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Membrane Transport, Structure, Function, and Biogenesis:

The MIT Domain of UBPY Constitutes a CHMP Binding and Endosomal Localization Signal Required for Efficient Epidermal Growth Factor Receptor Degradation

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The MIT Domain of UBPY Constitutes a CHMP Binding and Endosomal Localization Signal Required for Efficient Epidermal Growth Factor Receptor Degradation*

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We have identified and characterized a Microtubule Interacting and Transport (MIT) domain at the N terminus of the deubiquitinating enzyme UBPY/USP8. In common with other MIT-containing proteins such as AMSH and VPS4, UBPY can interact with CHMP proteins, which are known to regulate endosomal sorting of ubiquitinated receptors. Comparison of binding preferences for the 11 members of the human CHMP family between the UBPY MIT domain and another ubiquitin isopeptidase, AMSH, reveals common interactions with CHMP1A and CHMP1B but a distinct selectivity of AMSH for CHMP3/VPS24, a core subunit of the ESCRT-III complex, and UBPY for CHMP7. We also show that in common with AMSH, UBPY deubiquitinating enzyme activity can be stimulated by STAM but is unresponsive to its cognate CHMPs. The UBPY MIT domain is dispensable for its catalytic activity but is essential for its localization to endosomes. This is functionally significant as an MIT-deleted UBPY mutant is unable to rescue its binding partner STAM from proteasomal degradation or reverse a block to epidermal growth factor receptor degradation imposed by small interfering RNA-mediated depletion of UBPY.

Lysosomal degradation rates determine the levels of cell surface receptor tyrosine kinases, an important parameter in the control of cell growth (1, 2). Activated receptors are internalized and consequently committed to the lysosomal pathway by budding from the limiting membrane of the early endosome into lumenal vesicles, which define the multivesicular body (MVB).[3] Ubiquitination of receptors is required for their sorting into MVBs, which precludes their recycling to the plasma membrane (3, 4).

The constituents of the endosomal sorting machinery were initially identified as class E mutants in screens for vacuolar protein-sorting (VPS) defects in *Saccharomyces cerevisiae* (5, 6), characterized by an expanded pre-vacuolar compartment (7). These engage in a complex set of protein-protein interactions, which link four core complexes (endosomal sorting complexes required for transport, ESCRTs-0, I, II and III), and somehow impart directionality to the process (5, 8–11). It has been proposed that ESCRT-0 (comprising Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) and STAM (signal-transducing adaptor molecule)) may provide the first means of engagement with ubiquitinated receptor (12–15) through ubiquitin interaction motifs in both proteins, although both ESCRT-I and -II also contain ubiquitin-binding proteins (10, 16). In yeast, ESCRT-III is composed of four subunits (VPS2/Chm2, VPS24/Chm3, Snf7/Chm4, VPS20/Chm6), whereas mammals possess an expanded complement of isoforms, CHMP2A/B, CHMP3, CHMP4A/B/C, and CHMP6, providing the possibility for distinct ESCRT-III functions through combinatorial coding of core components. Two related proteins, Did2/VPS46/Chm1 and VPS60/MOS10/Chm5 (CHMP1A/B and CHMP5 in mammals), have poorly defined auxiliary roles in MVB sorting (17–19), and a further mammalian CHMP protein (CHMP7) has no yeast orthologue (20). All the CHMPs (charged MVB proteins) are characterized by a polarized distribution of charge: the N terminus is highly basic whereas the C terminus is highly acidic (17, 21).

Ubiquitination can be reversed by the action of deubiquitinating enzymes (DUBs), of which there are 84 predicted active members in the human genome (22, 23). Two mammalian DUBs (UBPY (ubiquitin-specific protease Y) and AMSH (associated molecule with the Src homology 3 domain of STAM)) compete for a common binding site on the Src homology 3 domain of the ESCRT-0 component STAM (24–26). Interestingly, siRNA-mediated depletion of each of these two DUBs has opposite effects on epidermal growth factor (EGF) receptor (EGFR) degradation; AMSH knock down promotes EGF and EGFR degradation (27, 28) whereas UBPY knock down is inhibitory (28–30). This effect of UBPY knock down is also in accordance with recent overexpression studies (31).

The last resolvable step of the MVB sorting pathway involves the AAA-ATPase VPS4 and may be coupled to disassembly of

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[3] The abbreviations used are: MVB, multivesicular body; VPS, vacuolar protein-sorting; ESCRT, endosomal sorting complex required for transport; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; STAM, signal-transducing adaptor molecule; DUB, deubiquitinating enzyme; siRNA, small interfering RNA; EGF, epidermal growth factor; MIT, microtubule-interacting and transport domain; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HEK, human embryonic kidney; PBS, phosphate-buffered saline.

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the ESCRT machinery (32–34). Both AMSH and VPS4 interact with ESCRT-III components through microtubule interacting and transport (MIT) domains (35–37), first identified in SNX15, spastin, and spartin (38). We have identified an MIT-like domain in UBPY. In this study we define the specificity of its CHMP protein interactions and show that it is necessary for the recruitment of UBPY to endosomes as well as for effective EGFR down-regulation.

EXPERIMENTAL PROCEDURES

Bioinformatics—All data base searches were performed as previously described (39) using a non-redundant data set constructed from current releases of SwissProt, TrEMBL, and Genpept. Generalized profile construction and searches were run locally using the *pftools* package. Profiles were constructed using the BLOSUM45 substitution matrix. The statistical significance of profile matches was derived from the analysis of the score distribution of a randomized data base.

Plasmids—UBPY-(1–133), UBPY-(1–438), UBPY-His_6, and UBPYΔMIT (lacking amino acids 1–123) were generated by PCR using PfuUltra HF (Stratagene) and pGEMT-UBPY (29) as a template (primer sequences available on request), sequenced, and subcloned into a yeast two-hybrid bait vector (pFBT9, a modified pGBT9), pGEX6P, and pEGFP-C1. AMSH bait and CHMP prey constructs have been described previously (35). The open reading frame of CHMP1B was PCR-amplified using pDEST-CHMP1B as template, sequenced, and subcloned into pET15b to generate an N-terminal His_6 tag. pET15b-VPS24 was a generous gift from Paul Whitley (40). pCDNA3-myc-CHMPs, GFP-UBPY, GFP-UBPY(C786S), siRNA-resistant GFP-UBPY*, GFP-AMSH, His_6-STAM, His_6-STAMΔSH3, His_6-STAMΔUIM (*i.e.* STAM(L175/L176A)) have been previously described (27, 29, 35). siRNA-resistant GFP-UBPY*(C786S) was generated by inserting a BbvI-BamHI fragment from GFP-UBPY* into GFP-UBPY(C786S) and GFP-UBPYΔMIT(C786S) by inserting an EcoRI-XmaI fragment from GFP-UBPY(C786S) into GFP-UBPYΔMIT. pGEXP-UBPY(C786S)-His_6 was generated by subcloning an EcoRI-StuI fragment from pEGFPC1-UBPY (C786S) into pGEX6P-UBPY-His_6. pGEX6P-UBPYΔMIT-His_6 was produced by subcloning a BamHI-EcoRI fragment from pCMV-Tag2B-UBPY-ΔMIT into pGEX6P-UBPY-His_6.

