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Dictyostelium discoideum and autophagy – a perfect pair

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ABSTRACT Autophagy is subdivided into chaperone-mediated autophagy, microautophagy and macroautophagy and is a highly conserved intracellular degradative pathway. It is crucial for cellular homeostasis and also serves as a response to different stresses. Here we focus on macroautophagy, which targets damaged organelles and large protein assemblies, as well as pathogenic intracellular microbes for destruction. During this process, cytosolic material becomes enclosed in newly generated double-membrane vesicles, the so-called autophagosomes. Upon maturation, the autophagosome fuses with the lysosome for degradation of the cargo. The basic molecular machinery that controls macroautophagy works in a sequential order and consists of the ATG1 complex, the PtdIns3K complex, the membrane delivery system, two ubiquitin-like conjugation systems, and autophagy adaptors and receptors. Since the different stages of macroautophagy from initiation to final degradation of cargo are tightly regulated and highly conserved across eukaryotes, simple model organisms in combination with a wide range of techniques contributed significantly to advance our understanding of this complex dynamic process. Here, we present the social amoeba *Dictyostelium discoideum* as an advantageous and relevant experimental model system for the analysis of macroautophagy.

KEY WORDS: *autophagy, ubiquitin-proteasome system (UPS), LC3-associated phagocytosis, proteaphagy*

Introduction

Macroautophagy, hereafter denoted as autophagy for simplicity, is the major lysosomal route for the clearance and turnover of damaged organelles and long-lived proteins (Stanley *et al.*, 2014). This cellular "self-eating" phenomenon was discovered in the sixties of the last century and the term "autophagy" was already coined in 1963 (De Duve and Wattiaux, 1966). Research in this process started slow, but is nowadays booming and increasingly fascinating. This was underlined by the award of the 2016 Nobel Prize in Physiology or Medicine to Yoshinori Ohsumi for his fundamental discoveries on the autophagic machinery in the budding yeast *Saccharomyces cerevisiae* (Levine and Klionsky, 2017, Tsukada and Ohsumi, 1993). Autophagy occurs in all eukaryotes at a basal level and is induced in response to cellular stresses such as starvation, the presence of protein aggregates or invading pathogens (Eskelinen and Saftig, 2009, Mizushima *et al.*, 2008). Most likely, autophagy evolved in unicellular organisms as a survival mechanism during starvation through the recycling of cellular building blocks (Wirawan *et al.*, 2012). The different molecular complexes act in a sequential way to deliver cytoplasmic cargo to the lysosome and crucial components were initially discovered and characterised in *S. cerevisiae* (Tsukada and Ohsumi, 1993). The hallmark of autophagy is the *de novo* formation of a double-layered vesicle, the so-called autophagosome, which can engulf parts of the cytoplasm, entire organelles or even pathogenic bacteria (Lamb *et al.*, 2013). The maturation of autophagosomes into autolysosomes is accomplished by fusion of the outer autophagosomal membrane with the lysosomal membrane. Then, the inner autophagosomal membrane and the cargo are degraded by lysosomal hydrolases (Stanley *et al.*, 2014). The proteins involved in autophagosome formation were named ATG, for AuTophaGy-related proteins, and are evolutionarily highly conserved across the eukaryotic lineage (Feng *et al.*, 2014). Autophagic dysfunction can result in a wide range of diseases, including neurodegeneration, cancer, muscular dystrophy, and lipid-storage disorders (Mizushima *et al.*, 2008, Schneider and Cuervo, 2014).

Autophagy has long been considered as a non-selective bulk degradation process of cytoplasmic components in response to nutrient starvation (Boya *et al.*, 2013). However, it is now clear that autophagy generally operates in a selective manner through recognition of the substrates by cargo specific autophagy recep-

Abbreviations used in this paper: LAP, LC3-associated phagocytosis; UPS, ubiquitin-proteasome system.

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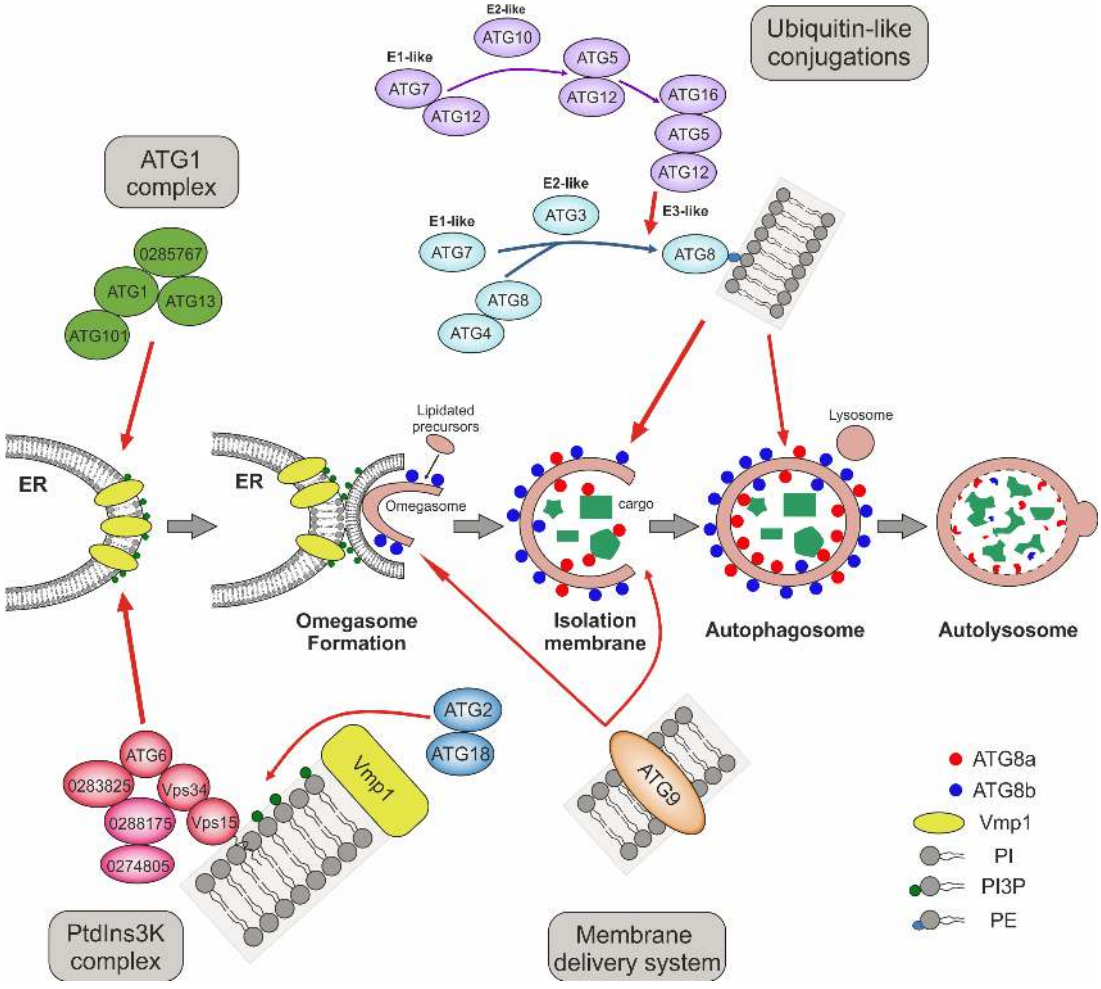

