THE DEUBIQUITINATING ENZYME USP8 PROMOTES TRAFFICKING AND DEGRADATION OF THE CHEMOKINE RECEPTOR CXCR4 AT THE SORTING ENDOsome.

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Running head: USP8 regulates CXCR4 Trafficking at Sorting Endosomes. 
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Reversible ubiquitination orchestrated by the opposition of ubiquitin ligases and deubiquitinating enzymes mediates endocytic trafficking of cell surface receptors for lysosomal degradation. Ubiquitin-specific protease 8 (USP8) has previously been implicated in endocytosis of several receptors by virtue of their deubiquitination. The present study explores an indirect role for USP8 in cargo trafficking through its regulation of the chemokine receptor 4 (CXCR4). Contrary to the effects of USP8 loss on EGFR, we find that USP8 depletion stabilizes CXCR4 on the cell surface and attenuates receptor degradation without affecting its ubiquitination status. In the presence of ligand, diminished CXCR4 turnover is accompanied by receptor accumulation on enlarged early endosomes and leads to enhancement of phospho-Erk signaling. Perturbation in CXCR4 trafficking, resulting from USP8 inactivation, occurs at the ESCRT-0 checkpoint, and catalytic mutation of USP8 specifically targeted to the ESCRT-0 complex impairs the spatial and temporal organization of the sorting endosome. USP8 functionally opposes the ubiquitin ligase AIP4 with respect to ESCRT-0 ubiquitination, thereby promoting trafficking of CXCR4. Collectively, our findings demonstrate a functional cooperation between USP8, AIP4 and the ESCRT-0 machinery at the early sorting phase of CXCR4 and underscore the versatility of USP8 in shaping trafficking events at the early-to-late endosome transition.

Endocytosis and trafficking of cell surface receptors is essential for organized signal transduction and maintenance of homeostasis. Malfunctions along the molecular pathways governing endocytosis can lead to a wide range of human pathologies including metabolic disorders, autoimmune diseases and cancer (1,2). Ubiquitination, a reversible post-translational modification of proteins (3), mediates spatial and temporal aspects of endocytosis by dictating macromolecular complex assembly and cargo fate (4). While poly-ubiquitination linked through Lys48 of ubiquitin targets substrates for proteasomal degradation (5), mono- and Lys63-linked ubiquitination signal cargo trafficking (6) and modulate function of endocytic sorting machinery (7). The reversible nature of ubiquitination is imparted by deubiquitinating enzymes (DUBs) and enables dynamic regulation of these ubiquitin-dependent processes in the cell (8).

Trafficking of endocytosed cargo for degradation by the lysosome occurs in a series of discrete selection stages that utilize the endosomal sorting complexes required for transport (ESCRTs) -0, -I, -II and -III. The ESCRT-0 complex, consisting of the adaptor proteins hepatocyte growth factor-regulated substrate (Hrs) and signal transducing adaptor molecule (STAM), resides at the sorting endosome and functions in the first step of cargo selection. ESCRT-0 partitions the endocytosed material arriving on the early endosomes between recycling and the multi-vesicular body (MVB) (9-11) and interacts with downstream ESCRT machinery (12,13) responsible for commitment of selected cargo for degradation (14). Due to its ability to influence both recycling and degradation (15), Hrs has been termed a “master regulator” of endocytosis. Although the mechanisms by which Hrs globally regulates endosomal sorting remain ambiguous, there is a clear phenotypic role for reversible ubiquitination as a signal directing ESCRT-0-mediated endocytosis.
trafficking through reversible ubiquitination of cargo as well as the sorting machinery itself. In yeast, the ESCRT-0 complex comprised of the Hrs and STAM orthologs, Vps27 and Hse1 respectively, associates with the ligase Rsp5 and the DUBs Ubp2 and Ubp7 to promote sorting into the MVB (16). Despite the low sequence similarity between their protein components, the yeast and human ESCRT-0 complexes exhibit highly conserved three-dimensional structures (17), suggesting that the mechanisms governing their function may be similar. The mammalian adaptor proteins STAM1/2 have been shown to interact with two DUBs, ubiquitin-specific protease 8 (USP8) and associated molecule with the SH3 domain of STAM (AMSH) (18-20). A conditional mouse knockout of USP8 is characterized by marked destabilization of Hrs and STAM (21). Moreover, USP8 depletion results in increased ubiquitination of the early sorting proteins (22,23) and incurs gross endocytic swelling, accompanied by the accumulation of ubiquitinated species on endosomes. These global effects of USP8 inactivation on the endocytic compartment are reminiscent of phenotypes resulting from perturbations in the function of Hrs (22-24). Collectively, the body of literature on USP8 implicates it as a regulator of Hrs function with consequences for the stability of receptors subject to Hrs-dependent endocytosis. On this basis, cooperation between USP8 and the ESCRT-0 complex is proposed to constitute a critical regulatory mechanism in endocytosis with profound consequences for cargo trafficking at the early-to-late endosome transition.

We have recently shown that USP8 associates with STAM to mediate direct deubiquitination of activated EGFR, thereby stabilizing the receptor against downregulation by the lysosome (18). The work presented herein investigates a different role of USP8 in endocytosis through the examination of its effects on trafficking and turnover of the chemokine receptor, CXCR4. CXCR4 is a prominent member of the seven transmembrane class of G protein-coupled receptors (GPCRs) that was first identified as a co-receptor for HIV (25) and has since been shown to fulfill critical roles in hematopoiesis, immune system organization and stem cell homing (26,27). By virtue of its overexpression in a variety of human cancers, CXCR4 constitutes a potential chemotherapeutic target. CXCR4-mediated chemotaxis along a gradient of its cognate ligand, stromal-derived factor 1α (SDF-1α/CXCL12), supports metastasis of cancer to the bone marrow (25,28). Ligand-induced lysosomal degradation of CXCR4 is critical for downstream signal attenuation and is mediated by the HECT-family E3 ligase atrophin-interacting protein 4 (AIP4/Itch) (29,30) and deubiquitinase ubiquitin-specific protease 14 (USP14) (31). CXCR4 activation has also been linked to ubiquitination of Hrs by AIP4, and its trafficking proceeds along an Hrs-dependent pathway (32), suggesting that alteration in Hrs ubiquitination status may modulate CXCR4 turnover. Despite these recent insights into the reversible ubiquitination machinery implicated in CXCR4 trafficking, the potential influence of other known endocytic regulators, including USP8, has not been addressed. USP8 and AIP4 have been shown to interact (33), and recent evidence has demonstrated cooperation between USP8 and AIP4 in reversible ubiquitination of a GPCR substrate, δ-opioid receptor (DOR) (34). As both enzymes impinge on Hrs ubiquitination, their opposition in this regard may regulate ESCRT-0 function and thereby control cargo transit from early to late endosomal compartments.