Yeast Two-hybrid—All bait constructs were transformed into the PJ69–4A MATa strain, while prey constructs were expressed in the complementary mating type-switched strain PJ69–4A MATα. Targeted yeast two-hybrid matrix experiments were performed as described previously (41). Selective growth of diploid yeast was assessed on (–) His/plates containing 2.5 mM 3-AT, and screens were repeated to ensure that growth phenotypes were reproducible. In each case, colony growth was recorded after 5 days of incubation at 30 °C.

Antibodies and Other Reagents—Primary antibodies used were as follows: mouse monoclonals anti-Myc (4A6) (Upstate), anti-EGFR RI (CRUK) (UK), anti-His_6 (Amersham Biosciences), anti-tubulin (Sigma), anti-GFP (Roche Applied Sciences), and polyclonal antibodies affinity-purified sheep anti-GFP (gift from Ian Prior, University of Liverpool), rabbit anti-ubiquitin (Sigma), rabbit anti-CHMP1B (gift from Evan Reid, University of Cambridge). Rabbit anti-Hrs and anti-STAM have previously been described (42, 43). Secondary horseradish peroxidase-coupled antibodies, horseradish peroxidase-coupled anti-GST, and Protein-G-agarose were obtained from Sigma. Donkey IR700 and IR800-coupled anti-mouse and anti-rabbit secondary antibodies were from Rockland, and AF594-coupled donkey anti-mouse antibodies were from Molecular Probes.

Protein Production—GST-UBPY-His_6, GST-UBPY(C786S)-His6, and GST-UBPYΔMIT-His6 expression in Rosetta (DE3) pLysS cells (Novagen) was induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (0.4 mM) for 4 h at 20 °C. The bacteria were lysed by sonication, and the protein was purified first with nickel-nitrilotriacetic acid-agarose (Qiagen) and then with glutathione-Sepharose (Amersham Biosciences), both used according to the manufacturer's instructions and dialyzed against 50 mM Tris, pH 7.2, 25 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol. GST-UBPY-(1–133), GST-UBPY-(1–438), GST-AMSH, His_6-CHMP1B, and His_6-CHMP3 were expressed in Rosetta (DE3) pLysS cells (Novagen) and purified with glutathione-Sepharose (Amersham Biosciences) (GST-tagged) or nickel-nitrilotriacetic acid-agarose (Qiagen) (His_6-tagged) according to the manufacturer's instructions.

Pulldown Experiments—Purified His_6-CHMP1B or His_6-CHMP3 (22 pmol) was incubated with 110 pmol GST, GST-UBPY-(1–133), GST-UBPY-(1–438), or GST-AMSH at 4 °C for 1 h in 300 μl of assay buffer (20 mM Hepes, pH 7.3, 120 mM KOAc, 0.1 mM dithiothreitol, 0.1% Triton X-100, and protease inhibitors). After brief centrifugation, the supernatants were incubated with 30 μl of glutathione-Sepharose at room temperature for 30 min. The beads were washed three times with buffer WB (20 mM Hepes, pH 7.3, 120 mM KOAc, and 0.1 mM dithiothreitol, 0.1% Triton X-100) and once with WB minus Triton X-100 before elution in SDS-PAGE sample buffer. *In vitro* translated Myc-CHMP proteins were produced with the TNT® Quick-coupled Transcription/Translation system (Promega) according to the manufacturer's instructions using pCDNA3-myc-CHMP constructs. Purified GST, GST-UBPY-(1–133), or GST-AMSH (220 pmol) were each incubated with 10 μl of *in vitro* translated product at 4 °C for 1 h in 300 μl of assay buffer. After brief centrifugation, the supernatants were incubated with 40 μl of glutathione-Sepharose at room temperature for 30 min and processed as above. Bound proteins were eluted with SDS-PAGE sample buffer and analyzed in parallel with a fraction of the input material by immunoblotting with anti-Myc or anti-His_6 antibodies, followed by IR800-coupled secondary antibodies. The Western blots were analyzed and the results quantified using a LI-COR Odyssey 2.1. Samples were reprobed with horseradish peroxidase-coupled anti-GST, developed by enhanced chemiluminescence (Pierce), and signals captured with a Uvichemi Gel documentation system (Uvitech) and quantified using Image J. The percentage of pulldown was calculated relative to the input after subtraction of the background signal attributable to GST alone.

Cell Culture, Transfection, and RNA Interference Experiments—HEK293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% non-essential amino acids, transfected

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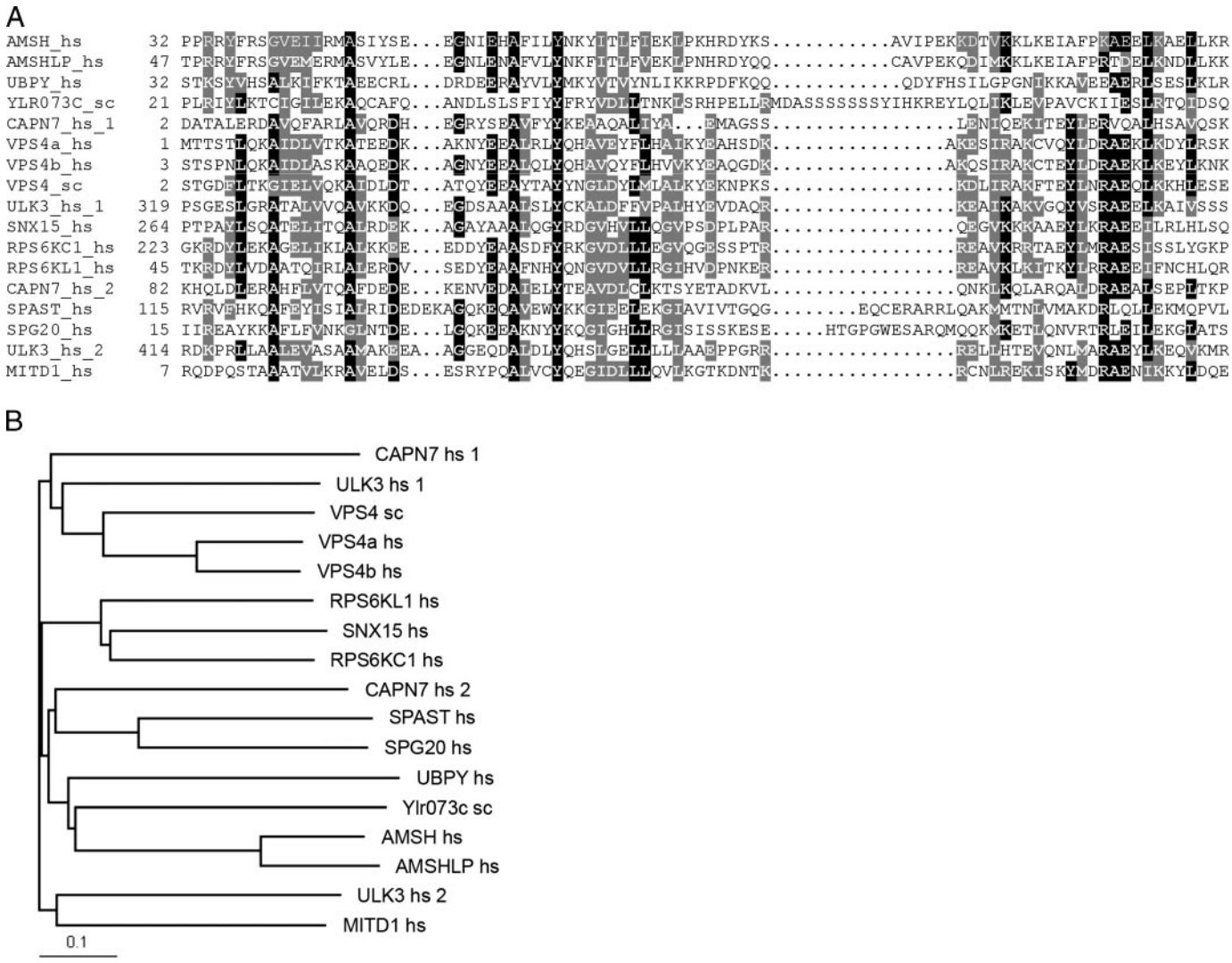

