APPENDIX

VCP/p97 cooperates with YOD1, UBXD1 and PLAA to drive clearance of ruptured lysosomes by autophagy

Chrisovalantis Papadopoulos[1], Philipp Kirchner[1], Monika Bug[1], Daniel Grum[1], Lisa Koerver[1], Nina Schulze[2], Robert Poehler[1], Alina Dressler[1], Sven Fengler[1], Khalid Arhzaouy[4], Vanda Lux[3], Michael Ehrmann[3], Conrad C. Weihl[4], Hemmo Meyer[1*]

[1] Molecular Biology I, [2] Imaging Center Campus Essen, [3] Microbiology,
Centre for Medical Biotechnology, Faculty of Biology, University of Duisburg-Essen, 45141 Essen, Germany
[4] Department of Neurology, Washington University School of Medicine, Saint Louis, MO 63110, USA.

Appendix Table of Contents:

Appendix Material and Methods

Generation and immortalization of MEFs, and stable U2OS cell lines.

Primary mouse embryonic fibroblasts (MEFs) were isolated from E13.5 embryos of $p97^{R155H/wt}$ mice (Badadani et al., 2010). The embryos were washed with PBS and the red organs were removed. The embryos were then minced with a scalpel and digested with 0.05% trypsin solution (Life Technologies) for 30 min at 37°C. The harvested cells were grown in DMEM supplemented with 10% FBS in the presence of penicillin/streptomycin at 37°C. After three passages, the MEFs were immortalized using SV40 large T antigen. Stable inducible U2OS cell lines expressing p97 mutants were described earlier (Ju et al., 2008). To induce lysosomal damage, MEFs were treated with 1 mM LLOMe, and U2OS with 1 mM for immunofluorescence or 2 mM for biochemical analysis.

Plasmids.

pcDNA5-p97-wt-myc-strep or E578Q were described previously (Ritz et al., 2011). pEGFP-C1-p97-wt or E578Q were generated by PCR cloning from pcDNA5-p97-wt-myc-strep or E578Q, respectively, using BamHI and XhoI. p97-wt or E578Q were cloned with a C-terminal mCherry into pIRESpuro2 (Invitrogen) using BamHI. Flag-HA-YOD1 was obtained via Addgene (#22554, W. Harper) and YOD1 was cloned into pEGFP-C1 (Clontech) using XhoI and EcoRI. pEGFP-YOD1-C160S, pEGFP-YOD1-MutS2 (I292Q and V295Q) and pEGFP-YOD1-MutS2-C160S were generated by site-directed mutagenesis. pmCherry-YOD1 was cloned from pEGFP-C1 into pmCherry-C1 (Clontech) with XhoI and EcoRI. The pmCherry-Gal3 construct was generated by PCR amplification of the Gal3 coding sequence from U2OS cDNA flanked by HindIII and EcoRI sites and cloned into pmCherry-C1. Full length PLAA cDNA was amplified from an IMAGE clone (IRATp970D0156D; Source Bioscience) and cloned into pEGFP-C1 using XhoI and BamHI. p47-GFP was described before (Kress et al., 2013; Ritz et al., 2011). UBXD1-GFP was cloned into pEGFP-N1 using EcoRI and BamHI. GFP-VCPIP1 was cloned into pEGFP-C1 from Flag-HA-VCPIP1 (#22592, W. Harper, Addgene) using XhoI and EcoRI. For generation of constructs with a C-terminal strep-HA the cDNA was PCR amplified from the constructs described above and integrated into pcDNA5/FRT/TO/SH/GW (Glatter et al., 2009) using gateway cloning (Invitrogen). LAMP1-RFP, GFP-LC3 and GFP-TAB2-NZF were gifts from J. Gruenberg (University of Geneva, Switzerland), T. Yoshimori (National Institute of Genetics, Osaka, Japan), and I. Dikic (Goethe University Frankfurt, Germany), respectively.

RNA Interference.

