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NEDD8 Ultimate Buster-1L Interacts with the Ubiquitin-like Protein FAT10 and Accelerates Its Degradation*

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FAT10 is an interferon-γ-inducible ubiquitin-like protein that consists of two ubiquitin-like domains. FAT10 bears a diglycine motif at its C terminus that can form isopeptide bonds to so far unidentified target proteins. Recently we found that FAT10 and its conjugates are rapidly degraded by the proteasome and that the N-terminal fusion of FAT10 to a long lived protein markedly reduces its half-life. FAT10 may hence direct target proteins to the proteasome for degradation. In this study we report a new interaction partner of FAT10 that may link FAT10 to the proteasome. A yeast two-hybrid screen identified NEDD8 ultimate buster-1L (NUB1L) as a non-covalent binding partner of FAT10, and this interaction was confirmed by coimmunoprecipitation and glutathione *S*-transferase pull-down experiments. NUB1L is also an interferon-inducible protein that has been reported to interact with the ubiquitin-like protein NEDD8, thus leading to accelerated NEDD8 degradation. Here we show that NUB1L binds to FAT10 much stronger than to NEDD8 and that NEDD8 cannot compete with FAT10 for NUB1L binding. The interaction of FAT10 and NUB1L is specific as green fluorescent fusion proteins containing ubiquitin or SUMO-1 do not bind to NUB1L. The coexpression of NUB1L enhanced the degradation rate of FAT10 8-fold, whereas NEDD8 degradation was only accelerated 2-fold. Because NUB1 was shown to bind to the proteasome subunit RPN10 *in vitro* and to be contained in 26 S proteasome preparations, it may function as a linker that targets FAT10 for degradation by the proteasome.

The covalent modification of proteins with lysine 48-linked polyubiquitin chains is a signal for their degradation by the 26 S proteasome (1, 2). The 26 S proteasome consists of a central proteolytic unit, the 20 S proteasome, and one or two copies of the 19 S regulator (also called PA700) that bind to the ends of the cylindrical 20 S protease. The 19 S regulator in turn can be dissociated into a proximal "base" that is formed by a ring of six ATPases (designated Rpt1–6) and a distal "lid" of at least 12 non-ATPase subunits (named RPN1–12) (3). Four main functions can be assigned to the 19 S regulator; it serves as a receptor for polyubiquitylated proteins (4–6); it unfolds proteins before degradation (7, 8); it cleaves off the polyubiquitin chains so that they can be recycled (9); and it opens the gate of the 20 S proteasome (10). Two subunits of the 19 S regulator, the non-ATPase subunit RPN10 (5) and the ATPase subunit Rpt5 (6), have been shown to bind polyubiquitylated proteins and are thought to be substrate-docking sites.

In addition to the direct binding of polyubiquitylated substrates to the proteasome, a second mode of substrate binding has been characterized. This association is mediated by adaptor proteins that possess ubiquitin-like domains that interact with the proteasome as well as ubiquitin-associated (UBA)[1] domains that are able to bind polyubiquitylated proteins. Examples of these adaptor proteins are the RAD23 protein (11, 12), the hPLIC1 protein (13, 14), and the protein NEDD8 ultimate buster-1 (NUB1) (15–17). Although the overexpression of these proteins has been shown to induce the degradation of non-covalently interacting proteins in several cases, it is still a matter of debate whether these adaptors are mediators of proteasomal degradation or whether they serve to regulate the degradation process in a negative manner possibly by competing with the 26 S proteasome for binding of polyubiquitylated substrates (18). Most interesting, it seems that UBA domains may also bind ubiquitin-like domains as has been shown for NUB1, which interacts *in vitro* with the ubiquitin-like protein NEDD8 and mediates its accelerated degradation (15–17).

It has often been emphasized that ubiquitin-like proteins, which become isopeptide-linked to target proteins through selective cascades of activating enzymes, conjugating enzymes, and ligases, serve purposes other than targeting for proteasomal degradation, as for instance changes of intracellular localization, alterations of enzymatic activity, or the modification of DNA binding. Recently, we and others have shown that a novel member of these so-called ubiquitin-like modifiers that was first described under the name of "diubiquitin" (19), and subsequently renamed to "FAT10," is rapidly degraded by the proteasome (20, 21). FAT10 consists of two ubiquitin-like domains that are joined by a short linker. It should be emphasized, however, that FAT10 is distinct from the ubiquitin-like modifier ISG15 (also called ubiquitin cross-reactive protein) that also consists of two ubiquitin-like domains (22).

The inducible expression of FAT10 in fibroblasts led to the appearance of the 18-kDa protein FAT10 as well as three closely migrating covalent conjugates at about 35 kDa which, like unconjugated FAT10, were degraded by the proteasome

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[1] The abbreviations used are: UBA, ubiquitin-associated; GST, glutathione *S*-transferase; HA, hemagglutinin; IFN, interferon; mAb, monoclonal antibody; NEDD, neural precursor cell-expressed developmentally down-regulated; NUB1, NEDD8 Ultimate Buster-1; TNF, tumor necrosis factor; GFP, green fluorescent protein.

with a half-life of 1 h (20). Mutation of the C-terminal diglycine motif of FAT10 abolished the conjugate formation, thus strongly suggesting that FAT10 becomes isopeptide-linked via its C-terminal diglycine motif in analogy to ubiquitin and other ubiquitin-like modifiers as SUMO, NEDD8, or ISG15, for example (22). In contrast to these well characterized protein modifiers, the putative activating and conjugating enzymes for FAT10 have not been identified. Most interesting, the fusion of FAT10 to the N terminus of the long lived green fluorescent protein (GFP) led to its rapid degradation in HeLa cells.[2] FAT10 and ubiquitin were equally efficient at targeting GFP for degradation when fused to its N terminus, thus underlining the potency of FAT10 as a degradation signal. Taken together, it seems that FAT10 in contrast to other ubiquitin-like modifiers targets its conjugation substrates for proteasomal degradation.

Although several interesting properties of FAT10 have been described, the biological function of FAT10 has so far remained elusive. FAT10 is encoded in the major histocompatibility complex class I region and is inducible in many different cell lines with the proinflammatory cytokines interferon (IFN)-γ and/or tumor necrosis factor (TNF)-α (21, 23). Moreover, FAT10 is up-regulated upon the activation of B cells and dendritic cells (24), which could hint at a function in antigen processing, but so far we have failed to substantiate this hypothesis experimentally. Interestingly, the induced expression of FAT10 causes apoptosis in fibroblasts, suggesting that it may be functionally involved in the TNF-α-triggered apoptosis pathway (20). In seeming contradiction to this hypothesis, the FAT10 mRNA has been found recently to be highly up-regulated in numerous gynecological and gastrointestinal tumors, but it was not reported whether the *fat10* gene was mutated in these tumors or not (25). It is therefore not yet clear whether FAT10 has the properties of an oncogene or a tumor suppressor. An involvement of FAT10 in cell cycle regulation was proposed by Liu *et al.* (21) who reported a non-covalent interaction of FAT10 with the nuclear spindle assembly checkpoint protein MAD2, but the physiological consequences of this association have remained unclear.

