

(20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM DTT, 0.2 mg/ml bovine serum albumin [BSA], and 0.005% P20) and captured on the antibody surfaces at densities of  $\sim 0.5$  kRU. Pure GST alone was also captured as a blank reference. Ubiquitin was purified (84), diluted in running buffer, and injected in duplicate or triplicate over the four flow cells at concentrations of 0 to 1,250  $\mu$ M. Responses were collected at a rate of 2 Hz during the 30-s association and dissociation phases. Binding isotherms from the equilibrium phases were fitted to simple 1:1 binding models to determine the dissociation constant and statistical fitting errors (see Fig. 4) (53). To obtain more accurate estimates for the dissociation constant and standard errors, the interaction between GST-EAP45<sub>1-139</sub> and ubiquitin was measured in six independent experiments, yielding values of  $410 \pm 90$   $\mu$ M. Biosensor binding experiments for EAP20 and GST-CHMP6 were performed as described for the EAP45-ubiquitin interaction, except that a BIA-CORE 3000 instrument was used and binding was examined in a buffer containing 50 mM Tris, pH 8.0, 300 mM NaCl, 10% glycerol, 1 mM DTT, 0.2 mg/ml BSA, and 0.005% P20. Anti-GST antibody was immobilized over all four flow cells ( $\sim 13,000$  RU/cell), and CHMP6, CHMP6<sub>1-100</sub>, and CHMP6<sub>101-200</sub> were captured to densities of  $\sim 2,700$  RU. Purified EAP20 was diluted in running buffer and injected in triplicate at 0.03 to 560  $\mu$ M concentrations.

**Immunofluorescence.** COS-7 cells grown on collagen-coated glass coverslips in 12-well plates were transfected with 0.5  $\mu$ g of pEGFP-VPS4A-KQ DNA and, in some experiments, 0.5  $\mu$ g pcDNA3.1-EAP20-myc DNA or pcDNA3.1-EAP30-myc DNA, using 2  $\mu$ l Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The cells were fixed 24 h posttransfection in 4% paraformaldehyde-phosphate-buffered saline (PBS), blocked in 10% fetal calf serum (FCS) in PBS containing 0.1% Triton X-100, and incubated at room temperature with murine monoclonal anti-Myc (9E10; Covance Inc.) to detect transfected Myc-tagged ESCRT-II proteins or with anti-EAP20 UT461 (1:1,000) or anti-CHMP6 UT463 (1:1,000) antibody to detect endogenous ESCRT proteins. Secondary antibodies were anti-mouse Alexa 594- or anti-rabbit Alexa 594-conjugated antibodies (Molecular Probes; 1:1,000). Images of 0.5- $\mu$ m-thick Z sections were collected as single wavelengths on an Olympus FV300 confocal fluorescence microscope, using Fluoview 2.0.39 software.

**Viral infectivity assays.** EAP20 and CHMP6 were silenced by transfecting 5  $\mu$ l of 20  $\mu$ M annealed siRNAs, together with 1  $\mu$ g of pSL1180 (carrier DNA) and 9  $\mu$ l Lipofectamine 2000, into 293T cells in six-well plates (22). The sense strands of the siRNAs were as follows (lowercase letters denote RNA, and uppercase letters denote DNA nucleotides [nt]): EAP20 (start site, nt 218), cgaucgagauug uuuagaTT; CHMP6-1 (start site, nt 257), ccaugguucagauuagaTT; and CHMP6-2 (start site, nt 284), agaucgaaugaagauuagaTT. TSG101 and INV (TSG101) siRNA constructs have been described previously (22). The levels of protein depletion were estimated by quantifying the intensities of bands in Western blots using NIH ImageJ software.

Viral-particle production was initiated during a second siRNA transfection ( $t = 24$  h) by replacing the 1- $\mu$ g carrier DNA either with an HIV-1 vector system or with an infectious R9 HIV-1 NL4-3 plasmid. Virus was harvested 24 h after the second transfection, and the titers were measured using single-cycle MAGIC assays (70). The HIV-1 vector system contained 0.38  $\mu$ g pCMV $\Delta$ R8.2 (54), 0.12  $\mu$ g pRSV-Rev (18), 0.12  $\mu$ g pMD.G (57), and 0.38  $\mu$ g pWPTS-nlsLacZ (all generous gifts from D. Trono, Swiss Federal Institute of Technology). HIV-packaged *lacZ* vectors were harvested 48 h later and titered on HeLaM cells as described previously (85).

**Western blotting.** Viral supernatants and cellular proteins from transfected 293T cells were prepared using standard procedures (82). For Western blots, virions or cytoplasmic proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose (TSG101) or polyvinylidene difluoride (all others) membranes in Tris-glycine-10% MetOH buffer, blocked in 1% (TSG101) or 5% milk, and incubated with antibodies diluted in Tris-buffered saline-0.1% Tween 20 buffer (anti-TSG101), or in 1% BSA or 5% nonfat milk in Tris-buffered saline-0.1% Tween 20. Proteins were detected by enhanced chemiluminescence (Pierce) or by imaging Alexa 680-nm (Molecular Probes; 1:10,000) or IRDye 800-nm (Rockland; 1:20,000) secondary antibody on an Odyssey scanner (Li-Cor Inc.). The primary antibodies used were rabbit anti-HIV CA (UT415 made against purified NL4-3 CA protein and affinity-purified; 1:15,000); rabbit anti-HIV MA from Didier Trono, Swiss Institute of Technology (1:25,000); rabbit anti-EAP20 UT461 (1:1,000); rabbit anti-CHMP6 UT463 or UT464 (1:1,000); and mouse anti-TSG101 (GeneTex; 1:1,000).