Following RNAi oligonucleotides were from Microsynth with the sequences: Ctrl (UUCUCCGAACGUGUCACGU), Luc (CGUACGCGGAAUACUUCGA), p47 (ACAAAGAGCU GGCUGAUGA), p62 (GCAUUGAAGUUGAUAUCGA), p97#1 (AAGUAGGGUAUGAUGACA UUG), p97#2 (GGAGUUCAAAGUGGUGGAAACAGA), PLAA#1 (CCAGUGAUGACCCUUG GUUAA), PLAA#2 (GGACAGACUCGUCUAAUCA), SEL1L (UUAACUUGAACUCCUCUCCC AUAGA), UBXD1#1 (CCAGGUGAGAAAGGAACUU), UBXD1#2 (UCAGAUACCACGUUGG UCCC), Ufd1 (GUGGCCACCUACUCCAAAU) (Riemer et al., 2014), VCPIP1 #1 (CCCGAUG AUUAUACUCCUG), VCPIP1#2 (CAGGGACAGACUUUAGUAA), YOD1#1 (GACCGUCAAA UUAGAGCUU), YOD1#2 (CAGCGUAACUUCCCUGAUC). Following RNAi oligo-nucleotides were from Metabion with the sequences: ATG5 (GCUAUAUCAGGAUGAGAUA), ATG7 (GG AACACUGUAUAACACCA).

Reagents.

NMS-873 was from Xcess Biosciences Inc., NH_4Cl from Fluka, bortezomib from Biomol and bafilomycin A_1 from Sigma.

Fluorescence microscopy.

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, blocked by 3% bovine serum albumin in PBS with 0.1% Triton X-100 and 0.1% saponin, stained by indirect immunofluorescence and mounted in ProLong Gold (Invitrogen). Confocal laser scanning microscopy was performed on a TCS SP5 AOBS system equipped with standard PMT detectors as well as sensitive HyD detectors (Leica Microsystems). Images were acquired using an HCX PL APO 63×/1.4NA oil-immersion objective or an HC PL APO 20×/0.7NA dry objective. Lasers used for excitation were HeNe 633 nm (Alexa Fluor® 633), DPSS 561 nm (Alexa Fluor® 568, mCherry), Ar 488 nm (Alexa Fluor® 488, EGFP) and Diode 405 nm (Hoechst). Acquisition and hardware was controlled by LAS AF software (Leica Microsystems). Confocal spinning disk microscopy used in Figure S5B was performed on an Eclipse Ti-E (Nikon) inverted microscope with a Andor AOTF Laser Combiner, a CSU-X1 Yokogawa spinning disk unit and an iXon3 897 single photon detection EMCCD camera (Andor Technology). Laser lines used for excitation of EGFP and mCherry were 488 nm and 561 nm, respectively. Images were acquired using an CFI APO TIRF 100×/1.49NA oil-immersion objective (Nikon). Acquisition was controlled by Andor IQ Software (Andor Technology). Live-cell imaging was performed at 37°C in imaging medium supplemented with 10% FCS.

Antibodies.

Anti-p97 (HME8), anti-UBXD1 (E43), anti-VCPIP1 (HME19), anti-p47 (HME22) and anti-Ufd1 (5E2) antibodies were described previously (Meyer et al., 2000; Ritz et al., 2011; Wang et al., 2004). Anti-K48 ubiquitin chain (clone Apu2), anti-K63 ubiquitin chain (clone Apu3), and mouse anti-ubiquitin antibody (clone FK2) were purchased from Millipore. Mouse anti-LAMP1 (sc-20011), rabbit anti-Gal3 (sc-20157), mouse anti-Gal3 (sc-32790), and anti-myc (sc-40) were purchased from Santa Cruz Biotechnology. Rabbit anti-HA (H6908), rabbit anti-ubiquitin (U5379), anti-YOD1 (ab2), rabbit anti-p62 (P0067), rabbit anti-LC3 (L7543) and anti-α-tubulin (T5168) were from Sigma-Aldrich. Mouse anti-p97 (58.13.3) was from Fitzgerald Industries, mouse anti-HA (HA.11) from Covance, anti-PLAA (Y102) from Abcam, mouse anti-p62 (H00008878-M01) from Abnova, anti-LC3 (PM036) from MBL, anti-ATG5 (NB110-53818) from Novus Biologicals, anti-ATG7 (AHP1651) from AbD Serotec, and rabbit anti-CAV3 from Affinity Bioreagents. Rabbit anti-GAPDH (14C10) and mouse anti-myc were from Cell Signaling Technology. HRP-coupled secondary antibodies were from Bio-Rad and Alexa Fluor-conjugated secondary antibodies from Invitrogen.