In order to learn more about the function of FAT10 and to possibly identify enzymes involved in FAT10 conjugation, a two-hybrid interaction screen was performed that identified NUB1L as a FAT10 interaction partner, and this firm non-covalent interaction was confirmed by coimmunoprecipitation and GST pull-down experiments. Strikingly, NUB1L expression led to an accelerated degradation of FAT10 suggesting that NUB1L may target FAT10 for proteasomal degradation.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents

The following antibodies were used: QIAexpress™ mAb anti-His_6 (Qiagen, Hilden, Germany), mAb anti-HA clone HA-7 (Sigma) mAb anti-Xpress™ (Invitrogen), and a FAT10-specific polyclonal antibody that was raised in rabbits by immunization with GST-FAT10 recombinant protein. For confocal microscopy, the following fluorescence-labeled mAbs were used: fluorescein isothiocyanate-coupled anti-HA (clone 3F10, Roche Applied Science) and Alexa Fluor™ 594-coupled goat anti-mouse IgG (H+L) (Molecular Probes, Eugene, OR). Horseradish peroxidase-coupled secondary antibodies were purchased from Dako (Glostrup, Denmark).

Expression Constructs and Recombinant Proteins

For generation of HA-NUB1L, His-NUB1L, GST, and GST-FAT10 proteins, NUB1L cDNA was PCR-amplified from the yeast two-hybrid prey plasmid pmg8-1 using 5′-CGGGAGGTACCTGGCGCAGGGATG-GCAC-3′ as forward and 5′-CTATCTAGATTAGTTTTTCTTTGTTGCT-GAC-3′ as reverse primer. The PCR product was cloned via Asp-718 and XbaI sites into the plasmid pCDNA3.1. The sequence between the HindIII and Asp-718 sites of the original vector was replaced by the sequence 5′-AAG CTT ACC ATG GCC TAC CCC TAC GAC GTG CCC GAC TAC GCC GCG GTA CC-3′ to create an in-frame fusion protein of influenza HA epitope and Nub1L (HA-NUB1L pcDNA). For the recombinant expression of a His_6-NUB1L fusion protein in *Escherichia coli*, the sequence between HindIII and Asp-718 was replaced by the sequence 5′-AAG CTT TAA GAA GGA GAT ATA AAT ATG GCC CGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT GCG GTA CCT-3′ (His_6-NUB1L RBS-pcDNA). This plasmid was transformed into a BL21 (DE3)pLysS *E. coli* host strain. The expression of the His-NUB1L fusion protein was induced with isopropyl-β-D-1-thiogalactopyranoside and purified by nickel-affinity chromatography using a HiTrap™ affinity column (Amersham Biosciences) as recommended by the manufacturer. In order to produce the GST-FAT10 fusion protein, the FAT10 cDNA was retrotranscribed from mRNA of JY B cells and amplified, using primers sense 5′-CCATGGATCCATGGCTCCCAATGCTTCCTGCCTC-3′ and antisense 5′-CCGTCTCGAGTCTCACCCTCCAATACAATAAGATGC-3′, and cloned via BamHI and XhoI sites into the expression vector pGEX-4T-3 (Amersham Biosciences). BL21 cells were transformed with this construct, and the GST-FAT10 fusion protein was induced with isopropyl-β-D-1-thiogalactopyranoside and purified by glutathione-Sepharose™ 4B chromatography (Amersham Biosciences) according to protocols provided by the supplier.

Generation of His_6-Xpress-FAT10-pBI, HA-FAT10-pBI, HA-NUB1L-pBI, His_6-Xpress-FAT10-HA-NUB1L-pBI, and HA-FAT10-HANUB1L-pBI—His_6-Xpress-FAT10-pcDNA3.1 and HA-FAT10-pcDNA3.1 have been described previously (20). The two differently tagged versions of FAT10 as well as HA-tagged NUB1L were excised from pcDNA3.1 via HindIII and XbaI and cloned into the vector pBK-CMV (Stratagene, Amsterdam). HA-NUB1L was excised from pBK-CMV with NotI and PstI and cloned into the MCSII of the vector pBI (Clontech). The two FAT10 clones were excised with BssHII and XbaI from pBK-CMV, and each of them was cloned into the multiple cloning site of pBI and HA-NUB1L-pBI via MluI and NheI.

Generation of GFP Fusion Proteins—In order to create fusion proteins of ubiquitin or *fat10* with the N terminus of GFP, we used the pcDNA3-based plasmids HA-FAT10 (20) and HA-ubiquitin.[3] The primer 5′-AGA CGG AAG CTT ACC ATG GCC TAC CCC-3′ was used as forward primer for all PCRs. For *fat10* we used the reverse primer 5′-ATT GCG GGA TCC GCC ACT GCA ATA CAA TAA G-3′ to replace the diglycine by Ala-Val in order to prevent a potential proteolytic processing. Ubiquitin was amplified with the reverse primer 5′-ATA TGG ATC CGC CAC TGC AGA GTC CGC TTC CTG-3′, which also replaces the diglycine by Ala-Val. After amplification, the PCR products were cloned via HindIII and BamHI restriction sites into pEGFP-N1 vector (Clontech). In order to obtain a SUMO-1 cDNA, we prepared human RNA from HeLa cells according to standard procedures. First strand cDNA synthesis and reverse transcriptase PCR was performed according to the instructions provided by the supplier (Roche Applied Science). We used the primers 5′-CGC CGC GGT ACC TAT GTC TGA CCA GGA G-3′ and 5′-ATA TGG ATC CGC CAC TGC CGT TTG TTC CTG ATA-3′ to mutate the C-terminal diglycine to Ala-Val. The PCR product was digested with Asp-718 and BamHI and cloned into pEGFP N1. All sequences were verified by dideoxy sequencing.

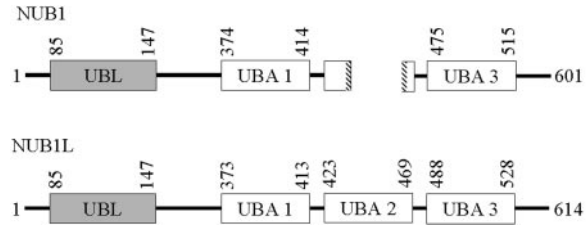


FIG. 1. **Scheme of domain composition of NUB1 and NUB1L.** The additional 14-amino acid insert in NUB1L creates a third UBA domain that spans residues 423–469. Both NUB1 and NUB1L contain a UBL and two other UBA domains, UBA1 and UBA3. NUB1 and NUB1L also contain a bipartite nuclear localization signal (NUB1-(414–431) and NUB1L-(413–430)) and a PEST domain at the C terminus.

[2] M. S. Hipp, S. Raasi, M. Groettrup, and G. Schmidtke, submitted for publication.

[3] M. Basler, unpublished data.