FIGURE 1. **UBPY contains a predicted MIT domain.** *A*, sequence alignment of predicted MIT domains within the human and *S. cerevisiae* genomes. The human genome contains 14 predicted MIT domains within 12 proteins while the yeast genome contains two MIT-containing proteins. Accession numbers (Uniprot) are, respectively, SPAST: Q9UBP0; VPS4a: Q9UN37; VPS4b: O75351; AMSH: O95630; AMSHLP: Q96FJ0; UBPY: P40818; SPG20: Q8N0X7; SNX15: Q9NRS6; RPS6KC1: Q5VUN8; RPS6KL1: Q9Y6S9; ULK3: Q8K1X6 (mouse); CAPN7: Q9Y6W3; LOC129531: Q8WV92; yeast (all SGD) VPS4: YPR173C; YLR073C: YLR073C. ULK3 and CAPN7 each contain two predicted MIT domains; in each case, the most N-terminal MIT is designated as "*1*" and the downstream MIT as "*2*". *B*, dendrogram of human and *S. cerevisiae* "MIT proteins" reveals the UBPY MIT domain to be most closely related to that of two other DUBs, AMSH and AMSH-LP, as well as a yeast protein (*Ylr073*) of unknown function lacking a DUB catalytic domain.

with GeneJuice (Merck Biosciences), and lysed or fixed for immunofluorescence 24 h post-transfection as previously described (29). For siRNA experiments, HeLa cells were treated twice over 96 h (24 and 72 h after the initial seeding) with either Control siRNA duplex (Nonspecific Control VII) or UBPY-specific siRNA duplex 1 (sense UGAAAUACGUGACUGUUUAUU, antisense 5′-PUAAACAGUCACGUAUUUCAUU; Dharmacon, Lafayette, CO) at 40.8 nM concentration using Oligofectamine (Invitrogen) in the absence of serum. Fetal bovine serum (10%) was added 4 h post-transfection. For rescue experiments, the cells were transfected 66 h before harvesting with pEGFPC1, siRNA interference-resistant GFP-UBPY (UBPY*), GFP-UBPYΔMIT, or catalytically inactive GFP-UBPY*(C786S). Cells were serum-starved for 16 h and stimulated with EGF (100 ng/ml) for 4 h to induce internalization and degradation of EGFR. Cells were either lysed and samples analyzed by Western blotting or fixed in paraformaldehyde and processed for immunofluorescence staining with anti-EGFR as described below. The percentage of GFP-positive cells that retained EGFR after 4 h of EGF treatment was determined manually by counting cells on an epifluorescence microscope.

Immunofluorescence—Cells were processed for immunofluorescence by fixation with 3% paraformaldehyde in PBS, permeabilization with 0.2% Triton X-100 in PBS, and preincubation in 10% goat serum in PBS. The cells were stained with anti-EGFR followed by Alexa-Fluor 594-coupled secondary antibodies, both diluted in 5% goat serum in PBS. Confocal images were captured with a Leica confocal SP2 AOBS (HCX PL APO CS 63.0 × 1.40 oil objective).

Cell Lysis and Immunoprecipitation—Cells were washed with ice-cold PBS and incubated for 10 min on ice in Nonidet P-40 lysis buffer (1% Nonidet P-40, 25 mM Tris/HCl, pH 7.5, 100 mM NaCl, 50 mM NaF). Lysates were cleared by centrifugation and incubated with sheep anti-GFP and Protein-G-agarose for

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2 h at 4 °C. Beads were washed three times with YP-IP buffer (0.1% Nonidet P-40, 25 mM Tris/HCl, pH 7.5, 150 mM NaCl), once with 10 mM Tris/HCl, pH 7.5, and resuspended in 1.5× SDS-PAGE sample buffer. Eluted proteins were resolved by SDS-PAGE followed by immunoblotting.

DUB Assay—GST-UBPY-His_6 (20 nM), preincubated with His_6-STAM or His_6-CHMP proteins (200 nM) for 30 min at 4 °C, was incubated with wild-type Lys-63-linked tetraubiquitin chains (0.4 μM; Boston Biochem) in 20 μl of DUB buffer (50 mM Tris/HCl, 25 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.2) for 2 h at 37 °C. Proteins were resolved on 4–12% NuPAGE gels (Invitrogen) and transferred to nitrocellulose. The membranes were boiled for 30 min in deionized water, blocked in 5% Fish Skin Gelatin, 0.1% Tween 20 in PBS, and probed with a rabbit antibody to ubiquitin (Sigma) and processed with secondary antibodies as described above.

RESULTS

UBPY Contains an MIT Domain—Fig. 1*A* shows an alignment of the N-terminal region of UBPY with VPS4 and other MIT domain-containing proteins, suggesting that like the other STAM binding DUB, AMSH, UBPY also contains an MIT domain. We have identified 14 predicted MIT domains within 12 proteins in the human genome. This represents a large expansion from the *S. cerevisiae* genome, which contains only VPS4 and Ylr073c, a protein of unknown function. The associated dendrogram shows that among MIT domain-containing proteins in the human genome, the UBPY-MIT most closely resembles AMSH and a related protein, AMSH-LP (Fig. 1*B*).

UBPY and AMSH Show Overlapping but Distinct CHMP Binding Profiles—We and others (35, 36, 44) have previously shown that AMSH interacts with multiple CHMP proteins and that the MIT domain is responsible for its interaction with the ESCRT-III component VPS24/CHMP3. We tested interactions of the UBPY MIT domain with ten human CHMP proteins using a directed yeast two-hybrid screen. When expressed from a bait construct, the N terminus of UBPY (residues 1–133) incorporating the MIT domain shows a strong interaction with CHMP1B but a greatly restricted CHMP interaction profile compared with AMSH (Fig. 2). Note that under these assay conditions CHMP6 was found to auto-activate the yeast two-hybrid (His) reporter. No interactions were seen when the UBPY MIT domain was expressed from a prey construct (not shown). This selectivity indicated by the two-hybrid screen was retained in a direct binding assay between proteins purified from bacteria. We tested the interaction of GST-AMSH and GST-UBPY-(1–133) or a longer fragment GST-UBPY-(1–438) (Fig. 2*A*) with His_6-tagged CHMP1B and CHMP3 (Fig. 3). The UBPY MIT domain directly interacts with CHMP1B but not with CHMP3, whereas AMSH interacts with both CHMPs as indicated in the yeast two-hybrid assay.

