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CELL SCIENCE AT A GLANCE

The LC3 interactome at a glance

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ABSTRACT

Continuous synthesis of all cellular components requires their constant turnover in order for a cell to achieve homeostasis. To this end, eukaryotic cells are endowed with two degradation pathways – the ubiquitin-proteasome system and the lysosomal pathway. The latter pathway is partly fed by autophagy, which targets intracellular material in distinct vesicles, termed autophagosomes, to the lysosome. Central to this pathway is a set of key autophagy proteins, including the ubiquitin-like modifier Atg8, that orchestrate autophagosome initiation and biogenesis. In higher eukaryotes, the Atg8 family comprises six members known as the light chain 3 (LC3) or γ-aminobutyric acid (GABA)-receptor-associated protein (GABARAP) proteins. Considerable effort during the last 15 years to decipher the molecular mechanisms that govern autophagy has significantly advanced our understanding of the functioning of this protein family. In this Cell Science at a Glance article and the accompanying poster, we present the current LC3 protein interaction network, which has been and continues to be vital for gaining insight into the regulation of autophagy.

KEY WORDS: Autophagy, LC3, GABARAP, Atg8, LIR motif, AIM

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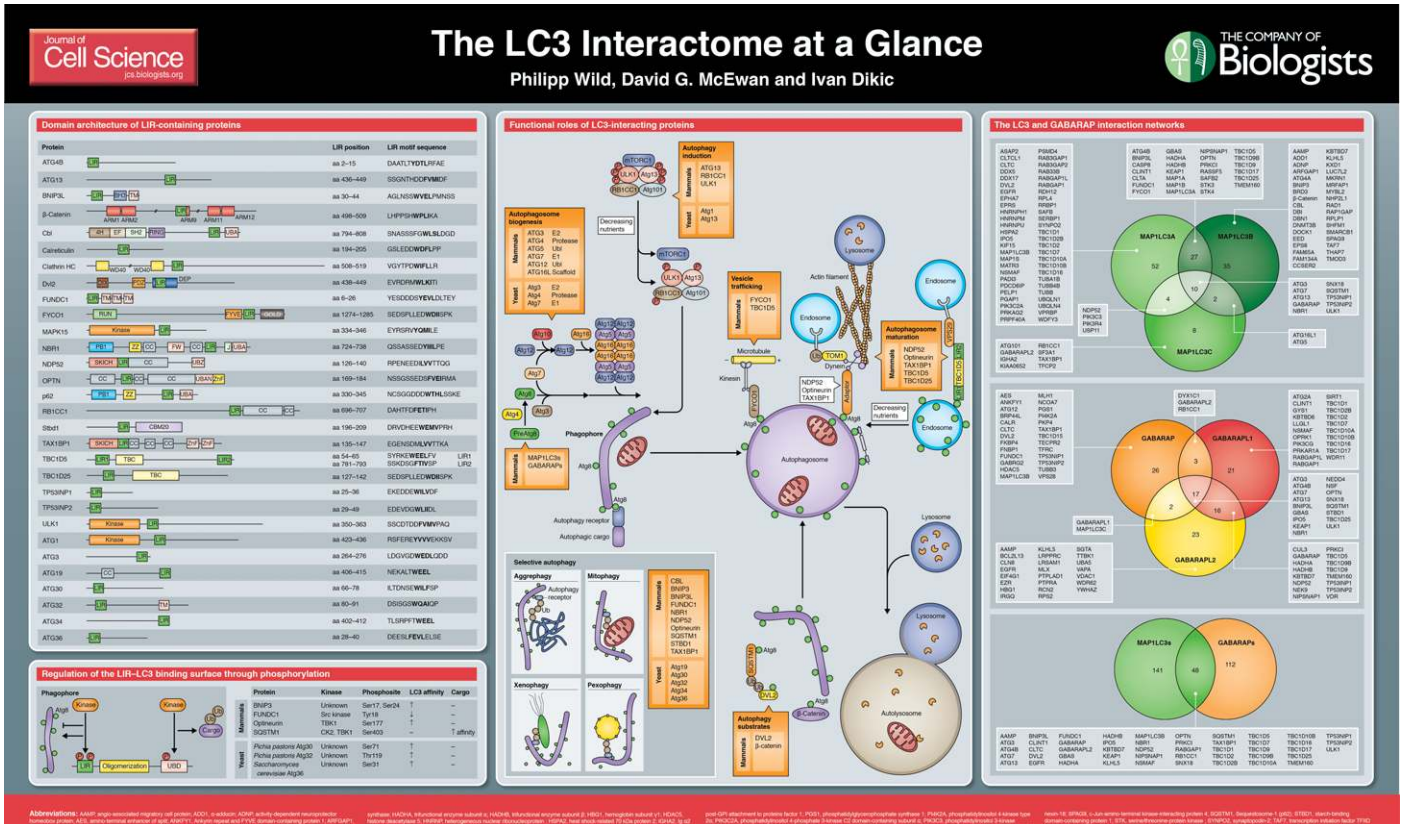
Introduction

