PREFACE

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1. INTRODUCTION

Autophagy is an evolutionary ancient biological process by which eukaryotic cells dispose of unwanted cytosolic entities through lysosomal degradation (Kaur & Debnath, 2015). Three distinct forms of autophagy have been characterized so far, differing from each other in the mechanisms through which autophagic substrates are delivered to lysosomes. In the course of *microautophagy*, cytosolic entities destined to disposal are taken up by lysosomes directly, upon invagination of the lysosomal membrane (Li, Li, & Bao, 2012). *Chaperone-mediated autophagy* relies on the recognition of cytosolic proteins bearing a KFERQ motif by members of the heat-shock protein (HSP) family, coupled to the lysosome-associated protein 2 (LAMP2)-dependent translocation of such cargo:chaperone complexes across the lysosomal membrane (Cuervo & Wong, 2014). *Macroautophagy* involves the progressive sequestration of autophagic cargoes by a double-membraned organelle (commonly known as autophagosome) that—upon sealing—becomes able to fuse with lysosomes (Lamb, Yoshimori, & Tooze, 2013). Although the pathophysiological relevance of microautophagy and chaperone-mediated autophagy is being increasingly recognized (Schneider & Cuervo, 2014; Uytterhoeven et al., 2015), macroautophagy arguably remains the best-characterized form of autophagy (and will be referred to as autophagy here below) (Galluzzi, Pietrocola, et al., 2015).

Autophagy continuously operates at low rates in virtually all eukaryotic cells, ensuring the degradation of normal by-products of life that may become dangerous upon accumulation, like damaged mitochondria and redox-active protein aggregates (Green, Galluzzi, & Kroemer, 2011). Such a baseline autophagic activity is critical for the maintenance of normal cellular functions, as demonstrated by the fact that defects in essential components of the autophagic machinery are associated with clinically relevant disorders including neurodegeneration, aging, and cancer (Choi, Ryter, & Levine, 2013; Galluzzi, Pietrocola, et al., 2015; Menzies, Fleming, & Rubinsztein, 2015). Moreover the autophagic flux, i.e., the actual degradation of autophagic substrates within lysosomes, increases in response to potentially lethal perturbations of intracellular and extracellular homeostasis, including nutritional, metabolic, physical, and chemical cues (Galluzzi, Pietrocola, Levine, & Kroemer, 2014; Green, Galluzzi, & Kroemer, 2014). In this setting, autophagy plays a key adaptive role as it supports the recovery of homeostasis and the survival of stressed cells. Accordingly, the pharmacological or genetic inhibition of essential constituents of the molecular machinery for autophagy generally (but not always) accelerates the demise of cells exposed to adverse microenvironmental conditions (Galluzzi, Bravo-San Pedro, et al., 2015). This said, autophagy can also contribute to cellular demise in a causal manner, both in developmental scenarios (e.g., in the maturation of salivary glands in *Drosophila melanogaster* larvae) (Berry & Baehrecke, 2007) and in cells responding to specific perturbations of homeostasis (e.g., neonatal neurons succumbing to hypoxia/ischemia) (Galluzzi, Bravo-San Pedro, Blomgren, & Kroemer, 2016). In both these cases, the pharmacological or genetic inhibition of autophagy de facto retards the cellular demise, warranting the use of the term "autophagic cell death" (Galluzzi, Bravo-San Pedro, et al., 2015). One particular instance of autophagic cell death that also impinges on the plasma membrane Na^+/K^+ ATPase is commonly known as autosis (Liu et al., 2013). In summary, autophagy mediates robust cytoprotective effects in multiple pathophysiologically relevant settings, but can also exert cytotoxic activity in some scenarios (Galluzzi, Bravo-San Pedro, et al., 2015).

Of note, autophagy can be relatively unselective or exquisitely specific (Sica et al., 2015). Rather unselective forms of autophagy dispose of various, nonessential cytoplasmic constituents and are generally activated by an increased demand of autophagic products (e.g., macromolecules for bioenergetic or anabolic purposes). Conversely, highly selective forms of autophagy target to lysosomal degradation-specific cytoplasmic entities and are often triggered by an increased availability in autophagic substrates

(e.g., mitochondria or bacteria decorated with ubiquitin moieties) and/or functions (e.g., the removal of damaged mitochondria or pathogens) (Okamoto, 2014). In particular, "mitophagy" specifically degrades damaged mitochondria, "reticulophagy" nonfunctional portions of the endoplasmic reticulum, "nucleophagy" nuclear buds containing damaged chromatin, "ribophagy" supernumerary ribosomes, "aggrephagy" redox-active protein aggregates, "xenophagy" invading bacteria that escape endocytic vacuoles, "virophagy" constituents of the viral capsid, and "lipophagy" lipid droplets destined to lipolysis (Sica et al., 2015). The existence of all these autophagic programs further underscores the importance of autophagy in the maintenance of cellular homeostasis (Galluzzi, Pietrocola, et al., 2015). In addition, autophagy is critically required for the optimal secretion of ATP by malignant cells undergoing so-called immunogenic cell death (Kroemer, Galluzzi, Kepp, & Zitvogel, 2013), hence playing a key role in the elicitation of anticancer immune responses triggered by some forms of chemotherapy (Galluzzi, Buque, Kepp, Zitvogel, & Kroemer, 2015). Thus, autophagy is also involved in cell-extrinsic mechanisms that ensure the preservation of organismal homeostasis.