We next directly compared the CHMP binding profile of bacterially expressed GST-UBPY-(1–133) and GST-AMSH toward the complete panel of 11 human Myc-tagged CHMP proteins, which were produced by *in vitro* translation. In this configuration, the UBPY MIT domain-(1–133) as well as a longer fragment (1–438, not shown) shows clear interactions with CHMP1A, 1B, 4C, and 7. This contrasts clearly with the binding profile for GST-AMSH, which does not bind CHMP7 but shows clear binding to CHMP3 (Fig. 4).

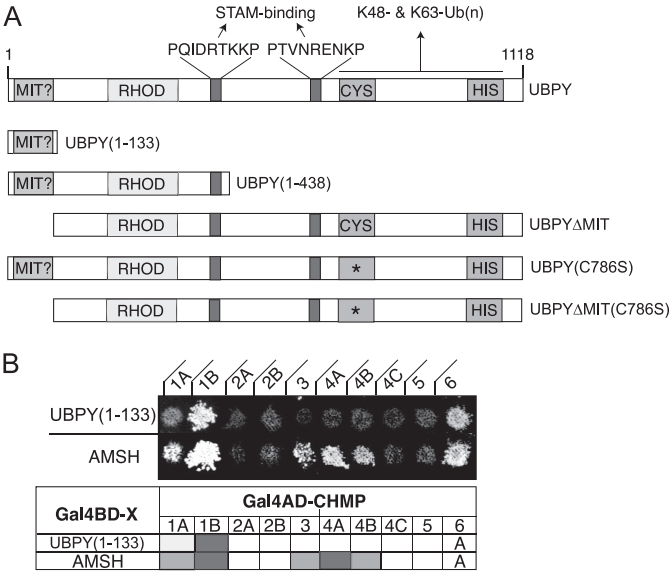

FIGURE 2. **CHMP interaction profile of AMSH and UBPY-(1–133) in a yeast two-hybrid assay.** *A*, schematic diagram of the domain structure of UBPY and the constructs used in this study. *RHOD*, Rhodanese domain. *B*, profiles for yeast two-hybrid interactions observed when strains expressing either the UBPY-(1–133) fragment or full-length AMSH were mated against strains expressing a collection of human CHMP proteins (*1A*, *1B*, *2A*, *2B*, *3*, *4A*, *4B*, *4C*, *5*, and *6*). *Shaded gray boxes* reflect relative colony growth on (–) His plates containing 2.5 mM 3-AT at 5 days post-replication. *Gal4BD-* and *Gal4AD-* indicate "bait" and "prey" constructs, respectively. *A* indicates auto activation/nonspecific promiscuous growth.

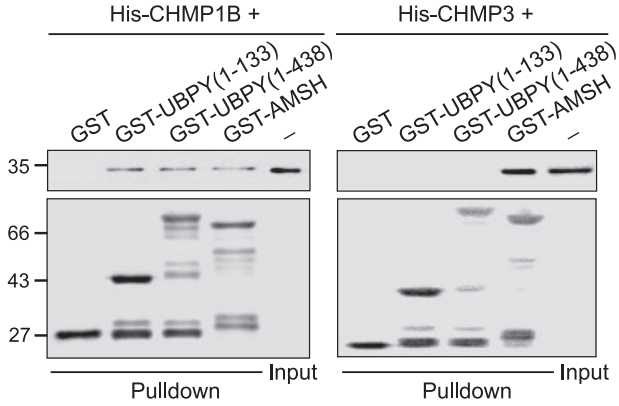

FIGURE 3. **Validation of direct CHMP1B-UBPY interaction using bacterially expressed proteins.** Purified GST, GST-UBPY-(1–133), GST-UBPY-(1–438), or GST-AMSH (110 pmol each) were co-incubated with His_6-CHMP1B and His_6-CHMP3 (22 pmol each) for 1 h on ice as described under "Experimental Procedures." Proteins were isolated with glutathione-Sepharose beads and analyzed in parallel with a sample of the input material (23% His_6-CHMP1B and 36% His_6-CHMP3) by immunoblotting with anti-His_6 and anti-GST. These data recapitulate results obtained in the two-hybrid assay (Fig. 2) wherein both AMSH and UBPY interact with CHMP1B but only AMSH interacts directly with CHMP3.

Finally, we confirmed the specific interaction of full-length UBPY with CHMPs 1A and 1B in cells by immunoprecipitation of GFP-UBPY from HEK293T cells co-expressing various Myc-tagged CHMP proteins. Also in the context of the full-length protein, selective binding was observed with CHMP1A, CHMP1B, and CHMP7 (not shown) but not with CHMP3 (Fig. 5*A*). Low level binding to CHMP5 is most likely indirect as CHMP5 interacts with CHMP1B in a yeast two-hybrid assay (9,

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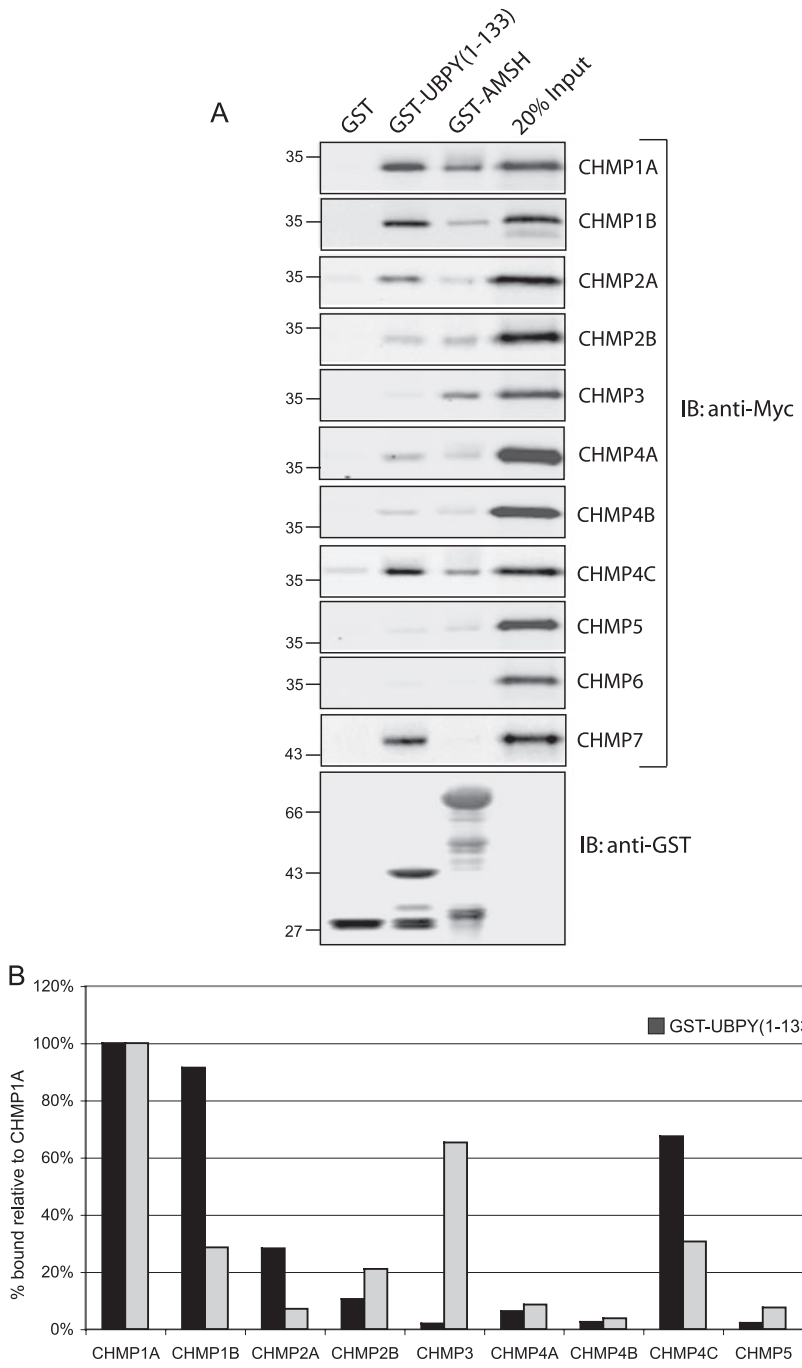

