ATG4 as a drug target for autophagy modulation in disease

Student: Stefano Lara González
Student number: S3027929
Master: Medical Pharmaceutical Sciences (MPS)
Course: Essay
Supervisor: Prof. Dr. Fulvio Reggiori
Department: Cell Biology, UMCG

1. Index

2. Abstract

Autophagy is a survival mechanism that is present in all eukaryotic cells, and it is positively regulated under stress conditions, e.g. during nutrient or oxygen deprivation. It has been linked to a number of diseases, including cancer, neurological disorders and infectious diseases. As a result, its regulation provides an interesting approach to target such diseases. The accurate modulation of autophagy is a challenging task, as many different mediators play a role in this pathway. Among these mediators, the ATG4 cysteine proteases show a lot of potential, as they are essential for autophagy, playing key roles in both the conjugation and deconjugation processes. Nonetheless, targeting ATG4 in order to modulate autophagy has shown to be of high complexity because it is tightly regulated by an intricate network of components, and further research is needed to help elucidate how the whole machinery works coordinately. Consequently, I mainly focused in this essay on the state of the art research regarding ATG4 functions, regulation and drug discovery.

3. Introduction

Autophagy is a genetically regulated, homeostatic multi-step degradation process that occurs in all eukaryotic cells as a survival mechanism.[1] In this process, damaged or superfluous organelles and long-lived proteins are catabolized during conditions of stress such as nutrient deprivation, hypoxia or drug treatment in order to produce ATP and sustain cell viability.[2] Autophagy can be divided into microautophagy and macroautophagy, both of which include nonselective and selective processes. In microautophagy, material is directly taken up by the corresponding degradative compartment (lysosome in mammals and vacuole in fungi and plants) via invagination of its limiting membrane, which leads to the formation of single-membrane vesicles that are released inside the vacuole/lysosome lumen for subsequent degradation.[3] In macroautophagy, on the other hand, there is a formation of a pre-autophagosomal structure (PAS), also known as phagophore assembly site, which is the precursor of an isolation membrane (or phagophore), a cistern that elongates and matures into an autophagosome (Figure 1). During its expansion, the isolation membrane sequesters cytoplasmic structures (Figure 1). Subsequently, complete autophagosomes fuse with vacuoles/lysosomes, where the sequestered cytoplasmic components are degraded by hydrolases (Figure 1). Finally, the resulting metabolites are released in the cytoplasm for recycling via efflux permeases (Figure 1).[4]

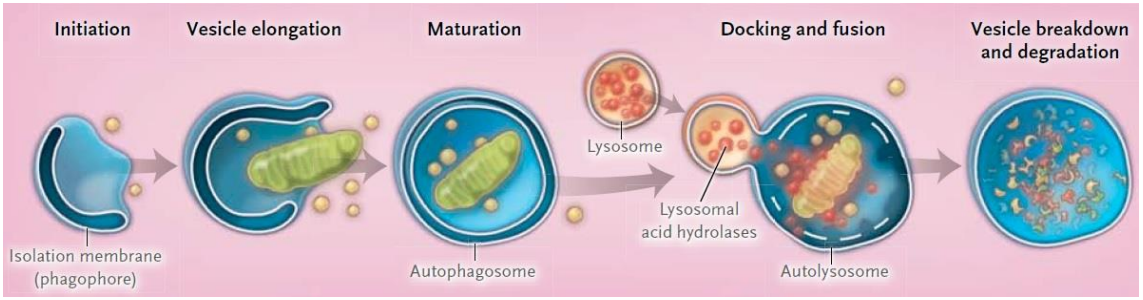

Figure 1: The macroautophagic pathway[4]

4. Physiological functions and pathological relevance of autophagy

Although the importance of autophagy is easily recognizable, it was not until that the AuTophaGy-related genes (*ATG*) were discovered in yeast, followed by the identification of homologues in higher eukaryotes, that the in-depth study of its physiological and pathological roles became possible.[5] It is now known that autophagy can degrade, among others, mitochondria (mitophagy), microorganisms (xenophagy), endoplasmic reticulum (reticulophagy), peroxisomes (pexophagy) and aggregated proteins (aggrephagy).[6] Autophagy has been associated to several physiological processes in mammals, which include maintenance of cellular and tissue homeostasis, intracellular quality control, cell differentiation and development, anti-aging, and innate and adaptive immunity.[7] Because of this, it is imaginable that any dysregulation in autophagy could eventually lead to the development or worsening of human diseases, and in the past two decades this pathway has indeed been linked to a number of pathologies. It has been found that it plays a role in the initiation and progression of cancer, where it provides tumor cells under metabolic stress, caused by poor vascularization and high proliferation rate, a survival advantage.[8] It has also been shown that it is dysregulated in the pathogenesis of neurodegenerative disorders, where faulty clearance mechanisms causes mitochondrial dysfunction and protein aggregates accumulation.[9] In Alzheimer's disease, for example, there is an increase in the accumulation of phagosomes.[10] Autophagy has also been linked to infections because it degrades many medically relevant pathogens—such as *streptococcus pyogenes* or *Mycobacterium tuberculosis*[11]—and, therefore, any impairment in the autophagic pathway could lead to an increased susceptibility for bacterial and viral infections.[12] Due to its link to various diseases, autophagy has increasingly become an attracting therapeutic target.

It is important to mention that, as the *ATG* genes products are highly conserved from yeast to higher eukaryotes, as a results finding obtained in yeast are highly relevant for human medicine.[3] Furthermore, the unified nomenclature for yeast autophagy-related genes[13] will be used in this article.

5. The mechanism and regulation of autophagy

The *ATG* genes encode for the Atg proteins, which compose the basic protein machinery required for autophagosome biogenesis and thus autophagy. Selective microautophagy and macroautophagy share most of the same machinery[3], while the machinery involved in nonselective microautophagy has not been well defined. The information related to macroautophagy will be discussed in this essay. There is a subset of Atg proteins that is essential for autophagosome formation, referred to as the 'core' Atg machinery.[14] Five major functional groups are included in this machinery: the Atg1 kinase complex, the class III phosphatidylinositol 3-kinase (PtdIns3K) complex I, two ubiquitin-like protein conjugation systems and the Atg9 complex.[15] In recent years, different forms of noncanonical autophagy have been discovered, which do not rely on all the components of the core Atg machinery.[7] The functions of the Atg proteins, along with the signaling pathways that regulate them, are described below, functionally relating them to the different steps of the autophagic pathway.

a. Autophagy induction

The Atg1 kinase complex, which is formed by Atg1, the regulatory protein Atg13, and the scaffold Atg17-Atg31-Atg29 subcomplex, is crucial for autophagy induction, as it participates in the

recruiting of other Atg proteins towards the PAS and in the activation of downstream targets via phosphorylation.[16] The Atg1 complex is activated by intracellular signaling cascades. One of the most characterized ones, is the one involving the inhibition of the target of rapamycin complex 1 (TORC1) as a consequence of nutrient starvation, in particular amino acid and/or nitrogen shortage (Figure 2).[17] The Atg1 complex is also activated when energy depletion is sensed by the Snf1/AMP-activated protein kinase (AMPK) (Figure 2).[4] On the other hand, protein kinase A (PKA) becomes activated by binding cAMP when glucose is present, leading to a negative regulation of the Atg1 kinase complex via phosphorylation of Atg1 and Atg13, which prevents localization of Atg13 to the PAS.[18] In mammals, growth factors, through stimulation of the class I PtdIns3K-AKT pathway, activate the mammalian TORC1 (mTORC1), which in turn negatively regulates the Unc-51-like kinase 1 (ULK1) complex (ULK1 is the mammalian homologue of yeast Atg1) (Figure 2).[4]

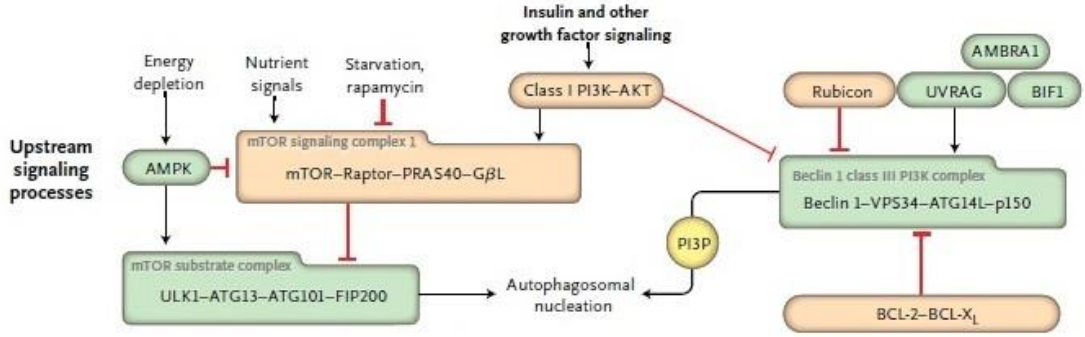

Figure 2: Machinery and regulation of the macroautophagic induction and nucleation in mammals.[4]