**EGFR degradation assays.** 293T or HeLa cells were plated on duplicate six-well plates in Dulbecco's modified Eagle's medium-10% fetal bovine serum, and transfected with either oligofectamine or Lipofectamine 2000 and specific siRNAs (see above). During the second siRNA transfection ( $t = 24$  h), 1  $\mu$ g R9 HIV plasmid was added to one set of cells (to control for knockdown efficiency and viral production), together with the second aliquot of 5  $\mu$ l siRNA. In this

case, virus and cells were harvested 48 h later ( $t = 72$  h) and analyzed by Western blotting and MAGIC infectivity assays. The other set of cells, used in EGFR assays, was transfected a second time with siRNA-oligofectamine alone. Twenty-four hours after the second transfection ( $t = 48$  h), the cells were reseeded on polylysine (Sigma)-coated coverslips and the next day were incubated in serum-free medium for 18 h. The cells were then incubated in the presence of 1  $\mu$ g/ml human EGF (Molecular Probes/Invitrogen) for 1 h (HeLa) or 2 h (293T cells); fixed in 3.7% formaldehyde-PBS, pH 7.2, at room temperature for 20 min; and incubated with mouse anti-human EGFR (1:100; Neomarkers) in 0.1% saponin-1% bovine serum albumin-PBS, pH 7.2, at room temperature for 60 min. The cells were washed extensively and incubated in Alexa 488-conjugated goat anti-mouse immunoglobulin G (1:750) (Molecular Probes/Invitrogen). The cells were imaged using an epifluorescence microscope with MagnaFire software or an Olympus FV300 confocal fluorescence microscope with Fluoview 2.0.3.9 software using 0.5- $\mu$ m sections. The fluorescence intensities were quantified using NIH ImageJ software.

**EGF-lysosome colocalization assays.** HeLa cells were plated on duplicate six-well plates in Dulbecco's modified Eagle's medium-10% fetal bovine serum and transfected twice with either oligofectamine or Lipofectamine 2000 ( $t = 0$  and  $t = 24$  h) with the siRNA oligonucleotides listed above and with EAP20-2 (start site, nt 477; gcacaagccgagaucaucTT). At  $t = 48$  h, the cells were reseeded on polylysine (Sigma)-coated coverslips, and at  $t = 72$  h, the cells were switched to serum-free media for 18 more hours while being incubated with 0.1 mg/ml Alexa 488-conjugated dextran (molecular weight, 10,000). The cells were then washed extensively in growth medium followed by a 4-h chase in dextran-free/serum-free medium, placed at 0°C, incubated with 1  $\mu$ g/ml EGF-Alexa Fluor 555-streptavidin complex (Molecular Probes/Invitrogen) for 60 min, and washed extensively in growth medium. Finally, the cells were incubated for 30 min at 37°C to allow time for EGF internalization and trafficking. Fluorescent images were captured with an Olympus FV300 confocal fluorescence microscope with Fluoview 2.0.3.9 software using 0.5- $\mu$ m sections and analyzed using NIH ImageJ software. Three to seven fields of cells were analyzed for each sample, and the EGF-lysosome localization was scored manually, with a positive score given to any cell showing significant colocalization of the EGF and dextran markers.

**KK3-mediated MHC-1 downregulation.** EAP20 and CHMP6 were silenced as described above with the addition of either 2  $\mu$ g of pTRACER-K3 or pTRACER empty control plasmid transfected at 24 and 48 h. Thirty-six hours after the second transfection,  $5 \times 10^5$  cells were incubated in suspension with anti-human HLA-ABC antibody (1  $\mu$ g/ $\mu$ l; eBioscience) for 1 h at 4°C in a solution of 10% FCS in PBS. The cells were washed once with 1 ml of PBS and then incubated with phycoerythrin-labeled goat anti-mouse immunoglobulin (1  $\mu$ g/ $\mu$ l; BD Biosciences) in 10% FCS-PBS, followed by an additional wash with PBS. The cells were pelleted by centrifugation and resuspended in 1 ml PBS, and surface HLA-ABC levels were analyzed using the FACScan system (BD Biosciences).

## RESULTS

**Protein interactions within the ESCRT-II complex.** To survey the protein interactions of human ESCRT-II, we tested for yeast two-hybrid interactions between each of the three human ESCRT-II proteins and all known human class E proteins (83). As shown in Fig. 1C, the following positive interactions were observed: EAP20-EAP30, EAP20-EAP45, EAP20-CHMP6, EAP30-EAP30, EAP30-EAP45, EAP30-TSG101, and EAP45-TSG101 (and TSG101-TSG101). All of these interactions were positive in both directions, and none of the ESCRT-II proteins interacted with control DNA binding domain (DBD) or activation domain (AD) constructs (Fig. 1C) or with any other class E proteins (data not shown). As noted above, ESCRT-II contains two copies of EAP20 and single copies of EAP30 and EAP45. Hence, all of the expected heteromeric interactions within the human ESCRT-II complex were observed, because EAP20, EAP30, and EAP45 all interacted with one another. The absence of EAP20 and EAP45 homomeric interactions can also be rationalized, because there is only a single copy of EAP45 in the complex and the two copies of EAP20 within ESCRT-II do not contact one another directly (30, 74). The observed homomeric EAP30-EAP30 interaction was unex-