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Transcriptional coregulator SNURF (RNF4) possesses ubiquitin E3 ligase activity

M. Häkli[a,1], K.L. Lorick[b,1], A.M. Weissman[b], O.A. Jänne[a,c], J.J. Palvimo[a,*]

[a] *Biomedicum Helsinki, Institute of Biomedicine, University of Helsinki, FIN-00014 Helsinki, Finland*
[b] *Laboratory of Protein Dynamics and Signaling, National Cancer Institute, Bldg 560, Rm 12-39, Ft. Detrick, Frederick, MD 21702-1201, USA*
[c] *Department of Clinical Chemistry, University of Helsinki and Helsinki University Central Hospital, FIN-00014 Helsinki, Finland*

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Abstract SNURF/RNF4 has been implicated in transcriptional regulation and growth inhibition in a RING finger-dependent fashion. In this work, we show that SNURF mediates its own ubiquitination in vitro in a ubiquitin-conjugating enzyme (E2)-selective manner: SNURF acts as an E3 ligase with UbcH5A and B, HHR6B (RAD6B), E2-25K, MmUbc7 and UbcH13, but not with UbcH3, UbcM4, MmUbc6 or E2-20K. In contrast, the well-characterized RING E3, AO7, functions only with members of the UbcH5 family. Furthermore, depending on the E2 used, the ubiquitin modification manifests as mono- or multi-ubiquitination. Mutation of conserved cysteine residues within the RING finger motif of SNURF abolishes the ubiquitination in vitro and in intact cells. Size fractionation of murine embryonal carcinoma F9 cell proteins shows that the majority of endogenous SNURF resides in salt-resistant ≥500-kDa complexes, suggesting that SNURF functions as a RING component in a multiprotein complex. Taken together, SNURF/RNF4 functions as an E3 ligase and this activity is closely linked to its transcription regulatory functions.

Key words: SNURF/RNF4; Transcriptional coregulator; E3 ubiquitin ligase; RING finger

1. Introduction

SNURF (RNF4) is a small nuclear protein that encompasses a C_3HC_4 (RING-HC)-type RING motif at its C-terminal domain [1]. RING fingers are conserved, cysteine-rich motifs which coordinate two zinc atoms in a 'cross-brace' fashion and have been shown to mediate protein–protein interactions and multi-protein complex formation [2,3]. SNURF was initially identified as a co-activator protein of steroid receptors [1]. In addition to steroid receptors, SNURF enhances the activity of promoter specificity protein 1 (Sp1)-regulated transcription [1,4,5]. SNURF can also act as a transcriptional co-repressor when associated with a novel POZ-AT hook-zinc finger protein (PATZ) [6,7]. The RING finger domain is mandatory for the ability of SNURF to coregulate Sp1, PATZ, and activator protein of stromelysin gene (SPBP) in transcription assays [4,7,8]. SNURF also interacts with goosecoid-like (Gsc1) homeodomain transcription factor and TRPS1 transcription factor (a repressor of GATA-mediated transcription), but as in the case of steroid receptors, regions N-terminal to the RING finger appear be important for the interactions [1,5,6,10]. Besides associating with transcription factors, SNURF can bind DNA and mononucleosomes in a RING domain-independent fashion. SNURF may, therefore, play a role in the assembly of nucleoprotein structures [11].

Recently, many RING finger proteins have been shown to mediate ubiquitin-conjugating enzyme (E2)-dependent attachment of ubiquitin (ubiquitination) to target proteins [12,13]. In particular, AO7 was used to show the necessity of the RING finger and its zinc-coordinating residues for interaction with E2s and subsequent ability to form ubiquitin chains [12]. Modification by ubiquitin is essential for the degradation of a large number of proteins, and it plays a central role in a variety of cellular processes, including cell cycle regulation, DNA repair, signal transduction, and transcriptional regulation [14,15]. The 76-amino acid ubiquitin polypeptide contains seven lysine residues where additional ubiquitins may be added to form multi-ubiquitin chains. The physiological consequence of ubiquitin modification depends on the nature of the ubiquitin chain being formed. Degradation is generally signaled by chain formation via lysine 48, while signal transduction and histone modification require mono-ubiquitination, and lysine 63 linkages are important for DNA repair and kinase activation [16]. Other ubiquitin chains (Lys6, Lys11, Lys29) have been described in vitro [17] and in vivo [18], but they are less well characterized. Ubiquitination of proteins is achieved in three enzymatic steps. The first step involves ATP-dependent activation of ubiquitin by a ubiquitin-activating enzyme (E1), resulting in formation of a thioester linkage between ubiquitin and E1. Activated ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2) and subsequently to a lysine residue of a substrate with the assistance of an E3 ubiquitin protein ligase. E3s bind substrates and thereby govern specificity of ubiquitination [19]. Two distinct classes of ubiquitin ligases have been established: HECT (homologous to E6-AP carboxy-terminus) domain E3s and RING finger E3s [19,20]. The HECT domain E3s form thioester intermediates with ubiquitin as a part of the process, whereas the RING finger E3s are thought to facilitate direct transfer of ubiquitin from E2 to substrate, probably without formation of covalent intermediates [12,19].

