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SPOTLIGHT

# Toward a standard model for autophagosome biogenesis

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**Two papers in this issue resolve a long-standing obstacle to a "standard model" for autophagosome biogenesis in mammals. The first, Olivas et al. (2023. *J. Cell Biol.* https://doi.org/10.1083/jcb.202208088), uses biochemistry to confirm that the lipid scramblase ATG9A is a bona fide autophagosome component, while the second, Broadbent et al. (2023. *J. Cell Biol.* https://doi.org/10.1083/jcb.202210078), uses particle tracking to show that the dynamics of autophagy proteins are consistent with the concept.**

Autophagy is responsible for maintaining cellular homeostasis under starvation, organelle damage, protein aggregation, intracellular infection, and other stressors. Autophagy proceeds by the de novo formation of a double membrane structure known as the phagophore, which engulfs its cytoplasmic contents, is sealed to become the autophagosome, and delivers its cargo to lysosomes for degradation. Groundbreaking genetics work identified a host of yeast autophagy-related (ATG) proteins that are critical for this process (1). The autophagy machinery is mostly conserved across eukaryotes, so it seems reasonable that the mechanism of autophagy itself should also be mostly conserved. In the past few years, the once-mysterious process of autophagosome initiation and growth in yeast and mammals has become much clearer (2, 3). Yet a few obstacles to a unified and conserved "standard model" of autophagosome biogenesis persist.

Two fundamental questions in autophagosome biogenesis concern the nature of the seeds from which autophagosomes are initiated, and the mechanism of membrane growth. In yeast, it has become clear that small vesicles containing the transmembrane protein Atg9 are the seeds of autophagosomes (4). Atg9 vesicles are competent to recruit other autophagy core complexes (4). These vesicles support the production of the key lipid PI(3)P and are covalently modified by the ubiquitin-like Atg8 protein (4).

Yeast Atg9 vesicles contain only a fraction of the phospholipid needed to build an autophagosome. For many years, the source of membrane supply and the mechanism of growth of the autophagosome was a black box. It is now clear that ATG2A (Atg2 in yeast) can serve as a high flux phospholipid channel for rapid lipid transfer from the ER (5–7). ATG2A transfers lipids into the proximal leaflet of the autophagosomal membrane, and thus a means for equilibrating phospholipids into the distal leaflet is needed. In 2020, the structures of yeast Atg9 and its mammalian ortholog ATG9A were determined (8–10), and these proteins were shown to serve as lipid scramblases capable of equilibrating lipids inbound from Atg2/ATG2 (8, 9). These studies established a model for yeast autophagy, in which Atg2 supplied phospholipid building blocks to Atg9 vesicles, where the resident Atg9 protein then equilibrated the lipids into the opposing leaflet. Since both Atg2 and Atg9 have orthologs in mammals, it was attractive to imagine a unified "standard model" of the process that could drive autophagosome formation in mammals, and indeed, all eukaryotes.

A critical prediction of this standard model is that ATG9A must reside within autophagosomal membranes in mammalian cells. Phospholipid equilibration is only possible when ATG9A spans the same membrane that it is responsible for equilibrating. This is not a process that can occur in trans, yet compelling live cell imaging studies of human cell lines had found that ATG9 formed transient puncta early in autophagy, which disappeared as autophagosomes matured (11). In this month's *JCB* issue, Olivas et al. (12) and Broadbent et al. (13) address the apparent discrepancy between the long-standing mammalian live cell imaging data on the one hand, and the yeast data and the attractive new conceptual model on the other. These groups use two orthogonal experimental approaches, the former by breaking down autophagic membranes into their constituents and scrutinizing the parts biochemically, and the latter using endogenously Halo-tagged ATG proteins to image autophagy with improved sensitivity. Strikingly, both

