Supplemental material

Identification of autophagosome-associated proteins and regulators by quantitative proteomic analysis and genetic screens

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Supplemental Experimental Procedures

Transfections
Fusion constructs of DsRed and RHEB (clone IRAKp961F0514Q; imaGenes) or FKBP1A (clone IRAUp969F0618D; imaGenes) were generated using vectors pDsRed-C1 and -N1, respectively, (Clontech, St-Germain-en-Laye, France) with the primers:
GGAGGgagctcAGATGCCGCAGTCCAAGTC (Sac1)
GAATAggtaCCCAGTGTCCTCAGGCTTTG (Kpn1) for RHEB
CGTCCgagctcGCCATGGGAGTGCAGGT (Sac1)
ATTCCggtaccTCCAGTTTTAGAAGCTCCACATCG (Kpn1) for FKBP1A.

Primary antibodies used for immune-detection
The primary antibodies used for immuneblot analysis included murine antibodies against early endosomal antigen (#PA1-065, Bioreagents, Rockford IL, USA), Golgin 97 (A-21270, Molecular Probes, Eugene, OR), Grp75 (SPA-825, Stressgen), Serca (564702, Calbiochem, La Jolla, CA, USA), GFP (B-2:Sc-9996, Santa Cruz Biotechnology, Inc, California, USA), FKBP12 (#H2008, Santa Cruz Biotechnology), PP70S6K(Thr389) (#9206, Cell signaling Technology, Inc, Danvers, MA) and GAPDH (Biogenesis). The following rabbit antibodies were used; Catalase (#ab1877, Abcam, Cambridge, USA), p62/SQSTM1 (PW9860, BIOMOL international, LP), RHEB (#4935, Cell Signalling Technology), and “core” subunits 20S proteasome (PW8155, BIOMOL international, LP). ECL immunoblotting reagent was from Amersham Biosciences, UK. The primary antibodies used for immunocytochemistry included mouse anti-LC3 (1:100, Nanotools, Teningen, Germany), Rabbit anti-RhoB (1:100, Santa Cruz), Rabbit anti 20S core (1:100, Biomol) or p62/SQSTM1 (1:250 Biomol).

siRNAs applied
The following siRNAs were applied against the human cDNA sequence of; Beclin1 (5´-CAGTTTGGCACAATCAATA-3´), RHEB1#1 (5´-CCTCAAATCCCTAGACCTT-3´), RHEB#2 (5´-TGGAAAGGCTGATCAGTTA-3´), VPS35#1 (5´-AGACAAAGAAGCTGATTTT-3´), CAP1#1 (5´-GGACAGAGCTGAGGCTATT-3´), EEF1G#1 (5´-GGGAAGTACTCCAGAGGCATT-3´), EEF1G#2
The following control siRNA (5'-CGACCGAGACAGCGCAAGAAG-3') were used. All were from Dharmacon Research.

**Primers applied for QRT-PCR**

The cDNA originating from cells transfected with the siRNA of interest were used as template in a duplex PCR reaction performing the amplification of the housekeeping gene porphobilinogen deaminase (PBGD) together with the gene of interest. The following forward and reverse primers were used against the human cDNA sequence of RHEB (5'-TAGCTCGATGTCCGTGCAG-3'; 5'-CTTTACAGCTGCTCTGTT-3'), VPS35 (5'-CGTGAAGATGGACCTGGAAT-3'; 5'-TCCACACGATCAGGGTAACA-3'), CAP1 (5'-GTGCTACACGCCAGCAAGAAA-3'; 5'-GCGGCATCATCTTTGATTTTT-3'), EEF1G (5'-CGCTTCCCTGAAGAATCTAC-3'; 5'-CAGTTTCGCCCATGTGTATG-3'), GNB2L1 (5'-TGAGTGTGCCCTCTCCTCCT-3'; 5'-GCTTGGCAGTTGATCCGGTGTC-3') and PBGD (5'-CATGTCTGGTAACGGCAATG-3'; 5'-AGGGCATGTCTCGTTCAAGCTC-3'). The applied annealing temperature was 58°C for all primers.

**Autophagosome purification and mass spectrometry**

Cells were washed three times in PBS, harvested, dissolved in 1 ml homogenization medium (HM; 0.25 M sucrose, 1 mM EDTA, 20 mM Hepes-NaOH, pH 7.4, protease inhibitor cocktail (CompleteTM tablets, Roche Diagnostics, Mannheim, Germany)) and lysed using a dounce tissue grinder (150 strokes). The lysate was fractionated by centrifugation: 1000 g for 10 min, taking the supernatant and centrifuging for 10 min at 3000 g, taking the supernatant and centrifuging for 15 min at 17,000 g, and re-suspending the pellet containing the vesicular fraction in 1 ml HM. The suspension was loaded on top of an iodixanol (Sigma) gradient made up of five 1.6 ml fractions iodixanol (5, 10, 16, 24, and 30%). Vesicles were separated on the gradient for 17 h at 100,000 g and 4°C and fractions of 1 ml were collected. Experiments were performed on an Agilent 1100 nanoflow system (Agilent Technologies, Boeblingen, Germany) coupled to a linear ion trap Fourier transform ion cyclotron mass spectrometer (LTQ-FT-ICR Ultra, Thermo Fisher Scientific, Bremen,
Germany) equipped with a nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark). The LTQ-FT-ICR Ultra was operated in data-dependent mode. For each cycle, a survey spectrum of $3 \times 10^6$ ions between m/z 350 to 1,600 was measured at a resolution of 100,000. In the linear ion trap, tandem mass spectra of the five most abundant multiply-charged ions were recorded using a collision energy of 30% and a target value of 30,000. Mass-spectrometric parameters were as follows: spray voltage 2.3 kV; no sheath and auxiliary gas flow; ion-transfer tube temperature 150 °C.