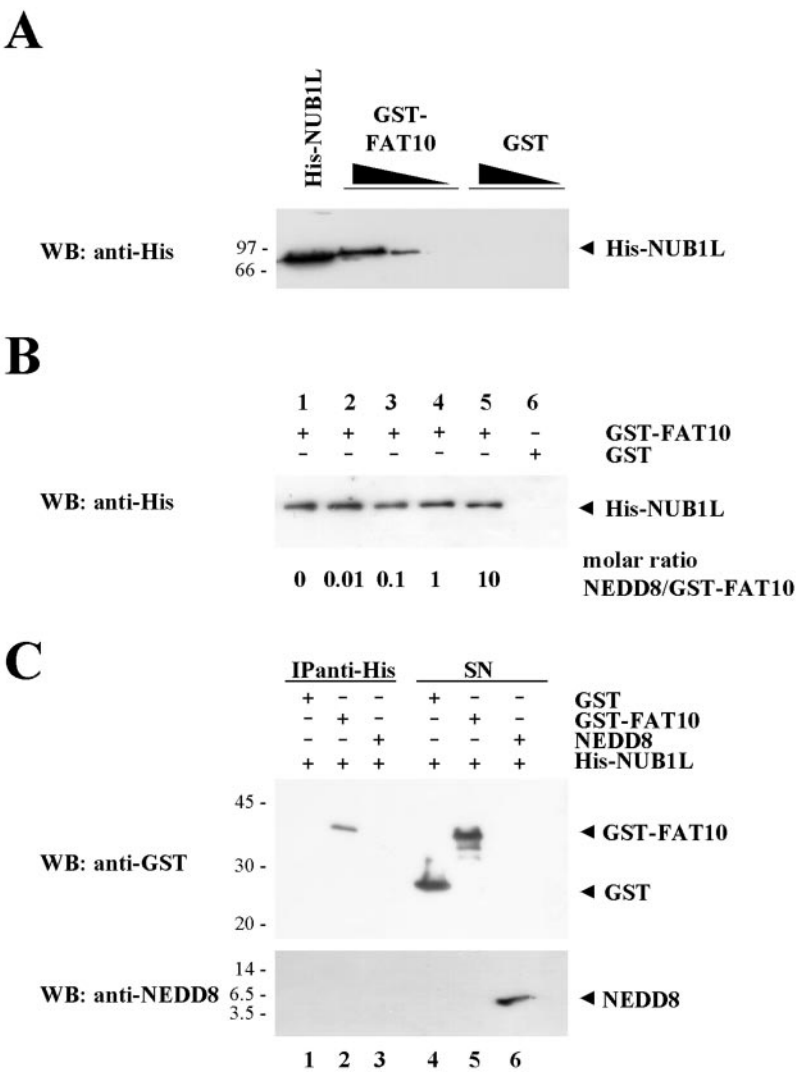


FIG. 2. **GST-FAT10 and NUB1L recombinant proteins directly interact *in vitro*.** *A*, purified recombinant GST and GST-FAT10 proteins were bound to glutathione-Sepharose™ 4B matrix and incubated with purified recombinant His-NUB1L for 2 h. After washing the beads, the interacting proteins were analyzed by anti-His Western blot (*WB*). Three different dilutions of the sample (100, 20, and 4%) were loaded. *Lane 1* contains the His-NUB1L input (100%). *B*, the same GST pull-down experiment as in *A* was performed but in the presence of increasing concentrations of recombinant NEDD8. The indicated molar ratios of NEDD8 and GST-FAT10 were estimated from Coomassie-stained SDS-PAGE. *C*, coprecipitation experiment to compare the association of GST-FAT10 and NEDD8 with His-NUB1L. Purified recombinant His-NUB1L was incubated with comparable concentrations of purified recombinant GST, GST-FAT10, and NEDD8. Subsequently, His-NUB1L was immunoprecipitated (*IP*) with an anti-His mAb, and the precipitates as well as 3% of the supernatants (*SN*) were analyzed by Western blots using anti-GST as well as anti-NEDD8 antibodies. Molecular mass markers are indicated in kDa at the *left*, and the positions of indicated proteins are marked with *arrowheads*. Representative examples out of three independent experiments are shown.

GST Pull-down Assay

GST-FAT10 and GST proteins were expressed in *E. coli* strain BL21. Bacteria were pelleted and lysed in phosphate-buffered saline plus 150 mM NaCl and 1% Triton X-100. After centrifugation the supernatant was incubated with 250 μl of glutathione-Sepharose™ 4B for 4 h at 4 °C on an end-over-end rotor. An aliquot was tested for similar binding of GST and GST-FAT10. After extensive washing, 575 μl of nickel-affinity purified NUB1L-His (50 μg/ml) was added and incubated for 2 h at 4 °C end-over-end. The beads were washed three times with phosphate-buffered saline before boiling in a sample buffer containing 5% SDS and 10% β-mercaptoethanol. Graded aliquots of the lysate were analyzed on Western blots probed with an anti-His_6 mAb.

Confocal Fluorescence Microscopy

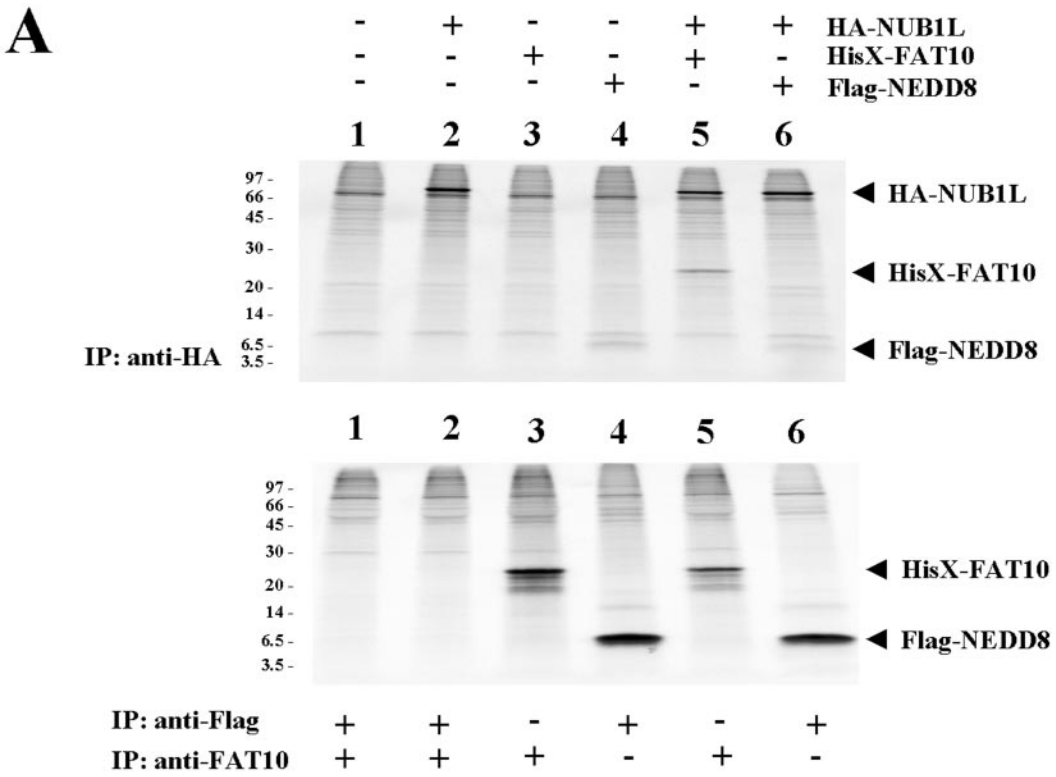
HEK293 cells were grown on coverslips to about 30% confluence at the time of transfection. The plasmids HA-NUB1L-pBI, His_6-Xpress-FAT10-pBI, and His_6-Xpress-FAT10-HA-NUB1L-pBI were introduced into different aliquots of cells together with the plasmid *p*-Tet-splice-tTA (26) to induce the expression of the genes. We used FuGENE™ transfection reagent (Roche Applied Science) for transfection at a reagent to DNA ratio of 3:1. After 16 h of incubation, the cells were fixed with 4% paraformaldehyde, permeabilized with phosphate-buffered saline containing 2 mM EDTA, 2% fetal calf serum, 2 mM azide, and 0.1% saponin. The differently transfected cells and the mock transfectants were stained with anti-HA-fluorescein isothiocyanate (the His_6-Xpress-FAT10-pbi transfectant served as negative control), anti-Xpress™, and Alexa Fluor™ 594 goat anti-mouse (the HA-NUB1L-pBI transfectant served as negative control) or with all three antibodies, followed by analysis with a laser scanning confocal fluorescence microscope (LSM 510; Zeiss, Oberkochen, Germany).