b. Autophagosomal membrane nucleation

The autophagosomal membrane nucleation is the process in which different membranous elements, possibly vesicles of different origin, needed for phagophore formation are recruited to the PAS upon autophagy induction, being a fundamental one the class III PtdIns3k complex I. This complex, which is composed of the lipid kinase vacuolar protein sorting (Vps) 34 (catalytically active subunit), the regulatory kinase Vps15, Vps30/Atg6, Atg14 and Atg38[7], uses phosphatidylinositol (PtdIns) to produce PtdIns-3-phosphate (PtdIns3P), which is important for the correct localization of the Atg2-Atg18 complex and elements of the two ubiquitin-like conjugation systems. This step allows the recruitment of Atg8, Atg9 and Atg12 to the PAS, all of them relevant proteins for phagophore expansion.[19] In mammals, the class III PtdIns3k complex I, called the Beclin 1 (ortholog of yeast Vps30/Atg6)-VPS34 complex, can be regulated by different cofactors: ultraviolet radiation resistance-associated gene protein (UVRAG), Bax-interacting factor 1 (BIF1) and activating molecule in BECN1-regulated autophagy protein 1 (AMBRA1) positively regulate its activity[20], while stimulation of the class I PtdIns3K-AKT pathway in response to growth factors, the Beclin 1-interacting protein Rubicon and the apoptosis-regulating proteins B-cell lymphoma 2 (BCL-2) and B-cell lymphoma extra-large (BCL-xL) negatively regulate this complex (Figure 2).[4]

c. Phagophore elongation

Two ubiquitin-like (Ubl) conjugation complexes, which involve the Ubl proteins Atg12 and Atg8, are essential for phagophore elongation and autophagosomal maturation.[21] Atg12 is

conjugated to Atg5 via the action of the ubiquitin-activating enzyme (E1) Atg7 and the ubiquitin-conjugating enzyme (E2) Atg10 (Figure 3). The resulting Atg12–Atg5 conjugate forms the Atg12–Atg5-Atg16 complex by noncovalent binding to Atg16, promoting the generation of an Atg12–Atg5-Atg16 complex dimer via Atg16 self-interaction (Figure 3). Atg8, on the other hand, is covalently conjugated to the phospholipid phosphatidylethanolamine (PE) in a process that involves the cysteine protease Atg4, the E1 enzyme Atg7 and the E2 enzyme Atg3. The Atg12–Atg5-Atg16 complex dimer is proposed to function as an E3 enzyme, but it is not absolutely required for conjugation to occur (Figure 3).[3] Interestingly, recent studies have shown that, at least in mammalian cells, the Ubl conjugation systems are not an absolute requirement for autophagosomal membrane biogenesis, although they play a very important role in promoting its efficient closure.[22] A detailed mechanism through which the conjugation systems elongate the phagophore and reach autophagosomal maturation is a topic currently being researched and the role that Atg4 plays in these processes is described in detail in chapter 4 of this essay.

Besides the Ubl conjugation complexes, the transmembrane protein Atg9 has been found to function as a membrane donor for the elongating phagophore, although there are no studies that directly prove this.[7] Nonetheless, this idea is supported by different facts: Atg9 is the only transmembrane protein that is essential for phagophore elongation,[23] it is highly mobile in the cytosol after rapamycin treatment[24], and it is capable of self-interaction, transiting to the PAS, apparently, as part of a complex[25]. Some of the components involved in Atg9 trafficking are Atg11, Atg23 and Atg27, which are thought to interact with Atg9 and be needed for its efficient transport to the PAS.[7]

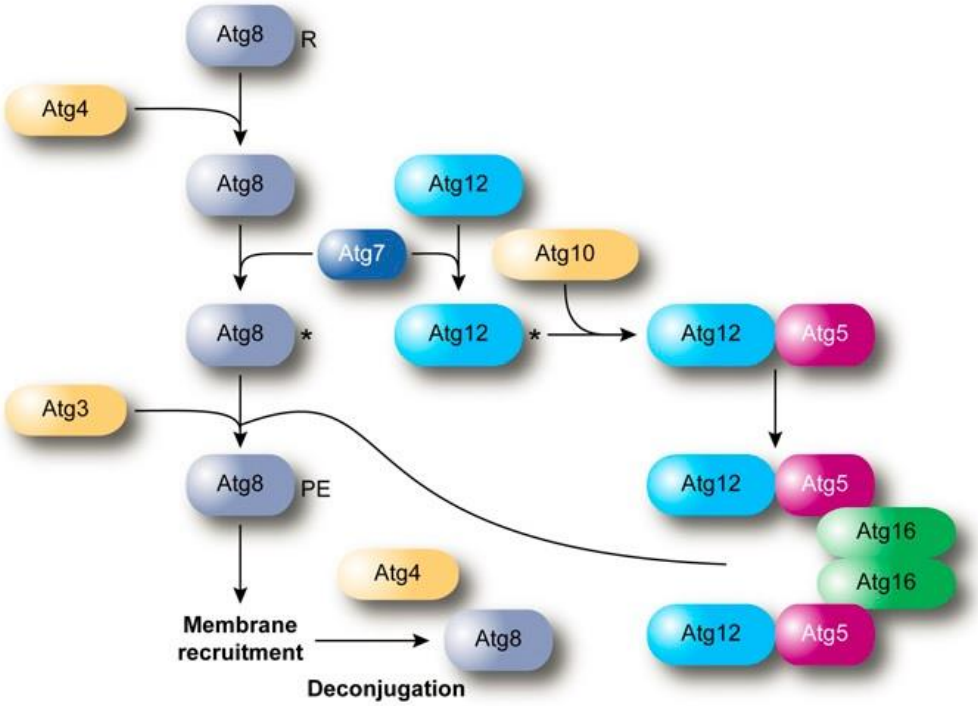

Figure 3: Schematic depiction of the ubiquitin-like conjugation systems.[3]

d. Autophagosome fusion

Autophagosome fusion is the process in which the outer membrane of the autophagosome docks and fuses with the vacuole/lysosome, forming an autophagic body/autolysosome. The mechanism controlling the timing of fusion is not fully elucidated, but there is knowledge about how premature fusion between the autophagosome and the vacuole is prevented.[7] For example, the deconjugation of Atg8–PE by Atg4 in yeast is a required step for fusion to occur[26], as this cleavage event apparently triggers autophagosome maturation. The maturation process is defined as the step preceding fusion where all of the Atg proteins are removed from the autophagosome surface after the closure of the isolation membrane.[27]

e. Cargo degradation and recycling

After fusion, the single-membrane vesicle engulfing the cargo is consumed by the putative lipase Atg15[28], and the cargo is subsequently degraded by hydrolases. The macromolecules produced are transported back to the cytosol through different efflux permeases, which include Atg22.[29]

6. The ATG4 cysteine proteases

a. ATG4 in autophagy modulation

Atg4 is involved in both the conjugation and deconjugation of Atg8 to PE (Figure 3).[3] The conjugation process, which is essential for autophagosomal formation, starts with the post-translational cleavage of an extra sequence at the C-terminus of a pro-ATG8 by Atg4, which generates the Atg8-I form. This form of yeast Atg8 possesses an exposed glycine on position 116 (Gly116) at the C-terminus.[4] Upon the induction of autophagosome formation, an amide bond is formed between Atg8-I and PE, which is present in the autophagosomal membranes, via the sequential action of Atg7, Atg3 and the Atg12–Atg5-Atg16 complex dimer (Figure 3).[3] This lipidation step is crucial for phagophore elongation, as studies have shown that, for example, the amount of Atg8 directly correlates with the size of the autophagosome[30], or that unclosed isolation membranes with abnormal morphology accumulate when the lipidation step is blocked by overexpression of an Atg4 dominant-negative mutant[31]. Atg8 is indispensable to normal development of the isolation membrane most likely because it has membrane tethering and hemifusion activities.[32] The Atg8 deconjugation takes place on autophagosome membranes when Atg4 hydrolyzes the Atg8–PE amide bond, which appears to occur together with the removal of PtdIns3P from the surface of the autophagosome by PtdIns3P phosphatases. This step is required for autophagosome maturation and fusion.[3327] It is important to mention that the Atg4 protease is not found at the PAS[34], and thus it must be recruited there. By expressing an Atg8 mutant that exposes the glycine residue at the C-terminus and therefore emulates the Atg8-I form, in the *atg4Δ* knockout strain, a previous study showed that when the deconjugation of Atg8–PE is blocked, smaller and fewer autophagosomes are produced and fusion is also defective.[26] Atg4 also recycles the Atg8–PE generated on inappropriate membranes to maintain a cytoplasmic pool of non-lipidated Atg8, which can re-enter the conjugation process and is required for formation of new autophagosomes at the PAS.[34]

