Ubiquitinated proteins can alternatively be delivered directly to the proteasome or via p97/VCP (valosin-containing protein). Whereas the proteasome degrades ubiquitinated proteins, the homohexameric ATPase p97/VCP seems to control the ubiquitination status of recruited substrates. The COP9 signalosome (CSN) is also involved in the ubiquitin/proteasome system (UPS) as exemplified by regulating the neddylation of ubiquitin (Ub). Here, we show that p97/VCP colocalizes and directly interacts with the CSN. The COP9 signalosome (CSN) is also involved in the ubiquitin/proteasome system (UPS) as exemplified by regulating the neddylation of ubiquitin (Ub). E3 ligases. Here, we show that p97/VCP colocalizes and directly interacts with the CSN (CSN5) in vivo and is associated with the entire CSN complex in an ATP-dependent manner. Furthermore, we provide evidence that the CSN and in particular the isopeptidase activity of its subunit CSN5 as well as the associated deubiquitinase USP15 are required for proper processing of polyubiquitinated substrates bound to p97/VCP. Moreover, we show that in addition to NEDD8, CSN5 binds to oligoubiquitin chains in vitro. Therefore, CSN and p97/VCP could form an ATP-dependent complex that resembles the 19 S proteasome regulatory particle and serves as a key mediator between ubiquitination and degradation pathways.

Many fundamental cellular functions such as membrane fusion, gene transcription, DNA replication, and repair are controlled by the covalent linkage of ubiquitin (Ub) to substrate proteins. Different Ub modifications serve as signaling-dependent regulators of protein interaction networks. Substrates that are polyubiquitinated via Lys48 are recognized by the 19 S regulatory particle of the proteasome that consists of a lid and a base complex. Upon recognition, substrates are unfolded by the base complex and hydrolyzed within the 20 S proteolytic chamber.

The COP9 signalosome (CSN) plays an essential role as mediator between signaling pathways and downstream mechanisms controlling developmental processes. This involves ubiquitin-dependent protein degradation of key regulatory molecules like the cell cycle inhibitor p27kip1, the tumor suppressor p53, and IkBα. The CSN is a highly conserved complex found in all higher eukaryotes. Like the proteasome lid, it contains eight core subunits (CSN1–8), and for each of them exists a paralogous subunit in the proteasome lid. CSN5 (also known as Jab1) and CSN6 possess a MOV34/PAD N-terminal (MPN) domain. The MPN domain of CSN5 harbors a metallopeptidase motif referred to as the Jab1/MPN domain-associated metallopeptidase (JAMM) motif that regulates the activity of E3 Ub-ligases by neddylation of the cullin component.

The chaperone p97 or valosin-containing protein (p97/VCP) has been recognized as another key player within the ubiquitin/proteasome system (UPS). p97/VCP extracts mono- or oligoubiquitinated substrates from complexes and presents them to the UPS. It is a member of the family of ATPases associated with various cellular activities (AAA+) and forms a homohexamer. Two AAA cassettes of each monomer build two consecutive stacked rings (D1 and D2 domain) in the hexamer underneath a ring of flexible N-terminal domains. Its structure is reminiscent of the proteasome base complex that contains a heterohexameric ring of the AAA+ ATPases Rpt1–6. After extraction of ubiquitinated substrates from larger complexes with the aid of substrate-recruiting cofactors, the segregated proteins can suffer three different fates depending on the involvement of a number of substrate-processing cofactors. They can (i) be polyubiquitinated promoting proteasomal degradation, (ii) stably maintain their ubiquitination status, or (iii) be deubiquitinated and released from the complex.
While investigating the interactome of macrophage migration inhibitory factor (MIF) by using stably transfected NIH 3T3 cells expressing MIF that is endogenously tagged with biotin (8), we identified p97/VCP as a new MIF-interacting protein. To clarify the role of MIF in the UPS, we at first explored the interaction of p97/VCP and CSN5. We discovered that both complexes form an ATP-dependent supercomplex resembling the proteasome-regulatory particle that could serve as a key UPS substrate-processing machine determining substrate fate.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The following antibodies were used: mouse anti-FLAG (M2) and mouse anti-β-actin (Sigma), mouse anti-p97/VCP (Affinity BioReagent), rabbit anti-CSN5, rabbit anti-p97/VCP, rabbit anti-ubiquitin (Santa Cruz Biotechnology), rabbit anti-CSN1 (Biomol,), mouse anti-UPS15 (Abnova), and rabbit anti-cullin 1 (Abcam). The c-Myc antibody (9E10) was a gift of Martin Eilers (Würzburg, Germany). Mouse anti-FLAG M2 agarose affinity gel was purchased from Sigma and K48-linked oligoubiquitin chains Ub(2–7) from Biomol International, LP.