Yeast Two-hybrid Screen

The yeast two-hybrid screen using the *fat10* bait was carried out by Dualsystems Biotech AG, Zurich, Switzerland. The bait construct for yeast two-hybrid screening was made by subcloning the full-length human *fat10* cDNA into the vector pLexA-DIR (Dualsystems Biotech AG, Zurich, Switzerland). The bait construct was transformed into the strain L40 (*MATa his3Δ200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ GAL4*) using standard procedures (27). Correct expression of the bait was verified by Western blotting of cell extracts using a mouse monoclonal antibody directed against the LexA domain (Santa Cruz Biotechnology, Santa Cruz, CA). The absence of self-activation was verified by cotransformation of the bait together with a control prey and selection on minimal medium lacking the amino acids tryptophan, leucine, and histidine (selective medium). For the yeast two-hybrid screen, the bait was cotransformed together with a human adult whole lymph node cDNA library (complexity 1×10^6, Clontech) into L40. A number of 8.2×10^5 transformants were screened, yielding 86 transformants that grew on selective medium. Positive transformants were tested for β-galactosidase activity using a filter assay (28). Out of 86 initial positives, 38 clones showed β-galactosidase activity and were considered to be true positives. Library plasmids were isolated from positive clones and retransformed into L40 with the bait plasmid and with a control bait encoding a LexA-lamin C fusion. Only 6 of 38 positives showed β-galactosidase activity when coexpressed with the bait but not when coexpressed with the control bait and were considered to be bait-dependent positive interactors. The identity of positive interactors was determined by sequencing.

Pulse-Chase Experiments and Immunoprecipitation

For the experiments presented in Fig. 3, we used the plasmids His_6-Xpress-FAT10-pBI, HA-NUB1L-pBI, and His_6-Xpress-FAT10-HA-



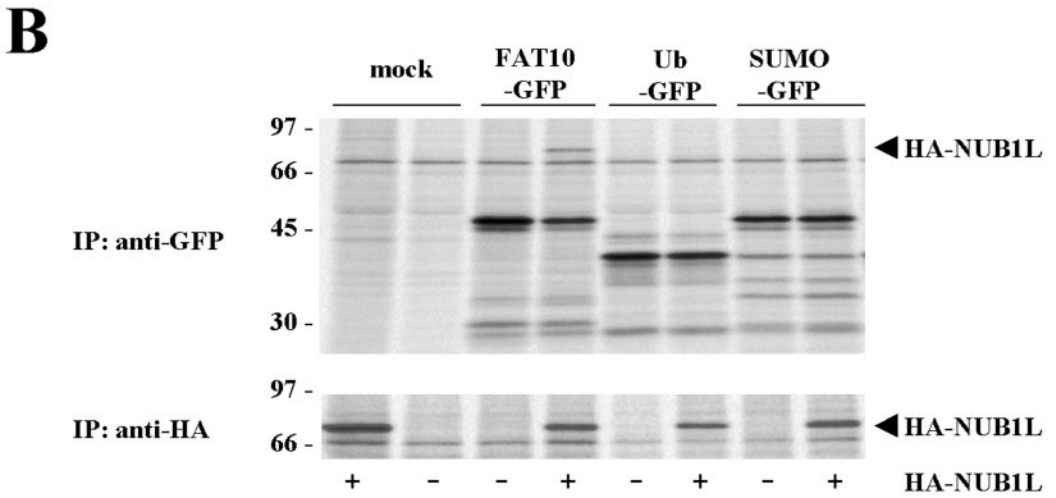


FIG. 3. **Coprecipitation of HisX-FAT10 and HA-NUB1L in transiently transfected cells.** *A*, His_6- and Xpress-tagged human FAT10 (*HisX-FAT10*), FLAG-tagged NEDD8 (*FLAG-NEDD8*), and HA-tagged human NUB1L (*HA-NUB1L*) were transiently expressed in HEK293 cells either alone or together as indicated at the *top*. The cells were labeled for 1 h with [^{35}S]Cys/Met prior to HA-specific immunoprecipitation, SDS-PAGE, and autoradiography. The positions of molecular mass markers in kDa are indicated at the *left*, and the positions of HA-NUB1L, HisX-FAT10, and FLAG-NEDD8 are shown at the *right*. From the supernatants of the experiment shown in the *upper panel*, proteins were immunoprecipitated with anti-FLAG and anti-FAT10 antibodies as indicated below the *bottom panel*. *B*, NUB1L specifically interacts with the ubiquitin-like protein FAT10. Expression constructs for FAT10-GFP, ubiquitin (*Ub*)-GFP, and SUMO-GFP were transiently transfected into HEK293 cells either alone or together with an HA-NUB1L expression plasmid as indicated *below each lane*. Cells were then radiolabeled and lysed prior to immunoprecipitation (*IP*) with anti-GFP and anti-HA mAb as indicated at the *left*. Shown are autoradiographies of the immunoprecipitates separated on SDS-PAGE. Representative examples out of 3–5 independent experiments are shown.

NUB1L-pBI. The data generated for Fig. 5 were obtained with the plasmids HA-FAT10-pBI, HA-NUB1L-pBI, and HA-FAT10-HA-NUB1L-pBI. HEK293 cells were transfected with the plasmids indicated in the figure legends and the inducer pTet-splice-tTa. 16 h after transfection the cells were starved for 1 h in Met/Cys-free RPMI 1640 medium (Sigma) supplemented with L-glutamine, penicillin/streptomycin, and 10% dialyzed fetal calf serum, followed by labeling for 1 h with 0.25 mCi/ml [^{35}S]Met/Cys (Translabel, Amersham Biosciences). Subsequently cells were washed three times, aliquoted, and chased for the indicated times. For the data in Fig. 3, the labeled cells were lysed in 20 mM Tris/HCl, pH 8.0, 0.1% Triton X-100 for 30 min on ice. After centrifugation for 15 min at 15,000 × *g,* 1 volume of 20 mM Tris/HCl, pH

8.0, 300 mM NaCl was added to the supernatant, and an aliquot was analyzed with a β-counter. For the experiment presented in Fig. 5, cells were lysed in RIPA Buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin) and centrifuged and counted as described above. Equal amounts of radioactivity were used for immunoprecipitation with either 5 μg of anti-HA antibody or 20 μg of anti-FAT10 antibody and protein G or protein A-Sepharose™ CL-4B (Amersham Biosciences). The samples were incubated overnight at 4 °C with agitation. After five washes with the respective lysis buffer, the immunoprecipitate was analyzed by SDS-PAGE/autoradiography on a Fuji BAS1500 radioimager.