Cloning and Expression of constructs for protein biochemistry.

Human p97 was cloned in pET15b (NdeI, BamHI), resulting in an expression construct containing an N-terminal His-tag. The E578Q mutant of p97 was generated via site-directed mutagenesis on this wild-type construct. Human cofactors YOD1 and UBXD1 were cloned in pET41b (NdeI, XhoI), resulting in a C-terminal His-tag. pET41b-YOD1-MutS2 (I292Q and V295Q) was generated by site-directed mutagenesis. Full-length PLAA (forward primer included a start codon and a His-tag) was cloned in pFL (BamHI, EcoRI) for expression in insect cells. GST was expressed from the pET41 vector. Ub-GST with a G76V mutation was described previously (Meyer et al., 2002). His-p97, GST and Ub-GST constructs were expressed in SoluBL21 cells. YOD1-His, UBXD1-His were expressed in Rosetta 2. Cells were grown at 37°C up to an OD600 of 0.7-0.8 (in case of UBXD1-His: OD600 = 2.0) prior to induction with IPTG. Expression of YOD1-His was induced with 0.5 mM IPTG for 2 h at 28°C. Expression of all other proteins was induced with 0.2 mM IPTG at 18°C for approximately 16 h. Pellets were washed with 50 mM HEPES, 150 mM KCl, 5 mM $MgCl_2$, 5% Glycerol, 20 mM Imidazole, pH 8.0 and either resuspended immediately in this buffer again or shock frozen and stored at -80°C prior to resuspension. The insect cell expression construct of full-length His-PLAA (in pFL) was transformed in EmbacY E. coli cells containing the bacmid for expression in insect cells. The bacmid was amplified in SF9 insect cells and finally expressed in Tnao38 cells (Hashimoto et al., 2010; Hashimoto et al., 2012) (27°C, 4 days, 115 rpm, SF900III medium).

Purification of recombinantly expressed proteins.

The cell pellets were resuspended in 50 mM HEPES, 150 mM KCl, 5 mM $MgCl_2$, 5% Glycerol, 20 mM Imidazole, pH 8.0. Afterwards PMSF (1 mM), DTT (0.4 mM) and Lysozyme were added. The suspension was incubated stirring for 45 min at 4°C prior to lysing the cells via sonication and centrifugation at 35,000×g for 30 min (4°C). The supernatant was loaded on one to three 5 ml HisTrap crude columns (GE Healthcare) and washed with 100 ml of 50 mM HEPES, 150 mM KCl, 5 mM $MgCl_2$, 5% Glycerol, 20 mM Imidazole, 0.4 mM DTT, 1 mM ATP, pH 8.0 and another 300 ml of 50 mM HEPES, 150 mM KCl, 5 mM $MgCl_2$, 5% Glycerol, 20 mM Imidazole, 0.4 mM DTT, pH 8.0 (binding buffer). Afterwards, the protein was eluted with elution buffer (like binding buffer, but 300 mM Imidazole). His-p97 and its mutant were immediately eluted on a 5 ml HiTrap Q HP column (GE Healthcare) connected to the HisTrap crude columns. Anion exchange chromatography was performed using gradient elution between buffer A (20 mM HEPES, 150 mM KCl, 5 mM $MgCl_2$, 5% Glycerol, 1 mM DTT, pH 7.2) and buffer B (like buffer A, but 1 M KCl). YOD1-His, UBXD1-His and His-PLAA were diluted with buffer C (20 mM HEPES, 25 mM KCl, 5 mM $MgCl_2$, 5% Glycerol, 1 mM DTT, pH 7.2) for anion exchange chromatography prior to loading on a 5 ml HiTrap Q HP (or in case of His-PLAA: MonoQ 5/50 GL column). Gradient elution was performed between buffer C and buffer D (like buffer C, but 200 mM KCl). After gradient elution of the proteins adjustment of the KCl concentration to 150 mM was performed with buffer B or C. Afterwards, the proteins were concentrated to the desired concentration, aliquoted, shock frozen and stored at -80°C. Analytical gelfiltrations of an aliquot were performed as a quality control before using any of the proteins for experiments.

