Degradation of Endocytosed Epidermal Growth Factor and Virally Ubiquitinated Major Histocompatibility Complex Class I Is Independent of Mammalian ESCRTII*

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Models for protein sorting at multivesicular bodies in the endocytic pathway of mammalian cells have relied largely on data obtained from yeast. These data suggest the essential role of four ESCRT complexes in multivesicular body protein sorting. However, the putative mammalian ESCRTII complex (hVps25p, hVps22p, and hVps36p) has no proven functional role in endosomal transport. We have characterized the human ESCRTII complex and investigated its function in endosomal trafficking. The human ESCRTII proteins interact with one another, with hVps20p (a component of ESCRTIII), and with their yeast homologues. Our interaction data from yeast two-hybrid studies along with experiments with purified proteins suggest an essential role for the N-terminal domain of hVps22p in the formation of a heterotetrameric ESCRTII complex. Although human ESCRTII is found in the cytoplasm and in the nucleus, it can be recruited to endosomes upon overexpression of dominant-negative hVps4Bp. Interestingly, we find that small interference RNA depletion of mammalian ESCRTII does not affect degradation of epidermal growth factor, a known cargo of the multivesicular body protein sorting pathway. We also show that depletion of the deubiquitinating enzymes AMSH (associated molecule with the SH3 domain of STAM (signal transducing adaptor molecule)) and UBPY (ubiquitin isopeptidase Y) have opposite effects on epidermal growth factor degradation, with UBPY depletion causing dramatic swelling of endosomes. Down-regulation of another cargo, the major histocompatibility complex class I in cells expressing the Kaposi sarcoma-associated herpesvirus protein K3, is unaffected in ESCRTII-depleted cells. Our data suggest that mammalian ESCRTII may be redundant, cargo-specific, or not required for protein sorting at the multivesicular body.

Multivesicular bodies (MVBs)‡ are formed following the inward budding of the outer endosomal membrane. Proteins included on the internal membranes are typically destined for the lysosome lumen, although in specialized cells containing secretory lysosomes the internal vesicles may be secreted from the cell. Proteins remaining on the outer MVB membrane are recycled to other compartments or transported to the limiting lysosome membrane. The sorting of proteins into the internal vesicles of MVBs is important for many essential cellular processes, including the down-regulation of growth factor receptors and antigen presentation. In addition, it has now become apparent that viruses such as the human immunodeficiency virus and Ebola virus exploit the cellular machinery required for MVB protein sorting for use in viral budding (for reviews, see Refs. 1, 2–4).

Over the last few years, significant progress has been made in identifying the signals and machinery involved in protein sorting into the luminal vesicles of MVBs. Much of this information has come from studies in the budding yeast Saccharomyces cerevisiae. The majority of yeast proteins studied so far use ubiquitin attached to their cytosolic domain as an MVB sorting signal, although there is also a ubiquitin-independent pathway (5–7). As well as identification of a sorting signal, yeast studies have provided the first clues to the molecular machinery of MVB protein sorting. Genetic screens in yeast identified 18 genes, the “class E” vacuolar protein sorting (VPS) genes, which encode proteins involved in MVB protein sorting (8–12, for review, see Ref. 13). Deletion of any class E VPS gene results in the secretion of the vacuolar hydrolase carboxypeptidase Y and the formation of a large, aberrant endosomal compartment in which proteins en route to the vacuole (the yeast lysosome) accumulate. 17 of the class E VPS genes encode soluble or peripherally membrane-associated proteins, 12 of which form 4 endosomal sorting complexes required for transport (ESCRT complexes 0, I, II, and III, see Table 1), and the remaining 5 are directly or indirectly associated with ESCRTII (5, 13–16). The sodium/proton exchanger Nhx1p is the only transmembrane class E VPS protein, and its role in MVB protein sorting remains unclear (17).

Current models for protein sorting at the MVB and the role of the ESCRT complexes in yeast suggest that ESCRT0 (Vps27p and Hse1p) binds to ubiquitinated cargo proteins and recruits ESCRTI (18–21). ESCRT1 binds both ESCRT0 and ubiquitinated cargo and recruits ESCRT complexes II and III (5, 14–16, 18, 20). Yeast ESCRTII is also capable of binding ubiquitin via an NZF motif in Vps36p, and thus the ubiquitinated cargo may be passed to ESCRTII en route to the luminal vesicles (22). Ubiquitin is removed from the cargo by the ESCRTIII-associated deubiquitinating enzyme Doa4p (23–25). Finally, ATP receptor: NZF, npl4 zinc finger; PBS, phosphate-buffered saline; TR, transferrin receptor; VPS, vacuolar protein sorting; GST, glutathione S-transferase; GFP, green fluorescence protein; EGFP, enhanced GFP; AMSH, associated molecule with the SH3 domain of STAM (signal transducing adaptor molecule); UBPY, ubiquitin isopeptidase Y; NlNTA, nickel-nitrotriacetic acid; siRNA, small interfering RNA.
hydrolysis by the ESCRTIII-associated ATPase Vps4p removes the ESCRT complexes from the membrane, and the cargo is sorted into the inward budding vesicle by a mechanism that remains to be clarified (for reviews see Refs. 3, 13, 26, and 27).

Mammalian homologues of all the soluble yeast class E Vps proteins have now been identified (see Table 1 for homologues of the ESCRT proteins) and have in some cases been shown to perform similar functions to their yeast counterparts. For example, components of ESCRTI (TSG101, hVps28p, and hVps37pA) are involved in mammalian MVB protein sorting (28–30). However, nothing is currently known about the role of the putative mammalian ESCRTII complex in endosomal sorting. The yeast ESCRTII complex is made up of two copies of Vps25p, and one each of Vps22p and Vps36p, and the structure of the core (i.e. without the N-terminal 395 amino acids of Vps36p) has been solved by x-ray crystallography at a resolution of 3.6 Å (31, 32). Yeast ESCRTII is a Y-shaped complex with a core consisting of eight winged-helix domains. By amino acid sequence alignments the mammalian homologues of the yeast ESCRTII proteins are the EAP-associated proteins, EAP20 (hVps25p), EAP30 (hVps22p), and EAP45 (hVps36p) (33, 34). ELL is an RNA polymerase II transcription factor that can increase the rate of transcription elongation by the polymerase from both promoter dependent and -independent templates (35). ELL can also negatively regulate polymerase activity in promoter-specific transcription (36). Yeast two-hybrid analysis suggests that hVps22p and hVps36p interact with TSG101 (an ESCRTI component) (37). In addition, two independent studies show yeast two-hybrid interactions between hVps22p, hVps25p, hVps36p, and hVps20p (also called CHMP6, an ESCRTII component) (37, 38). hVps25p and hVps20p have also been shown to associate in immunoprecipitation experiments, and this interaction was shown to be direct by pull-down assays using purified hVps25p and hVps20p (39). Evidence that human ESCRTII is found on endosomes comes from the finding that hVps25p colocalizes with overexpressed hVps20p on punctate endosomal structures, and hVps36p coexpressed with hVps25p in cells stimulated with epidermal growth factor (EGF) colocalizes with endosomal markers (39, 40).