RESULTS

Identification of NUB1 as a FAT10 Interaction Partner by Yeast Two-hybrid Screen—To identify putative novel interaction partners of human FAT10, we screened a human lymph node cDNA library using a LexA-based yeast two-hybrid system (29). Screening of 8.2×10^5 transformants yielded six bait-dependent clones that were analyzed further. Sequencing the insert of the plasmid recovered from one of the six transformants yielded the full-length in-frame cDNA of NUB1L.

NUB1 was first described by Yeh and co-workers as a protein of 601 amino acids and a molecular mass of 69.1 kDa which interacts with the ubiquitin-like protein NEDD8 (GenBank™ accession number AF300717) (15, 16). Sequencing of the NUB1 insert recovered from our two-hybrid screen revealed that the predicted amino acid sequence contained 14 amino acids more than the published sequence which were inserted between amino acid 451 and 452 of the published sequence for human NUB1 (Fig. 1). The original human NUB1 sequence contains two UBA domains of 37 amino acids spanning residues 376–413 and 477–514. Most interesting, the 14-amino acid insertion at residue 451 as predicted for our FAT10 interaction partner completes a partial UBA domain situated between residues 432 and 455 of human NUB1. It therefore appears that like the published NUB1 homologues of mice (GenBank™ accession number AF534114), *Drosophila* (GenBank™ accession number AE003752), and *Arabidopsis* (GenBank™ accession number AC007295), the human NUB1 protein variant found to interact with FAT10 contains three UBA domains in its C-terminal half. While we were preparing this manuscript, Yeh and co-workers (17) reported the existence of a longer splice variant of human NUB1, which they named NUB1L. Because our FAT10 interaction partner is identical to NUB1L except for an alanine residue at position 200, which was not found in our sequence, we adopted the name for this report.

Recombinant GST-FAT10 and His-NUB1L Fusion Proteins Interact Specifically in Vitro—NUB1L has interesting properties that are consistent with an interaction with FAT10. Like FAT10, it is inducible with IFN-γ, and it has three UBA domains that could potentially mediate binding to one or both of the ubiquitin-like domains of FAT10. We hence decided to focus on NUB1L as a FAT10-binding protein. At first we tried to confirm the yeast two-hybrid result in a GST pull-down experiment. A bacterial expression construct for a human GST-FAT10 fusion protein was generated, and the bacterially expressed GST-FAT10 protein was purified over a glutathione-Sepharose column. A His_6-tagged version of human NUB1L was cloned into a bacterial expression vector, expressed in bacteria, and purified over a nickel-Sepharose matrix. GST-FAT10 as well as GST protein as negative control were bound to glutathione-Sepharose and incubated for 2 h with purified His-NUB1L protein. The glutathione matrix was washed, and bound proteins were eluted and analyzed in 5-fold dilutions on anti-His Western blots. As shown in Fig. 2*A*, His-NUB1L specifically bound to the GST-FAT10 matrix but not to the GST control, thus indicating that FAT10 and NUB1L directly bind to each other *in vitro*.

Because NUB1 was originally described as a binding partner of NEDD8, we tested whether recombinant NEDD8 would be able to compete with FAT10 for binding to NUB1L. GST-FAT10 was immobilized on glutathione matrix and incubated with recombinant human NUB1L in the presence of commercially available recombinant human NEDD8 at molar ratios varying from 0.01 to 10 (Fig. 2*B*). Even a 10-fold molar excess of NEDD8 was unable to compete with FAT10 for binding to NUB1L. This result may indicate that NEDD8 binds to NUB1L at a different site than FAT10 or that NEDD8 binds to the same site of NUB1L but with much lower affinity than FAT10. In order to compare the binding of FAT10 and NEDD8 to NUB1L, we modified the binding assay in that His-NUB1L was incubated with comparable concentrations of GST, GST-FAT10, or NEDD8 followed by immunoprecipitation of His-NUB1L and Western analysis of the precipitate and the supernatant with anti-GST and anti-NEDD8 antibodies (Fig. 2*C*). In this assay, we consistently failed to demonstrate a specific interaction of recombinant NEDD8 and NUB1L, whereas binding of GST-FAT10 to NUB1L was readily observed. This result suggests that NEDD8 binds either not at all or much weaker to NUB1L as compared with FAT10.

FAT10 and NUB1L Strongly Interact Intracellularly—The intracellular localization of FAT10 and NUB1 is only partially overlapping as FAT10 has been shown to be predominantly localized in the cytoplasm (21, 20), whereas NUB1 as well as NUB1L are mainly found in the nucleus (17). In order to determine whether the two proteins can nevertheless substantially interact in intact cells and to confirm FAT10-NUB1L interaction in the intracellular environment, we performed co-immunoprecipitation experiments. Mammalian expression constructs for His_6 and Xpress-tagged human FAT10 (HisX-FAT10) and HA-tagged human NUB1L (HA-NUB1L) were generated and transiently transfected into HEK293 human embryonal kidney cells either alone or together. In addition, FLAG-tagged NEDD8 was transiently expressed alone or together with HA-NUB1L in order to investigate their intracellular interaction in HEK293 cells. The cells were metabolically labeled, and NUB1L was precipitated with anti-HA mAb. As depicted in Fig. 3*A*, HisX-FAT10 was prominently coprecipitated with HA-NUB1L when both proteins were coexpressed (Fig. 3*A*, *lane 5*), but no 23-kDa band corresponding to HisX-FAT10 was detected when either HA-NUB1L or HisX-FAT10 was expressed individually (Fig. 3*A*, *lanes 2* and *3*). A specific interaction of HA-NUB1L and NEDD8, in contrast, was not apparent in our experiments. A faint NEDD8 band appeared when immunoprecipitation was performed with the anti-HA mAb and a washing buffer containing 0.1% Triton X-100, but this band was equally prominent in the absence or presence of HA-NUB1L (Fig. 3*A*, *lanes 4* and *6*) suggesting unspecific binding. Using more stringent washing conditions, which were permissive for the NUB1L-FAT10 interaction (0.1% SDS, 1% Nonidet P-40), eliminated NEDD8 from all immunoprecipitates (data not shown) although the intensity of radiolabeled NEDD8 in the lysates was at least as high as that of FAT10 (Fig. 3*A*, *bottom panel*). In a series of coprecipitation experiments in HEK293 as well as HeLa cells, we determined the portion of HA-NUB1L-associated HisX-FAT10 to be about one-third of the total amount of HisX-FAT10, thus indicating that the association of FAT10 and NUB1L under the chosen experimental conditions is robust and extensive (Fig. 3*A*).[4]

To provide further evidence for the specificity of the NUB1L-FAT10 interaction, we generated FAT10-GFP, ubiquitin-GFP,

[4] M. S. Hipp, S. Raasi, M. Groettrup, and G. Schmidtke, unpublished data.