In this work, we show that SNURF possesses ubiquitin-

*Corresponding author. Fax: (358)-9-19125302.
E-mail address: jorma.palvimo@helsinki.fi (J.J. Palvimo).

[1] These authors contributed equally to this work.

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conjugating enzyme (E2)-selective E3 ligase activity that is dependent on its RING finger domain in vitro and in vivo. Furthermore, we show that this selectivity is not a general feature of RING proteins and results in differing patterns of ubiquitin modification that may explain the varied effects of SNURF on regulatory proteins. Size fractionation of protein complexes from F9 cells demonstrates that endogenous SNURF resides in macromolecular protein complexes, suggesting that SNURF functions as a RING component of a multi-subunit regulatory complex.

2. Materials and methods

2.1. Materials

The protease inhibitors phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin A, aprotinin and iodoacetamide, and ubiquitin were obtained from Sigma-Aldrich. MG-132 was purchased from Calbiochem. Glutathione-Sepharose 4B, pGEX-6P-1, Superose 6 HR 10/30 column, HMW gel filtration calibration kit, PreScission™ Protease, Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane and ECL detection reagents were from Amersham Biosciences. Horseradish peroxidase-conjugated anti-mouse IgG was from Zymed. RNA polymerase II antibody (N-20) was from Santa Cruz Biotechnology. HA.11 affinity matrix was obtained from Nordic BioSite AB, and GammaBind™ G-Sepharose was from Amersham Biosciences. Hemagglutinin (HA)-tagged ubiquitin expression vector (pMT123) was a gift from Drs. Dirk Bohmann and Mathias Treier. pcDNA-FLAG-SNURF, pcDNA-FLAG-SNURF mutants, and bacterial pGEX-5X-1-SNURF expression constructs have been described [5,11]. Mouse E1 enzyme was purified from baculovirus-infected insect cells, E2 conjugases (E2-25K, HHR6B, UbcH3, UbcM4, UbcH5B, MmUbc6, MmUbc7, E2-20K, UbcH13), and E3 ligase, glutathione *S*-transferase (GST)-AO7, were expressed in *Escherichia coli* as described [12,21–24]. E2-25K was the gift of Dr. Cecile Pickart [25], HHR6B was the gift of Dr. Art Haas [17], UbcH3, UbcM4, E2-20K and mouse E1 constructs were the gift of Dr. Kazuhiro Iwai [26,27], UbcH13 was the gift of Dr. Brian Druker [28]. GST-UbcH5A and rabbit E1 were purchased from Affiniti Research Products. [^{32}P]Ubiquitin was synthesized as described [29].

2.2. Cell culture and transfections

Murine embryonal carcinoma F9 and COS-1 cells were obtained from American Type Culture Collection, and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 25 U/ml of streptomycin and penicillin. F9 cells were cultured on 0.1% gelatin-coated cell culture dishes. For transfections, 3.5×10^5 COS-1 cells were seeded on 6-cm dishes 24 h before transfection. Four hours before the addition of plasmid DNA (2.5 μg/dish) and FuGene 6 transfection reagent (Roche Molecular Biochemicals), the cells received fresh medium. After 18 h of transfection, the medium was changed, and after 36 h of transfection 1.5 μM MG-132 was added. Cells were washed with phosphate-buffered saline (PBS) and harvested in PBS containing 10 mM iodoacetamide 48 h after transfection.

