

Extended Experimental Procedures

Plasmids and Cell Lines. ORF clones were obtained from the human ORFeome collection (Open Biosystems, Thermo Scientific) or cloned from available full-length cDNAs. All TBC1D5 plasmids were used as previously published [1]. HA-ULK1 was obtained from Dr. Masaaki Muramatsu [2]. Sequence validated ORF for AP2M1 protein was subcloned into pGEX6P1 vector (Clontech) using restriction free cloning and for ATG9A in pDONR223 was recombined into the Gateway destination vectors MSCV-Tet-FLAG-HA-IRES-PURO (which uses a tetracycline inducible CMV promoter; iTAP) for stable cell lines, and pDEST-CMV-N-Myc, pHAGE-N-GFP and pHAGE-CMV-N-mCherry for transient transfection using λ recombinase. Virus was produced for indicated ORFs by co-transfecting the respective iTAP, GAG/POL, and VSVG plasmids into 293T cells or by co-transfecting the respective plasmid together with Tat1b, Hgpm2, CMV-Rev and VSVG plasmids into 293T cells. After packaging, viral supernatants were used to infect U2OS cells or TReX-HeLa (Invitrogen) and subsequently selected for stable expression using 1 μ g/ml puromycin or 5 μ g/ml Blasticidin (pDEST-N-GFP), or both.

Stable and transient knock-down cell lines. U2OS cells stably expressing two different shRNA oligos targeting all 3 isoforms of human TBC1D5 (GAGAACGAACAGATCACCATT and GCAAAGGTGATGGACACTCAT), VPS29 (GCAACAGTTTGCCAGCTAAAT, CCTTTGCACCAAAGAGAGTTA and GCAACAGTTTGCCAGCTAAAT) were generated by Lentiviral infection. Virus was produced for each shRNA oligo (Open Biosystems, pLKO) by co-transfecting the respective pLKO plasmid together with Tat1b, Hgpm2, CMV-Rev and VSVG plasmids into 293T cells. After packaging, viral supernatants were used to infect U2OS cells and subsequently selected for stable expression using 1 μ g/ml puromycin. shRNA-resistant TBC1D5 variant was used as published [1]. Control shRNA cells were generated using MISSION non-target shRNA control Lentiviral particles obtained from Sigma Aldrich.

Transient knock-down of AP2 complex in U2OS cells was achieved by transfecting 2 different siRNA oligos (GCAAAGAGGCGGAAAUUAA or AGGCGGAAAUUAAGAGAAU) targeting human AP2A1, designed using Thermo Scientific software, and synthesized by MWG Biotech (Ebersberg, Germany). Control siRNA oligo was AllStars Control siRNA from QIAGEN (Cat. Nr. 1027281). All transient knock-downs were achieved by transfection of siRNA in final concentration of 40nM, and knock down was

inspected 4 days after transfection. Transfection of siRNA oligos was performed in siRNA-Max transfection reagent (Invitrogen) and OptiMEM (Gibco).

Antibodies. Antibodies used in this study are as published [1] and anti- ATG9A antibody rabbit monoclonal (NBP1-95342, Novus Biologicals), anti- Adaptin alpha mouse monoclonal (BD Transduction Laboratories, Cat. 610502), anti- AP2M1 (K-13) goat polyclonal (Santa Cruz) sc-49150, anti- clathrin mouse monoclonal (Research Diagnostics #RDI-67858a), anti- ULK1 rabbit monoclonal (Epitomics, Cat #5723-1), anti- pATG13 (Ser318) Rabbit polyclonal (Rockland, Cat.800-656-7625) and anti- LAMP1 (H4A3-c) mouse monoclonal (Developmental studies hybridoma bank).

