Supplementary Methods

Yeast strains, growth conditions and antibodies

Yeast strains are listed in Table S2. Yeast cells were grown in synthetic medium (SD: 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose, amino acids as required) or rich medium (YPD; 1% yeast extract, 2% peptone, 2% glucose). For starvation induction, cells in early logarithmic phase were washed and resuspended in starvation medium (SD-N: 0.17% yeast nitrogen base without amino acids, 2% glucose) for 2-4h, unless stated otherwise. Rapamycin was used at a final concentration of 220 nM.

The following antibodies were used in this study: mouse monoclonal anti-GFP antibody (Roche), rabbit polyclonal PAP antibody (Sigma), mouse monoclonal anti-GST antibody (Sigma), mouse monoclonal anti-Pgk1 antibody (Invitrogen), mouse monoclonal anti-myc (9E10), mouse monoclonal anti-His (penta) antibody (Qiagen). Polyclonal anti-Ape1 antibody was generated by immunizing rabbits with a synthetic peptide corresponding to amino acids 168-182. Anti-Atg11 antibodies were generated by immunizing rabbits with recombinant protein containing amino acids 685-1178 of Atg11 purified from *E. coli* and subsequent strip purification.

Plasmid construction

Plasmids are listed in Table S1. To generate Atg19 mutants, site-directed mutagenesis was performed and verified by sequencing. The promoter of *ATG11* was amplified from genomic DNA and ligated via SacI XbaI into a pRS415Gal1 vector, replacing the Gal1 promoter. The coding region of *ATG11* was amplified from genomic DNA and ligated into the SmaI site, generating an XhoI site 5' to the coding region. Monomeric GFP was then inserted via SpeI XhoI. To generate N-terminally tagged *ATG19*, the promoter and coding region of *ATG19* were amplified and used to exchange *ATG11* sequences, resulting in pCK770. The GFP tag was replaced by an HTB or an S-tag via XbaI/SpeI and SbfI, generating pCK773 and pCK776. The promoter and coding region of *ATG34* were amplified from genomic DNA and used to exchange the promoter and coding region of *ATG19* in pCK773 and pCK776, resulting in pTP50 and pTP36.

Purification of GST-Atg19 and 6xHis-Atg11

S. cerevisiae Atg19 wild-type and mutants thereof were cloned into pGEX4T1 resulting in N-terminal GST fusion proteins. Proteins were purified from *E. coli* Rosetta pLysS cells as described in [1]. The *S. cerevisiae* Atg11 fragment (amino acids 685-1178) with an N-terminal His-tag followed by a TEV protease recognition site was expressed from the pETDuet-1-His-TEV-Atg11 vector in the *E. coli* Rosetta pLysS strain. Cells were grown at 37°C in TB medium to OD_{600nm} of 0.8, induced with 0.5 mM IPTG and grown for further 16h at 18˚C. Harvested cells were resuspended in a buffer containing 50 mM HEPES pH 7.5, 300 mM NaCl, 10 mM Imidazole, 2 mM $MgCl_2$, 2 mM β-mercaptoethanol, Pefabloc (Carl Roth) and DNAse I (Sigma) and disrupted by freeze thawing followed by short sonication. After ultracentrifugation of the cell lysate (40000 rpm (Beckman Ti45 rotor) for 40 minutes at 4˚C) the cleared supernatant was loaded on a NiNTA column (GE Healthcare) and His-TEV-Atg11 was eluted by applying a step-wise imidazol gradient. The 150 mM imidazole fractions containing His-TEV-Atg11 were collected. The protein was concentrated and applied onto a Superdex 200 column (16/60 GE Healthcare) and eluted with a buffer containing 25mM HEPES pH 7.5, 150 mM NaCl and 1 mM DTT. Fractions containing pure His-TEV-Atg11 were pooled, concentrated and stored at -80°C.

Kinase assays

Kinase assays were performed as described [2]. For *in vitro* phosphorylation of Atg19 and Atg34, Atg1-TAP, Atg1-D211A-TAP and Hrr25-TAP were immunoprecipitated with 5 µl IgG coupled Dynabeads and incubated in the phosphorylation mixture with 2 µg soluble GST-tagged Atg19 and Atg34 proteins purified from *E. coli*. After 20 minutes incubation at 30ºC,

the Atg1 and Hrr25 bound beads were removed and the supernatant was assessed for radioactivity incorporation by phosphorimaging. As a kinase dead version of Hrr25 is not viable and could therefore not be used as a negative control [3], we used instead an immunoprecipitation of a non-tagged strain. The co-expression of a tagged Hrr25-kinase dead version with the endogenous wild type gene is also not an option as Hrr25 self-interacts, which would result in the co-precipitation of the active wild-type kinase in the kinase-dead samples.

Protein localization in yeast and quantitative live cell imaging

Fluorescent images were recorded with an applied precision PersonalDV microscope with a 60x oil immersion objective as stacks of 0.25 μm thick slices. Pictures from one figure panel are taken with the same imaging setup. Maximum intensity Z projections (Fig. 4C and S3C) or single slices (Fig. 2, 4D, S2A, S2B, S4B and S4C) of these stacks are shown. The contrast was linearly adjusted for each of the pictures. Quantification was performed using ImageJ software. Giant Ape1 assays were performed as previously described [4], using 3 hours of 250 μM $CuSO_4$ induction, with (Fig 2) or without (Fig 4D) 1 hour of 300 nM rapamycin treatment. For vacuolar staining, cells were treated with 16 μM FM4-64 for 1 hour, washed, and grown in medium without FM4-64 for at least 1 hour before imaging.

SILAC labelling, HTBeaq tandem affinity purification and mass spectrometric analysis.

SILAC labeling, protein purification and mass spectrometric analysis was based on methods described elsewhere [5]. Regulated protein phosphorylation events were determined as described in [5,6] with modifications: for phospho-mapping, purified proteins were digested using Trypsin (recombinant, proteomics grade, Roche), 5% w/w of the estimated protein amount. Digest was performed overnight at 37°C. MS analysis was performed using a reversed phase nano-HPLC (Ultimate 3000, Dionex, Sunnyvale CA, USA) directly coupled to an LTQ OrbitrapVelos mass spectrometer (Thermo Fisher Scientific) via a nanoelectrospray ion source (Proxeon). A phosphosite probability (calculated using phosphoRS 2.0) of 75% or higher was considered as confidently localized. For every experiment two biological replicates were analyzed.

Both Atg34 and Atg19 were analyzed both from rich and rapamycin treated cultures using SILAC. No difference could be detected in phosphorylation in the Atg11 binding region of Atg19. For Atg34 we did note a 6x upregulation of a doubly phosphorylated peptide: ADAL**SS**PDE**SS**IMSTPFK. One phosphorylation could be clearly assigned to S382 and the other phosphorylation was ambiguously on either S377 or S378. A peptide carrying a single phosphorylation on either S377 or S378 was increased only 2x upon rapamycin treatment. This suggests that S382 phosphorylation could account for a 3x increase. In non-quantitative mass spectrometric analyses we found S382 and S383 double phosphorylated peptides confirming their existence *in vivo*. Atg19 was furthermore analyzed from wild-type and *hrr25-ts* mutant cells using SILAC. A clear reduction of phosphorylation in *hrr25-ts* cells of around four fold was detected for peptides containing phosphorylated residues S390 and S391, as well as for S396 and a second site, which could not be clearly assigned. Additional doubly phosphorylated peptides harboring phospho-S391 and a second phosphorylation of ambiguous site allocation showed similar ratios. RAW files of all MS runs are available on request.

References

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