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The yeast *Saccharomyces cerevisiae*: An overview of methods to study autophagy progression

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ABSTRACT

Macroautophagy (hereafter autophagy) is a highly evolutionarily conserved process essential for sustaining cellular integrity, homeostasis, and survival. Most eukaryotic cells constitutively undergo autophagy at a low basal level. However, various stimuli, including starvation, organelle deterioration, stress, and pathogen infection, potently upregulate autophagy. The hallmark morphological feature of autophagy is the formation of the double-membrane vesicle known as the autophagosome. In yeast, flux through the pathway culminates in autophagosome–vacuole fusion, and the subsequent degradation of the resulting autophagic bodies and cargo by vacuolar hydrolases, followed by efflux of the breakdown products. Importantly, aberrant autophagy is associated with diverse human pathologies. Thus, there is a need for ongoing work in this area to further understand the cellular factors regulating this process. The field of autophagy research has grown exponentially in recent years, and although numerous model organisms are being used to investigate autophagy, the baker's yeast *Saccharomyces cerevisiae* remains highly relevant, as there are significant and unique benefits to working with this organism. In this review, we will focus on the current methods available to evaluate and monitor autophagy in *S. cerevisiae*, which in several cases have also been subsequently exploited in higher eukaryotes.

1. Introduction

1.1. An introduction to autophagy and its significance

Autophagy (or cellular "self-eating") plays an integral role in various aspects of cell physiology, whereas defects in this process are associated with numerous pathological conditions. Autophagy is ubiquitous in eukaryotes and occurs constitutively at a basal level; however, various stress conditions, including starvation or changing nutrient conditions, organelle deterioration, and pathogen infection, result in its upregulation. Importantly, aberrant autophagy is associated with diverse human pathologies, such as cancer, neurodegeneration, aging, cardiovascular, pulmonary and infectious diseases, macular degeneration, diabetes, and lysosomal storage disorders [1,2]. Thus, there is a need for ongoing work in this area to further understand the cellular factors regulating this inherently complex process.

In brief, the most obvious morphological feature of autophagy is the double-membrane autophagosome that contains either bulk cytoplasm or select cargo, depending on the inducing condition. However, the autophagosome is essentially the end product of the sequestration process (although it will fuse with the vacuole). The dynamic membrane compartment involved in autophagic sequestration is the phagophore, the autophagosome precursor. The mechanism of phagophore formation is unique, and is quite distinct from other vesicle-mediated processes involved in cargo trafficking. For example, in contrast to the secretory pathway where the vesicles bud off from pre-existing organelles already containing their cargo, the phagophore expands sequentially, providing autophagy with an extremely flexible capacity for cargo sequestration. In yeast, autophagosomes typically range from ~300 to 900 nm in diameter [3,4]. Nonetheless, efficient degradation of organelles such as peroxisomes and mitochondria involves their fission by the dynamin-related GTPase Dnm1 complex prior to, or during, engulfment by phagophores [5,6].

Abbreviations: 3-MA, 3-methyladenine; Atg, autophagy related; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; GFP, green fluorescent protein; PAS, phagophore assembly site; PKA, protein kinase A; PMN, piecemeal microautophagy of the nucleus; TEM, transmission electron microscopy; TOR, target of rapamycin; TORC1, TOR complex 1; VMA, vacuolar membrane ATPase.

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Upon completion of expansion, the phagophore seals to form the autophagosome. Flux through the pathway culminates in autophagosome–vacuole fusion, which releases the inner autophagosome single-membrane vesicle into the vacuole lumen. In yeast, the resulting inner autophagosome vesicles, which transiently reside within the vacuole, are known as autophagic bodies. To date, these structures have only been identified in yeast and are subsequently lysed, allowing degradation of the cargo by vacuolar hydrolases. The resulting macromolecules are subsequently transported into the cytosol via membrane permeases.

1.2. Saccharomyces cerevisiae is a fundamental model organism for the study of autophagy

The phenomenon of autophagy was first observed in mammalian cells using electron microscopy (EM) in the 1950s, and was officially termed as such by Christian de Duve in 1963 at the CIBA Foundation Symposium on Lysosomes (reviewed in [7,8]). However, autophagy was not described in yeasts until the 1980s [9]. *S. cerevisiae* and other fungi are fundamental model organisms for the study of autophagy. For example, much of our current understanding of autophagy is due to work conducted in *S. cerevisiae*, *Pichia pastoris*, and *Hansenula polymorpha* using genetic screens and biochemical methods. At present, 38 AuTophaGy-related (*ATG*) genes have thus far been identified in fungi [10]. Importantly, at least half of these genes are clearly conserved up to human, and analogous proteins are present for many others, reflecting a high degree of overall pathway conservation.