**Cell Culture and Transfections**—HEK 293T and NIH 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium, containing glutamine, 10% fetal calf serum, and antibiotics at 37 °C in 10% CO2. Cells were seeded in 6-well plates at a density of 2 × 10^5 cells 1 day before transfection and transiently transfected with 1 μg of expression plasmid using FuGENE 6 (Roche Applied Science) according to the instructions of the supplier. In some experiments, only 0.3 or 0.5 μg of DNA/well was used; however, the total amount of DNA/well was kept constant at 1 μg by adding empty vector DNA.

**Coimmunoprecipitation and Immunoblotting**—Washed cells were lysed in 500 μl of radioimmune precipitation assay buffer containing protease inhibitors (50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM K2HPO4, 1 mM v/v glycerol, 1% Igepal CA-630, 0.15% SDS, 1 mM Na3VO4, 1 mM sodium molybdate, 20 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride), passed through a 24-gauge needle, and centrifuged at 13,000 × g for 10 min at 4 °C. The supernatants were diluted (1:2) with immunoprecipitation (IP) buffer (20 mM Tris (pH 8), 150 mM NaCl, 2 mM EDTA, 1% Igepal CA-630, 20 mM NaF, and protease inhibitor mixture (Sigma)) and incubated on a rotating wheel (10 rpm) at 4 °C for 3 h to overnight with 30 μl of protein G-Sepharose 4B Fast Flow beads (GE Healthcare) preloaded with 1–2 μg of the cognate antibody. Beads were washed three times with 1 ml of ice-cold IP buffer, and immune complexes were collected by centrifugation, resuspended in 25 μl of 3X SDS-PAGE sample buffer, and incubated for 10 min at 95 °C. The IP samples were separated on an NuPAGE 4–12% Novex BisTris gel (Invitrogen) and blotted onto nitrocellulose membranes (GE Healthcare). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (pH 7.4) containing 0.05% Tween 20 and incubated with primary antibodies decorated with horseradish peroxidase-conjugated secondary antibodies according to the instructions of the manufacturers. Bound secondary antibodies were visualized by enhanced chemiluminescence (GE Healthcare).

**In Vitro Binding Assays**—1 μg of GST-CSN5 was immobilized on glutathione-Sepharose 4B beads (GE Healthcare) and incubated with His-tagged p97/VCP protein (0.5, 1, or 2 μg) for 2 h at 4 °C in binding buffer containing 25 mM Tris-HCl (pH 8.0), 200 mM KCl, 2 mM MgCl2, 1 mM ATP, 1 mM dithiothreitol, 5% glycerol, and 1% Triton X-100. After washing, bound proteins were analyzed by immunoblotting.

In the oligoubiquitin binding assay, glutathione-Sepharose 4B beads (GE Healthcare) were loaded with GST-CSN5 or N-NTA-agarose beads with p97/VCP and incubated with Lys48-linked oligoubiquitin chains Ub2–7 (Biomol International, LP) in binding buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) for 3 h at 4 °C. The beads were washed three times with binding buffer and resuspended in 3X SDS sample buffer. Bound proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to sequential immunoblotting using anti-ubiquitin, anti-CSN5, and anti-p97/VCP antibodies.

**Fluorescence Resonance Energy Transfer (FRET)**—Indirect double-labeling immunofluorescence was combined with confocal laser scanning microscopy (CLSM) and FRET analysis to identify associations of proteins as described previously (9). Cells were transfected with different plasmids using the same procedure as described above and fixed 24 h after transfection with 4% paraformaldehyde for 15 min. Both primary antibodies (rabbit anti-CSN5 and mouse anti-p97/VCP) were applied simultaneously and incubated overnight at 4 °C. For control of the specificity of the secondary reagents (donkey anti-rabbit Ig, F(ab’)_2, Cy5-conjugated, 1:400; donkey anti-mouse, Cy3-conjugated, 1:1000, both from Dianova, Hamburg, Germany) only the anti-CSN5 antibody and both secondary antibodies were applied.

