Supplementary Information for
In situ structural analysis reveals membrane shape transitions during autophagosome formation

Anna Biebera,b,c,1, Cristina Capitaniob,c,1, Philipp S. Erdmanna,d,2, Fabian Fiedlera, Florian Becka,f, Chia-Wei Leea,g, Delong Lie, Gerhard Hummerc,h,i, Brenda A. Schulmanc,b,2, Wolfgang Baumeistera,c,2, Florian Wilflinga,b,c,e,2

a – Department of Molecular Structural Biology, Max Planck Institute of Biochemistry, 82152 Martinsried, Germany
b – Department of Molecular Machines and Signaling, Max Planck Institute of Biochemistry, 82152 Martinsried, Germany
c – Aligning Science Across Parkinson’s (ASAP) Collaborative Research Network, Chevy Chase, MD 20815, United States
d – Fondazione Human Technopole, 20157 Milan, Italy
e – Mechanisms of Cellular Quality Control, Max Planck Institute of Biophysics, 60439 Frankfurt a. M., Germany
f – CryoEM Technology, Max Planck Institute of Biochemistry, 82152 Martinsried, Germany
g – Department of Molecular Metabolism, Harvard T. H. Chan School of Public Health, Boston, MA 02115, United States
h – Department of Theoretical Biophysics, Max Planck Institute of Biophysics, 60438 Frankfurt a. M., Germany
i – Institute of Biophysics, Goethe University Frankfurt, 60438 Frankfurt a. M., Germany

1These authors contributed equally to this work

2Corresponding authors:
E-mail: florian.wilfling@biophys.mpg.de
E-mail: schulman@biochem.mpg.de
E-mail: baumeister@biochem.mpg.de
E-mail: philipp.erdmann@fht.org

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**SI Methods**

**Data analysis – general notes**

All analyses of membrane structures and particle distributions were performed with custom written scripts in Python 3.7 ([https://www.python.org/](https://www.python.org/), RRID:SCR_008394). The scripts are available under [https://doi.org/10.5281/zenodo.6607748](https://doi.org/10.5281/zenodo.6607748) and on github ([https://github.com/Anna-Bieber/autophagy-tomo-analysis](https://github.com/Anna-Bieber/autophagy-tomo-analysis)). Major python packages used in this work include numpy 1.21.5 ([https://numpy.org/](https://numpy.org/), RRID:SCR_008633) (1), scipy 1.6.2 ([https://scipy.org/](https://scipy.org/), RRID:SCR_008058) (2), pandas 1.3.0 ([https://pandas.pydata.org/](https://pandas.pydata.org/), RRID:SCR_018214) (3), PyVista 0.27.4 (4), scikit-learn 0.23.2 ([https://scikit-learn.org](https://scikit-learn.org), RRID:SCR_002577) (5) (data analysis), matplotlib 3.3.0 ([https://matplotlib.org/](https://matplotlib.org/), RRID:SCR_008624) (6), seaborn 0.11.0 ([https://seaborn.pydata.org/](https://seaborn.pydata.org/), RRID:SCR_018132) (7), PyVista 0.27.4 (visualization), tifffile 0.15.1 ([https://pypi.org/project/tifffile/](https://pypi.org/project/tifffile/)) (8), mrcfile 1.3.0 ([https://pypi.org/project/mrcfile/](https://pypi.org/project/mrcfile/)) (9), starfile 0.4.11 ([https://pypi.org/project/starfile/](https://pypi.org/project/starfile/)) (10) (data I/O).
Ribosome analysis

Detection & averaging of ribosomes
Ribosome positions were determined by template matching on 2x binned tomograms (IMOD bin 4, 14.08 Å pixel size) using the StopGAP 0.7.0 (https://doi.org/10.5281/zenodo.3973664) (11) software package. In brief, a reference was constructed from ~300 manually picked ribosomes, which were aligned in StopGAP and used for template matching. For each tomogram, the positions of the 1500 highest scoring peaks were extracted and saved. Tomograms were exported from TomoMAN to Warp/M (12) for CTF estimation and locally reconstructing particles. Classification and refinement of 1x binned subtomograms (pixel size 7.04 Å) was performed in Relion 3.1.2 (https://www3.mrc-lmb.cam.ac.uk/relion/) (13), which yielded the final list of particles and a ribosome structure at 15.1 Å resolution (0.143 FSC criterion, SI Appendix, Fig. 2E).

Ribosome density
For ribosome density estimations, we produced complete filled segmentations of all organelles, large structures like glycogen granules and the volume outside the lamella in 5 example tomograms at 2x binning using Amira. For tomograms of autophagosomes and autophagic bodies, the autophagic content was segmented directly and the initial cytosol voxels were defined as all unlabelled voxels. For phagophores, a convex hull around the phagophore membrane points was calculated and all unlabelled voxels in the hull were subtracted from the cytosol and labelled as initial phagophore points. Next, the initial cytosol and phagophore volumes were cleaned with binary opening in scipy using a 3x3x3 voxel cube as structuring element and 2-3 iterations. The resulting cytosol and autophagic content segmentations were then used to assign ribosomes and calculate ribosome densities in- and outside the autophagic structures.

