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Tracking the transition from an ATG9A vesicle to an autophagosome

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ABSTRACT

Live-cell imaging is a well-established tool to gain insights into the dynamics of autophagosome formation. We have recently generated a panel of human cancer cell lines that express HaloTagged autophagy-related (ATG) proteins from their endogenous loci, providing a highly sensitive tool to study autophagosome biogenesis. Using these cell lines in combination with automated autophagic foci tracking we dissected the molecular mechanisms of phagophore initiation and expansion. Our work supports a model in which phosphorylations by the Unc-51-Like activating Kinases (ULK1/2) and phosphoinositide 3-kinases (PI3Ks) complexes trigger the recruitment of autophagy factors to mobile ATG9A-positive vesicles transforming them into phagophores. Only a small fraction, approximately 10–20%, of these phagophores expand into autophagosomes, suggesting that autophagosome biogenesis is inefficient. Finally, we demonstrate that ATG2A recruitment commits mobile phagophores to autophagosome formation via tethering to donor membranes and promoting the conjugation of human ATG8 homologues.

Abbreviations: ATG: autophagy-related proteins; ULK1/2: Unc-51-Like activating Kinases; PI3Ks: Phosphoinositide 3-Kinases; ATG2A: autophagy-related protein 2A; ATG5: autophagy-related protein 5; ATG16: autophagy-related protein 16; ATG8: autophagy-related protein 8; U2OS: human bone osteosarcoma epithelial cell; LC3B: microtubule-associated protein 1A/1B Light Chain 3B; GABARAPL1: GABA type A Receptor-Associated Protein Like 1; ATG9A: autophagy-related protein 9A; ATG13: autophagy-related protein 13; SQSTM1: Sequestosome-1/p62; WIPI2: WD repeat domain, Phosphoinositide Interacting 2; PI3P: Phosphoinositide-3-phosphate.

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The phagophore, the cellular precursor structure of autophagosomes, is a membranous cisterna that expands into an autophagosome that targets its content for degradation. The critical steps in autophagosome biogenesis are phagophore nucleation, which is triggered by the ULK and PI3K kinase complexes, phagophore expansion by ATG2-mediated lipid transfer, ATG8-like protein conjugation catalyzed by the ATG12–ATG5-ATG16L1 complex, cargo recruitment, autophagosome closure and finally fusion of the autophagosome with lysosomes. While the identity and biochemical activities of many of the proteins involved in autophagosome biogensis are well defined, the molecular nature of the phagophore remains uncertain. A model that has gained traction in recent years is that ATG9A-containing vesicles are the seed for autophagosomes, but evidence supporting this model in living mammalian cells was lacking. To characterize the molecular mechanisms of autophagosome biogenesis in human cells, we established and validated a panel of U2OS cancer cell lines that express HaloTagged ATG proteins from their endogenous loci using genome editing. The HaloTag can be covalently modified with bright, photostable and cell-permeable Janelia Fluor fluororescent dyes, providing a highly sensitive tool to visually monitor the tagged ATG proteins in living cells. Using these cell lines, we performed live-cell imaging, capturing the behavior of the tagged ATG proteins at the single-molecule level (examining one protein at the time) and analyzed their recruitment dynamics to the phagophores, which are detectable as autophagy-induced puncta. This approach allowed us to systematically characterize the fast diffusion dynamics of ATG proteins with high temporal and spatial resolution and the biogenesis of autophagosomes, from the phagophore-recruitment of the tagged ATG proteins to the accumulation of ATG8-like proteins, i.e., LC3B and GABARAPL1, or the cargo selective autophagy receptor SQSTM1 (Sequestosome-1/p62). With the exception of ATG9A, all proteins analyzed formed puncta, which subsequently co-localized with LC3B signals, suggesting that a minimal number of ATG9A molecules (less than detectable using our sensitive microscopy approach) are necessary for autophagosome formation. To demonstrate that ATG9A was required for autophagosome formation in our cell lines, we confirmed that knockout of *ATG9A* resulted in the complete disappearance of autophagy induced ATG2A puncta. These findings provide strong evidence for the critical role of ATG9A in phagophore formation and are consistent with the model that a vesicle containing a small number of ATG9A molecules forms the seed for autophagosome formation [1].