**Cloning of a Construct for Expression of mCSN**—A two-step PCR strategy was used for cloning an expression vector for CSN5 with point mutations in the JAMM domain (H140A/H142A) and incubated with His-tagged p97/VCP protein (0.5, 1, or 2 μg) for 2 h at 4 °C in binding buffer containing 25 mM Tris-HCl (pH 8.0), 200 mM KCl, 2 mM MgCl2, 1 mM ATP, 1 mM dithiothreitol, 5% glycerol, and 1% Triton X-100. After washing, bound proteins were analyzed by immunoblotting.
ATP-dependent Interaction of CSN and p97/VCP

The CLSM settings (TCS-SP2 AOB; Leica, Bensheim, Germany) for fluorescence detection were as follows: Cy3, 52% laser power at 543 nm, detection at 550–602 nm; Cy5, 20% laser power at 633 nm, detection at 642–705 nm. FRET was quantified by the acceptor photobleaching method, and FRET efficiency was shown as change of fluorescence intensity as described previously (9). Differences between experimental and control group ROIs were analyzed with the Kruskal–Wallis test followed by the Mann–Whitney test using SPSS software, version 12 (SPSS GmbH Software, Munich, Germany), with \( p \leq 0.05 \) being considered as significant and \( p \leq 0.01 \) as highly significant.

Gel Filtration—400 \( \mu l \) of HEK 293T lysate (3 mg of protein) was separated on a Sephacryl S-200 10/30 HR column (GE Healthcare) in a buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM ATP, and 10% glycerol that is used for proteasome purifications. Fraction size was 1 ml out of which 300 \( \mu l \) were precipitated with acetone, resolved in SDS sample buffer, and examined by SDS-PAGE and immunoblotting.

Discontinuous Native Gel Electrophoresis—1.4 \( \mu g \) of purified CSN from human erythrocytes (10) and 6 or 1.2 \( \mu g \) of bacterially expressed p97/VCP purified from the soluble fraction (detailed protocol available upon request) were incubated in 50 mM Tris-HCl (pH 7.2), 10% glycerol, 1 mM ATP, 2 mM MgCl\(_2\), in a 22-\( \mu l \) volume for 2 h at room temperature. The same volume of 2\( \times \) sample buffer (50 mM Tris-HCl (pH 7.2), 10% (v/v) glycerol, 150 mM KCl, 0.5% (w/v) Coomassie Blue G-250) was added, and after 30 min of incubation at room temperature samples were applied to a discontinuous native agarose-polyacrylamide composite gradient gel prepared according to Niepman and Zheng (11) and Suh et al. (12) as follows. 5 ml of 1% agarose (w/v) in water was boiled, equilibrated at 45°C, and mixed with 5 ml of prewarmed (45°C) 4% (w/v) acrylamide/bisacrylamide (37.5:1) solution in 0.75 M Tris-HCl (pH 8.8) and supplemented with 50 \( \mu l \) of 10% (w/v) ammonium persulfate (APS) and 5 \( \mu l \) of TEMED. 3 ml of this 0.5% agarose/2% acrylamide/bisacrylamide solution was drawn into a 22-\( \mu l \) pipette following 3 ml of a prewarmed 18% (w/v) acrylamide/bisacrylamide (37.5:1) solution in 0.75 M Tris-HCl (pH 8.8) with APS and TEMED (50 \( \mu l \) and 5 \( \mu l \), respectively, for a 10-\( \mu l \) solution) followed by 2–3 air bubbles. The 6-\( \mu l \) gel solution in the pipette was used to pour a minigel carefully at room temperature. After polymerization and maturation for 15 min at 4°C and 1 h at room temperature, samples were loaded, and electrophoresis was performed at 4°C and 15 V/cm for 16 h using 100 \( \mu l \) Tris-HCl (pH 8.8) as anode buffer and 25 \( \mu l \) Tris, 192 \( \mu l \) glycerine, 0.1% SDS, 0.002% Coomassie Blue G-250 as cathode buffer. Coomassie-containing cathode buffer was exchanged for cathode buffer without Coomassie after 5 h. Resolved proteins were transferred to nitrocellulose by wet blotting using a Trans Blot Cell (Bio-Rad) and 20 \( \mu l \) Tris, 163 \( \mu l \) glycerine, 5% methanol as transfer buffer.