Ribosome nearest neighbour analysis
For nearest neighbour analyses, we circumvented the time-consuming full segmentations by directly using TomoSegMemTV-generated segmentations of the autophagic membranes, only deleting parts of the automatic segmentation manually that deviated significantly from the actual membrane position. Convex hulls around the roughly segmented membranes were used to assign ribosomes to autophagic content or cytosol, and nearest neighbour distances within each area were calculated with KDTrees using scipy. Both 1) an unpaired Kruskal-Wallis test of mean and median nearest neighbour distances in cytosol, phagophores, autophagosomes and fusion structures, and 2) a Wilcoxon test treating measurements from the same tomogram as paired measurements revealed no significant differences between the ribosome nearest neighbour distances inside and outside of autophagic structures.

Contact site analysis
For an analysis of the contact sites of autophagic structures with other organelles, we excluded all tomograms from the ypt7Δ strain since the overall cellular architecture in this strain was disturbed by accumulation of medium-sized vacuoles (14) (SI Appendix, Fig. 1I). The nearest distances between different organelles (Fig. 3A) were measured manually in the tomograms using IMOD (estimated precision ~ 2 nm). For the preferred interaction areas, all contacts at the rim up to the dilation maximum were counted as rim contacts, while the back was defined as the area opposite of the rim. The orientation of phagophores relative to the vacuole was assessed by calculating the angle α between the rim plane
normal (pointing outwards) and the vector of the phagophore point closest to the vacuole to its nearest point in the vacuole.

For the phagophore deformations at contact sites (Fig. 3E), peak contacts were defined by a high curvature and a local increase in the phagophore intermembrane distance, while extended contacts do not show strong changes in the intermembrane distance, but are usually accompanied by a local flattening of the phagophore membrane. Global deformations are defined as a strong deviation from the usual spheroid-like shape of the phagophore towards the other organelle in the absence of a clear peak or extended contact. Finally, rim deformations are clear deviations of the rim tip out of the rim plane and/or the best-fitting rim circle. In an analysis of the maximum distance of rim points from the best-fitting rim plane, these phagophores stand out with high plane distances despite a phagophore orientation in which the rim is clearly visible, ruling out segmentation inaccuracies as cause of the deviation (SI Appendix, Fig. 3B).

Vacuole contact peak analysis

For a detailed analysis of peaks in the phagophore membrane towards the vacuole, we produced refined segmentations of the phagophore membrane at the relevant sites, determined the curvature using PyCurv (15) (radius hit 8 nm) and calculated for each phagophore point its nearest-neighbour distance to the segmented points of the vacuole. The resulting curvedness and vacuole distance values were visualized in Fig. 4 using Pyvista. Having tested different potential parameters for an automatic detection of the peaks in the outer phagophore membrane, the best-performing parameter was the product of the local gaussian curvature with the distance to the inner membrane. We calculated this value for all points in the outer phagophore membrane and applied a threshold of mean+3*std to get potential peak points. The resulting points were clustered with DBSCAN (ε = 10 nm) and all point clusters further than 10 nm from the segmentation border were included in the following analysis. For each peak, its full area was extracted by applying a cutoff to the distance to the original cluster points, which was determined as the distance at which the first derivative of the intermembrane distance vs distance to cluster points exceeds an empirically determined value (-0.15). The height $h$ of each peak was determined from the maximum intermembrane distance $d_{\text{max}}$ and the intermembrane distance of the whole segmented phagophore piece excluding the peak areas, $d_{\text{base}}$, as $h = d_{\text{max}} - d_{\text{base}}$. The width (FWHM) of each peak was estimated as the diameter of a circle fit into the points with $d_{\text{max}} - d_{\text{base}} = 0.5h$. Finally, the bending energy stored in the peak was estimated as difference in Helfrich bending energies $\Delta E_{\text{bend}} = E_{\text{bend,peak}} - E_{\text{bend,base}}$, where $E_{\text{bend,base}}$ is the bending energy of the base mesh normalized to the same membrane area as the peak area. To allow a better comparison of the different peaks as well as extended phagophore-vacuole contacts, we produced 2D elevation and vacuole distance maps (SI Appendix, Fig. 3F, G) using in-house scripts which are available upon request.

ER contact site analysis

For ten ER-phagophore contact sites, the local ER and phagophore membrane segmentations were refined carefully and the curvature was determined using Pycurv (radius hit 8 nm). Assuming a membrane thickness of around 5 nm (16) and an Atg2 protein length of 20 nm (17), we applied a cutoff distance of 25 nm to the ER-phagophore distance measured between membrane middles to analyze the size, local curvature (SI Appendix, Fig. 4A) and curvature change with interorganelle distance (SI Appendix, Fig. 4B) of potential contact regions. Potential Atg2 densities were identified visually and their
start and end coordinates were marked with IMOD. These coordinates were used to estimate the length of the density and find the closest cells in the phagophore and ER membrane meshes to report the local curvatures (SI Appendix, Fig. 4D).