We have investigated the role of the putative mammalian ESCRTII complex in MVB protein sorting in mammalian cells. hVps25p, hVps22p, and hVps36p interact with one another and with their yeast homologues. In addition, hVps25p can associate with endosomes in the presence of a dominant-negative mutant of hVps4Bp. Surprisingly, we find no evidence that mammalian ESCRTII plays a role in MVB protein sorting.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Enzymes used in DNA manipulations were purchased from New England Biolabs Ltd., except BsaI turbo DNA polymerase, which was obtained from Stratagene Europe. DNA oligonucleotides were synthesized by Sigma Genosys Ltd. All constructs were fully sequenced, either at the Department of Genetics, University of Cambridge, or the MRC genescience (Hinxton, England). hVPS25 was amplified from IMAGE clone 3614824 (MRC genescience), and the PCR product was inserted into pCRblunt II (Invitrogen). hVPS25 was then re-amplified to insert an EcoRI site before the start codon and a Sall site after the stop codon. These sites were used to subclone hVPS25 into pGEX 4T-1 (Amersham Biosciences) for expression of GST fusion proteins in bacteria or pGBDU-C3 and pGAD-C3 (41) for yeast two-hybrid analysis. For the expression of His6-tagged hVps25p in bacteria, full-length hVPS25 was amplified with an Ndel site before the start codon and an EcoRI site after the stop codon and subcloned using these sites into the plasmid pMW172H6. To create hVps25p with an N-terminal green fluorescent tag, hVPS25 was re-amplified to generate a BglII site before the start codon and an EcoRI site after the stop codon and subcloned using these sites into plasmids pEGFP-C1 (BD Biosciences). hVPS22 was amplified from IMAGE clone 3452153 and subcloned into pGBDU-C3 and pGAD-C3 as described for hVPS25. The IMAGE clone sequence of hVPS22 and our sequence were identical, but differed in 1 base from the published sequence of hVPS22 (33): our sequence has Gly in place of Cys at position 748. This change alters one amino acid: our sequence has Ala at 250 and the published sequence has Pro. Upon aligning our sequence with Vps22p sequences from several organisms, we found that Ala was present in the mouse protein at this position. We therefore decided not to mutate this residue in our sequence. To generate the N-terminal fragment of hVPS22p for two-hybrid analysis, bp 1–243 of hVPS22 were amplified by PCR with an EcoRI site before the start codon, and a stop codon engineered after base pair 243 followed by a Sall site. These restriction sites were used to subclone the fragment into plasmids pGBDU-C3 and pGAD-C3. To generate the C-terminal fragments of hVPS22p either bp 244–777 or 349–777 were amplified by PCR with an EcoRI site at the 5′-end and Sall site after the stop codon. These fragments were subcloned into pGBDU-C3 and pGAD-C3, hVPS36 was amplified from IMAGE clone 4814999 and subcloned into EcoRI sites and Sall sites into pGBDU-C3 and pGAD-C3 as described for hVPS25. As with hVPS22, the IMAGE clone sequence of hVPS36 and our sequence were identical but differed from the published sequence hVPS36 (33): our sequence has Cys in place of Ala at position 669 and Ala in place of Gly at 897. These changes alter one amino acid: our sequence has Ser at 223, and the published sequence has Arg. Upon aligning our sequence with Vps36p sequences from several organisms, we found that Ser was conserved at this position in both the mouse and fruit fly protein sequences. We therefore decided not to mutate this residue in our sequence. To generate the N-terminal fragment of hVPS36p for two-hybrid analysis, bp 1–375 of hVPS36 were amplified by PCR with an EcoRI site before the start codon and a stop codon engineered after base pair 375 followed by a Sall site. These restriction sites were used to subclone the fragment into plasmids pGBDU-C3 and pGAD-C3. To generate the C-terminal fragments of hVPS36p either bp 244–777 or 349–777 were amplified by PCR with an EcoRI site at the 5′-end and Sall site after the stop codon. These fragments were subcloned into pGBDU-C3 and pGAD-C3.

**Characterization and Function of Mammalian ESCRTII**

| ESCRT complex | Yeast protein | Human homologues |
|---------------|--------------|------------------|
| **ESCRT0**    | Vps27p       | Hxs (Hgs)        |
|               | Hse1p        | 1) STAM1         |
|               |              | 2) STAM2/HBP/EAST|
| **ESCRT1**    | Vps23p/Stp22p| TSG101           |
| Vps28p        | hVps28p      | 1) hVps37pAp     |
| Vps37p        | hVps37pBp    | 2) hVps37pCp     |
|               |              | 3) hVps37Bp      |
|               |              | 4) hVps37Dp      |
| **ESCRTII**   | Vps22p/Snf8p | hVps22p/EAP30    |
| Vps25p        | hVps25p/EAP20|                  |
| Vps36p        | hVps36p/EAP45|                  |
| **ESCRTIII**  | Vps2p/Dmdp/Ren1p | 1) hVps2Ap/CHMP2A |
| Vps24p        | hVps24p/CHMP3|                  |
| Vps20p        | hVps20p/CHMP6|                  |
| Vps32p/Snf7p  | 2) hVps32Ap/CHMP4A|
|               | 3) hVps32Bp/CHMP4B|
|               | 4) hVps32Cp/CHMP4C|

| Proteins of the yeast ESCRT complexes and their putative human homologues |
|--------------------------------------------------------------------------|
| For further information and references see Refs. 4, 13, 16, 28, and 83. |
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hVPS20 was amplified from IMAGE clone 3604039 and subcloned into pGBDU-C3 and pGAD-C3 as described for hVPS25. Yeast two-hybrid plasmids containing yeast VPS25, VPS22, VPS36, and VPS20 have been described previously (16).

