**Phase separation of initiation hubs on cargo is a trigger switch for selective autophagy**

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**Summary:**

Autophagy is a key cellular quality control mechanism. Nutrient stress triggers bulk autophagy, which non-selectively degrades cytoplasmic material upon formation and liquid-liquid phase separation of the Autophagy-Related Gene 1 (Atg1) complex. In contrast, selective autophagy eliminates protein aggregates, damaged organelles, and other cargo that are targeted by an autophagy receptor. Phase separation of cargo has been observed, but its regulation and impact on selective autophagy are poorly understood. Here, we find that key autophagy biogenesis factors phase separate into initiation hubs at cargo surfaces in yeast, subsequently maturing into sites that drive phagophore nucleation. This phase separation is dependent on multivalent, low-affinity interactions between autophagy receptors and cargo, creating a dynamic cargo surface. Intriguingly, high-affinity interactions between autophagy receptors and cargo complexes block initiation hub formation and autophagy progression. Using these principles, we converted the mammalian reovirus nonstructural protein µNS, which accumulates as particles in the yeast cytoplasm that are not degraded, into a neo-cargo that is degraded by selective autophagy. We show that initiation hubs also form at the endoplasmic reticulum during mitophagy in human cells. Overall, our findings suggest that regulated phase separation underscores the initiation of both bulk and selective autophagy in evolutionarily diverse organisms.

Autophagy is a highly versatile cellular degradation pathway that targets diverse components, ranging from membrane-bound organelles to distinct protein complexes. Bulk autophagy, also known as non-selective autophagy, involves the non-specific engulfment and degradation of cytoplasmic material within autophagosomes. Starvation induces the formation of the autophagy-initiating Atg1 complex 1,2. Under nutrient-rich conditions, autophagy and Atg1 complex formation are inhibited due to target of rapamycin complex 1 (TORC1)-mediated phosphorylation of Atg13, a member of the Atg1 complex 3. In contrast, starvation inhibits TORC1, promotes Atg1 complex assembly, and activates the Atg1 kinase 4–6. The Atg1 complex undergoes liquid-liquid phase separation and the resulting condensates are anchored to the vacuolar membrane via Atg13-Vac8 interactions 7–10. This process ultimately builds the phagophore assembly site (PAS), which is key for phagophore initiation 8. Thus, phagophore formation occurs without a templating cargo 11.

Unlike bulk autophagy, selective autophagy relies on autophagy receptors that recognize and bind to specific cargo, which templates membrane formation and thereby facilitates its exclusive packaging into autophagosomes for subsequent degradation. Before their degradation through selective autophagy, a variety of cargoes undergo phase separation 12. In budding yeast, for example, dodecamers of the aminopeptidase 1 (Ape1) proteins organize into a complex via phase separation, which is transported to the vacuole through a selective autophagy process known as the cytoplasm-to-vacuole targeting (Cvt) pathway. Amino acid substitutions in Ape1 that hinder phase separation also impede the Cvt pathway, suggesting that cargo liquidity is a critical determinant for selective autophagic turnover 13. Similarly, phase separation drives the formation of p62 (also known as SQSTM1) condensates and PGL granules, which both are selective autophagy cargos14–16. Autophagy receptors link their cargo to Atg8 on the growing autophagosome membrane, thus, phagophore formation in selective autophagy requires a templating cargo 17. In addition, cargo-bound autophagy receptors interact with the adapter and scaffolding protein Atg11 to activate the Atg1 kinase 18,19, 20.

We recently discovered that the endocytic protein Ede1 functions as a selective autophagy receptor in budding yeast 21. When endocytosis is impaired, Ede1 directly links early clathrin-mediated endocytosis proteins to Atg8 on the autophagosome. These Ede1-dependent endocytic protein deposits (ENDs) are degraded via autophagy 21. Intriguingly, selective autophagy of ENDs depends on the phase separation of Ede1 and its autophagy receptor function. Ede1 contains a low complexity region and coiled-coil domains, which promote the formation of higher oligomeric structures 22 and are required to form phase separations together with further END components. In addition, Ede1 contains several binding motifs that form interactions with autophagy machinery proteins. Disrupting the ability of Ede1 to undergo phase separation or its receptor function abolishes the Ede1-dependent autophagy pathway for endocytic proteins.

Overall, these findings reveal an interplay between phase separation and autophagy. Nevertheless, how phase separation influences phagophore initiation in selective autophagy is incompletely understood.

**Low-affinity cargo-receptor interactions promote receptor mobility and selective autophagy**

To address the role of phase separation in END turnover in more detail, we manipulated the END cargo properties. We generated two yeast strains expressing a double GFP-tagged Ede1 to visualize END condensation and degradation. In one strain, we also introduced a copper-inducible triple GFP-binding protein (3xGBP) fused to BFP 23. We treated cells with rapamycin to induce END turnover by autophagy 21,24. Although 2xGFP-Ede1-positive ENDs were eliminated in the control strain lacking BFP-3xGBP (-3xGBP), they persisted in the strain expressing BFP-3xGBP (+3xGBP) (Figure 1A, 1B, S1A and S1B). Intriguingly, fluorescence recovery after photobleaching (FRAP) revealed rapid exchange of 2xGFP-Ede1 between the bleached and non-bleached pool in the control strain but not in the strain expressing BFP-3xGBP (Figure 1C). These data suggest that Ede1 within the END compartment is liquid-like in the control strain but solidified in the strain expressing BFP-3xGBP, and this change in mobility altered its autophagic degradation. Despite their solidification, END assemblies still colocalized with the autophagy protein Atg8, suggesting that the autophagy receptor properties of Ede1 were not altered (Figure 1D and S1C). We infer that high-affinity interactions between 2xGFP-Ede1 and BFP-3xGBP render the receptor Ede1 immobile and that receptor mobility is required for selective END degradation.

To determine if receptor mobility is an important feature in selective autophagy more generally, we manipulated the Cvt pathway. First, we examined the affinity of the interaction between Ape1 cargo and the Atg19 receptor. Briefly, we immobilized a GST-BFP-tagged Ape1 propeptide (Ape1 residues 1-45, which interacts with Atg19) on GSH beads. We found that mCherry-Atg19 was recruited to the Ape1 propeptide, but this interaction was lost after washing, consistent with a low-affinity interaction. This suggests that receptor mobility in the Cvt pathway is established by low-affinity receptor-cargo interactions, allowing a high on- and off binding rate (Figure 1E and S1D). To examine the impact of cargo-receptor affinity on degradation, we co-expressed either untagged or GBP-tagged Atg19 in *atg19Δ* cells along with GFP-Ape1; these strains are predicted to form low- and high-affinity receptor-cargo interactions, respectively. Strikingly, free GFP was generated in *atg19Δ* cells expressing untagged Atg19 but not in those expressing Atg19-GBP (Figure 1F). These data suggest that the high-affinity cargo-receptor interaction blocked autophagic turnover of the GFP-Ape1. Together, these findings strongly suggest that receptor mobility is a key principle of selective autophagy.

**Autophagy biogenesis factors form initiation hubs at low-affinity cargo-receptor complexes**

The autophagy machinery initiates phagophore biogenesis at the cargo surface, selectively engulfing the cargo. A key step in this process is the recruitment of the scaffolding protein Atg11 to the autophagy receptor on the cargo surface. We examined the recruitment of Atg11 to the surface of diverse selective autophagy cargoes with different physical properties, including ENDs, Ape1, and mitochondria. Whereas mitochondria represent a membrane-delimited cargo, ENDs and Ape1 are membrane-less cargoes that undergo phase separation 13,22. Unexpectedly, Atg11 did not distribute uniformly around the cargo surface but instead accumulated in distinct foci (Figure 2A). In particular, Atg11 formed prominent foci around Ape1, as measured by the coefficient of variance of the fluorescence intensity (Figure 2B) 25. Important autophagosome biogenesis factors, such as the autophagy proteins Atg1 and Atg9, also clustered in these foci with Atg11 for both the Ape1 complex and the ENDs (Figure 2C and 2D). Because these clusters contain multiple factors that are involved in phagophore initiation, we termed them “initiation hubs”.

We noticed that Atg11 clustering was reduced in cells with increased affinity of receptor-cargo interactions, such as the strains co-expressing Atg19-GBP with GFP-Ape1 (Figure 2E) and those co-expressing 2xGFP-Ede1 with BFP-3xGBP (Figure 2F). Notably, single and combined deletion of Atg9 and Vac8 did not reduce Atg11 clustering on Ape1, suggesting that initiation hub formation is independent of downstream factors (Figure S2A). These data suggest that the elevated mobility of low-affinity cargo-receptor complexes allows Atg11 to establish initiation hubs and supports selective cargo degradation.

**Phase separation of Atg11 drives initiation hub formation**

Intriguingly, FRAP analysis revealed less mobility of the cargo GFP-Ape1 when compared to that of GFP-Atg11 (Figure 3A). Time-lapse microscopy analysis of GFP-Atg11 revealed that most of the Atg11 clusters on the surface of the Ape1 complex dynamically change their size and morphology (Figure 3B). These morphological changes resemble fusion and fission of individual clusters, consistent with phase separation *in vivo*.

To investigate phase separation of Atg11 *in vitro*, we purified GFP-Atg11 from insect cells and found that it formed round droplets in the presence of physiological salt concentrations (Figure 3C and S2B). Droplet formation was absent at low protein concentrations but became visible at around 0.05 µM, and droplet size increased with increased concentration of Atg11. FRAP experiments showed that GFP-Atg11 is largely mobile within the droplet (Figure 3D). In addition, we observed coalescence of individual droplets, another typical feature of phase-separating proteins (Figure 3E and S2C).

GFP-Atg11 was efficiently recruited to GST-BFP-Atg19 immobilized on GSH beads and remained bound after subsequent washes, consistent with a high-affinity interaction between the scaffold and receptor (Figure S2D). Moreover, GFP-Atg11 formed bright clusters on the Atg19-decorated beads (Figure S2D), suggesting phase separation *in vitro*. Given that increasing the affinity of the Atg19 receptor for the Ape1 cargo reduced Atg11 cluster formation (Figure 2E), we propose that low-affinity Atg19 receptor-cargo interactions enable phase separation of Atg11-Atg19 complexes and initiation hub formation. Such a high on- and off rate of cargo-receptor interactions represents an effective diffusion rate similar to the lateral diffusion in membranes. In general, this suggests that receptor mobility on cargo is critical for autophagy initiation and progression of the pathway.

Low-affinity interactions, such as those between the cargo and autophagy receptor, can be stabilized by increasing their number, resulting in avidity or high functional affinity 26. To assess the importance of the avidity-driven interactions for the assembly of initiation hubs, we developed a mathematical model of the Atg11-Atg19 subcomplex and cargo using the modeling framework cellular\_raza. We considered Atg11 and Atg19 as one entity due to their high-affinity interaction. We modeled a low-affinity interaction between Atg11 molecules, resembling its phase separation. We then mimicked very low affinity, low-affinity and high-affinity interactions between the cargo and the Atg11-Atg19 subcomplex. The model based on very low-affinity interactions produced a pattern of Atg11 clusters. However, these clusters are not located at the cargo (Figure 4A, Panel A, and supplementary movie 1). The model based on low affinity interactions also shows Atg11 cluster, however these are locatedon the cargo, because the avidity-mediated interactions are now strong enough to bind the clusters to the cargo, mirroring our *in vivo* observations (Figure 4A, Panel B, and supplementary movie 2). In contrast, a model based on individual high-affinity interactions produced uniform Atg11 binding on the cargo without large clusters, as observed *in vivo* (Figure 4A Panel C, and supplementary movie 3). This model suggests that low-affinity multivalent interactions between cargo and Atg11-Atg19 support the formation of initiation hubs.