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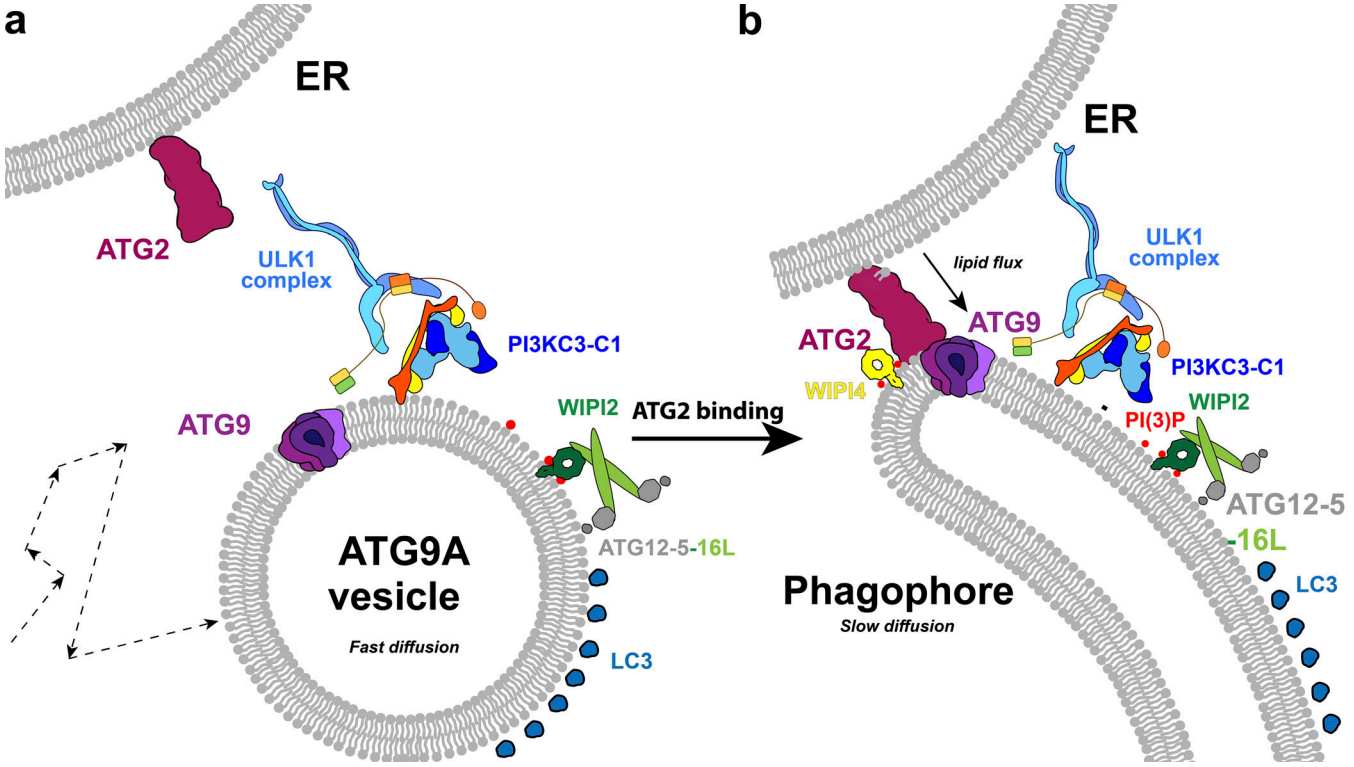


Figure 1. **ATG2 tethering slows diffusion of the ATG9A vesicle seeds of autophagosomes. (a)** Schematic of an untethered ATG9 vesicle prior to ATG2 recruitment. The dashed lines indicate the higher diffusivity random walk that ATG9 vesicles and other autophagy proteins undergo prior to ATG2 recruitment. **(b)** A phagophore following ATG2 recruitment. The stabilization of ATG9 vesicles at ER contact sites causes a dramatic reduction of diffusivity and allows for productive ATG2-mediated phagophore expansion.

approaches converge, showing that ATG9A vesicles can seed autophagosome biogenesis in mammals, too.

