When separation causes activation – biophysical control of bulk autophagy initiation

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Fujioka *et al.* uncovered liquid-liquid phase separation of the PAS, a key driver of autophagosome formation in yeast. Moreover, the authors demonstrated that liquid-like PAS controls autophagic kinase activation and is itself regulated by the phosphorylation status of its constituents.

Bulk autophagy is a homeostatic process which enables eukaryotic cells to engulf intracellular components in autophagosomes whose content is degraded upon fusion with lysosomes or vacuoles. In yeast, autophagosomes are generated at the pre-autophagosomal structure also known as the phagophore assembly site (PAS). Upon nitrogen starvation, the PAS is initiated by assembly of autophagy-related protein 1 (Atg1), Atg13, Atg17, Atg29 and Atg31 and activation of the Atg1 kinase domain. This leads to recruitment of other Atg proteins and formation of the autophagosome precursor termed phagophore. Besides this mechanistic understanding, the biophysical state of the PAS remained elusive.

Using fluorescence recovery after photobleaching and fluorescence correlation microscopy of starved or rapamycin-treated yeast cells expressing GFP-tagged Atg1 complex components, Fujioka *et al.* recently uncovered that the forming PAS exerts properties of liquid-like droplets, membrane-less organelles which form by liquid-liquid phase separation (LLPS), which can compartmentalize a variety of cellular processes (Snead and Gladfelter, 2019). Fujioka *et al.* furthermore demonstrated that purified Atg1, Atg13 and Atg17-Atg29-Atg31 jointly undergo LLPS upon mixing and form spherical droplets which increased in size upon coalescence and fell apart upon treatment with the LLPS inhibitor 1,6-hexanediol. Atomic force microscopy revealed that the PAS can vary from liquid-like to solid states depending on environmental parameters. LLPS of PAS components in vitro was found to be Atg1 independent, require specific interaction between Atg17 and Atg13 and to be negatively regulated by mTORC1-mediated hyper-phosphorylation of Atg13. Intriguingly, Fujioka *et al.* revealed a regulatory loop by which liquid-like PAS formation triggers Atg1 activation which in turn leads to the dissolution of the droplet unless the autophagy-promoting phosphatases Ptc2 and Ptc3 de-phosphorylate Atg1 and its substrate Atg13 (Figure 1). Lastly, Fujioka *et al.* showed that the liquid-like Atg1 complex can be tethered by Vac8 to the vacuole upon starvation in cells and to giant unilamellar vesicles upon mixing in vitro. The latter process was shown to require the Atg13-Vac8 interaction but to be Atg1-independent.

Phase separation is recognized to drive engulfment of a growing list of autophagic cargo in mammalian cells (Wang and Zhang, 2019) and in yeast (Yamasaki *et al.*, 2020). However, this process seemed to be restricted to selective autophagy pathways since bulk autophagosomes form in the absence of any specific cargo. The findings of Fujioka *et al.* changed this view by establishing a role of LLPS in the regulation of bulk autophagy. This opens exciting perspectives on bulk autophagy initiation and prompts several questions:

How does a liquid-like PAS promote phagophore formation? LLPS of PAS components seems to trigger Atg1 activation by increasing its local concentration. Yet, Atg1 autophosphorylation can also occur by targeting the kinase to the vacuole (Torggler *et al.*, 2014) where trans-activation of neighboring kinase domains happens as in many cellular signal transduction pathways. Hence, the liquid-like PAS may exert its unique features beyond Atg1 activation. Since synaptic vesicles are captured by phase-separated synapsin (Milovanovic *et al.*, 2018), we speculate that Atg9 vesicles are uniquely organized by a liquid-like Atg1 complex to prevent their dispersal away from the PAS. A similar hypothesis has been put forward for COPII transport carriers and their regulator TFG in mammalian cells (Hanna *et al.*, 2017). Here, phase separation of TFG is assumed to cluster transport carriers leading to their local retention at the ER/ERGIC interface.

How does the liquid-like PAS interact with endomembranes? Increasing evidence links cellular membranes to regulation of biomolecular condensates (Snead and Gladfelter, 2019). This stems from the fact that membrane surfaces limit the diffusion of reactants which undergo LLPS at a lower concentration threshold. Consistently, liquid-like PAS formation is expected to accelerate upon its Vac8-mediated vacuole tethering. However, the role of Vac8 in PAS LLPS has not been assessed in cells. Furthermore, the recruitment of Atg9 vesicles would likely increase the membrane surfaces surrounding the PAS. Atg9 vesicles might additionally help to maintain the liquidity of the PAS. Following Atg9 vesicle recruitment, the Atg2-Atg18 complex links the PAS to ER membranes (Kotani *et al.*, 2018). To what extent the latter influence the material state of the PAS remains elusive. Lastly, the phagophore is yet another membrane which potentially affects LLPS parameters at the PAS. The fact that these PAS sandwiching membranes differ in their lipid and protein composition may fine tune PAS condensate formation and maintenance upon bulk autophagy induction in addition to phosphorylation and dephosphorylation cycles.

How are hierarchical downstream PAS factors incorporated in the liquid-like PAS? Besides vesicle-embedded Atg9, the Atg5~Atg12-Atg16 complex is also recruited to the PAS. This step is mediated through direct binding between Atg12 and Atg17 and requires a fully assembled Atg1 complex (Harada *et al.*, 2019). Given that the Atg1 complex components retain high mobility within and outside of the liquid-like PAS, the latter might bind Atg5~Atg12-Atg16 in the cytosol and drag it into the PAS. Binding of these complexes might extend the interaction meshwork within the PAS since the dimeric Atg16 can link two high-order assemblies. Hence, recruitment of Atg5~Atg12-Atg16 into the PAS might contribute to maintenance of LLPS at the PAS. Knowing the stoichiometries of the components within the PAS condensate would certainly shed light on these issues.