2.3. Immunoprecipitation

COS-1 cells transfected with HA-ubiquitin and FLAG-SNURF expression plasmids and treated with or without 1.5 μM MG-132 were lysed in a buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Nonidet P-40, 15 mM $MgCl_2$, 50 μM $ZnCl_2$, 5 mM EDTA, 1 mM dithiothreitol (DTT), 10 mM iodoacetamide and 1:200-diluted protease inhibitor cocktail (Sigma). Cell lysates were clarified by centrifugation and pre-incubated with normalized mouse serum (Calbiochem) and GammaBind G-resin. After centrifugation and transfer of supernatants to new tubes, anti-HA affinity matrix was added and incubated at 4°C overnight. Resins were washed four times with lysis buffer and bound proteins were eluted with sodium dodecyl sulfate (SDS) sample buffer lacking β-mercaptoethanol at 65°C. Samples were centrifuged and β-mercaptoethanol was added to resin-free supernatants that were subsequently heated at 95°C for 5 min. Proteins were fractionated by 15% SDS–polyacrylamide gel electrophoresis (PAGE), electroblotted onto a Hybond ECL membrane, and SNURF was detected with anti-SNURF antibody and ECL detection system.

2.4. Gel filtration

SNURF coding sequence (*Bam*HI-blunt/*Sal*I) was cleaved from pGEX-5X-1-SNURF plasmid and cloned into pGEX-6P-1 vector. GST-SNURF was expressed in *E. coli* (BL21 CodonPlus DE3-RIL, Stratagene) and purified on glutathione 4B Sepharose as described [11]. The GST moiety was removed by using PreScission™ protease in a buffer containing 50 mM Tris–HCl (pH 7.5), 350 mM NaCl, 0.8% (v/v) Triton X-100, 0.1% (v/v) Nonidet P-40, 2 mM EDTA, 1 mM DTT, 50 μM PMSF, and 10 μM $ZnCl_2$, and the cleaved SNURF was dialyzed against GF buffer (50 mM Tris–HCl (pH 7.8), 1 mM EDTA, 450 mM NaCl, 0.1% (v/v) Triton X-100, 0.5 mM PMSF, and 10% (v/v) glycerol). F9 cells were lysed in the GF buffer by rotation for 30 min at 4°C and the cell lysates were centrifuged at $14\,000\times g$ for 20 min. Supernatants were filtered through Spin-X (0.22 μm, Costar) columns. The cell lysate (~5 mg protein) or purified recombinant SNURF (100 μg) was size-fractionated on a Superose 6 HR 10/30 column in GF buffer at 0.3 ml/min, and 1-min fractions were collected. In some experiments, the NaCl concentration of the F9 cell lysates was increased to 650 mM and GF buffer containing 650 mM NaCl was used as running buffer. Blue dextran 2000 (2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa) were used as molecular size markers. SNURF was detected from the eluate fractions by immunoblotting with a rabbit anti-SNURF antiserum raised against full-length SNURF produced in bacteria.

2.5. In vitro ubiquitination

Bacterially expressed GST-SNURF or its mutants were bound on glutathione 4B Sepharose and washed with 50 mM Tris–HCl (pH 7.4). GST-AO7 or GST-SNURFs (20 pmol) were incubated in a 30 μl reaction mixture containing the E2 enzyme (100 ng), the E1 enzyme (100 ng), ^{32}P-labeled ubiquitin (40 000 dpm), and 2 μl bacterial lysate from BL21 *E. coli* transformed with pET15b [12]. Reaction buffer consisted of 50 mM Tris–HCl (pH 7.4), 200 μM ATP, 0.5 mM $MgCl_2$, 0.1 mM DTT, 1 mM creatine phosphate, and 15 U of phosphocreatine kinase. Reactions were incubated at 30°C for 2 h and stopped by adding concentrated SDS–PAGE sample buffer. The proteins were separated on 15% SDS–PAGE gels and detected by autoradiography. GST-cleaved SNURF protein (600 nM) was incubated with GST-UbcH5A (200 nM) and E1 (30 nM), ubiquitin (1 μM) in 50 mM Tris–HCl (pH 7.4), 2 mM ATP, 5 mM $MgCl_2$, 2 mM DTT and 0.5 μg/μl lysate from BL21 *E. coli* transformed with pGEX-5X-1 in a reaction volume of 15 μl for 90 min at 30°C. Reactions were terminated by adding concentrated SDS–PAGE buffer, and analyzed by 15% SDS–PAGE and immunoblotting with anti-SNURF antibody. Thiol ester assay reactions were performed according to Jensen et al. [22]. After incubation at 30°C for 2 h, reactions were terminated by incubation in 25 mM Tris–HCl (pH 6.8), 5% (v/v) glycerol, 4% lithium dodecyl sulfate, 4 M urea at 30°C for 15 min. Samples were subjected to non-reducing SDS–PAGE, and radioactively labeled proteins were visualized by autoradiography.