Knock Downs—All siRNAs used here had been applied previously in several reports, which is documented by one citation per siRNA (see below). Furthermore, for CSN5 siRNA we show the reported functional consequences following knock down (hyperneddylolation of cullin 1, see Fig. 6C). Transfection of siRNAs (100 pmol) with Lipofectamine (Invitrogen) was performed in Opti-MEM according to the protocol of the supplier using 4 \( \mu l \) of transfection reagent/100 pmol of siRNA. siRNAs for validated negative control (AM4621), CSN1 (GAACCUGUAACGUGAAAUCAUtt) (13), CSN5 (GCUCAGAGUAUCGAGUGAAAtt) (14), and USP15 (GGACUGAUUAUCGAGUGAAUt) (15) were purchased from Ambion. 6 h after transfection the medium was replaced with Dulbecco’s modified Eagle’s medium containing 10% serum. After 72 h cells were harvested in lysis buffer (50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 \( \mu M \) leupeptin, 1 \( \mu M \) phenylmethylsulfonyl fluoride). In some experiments, the proteasome inhibitor MG132 (Calbiochem) was added (50 \( \mu M \)) to the medium 1 h before lysis.

Recombinant Proteins and in Vitro Binding Assays—Recombinant GST-CSN5 and His-p97/VCP were expressed in Escherichia coli BL21(DE3). Both proteins accumulated in inclusion bodies. GST-CSN5 inclusion bodies were purified and washed (Triton X-100 method) exactly as described (16) and denatured in phosphate-buffered saline containing 6 \( \mu M \) guanidine HCl, 10 \( \mu M \) dithiothreitol for 1 h at room temperature. After centrifugation (14,000 \( \times \) g, 30 min, 4°C) up to 2 ml of supernatant were added dropwise to 20 ml of renaturation buffer (100 mM Tris-HCl (pH 8.0), 10% (w/v) glycerol, 0.25 mM L-arginine) under stirring at room temperature (17). Refolding continued overnight at 4°C. The centrifuged (14,000 \( \times \) g, 30 min, 4°C) refolding solution was applied to a standard 1-ml glutathione-Sepharose column, and bound GST-CSN5 was eluted with 10 \( \mu M \) glutathione in 50 mM Tris-HCl (pH 8.0), snap frozen in liquid nitrogen, and stored at −80°C. His-p97/VCP inclusion bodies were purified and washed as described above. Denatured protein (6 \( \mu M \) guanidine HCl in phosphate-buffered saline) was purified by standard Ni-NTA-agarose chromatography. Bound protein was eluted with 300 \( \mu M \) imidazole and renatured by dialysis against phosphate-buffered saline containing 1 mM dithiothreitol (Fig. 1D). Alternatively, the protein was purified from the soluble fraction of the bacteriological lysate under nonnondenaturing standard conditions by metal chelate affinity chromatography (see Fig. 4C). Both preparations formed largely homohexamers in solution when analyzed by native gel electrophoresis. Detailed protocols are available on request. The in vitro binding assay was carried out essentially as described (18).

In the ubiquitin binding assay, glutathione-Sepharose 4B beads (GE Healthcare) were loaded with GST-CSN5 and incubated with Lys\(^{48}\)-oligoubiquitin chains Ub\(_2\)–7 (Biomol International, LP) in binding buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) for 3 h at 4°C. Washed beads were resuspended in 3× SDS sample buffer. Bound proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to sequential immunoblotting using antibodies against ubiquitin, CSN5/Jab1, and p97/VCP.

RESULTS

p97/VCP Interacts with CSN5 in Vivo and in Vitro—In a MIF interactome screen, we identified p97/VCP as a new MIF-interacting protein. The interaction of MIF and p97/VCP was indi-
rect and found to be mediated by CSN5, a well established binding partner of MIF (19). Overexpression of FLAG-tagged p97/VCP in NIH 3T3 cells followed by immunoprecipitation with anti-FLAG antibody coprecipitated large quantities of CSN5 (Fig. 1C). Likewise, His-p97/VCP specifically bound to immobilized GST-CSN5 in vitro (Fig. 1D). Moreover, double-labeling indirect immunofluorescence experiments combined with acceptor bleaching FRET-CLSM analysis in NIH 3T3 cells ectopically expressing p97/VCP and CSN5 (Fig. 2) revealed close proximity of both proteins (<10 nm) in the cytoplasm, indicating that physical interaction is possible. Importantly, endogenous CSN5/p97/VCP complexes were also coimmunoprecipitated in vivo from NIH 3T3 and HEK 293T cell lysates by using either anti-CSN5 (Fig. 1A) or anti-p97/VCP antibodies (Fig. 1B), but not by an isotype control antibody. Collectively, our data establish that p97/VCP and CSN5 do interact.

