USP32 regulates late endosomal transport and recycling through deubiquitylation of Rab7

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The endosomal system is a highly dynamic multifunctional organelle, whose complexity is regulated in part by reversible ubiquitylation. Despite the wide-ranging influence of ubiquitin in endosomal processes, relatively few enzymes utilizing ubiquitin have been described to control endosome integrity and function. Here we reveal the deubiquitylating enzyme (DUB) ubiquitin-specific protease 32 (USP32) as a powerful player in this context. Loss of USP32 inhibits late endosome (LE) transport and recycling of LE cargos, resulting in dispersion and swelling of the late compartment. Using SILAC-based ubiquitome profiling we identify the small GTPase Rab7—the logistical centerpiece of LE biology—as a substrate of USP32. Mechanistic studies reveal that LE transport effector RILP prefers ubiquitylation-deficient Rab7, while retromer-mediated LE recycling benefits from an intact cycle of Rab7 ubiquitylation. Collectively, our observations suggest that reversible ubiquitylation helps switch Rab7 between its various functions, thereby maintaining global spatiotemporal order in the endosomal system.
The endocytic pathway guards cellular homeostasis through a combination of controlled interactions with the extracellular world and regulated disposal of obsolete or harmful materials. Originating at the cell periphery, this pathway operates via a complex network of progressively maturing carrier vesicles. As early endosomes (EEs) move towards the interior of the cell, they acquire late endosomal (LE) characteristics and become poised to deliver select cargoes for degradation in the lysosome. To protect the endosomal system from the ravages of toxic lysosomal contents, the LE has evolved a gatekeeper function predicated on packaging cargoes destined for degradation into intraluminal vesicles (ILVs). The resulting multi-vesicular body (MVB) serves both as a platform for commitment of cargoes for degradation and as the last point of retrieval. In this way, the MVB constitutes the control center of the endosomal system, with its morphologic and functional integrity bearing directly upon the vesicular network as a whole.

Despite—or perhaps precisely because of—its central position within the endosomal system, cargo and membrane dynamics at the MVB are highly complex, and the manner in which different sorting and trafficking pathways are integrated to best serve its many functions is poorly understood. Over the years, reversible post-translational modification with ubiquitin, orchestrated through the opposition between ligases and deubiquitylating enzymes (DUBs), has become recognized as a powerful tool for spatial and temporal control of multi-protein complex assembly central to endosome biogenesis and function. This concept is best illustrated by the profound dependence of endosomal sorting complexes required for transport (ESCRT) on various ubiquitin signals, including ubiquitylation of cargoes as well as ESCRT proteins themselves. Cargo sorting to various destinations is further linked to vesicle trafficking carried out by small membrane-associated GTPases. These molecular switches, coupled to discrete vesicular maturation states, direct endosomal transport, fusion, and fission events, ensuring that this diverse system of vesicles moves and functions in an orderly fashion.

Ubiquitylation of several endosomal GTPases has been reported, including EE-bound Rab5 and LE/MVB-associated Rab7. Particularly in the case of Rab7—the principal director of membrane traffic to and from proteolytic compartments—the way(s) in which addition and removal of ubiquitylation inform various functions of this GTPase remain obscure. Once Rab7 takes residence on the limiting LE membrane, it can recruit a variety of effector proteins to facilitate diverse processes. These effectors include Rab7-interacting protein (RILP) utilized for anterograde vesicle transport (toward the nucleus) and pleckstrin homology domain-containing family M member 1, along with the associated homotypic fusion and protein sorting complex, for fusion. In addition to transport, Rab7 can also direct recycling from the LE membrane to the trans-Golgi network (TGN) and the plasma membrane by cooperating with the retromer complex. This begs the question of how Rab7 toggles between anterograde transport and recycling without plunging the MVB into chaos.

In this study, aiming to decipher this conundrum, we consider whether yet undiscovered layers of regulation of ubiquitin dynamics at the MVB membrane influence key decisions in this organelle’s biology. Ubiquitylation of Rab7 has recently been shown to promote its association with the retromer and result in extension of tubules from the limiting membrane of the MVB in opposition to ILV formation. Notably, however, no DUB has previously been reported to target Rab7. In a depletion screen for human DUBs affecting surface expression of the LE cargo receptor major histocompatibility class II (MHC-II), we identify USP32 as a powerful regulator of late compartment localization, morphology, and function. Using proteome-wide ubiquitin remnant profiling, we reveal Rab7 to be a key substrate of USP32 and go on to show that USP32 supports Rab7 functions in transport and recycling from the MVB by two different mechanisms. Taken together, our results underscore the nuanced ways in which reversible ubiquitylation can contribute to the ordered complexity of endosomal membrane dynamics.

Results

DUB screen for endosomal regulators identifies USP32. In pursuit of ubiquitin-dependent mechanisms in the regulation of endosomal processes, we performed a small interfering RNA (siRNA)-based screen for DUBs affecting surface levels of MHC-II receptor—a molecule known to traverse the endosomal tract, accumulating in MVBs—in human melanoma MelJuSo cells (Fig. 1a). A number of DUBs previously implicated in endosomal organization and cargo trafficking were picked up with this approach, including a key endocytic regulator USP8, depletion of which resulted in lower levels of MHC-II at the cell surface (Fig. 1a). After USP8, the top hit incurring diminished MHC-II surface levels was USP32 (Fig. 1a, Supplementary Fig. 1a, b)—a DUB from the same catalytic family whose cellular function was yet to be described. We hypothesized that DUBs whose loss results in lower receptor surface levels are likely to constitute regulators of endocytic traffic downstream of internalization.

To identify these, we further selected our DUB hits on the basis of two intracellular criteria: alterations in (i) distribution and (ii) size of endosomes (Supplementary Fig. 1c, d), taking advantage of the expansive endosomal system in MelJuSo cells, thereby converting into a crowded perinuclear (PN) vesicle cloud and a sparse peripheral contingent. Once again, a striking phenotype was observed with knockdown of USP32 characterized by profound dispersion and swelling of endosomes carrying MHC-II (Fig. 1b–d; Supplementary Fig. 1c, d). Swollen MHC-II endosomes were predominantly late in character, as evidenced by their positivity for the LE marker CD63 (as opposed to the EE marker EEA1), but also contained mannose-6-phosphate (M6PR) receptor (Fig. 1b–d; Supplementary Fig. 1e), which cycles between endosomes and TGN. Taken together, altered localization, size, and cargo profile of endosomes affected by the absence of USP32 pointed to broad-spectrum defects in the late compartment.

