RanBP2/Nup358 (3). SUMO-specific isopeptidases ensure reversibility of sumoylation (4). One of the two known SUMO isopeptidases in yeast (Ulp1; (5, 6) and two of eight SUMO isopeptidases in mammalian cells (SENP1 and SENP2; (7–10) are enriched at the basket of NPCs. The localization of E3 ligases and isopeptidases at the NPC (Fig. 1*A*) suggests mechanistic and/or functional links between sumoylation and nucleocytoplasmic trafficking (11).

NPCs mediate exchange of ions, metabolites, and macromolecules between the nuclear and cytoplasmic compartment (12–14). Most proteins and ribonucleoprotein particles require transport receptors of the importin/karyopherin β family to efficiently cross the NPC. The small GTPase Ran determines in which compartment transport complexes are assembled or disassembled, and thereby provides directionality to the transport of cargo. Like all GTPases of the Ras superfamily, Ran switches between two conformations, the GDP- and the GTP-bound state, with the help of auxiliary factors. These are the cytoplasmic Ran GTPase activating protein (RanGAP1 in mammalian cells) and the nuclear guanine-nucleotide exchange factor (RCC1 in mammalian cells). Their asymmetric distribution ensures that RanGTP concentration is high in the nucleus and low in the cytoplasm. RanGTP binds with high affinity to transport receptors of the importin β family, but the consequences of such an interaction differ: Whereas import receptors lose affinity to their cargo in the presence of RanGTP, *i.e.* in the nucleus, export receptors require RanGTP to form export complexes. Once export complexes are disassembled in the cytoplasm, RanGDP is re-imported into the nucleus by its dimeric import receptor, NTF2 (15, 16).

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[1] Current address: Dept. of Infectious Diseases, Virology, University Hospital Heidelberg, Heidelberg, Germany.
[2] To whom correspondence should be addressed: Zentrum für Molekularbiologie der Universität Heidelberg (ZMBH), Im Neuenheimer Feld 282, 69120 Heidelberg, Germany. Tel.: +49-6221-546804; Fax: +49-6221-545893; E-mail: f.melchior@zmbh.uni-heidelberg.de.
[3] The abbreviations used are: SUMO, small ubiquitin-related modifier; NPC, nuclear pore complex; RBD, Ran binding domain; ITC, isothermal titration calorimetry.

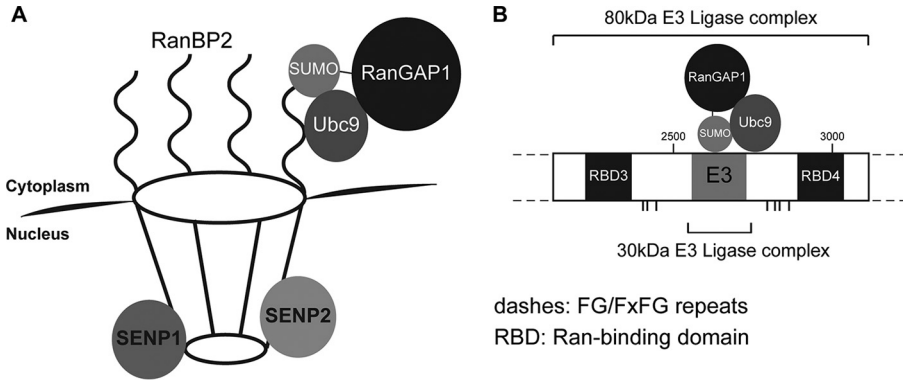


FIGURE 1. **Sumoylating enzymes at nuclear pore complexes.** *A*, multisubunit SUMO E3 ligase consisting of the 358 kDa protein RanBP2, sumoylated RanGAP1 and Ubc9 resides at cytoplasmic filaments of nuclear pore complexes. Two SUMO isopeptidases, SENP1 and SENP2, are enriched at nuclear baskets. *B*, schematic representation of recombinant RanBP2 complexes used in this study. The 80kDa RanBP2 fragment contains two Ran-binding domains (RBD3 and RBD4), the E3 ligase region and two clusters of FG repeats (*dashes*). Together with sumoylated RanGAP1 and Ubc9, it forms the 80 kDa RanBP2 complex. The 30 kDa RanBP2 complex is built with a RanBP2 fragment that lacks RBD3, RBD4, and FG repeats.

Although basic mechanisms of nucleocytoplasmic transport are conserved among all eukaryotes, higher organisms acquired additional features that may contribute to the efficiency of the process. One striking example is the re-localization of the Ran GTPase-activating protein from the cytoplasm in yeast to the cytoplasmic filaments of the NPC in plants and animals. In vertebrates, this requires sumoylation of RanGAP1 (17, 18), which allows it to form a stable complex with RanBP2 and Ubc9 (18–21). RanBP2 is the main component of cytoplasmic NPC filaments in vertebrates (22, 23). It has four Ran binding domains (RBDs) and numerous FG and FxFG repeats, which serve as low-affinity binding sites for nuclear transport receptors. Binding sites for sumoylated RanGAP1 and Ubc9 are situated between RBDs three and four (20, 24). Intriguingly, this area also comprises the SUMO E3 ligase activity (3, 25), and a reconstituted complex consisting of an 86 kDa RanBP2 fragment (named 80kDa RanBP2) spanning RBDs3–4, sumoylated RanGAP1 and Ubc9 is an active E3 ligase on model substrates *in vitro* (26).

At present, only two proteins are known whose sumoylation depends on the RanBP2 E3 ligase complex *in vivo*. These are the mitosis-specific SUMO substrates Topoisomerase IIα and Borealin (27, 28). If one considers RanBP2 domain organization and known binding partners, obvious candidate substrates for its E3 ligase activity are nuclear transport receptors and the GTPase Ran. Transport receptor sumoylation has recently been shown for *Saccharomyces cerevisiae* Kap114. However, yeast does not have RanBP2 and the responsible E3 ligase was shown to be Mms21 (29). Ran was identified as a SUMO target candidate in several mass spectrometry-based SUMO proteome screens (30–32). Convincing evidence for endogenous Ran Sumoylation in mammalian cells came from a recent SUMO linkage screen, which indicated that Ran is sumoylated on Lys-152 (33). Here, we aimed to investigate whether Ran is a target for the recombinant and endogenous RanBP2 SUMO E3 ligase complex, and to determine the influence of its nucleotide state and binding partners on this modification.

Experimental Procedures

Plasmid Constructs—Bacterial expression plasmids for Ran, Ubc9, the E1 enzyme subunits His-Aos1 and Uba2, SUMO1, SUMO2, His-RanBP2 (aa2304–3062), hRanGAP1, His-YFP-Sp100, and Gst-RanBP2ΔFG have been described previously (3, 26, 34, 35). Bacterial expression plasmids pET30a-Impβ, pQE32-Transportin, pQE60-Crm1, pET3-RanQ69L were kindly provided by Ralph Kehlenbach (Georg-August University of Göttingen, Göttingen). pET-NTF2 was a kind gift of Dirk Görlich (Max-Planck-Institute for Biophysical Chemistry, Göttingen). Bacterial expression plasmids pGEX-6P-1-PIAS1, pGEX-4T-1-PIASxα, pGEX-2TK-PIASxβ, pGEX-4T-1-PIAS3, and pGEX-4T-1-PIASy were kindly provided by Jacob S. Seeler (Institut Pasteur, Paris, France). Ran lysine mutants (the single variants K130R, K132R, K134R, K152R and the double mutant KK130,152RR) were generated by site-directed mutagenesis of the pET11d-Ran plasmid (34). NTF2-E42K and NTF2-W7A were created by site-directed mutagenesis using the pET-NTF2 plasmid (36). Imp β was PCR-amplified from pET30a-Impβ, introducing a 5′ BamHI and 3′ NotI restriction sites followed by cloning into pET23a. For generation of pET23a-RBD4-His, the coding sequence of the RBD4 of RanBP2 (aa 2902–3052) was PCR amplified from the pET23a-RanBP2$_{\mathrm{RB3-4}}$ construct (26) and cloned into the NdeI-XhoI sites of pET23a.

Antibodies—Mouse αRan and mouse αRCC1 were from BD Transduction Laboratories, rabbit αGFP and mouse αp53 were from Santa Cruz (sc-8334) (sc-126), respectively and rabbit αSENP1 was from Epitomics. Affinity-purified goat αRanBP2 and αUba2 antibodies have been described (37, 38). Sheep αSENP2 antibody was a kind gift from Ron T. Hay (University of Dundee). HRP-conjugated secondary antibodies were from Dianova. Fluorescent donkey anti-mouse secondary antibody was from Li-Cor Biosciences, Lincoln, NE.