For protein purification, full-length hVPS25 was subcloned into pMW172-GST such that the C terminus of hVPS25 was in-frame with a thrombin-cleavable GST tag. A fragment corresponding to residues 232-end of hVPS36 (the Cγ fragment), and a second fragment corresponding to residues 82-end (the Cε fragment) of hVPS22, each preceded by a ribosome-binding site were sequentially subcloned into pMW172-hVPS25-GST. hVps22p had an N-terminal His6 tag. This construct allowed co-expression of the three ESCRTII subunits from one plasmid under the control of a single T7 promoter. The dsRed-VPS4B E235Q construct was a kind gift from Prof. Wes Sundquist (Salt Lake City, UT) (37).

Antibodies—The rabbit polyclonal anti-calreticulin (PA3–900) antibody was from Affinity BioReagents, the mouse monoclonal anti-TSG101 (4A10) antibody was from Abcam, the mouse monoclonal anti-transferrin receptor antibody (H68.4) was from Zymed Laboratories Inc., the mouse monoclonal anti-LAMP1 antibody (H4A3) was from the Developmental Studies Hybridoma Bank, University of Iowa, the mouse monoclonal anti-clathrin (X22) was from Abcam, and the mouse monoclonal anti-EEA1 antibody was from BD Biosciences. The rabbit polyclonal antibodies against AMSH (antibody 850), Alix, and UBPy were generously provided by Sylvie Urbé (42), Harald Stenmark (43), and Pier Paolo Di Fiore (44), respectively. The anti-MHC class I mouse monoclonal W6/32 and rabbit anti-mannose 6-phosphate (MPR) antibodies have been described previously (45, 46).

To generate antibodies against hVps25p, a GST-hVps25p fusion protein was purified from bacteria as previously described (16) and used to immunize rabbits (Harlan Sera-Lab Ltd). Anti-hVps25p antibodies were affinity-purified using purified His6-hVps25p covalently bound to a CNBr-Sepharose column (Amersham Biosciences) and the method described in Mullock et al. (47).

Yeast Two-hybrid Analysis—Yeast were grown and transformed using standard genetic techniques and grown in rich medium (YEPD: 1% yeast extract, 2% peptone and 2% glucose) or synthetic dextrose minimal medium with appropriate amino acid supplements (SD: 0.67% yeast nitrogen base without amino acids and 2% glucose). Amino acid drop-out mixes were from Formedium Ltd. The yeast strains and two-hybrid plasmids have been described previously (41). Yeast strain p69-4a was transformed with GAL4-DNA binding domain plasmids (pGBDU) and plated onto SD without uracil. Yeast strain p69-4a was transformed with GAL4-DNA activation domain plasmids (pGAD) and plated onto SD without leucine. The α and a strains were mated on YEPD for 8 h at 30 °C, and then diploids were selected on SD without uracil and leucine. After replating onto SD without uracil and leucine, diploids were replica-plated onto SD without adenine. Interaction of the binding- and activation-domain fusion proteins was scored by growth of the yeast on SD without adenine over 8 days.

Protein Purification—pMW172 with hVPS25-GST, hVPS36 (Cγ), and His6-hVPS22 (Cε) were used to transform BL21(DE3)pLysS Escherichia coli (Novagen). Cells were grown at 37 °C until an A600 of 0.8 was reached, induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside, and grown overnight at 22 °C. Cells pellets were resuspended in 10 mM Hepes, pH 7.4, 200 mM NaCl with protease inhibitors and DNase, and lysed using a cell disruptor. Proteins were purified either on glutathione-Sepharose (Amersham Biosciences) or Ni-NTA (Qiagen) columns. Columns were washed extensively and eluted with 20 mM glutathione or 300 mM imidazole, respectively.

siRNA Knockdowns—The siRNA oligonucleotides were purchased from Dharmacon, Inc. The TSG101, Alix, and AMSH siRNA duplexes were as described (42, 49, 50). The hVps25p and UBPy depletions were performed using Dharmacon SMARTpools consisting of four oligonucleotides each. The target sequences of hVPS25 were CAGAACAC-TCCGTCCTTTA, GCCAAGGGCGGATCATC, GGGAACTCATACTATCGT, and GTCGATCCAGATTGATTA, and the target sequences of UBPy were GAAATGACATGACCAGTTT, TGAATAGCTGACGTGTTA, GGACAGGACGATAGATAGA, and AAATAAAGGTCACGAGAAA. The oligonucleotides all had 3′-UU overhangs and were synthesized using Dharmacon Option A4. siRNA oligonucleotides against hVPS22 and hVPS36 were Dharmacon SMARTpools with 3′-UU overhangs.

4 × 105 HeLa cells were seeded into 6-cm dishes and transfected the following day with siRNA oligonucleotides using the procedure described in Motley et al. (51). This is a double transfection procedure, with transfections on days 0 and 2. Following the first transfection, on day 1 the cells were transferred to 9-cm dishes. For TSG101 siRNA transfections, cells were transfected on days 1 and 2 to avoid the excessive cell death observed with the longer protocol (see Ref. 52).

Western Blotting—Cells were scraped into 5 ml of ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM NaH2PO4, 2 mM KH2PO4, pH 7.4) and pelleted at 1000 × g for 5 min at 4 °C. Cells resuspended in ice-cold lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 2 mM EDTA, 20 mM Tris, pH 8, with an EDTA-free protease inhibitor mixture (Roche Diagnostics Ltd.)). Following a 15-min incubation on ice, the lysates were spun for 20 min at 16,000 × g, and the postnuclear supernatant was removed to a fresh tube. Protein assays were carried out on the samples (using the BCA assay, Perbio Science UK Ltd.), and equal amounts of protein were loaded per lane on SDS-polyacrylamide gels. For ECL detection (Amer sham Biosciences), the proteins were transferred to nitrocellulose membranes, and the membranes probed using primary antibodies followed by goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich Co Ltd.).

Visualization of GFP and dsRed-tagged Proteins—For permeabilization before fixation: cells expressing GFP-hVPS25 and dsRed-VPS4B E235Q were washed once in cytosol extraction buffer (25 mM Hepes-KOH, pH 7.4, 25 mM KCl, 2.5 mM magnesium acetate, 5 mM EGTA, 150 mM l-glutamic acid) and then incubated in cytosol extraction buffer with 0.05% saponin for 30 s. Cells were fixed in 4% parafomaldehyde in PBS for 15 min at room temperature and permeabilized in PBS with 0.05% saponin. All antibody incubations and washes were carried out in PBS with 0.2% BSA and 0.05% saponin. Cells were stained with anti-MPR antibodies followed by an anti-rabbit secondary antibody conjugated to Alexa Fluor 647 (Invitrogen). Cells were visualized with a 63× Plan Apochromat objective (numerical aperture, 1.4) on a Zeiss Axiovert 200M inverted microscope with an LSM 510 confocal laser scanning attachment.