To test the functionality of the endocytic pathway and its proteolytic competency under suppression of USP32 activity, we examined ligand-mediated trafficking and degradation of the epidermal growth factor (EGF) receptor (EGFR). In HeLa cells treated with control siRNAs, following ligand stimulation, activated EGF receptor trafficking and degradation of its carrier endosomes occurs. These attributes were disrupted under conditions of USP32 knockdown, as evidenced by dispersion of the endosomal compartment induced by EGF stimulation (Fig. 2a, b) and redistribution of mature EGF-positive structures (i.e., those overlapping with the LE marker CD63) towards the periphery of the cell (Fig. 2a–c). Because proteolytic endosomes and lysosomes are known to largely reside in the PN region, we hypothesized that, in the absence of USP32, encounter of activated EGF with the principal proteolytic enzyme cathepsin D could be hampered. Indeed, a large proportion of EGF-positive endosomes were devoid of cathepsin D in cells depleted of USP32, unlike those in control cells (Fig. 2d, e). Consequently, ligand-mediated degradation of EGF was strongly inhibited by loss of USP32, leading to prolonged receptor activation (Fig. 2f, g; Supplementary Fig. 2). Taken together, these results implicate USP32 activity in the regulation of the endosomal system’s architecture, dynamics, and function.
**Fig. 1** Deubiquitylating enzyme (DUB) screen reveals USP32 as a regulator of endosome biology. 

**a** Small interfering RNA (siRNA)-based screen for DUBs affecting major histocompatibility class II receptor (MHC-II) surface levels. MelJuSo cells transfected with siRNAs targeting all human DUBs were analyzed for surface expression of peptide-loaded MHC-II by flow cytometry using monoclonal antibodies (L243-Cy3). Z-scores of DUBs whose depletion resulted in elevated (Z ≥ 3) or diminished (Z ≤ −3) levels of MHC-II on the cell surface are plotted, n = 3 biologically independent samples. Effect of USP32 depletion is highlighted by a red box.

**b** Effect of USP32 loss on the size and distribution of endosomes. Representative confocal overlays of fixed MelJuSo cells transfected with either control (siCtrl) siRNA or oligo #2 targeting USP32 (siUSP32_2) and immunostained against MHC-II (green) and vesicular markers or cargoes (magenta) are shown. EEA1: early endosome (EE) marker, CD63 late endosome (LE)/multi-vesicular body (MVB) marker, mannose-6-phosphate receptor (M6PR): trans-Golgi network (TGN) cargo. Transferrin receptor (TrfR): recycling endosome (RE) marker; PM: plasma membrane. Cell and nuclear boundaries are demarcated with dashed white lines, scale bars = 10 µm. Zooms Z1–Z3 are placed within a schematic of cargo flow.

**c** Percentage cells harboring enlarged MHC-II-positive vesicles, n = 3 independent experiments. Immunoblot of USP32 protein expression in response to depletion using two independent siRNA oligos (siUSP32_1 and siUSP32_2) is provided with actin as loading control.

**d** Vesicle dispersion expressed as fractional distance of MHC-II pixels (black open circles) along a straight line from the center of nucleus (0) to the PM (1.0). Red lines: mean, n = 2 independent experiments. Total number of cells analyzed per condition appears above each bar/scatter. Bar graphs report mean of independent sample values (black circles), error bars reflect ±s.d. All significant values were calculated using Student’s t test: **p < 0.01, ***p < 0.001, NS = not significant. See also Supplementary Fig. 1
**Fig. 2** Loss of USP32 disrupts cargo trafficking and lysosomal proteolysis. **a**–**e** Effect of USP32 depletion on ligand-mediated trafficking and degradation of epidermal growth factor (EGF) receptor (EGFR). a Representative confocal z-projections of fixed HeLa cells transfected as indicated, starved and stimulated with 100 ng/mL EGF-555 (white) for 120 min. Perinuclear (PN) and peripheral (PP) insets show overlays of EGF (green) with immunostained CD63 (magenta). b EGF-positive pixel distribution expressed as fractional distance along a straight line from center of nucleus (0) to the PM (1.0). Red lines: mean, n = 2 independent experiments. c Colocalization of EGF with CD63 in PN (left), PP (middle), and overall (right) in control cells (siCtrl, white bars) vs. those depleted of USP32 (siUSP32_2, gray bars), n = 2 independent experiments. d Representative confocal images of fixed HeLa cells transfected as indicated, starved and stimulated with EGF-555 (white) for 120 min. PN and PP insets show overlays of EGF (magenta) with immunostained cathepsin D (green). e Colocalization of EGF with cathepsin D in control cells (siCtrl, white bars) vs. those depleted of USP32 (siUSP32_2, gray bars), n = 3 independent experiments. All colocalization plots report Mander’s overlap quantified from multicell images (black circles). Cell and nuclear boundaries are depicted in dashed magenta and white lines, respectively. Scale bars = 10 μm. f, g Effect of USP32 depletion on ligand-induced degradation of EGFR. f Lysates from HeLa cells transfected as indicated, serum starved, and stimulated with EGF (25 ng/mL) for 0, 30, 60, or 120 min were analyzed by immunoblot against total EGFR (rabbit anti-EGFR) and phosphorylated (pY) EGFR (mouse anti-phosphotyrosine 4G10), with actin as a loading control. g Total (left graph, relative to t = 0) and activated (right graph, pY relative to t = 30) EGFR remaining at 120 min following stimulation in control cells (siCtrl) vs. those depleted of USP32 using different siRNA oligos (siUSP32_2 and siUSP32_3 + 4), n = 3 independent experiments. Bar graphs report mean of independent measurements (black circles), error bars reflect ±s.d. Total number of cells analyzed per condition appears above each bar/scatter. All significance calculated using Student’s t test: *p < 0.05, **p < 0.01, and ***p < 0.001. See also Supplementary Fig. 2.
USP32 is a membrane-associated catalytically active DUB. To understand the how USP32 functions in endosome biology, we began by investigating its cellular localization. Endogenous as well as ectopically expressed USP32 was found to associate with the perinuclear TGN and highly dynamic peripheral vesicles (Fig. 3a; Supplementary Fig. 3a, b and Movies 1, 2). We therefore examined the interplay between the LE and TGN as a function of USP32. While in normal cells frequent transient interactions between these compartments were readily observed (Supplementary Movie 3 and 4), loss of USP32 caused TGN-derived membranes to remain stuck on enlarged acidified endosomes (Supplementary Movie 5, 6). Similar findings were observed for depletion of USP8 (Supplementary Movie 7, 8), which has previously been implicated in regulating LE-to-TGN traffic. The vesicular pathway connecting the LE with the TGN is central to the biogenesis of proteolytic organelles and is frequented by the

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**Fig. 3** Catalytic activity of USP32 supports endosomal system’s architecture. 