Protein Expression and Purification—SUMO E1 enzyme, His-YFP-Sp100, untagged Ubc9, YFP-SUMO1, untagged SUMO, RanBP2 fragments and RanBP2 complexes were purified following previously published protocols (3, 26, 35). Purification of untagged wild type and mutant Ran proteins was based on (39) to generate Ran without defined nucleotide state, and extended to obtain Ran loaded specifically with GDP or GTP (40). In short, Ran was expressed in BL21(DE3) cells by induction with 0.6 mM IPTG for 3 h at 37 °C. Cells were lysed, centrifuged, and precleared by passing over DEAE column

(Sigma Aldrich). Upon ammonium sulfate precipitation (53% saturation), the precipitate was resuspended in the presence of 250 μM GDP, GTP, or GMP-PNP and further purified by gel filtration on Superdex200 (S200). Ran-containing fractions were subjected to anion-exchange chromatography on MonoQ (GE Healthcare). RanGDP eluted at ~60 mM NaCI, RanGTP eluted at ~130 mM NaCI. Finally, buffer was exchanged to TB (20 mM HEPES, pH 7.3, 110 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM EGTA, 1 mM DTT, and 1 μg/ml of each aprotinin, leupeptin, and pepstatin) by gel filtration on an S75 column (GE Healthcare). SUMO-Vme purification has been described elsewhere (41). NTF2 wild type, W7A, and E42K mutants were purified according to (15). Purification of His-tagged transport receptors and GST-tagged PIAS proteins followed standard pull-down procedures and included a final gel filtration step (S200, preparative column) in TB.

Mammalian Cell Culture—Adherent HeLa cells were cultured in DMEM supplemented with 10% (*v*/*v*) fetal bovine serum (FBS), and penicillin (100 units/ml)/streptomycin (100 mg/ml) at 37 °C and 5% CO_2. HeLa suspension cells were cultured in Jokliks medium supplemented with 5% (*v*/*v*) newborn calf serum (NCS), 5% (*v*/*v*) FBS and 2 mM glutamine.

In Vitro Sumoylation Assays with Recombinant Factors—*In vitro* sumoylation assays with purified recombinant proteins followed the general outline as in (35). Reactions were set up in 20 μl reactions. Unless specified differently, reaction mixtures included 70 nM Aos1/Uba2, 100 nM Ubc9, 15 μM SUMO1, and 5 mM ATP in TB supplemented with 0.05% Tween 20 and 0.2 mg/ml ovalbumin. Target proteins (RanGDP, RanGTP, YFP-Sp100, p53) were used in the range of 400 nM to 2 μM. RanBP2 fragments and complexes were typically used at 100 nM. Reactions were incubated at 30 °C or 37 °C for 0–120 min and stopped by 2× sample buffer. Samples were analyzed by immunoblotting with the indicated antibodies.

In Vitro Sumoylation Assays with Semi-permeabilized Cells—Semi-permeabilized Hela suspension cells were generated by treatment with 0.007% digitonin as described for nuclear transport assays (42). Reactions were set up in 60 μl reaction volumes and contained recombinant Ran (5 μM), an ATP-regenerating system (1.3 mM ATP, 3.2 mM creatine phosphate and 13 units/ml creatine phosphate kinase), an excess of YFP-SUMO1 and 3×10^6 semi-permeabilized cells in TB supplemented with 0.05% Tween 20 and 0.2 mg/ml BSA. Reactions were incubated at 30 °C for 30 min and stopped by adding 2× sample buffer.

siRNA Transfections—RanBP2 knockdowns were performed essentially as described in (43). For SENP1 and SENP2 knockdowns, HeLa cells were transfected with 10 nM siRNA (Ambion) against SENP1 (5′-GCUUAUAAUCCAAGCUAUUTT-3′) and against SENP2 (5′-GGAAAUCAGUAAUGCCCUATT-3′) by using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instructions. Cells were harvested and used for subsequent assays 96 h (for RanBP2) or 48 h (for SENP1 and SENP2) post-transfection.

Isothermal Titration Calorimetry (ITC)—Proteins were dialyzed extensively against 50 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM MgCl at 4 °C and centrifuged for 10 min at 20.000 g to remove aggregates. Before each measurement, samples were degassed and incubated for 10 min at room temperature. ITC experiments were run at 25 °C and set to deliver 20 injections at 90 s intervals. The titrations were performed with 200 μM RBD4 in the syringe and 15 μM RanGDP in the cell. Where indicated, the cell solution also contained 30 μM NTF2 or NTF2-E42K. ITC measurements were performed at least four times using an ITC 200 Microcalorimeter (MicroCal). ITC data were analyzed with the ORIGIN software package (MicroCal).

Identification of SUMO Acceptor Sites by Mass Spectrometry—Ran*SUMO1 containing gel slices were excised, reduced with 50 mM DTT, alkylated with 100 mM IAA and in-gel digested with modified trypsin (Promega) over night. Mass spectrometric analysis was performed on an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a nanoelectrospray ion source and coupled to an Agilent 1100 HPLC system (Agilent Technologies), fitted with a homemade C18 column with a 60 min LC gradient. Typical mass spectrometric conditions were: spray voltage, 1.8kV; heated capillary temperature, 150 °C; normalized CID collision energy 37.5% for MS/MS in LTQ. An activation q = 0.25 and activation time of 30 ms were used. The mass spectrometer was operated in the data dependent mode to automatically switch between MS and MS/MS acquisition. Survey full scan MS spectra (from *m*/*z* 350–2000) were acquired in the orbitrap with resolution *r* = 30,000 at *m*/*z* 400 (after accumulation to a target value of 10^6 in the orbitrap). The five most intense ions were sequentially isolated and fragmented in the linear ion trap using collision induced dissociation (CID) at a target value of 10^5. Identification of SUMOylated lysine residues was performed by ChopNSpice as described by Ref. 44.

Results

Sumoylation of Ran by the RanBP2 Complex Depends on Ran-binding Domains in the E3 Ligase—Most SUMO targets, with the notable exception of RanGAP1, cannot be sumoylated efficiently by physiologically relevant concentrations of the SUMO E1 and E2 enzymes (low nM concentrations); they require E3 ligases. *In vitro*, E3 ligase-dependence can often be circumvented by significantly increasing enzyme concentrations. This is typically the case for targets with a sumoylation consensus sequence (ΨKxE), as this motif is recognized by the E2 enzyme Ubc9 itself (45, 46). To test if and under which conditions Ran can be sumoylated, we turned to *in vitro* sumoylation assays with bacterially expressed and purified components. We first tested whether untagged Ran can be sumoylated in the presence of high concentrations of E1 (400 nM) and E2 (500 nM) enzymes. These experiments were performed both with SUMO1 and SUMO2, as some targets show preferential modification for one of these paralogues (47, 48). As shown in Fig. 2*A* for RanGDP, only a very small fraction of Ran was sumoylated with SUMO1 and even less with SUMO2 in a time-dependent manner, indicating that Ran is not an efficient SUMO target in the absence of E3 ligases. This is consistent with previous work that reported minimal sumoylation of GST-Ran with E1 and E2 enzymes (30), and is also not surprising considering the absence of a sumoylation consensus site in Ran. Next, we tested the effect of recombinant E3 ligases on Ran sumoylation in pilot experiments. PIAS E3 ligases, which are ubiquitously expressed in all eukaryotes, did not enhance Ran sumoy-