Immunofluorescence—24 h after the second siRNA transfection, HeLa cells were seeded onto glass coverslips and grown for a further 24 h. Cells were either fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 4 min (for EEA1, TIR, and clathrin antibody staining) or fixed and permeabilized by incubation with methanol for 5 min at −20 °C (for MPR and LAMP1 antibody staining). All antibody incubations were carried out in PBS with 0.2% BSA. The secondary antibody reagent was either goat anti-mouse or anti-rabbit Alexa Fluor 488 (Invitrogen). Coverslips were mounted on slides using Mowiol 4-88 (Merek Biosciences Ltd.) with 2.5% (w/v) 1,4-diazobicyclo[2,2,2]octane. Stained cells were viewed with a 63× Apochromat objective (numerical aperture, 1.4) on a Zeiss Axiosplan micro-
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RESULTS

Human ESCRTII Proteins Interact with One Another and with ESCRTIII—By multiple protein sequence alignment, the mammalian homologues of the yeast ESCRTII complex components are hVps25p/EAP20 and hVps36p/EAP43. A schematic representation of the yeast and human proteins is shown in Fig. 1. Yeast Vps36p is significantly larger than hVps36p (566 amino acids compared with 386) and includes two Npl4 zinc fingers (NZF motifs) in its N-terminal region that are not present in the human protein. One of these NZF motifs in yeast Vps36p has been shown to bind ubiquitin in vitro and to be required for the function of the protein in MVB protein sorting in vivo (22). More recently, mammalian Vps36p has been shown to bind ubiquitin via a novel “GLUE” domain, which is also thought to bind phosphoinositides (40). As shown in Fig. 1, yeast Vps36p also has a GLUE domain, but a region including the NZF motifs is inserted into it and replaces the ubiquitin-binding function.

Previously, yeast two-hybrid analysis has been used to study the interactions of the mammalian ESCRT protein homologues (37, 38). To examine the interactions of the human with the yeast ESCRTII and to deletion map the human ESCRTII protein interactions, we also employed the yeast two-hybrid system (41). Using this system, we have previously shown that the yeast ESCRTII proteins interact with one another, with Vps20p, a component of ESCRTIII, and with components of ESCRTI (16). Consistent with previous studies, we find that full-length fusions of the human ESCRTII homologues hVps25p, hVps22p, and hVps36p interact with one another and hVps25p interacts with hVps20p (also called CHMP6; see Fig. 2, a and b) (31, 37–39). However, there are some differences between our data and those of others. For example, we find that hVps36p interacts with hVps25p but detected no interaction of hVps25p with hVps22p or hVps36p. In contrast, Martin-Serrano et al. (38) found interactions of hVps25p, hVps22p, and hVps36p with hVps20p, although these interactions were weaker than that of hVps25p with hVps20p. These differences may be due to the different yeast two-hybrid systems used. In our yeast two-hybrid system...
we were unable to detect an interaction between hVps25p, hVps22p, or hVps36p and the transcription factor ELL.3

Yeast ESCRTII Proteins Bind Their Human Homologues—We found interactions between the yeast and mammalian ESCRTII proteins. As shown in Fig. 2b, hVps25p interacted with yeast Vps22p and Vps36p, hVps22p interacted with yeast Vps25p and Vps20p, and hVps36p interacted with yeast Vps25p and Vps20p. These data suggest that hVps25p, hVps22p, and hVps36p may indeed be the mammalian orthologues of the yeast ESCRTII components and may adopt similar conformations and/or have similar exposed binding domains to the yeast proteins. However, in separate experiments we found no evidence that the human proteins were able to complement the carboxypeptidase Y secretion phenotype of yeast strains lacking Vps22p, Vps25p, or Vps36p (vps22Δ, vps25Δ, or vps36Δ).3

Binding Domains of the ESCRTII Subunits—We tested the interactions with other ESCRTII proteins of different regions of hVps36p: the N-terminal domain (N), a C-terminal region containing the entire Vps36 domain (CA), or a fragment equivalent to the C-terminal portion

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present in the structure of the yeast ESCRTII complex (C8). As shown in Fig. 2, the Cα region of hVps36p was sufficient to bind both hVps25p and hVps22p. However, although the shorter Cα region of hVps36p interacted strongly with hVps25p, we were unable to detect an interaction between the Cα region and hVps22p. No interactions of the N-terminal region of hVps36p were found with any of the constructs tested. These data are summarized in Fig. 2c and are surprising, because in the structure of the yeast ESCRTII complex the C terminus of Vps36p (equivalent to Cα) shows a significant interaction with Vps22p (31, 32).

We also tested the interactions with other ESCRTII proteins of different regions of hVps22p: the N-terminal domain (N), a portion containing the entire EAP30 domain (C1) and a smaller portion containing most of the EAP30 domain (C2). The C-terminal region of hVps22p (either the C1 or C2 construct) was sufficient to bind hVps25p. However, as shown in Fig. 2, the full-length hVps22p was required to bind hVps36p. These data are summarized in Fig. 2c. We detected no interaction of the N terminus of hVps22p with any of the constructs tested. The recent structures of the yeast ESCRTII complex (31, 32) show that the N-terminal region of Vps22p protrudes from the core of the complex and makes little or no contact with Vps36p. However, although the N-terminal domain of Vps22p may not be required for binding hVps36p directly, it may maintain the C-terminal portion of hVps22p in the correct conformation to do so.

To investigate the interactions of the human ESCRTII complex in more detail, we used proteins purified from bacteria. We co-expressed hVps25p-GST, hVps36p (Cα), and His6-hVps22p (C1) from a single plasmid. All three proteins were eluted from a glutathione-Sepharose column after thrombin cleavage (this cleaves the GST tag from hVps25p and releases bound protein complexes) and passed over a Ni-NTA column. Lanes 1 and 2 show the proteins that fail to bind the Ni-NTA column, and lanes 3 and 4 show the material bound and eluted from this column. b, hVps25p-GST, hVps36p (Cα), and His6-hVps22p (C1) were co-expressed in E. coli. Following lysis, the soluble material was passed over an Ni-NTA column. Lane 1 shows the material bound to the column and subsequently eluted with 300 mM imidazole (eluate). Material that failed to bind the Ni-NTA column was subsequently passed over a glutathione Sepharose column and the material eluted from this is shown in lane 2.

