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Live-cell imaging for the assessment of the dynamics of autophagosome formation: Focus on early steps

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ABSTRACT

Autophagy is a cytosolic degradative pathway, which through a series of complicated membrane rearrangements leads to the formation of a unique double membrane vesicle, the autophagosome. The use of fluorescent proteins has allowed visualizing the autophagosome formation in live cells and in real time, almost 40 years after electron microscopy studies observed these structures for the first time. In the last decade, live-cell imaging has been extensively used to study the dynamics of autophagosome formation in cultured mammalian cells. Hereby we will discuss how the live-cell imaging studies have tried to settle the debate about the origin of the autophagosome membrane and how they have described the way different autophagy proteins coordinate in space and time in order to drive autophagosome formation.

1. Introduction

Macroautophagy (autophagy hereafter) is a membrane trafficking pathway that delivers cytosolic material for degradation to lysosomes via a specialized membrane compartment, the autophagosome. Cells activate autophagy under diverse stress conditions: either metabolic stress, such as scarcity of amino acids, low energy and absence of growth factors, or environmental stress, for instance changes in pH or temperature, hypoxia, and oxidative stress [1,2]. The type of autophagic cargo is dictated by the autophagic stimulus. For instance, amino acid starvation, which is the most common physiologic stimulus of autophagy, leads to the degradation of bulk cytosol. Autophagy is also activated to clear a variety of cellular waste, e.g. toxic protein aggregates and damaged organelles, which in turn protects tissues against degenerative diseases, like cancer and neurodegeneration [3,4]. Finally, autophagy is activated to defend cells against intracellular pathogens, like viruses and bacteria [5–8]. Degradation of the autophagic cargo in lysosomes produces anabolic building blocks that are recycled to the cytosol, therefore autophagy is fundamental for cell and organism regeneration [9].

The functional unit of autophagy is the autophagosome, a double membrane vesicle enclosing the cytosolic cargo. Genetic and biochemical studies have generated a hierarchical model of autophagosome formation that is widely accepted in the field [9–11]. The stages of autophagosome formation and the modules involved are the following [12,13]: (1) *Initiation stage*. Inactivation of the kinase mTOR (mechanistic – or mammalian – target of rapamycin) activates the complex containing the protein kinase ULK1 (UNC51-like kinase) [14], which translocates to the ER and activates the lipid kinase VPS34 (Vacuolar Protein Sorting) complex [15]. (2) *Nucleation stage*. The activated VPS34 complex mediates the localized synthesis of phosphatidylinositol-3 phosphate (PI3P) on an as yet unknown domain of the ER, nucleating a new omegasome [16]. The omegasome is a membrane platform connected to the ER, rich in PI3P and PI3P-binding proteins, like DFCP1 and the WIPI family. (3) *Elongation stage*. The omegasome provides a platform for the assembly of the machinery that catalyzes the covalent attachment of the ubiquitin-like protein LC3 to phosphatidylethanolamine. The lipidation of LC3 drives the expansion of the autophagosomal membrane (isolation membrane, IM) and provides a docking site for adaptor proteins, like p62, which drag the cytosolic cargo into the forming autophagosome. (4) *Fusion stage*. Finally, the edges of the flat IM fuse, creating a double membrane autophagosome that will subsequently fuse with a lysosome for the degradation of its cargo.

Microscopy is arguably the technique most commonly used in the study of autophagy [17]. Fluorescence microscopy of fixed cells has been routinely used to study the localization of endogenous or exogenous autophagy proteins, while electron microscopy (EM)

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has further improved the resolution [18]. Nevertheless, autophagosome formation is a highly dynamic process completed within a few minutes and none of these techniques can follow the intermediate stages over time. In contrast, live-cell imaging (LCI) overcomes this limitation as it allows to visualize autophagosome formation in real time [19]. Moreover, LCI monitors the autophagy proteins in their natural environment, without needing to fix and permeabilize the cells and risk creating artefacts. In this review we will not provide a protocol for LCI of autophagy; this was recently done in great detail in two publications [20,21]. We will instead focus on how LCI has been used to assess the dynamics of autophagosome formation in cultured mammalian cells. Owing to their size and spatial arrangement of their organelles such cells are the preferred model for these types of studies. They include the Human Embryonic Kidney 293 (HEK293) cells, the Mouse Embryonic Fibroblasts (MEFs), the HeLa cells, the COS7 cells and the Normal Rat Kidney (NRK) epithelial cells, to name a few of the most commonly used cell lines. Nevertheless, the recent development of new tools has made possible conducting sophisticated imaging studies in yeast [22,23]. More specifically, we will discuss how the field of autophagy has deployed LCI trying to answer two main questions: first, what is the origin of the autophagosome membrane, and second, what is the dynamic interplay between the proteins involved in autophagosome formation. We will also discuss the inherent limitations of LCI and the future developments that are required to achieve further progress.