**Mapping the Interface between CSN5 and p97/VCP.** —To elucidate the domain(s) involved in the interaction of both proteins, FLAG-tagged p97/VCP, wild type CSN5, or Myc-tagged CSN5 deletion mutants were ectopically expressed in HEK 293T cells. Following immunoprecipitation with anti-FLAG antibody, the Myc epitope was detected by immunoblotting (Fig. 3A). The results show that amino acids 1–110 of CSN5 are sufficient to mediate binding to p97/VCP, whereas amino acids 110–191 are not. The region 54–191 of CSN5 defines the MPN core domain and a C-terminal JAMM motif (129–175) whereas p97/VCP was mainly located in the cytoplasm.

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The latter appears not to be required for binding to p97/VCP. To confirm that binding is independent of a functional JAMM motif, a deneddylase-defective mutant (mCSN5; see also Fig. 6A) was expressed together with FLAG-p97/VCP. As mCSN5 was immunoprecipitated with anti-FLAG antibody, the JAMM motif could be excluded as being required for interaction with p97/VCP (Fig. 3B).

To map the binding interface on p97/VCP, a number of deletion constructs were expressed in HEK 293T cells (Fig. 3C). After immunoprecipitation of CSN5 neither the N domain alone, nor the individual D1 or D2 domains were coprecipitated. Only ND1 and D1D2 proteins were able to coprecipitate CSN5 albeit with less (ND1) or weak (D1D2) affinity compared with the wild type protein.
**p97/VCP and the CSN Form an ATP-dependent Complex**—Because CSN5 exerts its activities as part of the CSN complex, we subsequently investigated whether p97/VCP is interacting with the entire CSN. Total protein extracts from HEK 293T cells were separated by Sephacryl S-200 gel filtration chromatography. Immunoblot analyses revealed that p97/VCP, CSN1, and CSN5 are all present in fraction 5 (peak) that contains proteins and complexes with a molecular mass of about 660 kDa (Fig. 4A). When fraction 5 was used to immunoprecipitate CSN1, a subunit known to coprecipitate CSN2, 3, 5, and 8 (21), p97/VCP coprecipitated in addition to CSN5 (Fig. 4B). Further direct evidence for a high molecular weight CSN/p97/VCP complex resulted from biochemical studies. Bacterially expressed and purified p97/VCP and CSN purified from erythrocytes (10) were incubated together in the presence or absence of ATP and subsequently analyzed in native agarose-polyacrylamide composite gels (Fig. 4C). The CSN complex migrated as a well defined 450 kDa band (Fig. 4C, lane 1) and the majority of p97/VCP as an approximately 600-kDa complex, i.e. the expected molecular mass of the biologically active p97/VCP homohexamer (Fig. 4C, lane 5). Characteristically, only high concentrations of urea were able to dissociate the highly stable hexamer into monomers (Fig. 4C, lanes 6 and 7) as described previously (22). Only in the presence of ATP did the addition of excess p97/VCP lead to a supershift of the CSN and formation of a high molecular mass oligomeric complex considerably larger than 660 kDa that contained CSN5 and p97/VCP (Fig. 4C, lanes 2–4).

**CSN5 Binds to Oligoubiquitinated Chains and Substrates**—Next we wanted to test whether CSN5, apart from being a NEDD8 isopeptidase, can have other functions and potentially also bind Lys48-oligoubiquitin chains. Because CSN5 is very insoluble when produced in various expression systems, a strategy was developed to refold guanidine HCl-denatured GST-CSN5 by using the aggregation suppressor arginine and the folding enhancer sucrose (17). Refolded GST-CSN5 was immobilized on glutathione-Sepharose beads and incubated with Lys48-linked oligoubiquitin chains Ub(2–7) (Fig. 5A, lanes 2–4). CSN5 interacted with Ub(2–7) (Fig. 5A, lane 3) similar to His-p97/VCP that is known to bind oligoubiquitin (23) and was used as positive control (Fig. 5A, lane 5). The low molecular weight control protein secretoglobin 2A1 (SCGB2A1) (24) did not bind to glutathione-Sepharose-bound GST-CSN5 (data not shown), indicating that binding of oligoubiquitin chains is specific.