Protein concentration determination.

Protein concentrations were determined via measuring their absorbance at 280 nm and using the respective molar extinction coefficient. As it is known for p97 that there is always ADP bound in the D1 domains after purification from E. coli (Zhang et al., 2000), bleeding of nucleotide absorbance at 280 nm was measured and this value was added to the extinction coefficient of His-p97, resulting in a corrected extinction coefficient of 38,800 M-1cm-1 that was used for His-p97 and its mutant.

Appendix References

Badadani, M., Nalbandian, A., Watts, G.D., Vesa, J., Kitazawa, M., Su, H., Tanaja, J., Dec, E., Wallace, D.C., Mukherjee, J.*, et al.* (2010). VCP associated inclusion body myopathy and paget disease of bone knock-in mouse model exhibits tissue pathology typical of human disease. PLoS One *5*, e13183.

Glatter, T., Wepf, A., Aebersold, R., and Gstaiger, M. (2009). An integrated workflow for charting the human interaction proteome: insights into the PP2A system. Mol Syst Biol *5*, 237.

Hashimoto, Y., Zhang, S., and Blissard, G.W. (2010). Ao38, a new cell line from eggs of the black witch moth, Ascalapha odorata (Lepidoptera: Noctuidae), is permissive for AcMNPV infection and produces high levels of recombinant proteins. BMC biotechnology *10*, 50.

Hashimoto, Y., Zhang, S., Zhang, S., Chen, Y.R., and Blissard, G.W. (2012). Correction: BTI-Tnao38, a new cell line derived from Trichoplusia ni, is permissive for AcMNPV infection and produces high levels of recombinant proteins. BMC biotechnology *12*, 12.

Ju, J.S., Miller, S.E., Hanson, P.I., and Weihl, C.C. (2008). Impaired protein aggregate handling and clearance underlie the pathogenesis of p97/VCP associated disease. J Biol Chem *283*, 30289-30299.

Kress, E., Schwager, F., Holtackers, R., Seiler, J., Prodon, F., Zanin, E., Eiteneuer, A., Toya, M., Sugimoto, A., Meyer, H.*, et al.* (2013). The UBXN-2/p37/p47 adaptors of CDC-48/p97 regulate mitosis by limiting the centrosomal recruitment of Aurora A. J Cell Biol *201*, 559-575.

Meyer, H.H., Shorter, J.G., Seemann, J., Pappin, D., and Warren, G. (2000). A complex of mammalian Ufd1 and Npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. Embo J *19*, 2181-2192.

Meyer, H.H., Wang, Y., and Warren, G. (2002). Direct binding of ubiquitin conjugates by the mammalian p97 adaptor complexes, p47 and Ufd1-Npl4. Embo J *21*, 5645-5652.

Riemer, A., Dobrynin, G., Dressler, A., Bremer, S., Soni, A., Iliakis, G., and Meyer, H. (2014). The p97-Ufd1-Npl4 ATPase complex ensures robustness of the G 2/M checkpoint by facilitating CDC25A degradation. Cell Cycle *13*, 919-927.

Ritz, D., Vuk, M., Kirchner, P., Bug, M., Schütz, S., Hayer, A., Bremer, S., Lusk, C., Baloh, R.H., Lee, H.*, et al.* (2011). Endolysosomal sorting of ubiquitinated caveolin-1 is regulated by VCP/p97 and UBXD1 and impaired by VCP disease mutations. Nat Cell Biol *13*, 1116-1123.

Wang, Y., Satoh, A., Warren, G., and Meyer, H.H. (2004). VCIP135 acts as a deubiquitinating enzyme during p97-p47-mediated reassembly of mitotic Golgi fragments. J Cell Biol *164*, 973-978.

Zhang, X., Shaw, A., Bates, P.A., Newman, R.H., Gowen, B., Orlova, E., Gorman, M.A., Kondo, H., Dokurno, P., Lally, J.*, et al.* (2000). Structure of the AAA ATPase p97. Mol Cell *6*, 1473-1484.