For immuno-precipitation of autophagosomes by µMACS™ microbeads (magnetic beads coated with anti-GFP, MACS Miltenyi Biotec) cells from the three different SILAC conditions were harvested separately and pelleted by a 300 g centrifugation for 10min at 4°C. Cells were resuspended in HM and lyzed using 100 strokes in a dounce tissue grinder. The three cell lysates were differential centrifuged separately at 600 g for 10 min, 3000 g for 10 min and 17,000 g for 15 min. The pellets from the 17,000 g centrifugation were combined and diluted to 10mL in HM with 3% BSA. 200 µL µMACS™ microbeads were added and the solution incubated for 1h at 4°C with turning. Beads were washed on an LS Column in a MidiMACS™ Separator, removed from the separator and the captured components eluted by pushing 1.5mL HM with 3% BSA through the column. The suspension was centrifuged at 13,000 g for 2 min and the resulting pellet reduced and alkylated using dithiothreitol (DTT) and iodoacetamide (IAA), respectively. The reduced and alkylated protein solution was diluted in 50 mM ammonium bicarbonate and added 10 µg of trypsin followed by digestion at 37°C ON. Half of the digest was prepared for PISEP by desalting through a reverse phase C18 column and the peptides were then fractionated by PISEP in 11 fractions using the PISEP Stage-Tip protocol. All peptide fractions were analyzed using an LTQ Orbitrap XL (Thermo Fisher Scientific), with a nanoelectrospray ion source from proxeon (Odense) and coupled to an Agilent 1200 nanoflow system. The nanoscale reverse phase HPLC column was packed in a fused silica capillary (ReproSil-Pur® 3µm) with 75µm inner diameter and 15cm long. Peptides were loaded onto the LC-system by an autosampler and eluted by a 140 min. linear gradient of solvent A (0.5% acetic acid) and B (80% ACN and 0.5% acetic acid) and with a flow of 250nl/min. The peptides were eluted into the Orbitrap for a survey scan of $1 \times 10^6$ ions between m/z 350 to 1,600 was
measured at a resolution of 60,000 followed by fragmentation of the 5 most intense multiply charged ions in the LTQ by CID using 5000 ions.

**Proteasome activity**
The cells were lysed in lysis buffer (50 mM HEPES (pH 7.5), 5 mM EDTA, 150 mM NaCl and 1% Triton X-100 containing 1 mM ATP and 0.5 mM pefablock) on ice for 30 min. The lysate was transferred to an eppendorf tube and centrifuges at 15,000 rpm for 15 min at 4°C. From the supernatant the proteasome activity was measured using the 20S proteasome activity assay kit from Chemicon international (Cat. No. APT280, Temecula, CA, USA) according to manufacture’s instructions. The plate was measured on a Varioscan Flash plate-reader, Thermo Electron Corporation. The protein levels were determined in the supernatant using the bio-rad Dc protein assay kit (Bio-Rad laboratories) and a microtiter plate reader (VERSA max, Molecular Devices LTD, Crawley, UK).

**Yeast cell culture and autophagy measurements**
Experiments were carried out in BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and respective null mutants, obtained from Euroscarf. Proteasome mutants pre1-1, pre2-1, pre1-1/2-1 and respective Wild type strain (WCG4a) were kindly provided by Dr. Dieter H. Wolf (Heinemeyer et al., 1993). Strains were grown at 28°C on SC medium containing 0.17% yeast nitrogen base without amino acids (Difco), 0.5% (NH₄)₂SO₄ and 30 mg/l of all amino acids (except 80 mg/l histidine and 200 mg/l leucine), 30 mg/l adenine, and 320 mg/l uracil with 2% glucose (SCD) or on SCD medium lacking (NH₄)₂SO₄ and amino acids (SD-N). For induction of autophagy by nitrogen starvation, cells were inoculated from fresh overnight cultures to 0.4 OD₆₀₀ (~4 x 10⁶ cells/ml) in SCD, grown for 4-5 h to mid-log phase reaching ~1.5 OD₆₀₀, washed twice in ddH₂O and incubated in SD-N medium at 1 OD₆₀₀ for 3 hours. For induction of autophagy by rapamycin treatment, cells were grown to mid-log phase (~ 1OD₆₀₀) as above and rapamycin (AG Scientific) was added directly to the culture to a final concentration of 0.5 µg/ml (using 1 mg/ml stock solution in EtOH containing 1% Tween-20) preceding 3 hours of incubation. For the assessment of autophagic flux (ALP activity) upon
proteasome inactivation, yeast cell cultures were shifted to 37°C 45 min before rapamycin was added. Autophagy was determined in cellular extracts of 1 ml culture aliquots by alkaline phosphatase (ALP) activity according to Noda et al. 1995 (Noda et al., 1995) using respective strains transformed with and selected for stable insertion of pTN9 HindIII fragment containing the cytosolic form of Pho8p (Pho8p∆N60). In order to correct for intrinsic (background) ALP activity, strains without pTN9 insertion have been simultaneously processed and ALP activity subtracted. This was omitted in case of proteasome mutant experiments, since background activity was unaffected by pre mutations, but severely reduced upon Pho8p∆N60 insertion in WCG4a strains. For ALP activity assay 1.5 µg total protein was used as determined by BioRad Protein Assay (BIORAD). Notably, at least three different clones of each pTN9 transformant were employed for autophagy measurements to rule out clonogenic variations. Fluorescence readout of ALP assay at 485 nm (340 nm excitation) was performed with a GeniosPro plate reader (Tecan) and relative fluorescence of each knock out strain was normalized to wild type (100% activity).