To examine whether binding of oligoubiquitin as for Prp8p (25) is mediated by the JAMM motif, Myc-tagged CSN5 and truncation mutants 1–191, 1–110, and 110–191 were expressed in HEK 293T cells. Lysates were immunoprecipitated with anti-Myc antibody and immunoblotted to detect ubiquitin (Fig. 5B). The wild type protein coprecipitated a substantial fraction of ubiquitinated proteins (lane 2, set to 100%), and the 1–191 mutant containing the entire MPN domain (54–191) was still able to coprecipitate a significant amount of ubiquitinated proteins (lane 3, 52% compared with wild type). Importantly, also the 110–191 mutant harboring the JAMM motif (129–175) but lacking large parts of the MPN core (54–142) (20) plus the conserved glutamate 76 (26) still showed...
interaction with ubiquitinated proteins to a comparable extent (lane 5, 46% compared with wild type). In contrast, the mutant 1–110 that does not contain the JAMM motif virtually lost binding (lane 4, 5%). We conclude that in addition to conferring NEDD8 isopeptidase activity, the JAMM motif of CSN5 binds oligoubiquitin, but cannot depolymerize it.

CSN5 Regulates the Ubiquitination Status of Substrates Bound to p97/VCP—p97/VCP binds to polyubiquitinated substrates either directly with its N domain or indirectly via a number of cofactors. Because it controls the degree of ubiquitination of bound substrates, we examined whether CSN5 can modify this function. To replace wild type CSN5 with mCSN5, the JAMM mutant was expressed in HEK 293T cells in the presence of the proteasome inhibitor MG132 (27). Lysates were immunoprecipitated with anti-p97/VCP antibody and immunoblotted for the detection of ubiquitin (Fig. 6A). Ubiquitin-conjugated proteins bound to p97/VCP were found to accumulate in the mCSN5-transfected cells (Fig. 6A, lane 6, 1.3-fold more polyubiquitinated proteins compared with vector control), whereas overexpression of wild type CSN5 repeatedly led to a slight decrease of associated polyubiquitinated proteins (Fig. 6A, lane 5, 0.7-fold less polyubiquitinated proteins compared with vector control). Similarly, ubiquitinated proteins accumulated on p97/VCP when the CSN was destabilized by efficient knock down of CSN1 or CSN5 by RNA interference (Fig. 6B, lanes 6 and 7, respectively). In conclusion, the CSN5 JAMM motif and a functional CSN complex are required for the deubiquitination of ubiquitinated substrates bound to p97/VCP. Because the CSN is also associated with the deubiquitinase USP15 (28), we investigated its function by knock-down experiments. Silencing of USP15 resulted in the accumulation of ubiquitinated proteins bound to p97/VCP comparable with the effect in CSN1 and CSN5 knock downs (Fig. 6C, lane 8). The functionality of the knock-down experiments was exemplarily shown for CSN5 (Fig. 6C, upper panel). Knock down substantially increased the proportion of neddylated cullin 1 as the deneddylase activity of the CSN is impaired as a consequence of silencing CSN1 or CSN5 gene expression. The same effect was observed when mCSN5 was overexpressed (Fig. 6C, lower panel), demonstrating that the mutant protein is indeed compromising CSN deneddylase activity. Furthermore, IkBα accumulated in the CSN5 knock down (upper panel) due to hyper-neddylation of cullin 1 (15).

