Supplemental Information

Asymmetric Inheritance of Aggregated Proteins
and Age Reset in Yeast Are Regulated
by Vac17-Dependent Vacuolar Functions

Sandra Malmgren Hill, Xinxin Hao, Johan Grönvall, Stephanie Spikings-Nordby, Per O. Widlund, Triana Amen, Anna Jörhov, Rebecca Josefson, Daniel Kaganovich, Beidong Liu, and Thomas Nyström
Supplemental materials include:

Figures S1-S6
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Figure S1: A genome wide screen using Hsp104-GFP as a marker for protein aggregates identifies age asymmetry-generating genes (AGGs) 

**Related to Figure 1 and Materials and methods**

**A** Synthetic genetic array (SGA) crossings were used to create a mutant library containing a functional *HSP104-GFP* fusion as a marker for protein aggregates. Mutant arrays in the 96-well formats were subjected to a transient heat shock to induce protein misfolding and aggregation, followed by recovery at 30°C allowing new daughter cells to be generated. High content microscopy was used for automated image analysis to determine the inheritance of aggregates in newborn daughter cells. The mutants displaying a 25% increase in aggregate inheritance, as compared to wild type cells, were scored as being deleted in AGG genes. 

**B** The ranked top AGG hits (total of 111) are plotted from highest deviation to the lowest (top row), with their corresponding Hsp104-GFP intensity (bottom row), to illustrate that the AGG Hits identified in the high content microscopy screen was not identified due to an abnormally high (or low) signal intensity of Hsp104-GFP. 

**C** Mutants identified as having an improved asymmetry (>0.75 compared to WT values) were tested for enrichment of GO annotations in Biological Processes using the online tool at DAVID (p<0.01 Modified Fisher exact). 

**D** Chromosome location of the top 111 AGG hits identified as deficient in damage asymmetry shows an enrichment of hits on the right arm of chromosome III. The top 111 AGG hits were tested for enrichment of GO annotations in Biological Processes (Fig 1B), cellular components (E) and keywords (F) using the online tool at DAVID.
Figure S2:
Silver stain of Hsp104-GFP co-immunoprecipitation and inheritance of Htt103QP in vac17Δ cells
Related to Figure 1 and Figure 2
(A) 10% (0.8 mg) of each co-immunoprecipitation was run on SDS-PAGE and silver stained. Hsp104-GFP has an expected molecular weight of 140 kDa. Lane 1: Molecular weight marker. Lane 2: co-IP with Hsp104-GFP at 30°C. Lane3: co-IP with Hsp104-GFP at 37°C. Lane 4: empty. Lane 5: control co-IP at 30°C. Lane 5: control co-IP at 37°C. (B) In contrast to heat-induced aggregates, inheritance of the amyloid, disease-related, Huntingtin protein Htt103QP is not affected in vac17Δ cells.
Figure S3:
Localization of Vac17-3xGFP in WT and in cells overexpressing Vac17
Related to Figure 2

(A) Localization of Vac17-3xGFP in WT and VAC17 OE cells grown at 30°C (top row) or after 90 min of heat treatment at 38°C (bottom row). A WT strain lacking the Vac17-3xGFP but carrying the HSP104-mcherry construct was used as a control for bleed-through and autofluorescence. These cells were imaged with the same exposure time, and set to the same image display range as the WT Vac17-3xGFP strain. Scale bar = 5 µm. (B) Quantification of Vac17-3xGFP localization in WT and VAC17 OE cells growing at 30°C (nWT = 786 cells, nVAC17OE = 721 cells), and after heat treatment at 38°C for 90 min (nWT = 517 cells, nVAC17OE = 572 cells). The data presented as based on the average of N≥3 replicates ± SD.
**Figure S4:**

**Vac17 levels affect the rate of inclusion formation**

*Related to Figure 4 and Movies S1-S3*

(C) Figure is based on still images from Movies S1-S3, displaying inclusion formation in denoted strains. Vacuoles are stained by FM4-64 and visualized in red. HSP104-GFP in green is used as a marker for protein aggregates. Scale bar = 1 µM
**A**

Heat Stress

- WT
  - 0min
  - 30min
  - 65min

- vac17Δ
  - 0min
  - 30min
  - 85min

- VAC17 OE
  - 0min
  - 30min
  - 40min

**B**

IPOD formation (min)

|         | WT      | vac17Δ  | VAC17 OE |
|---------|---------|---------|----------|
| 0min    | 100     | 120     | 40       |
| 30min   | 60      | 100     | 60       |
| 65min   | 20      | 80      | 20       |

**C**

38°C, 90 min

- fab1Δ
  - VAC17 OE

- VAC17 OE
  - VPS16Δ

**D**

Inclusion formation (min)

- WT: 80 ± 10
- fab1Δ: 60 ± 10
- VPS16Δ: 60 ± 10
- VAC17 OE: 70 ± 10
Figure S5: Time-lapse imaging of inclusion formation in WT, vac17Δ and VAC17 OE strains during 38°C continuous heatshock.