3. Results

3.1. SNURF undergoes ubiquitination in an E2 conjugating enzyme-selective fashion in vitro

To determine whether SNURF can mediate ubiquitination, bacterially expressed, purified GST-SNURF was incubated with recombinant E1 enzyme and different recombinant E2s (HHR6B, UbcH3, UbcH5B, MmUbc6, MmUbc7, E2-20K, UbcH13, UbcM4, and E2-25K produced in bacteria) in the presence of ^{32}P-labeled ubiquitin and bacterial lysate. In this in vitro system, proteins present in the bacterial lysate and SNURF served as potential substrates. Reactions were analyzed by SDS–PAGE and autoradiography. As shown in Fig. 1A, addition of GST-SNURF to the reactions containing UbcH5B, MmUbc7, or E2-25K resulted in substantial multi-ubiquitination, while reactions with HHR6B and UbcH13 yielded predominately mono-ubiquitination. Also multi-ubiquitination could be seen with HHR6B and UbcH13 upon longer incubation times (data not shown). In contrast, no

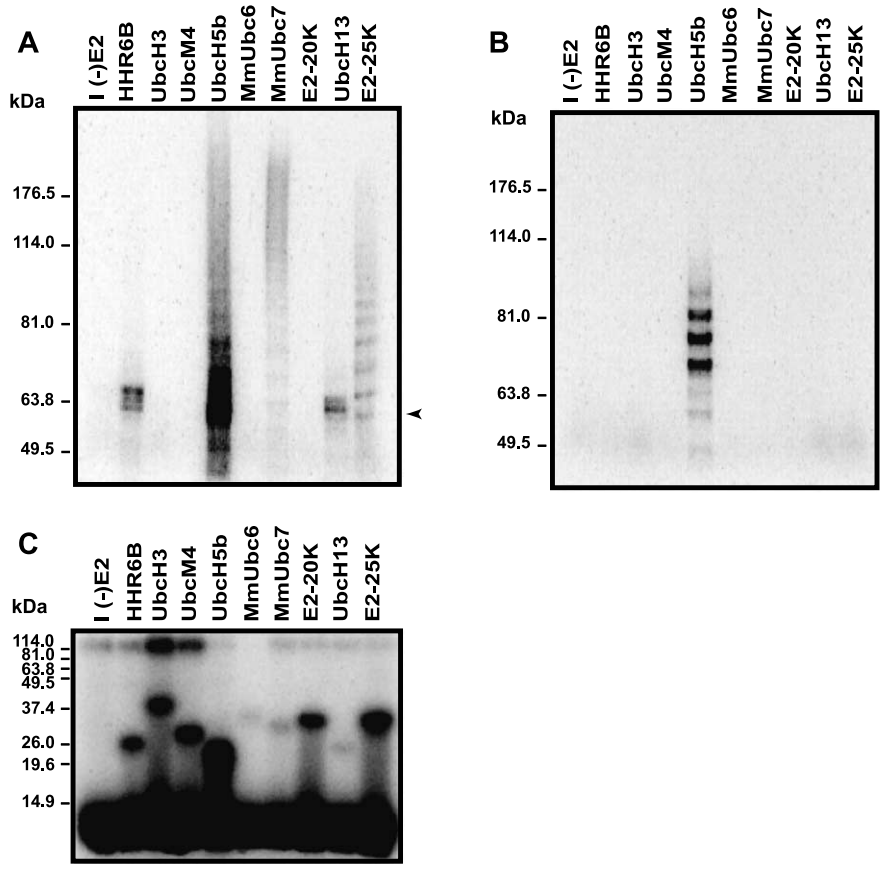

Fig. 1. SNURF mediates E2-dependent ubiquitination in vitro. Glutathione bead-absorbed GST-SNURF (A) or GST-AO7 (B) was incubated with ^{32}P-labeled ubiquitin, recombinant E1 and various recombinant E2 enzymes (HHR6B, UbcH3, UbcM4, UbcH5B, UbcM6, UbcM7, Ubc8, UbcH13, E2-20K, and E2-25K) for 2 h at 30°C as described in Section 2. The ubiquitinating mixture was removed and the protein on beads washed with 50 mM Tris–HCl, pH 7.4. Protein were separated by SDS–PAGE and ubiquitinated species were detected by autoradiography. C: E2s used in A and B were incubated with recombinant E1 and ^{32}P-labeled ubiquitin for 2 h at 30 °C and separated by SDS–PAGE under non-reducing conditions to show relative activity of individual E2s. The arrowhead in A shows the smallest ubiquitinated SNURF form.