Taken together, our results suggest that receptor mobility on cargo allows Atg11 condensation and the formation of initiation hubs, which promote autophagy.

**Initiation hubs coalesce at the vacuolar contact site to trigger phagophore initiation**

In selective autophagy, autophagosome formation is mediated through a spatial organization driven by numerous low-affinity but high-avidity-based interactions, with Vac8 coordinating these events at the vacuole at the site of the PAS 27. Vac8 colocalized with Atg11 clusters (Figure S3A and S3B), and *in vitro* reconstituted GFP-Atg11 phase separations efficiently interacted with purified vacuoles in a Vac8-dependent manner (Figure S3C), resembling the native avidity-mediated binding between these proteins 27. The interaction of Atg11 phase separations with Vac8 markedly deformed the GFP-Atg11 droplet at the condensate-vacuole contact site, as expected for a liquid-like droplet. As phase separations serve to elevate the local concentration of proteins and thus binding sites, they are an ideal platform for avidity-mediated interactions.

We noticed that some Atg11 clusters were largely immobile (Figure S3A and S3B). These clusters were mostly found at the contact site with the vacuole (Figure S4A and S4B) and were not only more stable compared to the surrounding clusters but also showed the highest fluorescence intensity. We hypothesized that all the Atg11 clusters coalesce into a single, stable initiation hub at the contact site with the vacuole, eventually maturing into the PAS, from which membrane formation is catalyzed. Indeed, cells lacking Vac8 also lost these high-intensity clusters (Figure S4C). Atg8, a marker for phagophore formation, colocalized with a single Atg11 initiation hub in wild type cells (Figure 4B). Atg9 and Atg1 colocalized with multiple Atg11 clusters, whereas Vac8 colocalized with only one (Figure 2C, S3A, S3B, S4A, and S4B). These data are consistent with multiple membrane seeds established by Atg9 at initiation hubs eventually relocating to the Vac8-dependent PAS, where downstream factors such as the PI3K complex I are recruited for phagophore formation 27. In line with this notion, similar to the phagophore membrane marker Atg8 (Figure 4B), Atg9 relocalized to only a single focus upon induction of membrane expansion by rapamycin treatment, suggesting that phagophore formation is initiated at this single site (Figure 4C). To visualize the sites of phagophore biogenesis at the PAS directly *in situ*, we applied a correlative cryo-electron tomography workflow. Consistent with the fluorescence microscopy results, this revealed a phagophore membrane initiated between the vacuolar membrane and the Ape1 complex (Figure 4D). Taken together, these results suggest that multiple initiation hubs coalesce at the vacuolar contact site and mature into the PAS, to trigger phagophore initiation.

**Ectopic formation of an Atg11 initiation hub triggers autophagic degradation of a neo-cargo**

Our work so far shows that receptor mobility on the surface of an autophagy cargo, rather than the biophysical nature of the cargo itself, is key to establish autophagy-competent Atg11-dependent initiation hubs. We hypothesized that artificially creating a low-affinity interaction with an autophagy receptor would trigger the degradation of a non-autophagic “neo-cargo”. To test this, we manipulated the reoviral nonstructural protein µNS. The mammalian reovirus protein μNS is foreign to yeast, self-assembles into particles 28–31, accumulates in the cytosol of yeast cells, and is not turned over by autophagy 27. These features of µNS make it an ideal putative cargo for selective autophagy.

To engineer a low-affinity interaction between the Atg19 receptor and µNS, we constructed an Ape1-propeptide-GFP-µNS (pp-GFP-µNS) fusion. Strikingly, pp-GFP-µNS displayed autophagic degradation (Figure 4E). In contrast, a direct Atg19-GFP-µNS receptor-cargo fusion, or an FK506-dependent high-affinity interaction between Cnb1-Atg19 receptor and FKBP-GFP-µNS cargo, did not trigger autophagic degradation (Figure 4E and S4D), consistent with the high-affinity cargo-receptor interactions generated above (Figure 1B, 1F, S1A and S1B). Moreover, only pp-GFP-µNS restored Atg11 clustering, highlighting, as predicted, that a low-affinity cargo-receptor interaction is necessary and sufficient for the formation of initiation hubs (Figure 4F and S4E). In summary, these findings suggest that targeting an autophagy receptor to a multimeric neo-cargo via a low-affinity interaction is sufficient to trigger its autophagic degradation.

**Initiation hubs for selective autophagy are conserved in human cells**

To test whether the principles of initiation hub formation during selective autophagy initiation in yeast are conserved in humans, we induced Parkin-dependent mitophagy in human U2OS cells by treating them with antimycin A and oligomycin (AO). We found that the ULK1 kinase, the human homologue of yeast Atg1, was diffuse in the cytoplasm of untreated U2OS cells but formed distinct foci at the mitochondria in AO-treated cells (Figure 5A and S5A). Similarly, LC3, a human homolog of yeast Atg8, localized in discrete foci on mitochondria in AO-treated cells (Figure S5B),suggesting that initiation hub formation is a conserved feature.

To assess mitophagy without inducing mitochondrial damage, we used the rapalog-inducible FRB-FKBP dimerization system in U2OS cells to target ULK1 to mitochondria 32. Specifically, we fused FRB to the tail anchor domain of the mitochondrial membrane protein FIS1 (FRB-FIS193-152, residues 93-152 of FIS1). Co-expression of an FKBP-GFP-ULK1 fusion enables its rapalog-inducible tethering to mitochondrial FRB-FIS193-152 and mitophagy induction (Figure 5B and S5C) 32,33. This tethering approach recruits FKBP-tagged proteins along the entire mitochondrial network, as observed for FKBP-GFP (Figure S5D). We hypothesized that targeting FKBP-GFP-ULK1 to the outer mitochondrial membrane would still promote the assembly of initiation hubs as it can diffuse laterally within the mitochondrial membrane and, therefore, rearrange. Indeed, treatment with rapalog resulted in the formation of distinct FKBP-GFP-ULK1 foci along the mitochondrial network (Figure 5C and S5E) that were not observed upon rapalog-dependent targeting of FKBP-GFP (Figure S5D), indicating a requirement for ULK1 and autophagy. FIP200, the human homologue of Atg11, undergoes phase separation during bulk autophagy induction 34, and we found that FIP200 co-localized in discrete foci with FKBP-GFP-ULK1 on the mitochondrial surface upon tethering (Figure S5F). Overall, these data suggest that initiation hub formation is a conserved feature of selective autophagy in human cells.

Autophagy components assemble on the ER surface in mammalian cells in bulk autophagy 35,36. We considered that autophagy machinery components also assemble on the ER surface during selective autophagy. To this end, we asked if mitochondrial initiation hubs form contact sites with the ER in U2OS cells. We performed peroxidase-catalyzed biotin proximity labeling by expressing the modified plant peroxidase APEX2 fused to either FKBP-ULK1 or FKBP alone (Figure S6A) 37,38. Biotinylated autophagy factors, including ATG3, ATG7, and GABARAPL2 were enriched in cells expressing FKBP-APEX2-ULK1 compared to those expressing FKBP-APEX2 (Figure 5D and S6B). Importantly, several biotinylated ER proteins were also enriched, including the ER-resident autophagic initiation factor DFCP1, and the lipid shuttling proteins ATG2A and ATG2B and VPS13A and VPS13C, suggesting that indeed the ER serves as an assembly platform during selective autophagy. FIP200 has been reported to link the ULK1 complex to the ER during bulk autophagy 36, and we found that the biotinylation of ER membrane proteins in cells expressing FKBP-APEX2-ULK1 depended on FIP200 (Figure 5E and Figure S6C). Together, these findings strongly suggest the conserved formation and function of initiation hubs during selective autophagy in mammalian cells and their PAS maturation at contact sites with the ER.

**A universal model of selective autophagy**

We propose that the mobility of receptors on selective cargo is essential for cargo degradability by selective autophagy, rather than the cargo property itself (Figure 5F). Rapid rearrangements of receptor molecules on the cargo surface support the recruitment of Atg11, promoting its phase separation and the formation of initiation hubs. These initiation hubs stabilize low-affinity interactions with the autophagy machinery through high avidity, ensuring proper spatio-temporal regulation of phagophore initiation. Rearrangements further allow the coalescence of multiple initiation hubs to establish the PAS, where phagophore formation is ultimately initiated. For membrane-delimited cargo, rearrangements are supported by lateral diffusion of proteins within the membrane itself. For membrane-less cargo, such mobility on cargo can either be achieved by the phase-separation of the cargo itself, or by low-affinity interactions between the cargo and receptors, which allow a high on-off rate and therefore an effective diffusion rate similar to the lateral diffusion in membranes. This together with other cargo properties such as size and shape will determine the cargo degradability. It also proposes that the introduction of a mobile surface on so-far nondegradable cargo might render these degradable and could open opportunities to engineer the targeting of aberrant structures such as amyloid fibers.

The best-known example of a balance between affinity and avidity is the effectiveness of antibodies in recognizing and responding to diverse pathogens. IgM antibodies are generated during the early phase of the immune response against pathogens. These antibodies possess ten low-affinity binding sites, which leads to high avidity and a high overall binding strength. This facilitates a swift screening of potential threats while allowing the reversible release of improperly bound non-antigens. In contrast, IgG antibodies produced in the later stages establish more stable and enduring bonds with antigens. This high-affinity mode of binding is essential for the sustained effectiveness of immune responses over an extended duration. Similarly, avidity-based interactions in autophagy are important at multiple stages throughout the pathway, and are likely key in the forward progression and self-organization of the process.

Our findings, therefore, suggest that the concept of low-affinity/high avidity is an underappreciated but important aspect in regulating the initiation of biological pathways.

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**Disclosure and competing interest statement**

The authors declare no competing financial interests.

**Methods**

**Yeast strains and plasmids**

Mammalian plasmids are listed in Table S1. Yeast, bacterial and insect cell expression plasmids are listed in Table S2. Yeast strains are listed in Table S3. Yeast genomic insertions and tagging were performed according to Janke et al. 2004 39, and multiple deletions were generated by PCR knock-out and/or mating and dissection. GFP-Atg11-containing strains were generated by crossing with yTB283 19, and GFP-ATG8-containing strains were crossed with yTB281, which had been generated by seamless tagging 40.

**Growth conditions**

Yeast cells were grown in a synthetic medium containing glucose (SD, 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose, and amino acids as required), or lactate medium (Slac, 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% lactic acid, 0.1% glucose, and amino acids as required), or rich medium (YPD, 1% yeast extract, 2% peptone, and 2% glucose), to mid-log phase. To induce bulk autophagy, cells were washed and resuspended in a nitrogen starvation medium (SD-N: 0.17% yeast nitrogen base without amino acids, 2% glucose) or treated with 220 nM (Figure 4C) or 100 nM (Figure 1B and Figure S1A and S1B) rapamycin. Yeast liquid cultures were incubated with shaking at 200 or 220 rpm at 30°C.

**Antibodies**

The following antibodies were used in this study: mouse monoclonal anti-GFP (1:100; 2B6, Merck), mouse monoclonal anti-GFP (1:5000; 7.1 and 13.1, Roche), IRDye 800CW Goat anti-Mouse (1:1000, 926-32210, Licor), anti-Pgk1 (1:1'10000, 22C5D8, Invitrogen).