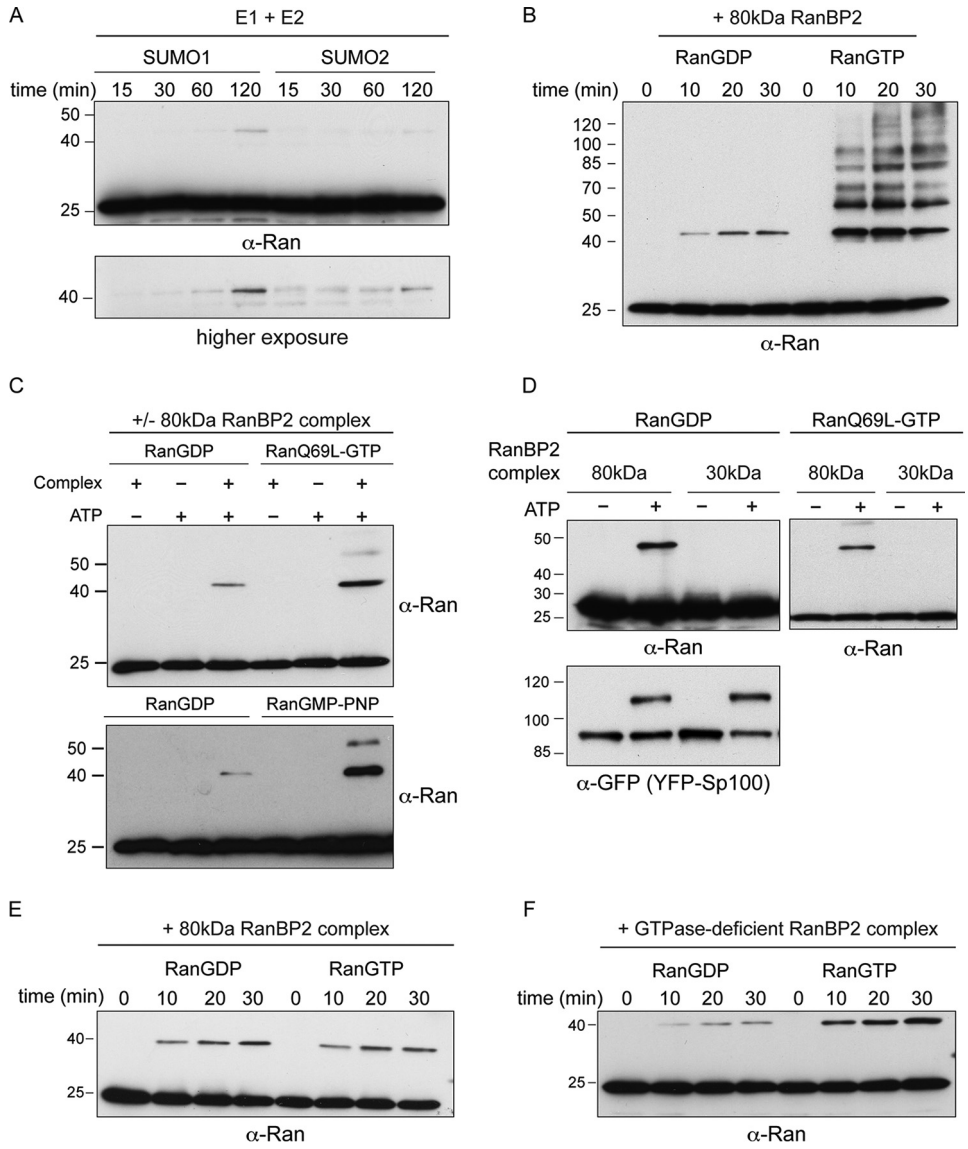


FIGURE 2. **The RanBP2 SUMO E3 ligase complex stimulates Ran sumoylation in dependence of Ran binding domains.** *A*, Ran is not efficiently sumoylated in the absence of an E3 ligase. *In vitro* sumoylation assays were performed with 2 μM RanGDP, high concentrations of E1 and E2 enzymes (400 nM and 500 nM, respectively), 3 μM SUMO1 (*left*) or SUMO2 (*right*) and 5 mM ATP at 37 °C. *B*, Ran is efficiently sumoylated in the presence of the 80 kDa RanBP2 fragment. *In vitro* sumoylation of 500 nM RanGDP or RanGTP with 70 nM E1, 56 nM E2, 15 μM SUMO1, 5 mM ATP, and 72 nM RanBP2 fragment at 30 °C. *C*, Ran sumoylation is stimulated by the 80kDa RanBP2 complex. *In vitro* sumoylation as in *B* but with or without 100 nM 80 kDa RanBP2 complex. *D*, 30 kDa RanBP2 complex is unable to stimulate Ran sumoylation. *In vitro* sumoylation of RanGDP or RanQ69L-GTP (*top panels*) and YFP-Sp100 (*bottom panel*) as in *B*, using 100 nM 80 kDa or 30 kDa RanBP2 complex as E3 ligase. *E* and *F*, RanGTP hydrolysis is faster than sumoylation. *In vitro* sumoylation of Ran was performed as in *B* with 400 nM RanGDP or RanGTP and 72 nM 80 kDa RanBP2 complex (*E*) or a complex variant that lacks RanGAP1's catalytic domain (*F*). *A–F*, reactions were stopped with 2× sample buffer at 30 min or at indicated time points. All samples were resolved by SDS-PAGE followed by immunoblotting with antibodies directed against Ran or GFP.

lation. In contrast, fragments of vertebrate specific RanBP2 stimulated Ran modification, especially with SUMO1 (data not shown). We thus turned to a systematic analysis of Ran sumoylation by the RanBP2 E3 ligase (Fig. 2, *B–F*). First, we tested the ability of the free 80 kDa RanBP2 fragment (Fig. 1*B*) to stimulate RanGDP and RanGTP sumoylation. Indeed, both forms of Ran were clearly sumoylated over a period of 30 min (Fig. 2*B*). Although RanGDP was only mono-sumoylated, a significant fraction of RanGTP acquired multiple SUMO1 moieties, either by attachment to multiple lysine residues of Ran or in the form of SUMO chains. Both are plausible, considering that RanBP2 is known to form SUMO1 chains on itself *in vitro* (3). We next repeated the experiments with the reconstituted RanBP2 complex, which consists of the 80 kDa RanBP2 fragment, full-length untagged sumoylated RanGAP1 and Ubc9 (Fig. 1*B*). In order to test the sumoylation efficiency of the complex on the GTP-bound conformation of Ran, we used two strategies: On one hand, we tested a Ran variant, RanQ69L, that locks Ran in the GTP conformation even in the presence of RanGAP1. On the other hand, we loaded wt Ran with the non-hydrolyzable GTP analog GMP-PNP. As shown in Fig. 2*C*, in both cases the RanBP2 complex stimulated sumoylation of Ran efficiently (Fig. 2*C*). However, both free RanBP2 and the complex stimulated sumoylation of the GTP-bound conformation of Ran more efficiently than the GDP-bound conformation, suggesting that the Ran binding domains may contribute to substrate

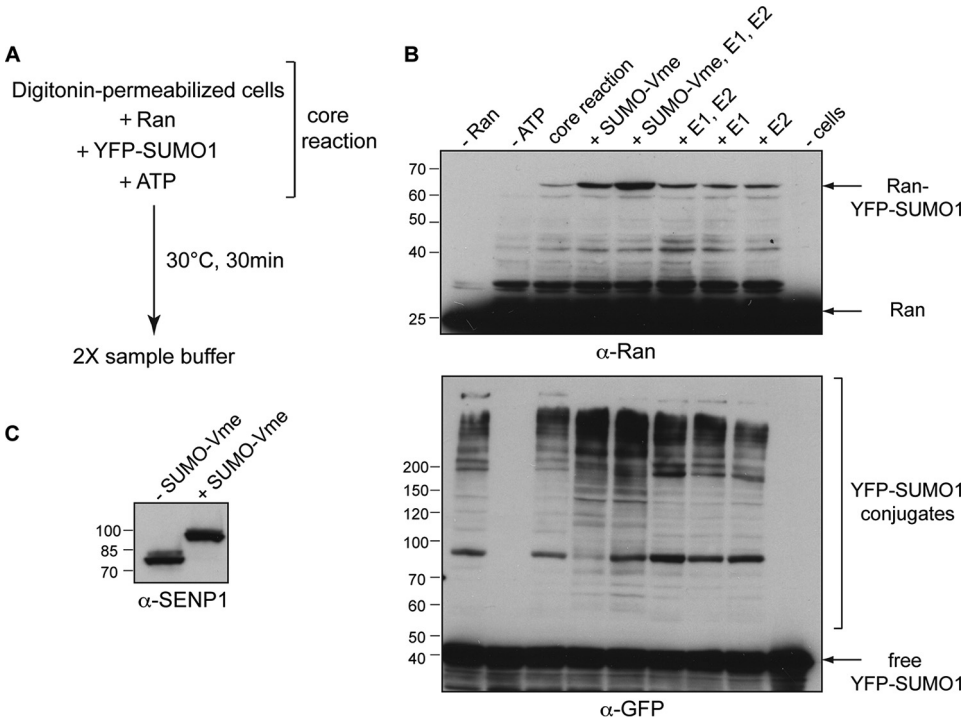


FIGURE 3. **Recombinant Ran can be sumoylated in semi-permeabilized HeLa cells.** *A*, flowchart for *in vitro* sumoylation with semi-permeabilized cells. HeLa suspension cells are treated with digitonin for 5 min on ice, washed and incubated with 5 μM RanGDP, 35 μM YFP-SUMO1 and an ATP-regenerating system at 30 °C for 30 min (core reaction). Reactions are stopped by 2× sample buffer and analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. *B*, *in vitro* sumoylation assays with semi-permeabilized HeLa cells were done as described in *A*. Modifications to the core reaction are indicated above the respective lanes. *E1*: addition of 120 nM recombinant E1 enzyme. *E2*: addition of 155 nM recombinant E2 enzyme. *SUMO-Vme*: pre-incubation of semi-permeabilized cells with SUMO-Vinylmethylester (SUMO-Vme) for 30 min on ice. Of note, all samples contain endogenous Ran, as it is partially retained in semi-permeable cells. *C*, efficiency of SUMO-Vme, which forms an irreversible adduct with the catalytic cysteine of SUMO isopeptidases, in semi-permeabilized HeLa cells was controlled by analyzing the mobility shift of the SUMO isopeptidase SENP1 by immunoblotting.

recognition. Both RanGDP and RanGTP can interact with Ran binding domains, yet their affinities differ dramatically: Whereas affinities of different RBDs for RanGTP are in the range of 1–10 nM (49), RanGDP binds to RanBP1 with an affinity of ~10 μM (50). To test whether the RBDs are required for Ran sumoylation, we reconstituted a smaller RanBP2 complex with a 30 kDa RanBP2 fragment lacking the RBDs (Fig. 1*B*). Both the larger and the smaller complex are known to be active on the model substrate YFP-Sp100 (26) and Fig. 2*D*, *lower panel*). However, the shorter complex was completely unable to stimulate Ran sumoylation (Fig. 2*D*, *upper panel*). This result is consistent with the idea that Ran sumoylation by the RanBP2 complex requires recognition of Ran by RanBP2's Ran-binding domains. Up to this point, our data suggested that RanGTP is a better substrate for the RanBP2 complex than RanGDP. However, RanGAP1 is a very fast enzyme (k_{cat} of 8 s^{-1} without and 10 s^{-1} with RanBP1, (51)) and it seemed very likely that hydrolysis would precede sumoylation. Indeed, when we used RanGTP rather than RanQ69L-GTP, it was sumoylated only at the rate of RanGDP (Fig. 2*E*). That this was due to hydrolysis was further confirmed by using a RanBP2 complex variant in which RanGAP1 was replaced by the GAP-deficient RanGAP1 tail (Fig. 2*F*). This complex stimulated RanGTP modification more efficiently than that of RanGDP (Fig. 2*F*). Together, these findings allow us to conclude that RanGTP is converted to RanGDP more rapidly than it is sumoylated. In consequence, RanGDP rather than RanGTP seems to be a relevant substrate for the RanBP2 E3 ligase complex.