FIGURE 4. **Interaction profile of GST-AMSH and GST-UBPY-(1–133) with *in vitro* translated (IVT) Myc-tagged CHMP proteins.** *A*, purified GST, GST-UBPY(1–133), or GST-AMSH (220 pmol) were incubated with *in vitro* translated Myc-CHMPs (*1A*, *1B*, *2A*, *2B*, *3*, *4A*, *4B*, *4C*, *5*, *6*, and *7*) at 4 °C for 1 h. The proteins were isolated with glutathione-Sepharose beads and analyzed in parallel with a sample (20%) of the *in vitro* translated input by immunoblotting with anti-Myc and anti-GST (a representative blot is shown for the latter in the *bottom panel*). *B*, quantification of the results shown in *panel A*. The efficiency of the pull down was assessed as percentage of the input bound to GST-UBPY-(1–133) and GST-AMSH after subtraction of the background signal (fraction bound to GST alone). The percentage bound to either UBPY or AMSH was then normalized to the strongest binder, CHMP1A, to allow direct comparison of the selectivity within the binding profiles. Note that UBPY and AMSH show both common (*CHMP1A*, *CHMP1B*, *CHMP4C*) and clearly distinct binding partners (*CHMP3* and *CHMP7*).

35) and does not show up as a UBPY binding partner in either of the other assays. Importantly, the MIT domain is clearly necessary for UBPY binding to CHMP1B as a GFP-UBPYΔMIT construct no longer co-immunoprecipitates with CHMP1B (Fig. 5*B*).

STAM, but Not CHMP, Interaction Stimulates UBPY DUB Activity—We have previously shown that the AMSH- and UBPY-interacting partner STAM can stimulate AMSH DUB activity in an *in vitro* assay in a manner dependent on the ubiquitin interaction motif domain of STAM (44). We now show that STAM can also stimulate UBPY DUB activity on enzymatically produced wild-type Lys63- and Lys48-linked tetraubiquitin chains in the same *in vitro* assay format (Fig. 6*A* and data not shown). In contrast, incubation with the newly identified UBPY-interacting partner CHMP1B had no effect on UBPY DUB activity either on its own or in combination with STAM, mirroring the failure of the AMSH binding partner CHMP3 to stimulate AMSH activity (Fig. 6*A*) (44). Removal of the MIT domain does not compromise the activity of UBPY in this *in vitro* setting or its ability to be stimulated by STAM (Fig. 6*B*).

The MIT Domain Is Required for Endosomal Localization of UBPY—GFP-UBPY is largely cytosolic in serum-starved cells but partially redistributes to early endosomes containing EGFR upon acute EGF stimulation (29, 45). No corresponding redistribution is observed with GFP-UBPYΔMIT despite the retention of two STAM binding sites in this protein, suggesting these are not sufficient for endosomal localization (Fig. 7, *A–F*). A catalytically inactive mutant of UBPY, GFP-UBPY(C786S), constitutively associates with endosomes that accumulate ubiquitin (29). Both endosomal localization (Fig. 7, *G–L*) and accumulation of ubiquitin on endosomes (not shown) are lost following deletion of the MIT domain from this mutant (GFP-UBPYΔMIT(C786S)).

The MIT Domain of UBPY Is Required for Maintenance of ESCRT-0 Stability and EGFR Degradation—A characteristic of UBPY-depleted cells is that STAM is no longer protected from proteasomal degradation by UBPY-mediated de-ubiquitination (29), resulting in the destabilization of both ESCRT-0 components, Hrs and STAM. Expression of siRNA-resistant GFP-UBPY* partially recovers

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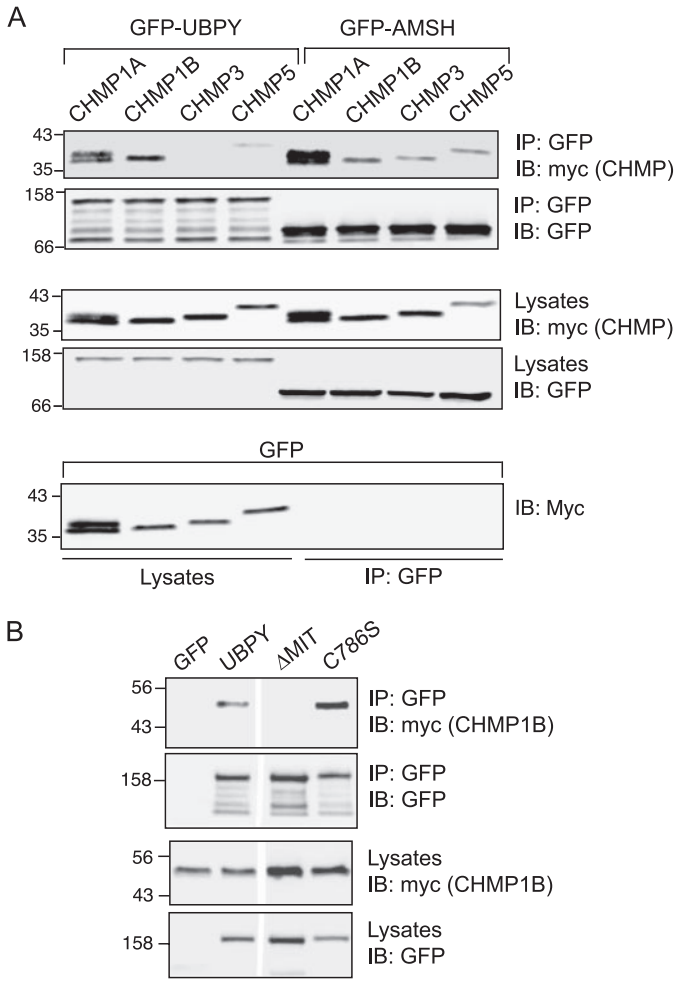

FIGURE 5. **Co-immunoprecipitation of full-length GFP-UBPY with CHMP1B from HEK293T cells.** *A*, GFP-UBPY or GFP-AMSH were co-expressed with various Myc-tagged CHMP proteins in HEK293T cells and immunoprecipitated with anti-GFP prior to blotting with anti-GFP and anti-Myc antibodies. As indicated in preceding assays both proteins interact with CHMP1A and CHMP1B, whereas only AMSH interacts with CHMP3. The interaction with CHMP5 seen in this assay may be indirect (see "Results"). *B*, co-immunoprecipitation between CHMP1B and UBPY is lost upon deletion of the MIT domain. Myc-CHMP1B and GFP-UBPY, GFP-UBPYΔMIT, or GFP-UBPY(C786S) were co-expressed in HEK293T cells and processed as described above.