**Standard biochemical assays**

Yeast cell cultures were either precipitated with 7% trichloroacetic acid (TCA) for 30 min on ice or overnight at -20°C or with 10% TCA for 20 min on ice. Precipitated proteins were either pelleted at 16,000 x g for 15 min at 4°C, washed with 1 ml acetone, air-dried, resuspended in urea loading buffer (120 mM Tris-HCl pH 6.8, 5% glycerol, 8 M urea, 143 mM β-mercaptoethanol, 8% SDS), boiled and analyzed by SDS-PAGE, or at 15,000 x g for 3 min at 4°C, washed twice with 1 ml ice-cold acetone, air-dried, resuspended in MURB buffer (50 mM Na2HPO4, 25 mM 2-[N-morpholino]ethanesulfonic acid (MES), pH 7.0, 1% SDS, 3 M urea, 0.5% 2-mercaptoethanol, 1 mM NaN3, and 0.05% bromophenol blue), vortexed for 5 min with acid-washed glass beads and boiled 42. Samples were loaded on 4-12% NuPAGE-SDS gels (Invitrogen), transferred to PVDF membranes and analyzed by western blotting.

**Tethering**

Tethering in exponentially grown cells was induced by the addition of 3 µM FK506 (LC Laboratories) for 1 h.

**Expression of Ape1 for clustering analysis by live microscopy**

Yeast cells were transformed with a plasmid containing GFP-Ape1 or BFP-Ape1 expressed under its endogenous promoter and a second copy of Ape1 under the copper-inducible CUP1 promoter. Cells were grown in a synthetic medium containing glucose (SD, 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose, and amino acids as required) to mid-log phase. Expression of Ape1 was induced by the addition of 50 µM CuSO4 overnight. This mild overexpression of Ape1 results in intermediate-sized Ape1 particles that are still degraded by autophagy (Figure 4E, Figure S4D). The degree of clustering for GFP-tagged proteins was measured by the coefficient of variance of the fluorescence intensity around the cargo (standard deviation divided by the mean GFP intensity).

**Mitochondria staining and mitophagy induction in yeast**

Exponentially growing cells were stained with MitroTrackerRed (Invitrogen) for 30 min at 30ºC. Cells were washed 1x with synthetic medium containing glucose and incubated for an additional 15 min at 30ºC. To induce mitophagy, stained cells were washed and resuspended in a nitrogen starvation medium (SD-N: 0.17% yeast nitrogen base without amino acids, 2% glucose). Yeast cultures were incubated with shaking at 220 rpm at 30°C for 3 h.

**Live-cell imaging of yeast**

Exponentially growing, nitrogen-starved or rapamycin-treated cells were placed on 35 mm glass bottom dishes (D35-20 1.5-N [In Vitro Scientific]) or microscopy slides pretreated with 1 mg/ml of concanavalin A type IV (Sigma-Aldrich), and live-cell imaging was performed at room temperature. Fluorescent microscopy images were recorded with a DeltaVision Ultra High Resolution microscope (GE Healthcare, Applied Precision) equipped with an UPlanSApo 100x/1.4 oil objective (Olympus), an sCMOS pco. edge camera (PCO), and a seven channel solid state light source (Lumencor) (Figure 1E, Figure S1D, Figure 3C, Figure 3E, Figure S2B, Figure S2C, Figure S2D, Figure S3C); or with a DeltaVision OMX Flex Microscope with UPlanSApo 60×/1.4 oil Olympus objective, using a PCO Edge 4.2 sCMOS camera. (Figure 2A, Figure 2B, Figure 2C, Figure 2E, Figure 3A, Figure 3B, Figure S2A, Figure S3A, Figure S3B, Figure 4B, Figure 4C, Figure 4F, Figure S4A, , Figure S4C, , Figure S4E); or with a Leica Stellaris 5 system with a 63x/1.40 oil (HC PL APO CS2) objective (Figure 1C), or with a Nikon Eclipse Ti2 with a 100x/1.49 oil (Apo TRIP) objective and a Hamamatsu C11440-22C camera (Figure 1B, Figure 1D, Figure 2A, Figure 2D, Figure S1A, Figure S1C, Figure 2F, Figure S4B).

Raw microscopy images acquired with the DeltaVision Ultra High Resolution microscope or with the DeltaVision OMX Super Resolution microscope were deconvolved using the softWorX deconvolution plugin (version R6.1.1 and version 7.2.1, respectively). Image analysis was performed using FIJI 43. Images from each figure panel were taken with the same imaging setup and are shown with the same contrast settings. Single focal planes of representative images are shown. For quantification, at least three independent replicates were analyzed and manual counting was performed blindly after randomizing image names.

Raw microscopy images acquired with Nikon Eclipse Ti2system were deconvolved using the NIS Elements Batch Deconvolution v5.20.00 and the chromatic shift was measured using fluorescent beads and corrected using Huygens 23.10.

The solidification of 2xGFP-Ede1 condensates was monitored by the colocalization of mTagBFP-3xGBP with 2xGFP-Ede1 after copper induction. For further colocalizations with ENDs, midsection images were acquired. For the quantification of mScarlet-Atg11 colocalization to ENDs conditions of the same day were compared with each other, and normalized to the amount of Atg11 colocalization observed in the -3xGBP strain.

For protein colocalization or analysis of GFP-Atg11 peak intensity and distribution on the surface of Ape1 images were generated by collecting a z-stack of 21 pictures with focal planes 0.25 μm apart. To quantify the degree of Atg11 clustering on different cargo structures, images were generated by collecting a z-stack of 21 pictures with focal planes 0.25 μm apart. For kymographs, time-lapse videos of at least 10 frames were collected with a time interval of 20 seconds.

***In vivo* FRAP analysis**

2xGFP-Ede1 FRAP analysis was performed on exponentially grown DF5 cells. Expression of mTagBFP-3xGBP constructs was performed in LoFl medium (low fluorescence synthetic growth medium (yeast nitrogen base without amino acids and without folic acid and riboflavin (FORMEDIUM)) supplemented with all essential amino acids and 2% glucose) by the addition of 1 mM CuSO4 for 6 h and incubation at 30ºC. The image resolution was set to 512x512 pixels, pixel size 11.1 pixels/micron, excitation wavelength to 488 nm, emission detection window to 490-750 nm, line time to 0.001 and line average to 1. For each sample, 420 frames were collected with a time interval of 0.518 seconds. Of those, 20 frames were collected before bleaching with 100% laser power (ROI bleaching) for 2 frames, and 400 frames were collected immediately after bleaching. For FRAP of mTagBFP-3xGBP-solidified ENDs only ENDs with a strong colocalization between 2xGFP-Ede1 and mTagBFP-3xGBP were chosen. Note that 2xGFP-Ede1 contains an N-terminal and a C-terminal GFP tag on Ede1.

GFP-Atg11 and GFP-Ape1 FRAP analysis was performed on exponentially grown BY474x cells. The image resolution was set to either 256x256 or 512x512 pixels, and the excitation wavelength to 488 nm. For each sample, at least 20 frames were collected with a time interval of 5 seconds and combined with a z-stack of 3 pictures with focal planes 0.25 μm apart. Of those, 3 frames were collected before bleaching, and at least 17 frames were collected immediately after bleaching.

To correct for drift or rotations in the samples, images were processed using the Fiji plugin StackReg 44. Double normalization was performed as described 45. For this, the average fluorescence intensities were recorded within either the bleached region, the entire Ede1-positive structures, the entire GFP-Atg11 or GFP-Ape1-positive structures using an in-house built analysis pipeline, and at a random cell-free spot for background subtraction before and immediately after photobleaching.

***In vitro* FRAP analysis of Atg11 droplets**

Purified GFP-Atg11 from Sf9 insect cells was diluted to a concentration between 0.005 µM and 2 µM in buffer containing 20 mM Hepes pH 7.5, 150 mM NaCl, and 5 mM DTT. For condensate formation, samples were incubated at room temperature for 20 min and transferred into a Chamber µ-Slide VI 0.1 (Ibidi). FRAP analysis of GFP-Atg11 condensates was performed at room temperature. The image resolution was set to 256 x 256 pixels, and the excitation wavelength to 488nm. For each sample, at least 61 frames were collected with a time interval of 3 sec. Of those, 2 frames were collected before bleaching, and at least 57 frames were collected immediately after bleaching. The average fluorescence intensities were recorded within either the bleached region, the entire GFP-Atg11-positive structures, and at a random condensate-free spot for background subtraction before and immediately after photobleaching. The analysis was done as described in the *in vivo* FRAP analysis section.

**Protein expression in *E. coli***

GST-mTagBFP, GST-mTagBFP-Ape11-45, GST-mTagBFP-Atg193D, and GST-mCherry-Atg19 fusion constructs were expressed from pGEX-4T.1 in *E. coli* BL21(DE3). Cells were grown in lysogeny broth (LB) medium supplemented with ampicillin at 37°C until an OD600 of 0.7, the temperature was reduced to 16°C, and expression was induced by the addition of 1 mM IPTG for 18 h. Cells were pelleted at 3,000 x g for 15 min at room temperature, resuspended in GST-lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5% glycerol, 1% Triton X-100, 1 mM PMSF, 1 mM DTT and cOmpleteTM protease inhibitor cocktail (Roche)), and lysed by sonication on ice. Cell lysates were cleared by centrifugation at 16,000 x gfor 10 min at 4°C.

***In vitro* binding assay**

The supernatants were incubated with Glutathione Sepharose 4B beads (GE Healthcare) for 1 h rotating at 4°C. Beads were washed three times by pelleting at 300 x g for 30 sec at 4°C and resuspension in GST-wash buffer (20 mM HEPES pH 7.5, 150 mM NaCl, and 5 mM DTT). mCherry-Atg19was elutedfrom GSH beads by the addition of 30 units of Thrombin protease and incubation at room temperature for 3 h and the purified protein was stored at -80˚C. GST-mTagBFP, GST-mTagBFP-Atg193D, or GST-mTagBFP-Ape11-45 coupled beads were incubated with purified GFP-Atg11 from insect cells or mCherry-Atg19 from *E. coli* for 20 min at room temperature. Unwashed samples were transferred into a Chamber µ-Slide VI 0.1 (Ibidi) and subjected to fluorescence microscopy. Afterwards samples were washed 8 times with GST-wash buffer (20 mM HEPES pH 7.5, 150 mM NaCl, and 5 mM DTT) and subjected to further imaging.

**Protein expression in Sf9 insect cells**

StrepII-GFP-Atg11 was expressed from baculovirus-infected Sf9 Spodoptera frugiperda insect cells (Expression Systems, cat # 94-001 F). The ATG11 ORF was subcloned with an N-terminal StepII-GFP tag into a pLIB library vector (reference). The recombinant bacmid carrying StrepII-GFP-Atg11 was assembled in DH10EMBacY *E. coli* strain (Geneva Biotech). For StrepII-GFP-Atg11 expression, Sf9 cells were grown in 1 l of ESF 921 Insect Cell Culture Medium (Expression Systems) supplemented with penicillin and streptomycin at 27°C to 1×106 cells/ml, infected by the addition of 1 ml of V3 virus, and grown for 4 days at 27°C. Cells were pelleted at 500 x g for 10 min at room temperature and washed with 1xPBS pH 7.4**.** Obtained pellets were resuspended in lysis buffer (500 mM Tris pH 7.4, 1.5 M KCl, 50 mM MgCl2, 10% glycerol, 5 mM β-mercaptoethanol, 1 mM PMSF, 0.1% Triton X-100, 10 mM imidazole, completeTM protease inhibitor cocktail (Roche)). Three freeze–thaw cycles were performed. Cell lysates were frozen in liquid nitrogen followed by thawing at 4°C**.** Benzonase® (25 U/ml; MERCK) was added and cells were incubated for 10 min on ice. Cell lysates were cleared three times by centrifugation at 15,000 x g for 10 min at 4°C and the supernatant was transferred to a new microfuge tube each time. The supernatants were recovered and applied to a Strep-Tactin®XT 4Flow®. The column was washed with Buffer W (100 Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0) and the protein was eluted with Buffer BXT (100 Tris-HCl, 150 mM NaCl, 50 mM biotin, 1 mM EDTA, pH 8.0).