Fig. 1. Schematic depiction of autophagosome formation in *D. discoideum*. *During autophagy, cytosolic material becomes engulfed by the cup-shaped isolation membrane, which elongates and closes to form a double-membrane autophagosome. After fusion with a lysosome, the cargo is degraded and recycled. Different components are mandatory for this process: the ATG1 complex, the PtdIns3K complex, the membrane delivery system and two ubiquitin-like conjugation systems. Activation of the ATG1 complex in the initiation stage results in phosphorylation of ATG6 (Beclin1) of the PtdIns3K complex. This leads to its recruitment to the omegasome. The recruitment process is also supported by Vmp1, which accumulates in subdomains of the ER. The signal lipid PtdIns3P is generated by the PtdIns3K complex and is required for binding of other autophagy proteins to the membrane such as ATG18 and ATG2. The balance between production and degradation of PtdIns3P regulates omegasome formation and elongation of the isolation membrane and the transmembrane protein ATG9 is thought to deliver the required membrane lipids. For efficient membrane elongation two ubiquitin-like conjugation reactions are crucial. The ATG12~5/16 complex regulates the conjugation of ATG8 (LC3 in mammals) to phosphatidylethanolamine (PE) at the isolation membrane. ATG8b (blue) joins the growing autophagosome before ATG8a (red). Furthermore, ATG8b is mainly localised at the outside and ATG8a at the inside of the autophagosome. In the final steps ATG8a and b at the outer membrane are cleaved by ATG4 (not shown), the autophagosome fuses with a lysosome and the inner membrane and the contents of the autophagosome are degraded in the autolysosome. The DDB_G numbers of the likely* D. discoideum *orthologues of the following mammalian proteins are provided: FIP200 (DDB_G0285767), ATG14 (DDB_G0283825), UVRAG (DDB_G0288175) and Bif-1 (DDB_G0274805). Components of the autophagic machinery are not drawn to scale. PI = phosphatidylinositol; PI3P = phosphatidylinositol-3-phosphate; PE = phosphatidyl-ethanolamine. Modified from (Mesquita* et al.*, 2017).*

tors (Farre and Subramani, 2016). They tether the cargo to the autophagosomal membrane by simultaneously binding the cargo and ATG8 family proteins (LC3 in mammals) present on the surface of the growing autophagosome (Zaffagnini and Martens, 2016). The interaction is mediated by different receptors, such as e.g. p62/SQSTM1, which contain ubiquitin-binding domains for the cargo and an LC3-interacting region (LIR), also called ATG8-interacting motif (AIM) (Boya *et al.*, 2013, Gatica *et al.*, 2018, Khaminets *et al.*, 2016). Based on the cargo to be destructed, selective autophagy is subdivided into ribophagy, mitophagy, aggrephagy, xenophagy, lipophagy, reticulophagy, nucleophagy, glycophagy, pexophagy, and proteaphagy (Marshall and Vierstra, 2018).

Dictyostelium discoideum an excellent model system for autophagy

Autophagy is intensively studied in vertebrates, e.g. *Mus musculus*, insects, e.g. *Drosophila melanogaster*, worms, e.g. *Caenorhabditis elegans*, fungi, e.g. *S. cerevisiae* and plants, e.g. *Arabidopsis thaliana*, to name just a few (Galluzzi *et al.*, 2017, Lv *et al.*, 2014, Mesquita *et al.*, 2017). Another of the well-established model organisms is the soil-living amoeba *D. discoideum* and in recent years, a large number of methods to monitor and quantify autophagy in this organism have been developed (Calvo-Garrido *et al.*, 2010, Domínguez-Martín *et al.*, 2017, Klionsky *et al.*, 2016). *D. discoideum* is a member of the phylum Amoebozoa and was first isolated and described by Kenneth Raper (Raper, 1935). *Dictyostelium* cells grow as unicellular amoebae that divide by binary cell fission and feed on bacteria by phagocytosis (Kessin, 1981). Upon depletion of the food source, up to 100,000 solitary amoebae aggregate by chemotaxis towards cAMP. The cell aggregate (or pseudoplasmodium) differentiates via distinct morphological states into a mature fruiting body, composed of a mass of spore cells supported by a thin, long stalk made of vacuolised dead cells

(Eichinger, 2003).