ubiquitination was evident with UbcH3, MmUbc6, UbcM4 or E2-20K. The RING finger E3 ligase AO7, however, was only active with UbcH5B (Fig. 1B). The relative activity of the E2s used is shown by thioester formation in Fig. 1C. Interestingly, while AO7 binds tightly to UbcH5B [12], stable complexes were not detected under in vitro conditions between GST-SNURF and any of the E2s tested (data not shown).

To test the role of SNURF's RING-HC motif for the apparent ubiquitin ligase activity, GST-SNURF proteins containing point mutations of the conserved cysteines in the RING finger motif (Fig. 2A) were studied in ubiquitination using UbcH5B as the E2 enzyme (Fig. 2B). Replacement of cysteines 136 and 139 by serines (C136/139S) in the putative coordination structure of the first zinc ion or comparable mutation in the second coordination structure (C177/180S) abolished the ubiquitination activity of SNURF. Likewise, deletion mutant 178–194 did not mediate ubiquitination. Interestingly, also deletion of the first 20 amino acids of SNURF resulted in attenuation of the ubiquitination activity (Fig. 2B). Comparable results were obtained when MmUbc7 was used as an E2 enzyme (data not shown). Taken together, these results indicate that SNURF can mediate ubiquitination in an E2 enzyme-selective fashion and that the intact RING finger motif is mandatory for SNURF's E3 activity.

Upon removal of GST-SNURF from the UbcH5B-containing reaction mixture with glutathione beads and washing of the unbound proteins away, the large majority of the ubiquitinated material remained bound to glutathione beads, indicating that GST-SNURF represents the major ubiquitinated species. To confirm that SNURF itself, and not the GST moiety, was conjugated by ubiquitin, SNURF was cleaved from the GST moiety and incubated with E1, UbcH5A and ubiquitin and analyzed the reactions by immunoblotting with anti-SNURF antibody. As shown in Fig. 2C, mainly mono- and di-ubiquitinated SNURF species were detected when both E1 and E2 were included in the reactions, confirming that SNURF is self-ubiquitinated.

3.2. SNURF is ubiquitinated in intact cells

To study whether SNURF is ubiquitinated in intact cells, we cotransfected COS-1 cells with expression plasmids encoding HA-tagged ubiquitin and wild-type or RING finger-mutated (C136/139/177/180S) SNURF. Cell lysates were immunoprecipitated with anti-HA antibody resin, and bound proteins were analyzed by immunoblotting with anti-SNURF antibody. When HA-ubiquitin was co-expressed with SNURF, higher molecular mass species indicative of ubiquitinated SNURF forms were immunoprecipitated with the res-