***In vitro* Atg11 analysis**

Purified GFP-Atg11 was diluted to a concentration between 0.005 µM and 2 µM in buffer containing 20 mM Hepes pH 7.5, 150 mM NaCl, and 5 mM DTT. For condensate formation and condensate coalescence, samples were incubated at room temperature for 20 min and transferred into a Chamber µ-Slide VI 0.1 (Ibidi) and subjected to fluorescence microscopy. Coalescence of GFP-Atg11 droplets was followed for 60 min with a time interval of 10 sec.

**Vacuolar purification**

Vacuoles from Vph1‐4xmCherry atg15∆ pep4∆ or Vph1-4xmCherry *vac8∆* atg15∆ pep4∆ cells were enriched. Cells were grown at 30°C, and a minimum of 1,000 OD600 units were harvested, washed, and treated in DTT-containing buffer (100 mM Tris–HCl pH 9.4; 10 mM DTT) for 20 min at 30°C. The cells were spheroplasted in YPD containing 600 mM sorbitol using recombinant lyticase for 30 min at 30°C. Spheroplasts were harvested at 1,500 x g for 10 min at 4°C, and the pellet was resuspended in 15% Ficoll (in PS200 buffer: 20 mM Pipes, pH 6.8; 200 mM sorbitol supplemented with cOmpleteTM protease inhibitor (EDTA‐free, Roche)) with 0.08 µg/OD600 unit DEAE‐Dextran. The samples were briefly centrifuged at 20,000 x g, 10 min, 4°C using an ultracentrifuge (Optima MAX‐130K Ultracentrifuge (Beckmann)). The pellet was taken up in 15% Ficoll and overlayed with 8%, 4% and 0% Ficoll solution. The gradient was centrifuged at 100,000 x g for 80 min, 4°C (Sorvall WX Ultracentrifuge). The enriched vacuoles at the 0–4% interface were collected and concentrated at 20,000 x g, 20 min, 4°C, and finally, the vacuoles were taken up in PS200 buffer.

**Mammalian cell lines, cell culture conditions and stable cell line generation**

The following mammalian cell lines were used in this study: cCE308: U2OS Flp-In T-REx cell line with stably integrated FRB-FIS193-152, used for the live fluorescent microscopy experiments. cCE377: U2OS Flp-In T-REx cell line with stably integrated FRB-FIS193-152, mt-mKeima and 2xFKBP-GFP-ULK1, used for the mKeima assay experiments. cRB7: stable HEK293 Flp-In T-REx cell line with stably integrated FRB-FIS193-152, mt-mKeima and 2xFKBP-APEX2-ULK1. cRB12: stable HEK293 Flp-In T-REx cell line with stably integrated FRB-FIS193-152, mt-mKeima and 2xFKBP-APEX2. Both cRB7 and cRB12 were used for the affinity purification/MS experiments.

All three cell lines, cCE377, cRB7, and cRB12, were created by integration of Flp-In expression vectors into either HEK293 (R78007, ThermoFisher Scientific) or U2OS (K650001, Thermo Fisher Scientific) Flp-In T-rex cells and are Hygromycin and Blasticidin resistant.

Both HEK293 and U2OS Flp-In T-REx cell lines were grown in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, D6429) containing 10% fetal bovine serum (FBS, Sigma, F7524-500ML), 5 U/ml penicillin (Sigma, P4333-100ML), and 50 μg/ml streptomycin (Sigma, P4333-100ML). The cells were incubated at 37°C in a humidified 5% (v/v) CO2-air atmosphere.

To generate the HEK293 and U2OS Flp-In T-REx cell lines, cells were seeded in six-well plates (Sarstedt, 83.3920), at a density of 500,000 per well and allowed to grow overnight in antibiotic-free medium. The following day, the cells were co-transfected with a 1:10 ratio of Flp-In expression vector (pCE70 for 2xFKBP-GFP-ULK1, pRB7 for 2xFKBP-APEX2-ULK1 and pRB30 for 2xFKBP-APEX2) to Flp-recombinase plasmid pOG44 (Thermo Fisher Scientific, V600520). After 24 h, the media was changed with one supplemented with 15 μg/ml Blasticidin (InvivoGen, ant-bl-05). Further 24 h later, cells were expanded from the six-well plates to p100 dishes (Sarstedt, #83.3902.300), and the media was changed to one containing 15 μg/ml Blasticidin (InvivoGen, ant-bl-05) and 100 μg/ml Hygromycin B Gold (InvivoGen, ant-hg-1). The Hygromycin B Gold treatment allowed for selection of the cells that stably integrated the construct. Selection media were then changed every five days. Colonies were then picked and transferred to 24-well plates (Sarstedt, 83.3922.005) and expanded. The cells were then checked for doxycycline-inducible target gene expression using Western blotting. All cells were periodically tested for mycoplasma contamination.

**siRNA treatment**

To knockdown the expression of endogenous FIP200, cells were treated with two different siRNAs for FIP200: ON-TARGETplus Human RB1CC1 siRNA (Dharmacon, J-021117-05-0010) and Human RB1CC1 siRNA HSS114818 (Invitrogen, 91044774) at a final concentration of 20 nM each. As a negative control, the wild type cell lines were treated with an ON-TARGETplus non-targeting siRNA #1 (Dharmacon, D-001810-01-05). siRNAs were transfected using ViaFect (E4981, Promega) according to the manufacturer’s instructions.

**Sample processing for mass spectrometry analysis**

HEK293 Flp-In T-REx cells were freshly thawed and seeded, and after 24 h, the cells were treated with the indicated siRNA (non-coding or siFIP200) for 72 h before harvesting. The day before harvesting, the cells were treated with 1 µg/ml of doxycycline (Sigma, D9891-10g) to induce the expression of the Flp-In integrated construct: 2xFKBP-myc-APEX2 for cRB12 and 2xFKBP-myc-APEX2-ULK1 for cRB7. On the day of the harvesting, 72 h after siRNA treatment, the cells were treated with 500 µM of biotin-phenol (IrisBiotech, 41994-02-9/LS-3500.5000) and, when indicated with 0.5 µM of rapalog (A/C Heterodimerizer, TaKaRa, 635056) for 1 h at 37°C. To induce the peroxidase activity of APEX2 and the formation of a biotinylated area, the cells were treated for 1 min with 1 mM H2O2 (Roth, CP26.5). Quickly after the 1 min H2O2 pulse, the cells were washed with DPBS (Sigma, D8537-500ML) and, to avoid further biotinylation from the APEX2, the cells were washed with quenching buffer (DPBS, 10 mM sodium ascorbate (Sigma, 11140-50G) and 5 mM Trolox (Sigma, 238813-25G)). The cells were then scraped, harvested, and lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% TritonX-100, 1x protease inhibitor cocktail (Roche, 05056489001)), supplemented with quenching reagents (10 mM sodium ascorbate, 1 mM Trolox). To clear the lysate, the cells were centrifuged at 10,000 x g at 4°C for 15 min.

Magnetic streptavidin beads (Pierce, Thermo Scientific, 88816) were chemically acetylated using Sulfo-NHS-acetate (Pierce, Thermo Scientific, 26777) to prevent contamination with co-digested streptavidin peptides 46,47. The supernatant of the cell lysate was incubated for 1 h at 4°C with the S-NHS-Ac treated magnetic streptavidin beads. After incubation, the samples were washed five times with Tris buffer (50 mM Tris, 150 mM NaCl) and three times with 50 mM ABC buffer (ammonium bicarbonate, Sigma, 09830-1KG). The beads were transferred to a new tube and resuspended in 50 µl 1 M urea and 50 mM ammonium bicarbonate. Samples were reduced with 2 µl of 250 mM dithiothreitol (DDT, Roche, 10708984001) for 30 min at room temperature and alkylated with 2 µl of 500 mM iodoacetamide (IAA, Sigma, I6125-5G) for 30 min at room temperature in the dark. The remaining IAA was quenched with 1 µl of 250 mM DTT for 10 min. Proteins were digested with 150 ng LysC (FUJIFILM Wako Pure Chemical Corp., 125-02543) at 25°C overnight. The supernatant was transferred to a new 0.2 ml vial and further digested for 5 h at 37°C by the addition of 150 ng trypsin (Trypsin Gold, Mass Spec grade, Promega, V5280). The digest was stopped by the addition of trifluoroacetic acid (TFA) to a final concentration of 0.5%, and the peptides were desalted using a 96-well OASIS HLB µElution plate (Waters, 30 µm particle size, 186001828BA) following the manufacturer’s protocol.

**Liquid chromatography-mass spectrometry analysis**

Peptides were separated on an Ultimate 3000 RSLC nano-flow chromatography system (Thermo-Fisher), using a pre-column for sample loading (Acclaim PepMap C18, 2 cm × 0.1 mm, 5 μm, Thermo-Fisher) and a C18 analytical column (Acclaim PepMap C18, 50 cm × 0.75 mm, 2 μm, Thermo-Fisher), applying a segmented linear gradient from 2% to 35% and finally 80% solvent B (80% acetonitrile, 0.1% formic acid; solvent A 0.1% formic acid) at a flow rate of 230 nl/min over 60 min.

Eluting peptides were analyzed on an Exploris 480 Orbitrap mass spectrometer (Thermo-Fisher), which was coupled to the column with a FAIMS pro ion-source (Thermo-Fisher) using coated emitter tips (PepSep, MSWil).The mass spectrometer was operated in DIA mode with the FAIMS CV set to -45, the survey scans were obtained in a mass range of 350-1200 m/z, at a resolution of 60k at 200 m/z and a normalized AGC target at 300%. 31 MSMS spectra with variable isolation width between 13 and 257 m/z covering 349.5-1200.5 m/z range, including 1 m/z windows overlap, were acquired in the HCD cell at 30% collision energy at a normalized AGC target of 1000% and a resolution of 30k. The max. Injection time was set to auto.

**Mass spectrometry data analysis**

MS raw data were converted to the htrms format using HTRMS converter (version 18.3, Biognosys) and processed with Spectronaut (version 18.5, Biognosys). The library-free DirectDIA+ workflow was employed for analysis of the htrms files, utilizing the *H. sapiens* 1 protein per gene reference proteome from Uniprot (Proteome ID: UP000005640, release 2023.03), concatenated with a database of 379 common laboratory contaminants (in-house database), and an entry for the 2xFKBP-APEX2 construct. The cleavage specificity was set to full trypsin specificity (Trypsin/P), allowing for 2 missed cleavages. The thresholds for precursor qvalue, precursor PEP, protein qvalue per experiment, protein qvalue per run, and protein PEP were all set at 1% 48. Carbamidomethylation of cysteine residues was set as fixed modification; methionine oxidation and protein N-terminal acetylation were considered as variable modifications. Cross-run normalization was disabled, and all other settings were used at their default values.