Analysis of membrane morphology of autophagic structures

All autophagic structures containing at least partially cytosolic cargo (visible ribosomes) were included in the following analysis, including structures observed in the eGFP-Ede1 ypt7Δ strain. We defined as phagophores all cytosolic autophagic structures with a visible opening and rim, the rest as autophagosomes. While some phagophores could have been mislabeled as “closed autophagosomes” if the rim was completely outside the lamella volume, the significant morphological differences between the autophagosomes and phagophores e.g. in sphericity and intermembrane distance, together with the fact that autophagosomes show a similar intermembrane distance as the subsequent fusion structures, clearly argues that most autophagosomes were assigned correctly. We used the automatically generated membrane segmentations for this analysis as described above, only deleting parts of the automatic segmentation manually that deviated significantly from the actual membrane position. Note that since the segmentations mark the middle of the membrane bilayer, all distances and fit results are reported with respect to the membrane middles.

Analysis of intermembrane distances

To analyse intermembrane distances, we devised a refined minimum distance algorithm that only uses minimum point distances and is robust against peaks and holes in either of the two surfaces. The algorithm is described below in detail. For the comparison of different double-membrane organelles, we segmented cortical and cytosolic ER sheets as well as mitochondrial membranes without cristae. The phagophore membranes were divided at the rim into inner and outer membrane. For the autophagosome-vacuole fusion structures, only the distances in the wrapped part were analyzed. This was done by automatically detecting the points at the border of the wrapped area, fitting a plane through these points and analysing only the membranes on the side of the plane that faces away from the vacuole (SI Appendix, Fig. 5C).

Intermembrane distance algorithm. For an automated analysis of the distance between two more or less parallel surfaces, the two easiest conceivable parameters are the minimum distance between points in the two surfaces (assuming sufficient sampling) and the distances calculated by following the normals of one surface until they intersect with the second surface (normal distance). As described by Kim and colleagues (18), both approaches can fail in the presence of peaks or holes in one membrane. Moreover, calculating consistent normals would necessitate refined segmentations (15). Based on the algorithm described by Kim et al., we devised a refined minimum distance algorithm that only uses minimum point distances and is robust against peaks and holes in either of the two measured surfaces. As illustrated in Supplementary Text Fig. 1, the major steps for finding the point-wise distances of a membrane A to a membrane B are:

1. For each point in A, find the nearest neighbor in B and record the distance (ii).
2. If a point in B was chosen by several points in A, keep the shortest connection and discard all other A-B pairs and their distances (ii).
3. For each point in B, find the nearest neighbor in A.
4. If a point in A that was discarded in step 2 is found as nearest neighbor by a point in B (step 3), add back the B-A pair and its distance to the final set of refined points and distances (iii+iv).

We tested this algorithm on various 2D examples and membrane segmentations and found that it is robust to peaks, holes and overhangs. All distance values reported in Fig. 2 were calculated in this way.

**Supplementary Text Fig. 1:** Illustration of intermembrane distance algorithm.

**Size and sphericity measurements**
To estimate the size and sphericity of whole autophagosomes and phagophores even though only a section of each structure is present in the tomograms, we fit ellipsoids into the inner and outer membrane segmentation points. We used an iterative ellipsoid fitting algorithm described in and adapted from Kovac et al. (19) which is more robust than simple least-squares approaches. To give a rough estimate of the overall dimensions of the structures, we report the volumes of the best-fitting ellipsoids for the inner membranes of both phagophores and autophagosomes. Since phagophores are incomplete, this volume is not the engulfed volume, but rather reflects roughly the expected final volume. To estimate how spherical the structures are, the best-fitting ellipsoids to the inner membranes were used to calculate the sphericity index as described in Cruz-Matías et al. (20). Additionally, we calculated the “classical” sphericity according to its original definition as ratio between the surface area of a sphere with volume equal to the structure of interest and the structure surface area (21) (SI Appendix, Fig. 5B). Finally, we also applied a least-squares algorithm for sphere fitting and used the root mean square error
(RMSE) of the sphere fit to the inner membrane as a third parameter for estimating how well phagophores or autophagosomes correspond to a sphere (SI Appendix, Fig. 5A).

**Membrane source contribution calculations**

The area-to-lumen ratios of autophagosomes were estimated based on the ellipsoid fits to the inner membrane. To ensure that the modelled autophagosome has the correct intermembrane distance, we modelled the outer membrane by adding the mean intermembrane distance determined for the respective autophagosome to all axes of the inner ellipsoid. The surface area of the inner and outer ellipsoid was estimated using the Knud Thomsen approximation (22) which gives a computationally inexpensive estimation of the ellipsoid area with a maximum error of ±1.061%. Since these ellipsoids correspond to the middles of the inner and outer membrane, we corrected the axis lengths with half the membrane thickness (0.5∗5 nm = 2.5 nm, addition to inner and subtraction from outer ellipsoid axes lengths) to calculate the intermembrane lumen. The same correction was performed for calculating the area/volume ratio of vesicles. The diameters of vesicles within a radius of distance of 100 nm from phagophores in the tomograms were measured manually in IMOD.