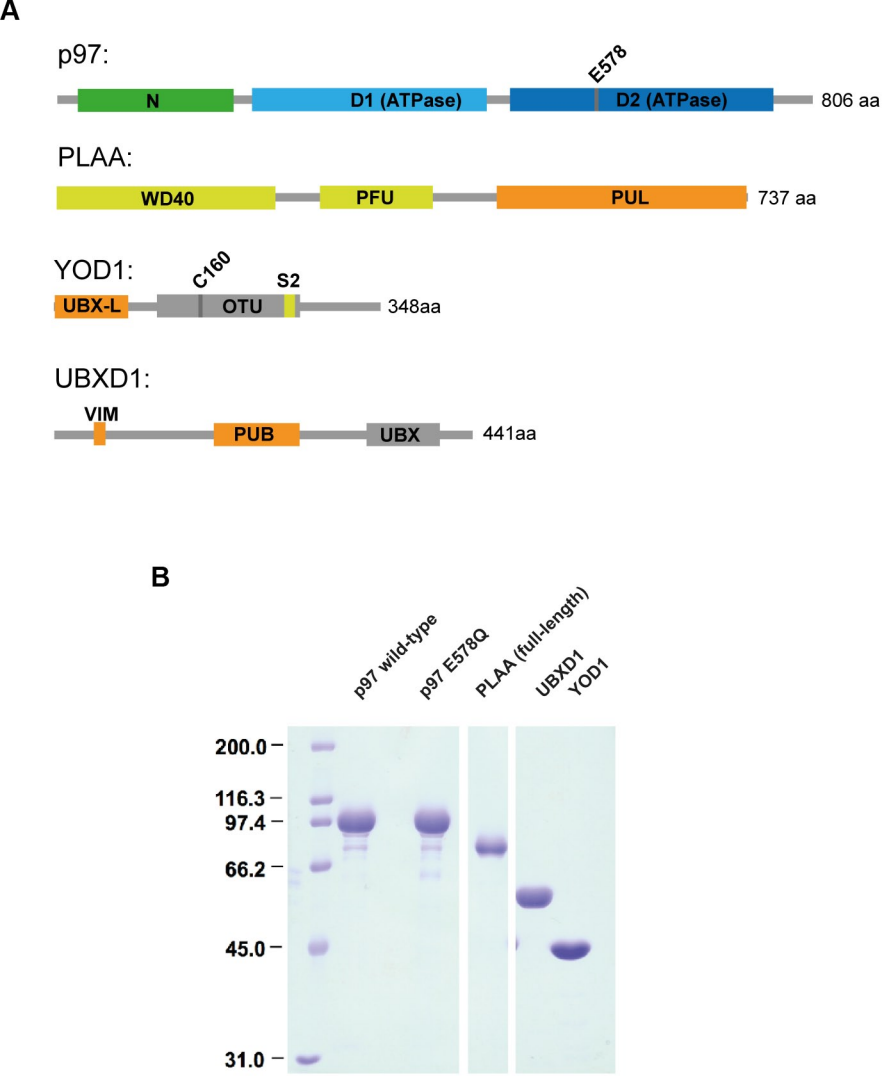
Appendix Figure Legends

Appendix Figure S1. Domain structure and purification of ELDR components, Related to Figure 3 and 6