Computational analysis was performed using Python and the in-house developed Python library MsReport (version 0.0.23, 47). Only non-contaminant proteins identified with a minimum of two peptides and being quantified in at least two replicates of one experiment were considered for the analysis. MS2 LFQ protein intensities reported by Spectronaut were used for the quantitative analysis. Intensities below 1000 were removed and treated as not quantified to exclude low-quality quantification. Intensities were log2 transformed and normalized across samples using the ModeNormalizer from MsReport. This method involves calculating log2 protein ratios for all pairs of samples and determining normalization factors based on the modes of all ratio distributions. Missing values were imputed by drawing random values from a normal distribution with μ = 9.96 and σ = 0.75.

Statistical analysis comparing experiments FKBP-APEX2-ULK1 (WT) with FKBP-APEX2 (WT), and FKBP-APEX2-ULK1 (siFIP200) with FKBP-APEX2 (siFIP200) was performed using the Linear Models for Microarray Analysis (LIMMA, version 3.54.2, 49) package in R. Moderated t-statistics were calculated using the limma-trend method, and multiple testing correction was applied using the Benjamini-Hochberg (BH) method. Gene ontology terms were obtained from Uniprot (release 2023.05). The in-house Python library XlsxReport was used to create a formatted Excel file summarizing the results of protein quantification (Table S1).

To assess the impact of FIP200 knockdown, ULK1 target proteins were selected based on an adjusted p-value < 0.01 and log2 ratio > 1 in FKBP-APEX2-ULK1 (WT) vs FKBP-APEX2 (WT). Average log2 protein intensities were calculated for each control, FKBP-APEX2 (WT) and FKBP-APEX2 (siFIP200). The mean of the respective control was subtracted from each replicate of the FKBP-APEX2-ULK1 WT and FKBP-APEX2-ULK1 siFIP200 samples, resulting in values denoted as "Signal over control [log2]". Focusing on ULK1-specific proteins associated with the GO term "ER membrane” (n=44), t-tests were calculated between FKBP-APEX2-ULK1 (WT) and FKBP-APEX2-ULK1 (siFIP200) experiments using log2 “signal over control” values. Multiple testing correction was applied using the Benjamini-Hochberg (BH) method with an FDR-controlled p-value cutoff of 0.05. The results are summarized in Table S1.

**Data availability**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE 50 partner repository with the data set identifier PXD047277 (Reviewer account Username: reviewer\_pxd047277@ebi.ac.uk Password: LVa33YXr).

**Fluorescence microscopy of mammalian cells**

For live-cell imaging, cells were grown in 35 mm glass-bottom dishes (Ibidi, 81156) and preserved in an environmental chamber at 37°C, 5% CO2 during image acquisition. Fluorescent signal from U2OS cells was imaged using a DeltaVision OMX Flex Microscope with UPlanSApo 60×/1.4 oil Olympus objective, using a PCO Edge 4.2 sCMOS camera. Cells were treated with 4 µM antimycin A (A8674, Sigma-Aldrich) and 4 µM oligomycin (75351, Sigma-Aldrich), for 1h, 0.5 µM rapalog (B/B Homodimerizer, TaKaRa, 635059) treatment was performed for 24 h. Mitochondria were stained with MitoTracker™ Deep Red FM (Invitrogen, M22426) according to the manufacturer’s instructions.

**Sample preparation for mKeima assay and sample analysis**

U2OS cells (cCE377) were seeded in 6-well plates at a density of 500,000 cells per well and cultured in DMEM media supplemented with 10% FBS. After 24 h, the media in the plates were replaced with one containing 1 µg/ml of doxycycline to induce the expression of 2xFKBP-GFP-ULK1. Additionally, in the specified samples, 0.5 µM of rapalog was added for a 24 h incubation period. Before harvesting, the indicated samples were treated with 200 nM Bafilomycin A1 (Cell Signaling Technology, 54645) and rapalog. Following the treatment, cells were washed with DPBS (Sigma-Aldrich, D8537) and detached with trypsin-EDTA (Sigma, T3924). The cells were then harvested in FACS medium (phenol-red free DMEM, Sigma, D1145-500ML, supplemented with 10% FBS). Cells were transferred to 1.5 ml tubes and centrifuged at 500 x g for 3 min at room temperature. The supernatant was removed, and the cell pellets were resuspended in 200 µl of FACS media and transferred to U-bottom 96-well plates (Greiner Bio-One, 650970). Flow cytometry experiments were performed with a CytoFLEX S (Beckman Coulter, B75408) using the CytExpert 2.3 analysis software.

Neutral pH mKeima and acidic pH mKeima were excited with the 405 nm and 561 nm lasers, respectively. For both mKeima forms, the emission was detected with a 610/20 bandpass filter. To detect GFP-positive cells, a 488 nm laser was used in combination with a 525/40 nm bandpass filter. For each sample, 200,000 events were collected and analyzed using FlowJo (FlowJo V.10.9.0 - May 5, 2023). The main population of live cells was selected via gating of SSC-A (Side Scatter - area) vs FSC-A (Forward Scatter - area) plots. Singlets were then selected in FSC-H (Forward Scatter - height) vs FSC-W (Forward Scatter - width) plots. Only GFP-positive cells were selected for the analysis, capturing a minimum of 80,000 cells per sample. Channel values of selected populations were then exported from FlowJo.

An in-house developed Python library mKeima (version 0.5.0, available at <https://pypi.org/project/mkeima>) was utilized for additional analysis. Channel values were treated in log space, and the mKeima ratio was calculated for each individual event by subtracting the acidic pH signal from the neutral pH signal. Subsequently, these ratios were standardized: first, by subtracting the mean of the bafilomycin-treated samples and then by dividing by the standard deviation of the bafilomycin samples. The population median of the standardized mKeima ratios was calculated and employed as the standardized mitophagy score.

**Statistics and reproducibility**

To assess statistical significance, one-tailed unpaired *t*-tests, two-tailed unpaired *t*-tests, one-way ANOVA, or two-way-ANOVA tests followed by a Dunnet post-hoc test were performed.

**Correlative cryo-electron tomography**

GFP-Atg19 cells expressing Ape1 under a copper inducible promoter were grown at 30°C in YPD supplemented with 250 µM CuSO4 for induction of Ape1 overexpression. Upon reaching OD600 = 0.8, rapamycin was added to the culture at a final concentration of 100 nM for 3 h to induce bulk autophagy. The culture was diluted to an OD600 of 0.8, and autofluorescent 1 µm diameter Dynabeads (Dynabeads MyOne carboxylic acid No. 65011, Thermo Fisher Scientiﬁc) were added at a 1:20 dilution prior to vitrification. EM grids (200 Mesh Au SiO2 R1/4, Quantifoil) were glow discharged for 90 sec on both sides using a Pelco easiGlow device. 3.5 µl of cell suspension was applied to each grid, and cells were vitrified using an EM GP2 grid plunger (Leica Microsystems) after 2 sec blotting time at 20°C and 90% relative humidity by plunge freezing in liquid ethane at -184°C. Grids were clipped using modified autogrid rings with cut-outs suitable for cryo-FIB milling. Cryo-confocal imaging of the plunge frozen samples was carried out on a TCS SP8 Cryo-CLEM (Leica Microsystems) equipped with a 50x/0.9 NA objective. For this, grids were initially mapped by collecting image stacks at 2x2 binning with a Z-spacing of 1.5 m in brightfield and widefield GFP fluorescence channels. Grid maps were used to identify intact grid squares containing cells with the target fluorescent signal. For confocal imaging of grid squares containing cells with the target fluorescent signal, image stacks with a Z-spacing of 300 nm and 84.4 nm pixel size were acquired with 488nm laser excitation. Grid mapping and cryo-confocal imaging were both carried out using the LAS X Navigator software (Leica Microsystems). Cryo-confocal stacks were deconvolved using Huygens Essential version 23.04.0p0 (Scientific Volume Imaging, http://svi.nl) and then resliced to achieve cubic voxels using Fiji 43. For FIB-milling with an Aquilos dual-beam cryo-focused ion beam-scanning electron microscope (cryo-FIB-SEM) (Thermo Scientific), equipped with a cryo-stage cooled to -180°C, grids were first coated with a protective organometallic platinum layer for 9 sec and then mapped by cryo-scanning electron microscopy (cryo-SEM). Grid maps were correlated to the cryo-fluorescence maps using MAPS Software (Thermo Scientific) and lamella sites were placed in grid squares in which cryo-confocal stacks had been collected. Semi-automatic FIB-milling of cells with the target fluorescent signal was then carried out at 8° milling angle using a Gallium ion beam and AutoTEM software (Thermo Scientific). The ion beam current and distance between milling patterns was decreased in a stepwise manner to achieve a final lamella thickness of 150-200 nm.

Tilt series were acquired on a Krios G4 (Thermo Scientific) cryo-TEM operated at 300kV and equipped with a Selectris X energy filter, an energy slit set to 10eV and Falcon 4i direct electron detector at a nominal magnification of 64000x, corresponding to a 1.197 Å pixel size, using SerialEM 51. A dose-symmetric acquisition scheme 52 was used with the start angle set to 8° and a nominal tilt range of 68° to -52° with 2° increments, a target defocus range of -1.5 to -4.5 µm and total dose of ~150e/Å2. Target areas for tilt series acquisition were identified via correlation of the cryo-confocal stacks with low-magnification TEM grid square images acquired at a nominal magnification of 470x, using 3D-Correlation Toolbox (https://3dct.semper.space/), with the Dynabeads serving as fiducial markers visible in both imaging modalities.

**Tomogram reconstruction and membrane segmentation**

Tilt series alignment and tomogram reconstruction via weighted back projection were carried out at bin4 using AreTomo version 1.3.3 53. Tomogram denoising at bin4 was done with cryoCARE (https://github.com/juglab/cryoCARE\_pip, 54) trained on tomograms reconstructed from odd/even tilt series frames with identical alignment parameters. Automatic segmentation of membranes in the denoised tomograms was carried out by MemBrain-Seg (https://github.com/teamtomo/membrain-seg, 55), followed by manual refinement in Amira 2022.2 (Thermo Scientiﬁc) and display in ChimeraX-1.6.1 (https://www.cgl.ucsf. edu/chimerax, 56).

**Modeling**

The mathematical modeling is described in the Supplementary material.

**Code availability**

We used cellular\_raza (https://github.com/jonaspleyer/cellular\_raza) to solve the equations of motion and implement neighbor counting. More details can be found in the supplementary material.