Related to Figure 4 and Movies S1-S7

(A) Formation of the peripheral IPOD inclusion, monitored by the Hsp42-GFP as a reporter. Scale bar = 1µm (B) Time for IPOD formation in denoted strains, as quantified from time-lapse images from (A). IPOD formation is significantly decelerated by VAC17 deletion (p=4.1E-05), whereas overproduction of Vac17naccelerates formation (p=2.5E-04). (C) Inclusion formation and vacuolar morphology in denoted strains. Vacuoles are stained by FM4-64 and visualized in red. HSP104-GFP in green is used as a marker for protein aggregates. Scale bar = 5 µm. (D) Time for inclusion formation in denoted strains, based on time-lapse imaging using Hsp104-GFP as a reporter. Deletion of FAB1 reduces inclusion formation (p=3.3E-03) and VAC17 overexpression cannot rescue this defect (p=3.1E-03 compared to WT). See also movies S1 and S6-S7. The data in the bar graphs is presented is an average of N≥3 replicates ± SD.
Figure S6: Vacuole number and fusion rate in relation to aggregate management

Related to Figure 3 and 4

The number of aggregates in a cell does not show a correlative relationship with the number of vacuoles, as tested during (a) heatshock (Pearson’s r=0.23 N=648 cells); (b) heatshock combined with salt stress to induce vacuolar fragmentation (Pearson’s r=0.28 N=728 cells); and (c) replicative aging (10-12 generations old, Pearson’s r=0.01 N=156 cells).

(d) Deletion of VAC17 does not influence rate of homotypic vacuole fusion, as observed through time-lapse imaging of vacuole morphology upon addition of 0.4M NaCl. Data in figure based on average values from >3 individual experiments. Linear regression of time points 0-40 min reveals no significant difference between the rate of vacuole fusion between the two strains (WT: 0.33±0.29, vac17Δ: 0.45±0.29, p=0.58).
Table S1: Top 11 Asymmetry Generating Genes (AGGs)

Related to figure 1
Summary of the 111 top hits identified in the screen, ranked from largest to lowest deviation from WT. Column 3 shows the bud/mother ratio obtained in the screen by dividing number of daughter cells with aggregates with the number of mother cells containing aggregates. An average of WT values are included in the last row of the table. Column 4 shows the fold change in asymmetry ratio, compared to WT values. Values over 1 indicate mutants with a higher bud:mother ratio, with an increase in number of daughters containing aggregates thus indicating a loss of damage asymmetry (Fig. 1A). A fold change of at least 1.25 was used as a cut-off to generate this list of top hits. Column 5 shows manual verification of asymmetry defect (Yes or No), empty means that the mutant had not been manually tested. The confirmation rate obtained when analysing 55 of the 111 mutants manually was 85%. Column 6 shows GO annotation for Biological Process.

Table S2: Mutants with increased asymmetry

Related to figure S1c
Summary of mutants identified in the screen as having an increased damage asymmetry, so that fewer daughter cells are found with protein aggregates. Column 3 shows the bud/mother ratio obtained in the screen by dividing number of daughter cells with aggregates with the number of mother cells containing aggregates. Column 4 shows the fold change in asymmetry ratio, compared to WT values. Values below 1 indicate mutants with a lower bud:mother ratio, with a decrease in number of daughters containing aggregates thus indicating an improved damage asymmetry (Fig. 1A). A fold change of 0.75 was used as a cut-off to generate this list of hits. Column 6 shows GO annotation for Biological Process. These mutants have not been confirmed or further tested as this work mainly focuses on mutants displaying a decreased asymmetry.