**In-gel digestion and mass spectrometric analysis**

Gel lanes were cut into 10 slices, samples in gel digested using trypsin (Shevchenko et al., 2006), resulting peptide mixtures were desalted using STAGE tips as described (Rappsilber et al., 2007), and analyzed by LC-MS/MS. The raw data was processed using MSQuant (Mortensen et al., 2010) or MaxQuant (Cox and Mann, 2008). Peak lists were searched against the human MSPI database containing 68,404 entries (Schandorff et al., 2007) using MASCOT 2.0 (Matrix Science, London, UK) with the following parameters: carbamidomethyl-cysteine was set as fixed modification, methionine oxidation, deamidation of asparagine and glutamine, and protein amino-terminal acetylation were set as variable modifications. Double or triple SILAC were chosen as quantification mode. Two miss cleavages were allowed, enzyme specificity was trypsin, precursor mass accuracy had to be within 30 ppm for FT-data and 7 ppm for Orbitrap data, and the fragment spectra mass accuracy was set at 0.6 Da. The identified peptides were recalibrated using MSQuant.
(http://msquant.sourceforge.net), results were combined using MGFcombiner (version 1.05), and re-searched using MASCOT 2.0 with above mentioned parameters, except that the precursor mass tolerance was set to 5 ppm. To determine the number of false-positive peptide hits the data was searched against a human MSIP1-decoy database essentially as described (Elias et al., 2004) and the MASCOT peptide score was adjusted to yield a number of false-positive peptide identifications of less than 1% (calculated as follows: FP rate [%] = reverse hits × 2 × 100/forward hits). For a protein to be counted as identified a minimum of two unique peptides (bold red hits, minimum length 7 amino acids) had to be sequenced and to fulfill the determined criteria. In order to yield the maximum number of peptides per identified protein, the data was researched against decoy-databases consisting of the identified proteins only. Thus, the MASCOT identification score yielding less then 1% FP could be dropped to 15, resulting in considerably more peptide IDs/protein, only considering proteins that were identified using a full-length decoy database and that did pass the stringent identification criteria stated above.

Cluster Analysis
Protein profiles were checked for apparent outliers/wrong assigned ratios by using the summed protein ratios over all measured fractions as an additional quality criterion. Outliers were defined by box plots (summed ratio > 1.5-fold of the interquartile range) and removed from datasets before cluster analysis. Also proteins with relative standard deviations of mass spectrometric quantitations higher than 300% in single gradient fractions were removed. Average rel. quantitation errors of further processed protein profiles (6 data points each) were: ConA 17±8% (max. 60%), Rapa 22±11% (max. 81%), and HBSS 24±13% (max. 116%).

To test the validity of the clusters it was required that major complexes like the ribosome, the exosome, and the proteasome located to a single cluster. In addition, the resolving power of the clusters was assessed by a principal component analysis (PCA). Proteins consistently localizing to clusters A, B, and C, were clearly separated (data not shown). In order to compare the three stimuli, respective gene IDs (NCBI) of the identified proteins where used. Thus, inconsistencies were avoided possibly arising
from different alleles or fragments identified in different experiments. There was no evidence that multiple splicing isoforms from the same gene existed within any of the individual data sets. Protein Center (Proxeon Biosystems) was used to investigate the GO identities of proteins in the generated clusters.

Functional analysis
The three large scale data sets were divided into seven different subsets depending on the overlap between the three stimuli (as illustrated in Figure 3A). Each subset was analyzed using the DAVID Functional Annotation Tool (Saeed et al., 2003) to identify enriched functional categories of proteins. DAVID was used to rank functional categories based on co-occurrence with sets of genes in a gene list measured against a human genome background. The categories with a Fisher Exact P-Value smaller than 0.05 were included using the most general unifying term. A number of redundant or obvious categories (like intracellular, cytosolic) were not included.

References