While only one Atg4 is present in yeast, four ATG4 homologues (ATG4A to ATG4D) are present in mammalian cells[35]. There are six human ATG8 homologues, all of them having a

conserved ATG4 cleavage site[35], and they belong either to the microtubule-associated protein 1 (MAP1) light chain 3 (LC3) protein subfamily (LC3A, LC3B and LC3C) or to the γ-aminobutyric acid receptor-associated protein (GABARAP) protein subfamily (GABARAP, GATE-16 (Golgi-associated ATPase enhancer-16)/GABARAPL2, and ATG8L/GABARAPL1) (Figure 4).[36] These ATG8 protein subfamilies seem to play different roles, i.e. while the members from the LC3 subfamily are involved in the elongation of the phagophore membrane and in the selective incorporation of specific cargo molecules into the autophagosome[37], the members from the GABARAP subfamily are involved in the maturation of the autophagosome.[38] Nonetheless, when conjugated, both LC3 and GABARAP proteins act as scaffolds for the recruiting of different autophagosome-associated proteins to the phagophore[39]. These proteins have ATG8 interacting region motifs, and are most likely dissociated and recycled when ATG4 mediated deconjugation takes place.[27]

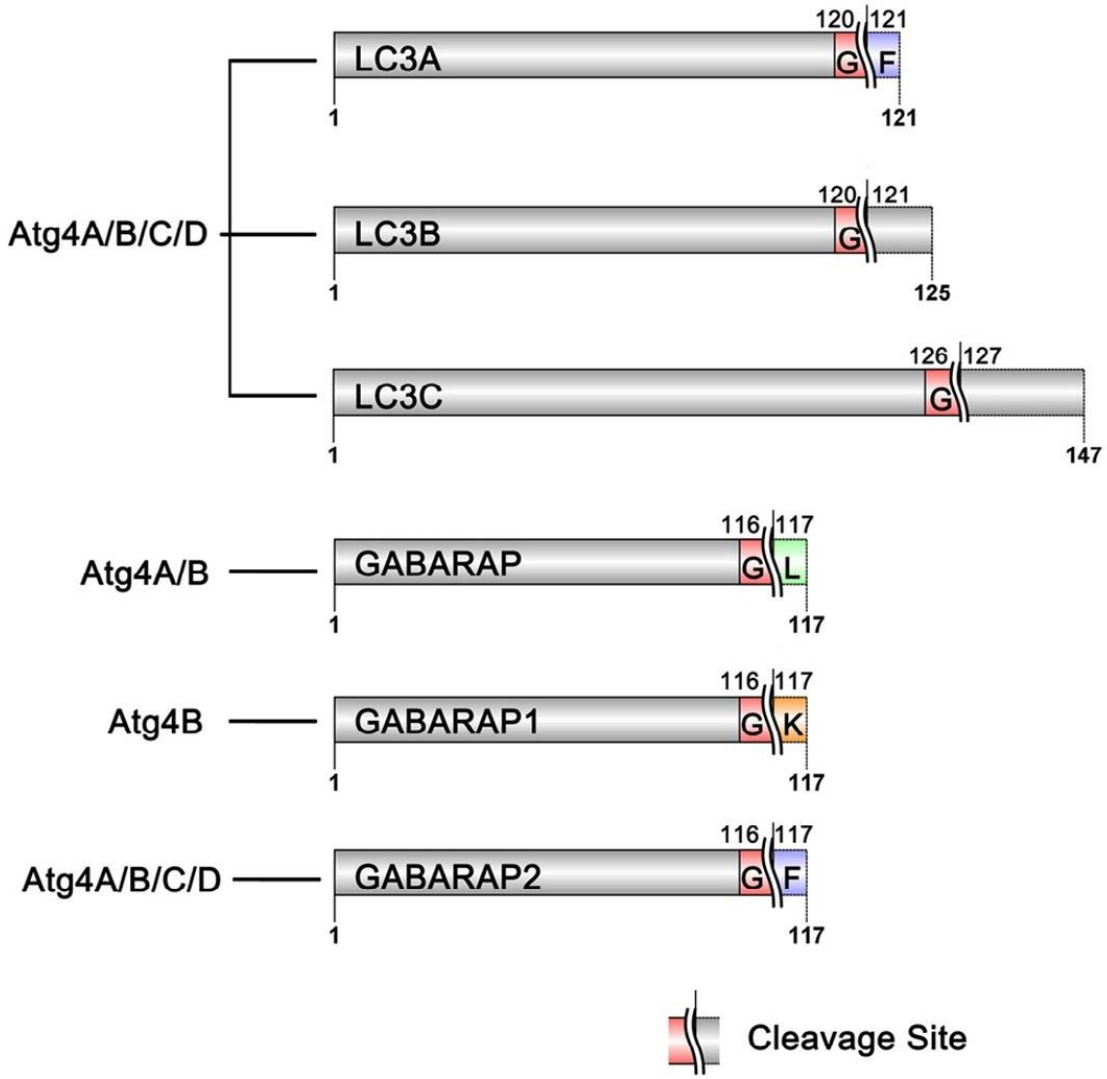

Figure 4: Preferred substrates of the four ATG4 homologues.[36]

The ATG4 mammalian homologues appear to have different substrate specificity (Figure 4).[36] While ATG4B is able to cleave most human homologues and ATG4A shows potent activity towards the GABARAP family, especially GATE-16, ATG4C and ATG4D seem to show minor activity

until they are cleaved N-terminally by a caspase.[35] The contributions to autophagy of each ATG4 mammalian homologue have not been fully elucidated, but some relevant information is available. ATG4B, for example, is considered a 'dominant' autophagy regulator in mammals, as it has been shown that its deletion results in pronounced autophagic defects. One example is a study that showed that *atg4b*$^{-/-}$ knockout mice have significant histopathological and neurological changes, which affect the development and survival of the animals.[40] When compared with *atg5*$^{-/-}$ knockout mice, these changes were of less prominent, which led to the idea that the rest of the ATG4 homologues could help attenuate the physiological effects caused by a lack of ATG4B through redundancy in their functions.[40] The ATG4C and ATG4D homologues, on a different note, have shown to play a crucial role in the survival response under conditions of stress such as nutrient deprivation, but they seem not to be essential for autophagy under normal conditions. For example, it has been shown that *atg4c*$^{-/-}$ knockout mice present normal autophagic activity under normal conditions, but when starved, they show decreased autophagic activity.[41] Also, it has been shown that *atg4d*$^{-/-}$ knockout HeLa cells are sensitized towards starvation-induced cell death, while *atg4b*$^{-/-}$ cells does not display such desensitization.[42] ATG4D has also been associated with mitophagic mitochondrial clearance during erythropoiesis.[43]

b. Dysregulation of ATG4 in disease

Development and progression of several pathologies have been associated to a dysfunctional autophagy process that in several cases, have also been linked to dysregulation of ATG4 proteins. For instance in various types of cancer, particularly the ones originated in female reproductive tissue, posttranslational alterations of the *ATG4* genes have been found.[44] ATG4A, for example, has been found to be hypomethylated in ovarian tumor-initiating cells and linked to poor prognostication of ovarian cancer patients.[45] Moreover, ATG4A expression has been found to be crucial for tumorigenic regulation of breast cancer stem cells *in vivo*.[46] On the same topic, *ATG4B* overexpression has been associated to cell necrosis induced in aggressive melanoma.[47] It is important to highlight that distinct patterns of *ATG4* genes alterations can be seen on the different types of human tumors.[48] In inflammatory bowel diseases (IBDs), low expression of the ATG4B protease has been seen in colon.[49] Furthermore, mutations of the *ATG4A* and *ATG4D* genes have been linked to the presence of granulomas in Crohn's disease.[50] The increased inflammatory response in IBDs has been associated with the fact that dysfunctional autophagy leads to a defective processing of bacterial components.[51] Infectious diseases have also been associated to different ATG4 proteases, i.e. the expression of ATG4D is induced during morphogenesis and propagation of HIV[52], and the replication of the Hepatitis C virus is initiated with the use of ATG4B and other autophagic components[53]. Other diseases related to ATG4 include type 1 diabetes mellitus, where ATG4C has been shown to play a role in neural injury of young patients when the diabetes is poorly controlled[54], or Huntington's disease, where the blocking of conventional autophagy via overexpression of ATG4B seems to influence this disease's progression in a transgenic mouse model of this disease.[55] Finally, *ATG4C* variants have been associated to aging free of major diseases and increased longevity.[56]

As the ATG4 protease family has been linked to a number of pathologies, and because its activity is essential and highly specific towards autophagy, the regulation of its different members provides an interesting approach as a drug target for autophagy modulation in disease. For instance, ATG4B has already been identified as a potential therapeutic target. In particular, a study has shown that in an osteosarcoma xenograft mouse model, the use of an ATG4B inhibitor effectively suppressed tumor growth and induces tumor regression.[57] In another investigation, the

authors showed that the knockdown of ATG4B impairs chronic myeloid leukemia (CML) stem/progenitor cells survival and sensitizes them to imatinib mesylate (IM) treatment.[58]

c. ATG4 regulation

Since autophagy plays a major role in a wide range of physiological functions, and as dysregulation of ATG4 is related to several diseases, the ATG4 homologues must be meticulously modulated by different regulators. Crystallographic studies of *Homo sapiens* (Hs) ATG4A and HsATG4B have been of notable help to understand the mechanics behind ATG4 regulation. HsATG4B was the first structure reported (Figure 5).[48] This protein is composed of the catalytic domain, which is conserved among papain-family cysteine proteases (C1 family), and the ATG4 specific short fingers domain, which is inserted into the catalytic domain.[59] The canonical catalytic triad of cysteine proteases is present in HsATG4B, and it is composed by Cys74, Asp278 and His280.