Table S3: Protein Interactors of Hsp104-GFP

Uweighted spectrum counts of all hits were compared to peptide counts in ProteinAtlas for S. cerevisiae. Statistical significance was calculated using a fisher’s exact test. Spectral counts for both controls and Hsp104-GFP co-IPs are in columns D, E and F, G respectively. Column H shows the number of observed peptides for the listed protein in the database. Column I shows total peptides observed in the Peptide atlas for the positive hits in each column D-F. Column J shows the number of peptides observed in each mass spectrometry run. K is relative abundance and column L is the statistical significance. The four sheets have calculations for each mass spectrometry run.
Supplemental experimental procedures

**Strains, plasmids, and growth conditions**

Strains with HSP104-GFP-LEU2 in the S228C SGA background were used in the aggregate asymmetry screen as well as in manual aggregate assays. Aggregate assays for vps16Δ was performed in BY4741 background with HSP104-GFP-HIS3, as this mutant was not included in the SGA library due to sporulation defects (Banta et al. 1988). Old cell isolations and lifespan assays were performed in BY4741 background, using deletion mutants from the YKO collection (EUROSCARF). See table S4 for detailed information of strains and plasmids. Yeast cells were grown in YPD or synthetic drop out media with corresponding antibiotics added.

**Strain construction**

The HSP104-GFP query strain was constructed by shifting the HIS3 marker used in yeast GFP collection to LEU2 marker. Subsequently, the complete HSP104-GFP-LEU2 cassette was amplified by PCR and transformed into the Y7092 SGA query strain background. The HSP104-GFP-LEU2 fusion was integrated into the yeast single deletion collection (SGA-V2), with Synthetic Genetic Array (SGA) methodology (Costanzo et al., 2010; Tong et al., 2001; Tong et al., 2004), using the Singer RoToR HAD system (Singer Instruments Co. Ltd.,). To produce a strain overexpressing VAC17, the GPD promoter was amplified from the pYM-N15 plasmid (Janke et al. 2004) and incorporated upstream of the VAC17 ORF. As the VAC17 ORF was not completely deleted in vac17Δ of the YKO collection (the last ~800 bp still present), a new deletion strain was produced: KanMX cassette was amplified from YKO mutant, with VAC17 flanking sequences, and transformed into WT. To overexpress VAC17 lacking the binding domain for Myo2 (vac17ΔBD), the ORF of the truncated VAC17 gene was amplified from a plasmid25 and then fused to a GPD promoter using overlap PCR. The cassette containing the truncated vac17ΔBD was incorporated into the VAC17 locus of a vac17Δ deletion mutant. The YCp50-MYO2 plasmid in the LWY2949 strain (Catlett et al. 2000) was replaced with a pRS413 plasmid carrying either the wildtype MYO2 allele, or the Myo2-N1304S allele lacking the binding domain for vac17 (Eves et al., 2012). Transformed cells were grown on selective media containing 5-Fluoroorotic acid (5-FOA) for counter selection. All transformations were performed using the LiAc transformation protocol and transformants were verified by PCR and sequencing. Standard mating and tetrad dissection protocols were used to construct ssa1Δ ssa2Δ and ssa1Δ ssa2Δ VAC17 OE strains.

**High-content microscopy screen for AGGs**

Cells were inoculated from agar plates into 96-well plates using Singer RoToR, and liquid cultures were grown at 30°C overnight. Pre-cultures were then diluted into 96-well plates with a final volume of 205µL/well and cultured for 12h at 30°C, upon which cells were transferred into 96-well PCR plates, subjected to heat treatment (34°C 2min, 38°C 2min and 42°C 26min) using a PCR machine (C1000™ Thermal Cycler). Following heat treatment, cells were allowed to recover at 30°C for 90min, at which point cells were fixed by adding a final concentration of 3.7% formaldehyde. To prepare cells for imaging, fixed cells were transferred into 96-well glass-bottled imaging plates with 200µL 1XPBS. Imaging was performed using a high-content imaging microscope (ImageXpress® Micro), acquiring a total of 25 images for each well. Acquired images were quantified using MetaXpress (Version 3.1) software. A MetaXpress sub-program was designed for automated image analysis, recognizing budding events, distinguishing mother- from daughter cells and analysing the occurrence of Hsp104-GFP foci in the respective cells (the journal for this image analysis is to be published elsewhere).