To probe whether USP8 may indirectly promote receptor degradation through its regulation of the ESCRT-0 machinery, the present study explores the role of USP8 in CXCR4 trafficking and offers novel insights into the regulation of ubiquitin-mediated events at the sorting endosome. Contrary to the effects of USP8 inactivation on EGFR, we find that USP8 loss-of-function leads to diminished CXCR4 turnover and induces receptor accumulation on enlarged Hrs-positive early endosomes. Catalytic activity of USP8 opposes that of AIP4 in the regulation of ESCRT-0 ubiquitination status and is essential for the maintenance of sorting endosome integrity. Furthermore, inactivation of USP8 bound to the ESCRT-0 complex exhibits colocalization with high ubiquitin content on disordered endosomes marked by Hrs and perturbs the early-to-late endosomal transition, resulting in impairment of CXCR4 trafficking. Our findings therefore demonstrate a functional cooperation between USP8, AIP4 and the ESCRT-0 machinery in
shaping receptor progression through the sorting endosome.

**MATERIALS AND METHODS**

**Expression constructs and DNA manipulation**—Mammalian expression constructs of murine USP8, HA- and Myc-ubiquitin (18) as well as HA-CXCR4, Myc-AIP4 and Myc-AIP4-C830A (32), all in pcDNA3.1, have been previously reported. Flag-ubiquitin, Myc-Cbl (BC032851) and Myc-Nrdp1ΔC (BC032637) constructs were generated by PCR and cloned into pcDNA3.1 at BamHI/EcoRI. USP8 and STAM2 constructs used in the BIFC assay (35) have also been previously described (18).

**Cell lines, transfection and siRNA**—HeLa or HEK293 cells were used for all experiments requiring cell culture in this study. Cells were grown in DMEM (CellGrow), supplemented with 10% FBS (Sigma-Aldrich). All transfections were performed using Lipofectamine-2000 (Invitrogen). Transient knockdown of USP8 and Hrs was accomplished by transfection of siRNA oligonucleotides. siUSP8-1 (18) and siUSP8-2 have been previously described (23), and a published siRNA sequence targeting human Hrs (32) was custom synthesized by Applied Biosystems (results were verified using another reported anti-Hrs sequence (12) purchased from Dharmacon).

**Western blotting and antibodies**—Western blots were visualized with LI-COR Odyssey Infrared imager and band intensities were quantified using Odyssey 3.0 software. Final image processing was performed in Adobe Photoshop CS3 and was limited to brightness enhancement and cropping. Contrast enhancement was used only to aide visualization of endogenous USP8 bands. All reported quantification is based on the number of independent experiments specified in the corresponding figure legends, with error bars representing SD. All graphical representations were generated using DeltaGraph7.5.5 software. The following primary mouse monoclonal antibodies were used for western blot analysis and immunofluorescence (IF): anti-HA (Covance), anti-Myc (CST or Santa Cruz), anti-β-Actin and anti-Flag (Sigma-Aldrich), anti-EGFR-528 (Santa Cruz), anti-TSG101 (Abcam), anti-EEA1 (BD Biosciences), anti-CD63 and anti-LAMP1 (Developmental Studies Hybridoma Bank, The University of Iowa). Rat monoclonal anti-CXCR4 antibody was purchased from BD Biosciences. The following primary rabbit polyclonal antibodies were used: anti-EGFR (Upstate), anti-phospho-Erk1/2 and anti-Erk1/2 (CST), anti-Flag (Stratagene), anti-STAM1 (SantaCruz) and anti-TSG101 (Abcam). Rabbit USP8 and Hrs antisera were generated against GST-fusion proteins of murine proteins as described (18). Goat secondary antibodies antimouse-AlexaFluor-800 and anti-rabbit-AlexaFluor-680 were purchased from LI-COR. Goat secondary antibody AlexaFluor-conjugates for anti-rat-488, anti-rabbit-568 and anti-mouse-488/-647 were purchased from Molecular Probes.

**CXCR4 abundance, signaling and degradation**—For endogenous CXCR4 stability, HeLa cells were seeded at 2.5 x 10^5 into 6-well plates and allowed to adhere overnight (O/N). Cells were transfected with 100 pmol of control or anti-USP8 siRNA and 48 hrs following transfection lysed in 2xSDS gel loading buffer containing 200 mM dithiothreitol (DTT). Samples were sonicated and subjected to western blot analysis as indicated. Quantification of relative protein abundance was carried out relative to actin controls.

For the signaling assay, cells were manipulated as above and treated with 1.25-2.5 nM SDF-1α (PeproTech) for 10 min or 1 hr, washed with ice-cold PBS, and samples were prepared as above. Signaling was assayed by western blot against phospho-Erk1/2 and quantified relative to unphosphorylated Erk1/2 control.

For turnover of transiently overexpressed HA-CXCR4 using siRNA, HeLa cells were seeded at 1.425 x 10^6 per 100 mm dish and allowed to adhere O/N. Cells were co-transfected with 1.5 µg of HA-CXCR4 and 600 pmol of siRNA. 24 hrs following transfection, cells were seeded into 6-well plates at 0.85 x 10^6 per well and allowed to adhere O/N. Cells were treated with 25 nM SDF-1α in the presence of 20-40 µg/ml cycloheximide (Sigma-Aldrich) for the indicated duration of time. Samples were prepared as described above and CXCR4 abundance was monitored by western blot against HA. Quantification of relative HA-CXCR4 abundance was carried out relative to actin controls.
For HA-CXCR4 degradation using overexpression of USP8 or mutants, HEK293 cells were seeded at 2 x 10^6 per 100 mm dish and allowed to adhere O/N. Cells were co-transfected with 1.5 µg of HA-CXCR4 and 7.5 µg of USP8 or mutant. 24 hrs following transfection, cells were seeded into 24-well plates, allowed to adhere O/N and treated with 10 nM SDF-1α in the presence of cycloheximide for the indicated duration of time. Samples were prepared and analyzed as described above.

Surface Biotinylation — For examination of surface CXCR4, HeLa cells were transiently co-transfected with HA-CXCR4 and relevant siRNA oligonucleotides in 100mm plates as indicated above, and biotinylation of membrane proteins under steady state growth conditions was performed as previously described (20).

Ubiquitination assays — CXCR4 ubiquitination was assayed using single-tag Flag-ubiquitin according to a published protocol (36), and EGFR as well as Hrs ubiquitination methods have been previously described (18). Receptor ubiquitination was quantified relative to total immunoprecipitated receptor control.