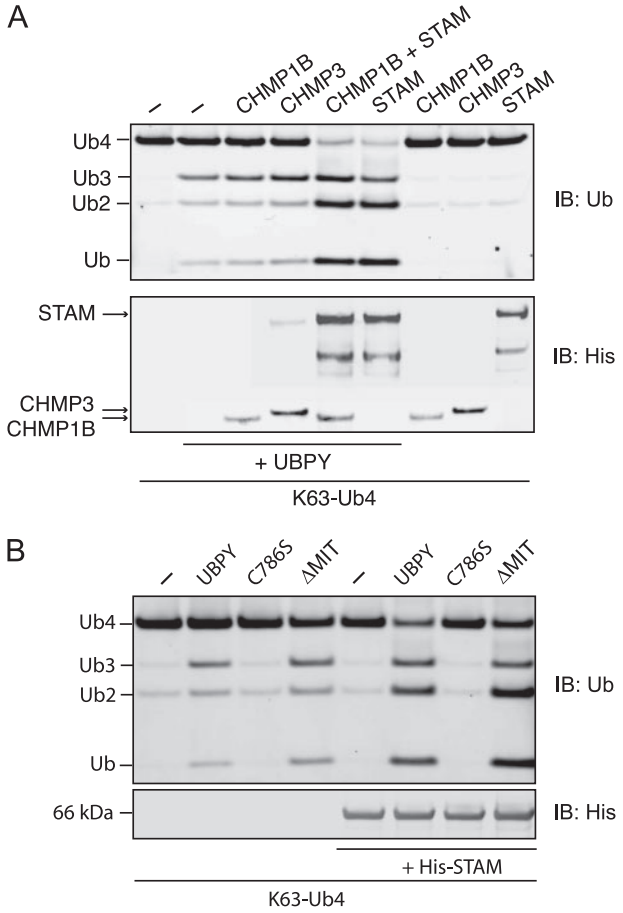

FIGURE 6. *A*, STAM, but not CHMP, interaction stimulates UBPY DUB activity. Full-length bacterially expressed and purified GST-UBPY-His_6 (20 nM) was preincubated for 30 min on ice with purified His_6-STAM, His_6-CHMP1B, or His_6-CHMP3 (all 200 nM) prior to incubation with Lys-63-linked tetraubiquitin chains (0.4 μM) for 2 h at 37 °C. The samples were analyzed by SDS-PAGE followed by immunoblotting with anti-ubiquitin (*top panel*) and anti-His_6 antibodies (*bottom panel*). *B*, the MIT domain is dispensable for UBPY DUB activity *in vitro*. Bacterially expressed and purified GST-UBPY-His_6, GST-UBPY(C786S)-His_6, or GST-UBPYΔMIT-His_6 (all at 24 nM) were preincubated for 30 min on ice with purified His_6-STAM (240 nM) and then incubated with Lys-63-linked tetraubiquitin chains (0.4 μM) for 2 h at 37 °C. The samples were analyzed by SDS-PAGE followed by immunoblotting with anti-ubiquitin (*top panel*) and anti-His_6 antibodies (*bottom panel*).

Hrs and STAM levels, whereas GFP-UBPYΔMIT or catalytically inactive GFP-UBPY*(C786S) are ineffective (Fig. 8) in this context. No significant changes in levels of the UBPY binding partner CHMP1B were observed following UBPY knock down and overexpression (Fig. 8).

Knock down of endogenous UBPY also leads to a failure to degrade EGF and EGFR (28–30). Prolonged EGF stimulation results in the loss of receptor from control cells as judged by immunofluorescence, but in UBPY-depleted cells the receptor is retained in clustered endosomal structures (29). We have used this assay to investigate the role of the MIT domain with respect to UBPY function. Expression of siRNA-resistant GFP-UBPY* in UBPY knockdown cells efficiently rescues this degradation defect, whereas neither GFP-UBPYΔMIT or GFP-UBPY*(C786S) can do so (Fig. 9). Overexpression of GFP-UBPYΔMIT in untreated or control siRNA-treated cells did not interfere with receptor down-regulation, suggesting that it does not act as a dominant negative mutant in this context.

DISCUSSION

The human genome encodes 14 predicted MIT domains within 12 proteins, of which several have previously been shown to interact with CHMP proteins. Some of these are quite promiscuous, such as AMSH (35, 36) or VPS4 (9, 11, 35), whereas others such as AMSH-LP have no apparent binding partners (35, 36). Although not so far confirmed through rigorous systematic analysis, available data suggest some specificity of spastin for CHMP1B (46) and of MITD1 for CHMP2A (35). Having identified UBPY as an MIT domain protein through sequence alignment, we could confirm the presence of an MIT domain signature of three α-helices by examination of a recently published study that provides a crystal structure for the N terminus of UBPY (47). The structure of the UBPY MIT domain is highly similar to that determined for VPS4 (37).

The MIT domain proteins AMSH, UBPY, and VPS4 all influence endosomal sorting and bind to CHMP proteins. It is important therefore to define the respective CHMP bind-

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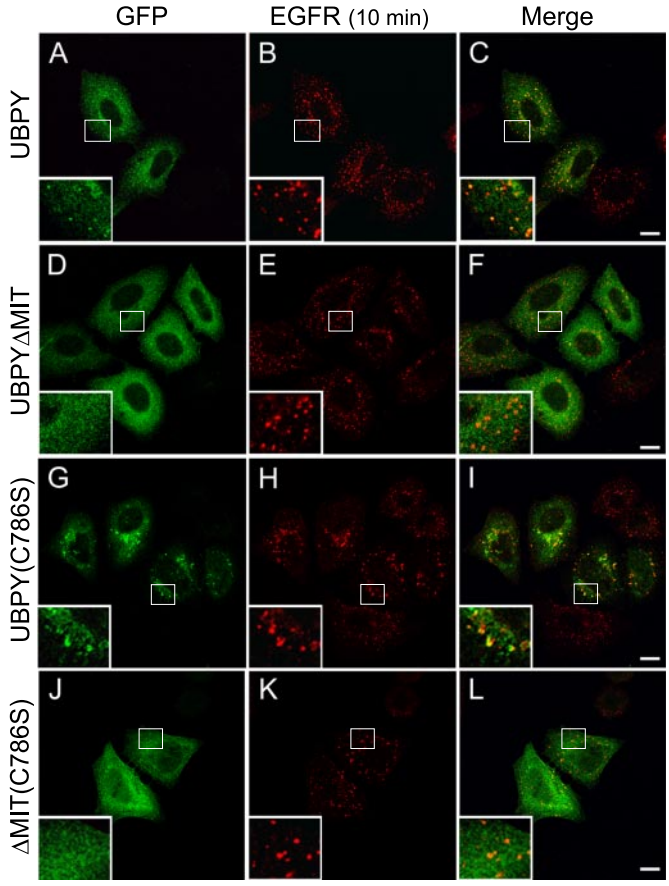