Recombinant Ran Can Be Sumoylated in Semi-permeabilized HeLa Cells—To address the question whether Ran can be sumoylated by the full-length RanBP2 complex in the context of intact nuclear pore complexes, we turned to semi-permeabilized HeLa cells. These are generated by treatment with low concentrations of digitonin, which selectively permeabilizes the plasma membrane but leaves the nuclear envelope and pore complexes intact (52). One advantage of this system is that one can add SUMO isopeptidase inhibitors such as SUMO-Vme, to prevent desumoylation. This is a recombinant SUMO protein modified with vinylmethylester at its C terminus (41, 53). We incubated semi-permeabilized cells with recombinant Ran and YFP-SUMO1 in the presence of ATP as outlined in Fig. 3*A*. Where indicated, cells were pre-treated with SUMO-Vme. After 30 min, samples were resolved by SDS-PAGE and immunoblotted with antibodies against Ran, GFP (to detect YFP-SUMO) or the SUMO isopeptidase SENP1. As known from our previous work (3), semi-permeabilized cells have sufficient sumoylation machinery to covalently link YFP-SUMO1 to unknown targets in an ATP-dependent manner (Fig. 3*B*, *lower panel*). Pre-treatment of cells with SUMO-Vme blocked SUMO isopeptidases as indicated by the mobility shift of SENP1 (Fig. 3*C*), and this was accompanied by a clear increase in overall sumoylation levels (Fig. 3*B*, *lower panel*, compare *lane 3* and *4*). Addition of 120 nM E1 and 155 nM E2 together or separately slightly boosted the sumoylation reaction. When the same samples were probed for Ran, Ran sumoylation was detectable even under core reaction conditions (Fig. 3*B*, *lane 3*).

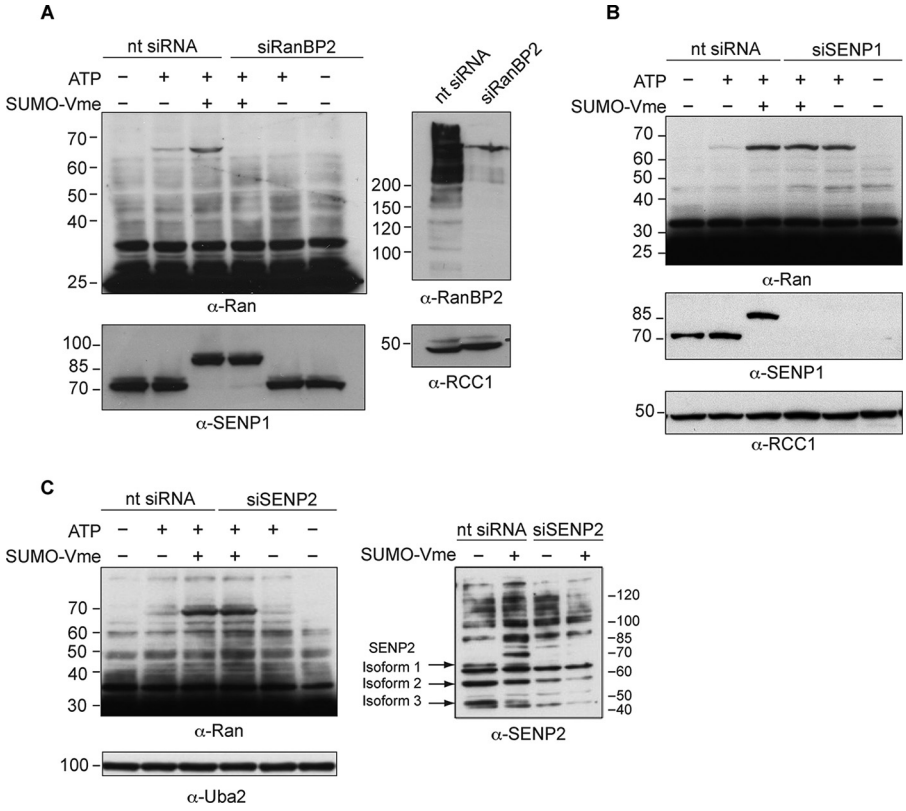


FIGURE 4. **RanBP2 and SENP1 are rate-limiting for sumoylation of Ran in semi-permeabilized HeLa cells.** *A* and *B*, adherent HeLa cells were transfected with siRNA against RanBP2 (*A*), SENP1 (*B*), or SENP2 (*C*). Following knockdown, cells were trypsinized and permeabilized with digitonin for 5 min on ice. Semi-permeabilized cells were incubated with 5 μM RanGDP and 35 μM (*A*) or 2.4 μM (*B-C*) YFP-SUMO1 at 30 °C for 30 min in the presence of an ATP-regenerating system. Reactions in *B-C* were also supplemented with 120 nM E1 and 155 nM E2. Where indicated, semi-permeable cells were pre-incubated with SUMO-Vme for 30 min on ice to inhibit endogenous SUMO isopeptidases. Reactions were stopped by 2× sample buffer and analyzed by immunoblotting with the indicated antibodies.

However, it was a very small fraction of the total Ran population. Blocking isopeptidases with SUMO-Vme or adding E1 and E2 enzymes clearly increased sumoylation of Ran (Fig. 3*B*, compare *lane 3* with *lanes 4*, *5*, *6*). These results not only demonstrate that recombinant Ran is sumoylated in semi-permeabilized cells in the presence of YFP-SUMO1 but also show that endogenous isopeptidases contribute to the low steady state levels of Ran*YFP-SUMO1 conjugate.

The Endogenous RanBP2 E3 Ligase Complex Is Required for Ran Sumoylation—Next, we wanted to investigate whether the endogenous NPC-associated RanBP2 E3 ligase complex is responsible for Ran sumoylation in semi-permeabilized cells. For this, we transfected HeLa cells with control (nt siRNA) or RanBP2 specific (siRanBP2) siRNAs. As shown in the *right panel* of Fig. 4*A*, RanBP2 knockdown was very efficient after 96 h. Of note, the 358 kDa RanBP2 is susceptible to limited proteolysis in cell lysates, which is the cause for the high MW smear detected by α RanBP2 immunoblotting. Subsequently, nt siRNA treated and RanBP2 depleted cells were permeabilized with digitonin and used in sumoylation assays as described above. Where indicated, SUMO-Vme was added (Fig. 4*A*, *lower panel*, shows efficient SENP1 inactivation). When recombinant Ran was incubated with control cells treated with nt siRNA, Ran sumoylation was observed as before and could be increased upon SUMO-Vme treatment (Fig. 4*A*, compare *lane 2 versus 3*). However, sumoylation of Ran was completely absent in cells that were lacking RanBP2, both in the absence and presence of SUMO-Vme. In conclusion, endogenous RanBP2 is responsible for Ran sumoylation in semi-permeabilized cells.

SENP1 Restricts Ran Sumoylation—Our finding that SUMO-Vme increases sumoylation in semi-permeabilized HeLa cells is a strong indicator for the presence of SUMO isopeptidases that revert Ran sumoylation. Excellent candidates are SENP1 and SENP2, as these are known to interact with nuclear pore complex proteins including the nuclear basket component Nup153 (7–10). To test their putative involvement, we depleted SENP1 and SENP2 from HeLa cells with the help of siRNA, permeabilized the cells with digitonin and employed them in Ran sumoylation assays as above. As shown in Fig. 4*B*, depletion of SENP1 significantly increased levels of sumoylated Ran (compare *lanes 2* and *5*). In contrast, knockdown of SENP2 did have no detectable effect on the levels of sumoylated Ran (Fig. 4*C*). Our observation that the levels of sumoylated Ran in SENP1-depleted cells could not be increased further by the addition of SUMO-Vme suggests that SENP1 is the major isopeptidase responsible for desumoylation of Ran in semi-permeabilized cells.