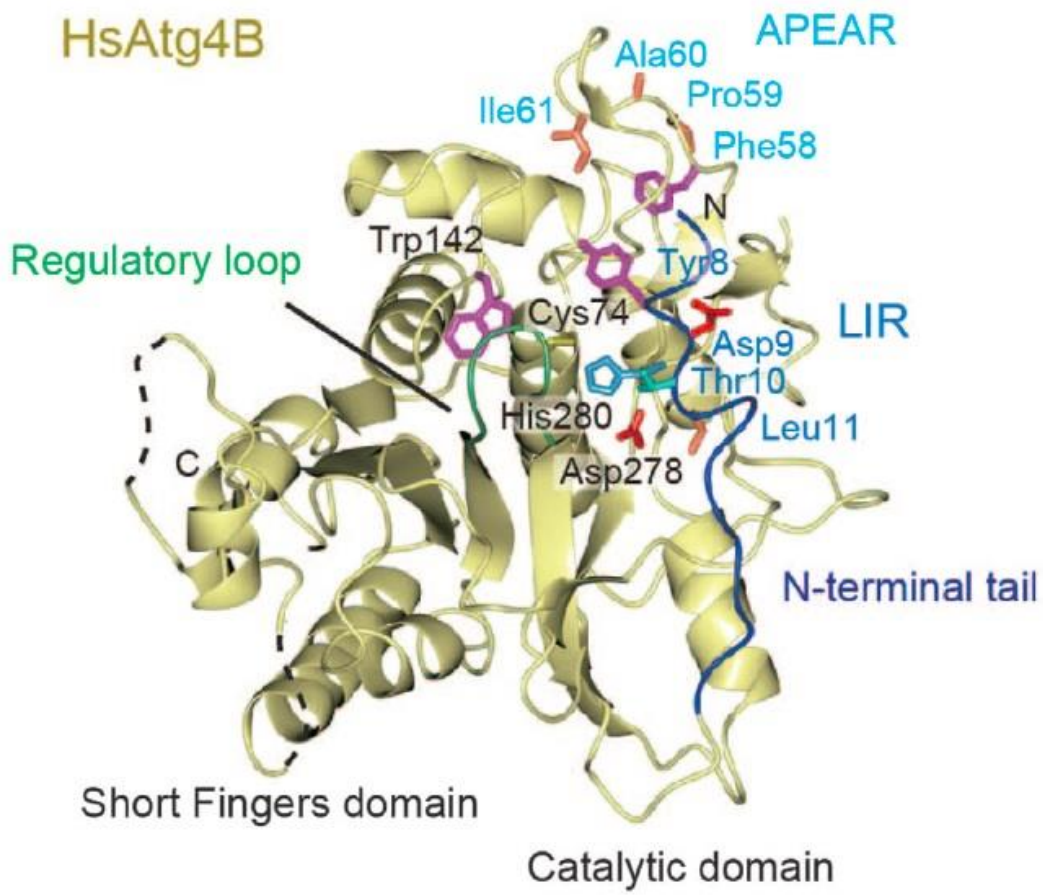

Figure 5: Crystal structure of HsATG4B and its most important components.[48]

An important feature of HsATG4B is its two structural auto-inhibited conformations in free form: the interaction between a regulatory loop and amino acid Trp142, which shields Cys74 from the solvent, and the binding of the N-terminal tail to the exit of the catalytic site.[59] In HsATG4A, Trp145 is involved in the shielding of Cys77 when it interacts with the regulatory loop; Trp145 and

Cys77 are the HsATG4A analog amino acids of HsATG4B Trp142 and Cys74, respectively.[48] These ATG4 homologues bind ATG8 using two different mechanisms: the first one, via the ATG8-interacting motif (AIM) or LC3-interacting region (LIR) placed at N- and C-terminal flexible tails, and the second one, via the core of this enzyme.[48] In HsATG4B a conformational change can be seen in both auto-inhibited regions upon LC3 binding, as Phe119 in LC3 lifts the regulatory loop, allowing the LC3 tail to be inserted into the active site, and the N-terminal tail of HsATG4B is detached from the enzyme core, which supposedly plays a role in enzyme access to LC3-PE bound in the membrane.[60] Regarding the residues associated to LC3 binding, HsATG4B Leu232 is not conserved in HsATG4A (Ile233 is the HsATG4A counterpart), and it has been suggested that this is the reason why HsATG4B can process LC3 more efficiently: Ile substitution of Leu232 causes a steric crush, while Leu substitution of Ile233 increases activity.[60] All these findings propose that ATG8, in a certain sense, regulates its own post-translational cleavage and delipidation process by regulating ATG4.

A significant step forward regarding ATG4 regulation was made when reactive oxygen species (ROS) were found to be essential for autophagy, and that they specifically regulated ATG4A and ATG4B activity.[61] Mitochondrial derived hydrogen peroxide (H_2O_2) generation is increased under nutrient deprivation conditions, and it reversibly inhibits ATG4A and ATG4B either by binding the catalytic Cys or its adjacent Cys, forming a reversible sulfenic acid, or by creating a disulfide bond between the same amino acidic residues via oxidation.[61] This process occurs through a PtdIns3K/BECLIN1 dependent pathway, and it is directed towards the de-conjugating activity of both homologues, allowing the accumulation of ATG8–PE (Figure 6).[6261]

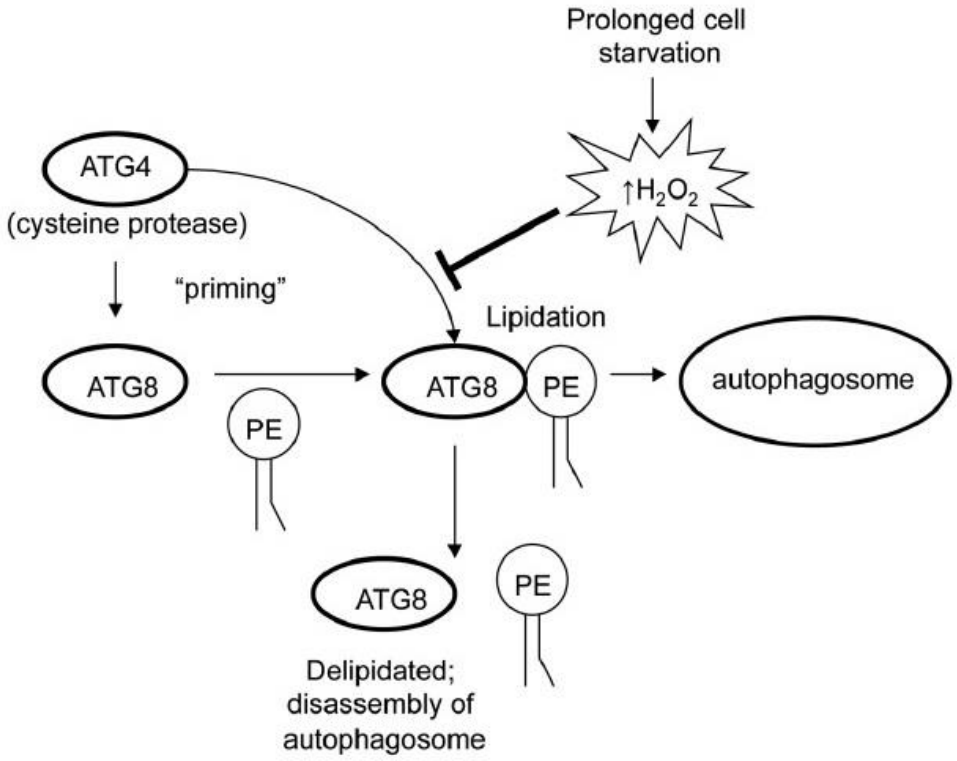

Figure 6: ROS-mediated induction of autophagy.[62]

This finding was of great relevance because, as mentioned before, ATG8 lipidation is a key step for autophagosome biogenesis and therefore, ATG4 must be inactivated after cleaving pro-ATG8 in order to avoid premature ATG8 deconjugation.[36] It has not been fully elucidated yet how this H_2O_2 mediated inhibition of ATG4A and ATG4B is interrupted, which would allow both homologues to carry out the crucial de-lipidation step, necessary for maturation and fusion of autophagosomes.[27] One hypothesis establishes that, as ROS are short-lived molecules, an oxidative gradient is created upon the transfer of mitochondrial ROS to the cytosol. This gradient would promote ATG8 conjugation by oxidizing ATG4 molecules in the mitochondrial proximity, and deconjugation would take place further away from the mitochondria as ATG4 would be active there (Figure 7).[63] On the same topic, another study suggests premature ATG8 deconjugation is avoided via the protection of lipidated ATG8 from ATG4 activity by the ATG12–ATG5-ATG16 complex and by the PtdIns3P binding proteins ATG18 and ATG21, as they are all required for ATG8–PE accumulation at the PAS.[64]