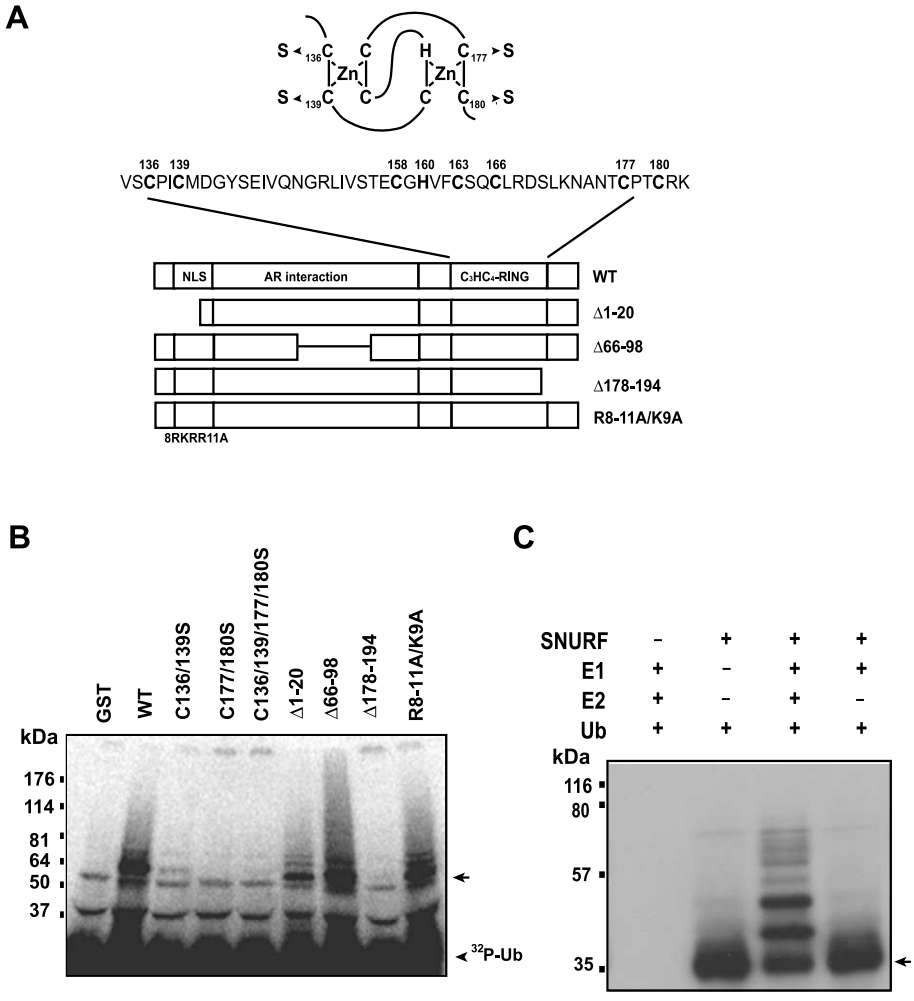

Fig. 2. Intact RING finger motif of SNURF is needed for ubiquitination. A: Schematic presentation of SNURF, SNURF RING finger, and SNURF mutants used in in vitro ubiquitination studies. Conserved cysteines and histidine of RING finger motif are underlined. The proposed 'cross-brace' RING C_3HC_4-type structure, where the zinc coordinating residues are pointed. Arrowheads indicate location of cysteine to serine mutations in the RING finger. B: GST, GST-SNURF and GST fusion of SNURF mutants C136S/C139S, C177/180S, C136/C139/C177/180S, Δ1–20, Δ66–98, Δ178–194, R8-11A/K9A were incubated with UbcH5B and analyzed as described in Fig. 1. C: Ubiquitination of GST-cleaved SNURF incubated with ubiquitin (Ub) in the presence (+) and absence (−) of recombinant E1 and GST-UbcH5A at 30°C for 90 min. The reactions were analyzed by immunoblotting with anti-SNURF antibody. Positions of GST-SNURF (B) and of SNURF (C) are depicted by arrows and free ubiquitin (^{32}P-Ub) by the arrowhead.

in (Fig. 3). In accordance with the cell-free experiments, the SNURF RING mutant showed no ubiquitination. Mainly mono- and di-ubiquitinated SNURF species were detectable in the absence of proteasomal inhibitor MG-132, and inclusion of the inhibitor rendered tri- and tetra-ubiquitinated SNURF species visible. While SNURF may target itself for degradation, this is not the sole function of its E3 activity, since the RING mutations did not stabilize the protein. Interestingly however, treatment with MG-132 allowed detection of higher levels of ubiquitinated SNURF (Fig. 3). Taken together, these results indicate that SNURF undergoes ubiquitination also in intact cells.

3.3. *Endogenous SNURF resides in large salt-resistant complexes*

Many RING finger proteins are subunits in multiprotein complexes [30]. To assess whether endogenous SNURF resides in macromolecular complexes, we studied endogenous SNURF protein in mouse embryonal carcinoma F9 cells in which SNURF is abundantly expressed. We size-fractionated F9 cell lysates by gel filtration and analyzed the presence of SNURF in the eluate fractions by immunoblotting with anti-SNURF antibody. In contrast to purified recombinant SNURF that eluted in low molecular mass fractions (Fig. 4A), the majority of endogenous SNURF in F9 cells resided in $\geq$500-kDa complexes that were stable at 0.45 M and 0.65 M NaCl (Fig. 4B and results not shown). The latter fractions also contained the vast majority of RNA polymerase II large subunit (Fig. 4C). A similar elution pattern for SNURF was observed when protein lysates of mouse testis were fractionated under identical chromatographic conditions (data not shown). In addition to unmodified SNURF migrating at ~35 kDa, immunoblotting of the F9 cell-derived fractions revealed the presence of higher molecular mass species of SNURF in $\leq$200-kDa gel filtration fractions (Fig. 4B), suggesting that endogenous SNURF is ubiquitinated and that the modification influences SNURF's protein–protein interactions.