**AGG screen data filtering**

Data from mutants with less than 100 mother cells and less than 100 daughter cells scored were removed from the final results, removing 394 from the initial 4309. The data was then filtered to remove data from 103 mutants, where less than 50% of mothers contained aggregates (WT had an average of 83% mother cells with aggregates). The aggregate asymmetry ratio for each mutant was determined by taking the percentage of daughter cells with aggregates divided by the percentage of mother cells with aggregates. The asymmetry ratios was then normalized to the WT value, so that values above 1 mean that more daughter cells are found containing aggregates, thus indicating a mutant
with a defect in establishing damage asymmetry (Fig. S1a, bottom; Table S1). The final list of 3812 mutants from the screen was then ranked according to the normalized asymmetry ratio. A top list of 111 AGG mutants was generated, which had a normalized ratio of at least 1.25 (Table S1). A list of the top hits displaying increased asymmetry (ratio of less than 0.75) can be found in Table S2.

**Enrichment analysis**

Enrichment analysis was performed on the top hits identified from the large-scale screen. These mutants were compared to a filtered background set (3812 mutants, those with more than 100 cells and more than 50% mother cells with aggregates), using Database for Annotation, Visualization and Integrated Discovery (DAVID) to analyze for functional enrichment (Huang da et al., 2009a, b). Enrichment tested for GO annotations in Biological process (GOTERM_BP_FAT), cellular compartment (GOTERM_CC_FAT) and keywords from Swiss-prot and Protein Information Resource Databases (SP_PIR_KEYWORDS), using a cut-off value of p<0.01 for significant enrichment (modified Fisher exact p-value).

**Interaction network**

The network of top AGG mutants with a known function/GO annotation was displayed in functional clusters (Fig. 1d) using Cytoscape (Shannon et al. 2003). Genetic and physical interactions of genes in the network were downloaded from BIOGRID database (Stark et al. 2006). Vac8 and fab1 mutants showed an asymmetry defect, but were filtered from the screen data due to the number of cells being below the set cut-off value for the screen. These mutants were however manually tested and verified and therefore added to the network.

**Hsp104 immunoprecipitation and mass spectrometry**

400 mL each of strains Hsp104-GFP and BY4741 were grown to OD ~0.5, split and allowed to grow for 1.5 hours at either 30°C or 37°C. Cells were harvested by centrifugation for 5 min at 3200 g (4000 rpm) and washed with cold Tris buffer (50 mM Tris, 200 mM NaCl, 1 mM EDTA pH 7.5) and re-suspended in 1 mL each of the same buffer with 1x protease inhibitor cocktail added (Complete mini, Roche). Cells were lysed with beads using a Fastprep (MP biomedicals). Lysate was collected and beads washed with an additional 0.5 mL of lysis buffer. Cells were cleared by spinning at 2000xg for 5 min at 4°C. 50 µL of a 50/50 slurry of anti-GFP beads (GFP-trap, Chromotek) were added to each prep and incubated with agitation for 2 hours at 4°C. Beads were then washed with 2 x 1 mL of lysis buffer, followed by 50 mM TEAB. Proteins were eluted with 2 sequential incubations with 100 µL 1% formic acid. 20 µL of each prep was run on SDS-PAGE (NuPage, Invitrogen) and silver stained (Pierce silver stain kit, Thermo Scientific).

Cysteins were reduced with 1 µL of 200 mM DTT (Biomol) in 50 mM HEPES (Biomol) for 30 min. at 57°C. The sample was cooled to 24°C and 2 µL of 400 mM IAA (Merck) dissolved in 50 mM HEPES was added and incubated in dark for 30 min. at 24°C. A novel protocol using paramagnetic beads, termed Single-Pot Solid-Phase-enhanced Sample Preparation (SP3) as described (Hughes et al. 2014) was used to prepare the samples for LC-MS/MS. The proteins were digested using trypsin (Promega) with an enzyme to protein ratio 1:50 at 37°C overnight.