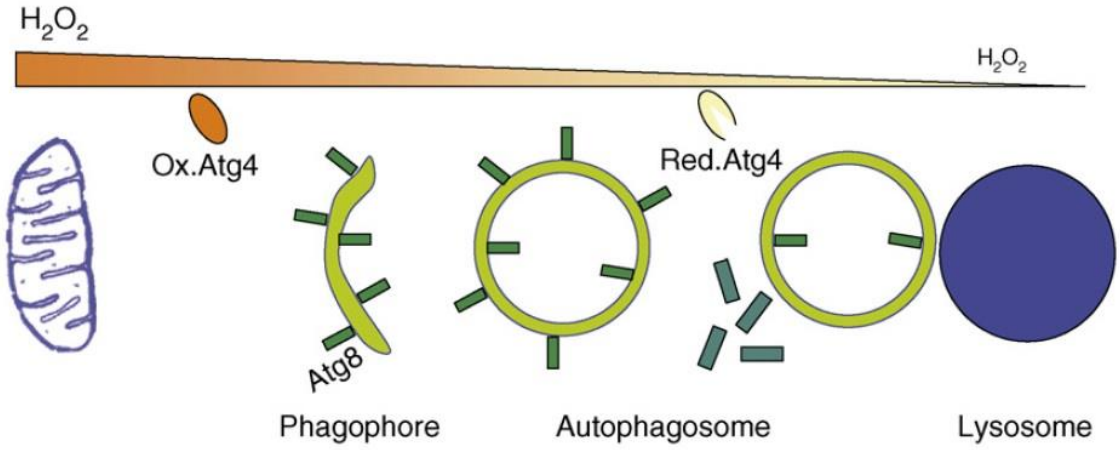

Figure 7: Influence of the H2O2 oxidative gradient on conjugation and deconjugation.[63]

ATG4 is also modulated by transcription factors, although it has been difficult to identify them due to the complex signal transduction pathways that ATG4 is part of. It has been shown that p53 plays a role in regulating *ATG4A* and *ATG4C* in response to DNA damage, which would induce a tumor-suppressing autophagic response by boosting p53-dependent apoptosis.[65] FoxO3 has shown to regulate *ATG4* genes in ovarian cancer cells and to upregulate *Atg4B* in the skeletal muscle of mice.[6667] The tissue-specific transcription factors EGR1 and C/EBPb have been associated with *HsATG4B* expression regulation in lung tissue and differentiating murine 3T3-L1 adipocytes, respectively.[6869] MicroRNAs (miR) also play a role in ATG4 regulation. It has been found that miR-376b regulates ATG4C intracellular levels, therefore modulating autophagy; tumor suppressor miR-101 targets ATG4D and inhibits autophagy; miR-34a targets ATG4B.[44] Another effector involved in ATG4 regulation is the membrane-associated E3 ligase RNF5, which recognizes ATG4B and induces its ubiquitination and subsequent proteasome-mediated degradation.[70] Furthermore, post-translational modifications are involved in ATG4B regulation. It has been shown that the activity of the mammalian ATG4B protease increases upon phosphorylation at Ser383 and Ser392 and also when the enzyme is modified with O-linked β-*N*-acetylglucosamine under metabolic stress, which supposedly promotes the maturation step.[48] A schematic depiction of the roles, highest affinity substrates and regulation of the ATG4 family is shown in Figure 8.[44]

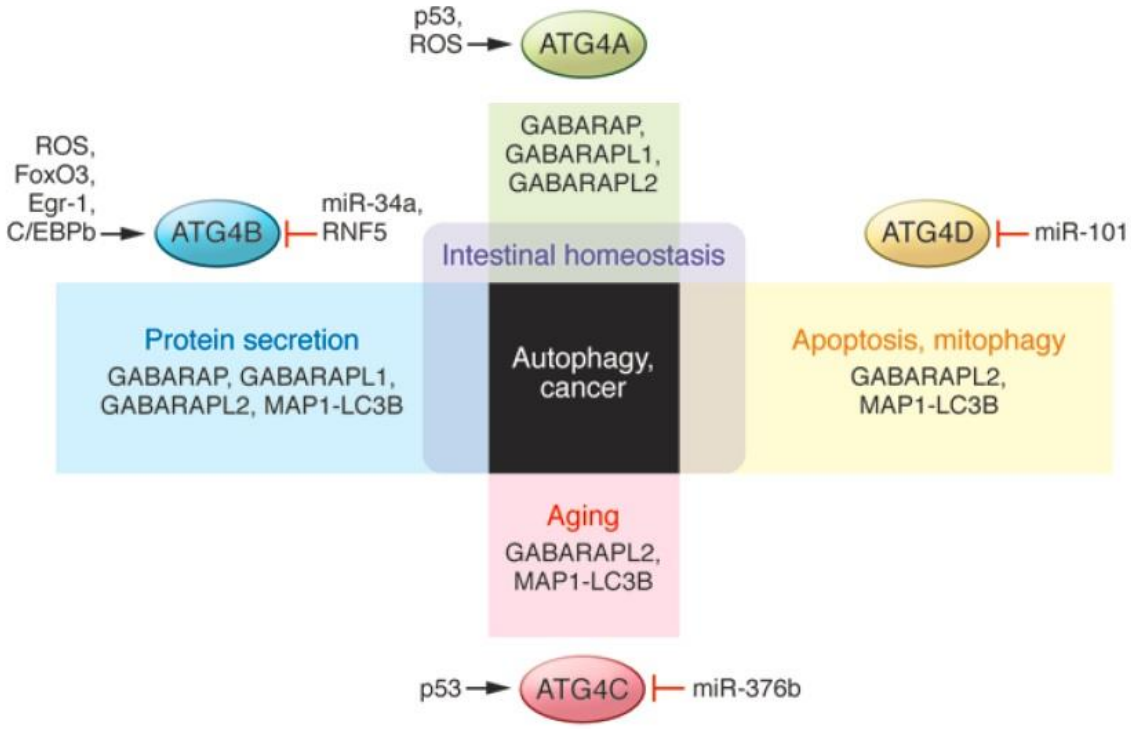

Figure 8: Roles, highest affinity substrates and regulation of the ATG4 cysteine protease family.[44]

Caspases seem to act on several HsATG proteins, which makes them a potential target for autophagy regulation. For example, caspases 3/-7/-8 target BECLIN1 during apoptosis, leading to a decrease in autophagy.[71] All the ATG4 proteases can be cleaved by caspase-3 *in vitro*, indicating that they might be involved in an apoptosis-autophagy crosstalk.[72] Unlike ATG4A and ATG4B, both ATG4C and ATG4D own a canonical DEVD caspase motif[42], and enhanced ATG4C and ATG4D enzymatic activity is seen (and, thus, increased autophagy) after caspase-mediated cleavage.[42] It is proposed that ATG4D is auto-inhibited and, upon caspase cleavage, the N-terminus is removed, which enables GABARAPL1 cleavage. In this context, ATG4D has been shown to work at an autophagy-apoptosis interface.[7374] Cleaved ATG4D is highly toxic as a result of exposing the BH3 domain, which leads to an interaction with BCL2 and subsequent apoptosis induction.[74] Moreover, ATG4D cleavage has been associated with mitochondrial import and oxidative stress.[73]

On a different note, a recent study has found that the activity of Atg4 on autophagosomal membranes might be regulated by the coordinated action of at least two conserved Atg8 recognition sites within Atg4. As the interaction of several proteins with Atg8 occurs via the LIR, potential LIR motifs were researched in the yeast Atg4 sequence. Four putative motifs were found: pLIR1, pLIR2, pLIR3 and pLIR4, where pLIR2 and pLIR4 are evolutionarily conserved, and pLIR1 and pLIR3 are yeast-specific. These sequence motifs were mutated in order to determine whether they played a relevant role in Atg8 binding and processing. Three of them, i.e. pLIR1, pLIR2 and pLIR4, were shown to be important *in vivo* for this function. Nonetheless, further experimentation showed that only pLIR2 (Phe102-Val103-Pro104-Ile105) plays a significant role in autophagy, more specifically being important for the recruitment of Atg4 to the PAS and consequent delipidation of Atg8–PE. Moreover and as it was probably expected, autophagosome formation was impaired

when pLIR2 was mutated, as it was shown by the decrease in the number and size of autophagic bodies compared to WT Atg4 and mutants of LIR1, pLIR3 and pLIR4 expressed in a *atg4*Δ *pep4*Δ strain (Pep4 is a major vacuolar protease). Subsequently, the specificity of pLIR2 towards membrane bound Atg8 was tested by performing immunoprecipitation with GFP-Atg8Δ, the primed Atg8-I form of Atg8, in *atg4*Δ *atg3*Δ cells expressing the different pLIR mutants, which showed that association to Atg8 was impaired in the pLR1 and pLR4 mutants, but not in Atg4^{pLIR2}. Later on, by analyzing the *in vitro* pLIR2 and pLIR4 mutants' interaction with GST-ATG8 via a pull-down assay, it was concluded that pLIR4 constitutively bound Atg8 and pLIR2 specifically bound Atg8–PE over non-lipidated Atg8. The pLIR4 mutant was used as control, since it had shown to be important for an efficient LC3/GABARAP cleavage by ATG4B.[75] These motifs were named, consequently, cLIR (C-terminal LIR) and APEAR (Atg8–PE association region). Finally, these two conserved Atg8 recognition sites were shown to work cooperatively by performing experiments using an Atg4 APEAR-cLIR double mutant which, when compared to single APEAR and cLIR mutants, showed a significant decrease in the Atg4-Atg8 association, a more noticeable defective Cvt pathway and an increased impairment in cleaving GFP from Atg8 and a practically inexistent Atg8–PE deconjugation from the autophagosomal membrane was seen when the double mutant was expressed in an *atg4*Δ strain. All these findings reaffirm how important Atg4 is for autophagy, as it is fundamental for the Atg8–PE deconjugation step.[7677]