**DISCUSSION**

Specific interaction of the homohexameric ATPase p97/VCP with subunit CSN5 of the COP9 signalosome and the whole CSN complex was demonstrated by five independent lines of evidence comprising (i) an in vivo interactome screen with biotin-tagged MIF, (ii) in vitro pulldown studies with overexpressed wild type and mutant proteins, (iii) in vivo FRET analyses with ectopically expressed proteins, (iv) biochemical demonstration of an ATP-dependent CSN/p97/VCP complex in composite native gels, and (v) reciprocal communoprecipitation of endogenous CSN5 and p97/VCP, i.e. without overexpression in vivo. Mapping the interface between CSN5 and p97/VCP by overexpression of wild type and mutant proteins revealed that the ND1 domains of p97/VCP are sufficient for

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**FIGURE 5. CSN5 binds to oligoubiquitinated proteins in vitro and in vivo.** A, oligoubiquitin chains Ub(2–7) were incubated with GST or GST-CSN5 immobilized on glutathione-Sepharose 4B or His-p97/VCP immobilized on Ni-NTA-agarose. Bound proteins were resolved by SDS-PAGE and identified by sequential immunoblotting. Protein samples of GST-CSN5 and His-p97/VCP were directly loaded in lanes 4 and 6, respectively. All lanes were on the same immunoblot; a few intervening lanes were omitted as indicated by vertical lines. B, HEK 293T cells transfected with empty vector (V) or Myc-CSN5 mutants were lysed and immunoprecipitated (IP) using a monoclonal anti-Myc antibody. Myc tag and ubiquitin were detected by immunoblotting (IB). Coprecipitated polyubiquitinated proteins were quantified with ImageJ (see numbers below the ubiquitin immunoblot (IB: Ub). Vector control was set to 0%, wild type (wt) Myc-CSN5 to 100%. The asterisk denotes IgG heavy chains.
specific binding, but that residues in the second ATPase domain D2 are contributing to binding affinity, which explains why binding of CSN5 to the ND1 protein is weaker than to the wild type protein. Weak binding to the D1D2 protein also shows that direct contacts with both ATPase cassettes are involved.

A plethora of cofactors have been described to interact with p97/VCP. Based on their domain structure they can be grouped into proteins with UBX domain and non-UBX proteins (29) or functionally into substrate-recruiting and -processing cofactors of different pathways (7, 30). Many cofactors bind to p97/VCP in homo- or heterooligomeric form and use the N domain of p97/VCP as interaction interface (7). Examples are the trimeric ubiquitin regulatory X (UBX)-protein p47 (31) and the ubiquitin fusion degradation1 (Ufd1)-nuclear protein localization 4 (Npl4) heterodimer (32). On the other hand, the UBX domain containing cofactor UBXD1 binds to the C terminus of p97/VCP with its peptide N-glycanase UBA/UBX (PUB) domain (33), similar to peptide N-glycanase that binds with its PUB domain (34) to the C-terminal 10 amino acids of p97/VCP (35).

As an ATPase, p97/VCP passes through cycles of ATP binding and hydrolysis. A number of results indicate that ATPase domain D2 is considerably more active in ATP hydrolysis than the D1 domain. Conformational changes caused by ATP hydrolysis mainly in domain D2 are subsequently transmitted to cofactors and substrate molecules (36). From at least one cofactor, Werner syndrome protein, it is also known that it binds to p97/VCP in an ATP-dependent fashion (37). Therefore, we conclude that conformational changes in p97/VCP originating from ATP binding and/or hydrolysis are required to enable CSN binding. This also explains why in addition to the important N domain, both ATPase domains are required for efficient binding.

Most p97/VCP cofactors use a UBX domain, which folds like ubiquitin, for binding to p97/VCP and a ubiquitin-associated domain that binds to ubiquitinated proteins (7, 30). Structural analysis of the p97 ND1 domains complexed with the p47 C-terminal domain revealed that the p47 UBX domain interacts with the p97/VCP N domain via a loop that is highly conserved in UBX domains, but is absent in ubiquitin (7). Other cofactors like the peptide N-glycanase use UBX-related domains such as the PUB-domain (35). Interestingly, valosin-containing protein/p47 complex-interacting protein p135 (VCIP135), a deubiquitinase (38), was also shown to interact with p97/VCP (39).

For CSN5, we identified a region containing an MPN domain responsible for interaction with p97/VCP. Part of the MPN domain is the JAMM motif that confers deneddylation activity to the signalosome (6). Our results show that for binding to p97/VCP, ubiquitin was detected in precipitates by immunoblotting. Asterisks denote IgG heavy chains, # indicates Myc-mCSN5. C, functional controls. Lysates of CSN5 knock-down cells were analyzed by immunoblotting for the presence of CSN5, cullin 1, β-actin, and β-actin (upper panel). Lysates of mCSN5-transfected cells were analyzed by immunoblotting for the presence of CSN5, cullin 1, and β-actin (lower panel).
activity do not interfere with binding. Therefore, the N-terminal half of the MPN domain, the so-called MPN core, and/or the N-terminal 54 residues of CSN5 are mediating binding to p97/VCP.