Immunofluorescence Microscopy — For knockdown studies, HeLa cells transfected with HA-CXCR4 and siRNA as described above, were seeded at 5 x 10^5 per well onto polylysine-coated glass coverslips (Corning) in 6-well tissue culture plates 24 hrs post-transfection and allowed to adhere O/N. Cells were treated with 25 nM SDF-1α for 5 hrs, fixed and immunostained as described below. For USP8 overexpression studies with HA-CXCR4, cells were seeded at 2.5 x 10^5 per well onto coverslips and co-transfected with 0.25 µg HA-CXCR4 and 1.25 µg vector control, Flag-USP8 or Flag-C748A for 24 hrs. Following transfection, cells were fixed and immunostained as indicated. Unless otherwise indicated (see below), cells were fixed in 4% paraformaldehyde (PFA) in PBS for 10-15 min at RT, followed by permeabilization in 0.1% Triton-X100/PBS for 10-15 min. All immunostaining, except for anti-CD63, was performed in 5% milk in PBS according to standard protocols. For immunostaining with anti-CD63, cells were also fixed in 4% PFA/PBS, however, permeabilization and antibody incubations were carried out in 3% BSA/PBS containing 0.05% saponin. For colocalization of CXCR4 with STAM1, cells were fixed and permeabilized in 1:1 methanol:acetone solution for 20 min at -20°C. Coverslips were mounted onto glass slides using ProLong Gold antifade mounting media with DAPI (Invitrogen).

All samples were imaged on a Leica SP5-2photon confocal microscope using 100x oil objective with 3x digital zoom. Images were collected and analyzed with LASAF software and final processing was performed using ImageJ64. Quantification of CXCR4-positive endosome size using IF was performed using 3 independent data sets, with 5-10 images per condition analyzed for each experiment. To enable direct comparison, all images within a given experiment were taken under the same magnification and laser intensity settings. For every cell analyzed, the area for 5-10 representative CXCR4-positive endosomes was quantified using ImageJ64 and average endosome size was expressed relative to that of the siControl samples. Colocalization was quantified using the JACoP plugin for ImageJ64, with 5-10 representative cells per condition collected from at least 2 independent experiments. Background corrections were specified manually in ImageJ64 prior to running JACoP analysis, with similar adjustments applied across all images per channel per experiment. For colocalization reported as a fraction of channel ‘A’ overlapping channel ‘B’ (labeled A:B), overlap was quantified using Mander’s coefficients calculated on the basis of automated threshold settings, with 0 and 1 corresponding to no colocalization and complete colocalization, respectively. For colocalization reported in the form of a coefficient (r), overlap was quantified using JACoP coefficients k1 and k2, where r^2=k1*k2.

Bi-molecular Fluorescence Complementation (BiFC) — BiFC was used in combination with conventional IF microscopy as previously described (18). For relevant fixation and immunostaining methods, as well as data analysis and representation of results, refer to the section above.

RESULTS

USP8 depletion results in enhanced CXCR4 stability and diminished SDF1α-mediated degradation — To examine the role of USP8 in the
regulation of CXCR4 stability, effects of transient USP8 ablation by short interfering RNA (siRNA) on receptor abundance were assessed. Efficient depletion of USP8 (80% decrease in USP8 protein levels) resulted in nearly a 2-fold increase in endogenous CXCR4 protein (Fig. 1A,B). By contrast, EGFR was drastically destabilized in the same cells. This is consistent with published evidence (18,21) and indicates that USP8 may function to exert opposing consequences on the stability of different cell surface receptors.

Steady-state accumulation of CXCR4 observed in the absence of USP8 suggests a role for USP8 in CXCR4 turnover. In the presence of activating ligand, SDF-1α (SDF1), CXCR4 is known to undergo endocytosis and ubiquitin-mediated degradation (29-32). Thus, the effect of USP8 depletion on the rate of ligand-dependent CXCR4 degradation was assessed. Cells transiently overexpressing HA-tagged CXCR4 (HA-CXCR4) and either control or anti-USP8 siRNA were treated with SDF1 in the presence of cycloheximide (used to block de novo protein synthesis) for the indicated duration of time and HA-CXCR4 abundance was monitored by western blot (Fig. 1C). At 5 hrs following the addition of SDF1, more than half of the total CXCR4 was downregulated in the control cells, while cells compromised for USP8 degraded only 30% of the starting CXCR4 material (Fig. 1D). Stabilization of CXCR4 against ligand-induced turnover was similarly observed using an alternate siRNA targeting USP8 in the same cells (Fig. S1A). The diminished rate of receptor degradation in the presence of SDF1 therefore indicated a requirement for USP8 in the establishment of ligand-mediated CXCR4 turnover.

To determine whether additional CXCR4 found in cells depleted of USP8 resides at the plasma membrane or is intracellular and thereby sequestered from the interaction with ligand, we examined CXCR4 surface levels as a function of USP8. In this assay, cells were surface modified with biotin and biotinylated proteins were isolated using neutravidin precipitation, followed by immunoblot against HA-CXCR4 (Fig. 2A). Comparison of protein levels in the surface fraction with the whole cell lysates revealed that in the absence of USP8, the change in total cellular CXCR4 was matched by a proportional accumulation of surface receptor (Fig. 2B). These findings demonstrate that the majority of additional CXCR4 material found in cells depleted of USP8 is available for ligand engagement at the plasma membrane.

Global changes in cellular receptor levels may impact ligand-mediated events downstream. Specifically, increase in surface CXCR4 abundance may give rise to enhanced and/or prolonged signaling in response to SDF1. Indeed, in cells expressing endogenous CXCR4, loss of USP8 resulted in increased phospho-Erk signaling following stimulation with SDF1 (Fig. 2C). The initial activation response (10 min) was 35% higher in USP8 knockdown cells compared to the control and sustained signaling (1 hr) increased by nearly 2.5-fold as a result of USP8 depletion (Fig. 2D). These changes in SDF1-induced phospho-Erk activation imply that USP8-dependent control of CXCR4 abundance and turnover directly affects signaling. Importantly, consistent with opposing effects of USP8 depletion on cellular abundance of CXCR4 and EGFR (Fig. 1A,B), augmentation of Erk signaling downstream of CXCR4 runs contrary to diminished EGF-induced activation of the same signaling cascade by EGFR, reported with both ablation as well as catalytic inactivation of USP8 (21,24). Furthermore, the prolonged effects on phospho-Erk following ligand stimulation suggest that USP8 may modulate receptor signaling not only at the plasma membrane, but perhaps also on signaling endosomes.

The evidence presented above suggests that the impairment in CXCR4 degradation observed with USP8 ablation may stem from a defect in receptor endocytosis. To address this hypothesis, the contribution of USP8 to ligand-mediated CXCR4 trafficking was assessed (Fig. 3). In USP8 depleted cells, HA-CXCR4, internalized following a 5 hr treatment with SDF1, accumulated on enlarged endosomes characterized by the early marker EEA1—a phenotype not observed in the control cells (Fig. 3A,D). These results correlate with enhanced stability of CXCR4 mediated by USP8 ablation under the same conditions (Fig. 1C,D), supporting a role for USP8 in promoting CXCR4 degradation. Previous work has established a role for Hrs in ligand-dependent turnover of CXCR4, as loss of Hrs blocks receptor degradation in response to SDF1 (32). We therefore compared the depletion effects of USP8
and Hrs on CXCR4 trafficking. Hrs knockdown (Fig. 3B) phenocopies loss of USP8 (Fig. 3A), leading to accumulation of CXCR4 on enlarged EEA1-positive endosomes (Fig. 3D). Moreover, in cells depleted for USP8, endosomes saturated with CXCR4 were also decorated with Hrs (Fig. 3C). Colocalization of both EEA1 and Hrs with these CXCR4-positive endosomes (Fig. S1B) indicates that the perturbation in CXCR4 trafficking resulting from USP8 ablation occurs at the Hrs-positive endosome. These studies therefore implicate USP8 as a critical regulator of CXCR4 endocytosis and suggest a functional cooperation between USP8 and Hrs in this regard.