To calculate the contribution of lipid transfer vs vesicle fusion to the final autophagosome membrane, we assumed that the intermembrane lumen of autophagosomes $V_{AP}$ corresponds to the combined lumen of all fused vesicles $V_{ves}$, and that the membrane area $A_{AP}$ is the sum of the membrane areas of the vesicles $A_{ves}$ and the area of lipids transferred e.g. from the ER, $A_{ER}$, thus:

$$V_{AP} = V_{ves} = 1$$
$$A_{AP} = A_{ves} + A_{ER} = A_{ER} = A_{AP} - A_{ves}$$

The contribution of lipid transfer to the final membrane, $A_{ER}/A_{AP}$, can thus be calculated from the area-to-volume ratios of the vesicles and autophagosome, $R_{ves} = A_{ves}/V_{ves}$ and $R_{AP} = A_{AP}/V_{AP}$, with:

$$\frac{A_{ER}}{A_{AP}} = 1 - \frac{A_{ves}}{A_{AP}} = 1 - \frac{A_{ves}/V_{ves}}{A_{AP}/V_{AP}} = 1 - \frac{R_{ves}}{R_{AP}}$$

**Analyzing the completeness of phagophores**

A comparison of all parameters considered to estimate the completeness of phagophores is given in Supplementary Note 1. The most robust parameter that we identified is the “rim opening angle” $\varphi$, defined as the angle between a plane through the phagophore rim and tangential planes to the phagophore membrane close to the rim. To measure the angle for each phagophore, a plane was fit through the roughly segmented points at the rim tip to give the rim plane. All inner membrane points within 50 pixels (~70 nm) to the rim plane were used to fit the tangent planes in the following manner: Using a circle fit through the rim points, these inner membrane points were divided into angular batches spanning 10° each. Next, planes were fit to each batch of points and the normals of these tangential planes were used for angle calculation with the rim plane normal. In practice, we noticed that this measurement sometimes leads to high variances of the angle, presumably due to errors in the determination of the rim plane normal or inherent asymmetry of the phagophore. To counteract this, we reasoned that the normals of the rim tangent planes should form a cone whose base plane should ideally be parallel to the rim opening plane. We therefore calculated the cone base planes for all rims in which the available points span more than 90° of the full cone circle, used the normals as corrected rim plane normals and reported the final rim opening angle as the mean of the angles between this vector and all tangential plane normals.
Correlation and bootstrap analysis to test for significance

Pair-wise correlation of different parameters was assessed with Spearman’s rank correlation coefficient. However, the input parameters were often mean values of different measurements and the simple correlation analysis did not consider the spread of raw data resulting in these mean values. To take the raw data into account and analyse the confidence of the reported correlation coefficients, we used a bootstrapping approach as described by Curran (23). In brief, for \(10^5\) iterations, we replaced each mean value by a randomly chosen value from its raw distribution and recalculated the Spearman correlation coefficient and p value. The resulting distributions of correlation coefficients and p values are shown in SI Appendix, Figures 5H and 6G-H.

Phagophore rim analysis

To analyze the phagophore rim shapes in detail, we produced refined segmentations of 26 rim segments and determined the local membrane curvatures using PyCurv (radius hit 8 nm). We next developed a strategy to extract the tip points and two sides of each rim segment and approximated the general shape of the phagophore at the rim segments by fitting a 3rd order polynomial surface through points lying in the middle between the two sides (see SI Appendix, Fig. 6A+D). The intermembrane spacing of the rims orthogonally to the overall rim shape was then calculated by ray tracing from one side of the rim to the other using the normals of the closest middle surface points as ray directions. To generate 1D and 2D histograms of distance and curvature values, all rim points were mapped to their nearest middle surface point, and we calculated for each middle surface point (I) its closest geodesic distance along the middle surface to the smoothed tip (“distance from the tip”) and (II) the geodesic distance of its closest tip point to the tip point with the lowest z (“distance along the tip”). Using these values as xy coordinates, we binned the points using 1-pixel bins for the distance from the tip (1 pixel of the bin4 tomogram = 1.408 nm), and 2-pixel bins for the distance along the tip to minimize the number of empty bins in the 2D histograms (SI Appendix, Fig. 6A-B).

Rim swelling analysis

Based on the 2D histograms of the intermembrane distance, we further analyzed the rim shapes by searching for minimum and maximum peaks with a prominence of at least 1 pixel in the histogram rows, smoothed slightly with a Savitzky-Golay filter (window length 9, polynomial order 2). We defined the peak detection frequency as fraction of complete histogram rows in which a peak was detected, a histogram of peak detection frequencies is shown in SI Appendix, Fig. 6E. The rim dilation factor was defined as ratio of the mean maximum peak height and the base distance, the intermembrane distance in the back of each rim, calculated by averaging the intermembrane distance values of all points further from the tip than 120 nm, where the rims are not dilated or constricted anymore.