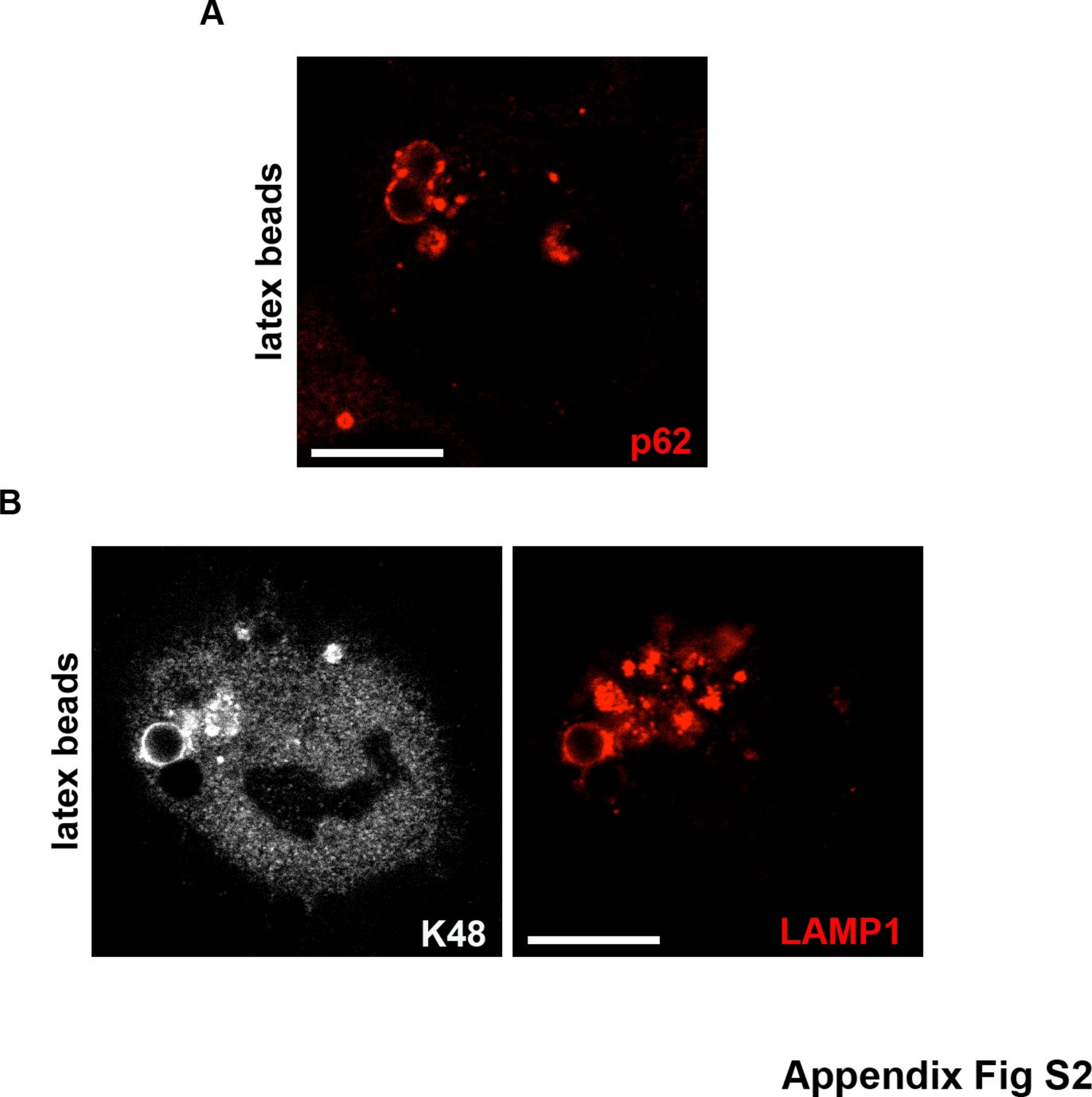
(A) Domain structure of p97, PLAA, YOD1 and UBXD1. Regions binding to p97 or ubiquitin are indicated in orange and yellow, respectively. WD40 (WD40 repeat domain), PFU (PLAA-family ubiquitin binding), PUL (PLAA, Ufd3 and Lub1 domain), UBX-L (ubiquitin domain-X-like), OTU (ovarian tumor DUB domain), S2 (ubiquitin binding site S2), VIM (VCP/p97-interacting motif), PUB (peptide N-glycosidase/ubiquitin-associated domain), UBX (ubiquitin domain-X). The UBX-L domain and the VIM bind to the N-terminal domain of p97, whereas PUL and PUB domains bind to the C-terminal tail of p97. **(B)** SDS-gel of proteins (5 µg each) used for biochemical characterization. PLAA (full length) was expressed in insect cells, all other proteins in E. coli.

Appendix Figure S2. Lipofectamine-coated latex beads damage lysosomes, Related to Figure 7

(A) HeLa cells were transfected with Lipofectamine-coated latex beads for 3 h and then fixed and stained for p62. **(B)** Cells treated as in (A) were stained for LAMP1 and K48 chains. Scale bars, 10 µm.



Appendix Fig S1



Appendix Fig S2