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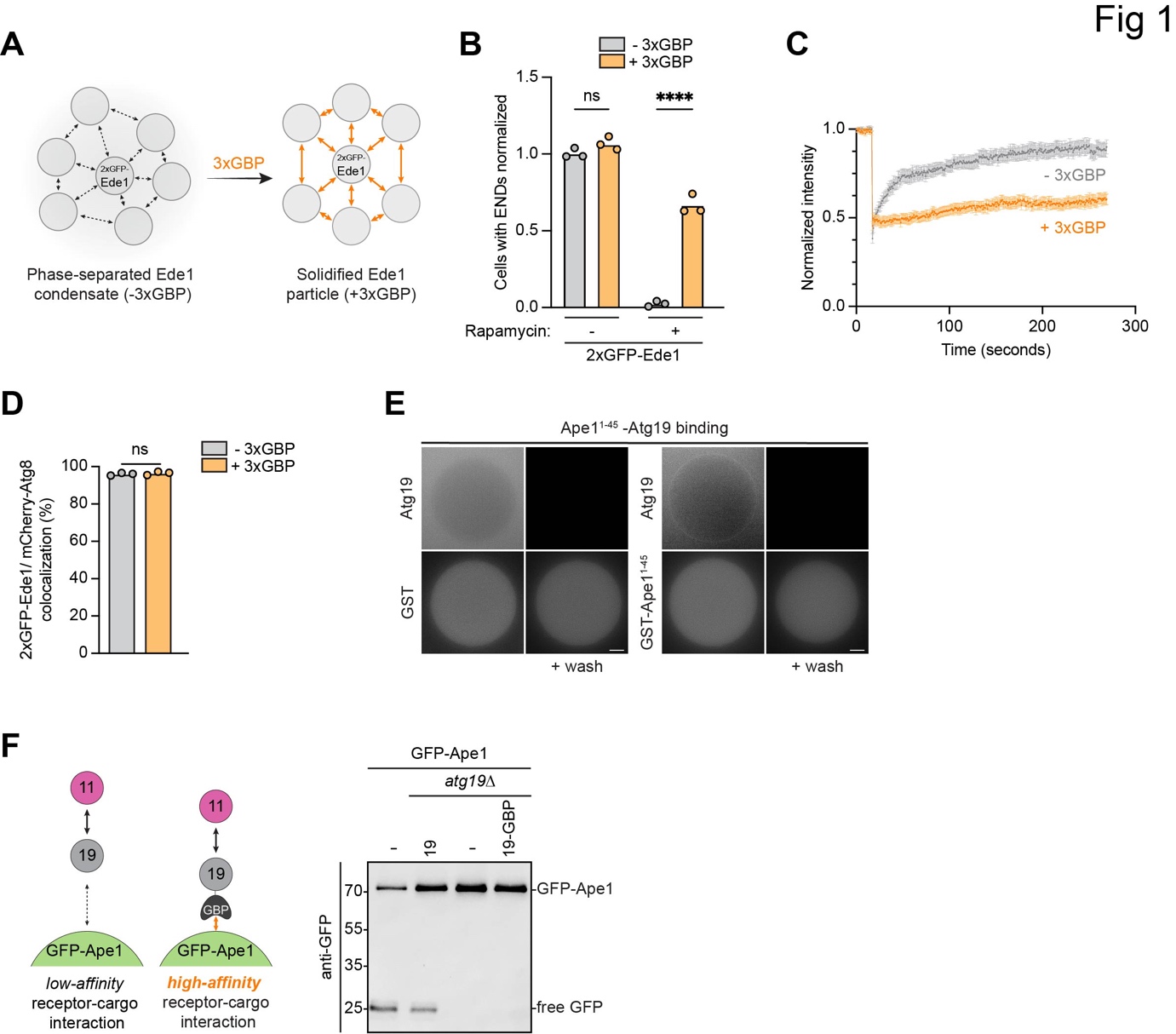
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**Figures and legends:**



**Figure 1: High-affinity receptor-cargo interactions impair selective autophagy**

A) Schematic of phase-separated END condensates containing 2xGFP-Ede1 in the absence of BFP-3xGBP (-3xGBP) and solidified END particles co-expressed with BFP-3xGBP (+3xGBP) with dashed black arrows representing low-affinity and orange arrows representing high-affinity interactions respectively.

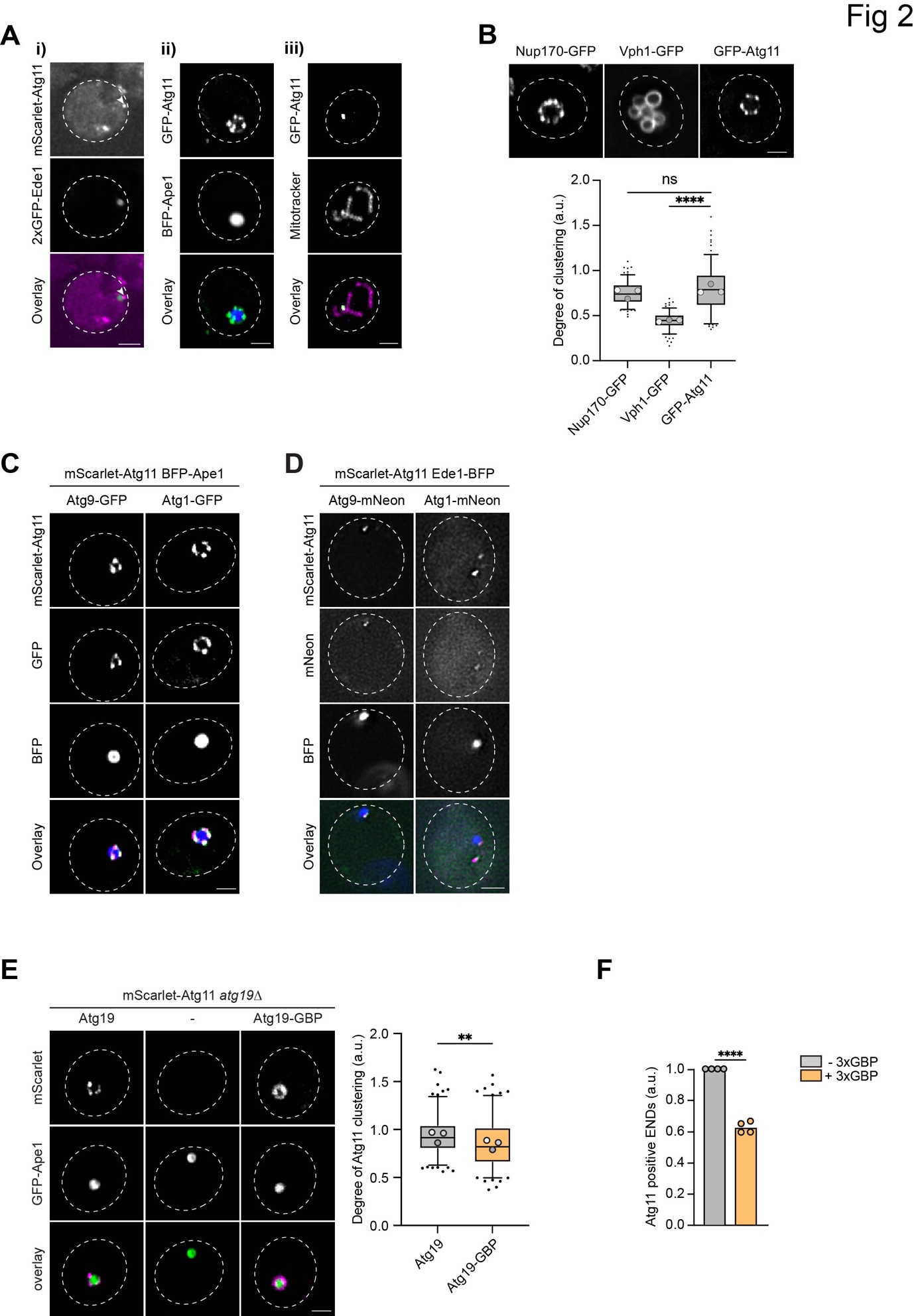
B) Cells expressing 2xGFP-Ede1 without (-3xGBP) or with (+3xGBP) BFP-3xGBP under the control of a copper-inducible promoter were grown to mid-log phase in the presence of 1 mM CuSO4 for 6 h. Afterwards, autophagy was induced by the addition of rapamycin. The number of cells with END condensates was quantified (n = 3 biological replicates). For each condition and replicate at least 250 cells were analyzed. The values of each replicate (circle) and the mean (bars) were plotted. Statistical analysis was performed using a two-tailed unpaired *t*-test. Significance is indicated with asterisks: \*\*\*p < 0.001, \*\*p < 0.01, \*p < 0.05, n.s. (not significant) p > 0.05.

C) The -3xGBP and the +3xGBP strains were grown to mid-log phase as described in Figure B). Ede1 assemblies were photobleached, and recovery of the signal was followed over time (1 frame/0.518 sec). Quantification shows the recovery of the GFP signal from a total of 30 ENDs for each strain from three independent replicates as mean SEM.

D) Both the -3xGBP and the +3xGBP strain co-expressing mCherry-Atg8 were grown to mid-log phase in the presence of 1 mM CuSO4 addition. The percentage of 2xGFP-Ede1/mCherry-Atg8-positive structures was quantified for three independent biological replicates. For each condition and replicate at least 100 END structures were analyzed. The values of each replicate (circle) and the mean (bars) were plotted. Statistical analysis was performed using a two-tailed unpaired *t*-test. Significance is indicated with asterisks: \*\*\*p < 0.001, \*\*p < 0.01, \*p < 0.05, n.s. (not significant) p > 0.05.

E) GST-BFP or GST-BFP-Ape11-45 was expressed in *E. coli* and bound to Glutathione Sepharose (GSH) beads. Protein-bound beads were incubated with *E. coli* cell lysates containing overexpressed mCherry-Atg19, and bound mCherry-Atg19 was analyzed before and after washing by fluorescence microscopy. Representative microscopy images from one out of three independent experiments are shown. Scale bar: 20 µm. Quantification is shown in Figure S1D.

F) (Left) Schematic of the experimental set-up. (Right) Wild type cells expressing GFP-Ape1 or *atg19∆* cells expressing GFP-Ape1 and Atg19, an empty control vector (-), or Atg19-GBP were grown to mid-log phase. Cell extracts were separated by gel electrophoresis and western blots were probed with anti-GFP.



**Figure 2: Receptor mobility enables the formation of initiation hubs**

A) i) *atg19∆* cells expressing 2xGFP-Ede1 and mScarlet-Atg11 were grown to mid-log phase. ii) GFP-Atg11 cells expressing endogenous BFP-Ape1 and copper-inducible untagged Ape1 were grown to mid-log phase in the presence of 50 µM CuSO4. iii) *atg19∆* cells expressing GFP-Atg11 were grown to mid-log phase. The mitochondrial network was stained with MitoTracker Red. Mitophagy was induced by nitrogen starvation. Representative fluorescence microscopy images of one out of three independent experiments are shown. Dashed lines indicate the contour of individual cells. Scale bar: 2 µm.