**USP8 modulates the integrity of the endocytic compartment at the ESCRT-0-positive sorting endosome**—Along with published evidence (19,22,23,37), the observations described above suggest that USP8 may function as a general regulator of Hrs-dependent trafficking at the sorting endosome. To investigate the cellular interactions between USP8 and the ESCRT-0 complex, Bi-molecular Fluorescence Complementation (BiFC) microscopy was employed. BiFC takes advantage of a GFP variant, Venus Fluorescent Protein (VFP) to enable visualization of protein-protein complexes as they form in living cells (35,38,39). In this assay, the VFP fluorophore is separated into the N- and C-terminal fragments (VN and VC, respectively), each fused to one of two proteins of interest (Fig. 4A). When co-expressed in cells, upon direct contact, the VN- and VC- fusion proteins form an irreversible fluorescent VFP complex that can be visualized by standard microscopic techniques. An irreversible complex between USP8 and ESCRT-0 would be expected to create a high concentration of DUB in specific regions in the cell marked by VFP. Manipulation of USP8 activity in this assay would therefore result in targeted alterations in the ubiquitination status of relevant USP8 substrates. This feature of BiFC was thus utilized to directly assay the relationship between USP8 function at ESCRT-0 and the efficacy of the sorting endosome.

Because BiFC requires exogenous expression of proteins, direct assessment of Hrs in this assay was not suitable, as its overexpression has been shown to negatively impact endocytosis (11). Instead, a previously described direct interaction between USP8 and the SH3 domain of STAM (18,19) was exploited as a marker of USP8 in complex with ESCRT-0. In the USP8/STAM BiFC assay (Fig. S2A), Flag-USP8 was fused to the VN- fragment (VN-Flag-USP8), and HA-STAM2 was incorporated into the VC- (VC-HA-STAM2). Reconstitution of the USP8/STAM interaction with BiFC yielded VFP fluorescence in cells co-expressing VN-Flag-USP8 or VN-Flag-ΔC with VC-HA-STAM2, while no fluorescence was observed in cells expressing an SH3 domain mutant of STAM2, E217A/E220A (VC-HA-STAM2-EEAA) (Fig. S2B). The VFP-positive puncta were marked by the co-staining of Flag- and HA-reactive antibodies corresponding to the epitope tags of individual BiFC protein components (Fig. S2B).

As the ESCRT-0 sorting endosome represents an intermediate between the early and late extremes of the endosomal continuum, the complex between USP8 and ESCRT-0 was expected to reside between endosomes populated by the early and late markers, EEA1 and CD63, respectively (Fig. 4B). Indeed, no significant overlap of the USP8/STAM2 VFP with either EEA1 or CD63 (Fig. 4C-E) was observed. Conversely, a catalytically inactive USP8 domain truncation mutant of USP8 (AC) in complex with STAM2 exhibited extensive overlap with EEA1 (Fig. 4C,D) and to a lesser extent with CD63 (Fig. 4C,E). Additionally, the morphology of ΔC/STAM2 VFP appeared different from the catalytically wild type VFP complexes, with irregular shapes and 'hollow circular ribbon' structures dominating the ΔC/STAM2 VFP population. The above observations were corroborated by changes in cellular STAM1 distribution as a function of USP8. Cells expressing no exogenous USP8 exhibited a punctate distribution of endogenous STAM1 protein, characteristic of normal endosomal localization (Fig. S2C). Under standard growth conditions, in the absence of SDF1, the majority of HA-CXCR4 expressed in these cells was found at the plasma membrane. Cells overexpressing wild type USP8 showed a more diffuse cellular pattern of STAM1 expression that did not appear to affect the receptor localization profile (Fig. S2C). Conversely, overexpression of the C748A mutant induced formation of enlarged and irregularly shaped STAM1-positive structures that accumulated HA-CXCR4 in the absence of ligand.
stimulation (Fig. S2C). Taken together, altered endosome morphology and distribution of STAM1 protein in the presence of catalytically inactive USP8 suggests a critical role for USP8-mediated deubiquitination in specifying the organization of the sorting endosome.

Catalytic inactivation of USP8 inhibits CXCR4 trafficking and downregulation without affecting receptor ubiquitination status—Stabilization of CXCR4 upon USP8 depletion described in Fig. 1 implicates the deubiquitinating activity of USP8 in promoting CXCR4 turnover. In agreement with the knockdown studies, overexpression of inactive USP8 mutants—a full-length catalytic point-mutant, C748A, or ΔC truncation—resulted in elevated CXCR4 levels and diminished receptor degradation in response to treatment with SDF1 compared to vector control and wild type USP8 (Fig. 5A-C, S3A). While both catalytic mutants of USP8 supported stabilization of CXCR4 (Fig. S3A), ΔC generally exhibited higher expression and therefore afforded a more robust phenotype (Fig. 5C). Contrary to the effects of USP8 inactivation on CXCR4, overexpression of either C748A or ΔC mutant resulted in a profound decrease of endogenous EGFR protein (Fig. S3B, C) and accelerated EGF-induced receptor downregulation (Fig. S3D,E).

Collectively, knockdown and overexpression studies demonstrate that USP8-mediated deubiquitination functions as a positive regulator of CXCR4, but not EGFR degradation. Because in the case of USP8 depletion, stabilization of CXCR4 in the presence of ligand was accompanied by receptor accumulation on enlarged early endosomes (Fig. 3A), we tested whether catalytic inactivation of USP8 would similarly affect CXCR4 trafficking. As expected, enlarged CXCR4-positive endosomes were observed in cells expressing C748A mutant compared to wild type USP8 (Fig. 5D). These abnormal endosomal structures were characterized by a high degree of colocalization with the early marker, EEA1 (Fig. 5D,F), but not with a late marker, LAMP1 (Fig. 5E,F), indicating that the deubiquitinating activity of USP8 is required for trafficking of CXCR4 between the early and late endosomal compartments.