Peptides were separated using the UltiMate 3000 RSLC nano LC system (Dionex) fitted with a trapping (Dionex Acclaim PepMap100, 75 µm x 2 cm, C18, 3 µm, 100 Å) and an analytical column (Dionex Acclaim PepMap RSLC 75 µm x 50 cm C18, 2 µm, 100 Å). The outlet of the analytical column was coupled directly to a Q-Exactive (Thermo) using the proxeon nanoflow source in positive ion mode. Solvent A was water, 0.1% formic acid and solvent B was acetonitrile, 0.1% formic acid. The samples (6.5 µL) were loaded using the µL pickup mode of the autosampler, with a constant flow of solvent A at 6 µL/min onto the trapping column. Trapping time was 5 minutes. Peptides were eluted via the analytical column a constant flow of 0.3 µL/min. During the elution step, the percentage of solvent B increased in a linear fashion from 4% to 7% B in 5 minutes, then from 7% to 25% in a further 105 minutes and finally from 25% to 40% in another 10 minutes. Column cleaning at 85% B followed, lasting 5 minutes, before returning to initial conditions for the re-equilibration, lasting 10 minutes. The peptides were introduced into the mass spectrometer (Q-Exactive, Thermo) via a PicoTip Emitter 360 µm OD x 20 µm ID; 10 µm tip (New Objective) and a spray voltage of 1.8 kV was applied. The capillary temperature was set at 250°C. Full scan MS spectra with mass range 300-1500 m/z were acquired in profile mode in the FT with resolution of 70000. The filling time was set at maximum of 32 ms with a limitation of 1x106 ions. DDA was performed with the resolution of the
Orbitrap set to 17500, with a fill time of 60 ms and a limitation of 5x10^5 ions. Normalized collision energy of 25 was used. A loop count of 15 with count 1 was used. Dynamic exclusion time of 30s was applied. An underfill ratio of 1%, corresponding to 8.3 x10^4 ions was used. The peptide match algorithm was set to 'off' and only charge states of 2+, 3+ and 4+ were selected for MS/MS. Isolation window was set to 2 m/z and 110 m/z set as the fixed first mass. MS/MS data was acquired in centroid mode. In order to improve the mass accuracy, a lock mass correction using a background ion (m/z 445.12003) was applied.

The raw files searched with Mascot (v.2.2.0.7) against the Uniprot_S.cerevisiae database including the two sequences of the fusion protein. Carbamidomethylation was specified as a fixed modification, and oxidation of methionine as variable in all searches. Trypsin was specified as protease with 2 missed cleavages allowed. Afterwards the Mascot .dat files were uploaded into Scaffold 3 and further processed. Unweighted spectral counts were compared to the PeptideAtlas S. cerevisiae database (http://www.peptideatlas.org, build March 2013) and significance tested using a Fishers Exact test. Proteins that were enriched in the BY4741 background datasets were removed from each of the Hsp104-GFP datasets (30°C and 37°C, respectively). Proteins were then analyzed for functional enrichment using DAVID, with a minimal count of 4 proteins and maximum EASE score of 0.1. For proteins unique to either the untreated or heat shocked sample, the 3 remaining datasets were used as background.

**Aggregate inheritance**

Cells were grown to exponential phase at 30°C, and were then subjected to 38°C heat shock for 90 min to induce aggregation. Live cells were then imaged in z-stacks and quantified for aggregate inheritance and aggregate fusion phenotype using Hsp104-GFP as a reporter for aggregates. This protocol was combined with FM4-64 staining for simultaneous analysis of vacuole inheritance.

**Aggregate Retention and removal**

To distinguish between the aggregate retention and aggregate removal (retrograde transport and/or degradation), heat treatment of cells was combined with a prior staining of the cell wall using Concanavalin A Tetramethylrhodamine conjugate as previously described (Hill et al., 2014). All buds present before the heat treatment will be stained by the dye, whereas buds that formed after staining and heat treatment will not be stained as their cell wall is composed of newly synthesized material. Retention efficiency was determined as the percentage of unstained buds that were free of aggregates, and removal efficiency was quantified by the percentage of stained buds that were aggregate-free.

**FM4-64 staining**

Cells were incubated in YPD with a final concentration of 0.01 μg/µL FM4-64 (N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide; Life technologies). Staining was performed at 30°C for 30 min, cells were then washed and resuspended in YPD, followed by a 90 min chase at 30°C. Live cells were imaged in z-stacks and vacuole inheritance was determined as percentage of budding vents with a visible vacuole in the bud.

**Fluorescence Microscopy**

Images were obtained using Zeiss Axio Observer Z1 inverted microscope, equipped with ApoTome 2, TempModule S and an AxioCam MRm camera, using Plan Apo 100X oil objective NA:1.4 and the following filtersets: 38 HE eGFP, 45 HQ TexasRed and 49 DAPI. Time lapse imaging was performed on cells attached to concanavalin A coated coverslips, mounted to a flow chamber for salt stress experiment or sealed for heat treatment.