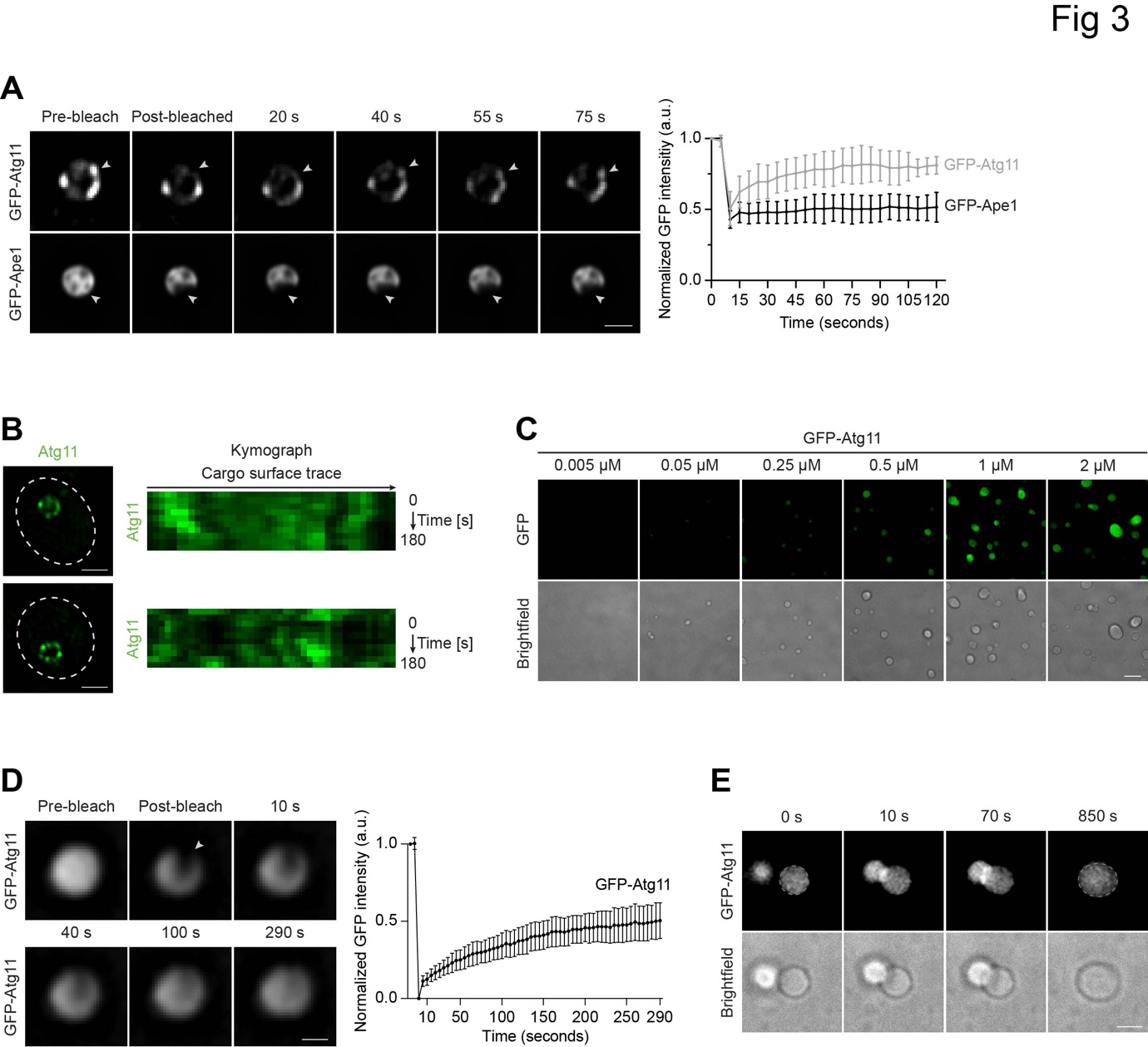
B) Nup170-GFP, Vph1-GFP *vac8∆ atg19∆*, and GFP-Atg11 cells (as above) were grown to mid-log phase. (Top) Representative fluorescence microscopy images from one out of three independent experiments are shown. Dashed lines indicate the contour of individual cells. Scale bar: 2 µm. (Bottom) The degree of GFP clustering was quantified in three independent biological replicates as the coefficient of variance (SD/mean GFP intensity) and displayed in a box and whisker plot. For each condition and replicate at least 50 structures were analyzed. The median degree of clustering (horizontal line) and the mean values of each biological replicate (circles) are indicated. Boxes represent the 25th to 75th percentiles, and whiskers expand to the 5th and 95th percentiles. Outliers are shown as black dots. Statistical analysis was performed using one-way ANOVA followed by a Dunnett post-hoc test. Significance is indicated with asterisks: \*\*\*p < 0.001, \*\*p < 0.01, \*p < 0.05, n.s. (not significant) p > 0.05. a.u. = arbitrary units.

C) Atg9-3xGFP *atg11∆* or Atg1-3xGFP cells expressing mScarlet-Atg11 and endogenous BFP-Ape1 and copper-inducible untagged Ape1 were grown to mid-log phase in the presence of 50 µM CuSO4. Representative fluorescence microscopy images of one out of three independent experiments are shown. Dashed lines indicate the contour of individual cells. Scale bar: 2 µm.

D) Cells expressing Ede1-BFP, mScarlet-Atg11, and either Atg1-mNeon or Atg9-mNeon were grown to mid-log phase. Representative fluorescence microscopy images of one out of three independent experiments are shown. Dashed lines indicate the contour of individual cells. Scale bar: 2 µm.

E) mScarlet-Atg11 *atg19∆* cells co-expressing either Atg19 or Atg19-GBP and endogenous GFP-Ape1 and copper-inducible untagged Ape1 were grown to mid-log phase in the presence of 50 µM CuSO4. Representative fluorescence microscopy images of one out of three independent experiments are shown. Dashed lines indicate the contour of individual cells. Scale bar: 2 µm. The degree of Atg11 clustering represented as the coefficient of variance (SD/mean mScarlet intensity) was analyzed in three independent biological replicates. For each condition and replicate at least 50 GFP-Ape1 positive particles were analyzed. Dark horizontal lines represent the median degree of Atg11 clustering; the box represents the 25th to 75th percentiles; whiskers expand to the 5th and 95th percentiles. Grey dots indicate the mean value of each biological replicate. Outliers are shown as black circles. Statistical analysis using two-tailed unpaired *t*-test. Significance is indicated with asterisks: \*\*\*p < 0.001, \*\*p < 0.01, \*p < 0.05, n.s. (not significant) p > 0.05. a.u. = arbitrary units.

F) *atg19∆* cells expressing mScarlet-Atg11 and 2xGFP-Ede1 without (-3xGBP) or with (+3xGBP) BFP-3xGBP under the control of a copper-inducible were grown to mid-log phase. The number of cells showing mScarlet-Atg11 clustering around ENDs was quantified and normalized to the 2xGFP-Ede1 *atg19∆* non-solidified sample. At least 386 END structures were analyzed per condition. The values of each replicate (circle) and the mean (bars) were plotted. Statistical analysis using two-tailed unpaired *t*-test. Significance is indicated with asterisks: \*\*\*p < 0.001, \*\*p < 0.01, \*p < 0.05, n.s. (not significant) p > 0.05.



**Figure 3: Phase separation of Atg11 drives initiation hub formation**

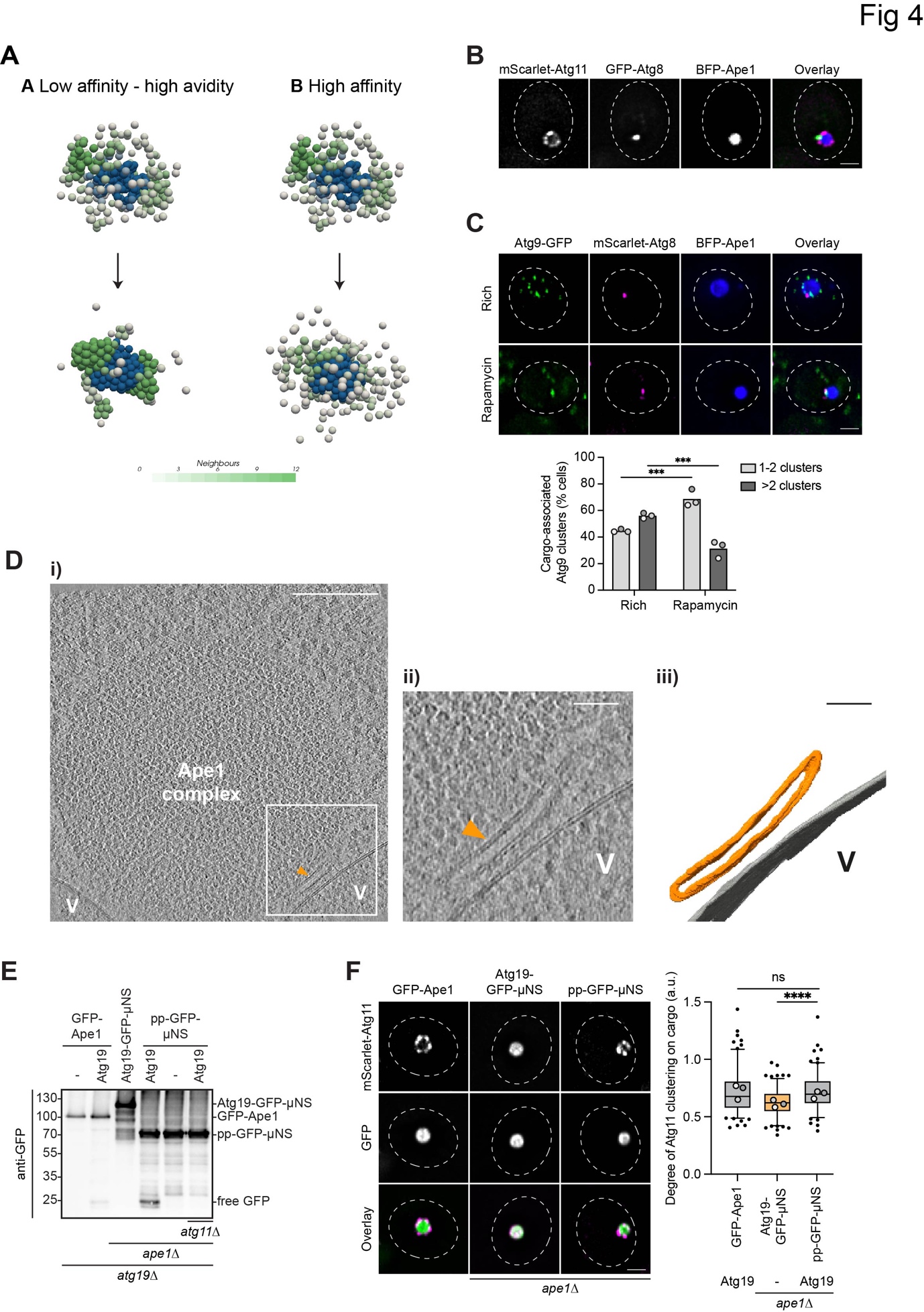
A) GFP-Atg11 cells expressing endogenous BFP-Ape1 and copper-inducible untagged Ape1 or wild type cells expressing GFP-Ape1 and copper-inducible untagged Ape1 were grown to mid-log phase in the presence of 50 µM CuSO4. GFP-Atg11 and GFP-Ape1 structures were photo-bleached, and recovery of the signal was followed over a time course of 120 seconds (1 frame/5 sec). Representative fluorescence microscopy images of one out of three independent experiments are shown. Scale bar: 2 µm. Quantification shows the recovery of the GFP signal from a total of 35 GFP-Atg11 and 33 GFP-Ape1 particles from three independent replicates as mean SD; white arrows indicate the bleached area.

B) GFP-Atg11 cells expressing endogenous BFP-Ape1 and copper-inducible untagged Ape1 were grown to mid-log phase in the presence of 50 µM CuSO4. The dynamics of GFP-Atg11 foci were monitored over a time course of 180 sec (1 frame/20 sec) and are represented as kymographs. Representative fluorescence microscopy images of one out of three independent experiments are shown. Dashed lines indicate the contour of individual cells. Scale bar: 2 µm.

C) GFP-Atg11 was purified from Sf9 insect cells, and droplet formation was monitored *in vitro* at different concentrations by fluorescence microscopy after 20 min incubation at room temperature in a buffer containing 150 mM NaCl. Representative microscopy images from three independent experiments are shown. Scale bar: 5 µm. Quantification is shown in Figure S2B.