Autophagy is an evolutionary conserved catabolic pathway that targets large protein complexes and organelles for degradation. Central to this process is the formation of double-membraned vesicles, autophagosomes, which sequester cargo and deliver it to the lysosome (Mizushima et al., 2008; Nakatogawa et al., 2009; Xie and Klionsky, 2007). This degraded material is subsequently recycled into the cytosol across the lysosomal membrane. As such, autophagy not only plays a vital role in achieving cellular protein homeostasis but also serves as a quality control system upon intracellular and extracellular stress conditions.



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Box 1. The lipid-conjugation machinery

An indispensable step for Atg8 and the mammalian orthologs to exert their function in autophagy is their covalent conjugation to the membrane lipid phosphatidylethanolamine (PtdEth). Similar to the ubiquitin ligases, Atg8 family members are initially processed by the cysteine protease Atg4 to expose a C-terminal glycine residue. Subsequently, attachment to PtdEth is mediated through the concerted action of the E1 enzyme Atg7 and the LC3- or GABARAP-specific E2-conjugating enzyme Atg3. The final step (the formation of the covalent bond between the carboxyl group of the glycine residue and the amino group of PtdEth) requires the Atg5–Atg12/Atg16 complex that acts as a scaffold for LC3 and GABARAPs – similar to the process for RING type E3 ubiquitin ligases – thus promoting their lipidation (see poster). Furthermore, as ubiquitylation, LC3 lipidation is reversible because the priming enzyme Atg4 also functions in the de-conjugation of LC3 from PtdEth. PtdEth-conjugated LC3 proteins (also known as LC3-II) localize to both sites of the isolation membrane and, importantly, remain associated with autophagosomes even after fusion with the lysosome, where they are degraded together with the captured material. This unique behavior and LC3–PtdEth conjugation are commonly exploited to monitor autophagy flux and autophagy induction or inhibition, respectively.

Although the initial discovery of autophagy dates back to the 1960s, its physiological importance has long been neglected, mainly owing to the lack of knowledge regarding its underlying molecular mechanisms and the view that cytosolic content is solely turned over in a random fashion (De Duve and Wattiaux, 1966). In the early 1990s, however, genetic screens using yeast mutants defective in autophagy led to the identification, to date, of 38 autophagy-related (Atg) genes, many of which are functionally conserved in higher eukaryotes (Nakatogawa et al., 2009; Tsukada and Ohsumi, 1993). Among these Atg genes, a subset of 17 genes constitutes the core autophagy machinery required for autophagosome formation and maturation. One of these genes encodes Atg8, which is characterized by a C-terminal ubiquitin-like domain that is preceded by a short N-terminal extension. The covalent attachment of Atg8 to phosphatidylethanolamine (PtdEth) at the autophagosomal membrane places it at a crucial juncture during autophagosome formation and cargo recruitment (Shpilka et al., 2012). Interestingly, the sole yeast Atg8-encoding gene has six human homologs comprising the microtubule-associated protein-1 light chain 3 (MAP1LC3) family (MAP1LC3A, MAP1LC3B, MAP1LC3C; short names LC3A, LC3B, LC3C, respectively, and collectively LC3) and the γ-aminobutyric acid (GABA)-receptor-associated proteins (GABARAP, GABARAPL1, GABARAPL2). The biological relevance of the expansion of Atg8 proteins in higher eukaryotes is largely unknown. In this Cell Science at a Glance article and the accompanying poster, we provide an overview of the currently established LC3 interactome based on the annotated interactions listed in the BioGRID, Mint and STRING databases. In addition, we also conducted a primary literature search on PubMed to account for any interaction partners that had been omitted. A comprehensive list of all reported Atg8 interactors can be found in the supplementary material Table S1.