USP8 has previously been shown to promote trafficking of the GPCR, protease-activated receptor 2 (PAR2), by virtue of direct deubiquitination (37). To assess whether CXCR4 is similarly regulated, effects of USP8 manipulation on receptor ubiquitination status were assessed. If impairment in CXCR4 trafficking resulted from inadequate USP8-dependent receptor deubiquitination, hyperubiquitination of CXCR4 would be expected in cells compromised for USP8. However, neither USP8 depletion nor overexpression of inactive mutants of USP8 was found to alter receptor ubiquitination status (Fig. 6A-C). Under the same assay conditions, overexpression of wild type AIP4 promoted enhanced ubiquitination of CXCR4, while its catalytic mutant, C830A, did not (Fig. 6B,C). Although exogenous expression of wild type USP8 resulted in decreased CXCR4 ubiquitination, this phenotype was also mirrored in the profound deubiquitination of the whole cell lysate relative to vector control (Fig. 6B). Given the negative results from C748A and ΔC overexpression, this likely represents a nonspecific consequence of elevated cellular levels of a broad specificity DUB (40).

By contrast to CXCR4, USP8 is known to mitigate ligand-induced ubiquitination of EGFR ((18,24); Fig. 6D, lanes 1-4). In this case, overexpression of catalytically inactive USP8 (ΔC) recapitulates receptor hyperubiquitination afforded by the cognate E3 ligase (41), Cbl (Fig. 6D, lanes 5,6,9,10), while overexpression of AIP4 exerts no effect (lanes 7,8). Furthermore, increased EGFR ubiquitination afforded by USP8ΔC is effectively countered by co-expression of an inactive Cbl RING domain truncation mutant, CblΔC (Fig. 6E, lanes 1-6), but remains impervious to exogenous expression of Nrdp1ΔC—an inactive mutant of another ubiquitin ligase known to associate with USP8 (42), but not involved in EGFR ubiquitination (lanes 7,8). Collectively, these results demonstrate that USP8 impinges on the ubiquitination of EGFR, but not CXCR4, indicating that USP8-mediated regulation of CXCR4 stability occurs indirectly and perhaps through deubiquitination of another substrate critical to receptor trafficking.

USP8-mediated deubiquitination of Hrs counteracts AIP4 to regulate ubiquitin dynamics and promote CXCR4 trafficking at the ESCRT-0-positive endosome—Ubiquitination is known to regulate activity of endosomal proteins (7) and Hrs has been shown to become ubiquitinated by AIP4.
in response to CXCR4 activation (32). Because USP8 depletion results in hyperubiquitination of Hrs (22), we examined whether catalytic activity of USP8 is explicitly required to support Hrs deubiquitination in the cell. Overexpression of wild type USP8 did not appear to significantly alter the prevalence of ubiquitinated Hrs species (Fig. 7A, lanes 2,3). Conversely, the USP8 truncation mutant, ΔC, elicited hyperubiquitination (Fig. 7A, lanes 7,9) and destabilization of Hrs and as well as its partner STAM1, but had no effect on the ESCRT-I protein, TSG101 (Fig. S4A-C), indicating specificity of USP8 toward the early ESCRT machinery. In agreement with published evidence (32), overexpression of wild type AIP4, but not its catalytic mutant, C830A, promoted excess ubiquitination of Hrs (Fig. 7A, lanes 5,6), while expression of Cbl in the same assay had no effect (lane 4). Additionally, co-expression of AIP4-C830A in the presence of USP8ΔC abrogated the USP8 loss-of-function phenotype (Fig. 7A, lanes 7-10), indicating that AIP4 and USP8 activities oppose one another in this regard (Fig. S5A). Consistent with these observations, AIP4 catalytic mutant rescued abnormal morphology of ubiquitin-rich STAM1-positive endosomes produced by overexpression of USP8ΔC (Fig. S5B). Taken together with Hrs ubiquitination profiling, these results suggest that reversible ubiquitination of Hrs by AIP4 and USP8 regulates the ESCRT-0 complex and sorting endosome integrity.

As Hrs dysfunction results in failed endocytic progression of cargo (10), it incurs accumulation of ubiquitinated species on endosomes (11,22). We therefore assessed the effects of targeted USP8 inactivation within the ESCRT-0 complex on endosomal ubiquitin dynamics. Cells expressing either VN-USP8 or VN-ΔC in combination with VC-STAM2 were co-transfected with Myc-ubiquitin and localization of VFP fluorescence to Hrs and ubiquitin was assessed (Fig. 7B). BiFC with both wild type and mutant USP8 exhibited extensive colocalization with endogenous Hrs (Fig. 7B,D), demonstrating association of the USP8/STAM complex with the ESCRT-0 sorting endosome. While the wild type USP8/STAM2 VFP did not appreciably colocalize with Myc-ubiquitin, the inactive DUB complex strongly overlapped with subcellular regions characterized by high ubiquitin content (Fig. 7B,D). In agreement with published data (22), these findings therefore demonstrate that USP8 inactivation profoundly affects endosomal ubiquitin dynamics at ESCRT-0.

To explore the connection between USP8-mediated deubiquitination and trafficking through the sorting endosome, we assessed the effect of USP8 inactivation in the context of the ESCRT-0 complex on trafficking of CXCR4. In BiFC cells expressing HA-CXCR4, the ΔC/STAM2 complex exhibited substantial colocalization with internalized CXCR4, whereas the wild type USP8/STAM2 complex did not (Fig. 7C,D). In agreement with these observations, colocalization between CXCR4 and endogenous STAM1 was found in the presence of USP8 mutant, C748A, but not in control cells or those expressing the wild type DUB (Fig. S2C). Collectively, these results provide a functional link between USP8 and the ESCRT-0 complex with respect to CXCR4 trafficking through the sorting endosome.

**DISCUSSION**

Reversible ubiquitination constitutes a key regulatory mechanism for cell surface receptor endocytosis and ligand-mediated degradation. Since DUBs impart reversibility to ubiquitination, they function as potent modulators of spatial and temporal features of ubiquitin-dependent cellular processes. USP8 is an example of a DUB with multiple cellular roles (43-45) and broad substrate specificity (21,40,42), differently implicated in the control of receptor tyrosine kinase (RTK) (18,21,24) and GPCR (34,37) endocytosis. Characterization of a mouse knockout has established a requirement for USP8 to stabilize growth factor receptors and support cellular proliferation (21). USP8 exerts these effects through direct deubiquitination (24) and consequent protection of RTKs, such as the EGFR, against lysosomal turnover (18), thereby prolonging receptor signaling (24,46). By contrast to growth factor receptors, USP8-mediated deubiquitination of two GPCRs, DOR and PAR2, has been shown to promote their degradation (34,37), indicating that USP8 is capable of affecting diverse outcomes in receptor endocytosis.
In addition to cell surface receptors, USP8 is implicated in the regulation of ESCRT-0 proteins, Hrs and STAM (21), and a critical role for USP8 in endocytosis is underscored by its ability to modulate endosomal ubiquitin dynamics (22,23). The present study explores whether regulation of ESCRT-0 ubiquitination by USP8 indirectly mediates trafficking and degradation of the chemokine receptor, CXCR4. Following ligand stimulation, CXCR4, a GPCR critically implicated in immune system function, HIV infection and cancer metastasis (25,47-49), undergoes ubiquitin-mediated lysosomal degradation required to turn off downstream signal transduction cascades (29). We find that depletion (Figs. 1-3) or catalytic inactivation (Fig. 5) of USP8 perturb endocytic trafficking of CXCR4, leading to diminished rate of receptor degradation and prolonged phospho-Erk signaling in response to activation with SDF-1α. These effects of USP8 on CXCR4 turnover (Fig. 1A and S3) and ligand-induced signaling are opposite of those observed for EGFR (18,24,46) and therefore imply that USP8 may contribute to the specificity underlying the determination of receptor fate.