2. Visualizing autophagosome formation by LCI

The use of genetically encoded fluorescent proteins revolutionized the field of autophagy by allowing to visualize autophagosome formation in live cells and in real time, almost 40 years after these structures were observed by EM for the first time [24]. At first, GFP-tagged LC3 was used in fixed cells to show that the mammalian homolog of Atg8 localizes on the autophagosome membrane, which is distinct from the lysosome membrane [25]. This was a major conceptual breakthrough that made this publication one of the most highly cited publications in the field of autophagy. At the same time, it introduced the use of fluorescent proteins in the field of autophagy, paving the way for the first LCI study in mammalian cells. LCI of autophagy was first reported one year later by Yoshimori and colleagues through the use of GFP-ATG5 [26]. The authors used the component of the conjugation machinery ATG5 as proxy to describe the dynamics of the IM during autophagosome formation in real time. They showed that, in contrast to the single phagophore-assembly site (PAS) in yeast, ATG5 appears and accumulates at multiple dot-like structures at random sites in the cytosol. The ATG5-decorated compartments progressively elongate and bend into cup-like structures, while ATG5 is eventually removed before their closure into vesicular autophagosomes. Therefore, the behaviour of the IM as visualized by LCI of GFP-ATG5 corroborated the patterns of autophagosome formation previously inferred by the EM studies.

3. Multi-colour LCI in the search of the origin of the autophagosome membrane

In the last 5 years, LCI has been the workhorse in the search for the origin of the autophagosome membrane in mammalian cells, pointing at three main potential sources, the ER, the mitochondria and the plasma membrane.

The timeline of studies using LCI to pinpoint the origin of the autophagosome membrane started in our lab, with the unexpected finding that the synthesis of the autophagy-related pool of PI3P occurs on the ER [16]. Using three different types of microscopy [wide field, confocal spinning disk and total internal reflection fluorescence (TIRF)], both the early (DFCP1) and the late (LC3) autophagy marker were visualized to stably associate with the ER throughout autophagosome formation. This relationship implied a physical connection between the two membranes, which was later confirmed by EM [27], and suggested that the ER might be the origin of the autophagosome membrane. The tight association between starvation-induced autophagosomes and ER was confirmed in an independent study conducted in MEFs [28]. The authors deployed dual colour LCI of a different autophagy marker (ULK1) to show that autophagosomes emerge and evolve in close proximity to the strands of the ER. The conclusions of the early qualitative studies were further supported by studies deploying more quantitative approaches that measured the distance between forming autophagosomes and ER in different cell lines. At first, LCI of ATG5 was deployed to show that starvation-induced autophagosomes in COS7 cells always emerge in tight association with the ER [29]. Our lab took a similar quantitative approach using LCI of ATG13, a component of the ULK1 complex, to show that starvation-induced autophagosomes in HEK293 cells always emerge close to ER [20]. This consensus was also extended to autophagosomes forming during basal autophagy. In HEK293 cells, LC3-positive autophagosomes forming during basal autophagy consistently appear on the ER [30]. Finally, a recent study conducted in primary neurons used LCI of ATG13 to show that autophagosomes forming under basal conditions at the distal tip of the neuron also emerge and evolve in association with the ER [31].

The relationship of forming autophagosomes with mitochondria has also been extensively studied using LCI. In NRK cells, LCI of two autophagy markers (ATG5 and LC3) was used to show that starvation-induced autophagosomes tightly associate with mitochondria [32]. More specifically, forming autophagosomes outgrow from mitochondria and retain their association despite the highly dynamic mitochondrial movements. However, other studies conducted in different cells and taking different approaches have reported a more loose association of early or late autophagic markers with mitochondria. In MEFs, most of the ULK1 puncta that are induced under starvation do not colocalize with the mitochondrial marker TOM70 [28]. In starved HEK293 cells, the distance between an emerging ATG13 particle and the most proximal mitochondrion is comparable with their distance from ER only for approximately half of the ATG13 particles [20]. In the same cells during basal autophagy, emerging LC3-positive autophagosomes also overlap with mitochondria only half of the times [30]. A similar trend is observed for autophagosomes forming under basal conditions at the distal tip of primary neurons: ATG13 particles occasionally colocalize with mitochondria, while more detailed analysis of the overlap between these compartments using kymographs has shown that their association is transient [31]. Yoshimori and colleagues provided a novel explanation for this discrepancy by performing LCI with three cameras in order to simultaneously visualize the IM marker ATG5 with ER and mitochondria [29]. They found that ATG5 particles almost always co-localize with the ER, but their proximity with mitochondria dynamically oscillates. Moreover, using LCI of ATG5 in combination with the ER–mitochondria contact site marker VDAC1, they suggested that autophagosome formation takes place at the mitochondria-associated ER membrane (MAM).

Although different experimental approaches in a number of studies have implicated plasma membrane in autophagosome formation, dual colour LCI of autophagy proteins in combination with a plasma membrane marker was used only in two studies and with different results. In HeLa cells, LCI of ATG16L1 and two plasma membrane markers (CellMask Orange or FM4-64) captured vesicles derived from the plasma membrane fusing with ATG16L1-positive vesicles near the plasma membrane [33]. However, LCI of