The six faces of the Atg8 family

The Atg8 protein family shares a common ubiquitin-like fold that is preceded by two N-terminal a-helices, which vary among the LC3 and GABARAP subfamilies, with this diversification of ubiquitin-like modifiers (Ubls) only found in metazoan animals and plants (Kabeya et al., 2000; Sagiv et al., 2000; Wang et al., 1999; Xin et al., 2001). These proteins are expressed ubiquitously in all tissues and display only slight variations between each family member (Kabeya et al., 2000; Sagiv et al., 2000; Wang et al., 1999; Xin et al., 2001). For example, the less abundant LC3C has pronounced expression in the lung, whereas GABARAPL1 and GABARAPL2 are predominant in the central nervous system (Xin et al., 2001). LC3B, the most extensively studied Atg8 protein to date, was initially reported to co-purify with microtubule-associated protein 1A and 1B and was proposed to influence their binding to microtubules (Kuznetsov and Gelfand, 1987; Mann and Hammarback, 1994). The GABARAP subfamily was first implicated in membrane trafficking processes. For instance, GABARAP and GABARAPL1 are involved in the translocation of transmembrane receptors from the Golgi complex to the plasma membrane (Chen et al., 2006; Leil et al., 2004), whereas GABARAPL2 participates in ER-to-Golgi as well as in intra-Golgi transport (Legesse-Miller et al., 1998). Supporting this notion is the reported interaction of the entire GABARAP subfamily with the SNARE fusion machinery-associated AAA ATPase *N*-ethylmaleimide-sensitive fusion protein (NSF), although their precise mode of action in intracellular protein trafficking remains to be elucidated (Chen et al., 2006; Kittler et al., 2001; Sagiv et al., 2000).

The lack of Atg8 paralogs in yeast leads to functionally defective autophagy when Atg8 is deleted, highlighting its crucial role during autophagy (Nakatogawa et al., 2009; Tsukada and Ohsumi, 1993). Since then, it has been shown that the Atg8 family members play a vital role during autophagosome biogenesis because these proteins are directly conjugated to lipids by the ubiquitin-like conjugation machinery (see Box 1 and poster).

However, the question that intrigues most researchers is why numerous LC3 and GABARAP proteins participate in autophagy in an apparently redundant manner. Weidberg et al. addressed the potentially distinct roles of LC3B and GABARAPL2 and suggested that LC3s act at an early stage of autophagy, during autophagosome elongation, whereas, further downstream, GABARAPs mediate autophagosome maturation, possibly involving the dissociation of the ATG5–ATG12/ATG16L1 complex (Weidberg et al., 2010). This study, however, did not address the contribution of the other LC3 and GABARAP subfamily members individually (i.e. LC3A, LC3C, GABARAP and GABARAPL1). During autophagosome biogenesis, LC3–PtdEth has been proposed to control the size of autophagosomes through its membrane tethering and hemifusion activities (Weidberg et al., 2011). Moreover, LC3 proteins play a key role in the selective recruitment of autophagic cargoes into autophagosomes, and serve as docking sites for adaptor proteins. Notably, both the core of the ubiquitin fold and N-terminal residues in the extension are indispensable for LC3-mediated cargo recognition.