Bending energy and reference rims

The Helfrich bending energy is defined as

\[
E_{\text{bend}} = 2\kappa \int (M - m)^2 \, dA
\]

for a membrane with bending rigidity \(\kappa\), mean curvature \(M\), spontaneous curvature \(m\) – assumed to be zero here – and area \(A\) (24). For the discretized \(n\) cells of the meshes produced from the segmented rims, we thus calculated the bending energy (25) from the local mean curvatures \(M_i\) and cell areas \(A_i\) as:

\[
E_{\text{bend}} = 2\kappa \sum_i (M_i - m)^2 A_i
\]
\[ E_{\text{bend}} = 2\kappa \sum_{i=1}^{n} M_i^2 A_i \]

Given that autophagic membranes are known to have high levels of unsaturated lipids\(^{(26)}\), we assumed a bending rigidity \( \kappa \) of 10 kBT for the calculations. For a comparison of different rim segments, the resulting energies were normalized by division through the tip length of the rim segment. This normalization should be sufficient since the main contribution to the bending energy comes from the tip area and the contribution in the back is close to zero. To analyze the effect of rim swelling on the bending energy, we generated a reference mesh for each rim mesh with the same overall shape, membrane area and tip length, but with no dilation and a half-toroid-like tip structure. The curvature and bending energy of the reference meshes were determined in the same way as for the original rim meshes.

**SI Note 1: Completeness parameters for phagophores**

One challenge in the analysis of phagophores was to find a parameter to estimate robustly the completeness of each observed structure. First, one could use an ellipsoid fit, cut it with a plane through the rim points and calculate e.g. the fraction of surface areas of the cut vs. complete ellipsoid (Supplementary Text Fig. 2A, strategy a). However, especially for early phagophores, ellipsoid fits did not always converge to reasonable final dimensions, even though the used iterative ellipsoid fitting algorithm is more robust to noise than a simple least-squares fit \(^{(19)}\). We thus searched for a parameter that does not rely on an ellipsoid fit, excluding also the bending angle which is used e.g. in Sakai et al. \(^{(27)}\) since this would require finding the center of the phagophore which would also necessitate an ellipsoid fit (Supplementary Text Fig. 2A, strategy b).

In the end, the most robust parameter that we identified is the “rim opening angle” \( \varphi \), defined as the angle between a plane through the phagophore rim and tangential planes to the phagophore membrane close to the rim. This angle should increase from 0° in an initial membrane disk to 180° just before phagophore closure (Supplementary Text Fig. 2A, strategy c).

An alternative parameter that does not rely on ellipsoid fits uses circle fits in planes parallel to the rim plane instead (Supplementary Text Fig. 2A, strategy d). For each phagophore, a plane parallel to the rim plane is moved from the rim towards the back in a stepwise fashion, and at each plane position, a circle is fit to the phagophore points close to the plane. The final parameter reported for each phagophore is the ratio of the maximum radius – the “belly” radius of the phagophore – divided by the radius at the rim. While this ratio should always equal 1 for initial phagophores, as soon as the phagophore reaches its characteristic cup shape, it should increase gradually until closure of the phagophore. After calculating the ratio for all phagophores, we first confirmed that it correlates positively with the rim opening angle (Supplementary Text Fig. 2B). As in the case of the rim opening angle, the intermembrane distance of phagophores correlates negatively with the ratio of maximum radius and rim radius (Supplementary Text Fig. 2C). The decrease of intermembrane distance during growth of the phagophore is thus detected through two independent parameters.
SI Note 2: Estimating the number of Atg2 molecules

To estimate the number of Atg2 molecules needed to build an autophagosome, we follow the argumentation presented in von Bülow & Hummer (28), inserting however the autophagosome dimensions obtained from the tomograms. For an average-sized autophagosome from the experimental data with a lipid bilayer area of 1.6 \( \mu \text{m}^2 \), an area per lipid (29) of 0.724 \( \text{nm}^2 \) and 58% of membrane contribution by lipid transfer (calculation with 40 nm vesicles), around 2.5 million lipids would need to be shuttled through Atg2 into the phagophore. Based on lipid transfer experiments from different groups (30, 31) the lipid transfer rate of Atg2 was estimated as 115 lipids/second or 750 lipids/second (28). Assuming a chemical potential difference between phagophores and ER of 1 k_BT and ten minutes of transfer time (28), the 2.5 million lipids could be transferred by 77 Atg2 molecules for the slow transfer rate, and only 12 Atg2 molecules for the fast transfer rate.
SI Figures