Immunoprecipitation. For western blot analysis: TReX-HeLa cells stably expressing various TBC1D5 constructs (iTAP or Myc), U2OS cells stably expression mCherry or GFP tagged ATG9A and 293T cells transiently expressing myc-VPS29 or iTAP-VPS35 were induced and transfected (GeneJuice, Novagen) for 16-20 hours, respectively. Whole cell lysates were incubated over night with anti-Flag M2 (Sigma Aldrich), anti-Myc (Santa Cruz) coupled resin, GFP/RFP Trap beads (ChromoTek) or respective endogenous antibody followed by incubation with protein G agarose (Roche). Samples were subjected to SDS-PAGE and immunoblotted. For total cell lysate analysis cell were lysed in RIPA Buffer (supplemented with protease inhibitors, 1% SDS and Benzodase (Novagen)) and for co-immunoprecipitation experiments cells were lysed in buffer containing 20mM CHAPS (AppliChem), 125mM NaCl, 50mM Tris-HCl pH 7.5, lysates were passed through U-40 insulin syringe 5 times and pre-cleared by centrifugation (13.000 rpm) for 10 minutes. Supernatant was used for incubation with respective antibodies or antibody-coupled beads.

Protein purification and binding assay. Indicated GST-fusion proteins were expressed in *E. coli* (BL21) cells and purified using GSH-Sepharose. Expression in *E. Coli* was induced with 0,5 M IPTG, on 16C, over night. For lysate analysis, plasmids were transiently transfected in 293T cells using GeneJuice. After 20 hours, whole cell extracts were incubated with the indicated GST-fusion protein purified from bacteria or with respective antibodies used for co-immunoprecipitation. Washed beads were subjected to SDS-PAGE and immunoblotting. For *in vitro* analysis, recombinantly purified GST tagged AP2M1 and TBC1D5 were incubated over night with anti- TBC1D5 antibody and G agarose in binding buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0,5% Triton-X). Agarose resin were washed 3 times with binding buffer, bound proteins subjected to SDS-PAGE and immunoblotted using anti-TBC1D5 and anti-AP2M1 antibodies.

Confocal microscopy and live cell imaging. Staining of U2OS cells transiently transfected or stably expressing mCherry, GFP, HA-Flag or myc tagged proteins was performed after the cells were fixed on coverslips with 2% PFA for 15 min., permeabilized with 0,2% Triton-X in PBS for 5 min., and blocked in PBS containing 5% BSA and 0,1% Tween for 1h on room temperature. Images were acquired with LSM510 confocal microscope (Zeiss), or Leica TCS SP8 confocal microscope and processed using ImageJ software. Filter settings on LSM510 for 4 multichannel acquisition were as follows: HeNe1 (HFT 488/543, NFT490, BP 560-615), HeNe2 (HFT 488/543/633, NFT 545, LP650), Argon (HFT 488, NFT490, BP 505-550) and UV laser (HFT 405/514, NFT545, BP 420-480). PMT collection ranges on Leica SP8 microscope were adjusted accordingly to the emission spectra of used fluorophores, in sequential scanning mode for 4 lasers (UV laser (PMT1 420-490nm), 488nm (PMT2 500-560nm), 552nm (PMT2 560-630nm) and 638nm (PMT3 650-750nm). Imaging of live U2OS cells, grown in 8-well Lab-Tek chambers, was performed on Visitron confocal spinning disc microscope, equipped with Yokogawa CSU-X1 Scanning head CSU X1-A1-5000rpm, single filtersets for GFP, mCherry, EBFP2 and HXP 120 Xenon Lamp for epi-fluorescence illumination. Movies were processed using ImageJ (NIH).

1. Popovic D, Akutsu M, Novak I, Harper JW, Behrends C, Dikic I (2012) Rab GTPase-activating proteins in autophagy: regulation of endocytic and autophagy pathways by direct binding to human ATG8 modifiers. *Molecular and cellular biology* **32**: 1733-1744
2. Yan J, Kuroyanagi H, Kuroiwa A, Matsuda Y, Tokumitsu H, Tomoda T, Shirasawa T, Muramatsu M (1998) Identification of mouse ULK1, a novel protein kinase structurally related to *C. elegans* UNC-51. *Biochem Biophys Res Commun* **246**: 222-227