**Protein extraction and immuno blotting**

Whole cell protein extraction was performed using NaOH and β-mercaptoethanol and protein concentrations were determined using the Bio-Rad Dc assay. SDS-PAGE electrophoresis was accomplished using Criterion precast Bis-Tris gels followed by blotting using Criterion wet tank transfer system (Bio-Rad). Vac17 levels were identified using sheep-α-Vac17 antibody, which was a kind gift from Lois Weisman.
Old cell isolation
Old cells were obtained using the magnetabind biotin-streptavidin system according to established protocols (Sinclair and Guarente, 1997; Smeal et al., 1996). Cells were grown to exponential phase at 30°C, washed in PBS and then labelled with EZ link Sulfo-NHS-LC biotin (Life Technologies) at a final concentration of 0.5mg/mL. Excess biotin was washed away, and cells were resuspended in growth medium for overnight culturing. The following day, cells were washed with PBS and incubated with 0.75 mg/mL MagnaBind streptavidin beads (Life technologies). Biotin-labeled cells were then isolated using a magnetic sorter and continuous washes with PBS+0.5% glucose. Cells were resuspended in growth medium and cultured overnight, followed by a second round of streptavidin addition and magnetic sorting. Median age of the old cells was determined by counting bud scars in z-stack images upon staining cells with 10 µg/mL Wheat Germ Agglutinin (WGA, Life Technologies).

Endocytosis assay
Endocytotic rate was determined by time-resolved uptake of FM4-64 according to established protocol (Baggett et al. 2003). Cells were stained with 0.02 µg/µL FM4-64 in cold YP, and resuspended in pre-warmed YPD and incubated at 30°C. Samples were obtained at set time points, washed with cold YP and kept on ice until imaging. Live cells were imaged in z-stacks and percentage of cells with stained vacuole was quantified for each time-point following staining.

Salt stress induced vacuolar fragmentation
To induce vacuolar fragmentation, a final concentration of 0.4M NaCl was added to the cell culture. Salt stress was combined with 38°C heat treatment for testing correlation between the number of vacuoles and the number of aggregates per cell. For time-lapse quantification of vacuole fusion; 0.4M NaCl was added to cells in a flow cell and cells were imaged immediately. Vacuole fusion rate was determined by analyzing the decrease in average vacuole number per cell in time points following salt addition.

pUbc9\textsubscript{ts} toxicity
Cells carrying a plasmid with Ubc9\textsubscript{ts}-RFP under a galactose inducible promoter (Kaganovich et al. 2008) were grown overnight in glucose, and then transferred into medium containing galactose to induce expression of Ubc9\textsubscript{ts}. Growth upon induction was monitored over four days using Bioscreen C equipment (Labsystems Oy, Helsinki, Finland)

Replicative lifespan
Yeast replicative lifespan was measured by using Singer MSM micromanipulator and following standard procedures (Egilmez et al., 1990). Briefly, exponentially growing cells were plated and placed in a grid using a dissection needle. Cells in the grid were allowed to grow for one generation, after which only the newly produced cells were kept in the grid. These cells represent virgin mothers and were followed through their subsequent divisions, keeping track of the number of produced daughter cells. Data was compared using a two-tailed Mann Whitney U test. All lifespans were tested in at least three separate sets (N) of experiments.

Fluorescence 3D Time-Lapse (4D Imaging)
For time-lapse imaging yeast cells were grown as described above and seeded on concanavalin A (Sigma) coated 4-well microscope plates (IBIDI). Confocal 3D movies were acquired using a dual point-scanning Nikon A1R-si microscope equipped with a PInano Piezo stage (MCL), using a 60x PlanApo VC oil objective NA 1.40. Movies were acquired in resonant-scanning mode. Image processing was performed using NIS-Elements software. Movies of Vps16\Delta and Vps16\Delta VAC17 OE were acquired using Zeiss AxioObserver Z1 as described above.

Statistical analysis
Data in bar graphs are presented as mean ±SD, and were analysed using unpaired two-tailed t-test, \(p<0.05\) considered statistically significant. Boxplots are illustrated as Interquartile range (IQR) containing 50% of all data points, with median value marked by a thick line. Outliers, depicted as dots, are
identified as values outside the 1.5xIQR range. Data in boxplots were analysed using Mood’s median test, p< 0.05 considered statistically significant. Replicative lifespan data was analysed as described above.
Supplemental References

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