D) *In vitro* formed GFP-Atg11 droplets were photo-bleached, and recovery of the signal was measured over a time course of 305 seconds (1 frame/5 sec). Representative microscopy images from one out of three independent experiments are shown. White arrows indicate the position of the photobleached area. Scale bar: 1 µm. Quantification shows the normalized recovery of the GFP signal from a total of 30 GFP-Atg11 condensates from three independent replicates as mean SD.

E) Coalescence of *in vitro* formed GFP-Atg11 droplets was monitored by time-lapse microscopy over a time course of 850 sec (1 frame/10 sec). Scale bar: 2 µm. Quantification is shown in Figure S2C.



**Figure 4: Initiation hubs coalesce at the vacuolar contact site to trigger phagophore initiation**

A) Panel A: Initial and final snapshots of a molecular simulation corresponding to a very low-affinity case of Atg11-Atg19 complexes binding to cargo. This case shows low avidity, since the complexes are not bound to the cargo and instead form independent of the cargo. The clustering is shown by visualizing the number of neighbors of Atg11 particles. Stronger colors indicate that more neighbors are in proximity and will result in a stronger coupling. Panel B: Initial and final snapshots of a molecular simulation corresponding to a low-affinity case of Atg11-Atg19 complexes binding to cargo. This case shows high avidity, shown by the binding of the Atg11-Atg19 complexes on the cargoPanel C: The same initial state evolved under high-affinity interactions.Uniform binding of the Atg11-Atg19 particles to the cargo is observed.

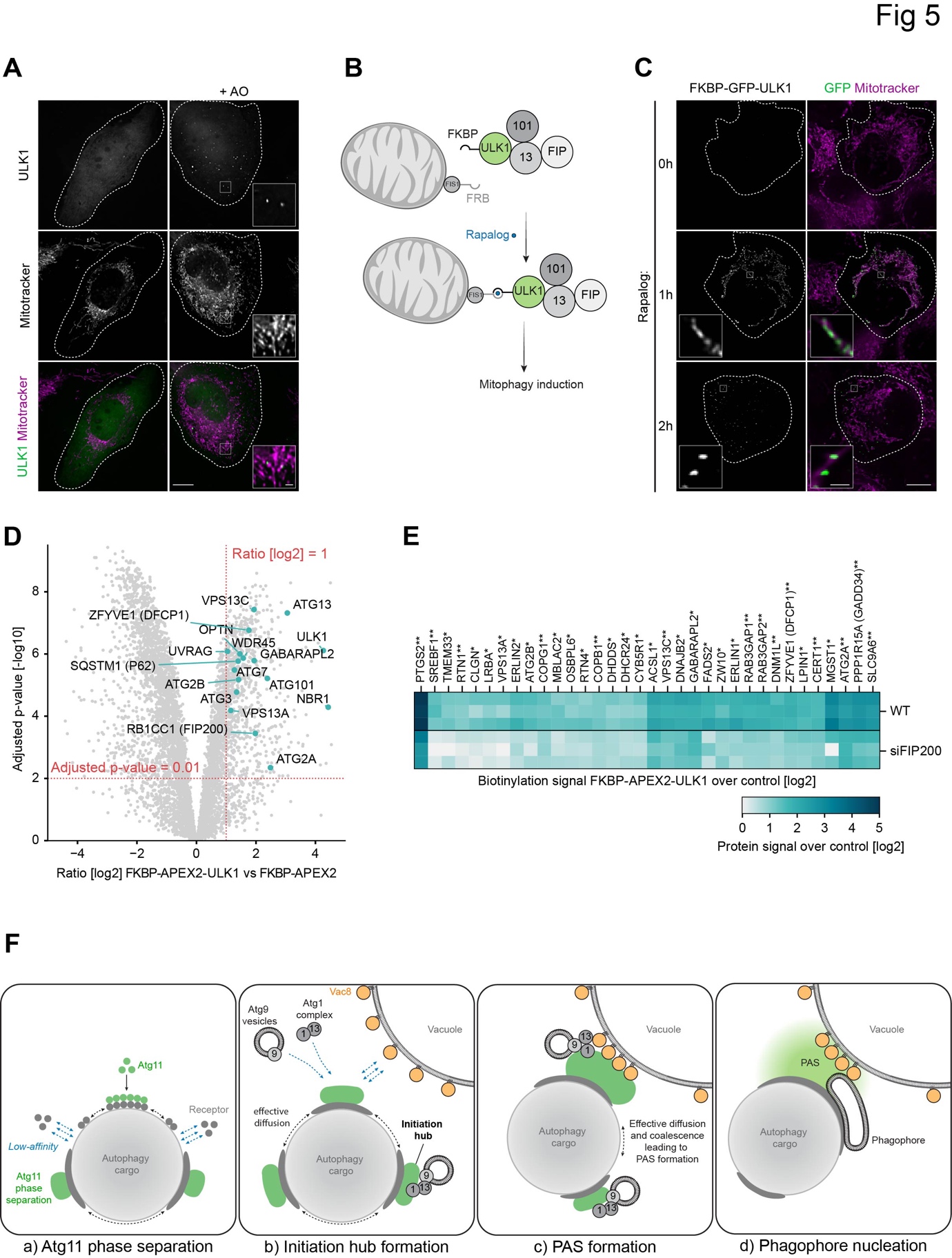
B) GFP-Atg8 cells expressing mScarlet-Atg11, endogenous BFP-Ape1 and copper-inducible untagged Ape1 were grown to mid-log phase in the presence of 50 µM CuSO4. Representative fluorescence microscopy images of one out of three independent experiments are shown. Dashed lines indicate the contour of individual cells. Scale bar: 2 µm.

C) Atg9-3xGFP mScarlet-Atg8 cells expressing endogenous BFP-Ape1 and copper-inducible untagged Ape1 were grown to mid-log phase in the presence of 50 µM CuSO4. Phagophore formation was induced by the addition of rapamycin 57. (Top) Representative fluorescence microscopy images of one out of three independent experiments are shown. Dashed lines indicate the contour of individual cells. Scale bar: 2 µm. (Bottom) The percentage of cargo-associated Atg9 clusters was quantified in three independent biological replicates. For each condition and replicate at least 50 BFP-Ape1 condensates associated with a mScarlet-Atg8 focus (representing early phagophore formation) were analyzed. The values of each replicate (circle) and the mean (bars) were plotted. Statistical analysis using two-tailed unpaired *t*-tests. Significance is indicated with asterisks: \*\*\*p < 0.001, \*\*p < 0.01, \*p < 0.05, n.s. (not significant) p > 0.05.

D) GFP-Atg19 cells overexpressing copper-inducible untagged Ape1 were grown to mid-log phase in the presence of 250 µM CuSO4. The contact site between the Ape1 condensate and the vacuolar membrane is captured by correlative *in situ* cryo-electron tomography. (i) The tomographic slice shows an Ape1 complex with a forming phagophore membrane (orange arrow) in close proximity to the vacuole (V); (ii) A different slice (+16.6 nm from (i)) shows a different plane of the phagophore membrane. (iii) Segmentation and 3D rendering of the tomographic volume. The phagophore is highlighted in orange and the vacuole membrane in grey. Scale bar: 200 nm (i), 50 nm (ii-iii).

E) Indicated strains expressing endogenous GFP-Ape1 and copper-inducible untagged Ape1, Atg19-GFP-µNS, or pp-GFP-µNS were grown to mid-log phase in the presence of 50 µM CuSO4. GFP cleavage was monitored by anti-GFP western blotting. pp = Ape11-45

F) Cells as in E) co-expressing mScarlet-Atg11 were monitored by fluorescence microscopy. Representative fluorescence microscopy images of one out of three independent experiments are shown. Dashed lines indicate the contour of individual cells. pp = Ape11-45. Scale bar: 2 µm. The degree of Atg11 clustering represented as the coefficient of variance (SD/mean mScarlet intensity) was analyzed in three independent biological replicates. For each condition and replicate at least 50 structures were analyzed. The median degree of clustering (horizontal line) and the mean values of each biological replicate (circles) are indicated. Boxes represent the 25th to 75th percentiles, and whiskers expand to the 5th and 95th percentiles. Outliers are shown as black dots. Statistical analysis was performed using one-way ANOVA followed by a Dunnett post-hoc test. Significance is indicated with asterisks: \*\*\*p < 0.001, \*\*p < 0.01, \*p < 0.05, n.s. (not significant) p > 0.05. a.u. = arbitrary units.



**Figure 5: Initiation hubs for selective autophagy are conserved in human cells**

A) U2OS cells transfected with FKBP-GFP-ULK1 and mCherry-Parkin were stained with MitoTracker DeepRed. Mitophagy was induced by antimycin A and oligomycin (AO) treatment. Representative fluorescence microscopy images of one out of four independent experiments are shown. Dashed lines indicate the contour of individual cells. Scale bar: 10 µm, scale bar inset: 1 µm. Quantification is shown in Figure S5A.

B) Schematic of the synthetic tethering setup in U2OS cells. FRB is targeted to the outer mitochondrial membrane by its fusion with the tail anchor domain of the mitochondrial membrane protein FIS1 (FRB-FIS193-152, residues 93-152 of FIS1). Expression of an FKBP-GFP-ULK1 fusion construct allows its inducible tethering to mitochondrial FRB-FIS193-152 by rapalog addition, resulting in mitophagy induction.

C) U2OS cells containing FRB-FIS193-152 and transfected with FKBP-GFP-ULK1 or FKBP-GFP were cultured in nutrient-rich medium and treated with rapalog. Mitochondria were stained with MitoTracker DeepRed. Representative fluorescence microscopy images of one out of three independent experiments are shown. Scale bar: 10 µm, scale bar inset: 1 µm.

D) Mass spectrometry analysis of APEX2-based proximity labeling in HEK293 cells stably expressing FKBP-APEX2-ULK1 or FKBP-APEX2. Cells were grown under nutrient-rich conditions and rapalog. Proximity labeling was induced by the addition of biotin phenol and a short pulse of H2O2. The volcano plot shows the enrichment of biotinylated proteins in FKBP-APEX2-ULK1 compared to FKBP-APEX2. Dashed red lines indicate the cut-offs used to identify ULK1-specific proteins (log2 ratio > 1 and adjusted p-value < 0.01). Autophagy hallmark proteins enriched in FKBP-APEX2-ULK1 are highlighted in cyan.

E) Heatmap displaying ULK1 specific ER membrane proteins with a significantly reduced signal upon knockdown of FIP200 (adjusted p-value < 0.05). The enrichment of protein signal in FKBP-APEX2-ULK1 over FKBP-APEX2 is shown for three individual replicates of wild type and FIP200 knockdown cells. Proteins with an adjusted p-value < 0.05 are marked with \* and < 0.01 with \*\*. P-values were adjusted for multiple testing using the Benjamini-Hochberg procedure.

F) A universal model of selective autophagy: a) Rapid rearrangements of cargo receptors on the cargo surface support the recruitment and phase separation of Atg11. For membrane-delimited cargo, rearrangements are supported by lateral diffusion of receptor proteins within the membrane itself. For membrane-less cargo, such mobility on cargo can either be achieved by the phase-separation of the cargo itself, or by low-affinity interactions between the cargo and receptors, b) Atg11 phase separations form initiation hubs by stabilizing low-affinity interactions with the autophagy machinery through high avidity. These interactions include autophagy machinery proteins such as Atg9 and the Atg1 complex, as well as Vac8, to establish vacuolar contacts.c) Rearrangements on the cargo further allow the coalescence of multiple initiation hubs to establish the PAS, to which further autophagy factors such as the PI3KC1 are recruited. d) From the matured PAS, ultimately, phagophore formation is initiated.