Since development takes place in the absence of external nutrients, *D. discoideum* cells must mobilise a large fraction of the required energy for biosynthetic needs and morphogenesis by autophagy and glycogenolysis (Mesquita *et al.*, 2017). In addition, autophagy is also required for signalling pathways relevant for the developmental process (Calvo-Garrido *et al.*, 2010). For example, the formation of fruiting bodies, which mainly consist of viable spores and vacuolised, cellulose-walled, dead stalk cells, requires autophagy (Mesquita *et al.*, 2017). For the differentiation from vegetative cells into stalk cells by autophagic cell death (ACD) at least two distinct stimuli are necessary. The first stimulus is starvation together with cAMP to induce autophagy and the second required stimulus is the main stalk cell differentiation-inducing factor DIF-1, a small dichlorinated molecule (Giusti *et al.*, 2009, Mesquita *et al.*, 2017). A very recent report showed that in the absence of the core autophagy proteins ATG5, ATG7 or ATG9 vacuolization of stalk cells still takes place, suggesting that ACD is not dependent on canonical autophagy but may still depend on certain non-canonical autophagy (Yamada and Schaap, 2019). Moreover, the unconventional secretion of AcbA, the precursor of the signalling peptide SDF-2 (spore differentiation factor 2), is indispensable for spore formation and depends on autophagy (Duran *et al.*, 2010). As a consequence, autophagy malfunction generally results in reduced cell survival upon nitrogen starvation and in developmental abnormalities in the affected strains, which also supports the identification of autophagy-deficient mutants in the laboratory (Fischer *et al.*, 2019, King *et al.*, 2013, Mesquita *et al.*, 2017, Otto *et al.*, 2003). The developmental phenotypes range from a complete lack of aggregation to the formation of extremely small and crippled fruiting bodies with drastically reduced spore viability (Calvo-Garrido *et al.*, 2010, Fischer *et al.*, 2019, Mesquita *et al.*, 2015, Messling *et al.*, 2017, Otto *et al.*, 2003, Otto *et al.*, 2004, Tung *et al.*, 2010, Xiong *et al.*, 2015). Differences in the importance of the respective autophagy proteins for the functioning of autophagy and additional non-autophagic functions for some of these proteins are likely responsible for the diverse observed phenotypes (Fischer *et al.*, 2019). Indeed, in recent years more and more non-autophagic functions of core autophagy proteins have been reported (Malhotra *et al.*, 2015, Mauthe *et al.*, 2016, Nam *et al.*, 2017, Xiong *et al.*, 2015). Recent excellent reviews have covered the study of autophagy in *D. discoideum* in the contexts of mechanical stress, human disease, infection with pathogens and cell death pathways (Calvo-Garrido *et al.*, 2010, King *et al.*, 2011, Mesquita *et al.*, 2017). Furthermore, currently established methods have been exquisitely summarized (Domínguez-Martín *et al.*, 2017, Mesquita *et al.*, 2013). In this review, we focus on the general features and mechanisms of autophagy, autophagy-dependent and -independent roles of ATG proteins, and the crosstalk between autophagy and the ubiquitin-proteasome system (UPS).

General features and mechanisms of autophagy

The autophagic process is tightly controlled by the cell's nutritional and energy level and can be subdivided into initiation, maturation, and lysosomal degradation phases. The responsible

TABLE 1

MAIN AUTOPHAGY-RELATED PROTEINS IN A SELECTION OF MODEL ORGANISMS

Homo sapiens	*Dictyostelium discoideum*	*Saccharomyces cerevisiae*	*Drosophila melanogaster*	*Caenorhabditis elegans*
		ATG1 protein complex subunits		
ULK1, ULK2	ATG1	ATG1	ATG1	UNC-51
ATG13	ATG13	ATG13	ATG13	ATG13
ATG101	ATG101	–	ATG101	EPG-9
FIP200	DDB_G0285767	ATG11, ATG17	ATG17	EPG-7
		PtdIns3K protein complex subunits		
Beclin-1	ATG6	VPS30	ATG6	BEC-1
PIK3C3	VPS34	VPS34	Pi3K59F	VPS-34
PIK3R4	VPS15	VPS15	VPS15	VPS-15
UVRAG	DDB_G0288175	VPS38	UVRAG	T23G11.7, Y34B4A.2
Bif-1	DDB_G0274805 *(ibrA)*	–	Endophilin B	F35A5.8
ATG14	DDB_G0283825	ATG14	ATG14	EPG-8
		Ubiquitin-like conjugation systems		
ATG3	ATG3	ATG3	ATG3	ATG-3
ATG4A, 4B, 4C, 4D	ATG4	ATG4	ATG4A, 4B	ATG-4.1, 4.2
ATG5	ATG5	ATG5	ATG5	ATG-5
ATG7	ATG7	ATG7	ATG7	ATG-7
GABARAP, GABARAPL1, L2, L3	ATG8b	ATG8	ATG8a	LGG-1
MAP1LC3A, B, C	ATG8a		*ATG8b*	LGG-2
ATG10	ATG10	ATG10	ATG10	ATG-10
ATG12	ATG12	ATG12	ATG12	LGG-3
ATG16L1, L2	ATG16	ATG16	ATG16	ATG-16.1, 16.2
		Membrane delivery system		
ATG2A, 2B	DDB_G0277419	ATG2	ATG2	ATG-2
ATG9A, 9B	ATG9	ATG9	ATG9	ATG-9
WIPI1, 2, 3 (Wdr45l), WIPI4 (Wdr45)	ATG18, Wdr45l	ATG18	ATG18a, 18b	ATG-18
Vmp1	Vmp1	–	Tango5	EPG-3

Standalone letters and numbers refer to the protein mentioned before; -, currently no obvious corresponding orthologue; italic, uncertain grouping.

core machinery for autophagosome formation comprises more than 20 proteins, that are evolutionary highly conserved and engaged in different molecular complexes (Fig. 1; Table 1) (Birgisdottir *et al.*, 2013, Calvo-Garrido *et al.*, 2010, Feng *et al.*, 2014, Ktistakis and Tooze, 2016). In mammalian and *D. discoideum* cells, several autophagosomes can be generated simultaneously at multiple cytoplasmic sites, whereas this occurs only at a single spot near the vacuole in *S. cerevisiae* and it has been suggested that yeast is "the odd one out" (King, 2012, Mesquita *et al.*, 2017). The exact origin of the autophagosomal membrane remains an enigma after more than 20 years of investigation. Recent observations indicate that the endoplasmic reticulum (ER) in interplay with the Golgi, endosomes, the plasma membrane and mitochondria serve as membrane source in mammalian cells (Wei *et al.*, 2018). The initial structure is a *de novo* generated subdomain of the ER, the socalled omegasome (PAS, phagophore assembly site or preautophagosomal structure in *S. cerevisiae*), which then becomes the isolation membrane (IM, phagophore in *S. cerevisiae*) (Axe *et al.*, 2008).