Trafficking of CXCR4 for lysosomal degradation in response to ligand activation requires receptor ubiquitination by AIP4 (32) as well as direct deubiquitination by USP14 (31)—a DUB of the same catalytic class as USP8. USP14 has been shown to regulate CXCR4 ubiquitination at the plasma membrane during the early phase of CXCR4 endocytosis without affecting Erk signaling (31). Unlike USP14, USP8 is not required for deubiquitination of CXCR4 (Fig. 6), and therefore functions to promote trafficking of this receptor through other means. Furthermore, because sustained Erk activation occurs largely on signaling endosomes (reviewed in (50)), the ability of USP8 to impinge on CXCR4/SDF-1α-induced signaling (Fig. 2C,D) is consistent with its endosomal localization ((22); Fig. 4) and suggests that USP8 may act to modulate the dynamics of signaling endosomes.

In addition to ligand-mediated endocytosis, CXCR4 is subject to constitutive internalization that controls cellular distribution and steady state receptor abundance (51). Since USP8 depletion stabilizes CXCR4 both in the absence and presence of ligand, loss of USP8 could in principle give rise to accumulation of CXCR4 either intracellularly or on the cell surface. Biotinylation of membrane proteins reveals a close correlation between changes in total and surface CXCR4 abundance observed in response to USP8 depletion (Fig. 2A,B), indicating that recycling of this receptor from the sorting endosome back to the plasma membrane is not mediated by USP8. Instead, these loss-of-function studies implicate USP8 as a positive regulator of CXCR4 trafficking for degradation.

Targeting of CXCR4 to the lysosome requires a sorting event mediated by Hrs (32). In agreement with published evidence (21,22), our results demonstrate that USP8 regulates the ubiquitination status and cellular protein abundance of Hrs (Fig. 7A; S4) and thus advocate a role for USP8 in Hrs-dependent trafficking. Diminished CXCR4 turnover in cells compromised for USP8 function is accompanied by receptor accumulation on enlarged endosomes (Fig. 3). The phenotype of Hrs knockdown in this context closely resembles that of USP8 (Fig. 3), implying that USP8 and Hrs function in the same pathway for CXCR4 trafficking. Moreover, in the presence of catalytically inactive USP8, intracellular CXCR4 accumulates on endosomes decorated with ESCRT-0 proteins (Fig. 7B-D; S2C), illustrating a requirement for USP8-mediated deubiquitination of Hrs in trafficking of CXCR4 through the ESCRT-0-positive sorting endosome.

The sorting endosome represents the transition between early endosomes and the MVB, where the ESCRT-0 complex partitions endocytosed cargo between recycling and degradation (14). The detrimental effects of Hrs loss-of-function on the integrity of the endocytic trafficking pathway have been described (10). Since USP8 modulates ubiquitination and stability of Hrs, this function may endow USP8 with the ability to mediate the spatial organization of the endocytic compartment. To directly illustrate the relationship between USP8 and the ESCRT-0 complex in this context, we chose to use bimolecular fluorescence complementation, or BiFC (Fig. 4). The irreversible nature of BiFC traps the interaction of interest as it forms in the cell (38), allowing the dissection of phenotypes specifically associated with manipulations targeted at the complex. A complex between USP8 and STAM would be expected to create a high
concentration of DUB on ESCRT-0-positive endosomes, leading to exaggerated alterations in the ubiquitination status of Hrs and STAM. This feature of BiFC was thus utilized to directly assay the relationship between ESCRT-0 ubiquitination and efficacy of trafficking at the sorting endosome. As visualized by BiFC, USP8 inactivation alters the morphology and localization profile of the ESCRT-0 complex (Fig. 4). While wild type USP8 and STAM BiFC localizes to Hrs-positive endosomes that are not characterized by either early or late endosomal markers, the catalytically inactive ΔC/STAM complex exhibits broad overlap with the early endosomes, in addition to Hrs (Fig. 4C-E). These observations are further supported by the ability of USP8 to influence cellular distribution of endogenous STAM1 (Fig. S2C). Although the individual localization profiles of Hrs, STAM and USP8 straddle the early-to-late endosomal transition, BiFC experiments suggest that USP8 specifically interacts with the ESCRT-0 complex between the early and late extremes of this continuum. Since ESCRT-0 function specifies cargo progression through the sorting endosome (9,10,52,53), deubiquitination of Hrs by USP8 may be required to partition the early and late endosomal compartments, thus enabling organized flow of trafficking events.

Ubiquitination of Hrs by AIP4 has been previously implicated in CXCR4 trafficking (32), and herein, we demonstrate that AIP4 and USP8 act in opposition to one another to regulate the ubiquitination state of ESCRT-0 (Fig. 7A; S5A). Imbalances in this dynamic directly impinge on the integrity of the sorting endosome. Specifically, overexpression of the catalytically inactive USP8ΔC induces malformations in the STAM1-positive endosome (Fig. S5B)—enlarged hollow structures similar to those observed with ΔC/STAM2 BiFC (Fig. 4) and characterized by high ubiquitin content. This swelling of the sorting compartment was effectively mitigated by co-expression of catalytically inactive AIP4, supporting the notion that the interplay between USP8 and AIP4 at ESCRT-0 is critical for endocytic function.

Endosomal accumulation of ubiquitinated species constitutes a hallmark of dysfunctional trafficking. USP8 has been previously implicated in the regulation of endosomal ubiquitin dynamics (22,23), though its connection to the ESCRT-0 machinery in this context was unclear. Using BiFC, we examined the relationship between USP8 activity and ubiquitin dynamics at the sorting endosome. USP8 inactivation gave rise to a broad cellular redistribution of ubiquitin to ESCRT-0-positive endosomes (Fig. 7B,C), altered STAM1 localization (Fig. S2C) and impaired CXCR4 trafficking (Fig. 5D-F and 7B-D). These findings imply that USP8-mediated deubiquitination of the ESCRT-0 complex critically impinges upon endosomal ubiquitin turnover and exhibits consequences for receptor trafficking through the Hrs-dependent sorting endosome. Conversely, despite also being subject to ESCRT-0-mediated sorting to the MVB (10), EGFR degradation is accelerated in the absence of USP8 ((18,24,46); Fig. 1A,B; S3). The contrast between the effects of USP8 on EGFR and CXCR4 illustrates that, depending on the identity of the receptor, USP8 may be critical for exit from the sorting endosome for either recycling or degradation. Specifically, when USP8 deubiquiticates the receptor directly, it may serve a protective role (EGFR; Fig. 6D,E) or instead act to promote trafficking for degradation (PAR3; (37)). Alternatively, as in the case of CXCR4, USP8 may indirectly regulate receptor trafficking through modulation of ESCRT-0 function.