Another recent study has shown that Atg1 phosphorylates Atg4 and inhibits its deconjugating proteolytic activity at the PAS. Three major findings in this study gave rise to this statement.[78] Firstly, it was found that Atg1 phosphorylates Atg4 and inhibits autophagy. An Atg4 sequence analysis showed seven Atg1 phosphorylation consensus sites, and a subsequent experiment showed that Atg4 could indeed be an Atg1 substrate. Next, the putative phospho-acceptor serines were individually mutated to alanine (non-phosphorylable) or aspartate (phospho-mimicking), in order to identify which residues were involved in autophagy regulation. After a set of experiments, it was demonstrated that S307 phosphorylation modulates Atg4-mediated Atg8–PE deconjugation. Secondly, it was determined that Atg4 phosphorylation affects its interaction with Atg8. Inhibition of Atg4 via phosphorylation at S307 could affect its activity either by interfering with the Atg8 interaction or by allosterically altering the catalytic site, so experimentation was carried out to solve this. The data obtained suggested that the S307 modulates the binding of Atg4 to Atg8. Lastly, it was concluded that Atg1 and Atg4 interact on autophagosomal membranes. It was shown that Atg4 phosphorylation at S307 blocks both processing and recycling of Atg8 and therefore, there must be a spatial regulation of Atg4 allowing the initial Atg8 cleavage but not premature Atg8–PE deconjugation. Subsequent experiments confirmed that the interaction between Atg4 and Atg8 occurs specifically on autophagosomal membranes. All these findings suggest that Atg1 provides a transient Atg4 inhibition at the PAS, protecting, in a certain sense, the Atg8–PE pool from the proteolytic activity of Atg4, which is essential for autophagosome formation. Disruption of the Atg1-mediated inhibition could thus be an important step on autophagosome maturation and fusion, as it would allow for Atg4 to act on autophagosomal membrane bound Atg8.[78] A similar story can be found regarding the mammalian ATG4 subfamily, which shows that ULK1 regulates the activity of ATG4B by phosphorylation of its Ser316.[79] This modification leads to the inhibition of the enzyme's catalytic activity *in vitro* and *in vivo*.[79] Moreover, phosphatase PP2A-PP2R3B is able to dephosphorylate Ser316, and it has been proposed that the actions of both enzymes, ULK1 and PP2A-PP2R3B, provide a phospho-switch regulation of ATG4B activity to control the processing of LC3.**Error! Bookmark not defined.**

d. ATG4 and drug discovery

ATG4 is essential for the autophagic process, which makes it an interesting drug target for autophagy modulation in disease. Special interest has been directed towards cancer as in general, it is considered that growing tumor cells activate autophagy to produce nutrients and escape stress.[48] As a result, targeting ATG4 proteases could help reduce cancer therapy resistance.[36] Among the ATG4 subfamily members, ATG4B has shown to have the highest activity and therefore ATG4B inhibitors have been widely developed to act as anti-cancer compounds. When screening ATG4B inhibitors, several new methods to measure ATG4 activity *in vitro*[35] and in living cells[8081] have been developed. A well-known ATG4 inhibitor is N-ethylmaleimide, which irreversible alkylates the key cysteine in the active site of several cysteine proteases and it has been shown to be an inefficient ATG4B inhibitor.[82] Via *in silico* docking, the compound NSC185058 was determined to be an ATG4B inhibitor with an IC50 of approximately 51 uM. NSC185058 inserts itself in the ATG4B catalytic pocket that contains aspartic acid 278 and histidine 280 using hydrophobic and hydrophilic binding interactions which are required for ATG4B proteolytic activity.[57] Another method that has been used is the Förster resonance energy transfer (FRET) assay, with which hypericin (IC50: 57 uM) and aurin trycarboxylic acid (IC50: 4,4uM) were identified as ATG4B inhibitors.[82] Likewise, time-resolved FRET, a method that combines FRET with time-resolved fluorometry, was used in the discovery of the potent ATG4B inhibitor Z-L-Phe-chloromethylketone, which has an IC50 of 0,1 uM.[83] The chloromethylketone moiety is the responsible for this potent inhibition because binding the catalytic Cys of ATG4B, but it also has high chemical reactivity that leads to cytotoxic activity.[83] With all the information about these active molecules, researchers created compounds containing a fluoromethylketone moiety and an LC3 C-terminal sequence-mimicking moiety, from which a naphthalene-1-carboxamide analog fluoromethylketone (FMK) 9a was designed. This compound showed the highest inhibitory potency so far (IC50: 80 nM) by covalently binding the catalytic Cys of ATG4B.[84] An important point to highlight is the fact that an ATG4 specificity improvement needs to be done, as all the compounds described also inhibit other cysteine proteases as cathepsin B and calpain, although they do not inhibit serine proteases, asparagine proteases, 20S proteasomes and metalloproteases.[48] A summary of the described ATG4 inhibitors is shown in Figure 9.[48]

Other compounds with anti-carcinogenic effects include the derivative asperphenamate N-Benzoyl-O-(N'-(1-benzyloxycarbonyl-4-piperidiylcarbonyl)-Dphenylalanyl)-D-phenylalaninol (BBP) and the anthelmintic flubendazole. BBP induces autophagic cell death of MCF-7 cells via a JNK-dependent ATG4 upregulation involving ROS production.[85] Flubendazole, is an agonist of ATG4B as it forms hydrophobic interactions with residues Lys259 and Glu17, inducing autophagic cell death in triple-negative breast cancer (TNBC).[86]

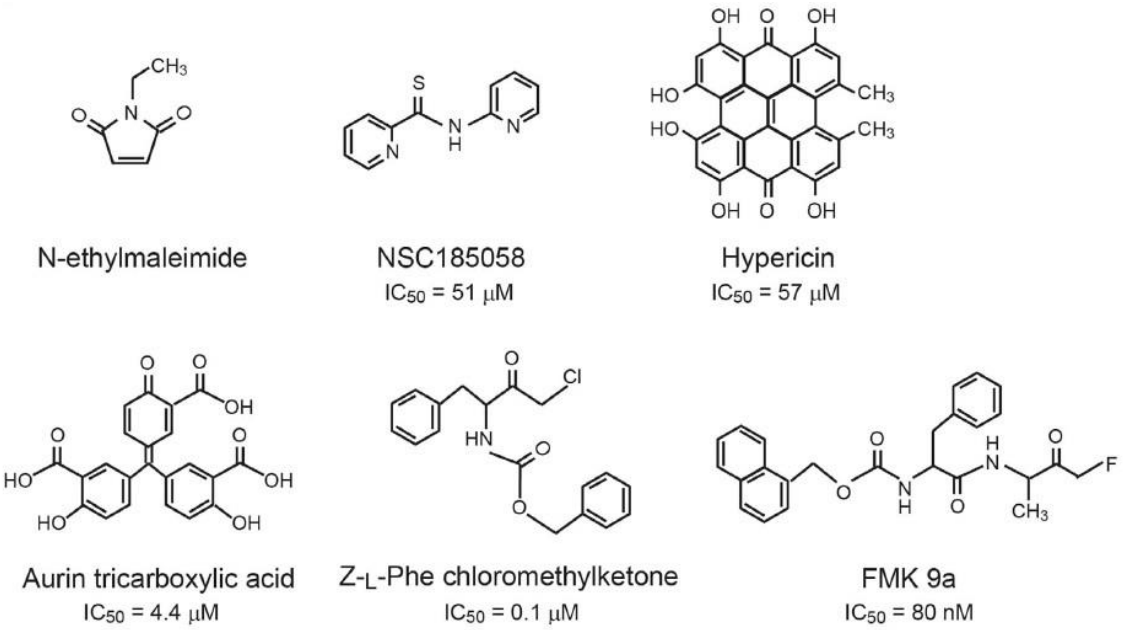

Figure 9: Summary of the current ATG4 inhibitors.[48]

7. Personal discussion

The pharmacological targeting of ATG4 proteins as a mean of modulating autophagy in disease is a promising approach, but much work has still to be done. One of the major hurdles to overcome is understanding the complex regulation of the ATG4 cysteine protease, which involves numerous components that are also modulated via intricate pathways. Several efforts are focused on the ATG4 protein spatiotemporal coordination in the conjugation and deconjugation steps, as both processes must be tightly regulated for normal autophagy progression, and many findings have already been done. Studies suggest different mechanisms , including the existence of different pools of ATG4 (cytoplasmic and at the PAS), modulation via mitochondrial generated ROS, protection of ATG8–PE from ATG4 activity by other autophagosome related proteins or conformational regulation of the ATG4 N-terminal tail via interaction with other proteins, among others, but they are still not enough to explain the whole process.