Which other cellular factors are part of the liquid-like PAS? The important roles of Ptc2 and Ptc3 in sustaining the liquid-like PAS make these two phosphatases prime candidates for additional PAS factors. Intriguingly, Ptc2 and Ptc3 both contain intrinsically disordered regions (IDRs) and interact with the Atg1 complex in immunoprecipitation experiments which are unlikely to capture transient enzyme-substrate relationships (Memisoglu et al., 2019). However, incorporation of these enzymes into the PAS together with Atg1 raises the question of how their activities are controlled in this liquid-like environment - particularly since the presence of the Atg5~Atg12-Atg16 complex in the PAS might further enhance the crowding and provide additional phosphosites for Atg1 and Ptc2/Ptc3. Hence, it is puzzling how

phosphorylation of specific Atg13 residues are actually detectable when phosphorylation and dephosphorylation reactions are seemingly in an equilibrium.

Does the mammalian counterpart of the Atg1 complex also phase separate? Intriguingly, except for ATG101, all ULK1 complex components and some of their regulators, such as SMCR8, contain IDRs. Since these are major determinants of LLPS, it will be important to examine the native ULK1 complex when exploring its material state. In contrast to yeast, the use of 1,6-hexandiol sensitivity as a readout for LLPS in mammalian cells proves to be highly problematic (Wheeler *et al*., 2016). Therefore, better tools are required for studying ULK1-related phase separation events *in vivo*.

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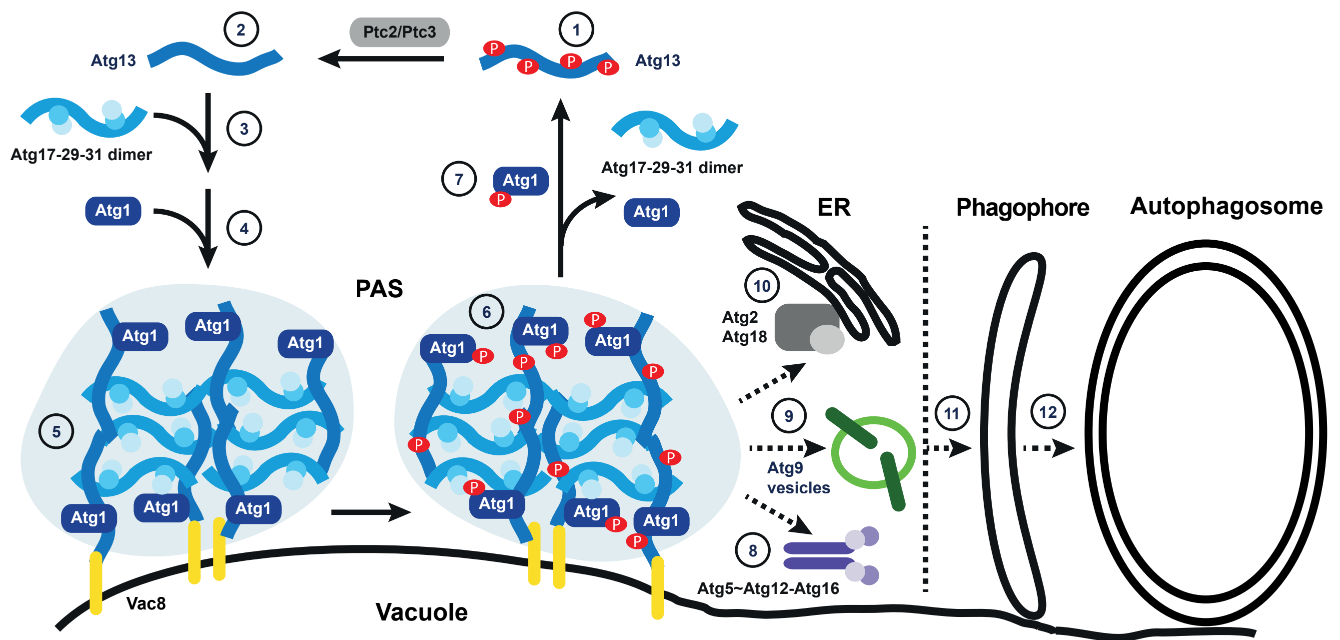
FIGURE LEGEND

Regulation of liquid-like PAS formation. In nutrient-rich conditions, TORC1 is active and phosphorylates Atg13 (1). Upon starvation, TORC1 is inhibited and PP2C phosphatases dephosphorylate Atg13 (2). Dephosphorylated Atg13 binds to Atg17-29-31 (3). Binding of Atg1 to Atg13 completes formation of the Atg1 complex (4). Higher-order assembly of Atg1 complexes leads to LLPS and a liquid-like PAS (5). Atg1 is activated by auto-phosphorylation (6). Activated Atg1 phosphorylates Atg13, which disrupts the Atg1 assembly (7). Downstream events whose relations to the liquid-like PAS remain to be determined: Recruitment of Atg5~Atg12-Atg16 (8), Atg9 vesicles (9) ER-tethered Atg2-Atg18 (10) as well as phagophore nucleation (11) and autophagosome closure (12).



Ptc2/Ptc3
Atg13
Atg13
Atg17-29-31 dimer
Atg1
Atg17-29-31 dimer
Atg1
Atg1
ER
Phagophore
Autophagosome
PAS
Atg2
Atg18
Atg9
vesicles
Atg5~Atg12-Atg16
Vac8
Vacuole