Characteristics of the LC3-interacting region

Many of the experimentally verified LC3-binding proteins contain a short hydrophobic LC3-interacting region (LIR), which in yeast is more commonly referred to as the Atg8-interacting motif (AIM) (Pankiv et al., 2007). The LIR sequence was first identified in the autophagic receptor p62/SQSTM1 (see below) and in yeast Atg19, and since then been established based on multiple LIR-containing interaction partners. The LIR motif is a WxxL sequence,

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N-terminally preceded by negatively charged residues (for examples, see poster) in which the aromatic residue appears to be the most crucial determinant (Alemu et al., 2012). The aromatic and the aliphatic amino acid in the last position are accommodated within two hydrophobic pockets (W and L pockets) of the LIR-docking site (LDS) on the Atg8 family member (Ichimura et al., 2008; Noda et al., 2008). A tryptophan residue is energetically favored over tyrosine or phenylalanine residue, whereas the lower affinity tyrosine or phenylalanine residue can be compensated for by the presence of acidic residues and/or or serine/threonine phosphorylation sites preceding the hydrophobic core motif. These have been shown to engage in electrostatic interaction with basic residues in the N-terminal extension and the Ubl domain of LC3 (R10, R11, K49 and K50) (Pankiv et al., 2007; Rogov et al., 2013; Shvets et al., 2008). It is worth noting that phospho-regulation of the LIR–LC3 interaction is reminiscent of that between small ubiquitin-related modifier (SUMO) and the SUMO-interacting motif (SIM) (Chang et al., 2011a; Hecker et al., 2006; Stehmeier and Muller, 2009). One atypical LIR motif regulates the interaction of the autophagy receptor NDP52 (also known as CALCOCO2) with LC3C. Consisting solely of the three aliphatic amino acids, LVV (termed CLIR), CLIR lacks the aromatic residue that typical LIRs contain. Although the LDS of LC3C enables its interaction with common LIR sequences, the CLIR appears to have evolved as an LC3C-exclusive binding motif, as rotation of the CLIR β-strand with respect to the canonical orientation creates additional hydrogen bonds with the LC3C surface (von Muhlinen et al., 2012). Finally, it should be pointed out that an interaction with Atg8 family members does not necessarily require a LIR sequence. Characterization of the Atg8 network, established by Behrends et al. by using LDS mutants, revealed that a substantial fraction of LC3-interacting proteins bind to LC3B and GABARAP independently of the LDS (Behrends et al., 2010).

Members of the core autophagy machinery that contain a LIR motif

The initial events of autophagosome formation are regulated by the mammalian serine/threonine UNC-51-like kinase (ULK) complex, which is functionally equivalent to the yeast Atg1 kinase complex. Recently, it has been shown that both ULK1 (as well as ULK2) and Atg1 kinase harbor canonical LIR motifs, which facilitate their association with autophagosomes (Alemu et al., 2012; Kraft et al., 2012; Nakatogawa et al., 2012). In the case of Atg1, this also results in its LIR-dependent degradation in the vacuole. Interestingly, Atg8-binding deficient variants of Atg1 are not impaired in the induction of autophagosome formation, yet overall autophagy is decreased, suggesting a role for Atg1 in later stages of autophagy (Nakatogawa et al., 2012). In addition, the ULK1 complex components Atg13 (yeast) and RB1CC1/FIP200 (mammals) contain functional LIR motifs that ensure the ULK complex is firmly tethered to the nascent autophagosome. Detailed analyses revealed that ULK complex members preferentially interact with the GABARAP subfamily members rather than the LC3 proteins, although the functional relevance of this remains elusive (Alemu et al., 2012).

The E2-like enzyme Atg3 (known by this name in both yeast and mammals) is another LIR-containing core autophagy component (Behrends et al., 2010; Yamaguchi et al., 2010). Interestingly, the functionally validated interaction of yeast proteins revealed a highly conserved LIR motif in Atg3 that is absent in the mammalian counterpart. Consistently though, this LIR sequence appears to be essential for the yeast-specific cytoplasm-to-vacuole (Cvt) pathway, but dispensable for starvation-induced autophagy (Yamaguchi et al., 2010). Remarkably, the Cvt pathway serves, as opposed to all other types of autophagy, a biosynthetic role by selectively delivering precursor enzymes to the vacuole.