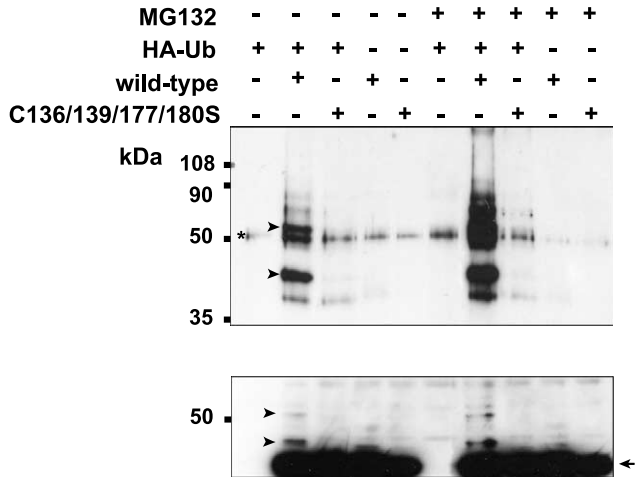

Fig. 3. SNURF is ubiquitinated in intact cells. COS-1 cells were co-transfected with wild-type or RING finger mutant (C136/139/177/180S) of FLAG-SNURF alone or with HA-ubiquitin expression plasmids as indicated by + and − signs. After 36 h transfection, cells were treated with (+) or without (−) MG-132 (1.5 μM) and harvested 12 h after that. Upper panel: Ubiquitinated proteins were immunoprecipitated with anti-HA antibody resin, eluted and analyzed by SDS–PAGE and immunoblotting with anti-SNURF antibody. Lower panel: Immunoblot analysis of the corresponding whole cell lysates using anti-SNURF antibody. Migrations of SNURF species modified by one or two ubiquitin moieties are indicated by arrowheads and an arrow shows the position of unmodified SNURF. The asterisk indicates the position of IgG heavy chain.

4. Discussion

Here, we show that the transcriptional coregulator SNURF/RNF4 possesses ubiquitin E3 ligase activity. As in the case of AO7, Mdm2, c-Cbl and ROC1 [12,21,31,32], an intact RING finger motif of SNURF was mandatory for the E3 ligase function; disruption by point mutations or deletion of the RING finger motif abolished the catalytic activity. RING finger ubiquitin ligases can be divided into single- and multi-subunit E3s. Single-subunit RING E3s, such as proto-oncoproteins Mdm2 (murine double minute 2) and c-Cbl [21,31], harbor the substrate recognition element and the RING finger in the same polypeptide. Multi-subunit RING E3s come in at least in three types: SCF (Skp-1–Cullin–F-box protein), APC (anaphase-promoting complex), and the VCB (von Hippel–Lindau–elongin C/elongin B) complexes, which all have a small RING finger protein, such as ROC1, and a member of the Cullin family of proteins as well as other subunits some of which recognize substrates [19,30]. The majority of endogenous SNURF was found in salt-resistant high molecular mass complexes in murine F9 cells, implying that this small nuclear RING finger protein may act as a RING component in a multi-subunit protein complex.

The E3 activity of SNURF was E2 enzyme-selective; SNURF was clearly active with HHR6B (RAD6B), UbcH5A, UbcH5B, MmUbc7, UbcH13 and E2-25K, whereas it showed no activity with UbcH3 (cdc34), MmUbc6, UbcM4 or E2-20K. While this multiple use of E2s is not unique to SNURF, it is clearly not a general feature of RING proteins, as other RING E3s mediate ubiquitination by a much more limited set of E2s; for example, AO7 (Fig. 1B), and BRCA1 [33] use only members of the UbcH5 family.