**a** Localization of USP32 to the trans-Golgi network (TGN). Left panels: representative confocal images of fixed MelJuSo cells stably expressing TGN46-GFP (green) and immunostained against endogenous USP32 (magenta). Cell and nuclear boundaries are demarcated with dashed white lines, scale bars = 10 µm. Right panels: Electron micrographs of sections co-labeled with anti-USP32 (10 nm gold) and anti-GFP (15 nm gold), scale bar = 0.25 µm. See also Supplementary Fig. 3 and Movies 1–8. 

**b** Top panel: schematic representation of USP32 domain organization: EF, calcium-binding domain; DUSP, domain found in ubiquitin-specific proteases (USP); UBL, ubiquitin-like domain; USP, catalytic domain harboring the principal catalytic residue C743. Bottom panel: in vitro cleavage of di-ubiquitin linkages (M1, K6, K11, K27, K29, K33, K48, and K63) by the catalytic domain (CD) of USP32. See also Supplementary Fig. 4. 

**c** DUB activity-based probe assay performed on lysates of HEK293T cells expressing USP32-HA or catalytic mutant C743A-HA in the absence (−) or presence (+) of Cy5-Ub-Prg probe. Reaction products were analyzed using in-gel fluorescence scanning followed by immunoblot against HA; * indicates USP32-HA labeling. 

**d** Representative confocal images of fixed MelJuSo cells transfected as indicated and immunostained against major histocompatibility class II (MHC-II) (green) and HA (magenta) are shown with the corresponding zooms. Cell and nuclear boundaries are demarcated with dashed white lines. Arrows point to HA-positive puncta juxtaposed to endosomes, scale bars = 10 µm. 

**e** Enlargement and dispersion of MHC-II-positive vesicles reported as % cells. Bars depict mean of n = 3 independent experiments (black circles), error bars reflect ±s.d., total number of cells analyzed per condition appears above each bar. Immunoblot against USP32 is shown with actin as a loading control. All significance was calculated using Student’s t-test: ***p < 0.001, NS = not significant.
proteolytic enzyme carrier mannose-6-phosphate receptor (M6PR)\textsuperscript{30,31}. In control cells pulsed with an antibody recognizing M6PR, efficient trafficking to the PN region was observed during the chase period. By contrast, in cells depleted of USP32, anti-M6PR remained dispersed in vesicular structures (Supplementary Fig. 3c, d), implying a defect consistent with mislocalization of M6PR to enlarged endosomes described in Fig. 1b, d. Taken together with perturbations in trafficking and downregulation of EGFR (Fig. 2), these results position USP32 at a crossroads of multiple vesicular pathways converging at the MVB.

To begin dissecting the nature of USP32 DUB function and its role in endosome biology, we performed biochemical characterization of USP32 catalytic activity. Both the full-length enzyme and its C-terminal fragment harboring the USP domain readily catalyzed mono- and di-ubiquitin substrates in vitro (Fig. 3b; Supplementary Fig. 4a, b)\textsuperscript{32}. Although USP32 was recently reported to interact preferentially with K6- and K29-linked ubiquitin chains\textsuperscript{33}, our analysis of all ubiquitin linkage types (M1, K6, K11, K27, K29, K33, K48, and K63) did not reveal striking cleavage preferences by this DUB (Fig. 3b; Supplementary Fig. 4b). As expected, wild-type USP32, but not its catalytic mutant C743A, ectopically expressed in mammalian cells, labeled with a DUB activity-based probe (Fig. 3c), and this catalytic determinant proved necessary to afford rescue of USP32 depletion with respect to both size and localization of endosomes carrying MHC-II (Fig. 3d, e). Unable to rescue loss of the endogenous enzyme, catalytically inactive USP32-C743A localized to discrete patches on enlarged MHC-II vesicles (Fig. 3d, e), suggesting that its activity likely targets endosomal constituents.

LE GTPase Rab7 is a substrate of USP32. To identify relevant USP32 substrates, we performed proteome-wide ubiquitome analyses under conditions of varying USP32 abundance. Stable isotope labeling of amino acids in cell culture (SILAC), followed by purification using antibodies recognizing Lys-$\varepsilon$-Gly-Gly remnants of ubiquitylated proteins subjected to trypsin digestion\textsuperscript{34}, was used to profile and compare ubiquitomes derived from different samples (Fig. 4a). Two complimentary perturbations were tested: (i) depletion and (ii) overexpression of USP32 (performed in MelJuso or HeLa cells, respectively). Given our interest in endosomes, we focused further analysis of the data sets on proteins known to function in this pathway. Interestingly, changes in the ubiquitination status of the LE master regulator small GTPase Rab7A, henceforth referred to simply as Rab7, were detected in response to altered USP32 abundance. Elevated ubiquitylation of its lysine 191 was observed with USP32 depletion using the SILAC strategy (Fig. 4b; Supplementary Fig. 3a),