A crucial prediction of the ATG9A vesicle seed model is that in the absence of ATG2, autophagosomes cannot form. Absent a lipid transporter, the lack of new membrane material from the ER means that the wholesale generation of a rapidly expanding lipid sheet is impossible. Olivas et al. (12) used an ATG2A/B double knockout (DKO) cell line expressing the autophagy marker GFP-LC3B and imaged sites of ATG9A vesicle accumulation by correlative light and FIB-SEM microscopy. The ATG2 DKO cells manifested extensive perinuclear aggregation of ATG9A and LC3 positive vesicles directly adjacent to the ER. This compartment contained numerous other autophagy factors, suggesting that they represent frustrated autophagosomes that fail to grow; yet, they still support the other biochemical reactions of autophagy, including PI(3)P synthesis and LC3 lipidation. These experiments showed that mammalian ATG9A-containing vesicles behave as expected for autophagosomal seeds, but they did not prove that ATG9A is actually incorporated into autophagosomes.

To determine whether ATG9A is physically present in completed autophagosomes, the authors turned to centrifugal membrane fractionation with a modern flavor. The authors separated cells to isolate an autophagosomal fraction that they treated with styrene-maleic acid (SMA) polymers to further fractionate the transmembrane protein content of this ATG9A cluster and autophagosomal fraction. SMA polymers are, in essence, amphipathic cookie cutters that punch holes in membranes, creating nanodiscs that contain transmembrane proteins in native lipids. By incubating both the ATG9A vesicle and autophagosomal fractions with SMA, then pulling down tagged ATG9A, the authors found that both frustrated and completed autophagosomal membranes contained ATG9A and the autophagosomal marker LC3B (Fig. 1 a). Controls were carried out to rule out the possibility that ATG9A and LC3B in different membranes were interacting with one another in trans. This innovative membrane biochemistry provides the smoking gun to show that ATG9A is in fact physically present in mammalian autophagosomes, albeit in trace amounts.

The reductionist biochemistry of Olivas et al. yields a strong affirmative answer to the central prediction of the ATG9A vesicle seeding model in mammals; the obvious gap in the study is its lack of live cell imaging. Given our new understanding that ATG9A is only present in trace amounts, a clear next step is to attempt to image endogenously tagged ATG9A using the brightest possible dyes and the most sensitive microscopy available. In a fortuitously but aptly timed study, uncoordinated with Olivas et al., Broadbent et al. generated a series of CRISPR-edited cell lines expressing HALO-tagged ATG proteins from endogenous loci. The sensitivity of this system offers two advantages compared with past efforts on this front. First, low-copy number proteins, like many in the autophagy pathway, can be accurately quantified and visualized. Second, image acquisition time, a strong function of the radiant flux of the fluorophore, is dramatically shorter with HALO ligands than genetically encoded fluorescent proteins,

opening the door to higher temporal resolution imaging, which is essential for imaging transient events.

The key experiment in the Broadbent et al. (13) study was the measurement of the diffusion of labeled ATG proteins under autophagy-inducing conditions. Consistent with the concept that ATG2A is both the phospholipid conduit and the tether to the ER, ATG2A behaved differently than most of the other factors analyzed and was not present at all autophagy initiation sites. Other soluble ATG proteins exhibited two types of puncta with distinct fast and slow diffusion coefficients, while ATG2A punctae had only slow diffusion. The fast diffusion mode is identified with soluble or ATG9A vesicle associated material, and the slow state with growing autophagosomes immobilized by tethering to the ER (Fig. 1 b). The authors then turned their attention to the role of ATG9A in autophagy. ATG9A knockout abolished the formation of the ATG2 punctae. In one limitation of the study, ATG9A seems to be present in such low numbers that it was still undetectable at autophagy initiation sites even with the enhanced sensitivity of the HALO tagging. It seems that beyond the first tiny seed, additional recruitment of ATG9A to the initiation site does not occur—in other words, the ATG9A you start with is all the ATG9A you get to grow an entire autophagosome.

A challenge presented by the new data is that they show a mere handful of ATG9A proteins reside in the autophagosome. This limited number of ATG9A molecules must scramble phospholipids at a high rate to keep up with ATG2. ATG2 itself must also transfer phospholipids at a high rate to keep up with the demands of autophagosome growth, which Broadbent et al. (13) report occurs even faster than previously believed. Theoretical considerations suggest this mechanism is feasible (14). A remaining task for the field is to confirm experimentally that transfer and scrambling do in fact occur at the needed rates.

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