Interactions of the Atg8 family members with all other components of the Atg8 lipidation system (ATG4, ATG5, ATG7, ATG16L1) have been reported (Behrends et al., 2010); however, the interaction of LC3 with the E1-like Atg7 and the cysteine protease Atg4 has been well characterized. The latter serves two roles during autophagosome biogenesis: (1) it primes Atg8 for conjugation to PtdEth by exposing its C-terminal glycine residue and (2) it retrieves PtdEth-conjugated Atg8–LC3B by cleaving off the PtdEth moiety. Crystal structures of the Atg4–LC3B complex in comparison to free Atg4 revealed that the LC3-bound enzyme undergoes a conformational change, which helps LC3B to reach out into the catalytic center of Atg4 (Satoo et al., 2009). Although experimental evidence is currently lacking, it is conceivable that this LIR motif-dependent interaction may promote the recruitment of Atg4, as well as its activity to removing the lipid moiety from Atg8.

Autophagy receptors

Autophagy had long been thought to be a bulk process without any apparent substrate selectivity. However, the identification of the first mammalian selective autophagy receptor p62/SQSTM1 sparked a deluge of new interest in selective autophagy (Bjørkøy et al., 2005; Komatsu et al., 2007; Pankiv et al., 2007). Selective autophagy refers to the specific removal of proteins, protein aggregates (aggrephagy), ribosomes, organelles (e.g. mitophagy, the selective autophagy of mitochondria, and pexophagy, the selective autophagy of peroxisomes) and bacteria (xenophagy), by the cell (Kirkin et al., 2009a; Komatsu et al., 2007; Kraft et al., 2010; Pankiv et al., 2007). Autophagy receptors are proposed to have a major role in selective autophagy by tethering cargo to the site of engulfing autophagosomes through their direct interaction with Atg8 homologs. Since the discovery of p62/SQSTM1, additional autophagy receptors have been characterized in both mammals and yeast (i.e. BNIP3, BNIP3L, FUNDC1, NBR1, NDP52, optineurin). A feature they all have in common is their ability to interact simultaneously with both autophagosomes through an interaction of their LIR motif with LC3, and the cargo substrate, which is often ubiquitylated. Indeed, several of the known receptors possess a ubiquitin-binding domain (UBD) that can sense different ubiquitin chain linkages and confers selectivity to the cargo that is destined for degradation, such as UBA domain in p62/SQSTM1 and NBR1, the ubiquitin-binding zinc finger in NDP52 and the ‘ubiquitin binding in ABIN and NEMO’ (UBAN) domain in optineurin (Kirkin et al., 2009b; Thurston et al., 2009; Wild et al., 2011). Additional domains might cooperatively target autophagy receptors to their respective cargoes. For instance, it has been demonstrated that the combination of both the non-specific membrane-binding J domain and the UBA domain of NBR1 facilitates its specific recruitment to and subsequent clustering of peroxisomes (Deosaran et al., 2013). Furthermore, the translocation of NDP52 to vacuole-escaped cytosolic *Salmonella* is initially triggered by its binding to galectin-8, which senses β-galactoside-containing glycans that are naturally absent in the cell interior (Thurston et al., 2012). Subsequently, ubiquitylation of the bacterial surface provides a key signal for the recruitment of three xenophagy receptors NDP52, p62/SQSTM1 and optineurin

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(Thurston et al., 2009; Wild et al., 2011; Zheng et al., 2009). In contrast, mitophagy receptors (Atg32 in yeast; BNIP3, BNIP3L and FUNDC1 in mammals) are outer mitochondrial membrane proteins that are already placed and primed for autophagic clearance of mitochondria (Hanna et al., 2012; Kanki et al., 2009; Liu et al., 2012; Novak et al., 2010; Okamoto et al., 2009). However, during Parkin-mediated mitophagy, outer mitochondrial surface proteins, such as VDAC1 or mitofusin, become ubiquitylated and BNIP3 has been shown to affect the translocation of Parkin to stressed mitochondria.