We also co-expressed hVps25p-GST, hVps36p (Cα), and His6-tagged hVps22p (C1), as above, but bound the soluble material first to a Ni-NTA column. As shown in Fig. 3b, lane 1, we were able to elute His6-hVps22p (C1) and hVps25p-GST from this column. The material that failed to bind the Ni-NTA column was subsequently passed over a glutathione-Sepharose column, and from this we were able to elute hVps25p-GST and hVps36p (Cα) with a very small amount of His6-hVps22p (C1) (Fig. 3b, lane 2). Again our results suggest that there is very little interaction of the hVps25p-hVps22p (C1) complex with the hVps25p-hVps36p (Cα) complex. To confirm this, we tried a GST-pull-down experiment with the purified hVps25p-GST-hVps36p (Cα) complex mixed with the purified His6-hVps22p (C1) subunit. We were able to detect only a very weak interaction between the two purified complexes.3 Thus, our yeast two-hybrid results (summarized in Fig. 2c) are supported by the data from studies with purified proteins and suggest that the N-terminal region of Vps22p is required for its stable interaction with Vps36p. Unfortunately, we were unable to confirm this directly, because attempts to purify full-length hVps22p were unsuccessful as the protein formed insoluble aggregates.3

**Full-length hVps25p, hVps22p, and hVps36p Form a Complex in Vivo**—Evidence that full-length hVps25p, hVps22p, and hVps36p form a complex in vivo comes from protein depletion experiments using small interfering RNAs (siRNAs). As shown in Fig. 4, depletion of hVps25p using siRNA results in significant loss of the protein as determined by Western blotting using antibodies to hVps25p. Transfection of siRNA against hVps22 or hVps36 also resulted in depletion of hVps25p but no depletion of a control protein, calreticulin. We have been unsuccessful to date in attempts to produce antibodies against hVps22p or hVps36p and so could not assess depletion of these proteins. However, these data suggest that loss of one human ESCRTII protein destabilizes the remaining ESCRTII proteins and is consistent with them forming a complex in vivo. Similar results were obtained in yeast: depletion of any one of the three ESCRTII-encoding genes destabilized the remaining ESCRTII proteins (14). It should also be noted that while depletion of one human ESCRTII protein destabilizes the complex, it does not affect the cellular levels of other ESCRT components such as TSG101 (ESCRTI) or Alix (an ESCRTIII-associated protein) (see Fig. 6d).

**Human ESCRTII Can Be Recruited to Endosomes**—The data presented above suggested that hVps25p, hVps22p, and hVps36p may be orthologues of the yeast ESCRTII components. If these proteins form human ESCRTII, we would expect them to associate with endosomes. Expression of an N-terminally tagged GFP-hVps25p in HeLa cells by transient transfection produced predominantly cytosolic and nuclear
staining. However, expression of dominant-negative versions of Vps4p in yeast or mammalian cells has been shown to increase the association of ESCRT components with endosomal membranes (5, 14, 15, 26, 37, 54, 55). We co-transfected HeLa cells with GFP-hVps25p and dsRed-hVps48p E235Q. This transfection produced enlarged, punctate structures in the cells as seen previously following overexpression of dominant-negative hVps4 constructs (55, 56). If the cytosolic pool of GFP-hVps25p was removed before fixation, the GFP-hVps25p was seen colocalized with the dsRed-hVps48p E235Q on the swollen structures, as well as in the nucleus (Fig. 5). To confirm that the swollen, punctate structures were endosomes, we costained them with antibodies against endosomal markers. Both the predominantly late endosomal protein MPR (Fig. 5) and the early endosomal protein EEA1 (53) partially colocalized with dsRed-hVps48p E235Q and GFP-hVps25p. These data suggest that hVps25p (and presumably the ESCRTII complex) can be recruited to endosomes in an hVps4p-dependent manner. Taken together, our data show that the human ESCRTII proteins associate with one another and are likely to form a complex in vivo that can be recruited to endosomes.

**EGF Degradation Does Not Require Mammalian ESCRTII**—To study the functional role of ESCRTII in mammalian cells, we studied degradation of EGF. EGF binds its receptor EGFR at the cell surface, the EGFR is mono-ubiquitinated at multiple sites and the EGFR-EGFR complex is internalized. Upon reaching MVBs, the EGFR complex is sorted into the inner vesicles and is degraded following fusion of the late endosome with the lysosome. The sorting of EGFR-EGFR at the MVb is dependent on ESCRTI and hVps4p, because cells depleted of TSG101 or hVps37Ap, cells microinjected with anti-hVps28p antibodies, or cells overexpressing dominant-negative versions of hVps4p are unable to degrade EGF or EGFR (28–30, 55). More recently, it has been shown that depletion of AMSH (a deubiquitinating enzyme that interacts with signal transducing adaptor molecule (STAM), a component of the ESCRT0) by siRNA transfection stimulates EGFR degradation (42). We measured iodinated EGF degradation in siRNA-transfected cells. As expected, depletion of TSG101 significantly inhibited EGF degradation, and depletion of AMSH increased EGF degradation (Fig. 6a). However, depletion of hVps25p had no effect on EGF degradation, and the kinetics of degradation was the same as seen in the mock transfected cells (Fig. 6a). The efficiencies of the depletions are shown by Western blotting in Fig. 6d. Transfection of siRNA directed against hVps25p also failed to affect EGF degradation. We found no significant differences in the initial rates of EGF internalization from the cell surface in any of the siRNA-treated cells tested.