Many questions arise when trying to put together all these pieces of information and coming up with new approaches to tackle the ATG4 regulation problem. It could be, for example, that because that ATG4C and ATG4D are crucial for survival under starvation because they play a more important role in the de-lipidation process. This could help explain how the de-lipidation process still occurs even when ATG4B and ATG4A are being inhibited by H_2O_2. Could it be that the caspase-mediated cleavage of ATG4C and ATG4D is induced when the autophagosome is completed, leading to de-lipidation of ATG8–PE? I might be that ATG4C and ATG4D are specifically recruited to the PAS for the deconjugation to take place. As mentioned before, caspase-mediated cleavage of Atg4D improves its activity towards the GABARAP protein subfamily, which could be one of the signals for maturation and fusion to occur. It could also be that, perhaps, the substrate specificity of the different homologues gives them a specific function within the regulation of ATG4, with the ones showing a higher affinity for LC3 or non-conjugated Atg8 being connected to the formation of the autophagosome and the ones with a higher affinity for the GABARAP

subfamily or conjugated Atg8 being connected to maturation and fusion. This substrate specificity could be provided by different autophagy-associated Atg8-interacting motifs distinctively expressed in the ATG4 subfamily members, and thus the higher or lower activity of the different homologues at different places in the cell could dictate the rate at which the conjugation and deconjugation processes are taking place. So for example, when autophagosomal formation is needed, specific ATG4 proteases are upregulated to help with this process, and this upregulation counterbalances the de-conjugating action of other ATG4 members, and vice versa.

It is important to keep in mind that, even if the whole ATG4 protease regulation process were to be elucidated, it would still not be easy to predict the outcome of the positive or negative regulation of ATG4, as it has been seen that autophagy plays a dual role in different diseases and various studies have shown that ATG4 modulation is context-dependent.[36] One study showed that inhibition of ATG4B in different prostate cancer cell lines could promote treatment resistance or amplify the action of both radiotherapy and chemotherapy.[87] Another investigation showed that inhibition of different ATG4 homologues in carcinoma cells would promote resistance or sensitize the cells depending on the culture conditions the cells.[88] Therefore, autophagy could promote cell survival or suppress tumorigenesis depending on the context.

As a final statement, our understanding of the function and regulation of the different ATG4 homologues is still limited, and connecting all the dots of this complex puzzle is still an ongoing project. Finding new components of the ATG4 regulation intricate network will, undoubtedly, give rise to better insight on how the whole machinery coordinately works and will help us elucidate the role of ATG4 in physiologic and pathologic processes. This latter point is of great importance, as both an increased and decreased autophagic activity could play either a beneficial or detrimental role depending on the context. As a result, new autophagy modulating drugs targeting ATG4 should be specific towards the different ATG4 homologues and the conjugation/de-lipidation steps in order to modulate autophagy in disease accurately and avoid eventual deleterious side effects.

8. References

[1] Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell 2008; 132:27-42.

[2] Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature 2008; 451:1069-1075.

[3] Reggiori F, Klionsky DJ. Autophagic processes in yeast: mechanism, machinery and regulation. Genetics 2013; 194(2):341-361.

[4] Choi A, Ryter S, Levine B. Autophagy in Human Health and Disease. N Engl J Med 2013; 368:651-662.

[5] Tsukada M, Ohsumi Y. Isolation and characterization of autophagy-defective mutant of *Saccharomyces cerevisiae*. Elsevier 1993; 333(1, 2):169-174.

[6] Johansen T, Lamark T. Selective autophagy mediated by autophagic adapter proteins. Autophagy 2011; 7(3):279-296.

[7] Yin Z, Pascual C, Klionsky DJ. Autophagy: machinery and regulation. Microb Cell 2016; 3(12):588-596.

[8] Yang S, et al. Pancreatic cancers require autophagy for tumor growth. Genes Dev 2011; 25:717-729.

[9] Jellinger KA. Basic mechanisms of neurodegeneration: a critical update. J Cell Mol Med 2010; 14:457-487.

[10] Ma JF, Huang Y, Chen SD, Halliday G. Immunohistochemical evidence for macroautophagy in neurons and endothelial cells in Alzheimer's disease. Neuropathol Appl Neurobiol 2010; 36:312-319.

[11] Grose C. Autophagy during common bacterial and viral infections of children. Pediatr Infect Dis J 2010; 29(11):1040-1042.

[12] Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. Nature 2011; 469:323-335.

[13] Klionsky DJ, et al. A unified nomenclature for yeast autophagy-related genes. Dev Cell 2003; 5(4):539-545.

[14] Xie Z, Klionsky DJ. Autophagosome formation: core machinery and adaptations. Nat Cell Biol 2007; 9(10):1102-1009.
[15] Yang Z, Klionsky DJ. Eaten alive: a history of automacrophagy. Nat Cell Biol 2010; 12(9):814-822.
[16] Papinski D, et al. Early steps in autophagy depend on direct phosphorylation of ATG9 by the ATG1 kinase. Mol Cell 2014; 53(3):471-483.
[17] Rabinowitz J, White E. Autophagy and metabolism. Science 2010; 330(6009):1344-1348.
[18] Stephan J, Yeh YY, Ramachandran V, Deminoff S, Herman P. The Tor and PKA signaling pathways independently target the Atg1/Atg13 protein kinase complex to control autophagy. PNAS 2009; 106(40):17049-17054.
[19] Obara K, Sekito T, Niimi K, Ohsumi Y. The ATG18-ATG2 complex is recruited to autophagic membranes via phosphatidylinositol 3-phosphate and exerts an essential function. J Biol Chem 2008; 283(35):23972-23980.
[20] He C, Levine B. The Beclin 1 interactome. Curr Opin Cell Biol 2010; 22:140-149.
[21] Ohsumi Y, et al. Molecular dissection of autophagy: two ubiquitin-like systems. Nat Rev Mol Cell Biol 2001; 2(3):211-216.
[22] Tsuboyama K, et al. The ATG conjugation systems are important for degradation of the inner autophagosomal membrane. Science 2016; 354(6315):1036-1041.
[23] Noda T, et al. Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation in the Cvt and Autophagy Pathways. J Cell Biol 2000; 148(3):465-480.
[24] Yamamoto H, et al. Atg9 vesicles are an important membrane source during early steps of autophagosome formation. J Cell Biol 2012; 148(3):219-233.
[25] Reggiori F, Shintani T, Nair U, Klionsky DJ. Atg9 cycles between mitochondria and the pre-autophagosomal structure in yeasts. Autophagy 2005; 1(2):101-109.
[26] Nair U, et al. A role for ATG8–PE deconjugation in autophagosome biogenesis. Autophagy 2012; 8(5): 780-793.
[27] Reggiori F, Ungermann C. Autophagosome maturation and fusion. J Mol Biol 2017; 429(4):486-496.
[28] Teter SA, et al. Degradation of lipid vesicles in yeast vacuoles requires function of Cvt17, a putative lipase. J Biol Chem 2001; 276(3):2083-2087.
[29] Yang Z, Huang J, Geng J, Nair U, Klionsky DJ. Atg22 recycles amino acids to link degradative and recycling functions of autophagy. Mol Biol Cell 2006; 17(12):5094-5104.
[30] Xie Z, Nair U, Klionsky DJ. Atg8 controls phagophore expansion during autophagosome formation. Mol Biol Cell 2008; 19(8):3290-3298.
[31] Fujita N, et al. An Atg4 mutant hampers the lipidation of LC3 paralogues and causes defects in autophagosome closure. Mol Cell Biol 2008; 19(11):4651-4659.
[32] Mizushima N, Yoshimori T, Ohsumi Y. The role of Atg proteins in autophagosome formation. Annu Rev Cell Dev Biol 2011; 27:107-132.
[33] Yu ZQ, et al. Dual roles of Atg8–PE deconjugation by Atg4 in autophagy. Autophagy 2012; 8(6):883-892.
[34] Nakatogawa H, Ishii J, Asai E, Ohsumi Y. Atg4 recycles inappropriately lipidated Atg8 to promote autophagosome biogenesis. Autophagy 2012; 8(2):177-186.
[35] Li M, Fu Y, Yang Z, Yin XM. Measurement of the activity of the ATG4 cysteine proteases. Methods Enzymol 2017; 587:207-225.
[36] Zhang L, Li J, Ouyang L, Liu B, Cheng Y. Unraveling the roles of ATG4 proteases from autophagy modulation to targeted cancer therapy. Elsevier 2016; 373(1):19-26.
[37] Noda N, et al. Structural basis of target recognition by Atg8/LC3 during selective autophagy. Genes to Cells 2008; 13(12):1211-1218.
[38] Weidberg H, et al. LC3 and GATE-16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis. EMBO J 2010; 29(11):1792-1802.
[39] Alemu EA, et al. ATG8 family proteins act as scaffolds for assembly of the ULK complex: sequence requirements for LC3-interacting region (LIR) motifs. J Biol Chem 2012; 287(47):39275-39290.
[40] Read R, Savelieva K, Baker K, Hansen G, Vogel P. Histopathological and neurological features of Atg4b knockout mice. Vet Pathol 2011; 48(2):486-494.
[41] Mariño G, et al. Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in AtgC4/autophagin-3. J Biol Chem 2007; 282(25):18573-18583.