The specificity of the LIR motif in autophagy receptors towards certain LC3 or GABARAP family members still remains an open issue. For example, the LIR sequence in NDP52 is optimized for binding to LC3C, whereas BNIP3L has been shown to interact with GABARAPL1 and only weakly with LC3B (von Muhlinen et al., 2012; Novak et al., 2010). Moreover, LC3 conjugation could be the factor that determines specificity, as it has been reported that p62 interacts with both GABARAPL2 and LC3B in their unlipidated form, but only the interaction with LC3B remains intact once attached to autophagosomes (Shvets et al., 2011). By comparing the binding of 28 LC3 and GABARAP interaction partners to either free or conjugated LC3 and GABARAP proteins, Behrends et al. found that 15 show either no difference or an enhanced association with the lipid-conjugated Atg8 proteins, whereas 13 interaction partners, including Atg3 and Atg7, display decreased affinity to the lipid-conjugated Atg8 proteins (Behrends et al., 2010). To clearly elucidate the specificity towards the different LC3 and GABARAP family members will require a concerted effort to determine how these family members are utilized (for instance, depending upon the stimulus) and the underlying molecular mechanisms.

LC3-binding proteins involved in vesicle trafficking and autophagosome maturation

The processes of autophagy and endosome maturation possess striking resemblances and, as such, also share multiple interaction partners that facilitate a crosstalk between both pathways. At the heart of endocytic pathways lie the small Rab GTPases that enable effector protein interaction, trafficking and fusion within the endocytic pathway. Therefore, some of these GTPases can also influence the autophagy pathway. For example, degradation of autophagy substrates is inhibited when the dominant-negative (constitutively GDP-bound) form of the late endosome/lysosome small GTPase Rab7 is overexpressed (Gutierrez et al., 2004). The Rab7-interacting protein FYCO1 (FYVE and coiled-coil domain containing protein 1) localizes to late endosomes and autophagosomes and interacts with LC3 and phosphatidylinositol 3-phosphate (PtdIns3*P*). Its interaction with LC3 is mediated by a W-type LIR, which is located between the FYVE and GOLD domains, and results in exclusive binding to PtdIns3*P* and coupling to kinesin motor proteins (Pankiv et al., 2010).

Another small GTPase that is involved in autophagy is the Golgi-resident Rab33B that interacts with ATG16L1 and is involved in recruitment of the ATG12–ATG5/ATG16 complex to pre-autophagosomal structures (Itoh et al., 2008). Small GTPases are activated through a cycling between their GDP- (‘off’) and GTP-bound (‘on’) state. They are switched off through the activation of their intrinsic GTPase activity, whereby GTP is converted into GDP, by the action of GTPase-activating proteins (GAPs). One family of GAPs is the TBC (Tre2, Bub2, Cdc16) family, which consists of ∼36 members, of which 14 can interact with LC3 and/or GABARAP proteins. TBC1D25, through its GAP activity towards Rab33B, can regulate the maturation of autophagosomes through a direct interaction with Atg8 homologs and its GAP activity towards Rab33B (Itoh et al., 2011). TBC1D5 is involved in retrograde trafficking and contains two LIR motifs, one at the N-terminus and the other at the C-terminus and both are required for efficient binding to, and co-localization with, mammalian Atg8 proteins (Popovic et al., 2012). Interestingly, its N-terminal LIR can also interact with VPS29, a component of the retromer complex, and can be out-competed by increasing concentrations of LC3, implicating TBC1D5 as a molecular switch between endosomal trafficking and starvation-induced autophagy (Popovic et al., 2012).