One simple explanation of our results is that there is redundancy in the mammalian MVB sorting system not seen in yeast. Therefore while ESCRTII may be essential for MVB sorting in yeast, in mammalian cells this essential function may be bypassed. Interestingly, analysis of mammalian ESCRT protein interactions has identified a link between ESCRTs I and III that we were unable to detect in the yeast system (16, 37, 38, 57). Alix, the mammalian homologue of Bro1p/Vps31p was found to interact with both TSG101 (ESCRTI) and hVps32A, -B, and -C (CHMP4A, -B, and -C; ESCRTIII) (37, 38, 57–59). We reasoned therefore that Alix may provide an alternative link between ESCRTs I and III that may bypass the requirement for ESCRTII in some situations. Therefore, depletion of both Alix and ESCRTII may be required to inhibit MVB sorting and thus degradation of EGF. To investigate this hypothesis, we transfected cells with siRNA directed against Alix and against both Alix and hVps25p. As shown in Fig. 6c, co-transfection of siRNAs against both hVps25p and Alix depleted levels of both proteins. However, depletion of hVps25p and Alix did not inhibit EGF degradation (Fig. 6b). In fact, depletion of Alix slightly increased EGF degradation, whereas depletion of Alix and hVps25p together had little effect on EGF degradation. Knock down of Alix in the double depletion situation (Alix and hVps25p) was never as efficient as a single Alix depletion (Fig. 6d), which may explain the smaller effect on EGF degradation observed. There are two alternative explanations for the effect of Alix depletion on EGF degradation: Firstly, we may be increasing the number of intraluminal vesicles formed and thus increasing the amount of EGF sorted for degradation. In this case, Alix would be a negative regulator of MVB formation, and this idea is consistent with the idea that Alix regulates the inward budding of MVB membranes in vitro via its association with the lipid lysobisphosphatidic acid (50). Secondly, depletion of Alix may allow a greater concentration of EGF in the inward-budding vesicles, again resulting in increased degradation. In this case, Alix would be a negative regulator of ESCRT complex function.

The increase in EGF degradation we see upon depletion of Alix is consistent with a previous report that suggested that knockdown of Alix promotes the degradation of EGF (60). However, our results differ from recently published results suggesting that Alix knockdown has no effect on EGFR degradation (43). These differences may be due to differences in the experiments: we have quantified EGF degradation using iodinated EGF at either 2.5 or 20 ng/ml (see Fig. 6, b and c), whereas Cabezas et al.
Alix, Alix and hVPS25 together, or UBPY. 
represent the UBPY siRNA transfected cells.
respectively, for mock, Alix, hVPS25 plus Alix, or UBPY. Error bars represent the ±S.E. over 9, 6, 3, or 7 experiments, respectively, for mock, Alix, hVPS25 plus Alix, or UBPY siRNA transfected cells.
degradation of 125I-labeled EGF (20 ng/ml) was measured as described under "Experimental Procedures." Cells were transfected with either water (Mock) or siRNA against TSG101, hVPS25, or AMSH. 
Mock, TSG101, hVPS25, or AMSH siRNA-transfected cells. b, same as in a except cells were transfected with either water (Mock) or siRNA against Alix, Alix and hVPS25 together, or UBPY. Error bars represent the ±S.E. over 9, 6, 3, or 7 experiments, respectively, for mock, Alix, hVPS25 plus Alix, or UBPY siRNA transfected cells. c, degradation of 125I-labeled EGF (20 ng/ml) was measured as described under "Experimental Procedures." Cells were transfected with water (Mock) or siRNA against TSG101, hVPS25, or Alix. Error bars represent the deviation from the mean over two separate experiments. d, Western blots showing the efficiency of protein depletions. Equal amounts of postnuclear supernatants from siRNA-transfected cells were run on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Western blots were developed using anti-hVps25p, anti-Alix, anti-AMSH, anti-TSG101, anti-UBPY, or anti-calreticulin antibodies. The levels of calreticulin show the equal protein loading of the samples (loading control). *, a nonspecific band.

As well as AMSH, we studied the effect of depleting another deubiquitinating enzyme, UBPY, on EGF degradation. Like AMSH, UBPY is thought to interact with ESCRT0 via STAM. We found that, unlike AMSH depletion (which causes an increase in EGF degradation, see Fig. 6a and Ref. 42), depletion of UBPY results in significant inhibition of EGF degradation (Fig. 6b). Our findings differ from those reported recently by Mizuno et al. (61), which suggest that depletion of UBPY slightly increases EGF degradation. The reason for this discrepancy is unclear. However, we feel that the reproducible, sensitive, and quantitative nature of our assay, together with the effects of UBPY depletion on endosomal morphology (see below and Fig. 9) provide compelling evidence that depletion of UBPY causes an inhibition of EGF degradation. We speculate that, although AMSH is thought to act directly on EGFR (42), UBPY may act on the MVB sorting machinery. One interesting possibility is that UBPY may act on the TSG101 component of ESCRTII, which is ubiquitinated (and inactivated) by the ubiquitin ligase TAL (62). De-ubiquitination of TSG101 by a protein such as UBPY may explain why depletion of either UBPY or TSG101 has similar effects on EGF degradation.

All our initial EGF degradation experiments were performed with a 2.5 ng/ml concentration of 125I-EGF. A recent report suggests that, at this low concentration of EGF, the EGF-EGFR complex is internalized via a clathrin-mediated route (63). However, upon stimulation with a higher concentration of EGF (20 ng/ml), the EGF-EGFR complex is internalized predominantly via a clathrin-independent caveolae pathway (63). To determine whether the route of EGF-EGFR internalization affects the downstream requirement for ESCRTII in MVB protein sorting, we performed EGF degradation experiments with 20 ng/ml concentrations of EGF. As shown in Fig. 6c, we again found no effect of hVps25p depletion on EGF degradation, even with this higher concentration of EGF. Transfection of the cells with siRNA against Alix slightly increased EGF degradation, as seen with the lower concentration of EGF. TSG101 depletion inhibited EGF degradation (Fig. 6c), demonstrating that EGF degradation is TSG101-dependent and ESCRTII-independent irrespective of its method of internalization.

To show that EGF was still targeted to lysosomes for degradation in the hVps25p-depleted cells, we internalized fluorescently labeled EGF. As shown in Fig. 7, EGF-Alexa Fluor 488 internalized for 1 h at 20 °C and 45 min at 37 °C in the presence of the protease inhibitor leupeptin in mock-transfected cells showed partial colocalization with the lysosomal marker lysosome-associated membrane protein 1 (LAMP1). Similar colocalization was seen following EGF-Alexa Fluor 488 uptake in the hVps25p-depleted cells. Our results suggest that EGF is taken up and degraded in lysosomes in hVps25p-depleted cells.