Transport Receptors of the Importin β Family and NTF2 Block Ran Sumoylation—Up to this point, we tested free Ran proteins, either in GTP- or GDP-bound conformations, for sumoylation. However, Ran has numerous interaction partners and is believed to exist largely in protein complexes at steady state. RanGTP interacts with all transport receptors of the

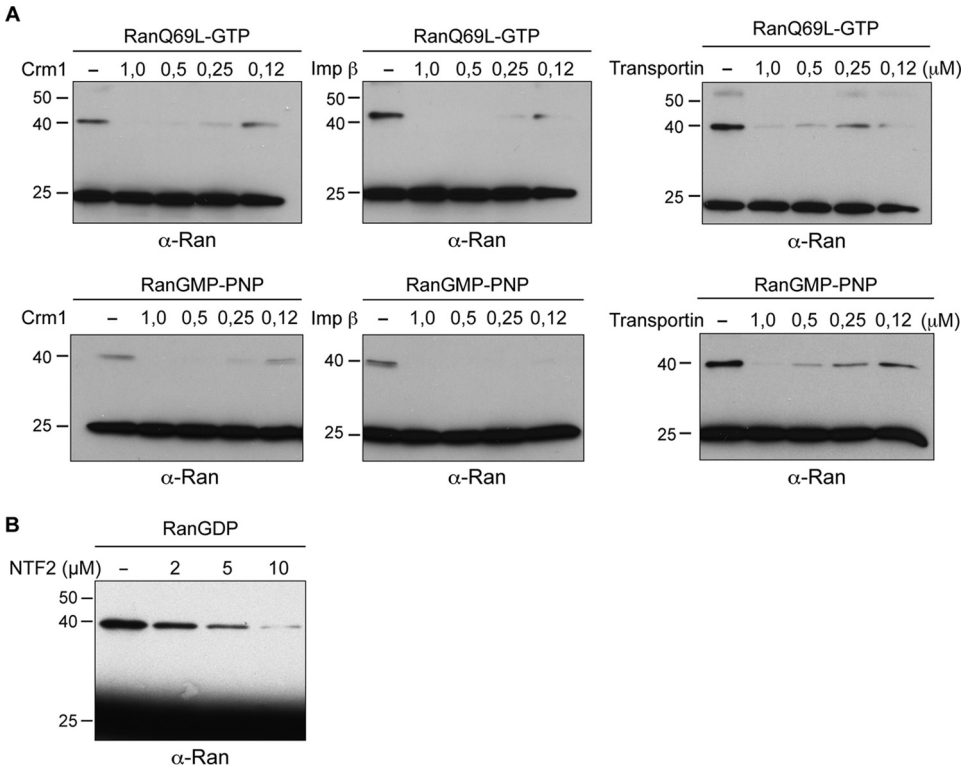
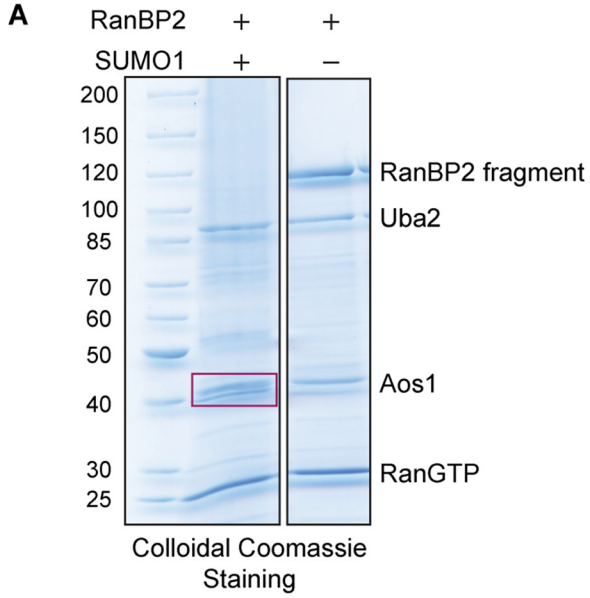


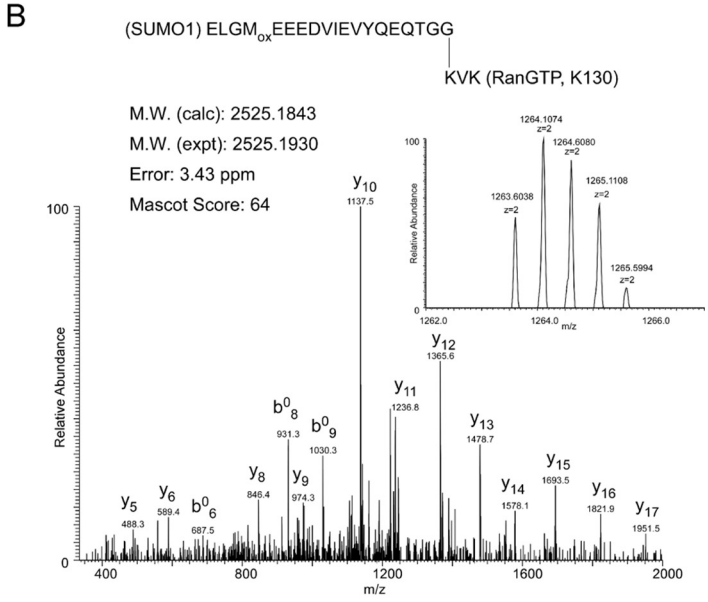
FIGURE 5. **Transport receptors including NTF2 inhibit sumoylation of Ran by the RanBP2 E3 ligase complex.** *A* and *B*, *in vitro* sumoylation assays were performed in the presence of 70 nM E1, 100 nM E2, 100 nM 80 kDa RanBP2 complex, 15 μM SUMO1, and 5 mM ATP at 30 °C for 30 min. 500 nM RanQ69L-GTP, RanGMP-PNP, or RanGDP were used as substrates. Crm1, Imp β, Transportin, and NTF2 were preincubated with Ran at the indicated concentrations (in μM) on ice for 1 h prior to sumoylation assays. Reactions were stopped with 2× sample buffer and analyzed by immunoblotting with antibodies against Ran.

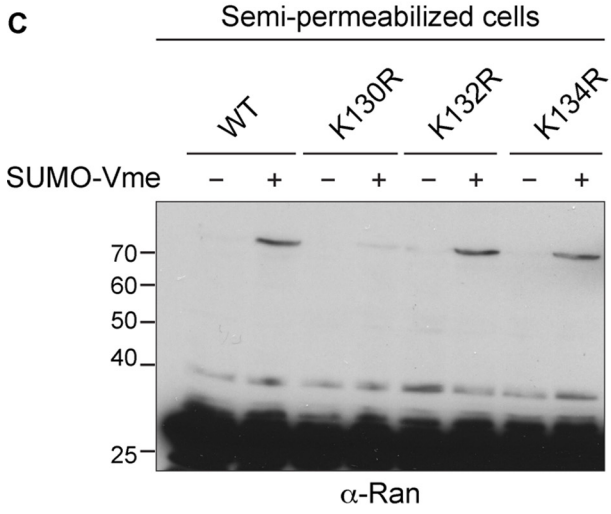
importin β superfamily, either to displace cargo molecules from import receptors or to allow export receptors to recognize their cargo (12–14). Although RanGTP/transport receptor complexes with or without cargo form in the nucleus, they are likely to encounter the RanBP2 complex upon translocation through the NPC. Moreover, RanGTP binds with high affinity to proteins that contain Ran binding domains (RBDs). In addition to RanBP2, this group includes, *e.g.* the vertebrate proteins RanBP1, RanBP3, and Nup50 (reviewed in (13)). NTF2 on the other hand interacts specifically with GDP-bound Ran (54–56). Complexes between RanGDP and NTF2 form in the cytoplasm and may encounter the RanBP2 complex on their way into the nucleus. NTF2 facilitates RanGDP transport into the nucleus by mediating interactions with FG-repeat containing nucleoporins (15, 16, 56, 57). In consequence, physiologically relevant substrates for the RanBP2 SUMO E3 ligase complex could either be free RanGDP, RanGTP in association with transport receptors or RanGDP in association with NTF2. To explore the possibility that RanGTP is sumoylated as part of nuclear export complexes, we incubated GTP-loaded RanQ69L as well as GMP-PNP loaded wt Ran with the prototypic transport receptors Crm1, Imp β, and Transportin prior to *in vitro* sumoylation. However, each of these transport receptors efficiently blocked RanGTP sumoylation (Fig. 5*A*). Transport receptors of the Imp β family cover a large fraction of Ran's surface area (58–60). In consequence, their inhibitory effect on Ran sumoylation could either be due to direct masking of relevant lysine residues or may be due to steric reasons (the charged E2-conjugating enzyme needs to simultaneously interact with RanBP2 and reach lysines in the substrate). Similar experiments were carried out to test the effect of NTF2 on RanGDP sumoylation. As shown in Fig. 5*B*, NTF2 blocked RanGDP sumoylation in a concentration-dependent manner (Fig. 5*B*). In light of NTF2's size and Ran's small NTF2 interaction surface, this clear effect was quite surprising (see below). Jointly, our findings have an important implication regarding Ran sumoylation: the only Ran species that may be sumoylated by the RanBP2 complex in a physiological context is free RanGDP.