The nutrient sensor target of rapamycin (TOR), a member of the phosphatidylinositol kinase-related family of serine/threonine protein kinases, receives and integrates extra- and intracellular signals, which mirror the energy and nutrient status of the cell (Calvo-Garrido *et al.*, 2010, Jung *et al.*, 2010, Noda, 2017). In its active form the mammalian TOR complex 1 (TORC1), composed of TOR, Raptor, Lst8 and Deptor, is the main inhibitor of autophagy. TORC1, the ATG1 and PtdIns3K complexes are well conserved in *D. discoideum* and evolutionarily close to the corresponding complexes in higher eukaryotes. Therefore, we think it is justified to infer that the corresponding activities, that were uncovered in different model organisms and are described in the following part, are also conserved in *D. discoideum*. In general, the activity of TORC1 is inhibited under starvation conditions. This results in rapid dephosphorylation of ATG13 and thereupon activation of the ATG1 complex (ULK1 in mammals), which consists of the ATG1 kinase, ATG13, ATG101 and the scaffold protein FIP200 (also known as RB1CC1 or ATG17; Fig. 1; Table 1). Interaction studies have demonstrated that ATG13 binds to both ATG1 and ATG101 and the latter in turn stabilises ATG13 (Mercer *et al.*, 2009, Mesquita *et al.*, 2015). The likely FIP200 homolog in *D. discoideum* is DDB_G0285767 (Li *et al.*, 2014, Mesquita *et al.*, 2017). The active ATG1 complex stimulates autophagy through phosphorylation of Beclin-1 (ATG6) in association with ATG14 of the class III phosphoinositide 3-kinase (PtdIns3K) complex (Park *et al.*, 2018). This leads to the generation of the phospholipid PtdIns3P by Vps34/PtdIns3K at the omegasome. The balance between production and degradation of PtdIns3P is pivotal for recruitment of further core autophagy proteins to the omegasome, such as the PtdIns3P binding proteins ATG18 (WIPI in mammals) and ATG2 (Lindmo and Stenmark, 2006, Obara *et al.*, 2008). Recently it was shown, that ATG2 is a multifunctional protein that tethers membranes and acts as a lipid-transfer protein. As ATG2 localises to the contact site between the enlarging IM and the ER it was suggested that ATG2 transfers phospholipids from the ER exit sites (ERES) to the IM during the process of autophagosome formation (Osawa and Noda, 2019, Valverde *et al.*, 2019). Furthermore, the highly conserved transmembrane proteins ATG9 and Vmp1 are needed for omegasome formation, PtdIns3P signalling and membrane delivery in *D. discoideum* and mammalian cells (Calvo-Garrido *et al.*, 2010, Calvo-Garrido *et al.*, 2014, Mesquita *et al.*, 2017, Tábara *et al.*, 2018). ATG9 is the only known integral membrane protein of the core autophagy machinery and resides in small vesicles that are involved in the delivery of membrane lipids to the growing autophagosome (Xie and Klionsky, 2007). Its knock-out resulted in a pleiotropic phenotype in *D. discoideum* (Tung *et al.*, 2010). In Vmp1-deficient *D. discoideum* cells PtdIns3P production and the subsequent recruitment of the autophagy machinery to the ER is intact, however, the autophagic flux is blocked. The ER-resident protein Vmp1 seems to generate an ideal ER microenvironment required for the correct structure of the omegasome, which allows the IM to elongate and to become a functional autophagosome (Calvo-Garrido *et al.*, 2010, Mesquita *et al.*, 2017, Tábara *et al.*, 2018). Further regulatory components of the PtdIns3K complex are the myristoylated protein kinase Vps15 (PIK3R4 in mammals), UVRAG and Bif-1 (Table 1).

For the expansion of the IM eight highly conserved core autophagy proteins are indispensable (Fig. 1; Table 1) (Geng and Klionsky, 2008). These proteins are involved in two ubiquitin-like conjugation reactions and similar to the ubiquitin system, the two ubiquitin-like proteins ATG12 and ATG8 (LC3 in mammals) are finally attached to their substrate via this enzymatic pathway. In the first ubiquitin-like reaction, ATG12 is activated by the E1-like enzyme ATG7 and then conjugated to the E2-like enzyme ATG10 (Geng and Klionsky, 2008). Subsequently, ATG12 is covalently attached to its target protein ATG5, and two ATG12~5 conjugates in turn associate non-covalently with an ATG16 dimer and form a hetero-tetrameric complex (Mizushima *et al.*, 1999). The ATG12~5 conjugation reaction seems to be irreversible, since so far no enzyme for the cleavage of the isopeptide bond between ATG12 and ATG5 has been identified (Geng and Klionsky, 2008). In the final step of the second ubiquitin-like reaction, ATG8 (LC3) is reversibly attached to the lipid phosphatidylethanolamine (PE) on the expanding autophagosomal membrane via the E3-like activity of the ATG12~5/16 complex. It is believed that the complex brings the ATG8-carrying E2-like enzyme ATG3 in proximity to PE and determines the exact site of ATG8-PE conjugation on the autophagosomal membrane (Fujita *et al.*, 2008, Sakoh-Nakatogawa *et al.*, 2013, Walczak and Martens, 2013). ATG8-PE is present on both the inner and outer membranes of the IM. In mammals there are seven paralogues of the single yeast ATG8. Three are grouped into the MAP1-LC3 (microtubule-associated protein 1 – light chain 3) and four into the GABARAP/GATE16 (Gamma-aminobutyric acid receptor-associated protein/Golgi-associated ATPase enhancer of 16 kDa) subfamily (Shpilka *et al.*, 2011). *D. discoideum* harbours two paralogues, ATG8a and ATG8b, which have distinct functions in canonical autophagy. It was shown that they associate to the autophagosome in succession and that ATG8b mainly localises at the outer membrane of the autophagosome while ATG8a is mainly present at the inner membrane (Matthias *et al.*, 2016, Messling *et al.*, 2017). This differential localisation supports a more prominent role for ATG8b as an adapter for the autophagy machinery and in autophagosome lysosome fusion while ATG8a appears to function mainly as a binding partner for autophagy receptors. On the basis of function and localisation it was inferred that ATG8b is likely the *D. discoideum* orthologue of the GABARAP and ATG8a of the LC3 subfamily in mammals (Table 1) (Messling *et al.*, 2017). The autophagosomal membrane further expands through the incorporation of membrane lipids, engulfs entire organelles or

parts of the cytoplasm and finally closes into a double-membrane structure, the autophagosome (Lamb *et al.*, 2013, Stanley *et al.*, 2014). After completion of autophagosome biogenesis and before fusion with the lysosome, ATG8 is cleaved from the outer membrane by ATG4, while ATG8 on the inner membrane is degraded inside the lysosome (Geng and Klionsky, 2008, Kirisako *et al.*, 2000). Autophagosomes eventually mature into autolysosomes upon fusion of the outer autophagosomal membrane with the lysosomal membrane. Finally, the inner autophagosomal membrane and the cargo are degraded by lysosomal hydrolases (Fig. 1) (Wirawan *et al.*, 2012). The molecular mechanism of autophagosome-lysosome fusion has so far not been investigated in *D. discoideum*. Results from different experimental systems showed, that it consists of two phases: the autophagosome migration phase and the fusion phase. In the migration phase, transport of autophagosomes to the location of the lysosomes in the perinuclear region occurs along microtubules in a dynein-dependent manner (Jahreiss *et al.*, 2008). In the autophagosome-lysosome fusion step, three sets of protein families are involved, Rab GTPases, membrane tethering complexes and soluble Nethylmaleimide-sensitive-factor attachment protein receptors (SNAREs) (Nakamura and Yoshimori, 2017).