**Fig. 4** Ubiquitome analysis reveals small GTPase Rab7 as a substrate of USP32. a Schematic representation of stable isotope labeling of amino acids in cell culture (SILAC)-based quantitative mass spectrometry (LC-MS/MS) workflow used to compare ubiquitylated proteomes of b control MelJuSo cells (siCtrl, green) vs. those where USP32 was knocked down (KD, siUSP32_2, magenta) and c HeLa cells overexpressing (OE) USP32-HA (magenta) vs. vector control (green). Cell growth media types: K0R0, light; K4R6, medium; K8R10, heavy. IP: immunoprecipitation, m/z: mass to charge ratio. b, c Volcano plots comparing abundance of detected peptides carrying a GlyGly (GG) modification expressed as Log 2 ratios of b USP32 knockdown (siUSP32_2, KD) vs. control (siCtrl, CTRL), n = 1 SILAC sample set independently validated using label-free quantitation (LFQ) with n = 2 biologically independent samples or c USP32-HA overexpression vs. vector control (Ctrl), n = 1 SILAC sample set. Small GTPases implicated in vesicular trafficking whose modified peptides were detected are labeled according to their respective Log 2 ratios: magenta $>$1; green $<$−1; blue between −1 and 1, not significant. Analysis was performed using MaxQuant and Perseus software tools as described in the Methods under ubiquitome analysis. All mass spectrometry (MS) data can be accessed via the PRIDE repository (PXD011899). d Ubiquitylation status of GFP-Rab7 vs. GFP-Rab5 as a function of USP32 catalytic activity. GFP-Rabs, immunoprecipitated (IP) from HEK293T cells coexpressing HA-Ub and either USP32, C743A, or neither, was assessed by immunoblot against HA; WCL: whole cell lysate. See also Supplementary Fig. 5.
and this alteration was confirmed in a label-free setting (PRIDE dataset identifier PXD011899). Conversely, ubiquitylation on the same residue diminished in the presence of overexpressed USP32 (Fig. 4c). To confirm Rab7 as a bona fide substrate of USP32, we performed an array of validation experiments. A discrete pattern of mono- and/or di-ubiquitin conjugates was observed on a proportion of affinity isolated GFP-Rab7 ectopically expressed in the presence of HA-tagged ubiquitin (HA-Ub). This modification strongly diminished upon coincubation with purified USP32, but not its family member USP30 (Supplementary Fig. 5b–d).

Furthermore, coexpression of GFP-Rab7 in cells with wild-type USP32, but not its enzymatically dead mutant C743A, ablated ubiquitylation on GFP-Rab7 when compared to vector control (Fig. 4d; Supplementary Fig. 5e). In contrast to Rab7 (and in agreement with the proteomic analysis above), no deubiquitylation of GFP-Rab5 by USP32 was observed under the same reaction conditions (Fig. 4d; Supplementary Fig. 5e).

**USP32 promotes endosomal transport via the Rab7/RILP axis.** Having established that USP32 can deubiquitylate Rab7 in vitro...
and in situ, we sought to understand the interplay between USP32 and Rab7 in endosome biology. Depletion of USP32 incurred dispersion and swelling of vesicular structures decorated with endogenous Rab7 (Fig. 5a, b; Supplementary Fig. 5f), similar to the effects observed for MHC-II and CD63 (Fig. 1b–d). These phenotypes were not seen for endosomes marked by Rab5 (Fig. 5a, b; Supplementary Fig. 5g). Besides the obvious increase in size (Fig. 5c, d; Supplementary Fig. 6a), Rab7-positive LEs affected by loss of USP32 appeared to exhibit abnormal intraluminal content (Supplementary Fig. 6b), consistent with aberrant delivery/retrieval of materials to/from the MVB. Additionally, depletion of Rab7 itself was associated with both mis-localization and enlargement of vesicles carrying MHC-II (Fig. 5e; Supplementary Fig. 7a), implying that Rab7 and USP32 regulate a common biological process. By contrast, loss of Rab5 did not result in swollen MHC-II endosomes (Fig. 5e; Supplementary Fig. 7a), indicating that late compartment swelling is not a general phenotype of endosomal Rab deficiency. Notably, depletion of USP32 did not alter cellular abundance of endogenous Rab7 (Fig. 5f), indicating that the phenotypes caused by USP32 loss are not due to ubiquitylation-dependent degradation of its substrate. On the other hand, silencing USP32 resulted in the accumulation of membrane-bound Rab7, as evidenced by elevated membrane-to-cytosol ratio relative to the control (Fig. 5g, h). This suggested that deubiquitylation of Rab7 by USP32 promotes release of Rab7 from the membrane to the cytosol, where a new functional cycle of this Rab can commence (Fig. 5i).

Based on the observations described above, we hypothesized that diminished availability of cytosolic Rab7 could inhibit Rab7-dependent LE dynamics. We therefore followed the behavior of Rab7-positive endosomes as a function of USP32 in living cells. As expected, under control conditions, vesicles carrying GFP-Rab7 partitioned between a crowded and relatively immobile PN vesicle cloud and a sparsely populated but highly motile peripheral fraction (Fig. 6a–c; Supplementary Movie 9). Loss of USP32 disrupted this PN/PP dichotomy and resulted in an inhibition of LE motility (Fig. 6a–c; Supplementary Movie 10). Loss of RILP co-isolated better with Rab7-2KR as compared to wild-type Rab7 (Fig. 6d, e). On the other hand, depletion of USP32 slightly diminished complex formation between RILP and wild-type Rab7 (Fig. 6f; Supplementary Fig. 7d). Taken together, these findings demonstrate that RILP favors Rab7 whose C-terminal lysine(s) are not modified with ubiquitin, providing a rationale for how Rab7 mutant lacking these moieties can partly restore LE localization and dynamics in cells compromised for USP32.

USP32 promotes membrane recycling from the Rab7 endosome. Among many roles of Rab7 at the LE/MVB facilitated by its canonical effectors such as RILP, this GTPase is also known to partner with the retromer complex to regulate recycling away from late compartments in agreement with a previous report, we found that USP32 interacts with VPS35, the principal cargo-selective component of the retromer, and partly localizes to structures positive for both VPS35 and Rab7 (Supplementary Fig. 8a, b). Additionally, silencing either VPS35, or its retromer partner VSP26, recapitulated LE dispersion and swelling observed with disruption of USP32 activity (Fig. 7a, b) similar to the effects observed for silencing of Rab7 (Fig. 5e; Supplementary Fig. 7a). As in the case of Rab7, depletion of USP32 had no effect on protein levels of either VPS35 or VPS26 (Fig. 7c) and, taken together with the observations above, signaled that the retromer complex and USP32 likely function in the same pathway with respect to membrane dynamics at the LE/MVB.