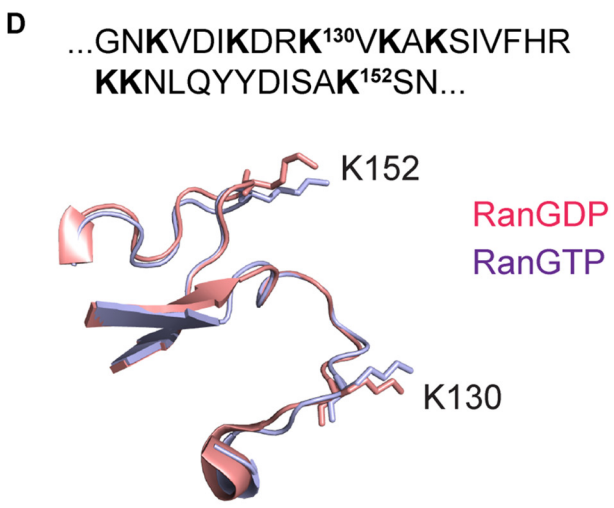
Lysine 130 of Ran Is the Major SUMO Acceptor Site—Intrigued by the observation that the 14 kDa NTF2 inhibited Ran sumoylation, we decided to map lysine residues in Ran that serve as SUMO acceptor sites by mass spectrometry. To ensure maximal sequence coverage, we set up large scale *in vitro* sumoylation reactions using conditions that ensured maximal efficiency of the modification: RanGTP was used as substrate and the 80kDa RanBP2 fragment rather than the complex was used as the E3 ligase. The sumoylation reaction resulted in several higher molecular weight Ran species that were visible upon colloidal Coomassie staining (Fig. 6*A*). These bands were excised, digested with trypsin and analyzed by mass spectrometry, which identified three consecutive lysines as SUMO acceptor sites: Lys-130, Lys-132, and Lys-134 (Fig. 6*B* and data not shown). To test the contribution of these three lysine residues to Ran sumoylation by the endogenous RanBP2 complex, we generated mutants in which each individual lysine was mutated to an arginine. These Ran variants were subjected to sumoylation in semi-permeabilized cells in the absence or presence of the isopeptidase inhibitor SUMO-Vme. Whereas the

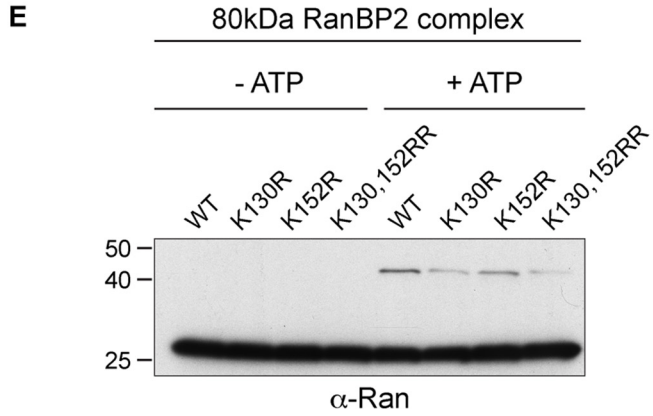
two Ran mutants Ran K132R and Ran K134R were modified like wild type Ran, Ran K130R sumoylation was strongly impaired (Fig. 6*C*). This suggested that Lys-130 is the major SUMO acceptor site in semi-permeabilized cells. However, a global screen for SUMO acceptor sites had identified Ran Lys-152 but no other lysine as an *in vivo* SUMO acceptor site in Ran (33). Why was Lys-130 not identified in the global screen? Inspection of Ran's sequence (Fig. 6*D*) offers one of several possible explanations: Digesting Lys-130 sumoylated Ran with LysC, which cleaves after lysines, generates a branched peptide that is too short to be unique. Intriguingly, Lys-130 and Lys-152 are rather close and similarly oriented both in RanGDP and in Ran GTP (Fig. 6*D*), which makes their use as alternative sumoylation sites plausible. To test their relative contributions in a purified sys-











tem, we created the respective single and double mutants and tested them with the recombinant 80 kDa RanBP2 E3 ligase complex. As shown in Fig. 6*E*, Lys-130 again appears the major site, but Lys-152 also contributes. In conclusion, two adjacent lysine residues could be identified in Ran that can serve as acceptor sites for sumoylation by the RanBP2 complex. Based on our assays with recombinant and endogenous complex, Lys-130 seems to be the favored site in cells.

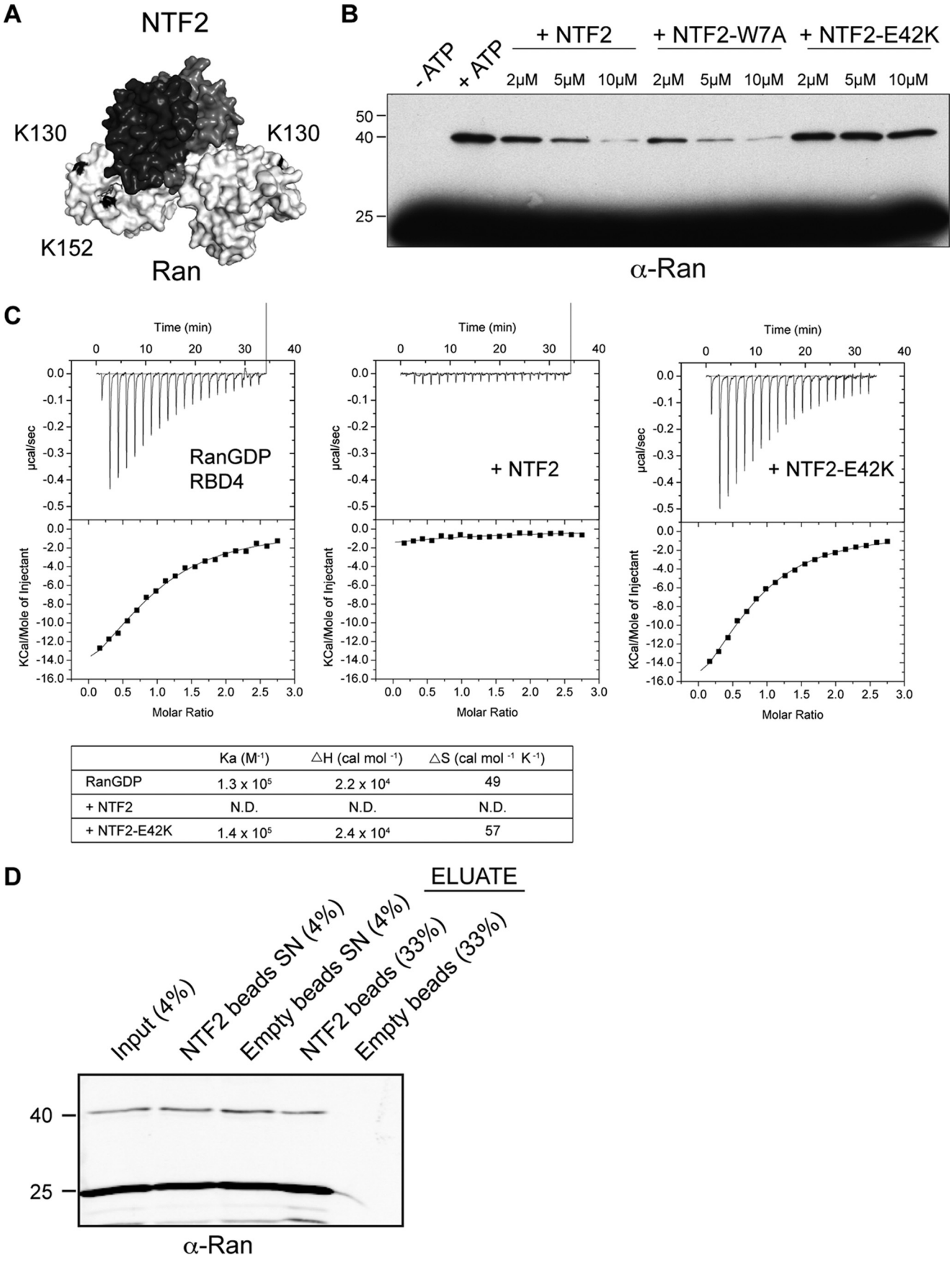
NTF2 Prevents Ran Sumoylation by Inhibiting RanGDP-RanBP2 Interaction—As described above, NTF2 blocked RanGDP sumoylation efficiently in *in vitro* assays. As neither of the two SUMO acceptor sites is masked upon NTF2 binding (Fig. 7*A*), we wondered what the underlying reason could be. Since NTF2 interacts not only with RanGDP but also with FXFG repeat containing nucleoporins, it may bind to these repeats in the RanBP2 complex and inhibit Ran sumoylation for steric reasons. To gain further insights, we utilized two well-known mutants of NTF2, namely W7A and E42K. Although NTF2-W7A has reduced affinity for FxFG repeat containing nucleoporins, it retains RanGDP binding (57). Conversely, NTF2-E42K interacts with FxFG repeats but fails to interact with Ran (61). We purified these NTF2 mutants and tested their effect on Ran sumoylation. Whereas NTF2-W7A was still able to block Ran sumoylation, NTF2-E42K has no inhibitory effect even at the highest concentration (Fig. 7*B*). In consequence, it is the NTF2 - Ran interaction itself that prevents Ran sumoylation. Since RanGDP can only be sumoylated by a RanBP2 complex that has Ran binding domains (Fig. 2*D*), we wondered whether NTF2 lowers Ran's affinity for these domains. To test this idea, we compared dissociation constants between Ran and RanBP2's RBD4 in the absence or presence of NTF2 variants by isothermal titration calorimetry (ITC). For this, purified recombinant RBD4 was injected into a solution of RanGDP, either alone or supplemented with NTF2 or NTF2-E42K (Fig. 7*C*). The mean K_d value from six independent experiments for RanGDP and RBD4 was determined to be 9.9 μM, in perfect agreement with the K_d determined for RanGDP - RanBP1 interaction (50). Similar values were obtained in the presence of NTF2-E42K, the mutant that cannot interact with Ran (Fig. 7*C*, *right panel*). The mean K_d value from four independent experiments is 10.4 μM. Strikingly, no binding at all could be observed between RanGDP and RBD4 when wt NTF2 was present (Fig. 7*C*, *middle panel*), which indicated that NTF2 decreases the affinity of RanGDP for RBD4 below the detection limit. This finding provides a clear explanation for NTF2's inhibitory effect in Ran sumoylation: NTF2 prevents RanGDP from interacting with the SUMO E3 ligase.