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SI Fig. 1. Autophagic structures revealed by correlative cryo-ET. (A) Cryo-fluorescence overlays on TEM maps at 470X and 6500X magnification for the phagophore shown in Fig. 1E. In this case fluorescence stacks were acquired on the final lamella by using a fluorescence microscope integrated in the FIB chamber (METEOR). (B) Cryo-fluorescence overlays on TEM maps for the phagophore in Fig. 1F. (C) Complete correlative workflow shown exemplary for the autophagosome shown in Fig. 1G. Fiducial-based registration of cryo-fluorescence volume data (projection image in upper left panel) is used to target FIB milling (bottom left) and tomogram acquisition (TEM overviews). (D) Cryo-fluorescence overlays on TEM maps for fusion structure shown in Fig. 1H. (E) Example of phagophore captured by using colocalization of eGFP-Ede1 and mCherry-Atg8 signal. (F-G) Comparison of structures from the three different yeast strains (eGFP-Atg8 (FWY0002), mCherry-Atg8 eGFP-Ede1 (FWY0085), and eGFP-Ede1 ypt7Δ (FWY0154)) employed in this study. Plots show mean intermembrane distance, standard deviation of the intermembrane distance, sphericity index and volume of the best fitting ellipsoid for phagophores (F) and autophagosomes (G). (H) Mean intermembrane distance of phagophores and autophagosomes compared separately for eGFP-Atg8 and EGFP-Ede1 mCherry-Atg8 strains. (I) Gallery of autophagic structures found in eGFP-Ede1 ypt7Δ strain. All scale bars: 470X, 10 μm; 6500X, 1 μm; tomograms, 200 nm. Statistical analysis: F and H, Mann-Whitney-U test, G: Kruskal-Wallis and pairwise Games-Howell post hoc test. ***p<0.001, **p<0.01, *p<0.05, n.s.: p≥0.05.
SI Fig. 2. Autophagic cargo under nitrogen starvation in yeast. (A) Gallery of autophagic structures engulfing selective cargo non-exclusively, i.e., together with cytosol. Last on the right example of structure engulfing Cvt cargo prApe1. All scale bars 200 nm. (B) Gallery of autophagic structures engulfing exclusive cargo. "?" = unknown membrane cargo. (C) Example of multilayer structure in which multiple autophagic structures are enwrapping each other. In the center an unperturbed open phagophore engulfing ribosomes. (D) Examples of open phagophores close to wrapped autophagic bodies (AB) in the vacuole. The last tomogram shows a putative remnant of a degraded AB. (E) Snapshots and FSC curve of ribosome average (1x binning, nominal pixel size 7.04 Å, resolution 15.1 Å) generated from the tomograms. (F) Ribosome densities (count/volume) determined in 5 example tomograms in the cytosol vs inside phagophores or autophagosomes. (G) Comparison of mean ribosome nearest neighbor distances in different compartments. Differences between cytosol and autophagic ribosome distances were analyzed with the Wilcoxon signed-rank test, treating values from compartments in the same tomogram as paired (n=77 tomograms).
SI Fig. 3. Analysis of phagophore-organelle contact sites. (A) Phagophore interaction area vs distance to different organelles. The color of each point indicates which phagophore area was closest to that single organelle. (B) Maximum distance of rim points from the best-fitting rim plane for phagophores with an obvious rim deformation vs other phagophores. While larger deviations in some “normal” rims can be explained by difficulties in segmentation caused by the missing wedge if the rim plane is close to parallel to the xy plane of the tomogram, the “rim deformation” examples show large deviations even with the rim clearly visible in all slices. (C) Histogram of angles indicating the orientation of the rim opening with respect to the vacuole. Angles are calculated between the normal of the phagophore rim plane (pointing outwards) and the shortest phagophore-vacuole vector. (D) Two examples of peak contact sites between vacuole (left) and phagophore (right). (E) Example of an extended contact site between an open phagophore (opening not visible in this slice) and the vacuole. (F) 2D maps and 1D profiles of peak contact sites. Colored peak elevation maps are overlaid with lines indicating the distance to the vacuole. (G) 2D maps and 1D profiles of extended contact sites. Grey scale maps indicate the distance to the vacuole.
**SI Fig. 4. Analysis of phagophore contact sites with the ER or NM.** (A) 3D views, colored by curvedness, of nuclear membrane (NM, left) and ER (right) contact sites with phagophore rims. Deformations of the phagophore rim are clearly visible in the NM-phagophore contact sites. (B) Local ER curvedness vs distance to the phagophore at ER-phagophore contact sites. The upper plot shows examples in which the ER curvedness increases at the contact site, the lower plot shows all other analyzed examples. The dotted line indicates the cutoff distance (corrected for a membrane thickness of 5 nm) which could potentially be spanned by Atg2. (C) Gallery of rim contact sites with nuclear membrane or ER. White arrowheads indicate connecting densities. Tomograms denoised with cryo-CARE. Scalebars 50 nm. (D) (i) Curvedness of the phagophore and ER membrane points closest to the segmented connecting densities; (ii) mean curvedness in the area within 20 nm of ER-phagophore distance (membrane surface to membrane surface) in structures for which connecting densities were observed; (iii) same as (ii) but for structures for which no connecting densities were observed. Differences analyzed with Wilcoxon signed-rank test.
SI Fig. 5. Analysis of autophagic membrane structures. (A-B) Alternative sphericity measurements confirm that autophagosomes are more spherical than phagophores. (A) Root Mean Square Error (RMSE) of best fitting spheres to phagophore vs autophagosome inner membranes. (B) "Classical" sphericity of phagophores and autophagosomes, calculated from best-fitting ellipsoids (fit to inner membrane points) as ratio between the surface area of a sphere with volume equal to the ellipsoid and the surface area of the ellipsoid. (C) The intermembrane distance of fusion intermediates (Fig. 5C) is measured only for the part wrapped by the vacuole membrane, by cutting the structures with a plane fit through the points at the wrapping border. (D) Diameters of vesicles observed within 100 nm to the phagophores. (E) Images of vesicles close to the phagophore membrane. Tomograms denoised with Cryo-CARE. Scalebars 50 nm. (F) Calculating the contribution of different membrane sources to the autophagosome. Given the combined area/volume (A/V) ratio of all fusing membrane sources (e.g. vesicles) on the x axis, the blue graph indicates the percentage of autophagosome membrane originating from lipid transfer (right y axis), e.g. from the ER. Blue line: calculation with mean autophagosome A/V ratio; blue area: calculation with mean ± standard deviation. For illustration, the magenta line (left y axis) shows the vesicle diameters corresponding to the A/V ratios of fusing membrane sources (x axis). Grey dotted lines indicate calculation results for vesicles with 40 nm diameter. (G) Standard deviation of intermembrane distance of phagophores vs opening angle $\phi$. (H) Plot showing standard deviations as error bars for the mean intermembrane distance vs opening angle plot showed in Fig. 5f. (I) Bootstrapping analysis for the correlation between intermembrane distance and $\phi$ shown in (H): distributions of Spearmans rho and p values obtained by bootstrapping from the raw data. (J) Mean intermembrane distance of autophagosomes vs autophagosome size, estimated by the radius of the best-fitting sphere to the inner membrane. The intermembrane distance shows no correlation with autophagosome size (Spearman's correlation, $p = 0.67$).
**A** Rim area separation and intermembrane distance determination through ray tracing