Post-translational modifications in the regulation of LC3 interactions

Structural studies of the LIR motif of p62/SQSTM1 in complex with LC3 have revealed that the acidic amino acids N-terminal of the hydrophobic core motif aid the LC3–LIR interaction. The LIR consensus sequence supports this notion, as acidic amino acids clearly predominate the upstream flanking region of the majority of known LIR motifs (Birgisdottir et al., 2013; Noda et al., 2008). Additionally, serine or threonine residues can be found directly preceding the critical aromatic amino acid in ∼25% of identified LIR sequences (Birgisdottir et al., 2013). These residues are prime candidates for post-translational modification (PTM) regulated activation (or inactivation) of autophagy receptors that can proceed rapidly and in a compartmentalized manner (McEwan and Dikic, 2011). Indeed, we have shown that phosphorylation of optineurin at Ser177 (the LIR is at 178–181) enhances its interaction with LC3B (Wild et al., 2011), which was confirmed by nuclear magnetic resonance (NMR) and crystallography studies that showed that the negatively charged phosphate group makes additional contacts with Lys51 and Arg11 in the N-terminal extension of LC3B (Rogov et al., 2013). Similarly, the yeast mitophagy and pexophagy receptors, Atg32 and Atg36 respectively, are regulated by phosphorylation of serine/threonine residues adjacent to their LIR motifs (Farré et al., 2013). In addition, the phosphorylation of both serine residues (Ser17 and Ser24) that flank the LIR motif of Bnip3, enhances its binding to Atg8, resulting in mitochondrial clearance. Conversely, in its unmodified state Bnip3 induces apoptosis (Zhu et al., 2013).

Importantly, phosphorylation of the LIR motif can also abrogate the interaction with LC3-interacting proteins. A subset of known LIR motifs contains a tyrosine residue in place of tryptophan, and phosphorylation at this site could potentially abrogate the interaction with Atg8 family members. Indeed, phosphorylation of the FUNDC1 LIR motif (YEVL) keeps it in an inactive state, whereas its dephosphorylation, which occurs under hypoxia, allows it to interact with Atg8, thus enabling the removal of stressed or damaged mitochondria by mitophagy (Liu et al., 2012).

Of note, phosphorylation sites in two members of the autophagy Ubl conjugation system, namely LC3A and LC3B, have been identified within their N-termini. The physiological role of these phosphorylation events needs further investigation but it appears to negatively affect LC3 function by inhibiting LC3 recruitment into autophagosomes (Cherra et al., 2010).

Interestingly, not only the LIR–LC3 interaction but also the interaction of autophagy receptors with their substrate can be regulated by phosphorylation. For example, phosphorylation of Ser403 within the UBA domain of p62 increases its affinity for ubiquitylated cargo, thereby promoting the degradation of autophagic substrates (Matsumoto et al., 2011). Taking into

account the above-discussed SUMO–SIM and LC3–LIR interactions, it is therefore conceivable that phosphorylation of Ubl-binding motifs as well as UBDs constitute a general mechanism to control binding to proteins that harbor a ubiquitin fold and regulate their trafficking, subcellular localization and/or degradation.

Conclusions and perspectives

The majority of the assembled LC3 interactome stems from high-throughput interaction studies and in particular the mammalian LC3 and GABARAP network that has been conducted by the Harper laboratory (Behrends et al., 2010). One should bear in mind that many of these reported interactions have not yet been substantiated biochemically and/or functionally. Although the very first studies of the LC3 proteins did not link this family to autophagy, the current LC3 interactome bears a strong bias towards this pathway. It is therefore important to not neglect autophagy-unrelated LC3 interacting partners and their functions, which are currently investigated less because of the overshadowing interest in autophagy. Having said this, the presented LC3 network is far from complete; in particular, fully elucidating PTM-driven interactions will require a substantial effort to determine the LC3-interaction network in time and space, and under various stress conditions (e.g. starvation, hypoxia, DNA damage, mitochondrial stress). There is also a clear lack of knowledge with regard to the diversification of the Atg8 family in higher eukaryotes, which most likely reflects both redundancy and functional specialization. Thus, future studies should be aimed at deciphering the role of the individual LC3/GABARAP proteins in autophagy.

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Competing interests

The authors declare no competing interests.

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