Autophagy-independent roles of ATG proteins

There is growing evidence in the literature for unconventional roles of ATG proteins, besides their function in canonical autophagy. RNA_{Seq} analysis of different mammalian cells in combination with a siRNA screen revealed that up to 36% of the autophagy-related genes encode proteins with additional unconventional functions (Mauthe *et al.*, 2016). For example in mouse embryonic fibroblasts, LC3-coated vesicles that differ from autophagosomes are necessary for the disposal of the ERAD effector protein EDEM1 in the process of ER-associated degradation (ERAD) (Calì *et al.*, 2008). Independent from its function in autophagy mammalian ATG7 has been implicated in nutrient deprivation induced cell cycle arrest via direct interaction and induction of p53 (Lee *et al.*, 2012). In addition, autophagy-independent functions have been reported in different organisms for ATG1, ATG2, ATG3, ATG4, ATG5, ATG6, ATG12, ATG16 and PtdIns3K (Bestebroer *et al.*, 2013, Schaaf *et al.*, 2016, Subramani and Malhotra, 2013). This list is likely not exhaustive and also in *Dictyostelium*, many of the core autophagy proteins appear to fulfil autophagy-independent functions in addition to their role in canonical autophagy (Xiong *et al.*, 2019, Xiong *et al.*, 2015).

LC3-associated phagocytosis

LC3-associated phagocytosis (LAP) is one such novel function for autophagy proteins and is a contributor to immune regulation and inflammatory responses across various cell and tissue types (Heckmann and Green, 2019). In contrast to canonical autophagy, LAP is not dependent on the AMPK–mTORC1–ULK1(ATG1) activation axis or nutrient status of the cells (Heckmann *et al.*, 2017, Martinez *et al.*, 2015). A plurality of ligands, including dying cells, immune complexes and pathogens, has been shown to facilitate the conjugation of LC3 to PE of the phagosome in this process. Several receptors that participate in cargo recognition have already been identified including toll-like receptors, immunoglobulin receptors or TIM4 (Heckmann and Green, 2019). Following an activating stimulus, LAP can be delineated into three phases, followed by lysosomal fusion. The first phase is the generation of the single membrane phagosome which serves as scaffold for the assembly of downstream regulatory complexes (Heckmann *et al.*, 2017). Secondly, the PtdIns3K complex composed of Vps34 (PtdIns3K), Vps15, Beclin-1 (ATG6 in e.g. yeast and *D. discoideum*), UVRAG and Rubicon generates membrane-localised PtdIns3P (Martinez *et al.,* 2015). Thirdly, the conjugation systems are recruited, which catalyse the conjugation of LC3 to PE of the phagosome (Fig. 2). The presence of LC3 on the phagosome, now termed LAPosome, mediates the fusion with the lysosome and the cargo is degraded (Heckmann and Green, 2019). LAP exists at the crossroads of phagocytosis and autophagy and is for example required for apoptotic corpse clearance during programmed cell death in multicellular organisms (Huang *et al.*, 2013, Martinez *et al.*, 2011). There is general agreement that LAP requires the entire ATG12~5/16 complex for LC3 recruitment (Lai and Devenish, 2012). Loss of any of the components of this complex led to a reduction or even abolishment of LAP function in mouse embryonic fibroblasts (MEFs) and mouse bone marrow-derived macrophages (Huang *et al.*, 2009, Kageyama *et al.*, 2011, Lai and Devenish, 2012). Investigation of the phagocytic and macropinocytic activity of the *D. discoideum* ATG9$^-$, ATG16$^-$, ATG9$^-$/16$^-$, ATG12$^-$ and ATG12$^-$/16$^-$ strains showed that the encoded proteins have functions in phagocytosis of bacteria and yeast and also in the uptake of nutrients via macropinocytosis (Fischer *et al.*, 2019, Tung *et al.*, 2010, Xiong *et al.*, 2015). However, this is only circumstantial evidence and thus far LAP has not been directly demonstrated in *D. discoideum*. There could also be an indirect connection as for example Vmp1 deficiency in *Dictyostelium* led not only to a simple block in autophagosome formation but rather to a disturbance in autophagy-dependent PtdIns3P signalling at the ER, which also indirectly influenced macropinocytosis (Calvo-Garrido *et al.*, 2014). Electron microscopy in combination with immunogold labelling could resolve, whether there is LAP also in *Dictyostelium*.

Mammals express two ATG16 isoforms of which ATG16L1 apparently plays a critical role in the defence against pathogens. By recruiting ATG16L1 to the bacterial entry site, bacterial sensing by NOD proteins is linked to the induction of autophagy (Travassos *et al.*, 2010). Furthermore, ATG16L1 is required for LAP as murine bone marrow-derived macrophages deficient in ATG16L1 failed to undergo both, canonical autophagy or LAP (Martinez *et al.*, 2015). More recently, it was reported that the C-terminal domain of ATG16L1, which is composed of seven WD40 repeats, is essential for LAP during non-canonical autophagy, but dispensable for canonical autophagy (Fletcher *et al.*, 2018). This finding opens the possibility for the detailed analysis of LC3 lipidation during LAP.