1. Remove entire tip region with a curvature cutoff and get separate sides
2. Get middle points halfway between nearest neighbor pairs of the two sides
3. Fit a smooth polynomial middle surface into the points and calculate its normal vectors
4. For each point in the tip region, calculate the angle between its surface normal and the normal of the nearest middle surface point
5. Tip points should have angles close to 90°. Use a spline fit to smooth original tip points.
6. Assign all other points in tip region to one of the sides and update the middle surface
7. To get intermembrane distance orthogonally to the rim, use normals of nearest middle surface points to do ray tracing from one side to the other

**B**

**C** Curvedness: 2D and 1D histograms

**D** Intermembrane distance: 2D and 1D histograms

**E** Peak detection frequencies

**F** Reference rim:
- same overall shape
- same membrane area
- same length along rim
- no dilation
- tip: half-toroid-like shape
- curvature measured in the same way as for experimental rim (pyrum, radius of 1 nm)

**G** Evaluation of correlation between distances and rim opening angle

**H** Evaluation of correlation between curvature and rim opening angle
SI Fig. 6. Phagophore rim analysis. (A) Steps for automated separation of rim areas and calculation of intermembrane distances by ray tracing orthogonally to the phagophore rim. (B) Illustration of Rim analysis steps as described in (A), shown for same example rim as in (C, D, F). Top left: Mesh of rim segment coloured by curvedness determined with PyCurv (radius hit 8 nm). Top right: Rim separated into tip and two sides, plotted with its corresponding middle surface (white). Bottom: Ray tracing vectors showing local intermembrane distances. (C) 2D and 1D histograms of the combined curvedness values of inner and outer membrane mapped against the distance from and along the tip for an example rim. (D) 2D and 1D histograms of mean intermembrane distance vs distance from and along the tip for the example rim. The 2D histogram is used for analysis of frequency and position of maximum and minimum peaks indicating rim dilation and constriction. (E) Detection frequency of maximum and minimum intermembrane distance peaks in the analyzed rims. A frequency of 1 indicates that a peak was found in every section moving along the tip, while 0.5 indicates that only half of the sections along the tip has a peak. All analyzed rims show maxima (dilation) in at least half of the analyzed segment, whereas minimum peaks (constriction) are not detected consistently. (F) 3D rendering of a model rim (left) and its corresponding reference rim, coloured by curvedness. Listed on the right are the criteria with which the reference is built. (G) Maximum and base intermembrane spacing plotted against the rim opening angle \( \phi \) with error bars indicating standard deviations of mean values. On the right bootstrapping analysis of Spearman correlation coefficients and p values. (H) Curvedness and first principal curvature \( \kappa_1 \) at the tip, plotted against \( \phi \) with error bars indicating standard deviations. On the right, corresponding correlation bootstrapping results.

**SI Tables**

**Table S1:** Phagophore peaks at vacuole contact sites, n=7

|            | Area [\(\text{nm}^2\)] | Height [\(\text{nm}\)] | Width [\(\text{nm}\)] | Min. vac. dist. [\(\text{nm}\)] | Max. curvedness [\(\text{nm}^{-1}\)] | \(\Delta E_{\text{bend}}\) [J] | Pearson’s \(\rho\) (peak elevation vs vac dist) |
|------------|--------------------------|--------------------------|--------------------------|-----------------------------------|-------------------------------------|-------------------------|-----------------------------------------------|
| mean       | 1916                     | 11.5                     | 24.2                     | 30                                | 0.081                               | 2.8E-19                 | -0.75                                         |
| std        | 1621                     | 10.6                     | 6.4                      | 14                                | 0.060                               | 4.6E-19                 | 0.17                                          |
| min        | 279                      | 3.6                      | 16.6                     | 19                                | 0.031                               | 1.5E-20                 | -0.89                                         |
| median     | 1212                     | 7.4                      | 23.7                     | 23                                | 0.061                               | 1.0E-19                 | -0.80                                         |
| max        | 4088                     | 33.2                     | 31.9                     | 53                                | 0.197                               | 1.3E-18                 | -0.43                                         |