Autophagy is regulated by a complex network of interacting signal transduction cascades (Galluzzi et al., 2014; Sica et al., 2015). One of the major regulators of autophagic responses is mechanistic target of rapamycin (MTOR) complex 1 (MTORC1), a supramolecular complex with protein kinase activity that tonically suppresses autophagy in physiologically conditions (Laplante & Sabatini, 2012). Various inducers of autophagy, including nutrient deprivation, operate indeed by shutting down MTORC1 signaling (Mihaylova & Shaw, 2011). Thus, when intracellular ATP levels drop, ADP and AMP accumulate and become able to activated AMP-dependent protein kinase (AMPK), which catalyzes the inactivating phosphorylation of MTORC1 and the activating phosphorylation of unc51-like kinase 1 (ULK1) (Laplante & Sabatini, 2012; Mihaylova & Shaw, 2011). ULK1 initiates a beclin 1 (BECN1)-dependent signal transduction cascade that ultimately results in the formation of autophagosomes (Egan et al., 2011). This process is generally accompanied by the formation of phosphatidylinositol-3-phosphate by phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3, best known as VPS34), as well as by the lipidation of microtubule-associated protein 1 light chain 3 (MAP1LC3, best known as LC3), which accumulates in the autophagosomal membrane to operate as an autophagic adaptor (Lamb et al., 2013). Additional proteins that are required for canonical autophagic responses are autophagy-related 4 (ATG3), ATG4, ATG5, ATG7, ATG9, ATG10, ATG12, and ATG16L1

(Lamb et al., 2013). ATG3, ATG4, and ATG7 cooperate to catalyze the lipidation of LC3 and other members of the Atg8 protein family. ATG7 also cooperates with ATG10 to generate an ATG5–ATG12–ATG16L1 complex that contributes to the elongation of forming autophagosomes. Finally, ATG9 appears to play a critical role in the first steps of autophagosome formation (Lamb et al., 2013). Of note, noncanonical BECN1- and VPS34-independent variants of autophagy have been reported (Codogno, Mehrpour, & Proikas-Cezanne, 2012; Niso-Santano et al., 2015), suggesting the existence of at least some degree of redundancy in the molecular mechanisms that initiate autophagic responses.

Not surprisingly, autophagy has been the subject of an ever more intense wave of investigation throughout the past 2 decades, a progression that has been accompanied by a significant methodological evolution (Ohsumi, 2014). Indeed, dozens of techniques are nowadays available for the characterization of virtually all aspects of autophagic responses, in vitro and in vivo (Klionsky et al., 2016). However, various conceptual/methodological problems that have permeated autophagy research in the past persist, and should be taken into attentive consideration when experimental determinations are designed as well as when results are interpreted. Consensus guidelines recently published in *Autophagy* provide a very comprehensive and detailed analysis of most (if not all) of these problems (Klionsky et al., 2016). Here, we would like to briefly recall three of them, which we feel still have a major negative impact on current studies on autophagy. First, investigators have tended (and still tend) to misemploy end-point biomarkers of autophagy, like the accumulation of autophagosomes in the cytoplasm or the lipidation of LC3, as indicators of an ongoing autophagic response (Klionsky et al., 2016). This is particularly problematic as many of these biomarkers can also accumulate when the last steps of the autophagic program are inhibited. Several approaches are currently available to properly measure autophagic flux in vitro, whereas it remains complicated to perform such measurements in fixed samples (Klionsky et al., 2016). Second, many of the chemicals that have been (and still are) employed to modulate autophagy in experimental settings, including the MTORC1 inhibitor rapamycin, the PI3K inhibitors 3-methyladenine and wortmannin, as well as the lysosomal inhibitor chloroquine, are relatively unspecific and affect various cellular processes other than autophagy, in an on-target or off-target manner (Klionsky et al., 2016). Genetic tools (e.g., homologous recombination, RNA interference, the CRISP/Cas9 system) provide a comparatively more robust

approach to ascribe a particular observation or phenotype to autophagy (Dominguez, Lim, & Qi, 2016; Ghildiyal & Zamore, 2009). However, (1) several components of the autophagic machinery exert autophagy-unrelated functions; and (2) the stable absence of a specific protein (as in knockout animals) may cause a considerable rewiring of signaling and metabolism, and/or the activation of compensatory processes. Genetically targeting at least two, if not three, distinct constituents of the autophagic machinery with RNA interference may be sufficient to circumvent the first of these issues (Ghildiyal & Zamore, 2009). Conversely, more refined strategies including the use of activatable Cre-coding constructs and the timely administration of (tissue-specific) Cre-encoding viral vectors are required to avoid cellular and organismal adaptation to the lifelong absence of one or more proteins (Masiero et al., 2009; Rao et al., 2014). We surmise that the development of novel, highly specific chemical inhibitors of autophagy and ever more refined strategies for the timed, tissue-specific genetic blockage of autophagic responses will shed new light on the implication of autophagy in several human disorders.

In *Molecular Characterization of Autophagic Responses* (an issue of the successful *Methods in Enzymology* series) world-leading experts in the field offer a comprehensive panel of techniques that can be used to measure virtually all aspects of autophagy, in vitro and in vivo. Thus, each of the 55 chapters of *Molecular Characterization of Autophagic Responses* provides a detailed description of one or a few protocols that are suitable for the characterization of autophagy in models as diverse as cultured human cancer cells, mice, fish, insects, worms, plants, and yeasts. The book (which consists of two parts, part A and part B) is equally addressed to expert investigators who may wish to expand their methodological competences, and to beginners in this exciting and rapidly expanding area of research.

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