UbcH5 family E2s have recently been shown to function with several RING finger E3s, including RLIM (RING finger

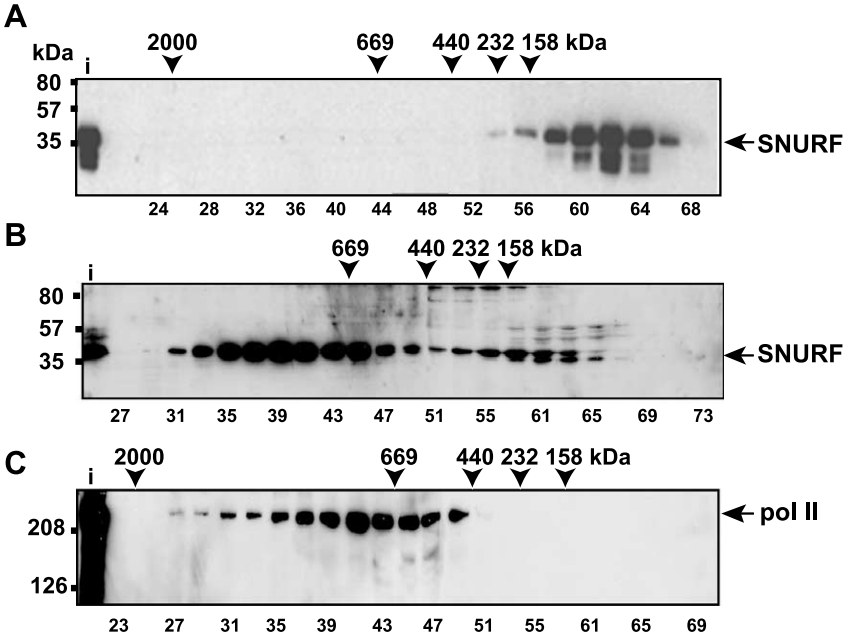

Fig. 4. Analysis of endogenous SNURF complexes of F9 cells by gel filtration chromatography. Purified recombinant SNURF (A) and whole cell extracts from F9 cells (B,C) were chromatographed on a Superose 6 HR10/30 column in a buffer containing 450 mM NaCl. The resulting 0.3-ml fractions (every other fraction as indicated) were subjected to immunoblot analysis with anti-SNURF antibody (A,B) or anti-RNA polymerase II (large subunit) antibody (C). Elution positions of size marker proteins (in kDa) analyzed under identical running conditions are depicted by arrowheads. (i, input sample.)

LIM domain-binding protein) and Mdm2 as well as multi-subunit RING E3s SCF and APC, and to ubiquitinate tumor suppressor p53, CLIM (a cofactor of LIM homeodomain transcription factors) [34–36]. HHR6 is a human counterpart of yeast DNA repair protein RAD6 which can ubiquitinate histones H2A, H2B and H3 and thereby regulate chromatin structure [37–39]. UbcH13, the human homologue of *Drosophila* bendless, has recently been shown to form lysine 63-linked ubiquitin chains that activate IκB kinase [28]. E2-25K is the huntingtin-interacting protein-2 (HIP-2) [25], whose physiological significance is not well defined. It can act as an E2 with RING2, an integrin-binding protein, but the latter E3 activity and integrin signaling have not been linked [40]. Furthermore, the p105 subunit of NF-κB is degraded in an E2-25K-dependent manner, but the E3 of the reaction is unknown [41]. Our results show that SNURF is an E3 with UbcH5 and E2-25K for which there are several possible substrates.

We have attempted to find a role for SNURF as an E3 ligase with its interaction partners Sp1 and AR, but have not found SNURF-dependent alteration in ubiquitination of these two proteins. However, our studies do not preclude a role for SNURF in the ubiquitination of other transcription factors. The mechanism by which RING finger proteins promote ubiquitination is not fully understood, but it is likely that the RING E3s serve as adapters between the E2 enzyme and the target protein [19]. Nevertheless, most of the RING finger proteins that have thus far been shown to mediate ubiquitination in cell-free systems lack defined substrates other than themselves. Also the significance of auto-ubiquitination of E3 ligases has remained elusive. Auto-ubiquitination may regulate the turn-over of E3s (MDM2, DIAP1) [21], or serve as a means to regulate their E3 activity or tumor suppressor function (BRCA1) [42].

SNURF showed mainly mono- and di-ubiquitination in intact cells. In contrast to poly-ubiquitination, mono- and di-ubiquitination do not serve as proteasome targeting signals [16]. This result, along with the finding that RING mutants of SNURF were not more stable than wild type protein, indicates that the E3 activity does not simply target SNURF to degradation. Instead, it is clearly connected to SNURF's function as a transcriptional and cell growth regulator, since mutations that inactivate the E3 function abolish both SNURF's coregulatory activity with many transcription factors and its ability to inhibit cell proliferation in germ cell tumor-derived cells [5,8,9,43]. Collectively, our results that SNURF possesses ubiquitin-conjugating enzyme-selective E3 ligase activity provide an important new insight into the function of this interesting transcriptional coregulator.

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