**Table S2:** ER-phagophore contact site analysis, n=10

|            | Min. dist. ph-ER [\(\text{nm}\)] | ER contact area [\(\text{nm}^2\)] | ph contact area [\(\text{nm}^2\)] | ph. mean curvedness [\(\text{nm}^{-1}\)] | ER mean curvedness [\(\text{nm}^{-1}\)] |
|------------|----------------------------------|-----------------------------------|-----------------------------------|------------------------------------------|---------------------------------------|
| mean       | 16.4                             | 3261                              | 3108                              | 0.055                                    | 0.029                                 |
| std        | 3.8                              | 4248                              | 4027                              | 0.028                                    | 0.015                                 |
| min        | 10.9                             | 323                               | 265                               | 0.016                                    | 0.008                                 |
| median     | 16.7                             | 1609                              | 1540                              | 0.062                                    | 0.029                                 |
| max        | 22.7                             | 14563                             | 13155                             | 0.085                                    | 0.058                                 |

**Table S3:** Rim dilation characteristics, n=26

|            | \(s_{\text{max}}\) mean [\(\text{nm}\)] | \(s_{\text{max}}\) SD [\(\text{nm}\)] | \(d_{\text{max}}\) mean [\(\text{nm}\)] | \(d_{\text{max}}\) SD [\(\text{nm}\)] | \(s_{\text{base}}\) mean [\(\text{nm}\)] | \(s_{\text{base}}\) SD [\(\text{nm}\)] | dilation factor |
|------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|----------------|
| mean       | 14.7                            | 0.78                            | 17.0                            | 4.3                             | 10.9                            | 0.72                            | 1.35                                       |
| std        | 1.8                             | 0.52                            | 7.0                             | 6.8                             | 0.5                             | 0.60                            | 0.15                                       |
| min        | 11.6                            | 0.19                            | 10.1                            | 0.7                             | 9.8                             | 0.24                            | 1.11                                       |
| median     | 14.5                            | 0.61                            | 15.6                            | 2.6                             | 10.8                            | 0.47                            | 1.32                                       |
| max        | 18.3                            | 2.04                            | 47.2                            | 36.9                            | 12.0                            | 2.70                            | 1.76                                       |
### Table S4: Curvature at the rim tip, n=26

|        | \( \kappa_1 \) mean [nm\(^{-1}\)] | \( \kappa_1 \) SD [nm\(^{-1}\)] | \( \kappa_2 \) mean [nm\(^{-1}\)] | \( \kappa_2 \) SD [nm\(^{-1}\)] | Curvedness mean [nm\(^{-1}\)] | Curvedness SD [nm\(^{-1}\)] |
|--------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|-------------------------------|-------------------------------|
| mean   | 0.153                           | 0.018                           | 0.020                           | 0.024                           | 0.111                         | 0.013                         |
| std    | 0.019                           | 0.003                           | 0.014                           | 0.008                           | 0.014                         | 0.002                         |
| min    | 0.112                           | 0.013                           | -0.001                          | 0.009                           | 0.082                         | 0.009                         |
| median | 0.154                           | 0.017                           | 0.020                           | 0.023                           | 0.110                         | 0.013                         |
| max    | 0.193                           | 0.023                           | 0.060                           | 0.037                           | 0.143                         | 0.018                         |

### Table S5: Yeast strains used in this study

| Name        | Relevant genotypes                                                                 | Reference                      |
|-------------|-----------------------------------------------------------------------------------|--------------------------------|
| FWY0001     | MAT\( \alpha \), his3-\Delta200, leu2-3,2-112, lys2-801, trp1-1(\( am \)), ura3-52 | Wilfling et al. 2020 (32)       |
| FWY0002     | natNT2::pADH::EGFP::Atg8                                                           | Wilfling et al. 2020 (32)       |
| FWY0085     | natNT2::pADH::EGFP::Ede1, pRS305::pADH::mCherry-Atg8                               | Wilfling et al. 2020 (32)       |
| FWY0154     | natNT2::pADH::EGFP::Ede1, ypt7A::hphNT1                                            | in this study                   |
| FWY0155     | pRS305::pADH::mCherry-Atg8                                                          | in this study                   |
| FWY0156     | pRS305::pADH::mCherry-Atg8, Vph1::EGFP::kanMX4                                     | in this study                   |
| FWY0157     | pRS305::pADH::mCherry-Atg8, Idh1::EGFP::HIS3MX6                                    | in this study                   |
| FWY0158     | pRS305::pADH::mCherry-Atg8, Sec16::EGFP::HIS3MX6                                   | in this study                   |
| FWY0159     | pRS305::pADH::mCherry-Atg8, Sec61::EGFP::HIS3MX6                                   | in this study                   |

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