[42] Betin VM, Lane JD. Caspase cleavage of Atg4D stimulates GABARAP-L1 processing and triggers mitochondrial targeting and apoptosis. J Cell Sci 2009; 122(Pt 14):2554-2566.
[43] Betin VM, Singleton BK, Parsons SF, Anstee DJ, Lane JD. Autophagy facilitates organelle clearance during differentiation of human erythroblasts: evidence for a role for ATG4 paralogs during autophagosome maturation. Autophagy 2013; 9(6):881-893.
[44] Fernández Á, López-Otín C. The functional and pathologic relevance of autophagy proteases. J Clin Invest 2015; 125(1):33-41.
[45] Liao YP, et al. Hypomethylation signature of tumor-initiating cells predicts poor prognosis of ovarian cancer patients. Hum Mol Genet 2014; 23(7):1894-1906.
[46] Wolf J, et al. A mammosphere formation RNAi screen reveals that ATG4 promotes breast cancer stem-like phenotype. Breast Cancer Res 2013; 15(6):R109.
[47] Chunrong H, et al. A pilot study on morphology and the mechanism involved in linearly patterned programmed cell necrosis in melanoma. Oncol Lett 2010; 1(5):821-826.
[48] Maruyama T, Noda NN. Autophagy-regulating protease Atg4: structure, function, regulation and inhibition. J Antibiot (Tokyo) 2017; 1-7.
[49] Cabrera S, et al. ATG4B/autophagin-1 regulates intestinal homeostasis and protects mice from experimental colitis. Autophagy 2013; 9(8):1188-1200.
[50] Brinar M, et al. Genetic variants in autophagy-related genes and granuloma formation in a cohort of surgically treated Crohn's disease patients. J Crohns Colitis 2012; 6(1):43-50.
[51] Cho JH. The genetics and immunopathogenesis of inflammatory bowel disease. Nat Rev Immunol 2008; 8(6):458-466.
[52] Wang X, et al. HIV-1 and HIV-2 infections induce autophagy in Jurkat and CD4+ T cells. Cell Signal 2012; 24(7):1414-1419.
[53] Dreux M, Gastaminza P, Wieland SF, Chisari FV. The autophagy machinery is required to initiate hepatitis C virus replication. Proc Natl Acad Sci USA 2009; 106(33):14046-14051.
[54] Hoffman WH, Shacka JJ, Andjelkovic AV. Autophagy in the brains of young patients with poorly controlled T1DM and fatal diabetic ketoacidosis. Exp Mol Pathol 2012; 93(2):273-280.
[55] Proenca CC, et al. Atg4B-dependent autophagic flux alleviates Huntington's disease progression. PLoS One 2013; 8(7):e68357.
[56] Walter S, et al. A genome-wide association study of aging. Neurobiol Aging 2011; 32(11):2109.e15-28.
[57] Akin D, et al. A novel ATG4B antagonist inhibits autophagy and has a negative impact on osteosarcoma tumors. Autophagy 2014; 10(11):2021-2035.
[58] Rothe K, et al. The core autophagy protein ATG4B is a potential biomarker and therapeutic target in CML stem/progenitor cells. Blood 2014; 123(23):3622-3634.
[59] Sugawara K, et al. Structural basis for the specificity and catalysis of human Atg4B responsible for mammalian autophagy. J Biol Chem 2005; 280(48):40058-40065.
[60] Satoo K, et al. The structure of Atg4B-LC3 complex reveals the mechanism of LC3 processing and delipidation during autophagy. EMBO J 2009; 28(9):1341-1350.
[61] Scherz-Shouval R, et al. Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. EMBO J 2007; 26(7):1749-1760.
[62] Essick EE, Sam F. Oxidative stress and autophagy in cardiac disease, neurological disorders, aging and cancer. Oxid Med Cell Longev 2010; 3(3):168-177.
[63] Scherz-Shouval R, Elazar Z. ROS, mitochondria and the regulation of autophagy. Trends Cell Biol 2007; 17(9):422-427.
[64] Nair U, Cao Y, Xie Z, Klionsky DJ. Roles of the lipid-binding motifs of Atg18 and Atg21 in the cytoplasm to vacuole targeting pathway and autophagy. J Biol Chem 2010; 285(15):11476-11488.
[65] Kenzelmann Broz D, et al. Global genomic profiling reveals an extensive p53-regulated autophagy program contributing to key p53 responses. Genes Dev 2013, 27(9):1016-1031.
[66] Lu Z, et al. ARHI (DIRAS3) induces autophagy in ovarian cancer cells by downregulating the epidermal growth factor receptor, inhibiting PI3K and Ras/MAP signaling and activating the FOXo3a-mediated induction of Rab7. Cell Death Differ 2014; 21(8):1275-1289.
[67] Mammucari C, et al. FoxO3 controls autophagy in skeletal muscle *in vivo*. Cell Metab 2007; 6(6):458-471.

[68] Chen ZH, et al. Egr-1 regulates autophagy in cigarette smoke-induced chronic obstructive pulmonary disease. PLoS One 2008; 3(10):e3316.
[69] Guo L, et al. Transactivation of Atg4b by C/EBPβ promotes autophagy to facilitate adipogenesis. Mol Cell Biol 2013; 33(16):3180-3190.
[70] Ersheng K, et al. Regulation of ATG4B Stability by RNF5 Limits Basal Levels of Autophagy and Influences Susceptibility to Bacterial Infection. PLoS Genet 2012; 8(10):e1003007.
[71] Cho DH, et al. Caspase-mediated cleavage of ATG6/Beclin-1 links apoptosis to autophagy in HeLa cells. Cancer Lett 2009; 274(1):95-100.
[72] Norman JM, Cohen GM, Bampton ET. The *in vitro* cleavage of the hAtg proteins by cell death proteases. Autophagy 2010; 6(8):1042-1056.
[73] Betin VM, MacVicar T, Parsons SF, Anstee DJ, Lane JD. A cryptic mitochondrial targeting motif in Atg4D links caspase cleavage with mitochondrial import and oxidative stress. Autophagy 2012; 8(4):664-676.
[74] Betin VM, Lane JD. Atg4D at the interface between autophagy and apoptosis. Autophagy 2009; 5(7):1057-1059.
[75] Rasmussen MS, et al. ATG4B contains a C-terminal LIR motif important for binding and efficient cleavage of mammalian orthologs of yeast Atg8. Autophagy 2017; 13(5):834-853.
[76] Abreu S, et al. Conserved Atg8 recognition sites mediate Atg4 association with autophagosomal membranes and Atg8 deconjugation. EMBO Rep 2017; 18(5):765-780.
[77] Kriegenburg F, Reggiori F. LIR and APEAR, two distinct Atg8-binding features within Atg4. Oncotarget 2017; 8(47):81717-81718.
[78] Sánchez-Wandelmer J, et al. Atg4 proteolytic activity can be inhibited by Atg1 phosphorylation. Nature communications 2017; 8(1):295.
[79] Pengo N, Agrotis A, Prak K, Jones J, Ketteler R. A reversible phospho-switch mediated by ULK1 regulates the activity of autophagy protease ATG4B. Nat Commun 2017; 8(1):294.
[80] Choi KM, et al. A monitoring method for Atg4 activation in living cells using peptide-conjugated polymeric nanoparticles. Autophagy 2011; 7(9):1052-1062.
[81] Zhenhong Ni, et al. AU4S: A novel synthetic peptide to measure the activity of ATG4 in living cells. Autophagy 2015; 11(2):403-415.
[82] Nguyen TG, et al. Development of fluorescent substrates and assays for the key autophagy-related cysteine protease enzyme, ATG4B. Assay Drug Dev Technol 2014. 12(3):176-189.
[83] Xu D, et al. Identification of New ATG4B Inhibitors Based on a Novel High-Throughput Screening Platform. SLAS Discov 2017; 22(4):338-347.
[84] Qiu Z, et al. Discovery of Fluoromethylketone-Based Peptidomimetics as Covalent ATG4B (Autophagin-1) Inhibitors. ACS Med Chem Lett 2016; 7(8):802-806.
[85] Li Y, et al. JNK-dependent Atg4 upregulation mediates asperphenamate derivative BBP-induced autophagy in MCF-7 cells. Toxicol Appl Pharmacol 2012; 263(1):21-31.
[86] Zhang L, et al. Systems biology-based discovery of a potential Atg4B agonist (Flubendazole) that induces autophagy in breast cancer. Mol Biosyst 2015; 11(11):2860-2866.
[87] Tran E, et al. Context-dependent role of ATG4B as target for autophagy inhibition in prostate cancer therapy. Biochem Biophys Res Commun 2013; 441(4):726-731.
[88] Apel A, Herr I, Schwarz H, Rodemann HP, Mayer A. Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. Cancer Res 2008; 68(5):1485-1494.