We next examined whether USP32 regulates the interplay between Rab7 and the retromer complex on endosomes. Depletion of USP32 gave rise to swelling of the VPS35-positive vesicular compartment and accumulation of VPS35 on endosomes carrying MHC-II (Fig. 7d, e), suggesting that increased ubiquitylation on Rab7 stabilizes VPS35 on endosomes. This
supposition was reinforced by the observation that in cells depleted of endogenous Rab7, endosomes marked by GFP-Rab7-2KR exhibit less contact with structures harboring VPS35, as compared to those with wild-type GFP-Rab7 under the same conditions (Supplementary Fig. 8c–e). To probe whether ubiquitylation of Rab7 affects its relationship with VPS35, we fused a promiscuous biotin ligase domain BirA 40 to the N terminus of Rab7 or its mutant 2KR (following the GFP tag) and co-expressed these fusion constructs with RFP-VPS35. Following biotin addition, biotinylation of VPS35 was detected in the presence of GFP-BirA-Rab7 above the GFP-BirA control (Fig. 7f), which diminished when GFP-BirA-2KR was expressed instead (Fig. 7f, g). Conversely, depletion of USP32 improved biotinylation of VPS35 in the presence of GFP-BirA-Rab7 (Fig. 7h; Supplementary Fig. 8f). In the same experiment, labeling of endogenous USP32 with biotin was also observed, demonstrating
Deubiquitylation of Rab7 by USP32 promotes late endosome transport. a Late endosome (LE) organization and dynamics as a function of Rab7 ubiquitination status. Top panels: representative confocal images of live MelJuSo cells stably expressing GFP-Rab7 or GFP-Rab7-2KR (GFP-2KR) (white) taken at the start of time-lapse, f = 0. Scale bars = 10 μm. Bottom panels: vesicle displacement rates depicted on a rainbow color scale (blue: immobile; red: maximum mobility per time interval) tracked over 250 s at 5 s per frame. Cell and nuclear boundaries are depicted in dashed white lines, boxed zoom-ins highlight selected perinuclear (PN) and peripheral (PP) regions. b Vesicle dispersion expressed as fractional distance of GFP pixels along a straight line from center of nucleus (O) to the PM (1.0). Red lines: mean, n = 2 independent experiments. c Quantification of vesicle motility calculated using TrackMate for Fiji (for details see the Methods section), n = 3 independent experiments. Plots report mean velocities of Lysotracker-positive structures calculated from multicolor time-lapses of control cells (siCtrl, open circles) vs. those depleted of USP32 (siUSP32_2, closed circles). See also Supplementary Fig. 7b, c and Movies 9–12. d–f Co-immunoprecipitation (Co-IP) of Rab7-interacting protein (RILP) with GFP-Rab7 as a function of Rab7 ubiquitination status. e Immunoblot of Co-IP from HEK293T cells transfected and treated as indicated. f Quantification of Co-IP for HA-RILP with GFP-Rab7 (white bars) vs. GFP-2KR (gray bars), n = 3 independent experiments, is shown along with a schematic summary. g Quantification of Co-IP for HA-RILP with GFP-Rab7 from control HEK293T cells (siCtrl, white bars) vs. those depleted of USP32 (siUSP32_2, gray bars) normalized to control, n = 3 independent experiments. See also Supplementary Fig. 7d. Bar graphs report mean of independent measurements (black circles), error bars reflect s.d. Where applicable, total number of cells analyzed per condition appears above each bar/scatter. All significance was calculated using Student’s t test: *p < 0.05, **p < 0.01, ***p < 0.001, NS = not significant.

Discussion

Ubiquitylation provides key signals in endocytosis by directing cargo for lysosomal degradation and modulating the functionality of sorting machineries. In turn, deubiquitylation offers a necessary counterforce, imparting spatiotemporal controls to these dynamic processes. Strikingly, out of nearly 100 human DUBs, only a handful have been implicated in the endocytic pathway. To evaluate whether additional DUBs are entrusted with its upkeep, we performed a depletion screen, leading to the identification of USP32 as a potent regulator of the endolysosomal compartment. In cells lacking USP32, these vesicles exhibit an array of architectural and functional defects, including aberrant localization, structure, and motility, as well as compromised resolution of recycling tubules and attenuated cargo proteolysis. Collectively, these deficiencies exemplify the multifaceted impact of USP32 on membrane traffic and underscore the utility of reversible ubiquitylation in the regulation of dynamic cellular processes.
ubiquitylation and subsequent deubiquitylation of Rab7 work in concert to mediate efficient recycling from the LE membrane. Two other DUBs—USP8 and USP7—have also been implicated in various aspects of retromer-associated extraction of cargoes from endosomes. Our study reveals an additional regulatory mechanism for this complex and dynamic process, exemplifying the diversity of spatiotemporal regulation through reversible ubiquitylation.

Collectively, our findings establish USP32 as a key component of the molecular repertoire entrusted with guarding the health of the endolysosomal system. Through investigating the effects of USP32 on its substrate Rab7, we show that Rab7-associated endosomal processes depend not only on its GTP-based state but also on its modification(s) with ubiquitin. In addition to Rab7-driven processes of transport and recycling, the severe nature of USP32 loss-of-function phenotypes leaves open the possibility that additional facets of endosomal traffic and membrane dynamics could be affected. Recently, a number of studies have implicated USP32 in the pathogenesis of various cancers, as well as Parkinson’s disease. Since the latter, in particular, is associated with defects in endosomal membrane dynamics, our study may set the groundwork for understanding and ultimately
targeting the involvement of USP32 in this context. On the other hand, association of USP32 with diverse ailments suggests far-reaching cellular activities, possibly independent of Rab7, and elucidating these in the future could provide new avenues for drug discovery.