FIGURE 7. **Recruitment of UBPY to endosomes is contingent on an intact MIT domain.** HeLa cells transfected with GFP-UBPY, GFP-UBPYΔMIT, or GFP-UBPY(C786S) were serum-starved for 16 h before stimulation with EGF (100 ng/ml) for 10 min at 37 °C. The cells were fixed, permeabilized, and stained with anti-EGFR followed by AlexaFluor 594-labeled secondary antibodies and analyzed by confocal microscopy 24 h post-transfection. GFP-UBPY translocates from the cytosol to EGFR-containing endosomes in response to EGF (*panels A–C* and Ref. 29). This redistribution is not seen when the MIT domain is deleted (*GFP-UBPYΔMIT*) (*D–F*). The catalytically inactive mutant GFP-UBPY(C786S) constitutively associates with endosomes (*G–I*), but again this localization is lost by removal of the MIT domain (*GFP-UBPYΔMIT(C786S)*) (*J–L*). *Insets* show a 3-fold enlargement of the selected area. *Scale bars*, 10 μm.

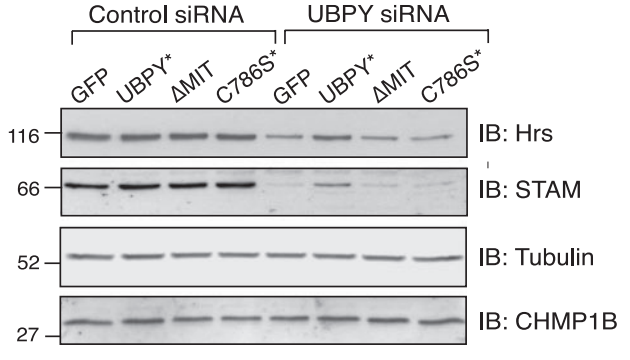

FIGURE 8. **The MIT domain of UBPY is required for stabilization of ESCRT-0 components.** HeLa cells were treated with UBPY siRNA or control siRNA prior to transfection with GFP or GFP-tagged constructs expressing siRNA-resistant UBPY*, UBPYΔMIT, and UBPY*(C786S). Note that the UBPY-specific siRNA targets a region corresponding to the MIT domain. Cells were lysed, and samples analyzed by SDS-PAGE and immunoblotting with Hrs, STAM, CHMP1B, and tubulin antibodies.

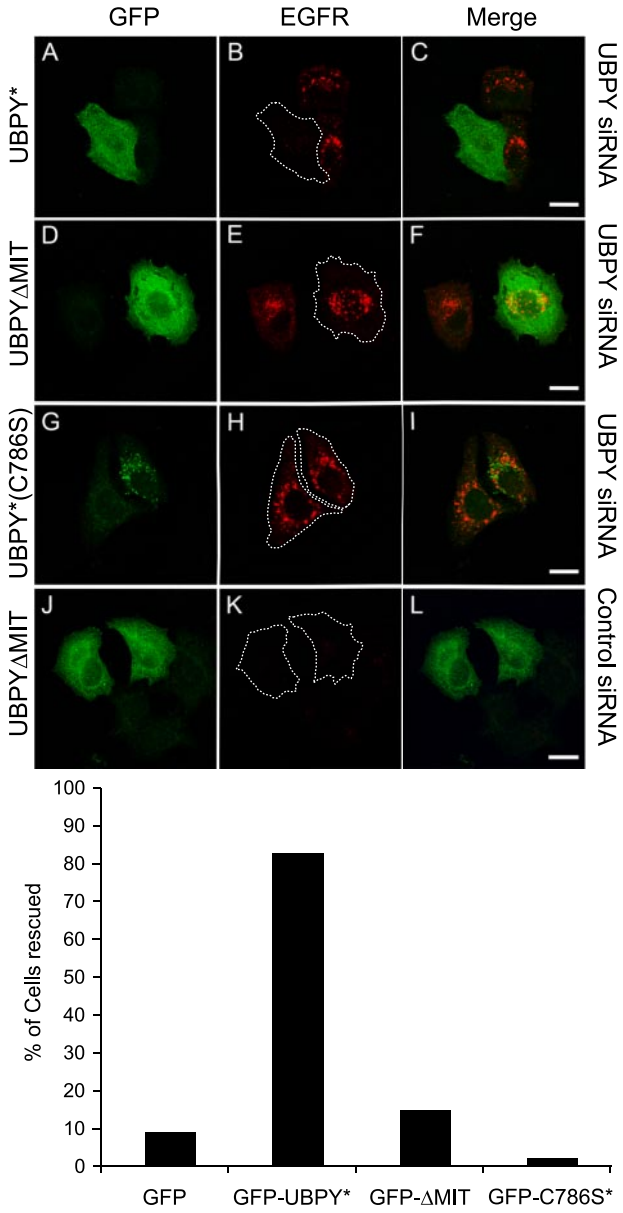

FIGURE 9. **The MIT domain of UBPY is required for regulation of EGFR degradation.** HeLa cells were treated with UBPY siRNA (*A–I*) or control siRNA (*J–L*) and subsequently transfected with GFP-tagged constructs expressing siRNA-resistant forms of UBPY and mutants thereof: UBPY* (*A–C*), UBPYΔMIT (*D–F* and *J–L*), and UBPY*(C786S) (*G–I*). Cells were serum-starved overnight and stimulated for 4 h with EGF, which promotes the internalization and degradation of EGFR in control cells but results in retention of EGFR in clustered endosomes in UBPY-depleted cells. Cells were fixed, permeabilized, and stained with anti-EGFR followed by AlexaFluor 594-labeled secondary antibodies and analyzed by confocal microscopy. Expression of GFP-UBPY* restores EGFR degradation in cells treated with UBPY-specific siRNA (*A–C*), but this is not the case for UBPYΔMIT (*D–F*) or the catalytically inactive form UBPY*(C786S) (*G–I*). Overexpression of GFP-UBPYΔMIT in control cells does not interfere with EGFR degradation (*J–L*). *Scale bars*, 15 μm. The degree of rescue was assessed by counting the number of GFP-positive cells (*outlined in white*) that retained EGFR for each of the constructs in a representative experiment. Number of cells counted: GFP, 780; GFP-UBPY*, 754; GFP-UBPYΔMIT, 338; GFP-UBPY*(C786S), 46.

ing profiles of each MIT domain. Our two-hybrid analysis indicated common binding of AMSH and UBPY to CHMP1B (also shared by VPS4) but in addition a specific preference of AMSH for other CHMPs, including CHMP3. In fact, the AMSH MIT domain is the only one so far characterized to display CHMP3 binding activity. In contrast to CHMP1B, CHMP3 is a core component of the ESCRT-III complex that is thought to act at a late stage in the sorting of membrane

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proteins into multivesicular bodies, although the molecular details of the mode of action of these proteins are still unclear.