Because at least a large fraction of CSN5 is found in the signalosome (for a detailed discussion, see reference 40) and ectopically expressed CSN5 variants and mutants replace the wild type CSN5 within the signalosome (27), it cannot be excluded that signalosome subunits other than CSN5 contribute to CSN-p97/VCP interaction.

It is known that JAMM motifs can have diverse functions. Whereas the JAMM motif of CSN5 acts as a NEDD8 isopeptidase, the motifs of RPN11 and AMSH function as ubiquitin isopeptidases (41). Contrary to these enzymatic activities, the JAMM motif of MOV34 is unable to complex a zinc ion and is thought to have primarily a structural role (42) similar to the pre-mRNA splicing factor Prp8p, whose inactive JAMM motif acts as a new ubiquitin binding domain (25).

Experiments described by Groisman et al. (27) indicated that in addition to its isopeptidase activity that deconjugates NEDD8 from neddylated cullins, CSN5 could possess an isopeptidase activity that deconjugates ubiquitin. Hetfeld et al. (28) reported that CSN purified from erythrocytes binds gold-Ub(4) particles. But it remained unclear whether binding is mediated by the metalloprotease subunit CSN5 or the associated deubiquitinate USP15. Because CSN5 accumulates in insoluble form when expressed in different systems, we developed a strategy to renature denatured CSN5. Using this approach, we were able to show specific binding of oligoubiquitin to recombinant CSN5, whose inactive JAMM motif acts as a new ubiquitin binding domain (25).

p97/VCP controls the degree of ubiquitination of bound substrates that are either bound directly or indirectly via a plethora of cofactors. Here, we could show that the CSN interacts with p97/VCP and regulates the amount of polyubiquitinated proteins bound to p97/VCP. When the CSN was inactivated by knock-down of CSN1 or CSN5, the amount of polyubiquitinated proteins bound to p97/VCP increased, indicating that the CSN is required for proper processing of substrate proteins bound to p97/VCP. Because the same effect could be observed when a CSN5 mutant with defective JAMM motif was transfected, we further conclude that a functional isopeptidase activity of CSN5 is required for substrate processing by p97/VCP.

Based on the newly established interaction of CSN and p97/VCP, we propose that CSN and p97/VCP could form a large ATP-dependent oligomeric complex we name CSN regulatory particle (RP). The analogy is based on pairwise sequence homologies between all CSN and RP lid subunits on the one hand (43) and structural homologies between the homohexameric AAA⁺ ATPase p97/VCP and the AAA⁺ ATPases Rp1–6 of the RP base that form a heterohexamer on the other hand (44). This implies that the proteasomal proteins RPN1, 2, and 10 or paralogous proteins could be part of the CSN RP as well. Until recently, p97/VCP was known mainly for its role in endoplasmic reticulum-associated protein degradation. Together with new results showing that p97/VCP associates with all mammalian UBX domain proteins linking it to dozens of E3 ligases and their cytoplasmic substrates (45), our results further suggest that not only p97/VCP itself, but the CSN RP plays a global regulatory role in protein turnover. p97/VCP was termed a molecular “gearbox” that in conjunction with substrate-processing cofactors is regulating the ubiquitination status of substrates (7). Based on our findings that the deneddylase activity of CSN5 and the deubiquitinate USP15 are involved in regulating the ubiquitination status of proteins recruited to p97/VCP, we suggest that both activities are decisive for “switching gears.” Other deubiquitinases could also be involved because the CSN is associated with at least two different enzymatic deubiquitinase activities (27, 28). A further level of control originates from the association of protein kinases CK2 and D with the CSN that potentially could regulate the association of substrate-processing cofactors with p97/VCP through phosphorylation. Therefore, what emerges is the picture of a molecular machine that extracts ubiquitinated and abnormal folded proteins from larger protein complexes or membranes and determines their fate by using the N-terminal domain of p97/VCP and the CSN as a hub for substrate-processing cofactors and regulatory enzymes. Because the interaction of p97/VCP with the CSN originated from an interactome screen for the cytokine MIF, our results also suggest that MIF may not only be involved in regulating the deneddylase activity of CSN5 (46), but appears to control the UPS on a much broader scale.

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