Further autophagy-independent functions of ATG5, ATG12 and ATG16

In silico analyses showed that *D. discoideum,* yeast and human ATG5 harbour one helix-rich domain and two ubiquitin-like domains and ATG12 one ubiquitin-like domain. Although ubiquitin, ATG5, and ATG12 do not share sequence similarity, the 3D structures of the ubiquitin-like domains are highly similar, especially with respect to the α-helices and β-strands (Fischer *et al.*, 2019, Geng and Klionsky, 2008, Tsukada and Ohsumi, 1993). *D. discoideum* ATG16 is composed of three distinct regions, as is the case for the ATG16 orthologs in higher eukaryotes: the N-terminal domain which is responsible for binding to ATG5, followed by a coiled-coil domain (CCD) which mediates homo-dimerisation and seven WD40

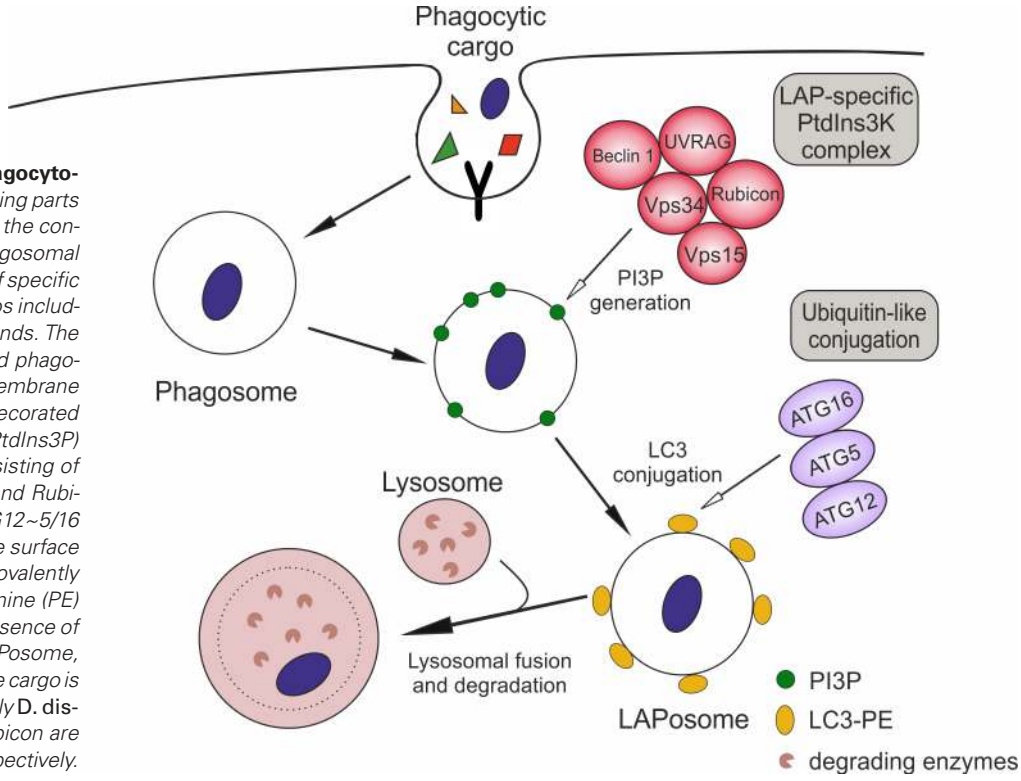

Fig. 2. Mechanism of LC3-associated phagocytosis (LAP). *LAP is characterised by employing parts of the canonical autophagy machinery for the conjugation of LC3 family proteins to the phagosomal membrane. It is initiated by engagement of specific receptors that recognise a variety of cargos including pathogens, dying cells or soluble ligands. The cargo is internalized by receptor-mediated phagocytosis and engulfed within a single-membrane phagosome. This compartment is rapidly decorated with phosphatidylinositol-3-phosphate (PtdIns3P) generated by the PtdIns3K complex consisting of Vps15, Vps34, Beclin-1 (ATG6), UVRAG and Rubicon. Autophagy proteins including the ATG12~5/16 complex are subsequently recruited to the surface of phagosomal membrane, which in turn covalently link LC3 (ATG8) to phosphatidylethanolamine (PE) on the surface of the phagosome. The presence of LC3 on the phagosome, now termed LAPosome, mediates fusion with the lysosome and the cargo is degraded. The DDB_G numbers of the likely* D. discoideum *orthologues of UVRAG and Rubicon are DDB_G0288175 and DDB_G0293570, respectively.*

repeats in the C-terminal half of the protein, which are predicted to form a β-propeller structure (Xiong *et al*., 2019). This structure serves as a hub for protein-protein interactions, which appear to be crucial for autophagy-dependent or -independent functions of ATG16 (Xiong *et al*., 2019). The C-terminal domain is missing in yeast ATG16 and recently, the crystal structure of the yeast ATG12~5/16 complex has been solved, showing that ATG12 and ATG16 are located on opposite sides of ATG5 and that there is no molecular interaction between ATG16 and ATG12 (Suzuki *et al*., 2017).

The activity of the conserved hetero-tetrameric ATG12~5/16 complex is indispensable for autophagosome formation. During canonical autophagy it localises to the outer membrane of the expanding IM and is released shortly before or after autophagosome completion (Mizushima *et al*., 2001). The association of the ATG12~5 conjugate with ATG16 apparently unmasks a membrane-binding site in ATG5 and the membrane tethering ability of ATG5 is also stimulated by ATG12 (Walczak and Martens, 2013). Within the ATG12~5/16 complex, ATG16 is required for correct localisation and the ATG12~5 conjugate possesses the E3 ligase activity that promotes the conjugation of ATG8 to PE of the autophagosomal membrane (Fujita *et al*., 2008, Hanada *et al*., 2007). Mice lacking ATG5, ATG12 or ATG16L1 survive the embryonic phase, but die one day after birth, corroborating the importance of an intact ATG12~5/16 complex for postnatal survival (Kuma *et al*., 2004, Malhotra *et al*., 2015, Saitoh *et al*., 2008). With respect to functions in non-canonical autophagy the situation is complicated and it is often not clear whether these are mediated by ATG5, ATG12, ATG16, the ATG12~5 conjugate, ATG5/16 or the ATG12~5/16 complex. In MEFs the ATG12~5/16 complex contributed to pneumolysin toxin resistance and restriction of cell-to-cell spread of *Listeria monocytogenes* through a pathway independent of macroautophagy. The authors showed that in MEFs deficient in ATG16L1, as well as ATG5 or ATG12, cholesterol accumulated in lysosomes. This resulted in less efficient lysosomal exocytosis, which is needed for efficient plasma membrane repair. Interestingly, MEFs harbouring the ATG16L1 T300A allele, which was previously found to increase the risk of Crohn's disease (CD), were also less efficient in plasma membrane repair (Serramito-Gómez *et al*., 2016, Tan *et al*., 2018). There is also evidence that ATG16L1 modulates the balance between NOD2-induced xenophagy versus cytokine production. This may explain the effects of this polymorphism on the inflammatory process in CD (Plantinga *et al*., 2011). In addition, the autophagy machinery including components of the ubiquitin-like conjugation systems also has an important role in maintaining membrane integrity during mycobacterial infection (Cardenal-Muñoz *et al*., 2017). In *D. discoideum*, membrane damages caused by *Mycobacterium marinum* activate an autophagic defence response reflected by an up-regulation of autophagy genes, stimulation of autophagosome formation and recruitment to the mycobacteria-containing vacuole (MCV). The autophagic flux is simultaneously repressed resulting in the accumulation of cytoplasmic material in the MCV, which supports bacterial survival within the niche (Cardenal-Muñoz *et al*., 2017). On the other hand, the membrane generated by the autophagic machinery at the distal pole of the ejecting bacteria prevents plasma membrane leakage and cell death of *D. discoideum* cells (Gerstenmaier *et al*., 2015). In *Drosophila*, ATG16 is crucial for the differentiation of intestinal stem cells into enteroendocrine (EE) cells. Expression of ATG16 lacking the WD40 repeat domain led to morphological changes in the intestine that resembled inflammatory bowel disease (Nagy *et al*., 2017). Furthermore, the C-terminal WD40 domain is important in xenophagy (Xiong *et al*., 2019). During infection of intestinal epithelial cells with *Salmonella typhimurium*, damage of the en-