Depletion of Mammalian ESCRTII Does Not Affect KK3-mediated Down-regulation of MHC Class I Molecules—As described above, depletion of ESCRTII did not inhibit EGF degradation. Therefore, it is possible that ESCRTII may be cargo-specific, and we decided to study another cargo of the MVB sorting pathway. The Kaposi's sarcoma-associated herpesvirus (KSHV) protein K3 (KK3) is a ubiquitin ligase (52). This protein is involved in the evasion of the immune system by virally infected cells. Expressed alone in HeLa cells this protein polyubiquiti- nates and causes down-regulation from the cell surface of MHC I molecules (52, 64). The down-regulation of MHC I in KK3-expressing cells is dependent on TSG101 and hVps4p (37, 52). We assayed the down-regulation of MHC I in HeLa cells or in HeLa cells stably expressing KK3 (HeLa-KK3). >99% of HeLa cells had MHC I on the cell surface, as determined by cytofluorometric analysis (Fig. 8a). This was unchanged
in HeLa cells transfected with siRNA against hVps25p or TSG101 and consistent with the cell-surface staining of MHC I seen in these cells by immunofluorescence (Fig. 8b). In HeLa-KK3 cells, few cells had cell-surface MHC I (Fig. 8, a and b, HeLa-KK3 mock). In contrast, in HeLa-KK3 cells transfected with siRNA against TSG101, MHC I now appeared on the cell surface, as seen by a shift in the trace in Fig. 8a to the right and cell-surface fluorescence in Fig. 8b. However, HeLa-KK3 cells transfected with siRNA against hVPS25 showed identical results to the mock transfected cells (Fig. 8, a and b) suggesting that depletion of ESCRTII did not affect K3-mediated MHC I down-regulation. The transfection of HeLa-KK3 cells with siRNA against hVPS25 or hVPS36 produced similar results to those obtained with siRNA against hVPS25.

Depletion of Mammalian ESCRTII Does Not Affect Gross Endosomal Morphology—Data from both the yeast and mammalian systems have previously suggested that deletion or expression of dominant negative constructs of ESCRT machinery components causes dramatic changes to endosomal morphology. For example, deletion of any one of the yeast genes encoding the ESCRTII proteins causes the enlarged endosomal “class E” compartment characteristic of the yeast vps class E mutants. In addition, expression of dominant-negative versions of hVps4p or depletion of TSG101 in mammalian cells causes enlarged endosomal structures to form (Fig. 5 and Refs. 29 and 56). Thus if ESCRTII plays a role in MVB protein sorting in mammalian cells we might expect significant changes in endosomal morphology on depletion of the complex. We depleted hVps25p from HeLa cells using siRNA and looked at various endosomal markers by immunofluorescence. As a control, we also depleted TSG101 using siRNA. In the TSG101-depleted cells our markers for early recycling endosomes (transferrin receptor, TfR), early sorting endosomes (early endosomal autoantigen 1, EEA1), late endosomes (MPR), and late endosomes/lysosomes (LAMP1) all showed enlarged endosomal structures compared with mock transfected cells (Fig. 9). TSG101 depletion did not affect clathrin distribution. Depletion of UBPy, which, like TSG101 depletion, significantly inhibited EGF degradation in our assay (Fig. 6), also caused dramatic swelling of early and late endosomal structures (Fig. 9). No effect of UBPy depletion was seen on clathrin distribution. Unlike TSG101 or UBPy depletion, hVps25p depletion resulted in no differences in the staining patterns of TfR, clathrin, MPR, or LAMP1 from the mock transfected cells (Fig. 9). The only significant difference we observed was in the level of EEA1 staining in hVps25p-depleted cells. Further experiments proved that this reduction in staining was a 50% reduction in total cellular level of the EEA1 protein. However, although this effect was seen with two non-overlapping siRNA oligonucleotide duplexes against hVps25p, it was not seen with a further two siRNA duplexes. Because all four individual oligonucleotide duplexes efficiently deplete hVps25p, we are unable to conclude that this reduction in EEA1 levels is specific to hVps25p depletion. EEA1 staining in cells depleted for hVps25p using an siRNA duplex that did not reduce EEA1 levels was similar to that seen in untreated cells.

We conclude that there are no gross morphological changes on endosomal compartments in cells lacking mammalian ESCRTII.

DISCUSSION

The protein machinery responsible for the sorting of proteins at the MVB in yeast appears to be conserved in mammalian cells. Current
models for mammalian MVB sorting mirror those proposed in the yeast system (1–3). However, the putative mammalian orthologues of ESCRTII were identified as part of a complex with the RNA polymerase II transcription factor ELL, and the role of these proteins in MVB sorting was unclear (33, 34). The main purpose of this study was to investigate the functional role of the putative human ESCRTII complex in mammalian MVB sorting. We found evidence that hVps25p, hVps22p, and hVps36p make up the human ESCRTII complex, which can be recruited to endosomes. However, depletion of human ESCRTII in siRNA experiments did not affect EGF degradation in HeLa cells, MHC I down-regulation in cells expressing the KSHV protein K3, or gross endosomal morphology.

**Mammalian ESCRTII Complex: hVps25p, hVps22p, and hVps36p**—We have shown that the human homologues of the yeast ESCRTII complex proteins, hVps25p, hVps22p, and hVps36p are likely to make up a mammalian ESCRTII complex. Consistent with the findings of others, we have found that hVps25p, hVps22p, and hVps36p bind to one another and to hVps20p (37–39). In addition, we have also demonstrated interactions of the human proteins with their yeast homologues, and with yeast Vps20p. These data, together with the recruitment of human ESCRTII to endosomes in cells expressing a dominant-negative hVps4p construct, suggest that hVps25p, hVps22p, and hVps36p are the orthologues of the yeast ESCRTII proteins.

From the yeast ESCRTII structure, the interaction of Vps20p with the complex is via Vps25p (31). Our data are consistent with a similar interaction in human ESCRTII, because we find that hVps25p binds hVps20p while hVps22p and hVps36p do not. The yeast ESCRTII structures were lacking the N-terminal 395 amino acids of Vps36p, suggesting that the C-terminal domain is sufficient to interact with Vps25p and Vps22p (31, 32). Consistent with the yeast data, we have shown that the C-terminal domain of hVps36p is sufficient to bind both hVps22p and hVps25p. However, our analysis with the human proteins suggests that the C-terminal region of hVps36p required for binding to hVps22p is larger than that predicted by the yeast structure. As summarized in Fig. 2c, we find that a construct containing amino acids 126–386 (C terminus) of hVps36p is capable of binding both hVps25p and hVps22p, but a construct corresponding to the shorter part of yeast Vps36p in the structure (hVps36p amino acids 232–386, C terminus) only binds Vps25p. This implies that amino acids 126–231 of hVps36p may be required either to mediate or strengthen the interaction between hVps36p and hVps22p in the mammalian complex. Also unexpected from the yeast ESCRTII structure was the finding that the N-terminal region of hVps25p was required for the binding of hVps36p. (It should also be noted that yeast Vps36p is significantly larger than human Vps36p.) Our yeast two-hybrid and purified protein interaction data suggest that human ESCRTII may have a modified structure compared with yeast ESCRTII, which may account for its unexpected redundancy or cargo-specificity in MVB protein sorting (see below).