Methods

Cell culture and transfections. MelSuSo (human melanoma) cells, kindly provided by Prof. G. Riemthuller (LMU, Munich), were cultured in Iscove's modified Dulbecco's medium (IMDM, Gibco) supplemented with 7.5% fetal calf serum (FCS, Greiner). Human HEK293T (Cat# ATCC®) cell lines stably expressing GFP-TGN46 or GFP-Rab7 were generated by clonal screening by way of the retromer complex. Representative confocal images of fixed MelSuSo cells transfected with the indicated siRNAs and immunostained against major histocompatibility class II (MHC-II) (white) are shown with the corresponding immunoblot analyses; targeting small interfering RNA (siRNA): (+) control siRNA; (−): Cell and nuclear boundaries are depicted in dashed magenta and white lines, respectively. Percent cells harboring dispersed (gray bars) and/or enlarged (white bars) MHC-II-positive vesicles in response to VPS35 and VPS26 depletion, n = 3 independent experiments. **Effects of USP32 depletion on cellular abundance of endogenous VPS35 and VPS26, as assessed by immunoblot. d, Effects of Rab7 ubiquitylation status on the retromer compartment. d, Representative confocal images of fixed MelSuSo cells transfected with the indicated siRNAs and immunostained for endogenous VPS35 (white). Boxed perinuclear (PN) and peripheral (PP) region overlays of VPS35 (magenta) with MHC-II (green) highlight retromer/LE interactions. Cell and nuclear boundaries depicted in dashed magenta and white lines, respectively. e, Colocalization between VPS35 and MHC-II in control cells (white bars) vs. those depleted of USP32 (gray bars). Plots report Mander's overlap calculated from multicolor images (black circles) taken from n = 3 independent experiments. See also Supplementary Fig. a-e. h, Effect of Rab7 ubiquitylation status on its interaction with VPS35 as measured by proximity-based labeling with biotin. i, Biotinylation of GFP-VPS35 in the presence of free GFP-BirA (−), GFP-BirA-Rab7 (WT) vs. GFP-BirA-2KR (2KR) assayed in HEK293T cells. j, Biotinylation of GFP-VPS35 by GFP-BirA-Rab7 (white bar) vs. GFP-BirA-2KR (gray bar) above GFP-BirA background control, n = 3 independent experiments. k, Quantification of endogenous VPS35 and VPS26 and VPS35 biotinylation (combined) by GFP-BirA-Rab7 above GFP-BirA control in HeLa cells (siCtrl, white bar) vs. those depleted of USP32 (siUSP32_2, black bar), n = 5 independent experiments. Bar graphs report mean, error bars reflect s.d. See also Supplementary Fig. b.f. All significant values were calculated using Student's t-test: *p < 0.05, **p < 0.01, NS = not significant. Sale bars = 10 µm.

Antibodies and fluorescent dyes. The following antibodies were used for confocal microscopy: mouse anti-USP32 A-10 (Santa Cruz Biotechnology, Cat sc-37445; 1:100), rabbit anti-human HLA-DR (1:300) for MelJuSo cells, mouse anti-TRK (Abcam, Cat# ab63759; 1:500), rabbit anti-USP32 A-10 (Santa Cruz Biotechnology, Cat# sc-37445) in (Santa Cruz Biotechnology, Cat# sc-374465), rabbit anti-VPS26 (Abcam, Cat# ab2733; 1:500), rabbit anti-CI-M6PR (Abcam, Cat# ab2733; 1:500), mouse anti-CI-M6PR (Abcam, Cat# ab30109; 1:500), mouse anti-cathepsin D (Abcam, Cat# ab75852; 1:50), and rat anti-HA (Roche, Cat# 3F10, 1:300). For detection by confocal microscopy, primary antibody incubation 1 h at RT was followed by incubation with appropriate secondary goat anti-mouse Alexa 488/568/647 (Thermo Fisher Scientific, Cat# A-11004, 1:2136, respectively), donkey anti-rabbit Alexa 488/568/647 (Thermo Fisher Scientific, Cat# A-11004/A-31571/A-21245, respectively), donkey anti-mouse Alexa 488/647/750 (Thermo Fisher Scientific, Cat# A-21206/A-31571, respectively), donkey anti-rabbit Alexa 488/647 (Thermo Fisher Scientific, Cat# A-21206/A-31571, respectively), donkey anti-gold Alexa 568 (Thermo Fisher Scientific, Cat# A-11057), and donkey anti-rat Alex 568 (Bioviz, Cat# 20092) in a 1:300 dilution. Lysotracker DeepRed (Thermo Fisher Scientific, Cat# L12492, 1:10,000) was used for detection of acidified compartments by confocal microscopy in live samples. One hundred nanograms per milliliter final concentration of EGFP-Alexa 555 (100 µg, Thermo Fisher Scientific, Cat# E35350) was used in endocytosis assays. The following antibodies were used for detection of endosomal antigens and overexpressed proteins by Western blot analysis in a 1:1000 dilution: mouse anti-USP32 A-10 (in Santa Cruz Biotechnology, Cat sc-374465), rabbit anti-VPS26 (Abcam, Cat# ab181352; 1:1000), goat anti-VPS35 (Abcam, Cat# ab10099; 1:50), mouse anti-CI-M6PR (Abcam, Cat# ab2733; 1:100), rabbit anti-Rab7 (Cell Signaling, Cat# 9367c, 1:100), rabbit anti-Rab5 (Cell Sig-

DNA constructs. USP32-GFP construct was used as a template to amplify USP32 for cloning into mGFP-N1 and 2xHA-N1 mammalian expression vectors using BamHI/HindIII restriction sites. For in vitro protein expression, USP32 full-length (FL) and catalytic domain (CD) were cloned into pFastBacNK-His-3C-LIC-amp using ligation-independent cloning. HA-Ub, GFP-Rab7, HA-
Cat# MMS-101R), and anti-mRFP®. Mouse anti-β-actin (Sigma-Aldrich, Cat# A5441) was used as a loading control in a 1:10,000 dilution for Western blot.

Secondary IRDye 680LT donkey anti-goat IgG (H+L) (LI-COR, Cat# 926-68024, 1:20,000), IRDye 800CW goat anti-rabbit IgG (H+L) (LI-COR, Cat# 926-32211, 1:5000), IRDye 800CW goat anti-mouse IgG (H+L) (LI-COR, Cat# 926-32210, 1:5000), IRDye 680LT goat anti-rabbit IgG (H+L) (LI-COR, Cat# 926-68021, 1:20,000), and IRDye 680LT goat anti-mouse IgG (H+L) (LI-COR, Cat# 926-68020, 1:20,000) were used for detection using the Odyssey Classic imager (LI-COR).

siRNA-based DUB screen. All human DUBs were individually depleted from MelJuSo cells by transfection with pools of siRNAs in 96-well format as follows: 96-well plates—20 µL transfection mix plus 80 µL cell suspension. After 72 h, cells were analyzed for surface expression of peptide-loaded MHC-II by flow cytometry.
using L243-Cy3 monoclonal antibody. The data were normalized and converted into Z-scores. Those DUBs whose depletion was found to perturb MHC-II surface levels at or above the chosen Z-score threshold of ±3 were subsequently analyzed for intracellular MHC-II phenotypes by microscopy scored on two criteria: (i) size and (ii) distribution of vesicles carrying MHC-II. Those DUB hits, which showed vesicle enlargement and/or redistribution, were followed upon with deconvolution using phenocopy by at least two independent siRNA oligos as minimal criteria.