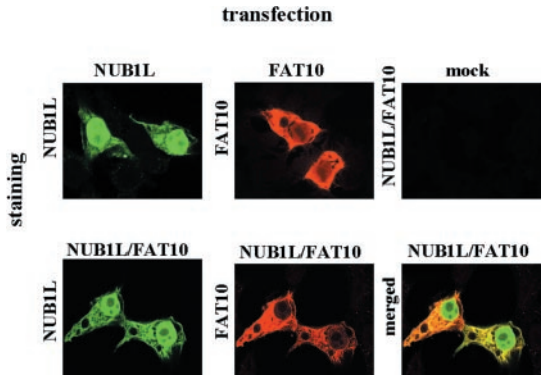


FIG. 4. **FAT10 and NUB1L do not mutually affect their intracellular localization.** HEK293 cells were transiently transfected with either HA-tagged human NUB1L or His_6- and Xpress-tagged human FAT10 or mock-transfected (single transfectants, *top panels*). Alternatively, tagged NUB1L and FAT10 were coexpressed in HEK293 cells from a bidirectional vector (double transfectants, *bottom panels*). Antibodies against the Xpress or HA tag were used to visualize the intracellular localization of HA-NUB1L (*green*) and HisX-FAT10 (*red*) by confocal fluorescence microscopy. The *rightmost panel* on the *bottom* is a merge of the two images to the *left*.

and SUMO1-GFP expression constructs and transfected each of them either alone or together with the HA-NUB1L expression construct into HEK293 cells. The cells were metabolically labeled, and the GFP fusion proteins were immunoprecipitated with an anti-GFP antibody in order to monitor whether the HA-NUB1L protein would associate with the respective fusion proteins. The autoradiography shown in Fig. 3*B* illustrates that the FAT10-GFP precipitate but neither the ubiquitin-GFP nor the SUMO-GFP precipitate contained bound NUB1L, although the NUB1L expression was comparable in the transfected cells (Fig. 3*B, bottom panel*). Taken together, our data indicate that the intracellular binding of NUB1L to FAT10 is highly specific.

The Coexpression of FAT10 and NUB1L Does Not Mutually Change Their Intracellular Localization—Because other ubiquitin-like modifiers such as SUMO-1, for example, alter the intracellular localization of their target proteins, we examined whether FAT10 and NUB1 would mutually influence their localization. Previously, we and Liu *et al.* (20, 21) have shown in mouse fibroblasts and human B cells that FAT10 is predominantly localized in the cytoplasm. This finding was recently challenged by Lee *et al.* (25) who found that FAT10 was localized in the nucleus of the transiently transfected human embryonic liver cell line WRL68 and in murine NIH/3T3 fibroblasts adenovirally transfected for FAT10 expression. In order to reexamine this issue, we transiently transfected HEK293 cells with expression constructs encoding either His-Xpress-tagged human FAT10 or HA-tagged human NUB1L. In addition, we coexpressed both proteins together from a vector encoding both proteins under a bidirectional promoter. The analysis of the cells with confocal microscopy revealed that in agreement with our previous studies, FAT10 was predominantly localized in the cytoplasm irrespective of whether it was expressed alone or together with NUB1L (Fig. 4). The FAT10 immunoreactivity in the nucleus was faint but clearly detectable, whereas nuclear bodies that had the appearance of nucleoli were spared. NUB1L, in contrast, was clearly more prominent in the nucleus than in the cytoplasm but was also absent from nuclear bodies. However, similar to FAT10, the distribution of NUB1L was not exclusively confined to one compartment as the cytoplasmic staining for NUB1L was easily detectable in contrast to mock-transfected controls. We therefore conclude that the localization of NUB1L and FAT10 is predominant in the nucleus and cytoplasm, respectively, but that a significant overlap prevails as illustrated by the merged images. This overlap is consistent with their interaction observed by coimmunoprecipitation in the same cells (Fig. 3). Nevertheless, an impact of FAT10 coexpression on the intracellular localization of NUB1L and vice versa was not apparent when comparing the single and double transfectants shown in the *upper* and *lower panels* of Fig. 4.

NUB1L Strongly Down-regulates FAT10 Expression Levels by Accelerating Its Degradation—NUB1L contains a ubiquitin-like domain and was shown to bind to the RPN10 subunit of the 26 S proteasome *in vitro* (15). Moreover, NUB1L was reported to accelerate the degradation of the ubiquitin-like modifier NEDD8. We hence investigated whether NUB1L would also increase the degradation rate of FAT10. HEK293 cells were transiently cotransfected with an expression construct for HA-FAT10 or HA-tagged NEDD8 either alone or together with an HA-NUB1L expression construct. To ensure that FAT10 and NUB1L were expressed in the same cells, both mRNAs were transcribed from the same plasmid under the control of a bidirectional promoter in some of the experiments. The cells were pulse-labeled with [^{35}S]methionine for 1 h and then chased for up to 4 h. Immunoprecipitation with anti-HA mAb revealed that the coexpression of human HA-NUB1L led to a strong down-regulation of HA-FAT10 protein levels and to an ~8-fold acceleration of HA-FAT10 degradation (Fig. 5*A*). Quantification of the FAT10 signal on a radioimager revealed that the coexpression of HA-NUB1L reduced the half-life of HA-FAT10 in HEK293 cells from 4 h to less than 30 min (Fig. 5*B*). Also for NEDD8 we consistently observed an acceleration of degradation when NUB1L was coexpressed (Fig. 5*C*), but the effect was much less prominent with an ~2-fold increase in degradation rate (Fig. 5*D*). Taken together, our data suggest that the rapid degradation of FAT10 is a potential functional consequence of the herein described interaction of FAT10 with NUB1L.

DISCUSSION

In this study we identify NUB1L as a new non-covalent interaction partner of the ubiquitin-like modifier FAT10. This interaction was initially found in a yeast two-hybrid screen using full-length human FAT10 as a bait and subsequently confirmed by coimmunoprecipitation from NUB1L- and FAT10-coexpressing cells. The binding of FAT10 and NUB1L appears to be direct because recombinant purified GST-FAT10 and His-NUB1L interacted in GST pull-down assays. The functional consequence of FAT10-NUB1L interaction does not seem to be a change in localization because no such change was observed by confocal microscopy when both proteins were coexpressed. Instead, NUB1L coexpression led to an 8-fold acceleration of FAT10 degradation resulting in a fulminant down-regulation of FAT10 expression level in NUB1L-coexpressing cells.