The complexity of DUB biology in endocytosis and their relationships to other members of the reversible ubiquitination machinery is only starting to emerge. Considering the differences between USP8-mediated regulation of CXCR4 and EGFR turnover, this work therefore contributes to the growing appreciation of versatility as well as specificity of USP8 function in the determination of plasma membrane receptor fate. As evidenced by the dependence of CXCR4 turnover on the catalytic activity of USP8, the regulation of receptor trafficking through the ESCRT-0-positive endosome is intricately connected to the ubiquitin-dependent events mediated by USP8. Collectively, the results of our study demonstrate that USP8 modulates endosomal ubiquitin dynamics and cargo trafficking through the ESCRT-0-positive endosome as part of a mechanism responsible for controlling spatial and temporal progression of endosomal subcompartments. On this basis, these results extend our understanding of the mechanism...
of USP8 action as well as critically implicate USP8 in control of a highly topical and medically relevant plasma membrane receptor.

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REFERENCES

FIG. 1. USP8 depletion leads to enhanced CXCR4 stability and diminished ligand-mediated turnover. (A) Effects of transient USP8 depletion on endogenous protein abundance of EGFR and CXCR4. Whole cell lysates from HeLa cells transfected with either vehicle alone (Mock), control siRNA (siControl) or siRNA directed against human USP8 (siUSP8) were analyzed for endogenous proteins by western blot, as indicated. (B) Quantification of relative protein abundance is expressed as percent of control relative to the actin control. Statistical analyses were carried out using student t-test; **** p<0.0001; n=3. (C) Degradation of CXCR4 in response to treatment with SDF-1alpha (SDF1). Cells transfected with HA-CXCR4 and either siControl or siUSP8 were treated with 25 nM SDF1 in the presence of cycloheximide for the indicated length of time. Western blot analysis of HA-CXCR4 and transferrin receptor (TrfR) abundance throughout the treatment time-course is shown. (D) Plot of HA-CXCR4 abundance along the SDF1 time-course relative to untreated cells (time=0); n=3.

FIG. 2. USP8 ablation leads to accumulation of CXCR4 on the cell surface and supports prolonged Erk activation. (A) Comparison of changes in CXCR4 surface levels with total cellular accumulation of CXCR4 resulting from USP8 knockdown using biotinylation of membrane proteins. Cells, transfected with HA-CXCR4 and either siControl or siUSP8, were biotinylated and surface proteins were isolated by precipitation with Neutravidin beads. HA-CXCR4 abundance was assayed by western blot against HA. (B) Quantification of HA-CXCR4 in whole cell lysates (WCL) and on the cell surface (Neutravidin ppt.) relative to the control; **** p<0.0001, n=3. (C) Analysis of phospho-Erk signaling downstream of endogenous CXCR4. Cells transfected with either siControl or siUSP8 were treated in the presence of 1.25 nM SDF1 for the indicated length of time. SDF1-mediated activation of Erk1/2 was analyzed by western blot against phospho-Erk1/2. (D) Quantification of Erk activation in response to SDF1 stimulation relative to untreated (0’) control cells and corrected for unphosphorylated Erk abundance. *** p<0.001; n=3.

FIG. 3. USP8 depletion inhibits ligand-induced trafficking of CXCR4 through the Hrs-positive endosome. Accumulation of CXCR4 on early endosomes, characterized by EEA1, in response to ligand treatment in cells depleted for (A) USP8 or (B) Hrs. (C) Colocalization of accumulated CXCR4 with Hrs-positive endosomes in USP8-depleted cells. HeLa cells, transfected with HA-CXCR4 and either control siRNA (siControl), siRNA directed against human USP8 (siUSP8) or Hrs (siHRS) as indicated, were treated in the presence of 25 nM SDF1 for 5 hrs. Following treatment, cells were fixed and immunostained against CXCR4 (green) and either EEA1 (red) or Hrs (red). All scale bars correspond to 10 microns and the insets represent 9x magnification of the region selected within the original image. (D) Size comparison of endosomes populated by CXCR4 in cells depleted for USP8 or Hrs. Relative size of CXCR4-positive endosomes was calculated as described in the Materials and Methods; **** p<0.0001, n=3.

FIG. 4. USP8 catalytic activity modulates the integrity of the endocytic compartment at the ESCRT-0-positive sorting endosome. (A) Schematic representation of Bimolecular Fluorescence Complementation (BiFC) between USP8 and STAM2 (characterization of the BiFC complex formation is shown in Fig. S2). (B) Model of subcellular localization profile of the USP8/ESCRT-0 complex at the sorting endosome, between the early and late extremes. (C-E) Analysis of USP8 catalytic function in the context of the ESCRT-0 complex. HeLa cells co-transfected with VN-USP8 or its catalytic USP domain truncation mutant, ΔC, in combination with VC-STAM2 were fixed following incubation adequate for visualization of VFP fluorescence (7.5-9 hrs) and immunostained for early endosomal marker (D) EEA1 or (E) late endosomal marker CD63 (red) as indicated. ΔC mutant, containing amino acids 1-735 of murine USP8, was constructed on the basis of available structural data (54) and has been previously described (18). USP8/STAM BiFC complex was visualized by Venus Fluorescent Protein (VFP; green). All scale bars correspond to 10 microns and the insets represent 9x magnification of the region selected.
within the original image. (C) Colocalization of the USP8/STAM BiFC complex with early and late endosomal markers; n=3. Degree of overlap, expressed as a fraction of VFP colocalized with EEA1 or CD63, was calculated as described in Materials and Methods; **** p<0.0001, * p<0.5; n=3.

FIG. 5. Catalytic activity of USP8 promotes CXCR4 trafficking and downregulation. (A-C) Effects of wild type and catalytically inactive USP8 overexpression on cellular abundance and ligand-induced turnover of CXCR4. HEK293 cells co-transfected with HA-CXCR4 and either empty vector (Vector), wild type (WT), catalytically inactive point-mutant (C748A) or catalytic domain truncation mutant (ΔC) of USP8 were treated with 10 nM SDF1 in the presence of cycloheximide for the indicated length of time. CXCR4 abundance was monitored by western blot against HA. Western blot data for ΔC overexpression is provided in Fig. S3B. (B) Quantification of total HA-CXCR4 abundance expressed relative to untreated (time=0) control cells (Vector); **** p<0.0001, *** p<0.001, n=5. (C) CXCR4 degradation following 3-4 hrs of treatment with SDF1 was calculated as % of total HA-CXCR4 in untreated cells; n=3. (D-G) Effects of USP8 catalytic activity on the endocytic trafficking of CXCR4. HeLa cells transfected as above were incubated under standard growth conditions, fixed and immunostained against CXCR4 (green), Flag (not shown) and either (D) EEA1 (red) or (F) LAMP1 (red). All scale bars correspond to 10 microns and the insets represent 9x magnification of the region selected within the original image. Colocalization between CXCR4 and (E) EEA1 or (G) LAMP1 is expressed in the form of overlap coefficient, r, calculated as described in Materials and Methods; **** p<0.0001, ** p<0.01 n=2.