**Functional Consequences of ESCRTII Depletion**—In our experiments, depletion of human ESCRTII had no effect on EGF degradation. This was surprising, because previous studies have shown that ESCRTI and hVps4p are essential for this process (28–30, 55). Yeast studies also imply that ESCRTII is essential for MVB protein sorting, and current models imply that yeast ESCRTII is required in stoichiometric amounts (14, 16). In addition, recent studies show that mutation or depletion of Drosophila Vps25p results in enlarged endosomal structures where the
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receptors Notch and Dpp accumulate (65, 66). A similar phenotype was seen in patches of Drosophila tissue expressing mutant forms of the erupted gene product, the orthologue of mammalian TSG101 (67). However, although vps25 or erupted mutant Drosophila cells show ectopic Notch activation, cells expressing mutant forms of Hrs did not. Cells from hrs mutant Drosophila failed to degrade EGF leading to enhanced signaling (68). In contrast, Drosophila cells expressing mutant vps28 showed little or no effect on the trafficking of the Sevenless receptor or its ligand Boss, on EGFR trafficking, or on the trafficking of Delta (a ligand of Notch) (69). The different functional effects of mutating ESCRT subunits in Drosophila therefore make it difficult to fit the fly data with the current yeast model of MVB sorting. Nevertheless, it is significant that Drosophila ESCRTII appears important in endosomal sorting. Also of note is the finding from siRNA analysis in the Caenorhabditis elegans system: depletion of the VPS class E homologues resulted in a variety of different phenotypes (70). Depletion of the Vps36p homologue was larval-lethal, whereas depletion of the Vps22p or Vps25p homologues resulted in no observable phenotype.

By Western blotting, we were typically able to knock down expression of hVps25p by >90%. We were also able to show that transfection of siRNA oligonucleotides against hVps22p or hVps36p significantly reduced the level of hVps25p (Fig. 4). Thus knock down of one ESCRTII component destabilized the complex, and our cells had very little ESCRTII. However, the <10% ESCRTII remaining after siRNA transfection using our double transfection procedure is likely to be the result of ~90% transfection efficiency of the siRNA. Thus we produce a mixed population of cells, ~10% of which have relatively normal levels of ESCRTII and 90% of which have almost no ESCRTII. There are several possible explanations for our lack of MVB sorting phenotype on depleting mammalian ESCRTII: (i) Human ESCRTII may be cargo-specific. To address this issue, we studied the down-regulation of MHC I in cells expressing the KSHV ubiquitin ligase K3. K3-mediated MHC I down-regulation is dependent on TSG101 (ESCRTII) and hVps4Bp (37, 52). As with EGF degradation, we saw no effect of ESCRTII depletion on MHC I trafficking in KK3-expressing cells. It should be noted that the EGFR is mono-ubiquitinated at multiple sites, whereas MHC I is poly-ubiquitinated by the KK3 E3 ubiquitin ligase (52, 71). Thus for two differently ubiquitinated cargo proteins we saw no requirement for ESCRTII in MVB protein sorting. It should also be noted that overexpression of ESCRTII proteins had no effect on HIV budding, whereas overexpression of other ESCRT proteins had a dominant-negative effect (38). However, our results do not rule out ESCRTII being specific for a cargo we have not yet tested. Interestingly, a recent report has suggested the presence of more than one type of MVB, with only a subpopulation of MVBs capable of sorting EGF (72). Therefore, ESCRTII may be required for protein sorting at the MVBs that do not sort EGF/EGFR (or virally ubiquitinated MHC I) and the other ESCRT complexes in a similar way to its yeast counterpart. As mentioned above, the mammalian MVB sorting is likely to be more complex than the yeast system. This is necessarily so, because cells of multicellular organisms can be specialized for a huge number of different functions. Because we find the interactions of the human ESCRTII proteins with each other and with ESCRTIII, the human ESCRTII proteins bind the yeast proteins, and human ESCRTII can be recruited to endosomes, we suggest that human ESCRTII is likely to interact with the other ESCRT complexes in a similar way to its yeast homologue. However, instead of the essential role played by yeast ESCRTII in linking ESCRTs I and III and binding ubiquitinated cargo proteins, mammalian ESCRTII may play a regulatory role, perhaps in coordinating MVB sorting with other endosomal sorting events. (ii) Our ESCRTII depletions by siRNA techniques may not be sufficient to knock out ESCRTII function. As discussed above, we were typically able to knock down expression of mammalian ESCRTII almost completely in ~90% of cells in the population. Similar levels of protein depletion were achieved with siRNAs against TSG101, UBPy, and AMSH, and yet these depletions were sufficient to produce either significant inhibition or stimulation of EGF degradation. Nevertheless, we cannot rule out the possibility that ESCRTII is required for MVB sorting at extremely low (non-stoichiometric) or catalytic levels.

ESCRTII or EAP Complex?—Our data and those of others suggest that hVps25p, hVps22p, and hVps36p make up the mammalian ESCRTII complex. However, do the same proteins (in a complex with the transcription factor ELL) have another role in the nucleus as part of the EAP complex? Several other mammalian class E Vps protein homologues are thought to play additional roles in the cell nucleus. Mammalian Vps46p (CHMP1) plays a role in chromatin remodeling: TSG101 binds the glucocorticoid receptor and inhibits receptor transcriptional activation; and mammalian Vps24p (CHMP3) was localized to the nucleus in addition to endosomes (73–75). In addition, other components of the endocytic protein sorting machinery have been shown to link endocytic sorting to transcriptional regulation (reviewed in Ref. 76). For example, Eps15, epsin, and β-arrestin 2 (components of clathrin-coated pits) undergo nucleocytoplasmic shuttling (77–80). In addition, the Rab5 effectors APPL1 and APPL2 dissociate from early endosomal membranes and translocate to the nucleus following prolonged treatment of cells with EGF (81). In the nucleus, the APPL proteins bind the nucleosome remodeling and histone deacetylase complex NuRD/MeCP1. Therefore, the ESCRT proteins may provide a further link between transcriptional regulation and endocytic protein sorting.

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