The field of autophagy research has grown exponentially in recent years, and although numerous model organisms exist to investigate autophagy, *S. cerevisiae* continues to be an important system for studying this process. In addition to the high degree of conservation, there are significant and unique benefits to working in this organism, including the capability to do *in vivo* genetics work quickly and relatively easily, and the myriad unique assays that are available to monitor different steps of autophagy. This review will focus on an overview of the current methods that can be used to evaluate and monitor the various stages of autophagy in *S. cerevisiae* (see the accompanying article by Guimaraes et al. for additional information and protocols).

1.3. Overview of autophagy in S. cerevisiae

There are two major forms of autophagy in yeast—macroautophagy and microautophagy (reviewed in [11]); this review focuses on macroautophagy. Briefly, microautophagy can be selective or nonselective, and is morphologically distinct from macroautophagy [11–13]. During the process that is strictly defined as microautophagy, tubules invaginate directly from the vacuolar membrane into the lumen [12]. Following scission, these tubules release single-membrane vesicles that may appear similar to autophagic bodies, although the mechanism of formation is completely distinct; microautophagy does not directly involve the Atg proteins, and its physiological function is not fully understood. In contrast to microautophagy, there are also microautophagy-like processes such as micropexophagy (the selective microautophagic degradation of peroxisomes), micromitophagy (which is used to eliminate mitochondria), and piecemeal microautophagy of the nucleus/PMN or micronucleophagy (to remove small portions of the nucleus). These types of sequestration involve direct uptake at the vacuolar membrane, and require the Atg proteins (for further reviews on microautophagy, see [11–14]).

There are four main stages of autophagic activity, enabling progression (otherwise known as flux or autophagic turnover) through the pathway (Fig. 1). As a number of excellent reviews already exist detailing the complex molecular interactions that occur throughout the various stages of autophagy [8,11,13,15–17], and as this review primarily focuses on methods, we will only concisely discuss each phase of autophagic activity before moving on to the most common assays that may be used to assess each.

2. Induction and nucleation of the phagophore

2.1. Background

During the induction and nucleation phase, a physiological stimulus such as nutrient deprivation, or a change in nutrient conditions, results in a shift from basal, constitutive autophagy to induced autophagy (Fig. 2). Stimulation of autophagy can also be achieved through genetic or pharmacological means such as occurs following treatment with rapamycin, which inhibits the activity of TOR (target of rapamycin), a serine/threonine kinase that is a major regulator of cellular metabolism, and a negative regulator of autophagy [18,19]. In yeast, the intracellular location of autophagosome formation is the phagophore assembly site (PAS), which localizes adjacent to the vacuole [15]. The "core" Atg proteins assemble at the PAS to initialize phagophore nucleation [17]. The initial protein complex recruited to the PAS is comprised of Atg1, Atg13 and the Atg17-Atg31-Atg29 ternary subcomplex [13,20,21]. Atg1 is another serine/threonine kinase, and its activity is regulated by Atg13 [17,22–24] and Atg17-Atg31-Atg29 [8,20,25,26]. Following recruitment of the Atg1 complex, Atg9 (and interacting proteins including Atg2 and Atg18) localize to the PAS [8]. Thus far, Atg9 is the only integral membrane protein that is absolutely essential for autophagosome formation, although the exact role of Atg9 has not yet been determined [27]. The Pho23-Rpd3 complex regulates *ATG9* transcription, and the expression level of Atg9 controls the frequency of autophagosome formation [28,29], which fits with a general model whereby Atg9 directs the delivery of membranes to allow formation of the phagophore. Furthermore, various SNARE proteins that control the localization of Atg9 have been implicated in autophagosome biogenesis [30,31].

Although the detailed mechanism is not understood, the PAS may be a nucleation site that is converted into a phagophore. In yeast and mammals, various intracellular compartments have been identified as the probable source(s) of the phagophore membrane, including the ERGIC (ER–Golgi intermediate compartment) [32,33], the ER [34–36], the Golgi apparatus [37], the mitochondrial-associated membrane (MAM) at ER–mitochondria contact sites [38], the mitochondria [39], the plasma membrane [40,41] and recycling endosomes [42]. However, it is possible that the source varies according to the type of autophagy that the cell is undergoing (nonselective versus selective and what form of selective), the organism, and the signaling pathway(s) involved.

2.2. Methods to induce autophagy

There are various methods available to initiate autophagy (Fig. 2 and Table 1). As mentioned above, rapamycin can be used to induce TOR-dependent autophagy [18,43]; however, in higher eukaryotes TOR-independent autophagy pathways have also been identified [44–48]. Furthermore, rapamycin does not appear to induce autophagy as strongly as when nitrogen starvation is used as a stimulus [43]. Culturing cells in nitrogen starvation medium (SD-N; synthetic medium with dextrose, minus nitrogen: 0.17% yeast nitrogen base without ammonium sulfate or amino acids, containing 2% glucose) for 2–4 h also initiates nonselective autophagy.

Alterations in the type of culture medium may be used to induce various forms of selective autophagy. Ribophagy, a selective