Confocal microscopy. For fluorescence confocal microscopy of fixed samples, cells were seeded into 24-well plates (Costar, Cat# 3524) containing glass coverslips (Menzel Gläser, Cat# MENZCB00130RAC) and transfected as indicated. Fixation was performed in 3.7% formaldehyde (acid-free, Merck Millipore) in phosphate-buffered saline (PBS) for 20 min. After washing 3× with PBS, samples were permeabilized using 0.1% Triton X-100 (T8787, Sigma-Aldrich) in PBS for 10 min, followed by two washes with PBS. After permeabilization, cells were blocked with 5% (w/v) skim milk powder (LP0031, Oxiod) in PBS for 30 min and incubated with the appropriate primary antibodies diluted in blocking buffer at dilutions described above for 1 h at RT. Samples were then washed in PBS (three times for 5 min) and incubated with the appropriate secondary anti-rabbit/mouse/rat Alexa-dye-coupled antibodies (Invitrogen) in blocking buffer for 30 min. After washing in PBS (three times for 5 min), cells were mounted using ProLong Gold antifade Mounting medium with DAPI (Life Technologies, Cat# P36941). Samples were imaged using confocal microscopy.

**Fig. 8** USP32 regulates extraction of membranes from Rab7-positive endosomes. **a, b** Bud/tubule resolution from Rab7 endosomes as a function of USP32 depletion visualized by confocal microscopy. Scale bars = 5 µm. See also Supplementary Movies 13–16. c Alterations in late endosome (LE) morphology in response to USP32 depletion as visualized by correlative light and electron microscopy (CLEM). GFP-Rab7-2KR (GFP-2KR) fluorescence (green) and transmission electron micrographs (TEMs) are shown; scale bars = 0.25 µm. d Comparison of GFP-Rab7-2KR-positive LE profile in siCtrl (black line) and siUSP32_2 (red line); x-axis: LE diameter in µm; y-axis: number of LE profiles. See also Supplementary Fig. 9.
Leica SP5 or SP8 microscopes equipped with appropriate solid-state lasers, HCX PL 63 times magnification oil emission objectives and HyD detectors. Data were collected with a total integration time of 1024 × 1024 pixels and post-processing with line averaging. Post-collection image processing and colocalization analyses were performed using the Fiji software. Colocalization was reported as Manders’ overlap or Pierson’s coefficient, as indicated. Fractional distances were calculated as follows. Fluorescence intensities (above automated background threshold) were measured along the longest intracellular line ROI (right media interest) drawn from the center of a cell’s nucleus (fractional distance = 0) to the plasma membrane (fractional distance = 1.0) using the line profile tool in the LAS-AF software, and their absolute distance to the center of the nucleus was expressed relative to the center of the line\textsuperscript{14}. Fractional distances are reported in scatter plots along with the median distance value (red line) within the sample and the total number of cells analyzed.

For fluorescence confocal microscopy of live cells, samples were seeded into 35 mm glass bottom dishes (MatTek, Cat# P35GC-15-14-C) and transfected as indicated. Samples were incubated with Lysotracker DeepRed (1:10,000) 30 min prior to imaging. Imaging was performed on a Leica SP5 microscope with solid-state lasers or Leica SP8 microscope with a white light laser, both equipped with HyD detectors and a climate chamber. Data were collected using 63× oil immersion objectives in combination with 1.5–2.5 digital zoom in 1024×1024 scanning format at 3 s intervals with line averaging. Tracking of Lysotracker-positive vesicles was performed using TrackMate for Fiji\textsuperscript{24}. Fiji was also used for post-collection image processing.

**Correlative light and electron microscopy.** Cells were fixed at room temperature in 2% paraformaldehyde and 0.2% glutaraldehyde in culture medium for 2 h, embedded in 12% gelatin, infused with 2.3 M sucrose, and frozen in liquid nitrogen. Ultrathin sections (75 nm) of the frozen cells were produced in a Leica UC6 cryo-ultramicrotome and transferred onto a Leica EM A100 (Carl Zeiss), carrying a Formvar support film coated with carbon and 100 nm blue fluorescent microspheres (Life Technologies). The sections attached to the grid were rinsed in PBS at 37°C to remove the gelatin and sucrose, and stained with DAPI (60 ng/mL; Sigma-Aldrich) for 5 min. The grid with the attached sections was imaged in 30% glycerol in a confocal microscope using a 100× objective. For correlative confocal microscopy imaging, the grid with the attached sections was rinsed in distilled water and stained in 1.8% methylene blue containing 0.4% uranyl acetate and subsequently air-dried before imaging in the TEM. Confocal stacks were deconvoluted with theoretical point spread functions using the Huygens Essential deconvolution software (Huygens Essential, Hilversum, Netherlands). Electron microscopy images were recorded using a Tecnai 12 TEM (FEI company) equipped with an Eagle 4 × 4 × 4K digital camera using a magnification of ×13000. Superimposition and correlation of confocal and electron microscopy images was performed in the Adobe Photoshop software on basis of the signal of the DAPI stain and fluorescent microspheres.

The diameter of GFP-Rab7-positive endosome profiles were measured on 75-nm-thick sections in ImageScope using montages of electron microscopy images produced by stitching software.

**Ubiquitination analysis.** MelJuSo cells were grown in IMDM medium supplemented with 10% FCS, 1% penicillin/streptomycin for label-free quantitation (LFQ) experiments. HeLa or MelJuSo cells were grown either in DMEM or IMDM, respectively, supplemented with six passages in isotope-labeled media in 175 cm\textsuperscript{2} flasks containing 50% D4 lysine/arginine (Thermo Fisher Scientific) supplemented with 7.5% dialyzed FCS (Thermo Fisher Scientific), 1% penicillin/streptomycin, and either light lysine/arginine (K0R0), medium (4,4,5,5-D\textsubscript{4}Lys,13C\textsubscript{6}Arg–K0R0); MelJuSo cells transfected with siRNAs targeting USP32 and HeLa cells transfected with 10% FCS, 1% penicilin/streptomycin for label-free quantitation (LFQ) Ubiquitome analysis (Thermo Fisher Scientific) K8R10) (Thermo Fisher Scientific), or heavy (13C\textsubscript{6}15N\textsubscript{2}Lys,13C\textsubscript{6}15N\textsubscript{4}Arg–K0R0). Experiments were conducted as described in a previous study\textsuperscript{20}. Interestingly, the Ubiquitome (Thermo Fisher Scientific) Ubiquitome analysis (Thermo Fisher Scientific) was used for the lysosomal Ubiquitin mass spectrometer (Thermo). The QEx instrument was set to pick the 15 most intense features for subsequent tandem mass spectrometry analysis at a resolution of 17,500, a maximum acquisition time of 128 ms, an AGC target of 1 × 10\textsuperscript{6}, an isolation width of 1.6 Th, and a dynamic exclusion of 27’s (Fusion Lumos 12.0). The orbitrap Fusion Lumos was used for SILAC samples and QEx for LFQ samples. Raw data were converted to Mascot generic files using mconvert30, and database searches were performed with MASCOT\textsuperscript{31}. Alternatively, for quantitative analysis of all SILAC samples combined, raw MS data were processed using MaxQuant (v1.5.3.1) and subsequently analyzed using the Perseus software (v1.5.3.1). The MS data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD0011899.