Recently, we have determined the half-life of FAT10 and a covalent conjugate of FAT10 to be in the order of 1 h, and this degradation was prevented by proteasome inhibitors thus strongly suggesting that the proteasome is in charge of FAT10 catabolism.[2] Moreover, the fusion of FAT10 to the long lived protein GFP led to its rapid degradation indicating that FAT10 may serve as a degradation tag. Ubiquitin-like proteins, as for instance RAD23 and hPLIC1/2, were shown to act as adaptors by associating with the 26 S proteasome via their ubiquitin-like domains (30, 31), and it is possible that NUB1L serves a similar purpose for FAT10 and, to a lesser degree, for NEDD8. NUB1 appears to copurify with the 26 S proteasome, and an interaction of NUB1 recombinant protein with an RPN10 fusion protein has been shown by Kamitani *et al.* (15) in GST pull-down assays. NUB1 contains a ubiquitin-like domain in its

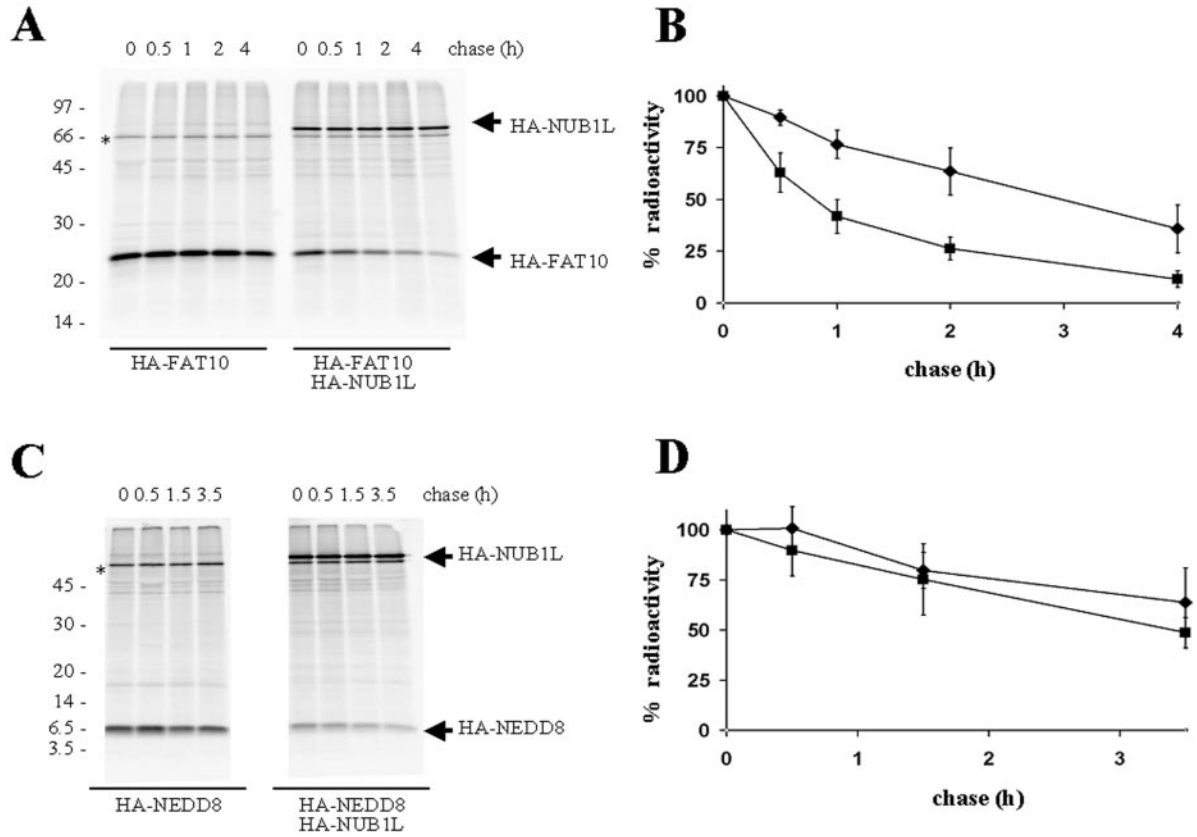


FIG. 5. **NUB1L expression reduces the half-life of FAT10.** *A*, HA-FAT10 either alone (*left panel*) or together with HA-NUB1L (*right panel*) was transiently expressed in HEK293 cells. The cells were labeled for 1 h with [^{35}S]Cys/Met and chased for the indicated times prior to HA-specific immunoprecipitation, SDS-PAGE, and autoradiography. Molecular mass markers are indicated to the *left* in kDa, and immunoprecipitated proteins are indicated with an *arrowhead*. The *asterisk* denotes an unspecific band. *B*, the HA-FAT10 bands from three experiments were quantified on a radioimager and plotted as percent radioactivity based on values of the pulsed cells. *Filled boxes* denote degradation in the presence of NUB1L; *filled diamonds* denote degradation in the absence of NUB1L. *C*, HA-NEDD8 either alone (*left panel*) or together with HA-NUB1L (*right panel*) was transiently expressed in HEK293 cells, metabolically labeled, chased as indicated, and immunoprecipitated with anti-HA mAb. *D*, quantitative evaluation of three autoradiographies similar to that shown in *C* with a radioimager. Typical experiments out of 3–4 independent experiments are shown.

N terminus, and this could potentially bind to the ubiquitin interaction motif of RPN10 or the leucine-rich repeat-like domain of the base component RPN1 which is bound by the ubiquitin-like domains of the adaptors hPLIC1/2 and RAD23, respectively (13, 32). In its C-terminal domain, NUB1L contains three UBA domains. Domains of this class have been shown to bind to polyubiquitylated proteins and are therefore likely candidates for FAT10 interaction domains. We hence hypothesize that NUB1L bridges FAT10 and its covalent conjugates to the 26 S proteasome and mediates their degradation. If this assumption is valid, FAT10-proteasome interaction may be inapparent when the expression levels of NUB-1 are low as seems to be the case in most tissues (16). Our results therefore predict that the rate of FAT10 degradation in tissues and cell lines is regulated by NUB1.

In this respect it is interesting that NUB-1 is inducible with the antiviral cytokines IFN-β and IFN-γ (16). FAT10 is induced in a broad array of cell lines with TNF-α and IFN-γ but not with IFN-α/β (23). This overlapping but not identical pattern of cytokine inducibility implies that the T cell-derived cytokines TNF-α and IFN-γ induce at the same time high levels of the ubiquitin-like protein FAT10 as well as the putative adaptor NUB-1 which facilitates the rapid degradation of FAT10. This scenario is very compatible with a role of FAT10 in selectively targeting so far unidentified substrate proteins for proteasomal degradation in a situation where cells are stimulated by T helper type 1-derived cytokines. The pattern of inducibility also suggests that NUB-1 must have a function other than FAT10 degradation in a situation where cells produce type I interferons (IFN-α and IFN-β) leading to the induction of NUB-1 but not of FAT10. This prediction is also consistent with the fact that NUB1 is expressed in organisms like *Arabidopsis* or *Drosophila* which lack a specific immune system, whereas FAT10 has only been found in mammals endowed with a specific immune system. The promotion of NEDD8 degradation is one example of a FAT10 unrelated function of NUB1 given that homologues of NEDD8 are also expressed in lower eukaryotes.