dosomal membrane by bacteria residing in the endosome leads to the exposure of endosomal proteins, which are ubiquitinated. ATG16L1 is recruited to the *Salmonella*-containing endosomes by a direct interaction between the WD40 repeat domain and ubiquitin (Fujita *et al.*, 2013, Xiong *et al.*, 2019). ATG16 is also involved in Cullin-3-mediated ubiquitination and proteasomal degradation of the selective autophagy adaptor p62/SQSTM1 (Xiong *et al.*, 2019). In *Dictyostelium*, ATG16 carries out additional functions in proteasomal activity, axenic growth and macropinocytosis (Xiong *et al.*, 2018, Xiong *et al.*, 2019, Xiong *et al.*, 2015). Since the *Dictyostelium atg16* knockout mutant displayed strongly reduced proteasomal activity, ATG16 is crucial for optimal UPS function (Xiong *et al.*, 2015). Moreover, a strong defect of *Dictyostelium* ATG16$^-$ cells in macropinocytosis, as evidenced by their reduced growth in liquid medium and reduced uptake of TRITC-labelled dextran, which could be caused by a disturbance of recycling endosomes, was observed (Fischer *et al.*, 2019, Xiong *et al.*, 2015).

Several autophagy-independent functions have been described for ATG12 in different organisms. Under certain nutrient-limiting conditions, ATG12 interacts with ATG3 in maintaining mitochondrial homeostasis and preventing cell death in MEFs, HeLa and HEK293 cells. In this process, ATG12 serves as a positive mediator of mitochondrial apoptosis and directly regulates the apoptotic pathway by binding and inactivating pro-survival Bcl-2 family members, including Bcl-2 and Mcl-1 (Radoshevich *et al.*, 2010, Rubinstein *et al.*, 2011). Furthermore, ATG12 is involved in endosomal trafficking and IFNγ-mediated host defence against murine norovirus (MNV) infection as shown in HeLa cells and bone marrow-derived macrophages (Hwang *et al.*, 2012, Murrow *et al.*, 2015). In viral protein translation, ATG12 is crucial for translation of hepatitis C RNA, virus replication and egress from cells (Dreux and Chisari, 2011). In *D. discoideum* we found massive transcriptional changes and complex phenotypes of varying severity for ATG12$^-$, ATG16$^-$ and ATG12$^-$/16$^-$ knock-out cells implying that ATG12 and ATG16 have, in addition to their role in canonical autophagy, autophagy-independent functions (Fischer *et al.*, 2019). The developmental phenotypes of the ATG12$^-$, ATG16$^-$ and ATG12$^-$/16$^-$ strains were similar to those previously reported for ATG5$^-$, ATG7$^-$ and ATG16$^-$ mutants (Otto *et al.*, 2003, Xiong *et al.*, 2015). Loss of either of these proteins led to severe impairments in the tipped mound stage, in the slug stage, and in fruiting body formation. The ATG12$^-$, ATG16$^-$ and ATG12$^-$/16$^-$ strains displayed similar defects in autolysosome formation and cellular viability in response to amino acid starvation, implying that ATG12 and ATG16 act together with ATG5 as a functional unit in canonical autophagy. Interestingly, an incremental increase in the severity of the phenotype from ATG12$^-$ to ATG16$^-$ cells to the double knock-out strain was the case for spore viability and maximal cell titre in liquid culture. This suggests that ATG12 and ATG16 fulfil an independent function in these cellular processes or that the ATG12~5/16 complex without either ATG12 or ATG16 has still some residual activity. In contrast, for proteasomal activity, axenic growth, and macropinocytosis the phenotypes of ATG16$^-$ cells were in comparison to ATG12$^-$ cells slightly more severe. Therefore, ATG16 either fulfils an additional function in these cellular processes or there is still some ATG5/16 complex with residual activity formed in the absence of ATG12. Even though the exact link between autophagy and phagocytosis is currently not clear in *D. discoideum*, the results of the clearing assay for *K. aerogenes* suggest that ATG16 has an inhibitory and ATG12 a stimulatory effect on the clearing of *K. aerogenes* by phagocytosis (Fischer *et al.*, 2019).

ATG5, as also is the case for ATG16L1, is important for the biology of the Paneth cells of the mouse ileal epithelium since ATG5-deficient Paneth cells have notable abnormalities in antimicrobial peptide secretion (Cadwell *et al.*, 2008). In osteoclasts, ATG5 is involved in the polarised secretion of lysosomal contents (DeSelm *et al.*, 2011). Moreover, ATG5 also participates in autophagy-independent defence mechanisms against the intracellular protozoan parasite *Toxoplasma gondii*. This parasite persists in the vacuole of non-activated macrophages by preventing the fusion of the modified parasitophorous vacuole, in which it replicates, with the lysosome. In particular, *T. gondii*-infected *atg5*-deficient macrophages are unable to clear the parasite (Bestebroer *et al.*, 2013). Apoptotic stimuli, like anticancer drugs stimulate the calpain-mediated ATG5 cleavage and the resulting truncated form of ATG5 associates with mitochondria and triggers cytochrome c release and subsequent caspase 3 activation. Therefore, ATG5 represents a molecular link between autophagy and apoptosis — a finding with potential importance for clinical anticancer therapies (Bestebroer *et al.*, 2013, Ye *et al.*, 2018).