Discussion

The physical proximity between RanBP2's E3 ligase region, its Ran binding domains and the Ran GTPase-activating enzyme RanGAP1 in the RanBP2 SUMO E3 ligase complex has long suggested that the GTPase Ran could be a target for RanBP2-dependent sumoylation. However, sumoylated species of Ran have not been detected by conventional methods in cells or extracts. When Ran Lys-152 was found conjugated to SUMO in a mass spectrometry, based screen for SUMO acceptor sites (33), we decided to investigate this question by biochemical means. Our analysis reveals that Ran can indeed be sumoylated by reconstituted 80 kDa RanBP2 complexes and by NPC-associated full-length RanBP2 complexes in semi-permeabilized cells. These findings add Ran to the very small list of bona fide RanBP2 targets. Sumoylation of Ran, but not that of the model substrate YFP-Sp100, requires RanBP2's Ran binding sites (Fig. 2*D*), which reveals a novel mechanism of target-specific recruitment. Our analysis revealed several reasons for the very low steady - state levels of Ran sumoylation in cells: On the one hand, Ran sumoylation is inhibited when Ran is in complex with transport factors such as importin β, Crm1 and NTF2. On the other hand, SUMO can be efficiently removed from Ran by the intranuclear SUMO isopeptidase SENP1. An additional reason for low endogenous Ran sumoylation may be that Ran is acetylated *in vivo* on several lysine residues including Lys-130 and Lys-152 (62, 63). Switches between SUMOylation and acetylation on specific lysine residues have been described for several SUMO targets and are believed to serve important regulatory roles (reviewed in Ref. 2). Consistent with the idea that Lys-130 and Lys-152 are important for Ran regulation is the striking degree of their evolutionary conservation: Ran Lys-152 is highly conserved in animals, fungi and plants, whereas Ran Lys-130 is strictly conserved only in animals and fungi. Intriguingly, Lys-130 is not present in plants, instead they have a glutamine, which resembles an acetylated lysine. Together, these findings suggest that animals require both Lys-130 and Lys-152 to allow regulation of Ran by lysine-specific modifications. Although an important physiological role for Ran sumoylation is still at large, our detailed mechanistic insights allow to speculate about possible functions for this modification.

RanGDP Is the Relevant Substrate for the RanBP2 E3 Ligase Complex—Recognition of Ran by the RanBP2 E3 ligase depends on RanBP2's Ran binding domains, which explains why RanGTP is much more efficiently sumoylated than RanGDP by the 80 kDa RanBP2 fragment *in vitro* (Fig. 2). However, several

FIGURE 6. **Lys-130 is the major SUMO acceptor site in Ran.** *A*, 1 μM RanGTP was sumoylated in the presence of 70 nM E1, 83 nM E2, 148 nM RanBP2 fragment and 5 mM ATP with or without 15 μM SUMO1 in a total volume of 200 μl. Proteins were separated by SDS-PAGE and stained with colloidal Coomassie. Protein markers are indicated as kDa. Of note, the RanBP2 fragment undergoes massive autosumoylation and disappears in the + ATP sample. The indicated gel slice (*red box*) was cut, subjected to tryptic digest and analyzed by mass spectrometry. *B*, ESI-MS/MS spectra of the C-terminal tryptic SUMO1 peptide conjugated to Ran peptides around Lys 130. Y- and b-type ions are shown in the spectra at their respective positions in the conjugated peptides. *C*, Ran K130R is severely impaired for sumoylation in semi-permeabilized HeLa cells. Sumoylation assays with semi-permeabilized HeLa cells were performed as described in Fig. 3*A* using RanGDP variants at 1 μM concentration. Where indicated, cells were treated with SUMO-Vme prior to sumoylation assays. *D*, amino acids 121–154 of Ran are represented as a linear sequence and the amino acids 118–136, 146–159 are found in structures of RanGDP (*red*) (74) and of RanGMP-PNP (*blue*) in complex with RanBD1 (75). Lys-130 (identified as a SUMO acceptor site here) and Lys-152 (identified in Ref. 33 are *highlighted*. *E*, sumoylation of Ran variants with recombinant enzymes. Assays with 500 nM wt or variant RanGDP, 70 nM E1, 100 nM E2, 100 nM 80kDa RanBP2 complex, and 15 μM SUMO1 with or without ATP were incubated at 30 °C for 30 min. Reactions were stopped with 2× sample buffer and analyzed by immunoblotting with Ran antibody.



	Ka (M^{-1})	△H (cal mol^{-1})	△S (cal mol^{-1} K^{-1})
RanGDP	1.3×10^5	2.2×10^4	49
+ NTF2	N.D.	N.D.	N.D.
+ NTF2-E42K	1.4×10^5	2.4×10^4	57

lines of argument suggest that RanGDP (but not RanGTP) is sumoylated in cells. First, one needs to consider that RanBP2 is quantitatively associated with RanGAP1 in HeLa cells (26). In consequence, any free RanGTP that may encounter RanBP2 will also encounter RanGAP1. Since hydrolysis is much faster than sumoylation, RanGTP will be converted to RanGDP prior to modification (Fig. 2, *E* and *F*). In interphase, RanGTP is generated in the nucleus and reaches the cytoplasm only in association with transport receptors. Whereas transport receptors can protect Ran from GTP hydrolysis, they also inhibit Ran sumoylation (Fig. 5). RanGTP sumoylation is conceivable in mitosis, when RanGTP can encounter RanBP2 without transport receptors, but only under currently unknown conditions of reduced RanGAP1 levels or activity. RanGDP on the other hand is generated directly on the Ran binding domains of RanBP2, as a consequence of export complex disassembly and hydrolysis at these sites. Although RanGDP's affinity for Ran binding domains is only in the range of 10 μM (Fig. 7*C* and Ref. 50), RanBP2's local concentration at nuclear pore complexes in interphase cells (32 copies per NPC; (64) should be sufficiently high for repeated interactions. In consequence, as long as RanGDP is not trapped by NTF2, it can be sumoylated by the RanBP2 complex.

NTF2 Inhibits RanGDP Sumoylation by Preventing Ran from Binding to RBD's—Following up on our observation that NTF2 inhibits Ran sumoylation, we discovered that NTF2 lowers the affinity of RanGDP for Ran binding domains from 10 μM to undetectable levels (Fig. 7*C*). Mechanistically, the most likely explanation is that the switch loops of RanGDP may have to adopt a GTP-bound conformation when interacting with RBD's, and that NTF2 prevents this conformational change by stabilizing the GDP conformation (65). This idea is consistent with RanBP1's effect on RanGDP interaction with RanGAP1 and with nuclear transport receptors (49, 51, 66, 67). Whether NTF2's ability to prevent RanGDP from RBD binding plays a significant role in the efficiency of transport processes remains to be seen. However, in light of the high local concentration of RBD's, NTF2 may well be needed to prevent cytoplasmic RanGDP from blocking these sites. This idea is in line with findings suggesting that nearly all RanGDP at the NPC is bound by NTF2. (68)

Ran Sumoylation, a Side Reaction of an Active E3 Ligase?—A likely scenario in which Ran encounters the RanBP2 E3 ligase complex is as part of nuclear export complexes. It is unclear how RanBP2 recognizes its SUMO targets, and we speculate that nuclear export complexes could be used for this purpose (for a more detailed discussion see (26)). In such a model, transport receptors and the GTPase Ran would have functions similar to those of F-box proteins in the context of SCF ubiquitin E3 ligases. It is well known that F-box proteins are heavily ubiquitiylated in a mechanism known as auto-ubiquitylation (*e.g.* (69, 70). This is considered to be a regulatory mechanism that helps adjusting the required levels of the E3 ligase. One possible reason for Ran sumoylation may thus be that it is a transient component of an active SUMO E3 ligase and that it is sumoylated in a (relevant or unwanted) side reaction.