NUB1 was discovered 2 years ago by Yeh and co-workers (16) as a protein that interacts with the ubiquitin-like modifier NEDD8 *in vitro* and accelerates its proteasome-dependent degradation. It is therefore somewhat surprising that now a second ubiquitin-like modifier, FAT10, is down-regulated by the same protein. Both in our GST pull-down experiments (Fig. 2) as well as in our coimmunoprecipitation experiments (Fig. 3), we failed to detect a specific interaction between human NUB1L and human NEDD8 even when using buffers without detergents and physiological salt conditions. Yeh and co-workers (17) reported a specific interaction of RH-tagged NUB1 as well as RH-NUB1L with GST-NEDD8 but not GST when using a washing buffer containing 0.1% Nonidet P-40 as a detergent in GST pull-down assays. When we used buffers containing 0.1% Nonidet P-40, we lost the interaction of His-NUB1L with untagged NEDD8. We have used different tags than Yeh and co-workers and slightly different conditions so it may be that for technical reasons we missed a significant interaction of NUB1L and NEDD8. Hence, we cannot claim that the NUB1L-

NEDD8 interaction does not exist at all. We can, however, conclude from our experiments that the binding of NEDD8 to NUB1L is too weak to compete with the FAT10-NUB1L interaction and that even a 10-fold excess of NEDD8 does not affect the amount of His-NUB1L that can be pulled down with GST-FAT10 (Fig. 2*B*). It appears that the NUB1L-FAT10 interaction that withstands even washing with RIPA buffer (containing 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS) is much more robust and extensive than the NEDD8-NUB1L interaction. These results from binding assays are also in accordance with our finding that NUB1L coexpression accelerates the degradation of FAT10 in HEK293 cells by a 4-fold greater factor than that of NEDD8 (Fig. 5).

Tanaka *et al.* (17) have very recently performed an extensive analysis of domains within NUB1L that are responsible for NEDD8 interaction and degradation. Most interesting, it is only the second of three consecutive UBA domains in NUB1L that can bind to NEDD8 thus leaving the first and third UBA domain as potential interaction domains for FAT10. We are currently performing numerous domain shuffling experiments in order to find out which parts of NUB1L and FAT10 are responsible for their binding. We may be in for surprises given that Yeh and co-workers (17) unexpectedly found that NEDD8 binds to the PEST sequence in the ultimate C-terminal part of NUB1L as well, and that interaction via the PEST sequence is pivotal for NEDD8 degradation. Another important question that we are currently addressing is whether NUB1L is able to target FAT10 for binding and degradation by the isolated 26 S proteasome *in vitro*. This experiment will also help to clarify whether polyubiquitylation of FAT10 is required for its degradation or whether it becomes degraded in a ubiquitin-independent manner as has been shown previously (33) for ornithine decarboxylase. In the latter case, the covalent modification with FAT10 would be a cytokine-inducible and self-limiting alternative to polyubiquitylation as a marker for proteasomal degradation.

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REFERENCES

1. Voges, D., Zwickl, P., and Baumeister, W. (1999) *Annu. Rev. Biochem.* **68,** 1015–1068
2. Hershko, A., Ciechanover, A., and Varshavsky, A. (2000) *Nat. Med.* **6,** 1073–1081
3. Glickman, M. H., Rubin, D. M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V. A., and Finley, D. (1998) *Cell* **94,** 615–623
4. Deveraux, Q., Ustrell, V., Pickart, C., and Rechsteiner, M. (1994) *J. Biol. Chem.* **269,** 7059–7061
5. van Nocker, S., Deveraux, Q., Rechsteiner, M., and Viersta, R. D. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 856–860
6. Lam, Y. A., Lawson, T. G., Velayutham, M., Zweier, J. L., and Pickart, C. M. (2002) *Nature* **416,** 763–767
7. Braun, B. C., Glickman, M., Kraft, R., Dahlmann, B., Kloetzel, P. M., Finley, D., and Schmidt, M. (1999) *Nat. Cell Biol.* **1,** 221–226
8. Strickland, E., Hakala, K., Thomas, P. J., and DeMartino, G. N. (2000) *J. Biol. Chem.* **275,** 5565–5572
9. Verma, R., Aravind, L., Oania, R., McDonald, W. H., Yates, J. R., Koonin, E. V., and Deshaies, R. J. (2002) *Science* **298,** 611–615
10. Köhler, A., Cascio, P., Leggett, D. S., Woo, K. M., Goldberg, A. L., and Finley, D. (2001) *Mol. Cell* **7,** 1143–1152
11. Chen, L., and Madura, K. (2002) *Mol. Cell. Biol.* **22,** 4902–4913
12. Rao, H., and Sastry, A. (2002) *J. Biol. Chem.* **277,** 11691–11695
13. Walters, K. J., Kleijnen, M. F., Goh, A. M., Wagner, G., and Howley, P. M. (2002) *Biochemistry* **41,** 1767–1777
14. Gao, L., Tu, H., Shi, S. T., Lee, K. J., Asanaka, M., Hwang, S. B., and Lai, M. M. C. (2003) *J. Virol.* **77,** 4149–4159
15. Kamitani, T., Kito, K., Fukuda-Kamitani, T., and Yeh, E. T. H. (2001) *J. Biol. Chem.* **276,** 46655–46660
16. Kito, K., Yeh, E. T. H., and Kamitani, T. (2001) *J. Biol. Chem.* **276,** 20603–20609
17. Tanaka, T., Kawashima, H., Yeh, E. T. H., and Kamitani, T. (2003) *J. Biol. Chem.* **278,** 32905–32913
18. Raasi, S., and Pickart, C. M. (2003) *J. Biol. Chem.* **278,** 8951–8959
19. Fan, W., Cai, W., Parimoo, S., Lennon, G. G., and Weissman, S. M. (1996) *Immunogenetics* **44,** 97–103
20. Raasi, S., Schmidtke, G., and Groettrup, M. (2001) *J. Biol. Chem.* **276,** 35334–35343
21. Liu, Y., Pan, J., Zhang, C., Fan, W., Collinge, M., Bender, J. R., and Weissman, S. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4313–4318
22. Jentsch, S., and Pyrowolakis, G. (2000) *Trends Cell Biol.* **10,** 335–342
23. Raasi, S., Schmidtke, G., Giuli, R. D., and Groettrup, M. (1999) *Eur. J. Immunol.* **29,** 4030–4036
24. Bates, E. F. M., Ravel, O., Dieu, M. C., Ho, S., Guret, C., Bridon, J. M., AitYahia, S., Briere, F., Caux, C., Banchereau, J., and Lebecque, S. (1997) *Eur. J. Immunol.* **27,** 2471–2477
25. Lee, C. G., Ren, J., Cheong, I. S., Ban, K. H., Ooi, L. L., Yong Tan, S., Kan, A., Nuchprayoon, I., Jin, R., Lee, K. H., Choti, M., and Lee, L. A. (2003) *Oncogene* **22,** 2592–2603
26. Shockett, P., Difilippantonio, M., Hellman, N., and Schatz, D. G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 6522–6526
27. Gietz, R. D., and Woods, R. A. (2001) *BioTechniques* **30,** 816–828
28. Serebriiskii, I. G., and Golemis, E. A. (2000) *Anal. Biochem.* **285,** 1–15
29. Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. (1993) *Cell* **75,** 791–803
30. Schauber, C., Chen, L., Tongaonkar, P., Vega, I., Lambertson, D., Potts, W., and Madura, K. (1998) *Nature* **391,** 715–718
31. Kleijnen, M. F., Shih, A. H., Zhou, P. B., Kumar, S., Soccio, R. E., Kedersha, N. L., Gill, G., and Howley, P. M. (2000) *Mol. Cell.* **6,** 409–419
32. Elsasser, S., Gali, R. R., Schwickart, M., Larsen, C. N., Leggett, D. S., Muller, B., Feng, M. T., Tubing, F., Dittmar, G. A. G., and Finley, D. (2002) *Nat. Cell Biol.* **4,** 725–730
33. Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K., and Ichihara, A. (1992) *Nature* **360,** 597–599