The plurality of recent studies on autophagy-independent functions of ATG proteins emphasizes the importance of revisiting phenotypes and functions, that to date have been attributed to canonical autophagy based on the genetic analysis of a single individual ATG protein. Autophagy-related or -independent functions in different organisms include secretion, trafficking of phagocytosed material, replication and egress of viral particles, and regulation of inflammatory and immune signalling cascades. The multitude of autophagy-independent processes in diverse model organisms also clearly shows that the non-metazoan social amoeba *Dictyostelium* can only shed light on some of the multifaceted roles of ATG proteins in non-canonical autophagy. This exciting new facet of autophagy research deepens our understanding of autophagy-related functions and signalling pathways mediated by single ATG proteins as well as entire cellular processes using components of the autophagy machinery. Henceforth, it will be challenging to mechanistically separate autophagy from these related pathways.

Crosstalk between autophagy and the ubiquitin-proteasome system (UPS)

Until recently, the UPS and autophagy were considered to be two independent protein degradation machineries with no point of interaction, since both systems have different substrate preferences and separate molecular mechanisms (Korolchuk *et al.*, 2009, Korolchuk *et al.*, 2010). The proteasome is extremely efficient in degrading smaller short-lived, abnormal or damaged proteins, which require temporal control such as cell cycle-related proteins (Ciechanover and Kwon, 2015). In contrast larger long-lived proteins, protein aggregates or even whole organelles are delivered to and degraded in lysosomes via the autophagosomal route (Ciechanover and Kwon, 2015). However, the observation that more than 40 proteins are shared as either substrates or regulators of both autophagy and the UPS, among them core autophagy proteins like ATG5, ATG7, ATG8, and ATG16, has generated interest in analysing the crosstalk between these two pathways (Gao *et al.*, 2010, Komatsu *et al.*, 2005, Mizushima and Levine, 2010, Nam *et al.*, 2017, Xiong *et al.*, 2018). The most

crucial common denominator of both degradative pathways is ubiquitination as degradation signal on substrates (Kraft *et al.*, 2010). The mode of degradation is determined by the nature of ubiquitin chains, with mono-ubiquitinated substrates and K63-linked chains being preferably degraded by autophagy and K48-linked chains by the UPS (Kwon and Ciechanover, 2017). To connect ubiquitin and autophagy, selective autophagy requires ubiquitin-binding receptors such as p62/SQSTM1, NBR1 or HDAC6 (Schreiber and Peter, 2014). The strongest evidence for a functional interaction between the UPS and autophagy came from the finding that the autophagy pathway is activated in response to a decrease in UPS activity (Shen *et al.*, 2013). The up-regulation of autophagy genes is mainly mediated by the unfolded protein response (UPR) resulting in the activation of the transcription factor ATF4 and by mitochondria-originating ROS, which triggers autophagy through activated AMPK (B'Chir *et al.*, 2013, Zhao *et al.*, 2016). Whether autophagy inhibition triggers a compensatory increase of the UPS activity is still controversial. There have been contradictory reports describing compensatory up-regulation of the UPS, no change in UPS activity or even a decrease in the UPS activity (Korolchuk *et al.*, 2009, Liu *et al.*, 2016, Wang *et al.*, 2013). It must be noted that the majority of autophagosomal substrates are too large to be degraded through the cylindrical proteasome (Park and Cuervo, 2013). In recent years a number of studies reported that mice and *D. discoideum* strains lacking core autophagy genes, such as ATG5, ATG7, ATG8a, ATG12 and ATG16, showed an accumulation of ubiquitinated protein aggregates. In addition, the *D. discoideum* autophagy mutants displayed a significant decrease in their proteasomal activity in comparison to wild-type cells (Arhzaouy *et al.*, 2012, Fischer *et al.*, 2019, Komatsu *et al.*, 2005, Messling *et al.*, 2017, Mizushima and Levine, 2010, Xiong *et al.*, 2015). Interestingly, in mammals the half-life and cellular concentration of ATG8, ATG12 and ATG16 themselves appear to be regulated by the UPS and, *vice versa*, proteasomal subunits were found to be degraded by lysosomes (Cuervo *et al.*, 1995, Fujita *et al.*, 2009, Gao *et al.*, 2010, Haller *et al.*, 2014).

The close interconnection between the UPS and autophagy was further substantiated by the discovery of proteaphagy, a novel type of selective autophagy, in *S. cerevisiae*, HeLa cells and the mouse-ear cress *A. thaliana* (Cohen-Kaplan *et al.*, 2016, Marshall *et al.*, 2015, Marshall *et al.*, 2016, Waite *et al.*, 2016). In *A. thaliana*, the autophagic degradation of the 26S proteasome is mediated by the 19S proteasomal subunit RPN10 (PSMD4) via ubiquitin and ATG8 (Marshall *et al.*, 2015). In yeast the ubiquitin receptor Cue5 and the Hsp42 chaperone are responsible for degradation (Marshall *et al.*, 2016). In contrast, in mammals, stress-induced proteaphagy was dependent on p62/SQSTM1 and ubiquitin (Cohen-Kaplan *et al.*, 2016). It appears therefore that proteaphagy is universal in eukaryotes, however, the mechanism and the autophagy receptors vary from organism to organism. There is evidence in *D. discoideum* for a direct interaction of ATG16 with PSMD1 and PSMD2, two subunits of the 19S regulatory particle of the proteasome. It was also shown that PSMD1/2 get degraded in lysosomes. However, it is currently not clear how the interaction between ATG16 and PSMD1/2 is regulated and whether this interaction is responsible for proteaphagy in *D. discoideum* (Xiong *et al.*, 2018). Taken together, the data suggest that in autophagy-compromised *D. discoideum* strains less active or inactive proteasomes accumulate, which would cause the observed dramatic decrease in proteasomal activity (Arhzaouy *et al.*, 2012, Fischer *et al.*, 2019, Messling *et al.*, 2017, Xiong *et al.*, 2015).

Conclusions

The "professional" phagocyte *D. discoideum* is an extremely suitable, non-mammalian model organism for the study of autophagy in the context of a whole organism due to its unique life style, with vegetative unicellular and multicellular stages. The haploid genome of *D. discoideum* is completely sequenced and annotated, the generation of single or multiple gene replacement mutants and of strains expressing tagged proteins is generally straightforward and a rich collection of biochemical, cell biological and microscopical methods is available to study mutant phenotypes. These advantages and the close similarity of its autophagic system to higher eukaryotes including man make *D. discoideum* a perfect system for the investigation of unanswered questions in canonical autophagy, for the analysis of autophagy-independent roles of ATG proteins and also for the discovery of new players in the fascinating autophagy world.

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