FIG. 6. USP8 inactivation does not affect the ubiquitination status of CXCR4. (A-C) Effects of USP8 inactivation on CXCR4 ubiquitination. (A) HEK293 cells co-transfected with HA-CXCR4, Flag-ubiquitin (Flag-Ub) and either siControl or siUSP8 were serum-starved and treated in the absence (-) or presence (+) of 100nM SDF1 for 30 min. HA-CXCR4 was immunoprecipitated and its ubiquitination status was assessed by western blot against Flag-Ub. (B) CXCR4 ubiquitination profile as a function of AIP4 and USP8 catalytic activities. HEK293 cells co-transfected with HA-CXCR4, Flag-Ub and vector, Myc-tagged AIP4 or its catalytic point-mutant, C830A, wild type USP8 or inactive mutant ΔC were treated as indicated and ubiquitination of HA-CXCR4 was evaluated as above. (C) Quantification of CXCR4 ubiquitination relative to control; **** p<0.0001, n=3. (D) Effects of USP8 manipulation on ligand-mediated ubiquitination of EGFR. HeLa cells co-transfected with HA-ubiquitin (HA-Ub) and vector control, USP8, ΔC, AIP4 or Cbl as indicated were serum-starved and treated in the absence (-) or presence (+) of 10 ng/ml EGF for 10 min. EGFR was immunoprecipitated and ubiquitination was analyzed by western blot against HA. (E) Effects of interplay between catalytic activities of USP8 and Cbl on EGFR ubiquitination status. HeLa cells were co-transfected with HA-Ub and vector or USP8ΔC alone or in combination with either catalytically inactive mutants of ubiquitination ligases, Cbl (CblΔC) or Nrdp1 (Nrdp1ΔC); EGFR ubiquitination was assayed as in D.

FIG. 7. USP8 modulates Hrs ubiquitination to regulate endosomal ubiquitin dynamics and trafficking through the ESCRT-0 checkpoint. (A) Effects of USP8 catalytic activity on ubiquitination of endogenous Hrs. HeLa cells co-transfected with HA-ubiquitin (HA-Ub) and vector, AIP4 or mutant C830A, USP8 or ΔC, as indicated; Hrs was immunoprecipitated and its ubiquitination status was assessed by western blot against HA-Ub. (B) Ubiquitin dynamics on ESCRT-0 endosomes as a function of USP8 catalytic activity. HeLa cells expressing Myc-ubiquitin, VC-STAM2 and either VN-USP8 or VN-ΔC constructs were fixed and immunostained with anti-Hrs (red) and anti-Myc (blue) antibodies. USP8/STAM2 complex was visualized by VFP (green) fluorescence. (C) Localization of internalized CXCR4 to dysfunctional endosomes as a result of USP8 inactivation targeted to the ESCRT-0 complex. HeLa cells co-expressing HA-CXCR4, VC-STAM2 and either VN-USP8 or VN-ΔC were fixed and immunostained with anti-CXCR4 (red) and anti-EEA1 (blue) antibodies. (D) Degree of overlap between the USP8/STAM2 complex and Hrs, Ubiquitin and CXCR4 as a function of USP8-mediated deubiquitination expressed as a fraction of total VFP; **** p<0.0001, *** p<0.001, n=3. 3 channel
overlays are displayed in white. All scale bars correspond to 10 microns and the insets represent 9x magnification of the region selected within the original image.
FIG. 1

A. Western blot analysis showing the levels of EGFR, USP8, CXCR4, and Actin in mock, siControl, and siUSP8 treatments.

B. Bar graph comparing the relative protein abundance of USP8, EGFR, and CXCR4 between siControl and siUSP8 treatments.

C. Western blot analysis of SDF1-treated cells showing the levels of HA-CXCR4, USP8, TrfR, and Actin for siControl and siUSP8 treatments.

D. Graph showing the abundance of CXCR4 over time for siControl and siUSP8 treatments.
FIG. 2

A. Steady-state

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<th>Neutavidin Ppt.</th>
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B. CXCR4 abundance (% of untreated)

- Total: siControl: 100, siUSP8: 250
- Surface: siControl: 50, siUSP8: 250

D. Relative pERK

- 10 min: siControl: 1.4, siUSP8: 0.3
- 60 min: siControl: 0.3, siUSP8: 0.3
FIG. 3

A. CXCR4  EEA1  MERGE
   siControl
   siUSP8

B. CXCR4  EEA1  MERGE
   siHRS

C. CXCR4  HRS  MERGE
   siControl
   siUSP8

D. Relative CXCR4-positive endosome size

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<td>siUSP8</td>
<td>4.5***</td>
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<tr>
<td>siHRS</td>
<td>4.5***</td>
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</table>

*Significant differences compared to siC control.

**** p < 0.0001
FIG. 4

A. USP8/STAM Complex

B. Recycling, Sorting Endosome, Early Endosome (EEA1), ESCRT-I/II/III, MVB, Late Endosome (CD63), USP8

C. Colocalization (fraction of overlap)

D. VN-USP8, VC-STAM2, VN-AC, VC-STAM2

E. VN-USP8, VC-STAM2, VN-AC, VC-STAM2
FIG. 5

A. SDF1-treated

<table>
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B. CXCR4 abundance (% of control)

![Graph](image10.jpg)

C. % CXCR4 degraded

![Graph](image11.jpg)

D. CXCR4, EEA1, MERGE

![Images](image12.jpg)

E. CXCR4, LAMP1, MERGE

![Images](image13.jpg)

F. Overlap coefficient 'r'

![Graph](image14.jpg)
FIG. 6

A.

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<tr>
<td>SDF1</td>
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</tr>
<tr>
<td>Flag-Ub</td>
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<tr>
<td>HA-CXCR4</td>
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B.

SDF1

Flag-Ub

IP: HA-CXCR4

HA-CXCR4

Myc

C.

![Graph showing CXCR4 ubiquitination](image)

D.

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E.

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FIG. 7

A.

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B.

VFP

HRS

Myc-ubiquitin

MERGE

C.

VFP

CXCR4

EEA1

MERGE

D.

Overlap (fraction of VFP)

VFP : Hrs

VFP : Ub

VFP : CXCR4
The deubiquitinating enzyme USP8 promotes trafficking and degradation of the chemokine receptor CXCR4 at the sorting endosome
Ilana Berlin, Katherine M. Higginbotham, Rebecca S. Dise, Maria I. Sierra and Piers D. Nash

J. Biol. Chem. published online September 27, 2010

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