To investigate autophagy factor recruitment to the phagophore in an unbiased way, we carried out automated tracking of ATG2A, ATG5, ATG13, ATG16, WD-repeat domain phosphoinositide interacting 2 (WIPI2), and ULK1 puncta formed upon amino acids starvation. This approach allowed us to analyze the puncta lifetime, their movement patterns, and co-localization

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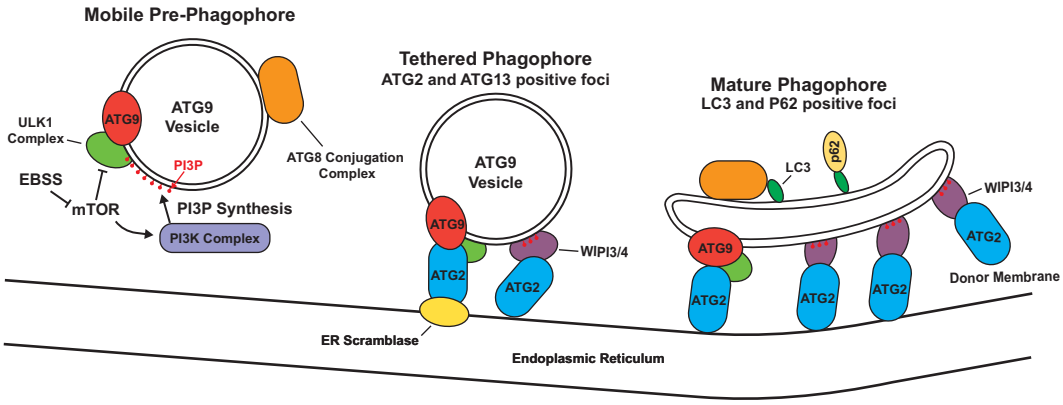

Figure 1. Model depicting the formation and maturation of the phagophore. ATG9A vesicles are activated through ULK1 signaling and PI3P synthesis, which promote the recruitment of the ATG machinery, forming mobile pre-phagophores. A possible increase in the PI3P synthesis then recruits ATG2A, allowing the tethering of the pre-phagophore to the ER membrane and committing it for expansion and closure into an autophagosome.

with LC3B, GABARAPL1 or SQSTM1. Analysis of the movement patterns of autophagic puncta revealed two distinct classes of phagophores that differed in their mobility for all examined proteins except ATG2A. We hypothesized that the fast moving population represented the cytosolic ATG9A vesicles that have initiated ATG protein recruitment. Consistent with this hypothesis, the diffusion coefficient observed for this population was comparable to a sub-population of ATG9A molecules observed in our single-molecule imaging experiments. The diffusion coefficient if the slow-moving puncta matched the single population of ATG2A foci, indicating that they represent ATG9A vesicles tethered to a lipid donor membrane by ATG2A. To validate this observation, we performed dual-color imaging of ATG2A and ATG13. The results demonstrated that ATG13 puncta that co-localized with ATG2A exhibited slower diffusion than ATG13 foci negative for ATG2A. These findings provide compelling evidence for the initial accumulation of ATG proteins on mobile ATG9A containing vesicles, which are immobilized by ATG2A mediated tethering to a lipid donor membrane, probably facilitating phagophore expansion (Figure 1).

In our imaging experiments. we observed that only a small fraction of ATG protein-positive puncta proceeded to co-localize with LC3B, GABARAPL1 or SQSTM1, approximately 10–20%, indicating frequent phagophore expansion failure. Consistent with this interpretation, puncta that failed to recruit LC3B were very short lived, approximately 25 s, while phagophores that matured to the point of LC3B accumulation were longer lasting, approximately 100 s. Together these experiments defined the timing of phagophore expansion and highlighted the frequency of autophagosome biogenesis as a potentially important regulatory step in overall autophagic flux.

While our findings support the notion of ATG9A-positive vesicles serve as the foundation for autophagosome biogenesis, they also raise intriguing questions about the fate of the majority of phagophores that never mature into autophagosomes. Defining the molecular mechanisms and signals that govern the decision between phagophore progression/expansion or disassembly will be a crucial focus of future investigations. It is essential to determine whether this inefficiency extends beyond cancer cells, potentially to neurons, and whether aging affects the efficiency of autophagosome biogenesis. Finally, we hope that our panel of U2OS cell lines expressing HaloTagged ATG proteins from their endogenous loci will be a valuable tool for the autophagy community and we look forward to sharing these cells with all research labs that would like to use them in their studies.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Reference

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