**Ubiquitination assays.** Ubiquitination status of GFP-tagged proteins was assessed by using ubiquitination assay\textsuperscript{34}. HEK293T cells, transfected with HA-ubiquitin, GFP-substrates, and USP32 or its mutant as indicated, were lysed in 300 µL lysis buffer 1 (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100, 10 mM N-ethylmaleimide (general DUB inhibitor diluted in DMSO, freshly added) and protease inhibitors (Roche Complete EDTA-free, freshly added). Samples were incubated with 6 µL GFP Trap_A beads (Chromotek) for 2 h at 4°C. Beads were washed three times with lysis buffer. During the fourth washing step, 30 µL Protein G4 fast flow (GE Healthcare) was added and all liquid was removed prior to the addition of SDS sample buffer (containing 10 mM DTT). Proteins were denatured by heating at 95°C for 15 min, subjected to 8% SDS-PAGE, and detected by Western blotting, as indicated.

**Protein expression and purification.** Spodoptera frugiperda Sf9 insect cells were used as hosts for the baculovirus. SF900 II SFM medium (Gibco, Cat# 10902096) containing 1% penicillin–streptomycin (Gibco, Cat# 15140122) was used for insect cell culturing. Suspension insect cells were grown under serum-free conditions at 27°C with shaking. Sf9 cells insect cells were transfected with 10 µg bacmid DNA purified from DH10Bac-competent cells (transformed with pFastNKi-haS-3C-LC- Ubiquitin and pFastNKi-haS-3C-LC-Ubiquitin) in 2 ml DH10Bacfectin (BaculoFast cocktail) and sonication. The lysates were centrifuged at 20,000 × g for 30 min at 4°C. The supernatants were incubated with 6 µL GFP Trap_A beads (Chromotek) overnight at 4°C (Cat# 10362100). Three days after transfection, PI baculovirus stock was collected from the culture medium. A total of 1 × 10\textsuperscript{6} cells/mL in 30 mL medium was infected with PI baculovirus stock to prepare P2 baculovirus stock. Baculovirus stocks were stored at 4°C and protected from light. A total of 1 × 10\textsuperscript{8} Sf9 insect cells were infected using a low MOI (multiplicity of infection ratio of infectious virus particles) to infect the cells. The cells were harvested 72 h after the baculovirus infection. Cells were lysed with lysis buffer 3 (20 mM Tris, pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol, 10 mM imidazole, and protease inhibitor cocktail) and sonication. The lysates were centrifuged at 21,000 × g for 30 min at 4°C. The supernatants were incubated with 10 µL Ni-NTA-agarose (Clontech Inc., Palo Alto, CA, USA) for 20 min at 4°C and the beads were washed with wash buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol, and 10 mM imidazole). Protein was eluted with elution buffer containing 20 mM Tris, pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol, and 250 mM imidazole. Protein was dialyzed to remove imidazole and purified further with a size exclusion column (S200 16/60 column) using a AktaPrime purifier. All proteins were stored at –80°C.

**Enzyme activity assays.** Di-ubiquitin hydrolysis assay was performed in a buffer containing 50 mM HEPEs (pH 7.5), 100 mM NaCl, 5 mM DTT, and 75 mM enzyme: USP32-FL or USP32-C2 and 5 µg synthetic di-ubiquitin of specific linkage generated in our lab (M1, K6, K11, K27, K93, K48, and K63) for each reaction. Hydrolysis reaction was performed at 37°C in 60 min and stopped by the addition of SDS-containing sample loading buffer and heat denaturation. Samples were analyzed by SDS-PAGE on 4–12% Bis-Tris NuPage gel.
Subcellular fractionation. MeltusCo cells stably expressing GFP-Rab7 were transfected in 10 cm dishes with the indicated siRNA. Seventy-two hours after transfection, cells were harvested in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM MgCl2, and protease inhibitors (Roche, complete EDTA-free, Cat# 05056489001). Cell suspension was passed through a 21 G needle 10 times or more (until near complete lysis) using a 5 mL syringe. Samples were centrifuged at 1000×g for 10 min. The supernatant was transferred into fresh tubes and centrifuged at 2000×g for 20 min. The resulting supernatant (containing membrane and cytosolic fraction) was transferred into fresh tubes and centrifuged at 20,000×g for 30 min. The supernatant containing the cytosolic fraction was transfected into a new tube, and the pellet was resuspended with a lysis buffer containing 0.5% NP-40, 20 mM Tris-HCl (pH 7.5), 1 mM MgCl2, and protease inhibitors, and then incubated on ice for 20 min. Resulting cytosolic and membrane fractions were incubated with 6 µL GFP_Trap_A beads (Chromotek) overnight at 4 °C. Beads were washed three times with lysis buffer. During the fourth washing step, 30 µL Protein G4 fast flow beads (GE Healthcare) was added and all liquid was removed prior to the addition of SDS sample buffer containing 10 mM DTT. Proteins were denatured by heating at 95 °C for 15 min, subjected to 8% SDS-PAGE and detected by Western blotting, as indicated.

Statistical analysis. Statistical evaluations report on Student’s t test (two-tailed distribution) with *p<0.05, **p<0.01, and ***p<0.001, NS: not significant. All error bars correspond to the mean ± SD.

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prepared samples. B.M.K. and R.K. carried out quantitative mass spectrometry experiments and analyzed the ubiquitome data using prepared samples. A.E.E.-B. coordinated biochemical experiments. J.J.A prepared GFP-BirA-Rab7 constructs for biotinylation assay. J.N. advised on the project. H.O. coordinated the study. I.B. and A.S. wrote the manuscript with the help of J.N. and H.O.

**Additional information**

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