Possible Roles for Ran Sumoylation—Lys-130 and Lys-152 are the major Ran sumoylation sites for the endogenous RanBP2 complex (Fig. 6 and Ref. 33). Neither of these lysines is essential for Ran's function in *in vitro* transport assays with importin α/β and an artificial model cargo (data not shown). In consequence, RanBP2-dependent Ran sumoylation does not seem to be required for the basic transport mechanism. Considering that RanBP2 is neither present in yeast nor in plants, this is not surprising. This does however not exclude that Ran sumoylation/desumoylation cycles may contribute to the efficiency of (specific) transport pathways in cells. Precedence for a role of SUMO in nuclear import complex disassembly comes from work on the sumoylation of the transport receptor Kap114 in yeast (29). SUMOylation can either block or mediate protein interactions, the latter usually via SUMO interaction motifs that are found in interaction partners (reviewed in Ref. 2). Lys-130 and Lys-152 lie on the same surface of Ran (Fig. 6*C*), indicating that Ran sumoylation on either residue may have similar consequences. Lys-130 and Lys-152 have been implicated in RanGAP1 (51) and RCC1 (71) interactions, and SUMOylation of these sites could theoretically interfere with these. However, this is not consistent with our finding that Ran is sumoylated after GTP hydrolysis in the cytoplasm and desumoylated by SENP1, which likely takes place prior to encountering RCC1. Moreover, physiologically relevant inhibition would only be plausible if a significant fraction of Ran would be sumoylated at a given time. Other known interactions of Ran are unlikely to be affected by sumoylation, also because SUMO is attached to its targets via a very flexible isopeptide linkage. In fact, NTF2 can bind both unsumoylated and sumoylated Ran (Fig. 7*D*). More likely seems the idea that SUMO allows Ran to engage in novel interactions. Ran sumoylation at the cytoplasmic side of the NPC and desumoylation by SENP1 inside the nucleus may well contribute to directed movement of specific cargo through the NPC (Model in Fig. 8), for example in the context of Ran's recently discovered novel role as a

FIGURE 7. **NTF2 inhibits Ran sumoylation by preventing its interaction with Ran Binding domains.** *A*, Lys-130 and Lys-152 are accessible in the Ran/NTF2 complex. Shown is a crystal structure of Ran (*white*) in complex with NTF2 dimers (black/gray) in which Lys-130 and Lys-152 are labeled in *black* (PDB accession code 1A2K (65). *B*, inhibitory effect of NTF2 is mediated via NTF2-Ran interaction. *In vitro* sumoylation of 500 nM RanGDP was performed as in Fig. 5. Wt NTF2, the variant NTF2-W7A (fails to bind FG repeats) or the variant NTF2-E42K (fails to bind Ran) was preincubated with Ran at the indicated concentrations on ice for 1 h prior to sumoylation assays. Reactions were stopped with 2× sample buffer, and samples were analyzed by immunoblotting with α-Ran. Of note, *lanes 2–5* are identical to those shown in Fig. 5*B*. *C*, NTF2 lowers the interaction of Ran with RBD4 to undetectable levels. ITC titrations were performed in an ITC 200 Microcalorimeter at 25 °C with 200 μM RBD4 in the syringe and 15 μM RanGDP in the cell. Where indicated, the cell solution also contained 30 μM NTF2 WT or NTF2-E42K mutant. Table: Values for the specific ITC experiments shown here. Standard errors for all ΔH and K_a values were below 12%. *D*, immobilized NTF2 and control beads were incubated with the *in vitro* sumoylation reaction of 500 nM RanGDP in the presence of 100 nm E1, 100 nM E2, 100 nM RanBP2 complex, 15 μM SUMO1, and 5 mM ATP for 1 h at 37 °C. Bound proteins were compared with 4% of the input by immunoblotting with Ran antibodies, fluorescent secondary antibodies, and analysis with an Odyssey infrared imaging system. Shown is one of three independent experiments. Quantification revealed no significant differences in NTF2 binding for unmodified and sumoylated Ran (unmod. Ran/Ran*SUMO in input = 24 ± 8; unmod. Ran/Ran*SUMO in bound fraction = 33 ± 9).

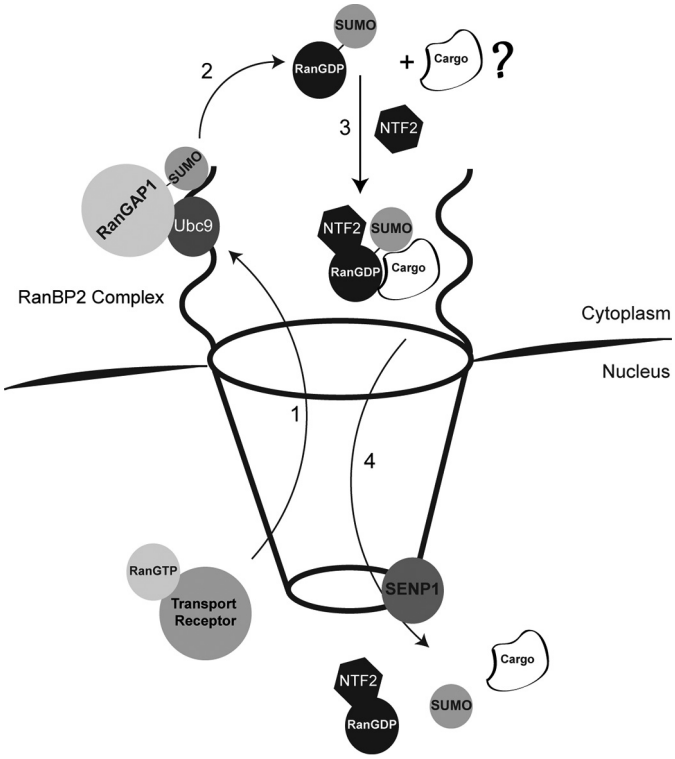


FIGURE 8. **A hypothetical role for RanGDP sumoylation by the RanBP2 complex.** As detailed in the discussion, our findings indicate that RanGDP, rather than RanGTP, is the physiologically relevant substrate for the RanBP2 complex. In interphase, RanGDP sumoylation can only take place after RanGTP exits the nucleus as part of a complex with transport receptors (1). Such a complex can interact with the RanBP2/RanGAP1*SUMO1 complex at the cytoplasmic filaments of NPCs. RanGAP1 and RanBP2's RBDs contribute to complex disassembly and RanGTP hydrolysis. In consequence, RanGTP is converted to RanGDP while sitting on Ran Binding domains of RanBP2. Two fates are then conceivable. Either RanGDP dissociates, encounters NTF2, which prevents rebinding, and is rapidly translocated into the nucleus. Alternatively, RanGDP is sumoylated on RanBP2 (2). As NTF2 can interact with sumoylated Ran (Fig. 7*D*), this may allow formation of import competent complexes consisting of Ran*SUMO1, NTF2 and hypothetical cargo proteins that may selectively bind to sumoylated Ran (3). In such a scenario, sumoylated Ran serves as a specific substrate receptor and NTF2 as the import receptor. Upon translocation into the nucleus, the NTF2/Ran*SUMO1/cargo complex encounters SENP1, which reverts the modification and thereby induces cargo release (4).

transport receptor (72, 73). Addressing such questions will be an exciting future challenge.

Author Contributions—V. S. designed and carried out most experiments and wrote the manuscript. S. M. R. designed and carried out several experiments and wrote the manuscript. H. H. H. and H. U. carried out mass spectrometry analyses, F. M. guided the project, designed experiments and wrote the manuscript.

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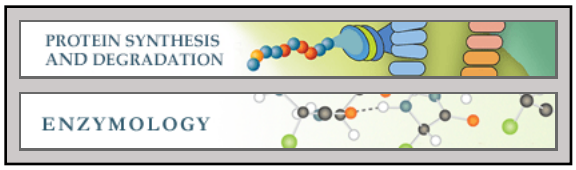
Protein Synthesis and Degradation:

Sumoylation of the GTPase Ran by the RanBP2 SUMO E3 Ligase Complex



Volkan Sakin, Sebastian M. Richter, He-Hsuan Hsiao, Henning Urlaub and Frauke Melchior

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