A more comprehensive, quantitative, and direct analysis of binding of all Myc-tagged CHMP proteins to GST-AMSH and GST-UBPY-(1–133), while broadly in line with the two-hybrid data, revealed further common binding partners in CHMP1A and CHMP4C. Remarkably, UBPY showed a high degree of specificity (compared with AMSH) for CHMP7. This recently characterized member of the CHMP family inhibits EGFR degradation on overexpression (20) but has hitherto not been included in any of the screens for CHMP binding to MIT domains.

The emerging picture is one of overlapping but distinct binding profiles of MIT domain proteins. Thus, some of these proteins may compete with each other for the same binding sites within the ESCRT machinery, while others will be more selective. The role of such specific interaction profiles is currently unclear, as we still know too little about the functional contingencies and redundancies within the family of CHMP proteins. This may, however, ensure that association of each MIT protein with particular endosomes is governed by the CHMP repertoire at that location.

Our observation that the UBPY MIT domain is essential for endosomal localization is surprising, given that UBPYΔMIT retains at least two binding sites for the endosomal protein STAM (24). For AMSH, endosomal localization is maintained upon deletion of either the STAM binding site (44) or the MIT domain (48), although deletion of both has not been tested. Nevertheless, STAM stimulates UBPY activity as previously observed for AMSH (44). We propose a model in which the primary endosomal localization signal for UBPY involves MIT-CHMP interactions while the STAM interaction primarily serves to present ubiquitinated substrates captured by STAM. Interestingly, while both UBPY and STAM are also present in cytosolic pools, the MIT domain, and by extension CHMP binding, is required to protect STAM from proteasomal degradation. This may suggest that in the cellular context CHMP binding is important to direct UBPY activity toward specific endosomal substrates.

Does endosomal localization of UBPY matter? UBPY knock down has global effects on cellular protein ubiquitination patterns (29, 49). However, we now show that its role in EGFR trafficking requires endosomal localization through its MIT domain. Rescue of an inhibitory effect on EGFR degradation in UBPY-depleted cells requires both catalytic activity and an intact MIT domain. In this respect, there are striking parallels with the *S. cerevisiae* endosomal DUB, Doa4, which requires endosomal association through its N-terminal domain to undertake de-ubiquitination of protein cargo at the MVB (50, 51).

In conclusion, we have demonstrated the importance of the N terminus of UBPY encompassing the MIT domain for both endosomal localization and physiological function. Endosomal localization of UBPY is itself under the control of cell signaling events, and it will be important to understand how this interaction is regulated.

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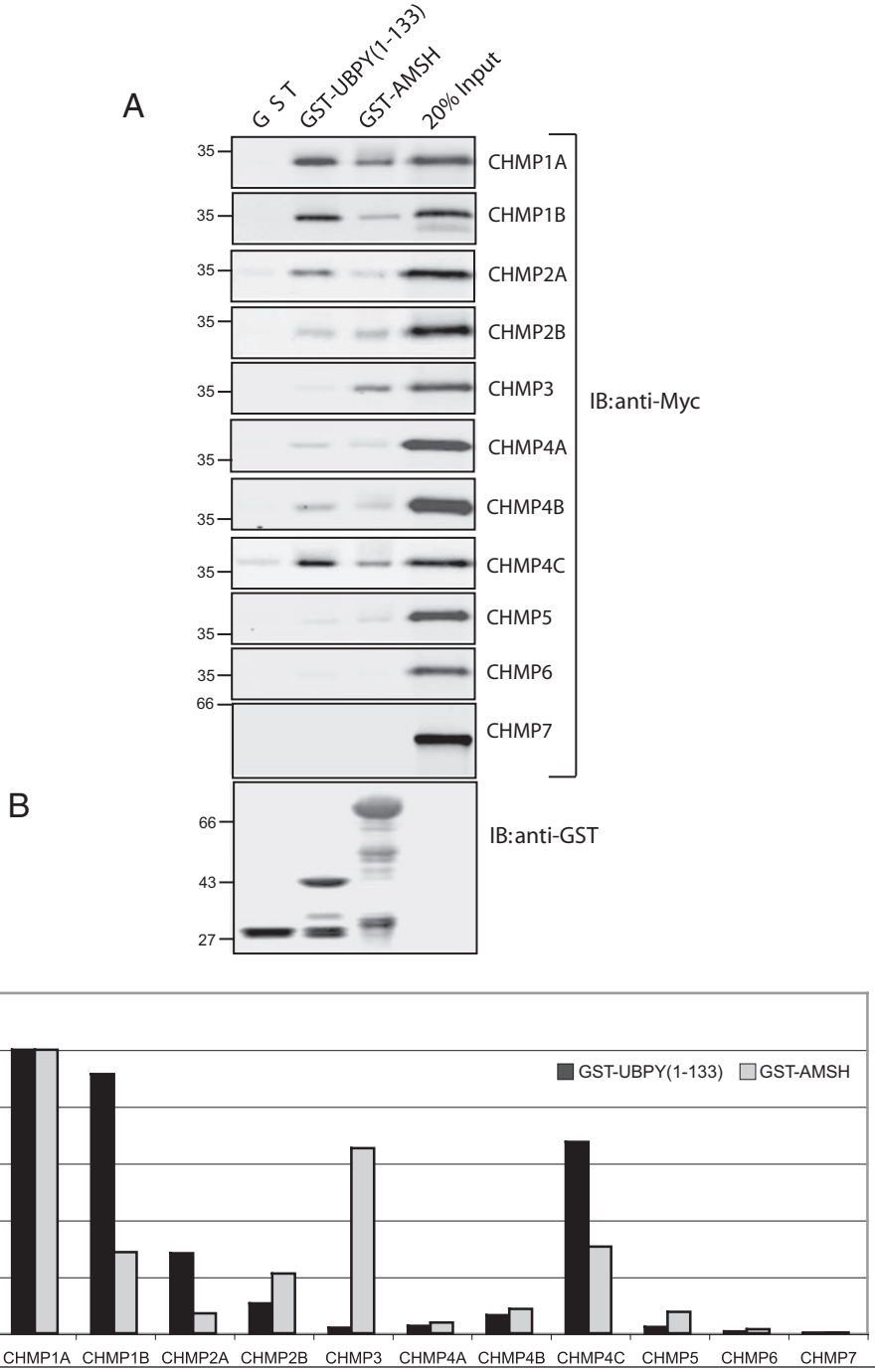
The MIT domain of UBPY constitutes a CHMP binding and endosomal localization signal required for efficient epidermal growth factor receptor degradation.

Paula E. Row, Han Liu, Sebastian Hayes, Rebecca Welchman, Panagoula Charalabous, Kay Hofmann, Michael J. Clague, Christopher M. Sanderson, and Sylvie Urbé

On page 30933, note that the original Fig. 4 suggested a specific interaction between CHMP7 and UBPY, which was based on experiments performed with a misidentified construct, resulting from an error in clone distribution between labs. We have since repeated the experiments with two new independently cloned CHMP7 constructs and did *not* find any interaction between CHMP7 and UBPY. We sincerely apologize for any confusion that this may have caused.



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