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# Lipidation Status of Single Membrane-Associated ATG8 Proteins

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## Q2 Spotlight

3 Lipidation status of single  
4 membrane-associated  
5 ATG8 proteinsQ4 Q3 Christophe Viret<sup>1,\*,@</sup> and  
7 Mathias Faure<sup>1,2,\*</sup>

8

9 **ATG8 are core autophagy pro-**  
10 **teins, the lipidated forms of which**  
11 **decorate double-membraned auto-**  
12 **phagosomes, as well as single-**  
13 **membraned organelles such as**  
14 **endolysosomes. Recent studies**  
15 **from the Florey and Münz laborato-**  
16 **ries delineate the status of single**  
17 **membrane-associated ATG8 pro-**  
18 **teins by indicating that their**  
19 **membrane anchoring can involve**  
20 **phosphatidylserine conjugation and**  
21 **their stabilization depends on ATG4**  
22 **protease inhibition.**

23 Autophagy is a highly conserved process  
24 that engages specialized proteins (ATGs)  
25 to degrade cytosolic compounds via  
26 the lysosomal pathway. While the ULK1  
27 kinase complex and the class III PI3K  
28 complex I regulate early biogenesis of  
29 autophagic membranes (phagophore),  
30 the ATG12-ATG5-ATG16L1 conjugation  
31 complex allows for phagophore elonga-  
32 tion and recruitment of lipidated forms  
33 of ATG8 proteins (LC3A, B, and C and  
34 GABARAP-L1, L2, and L3) that are an-  
35 choring points for autophagy receptor-  
36 cargo complexes. Autophagic cargoes  
37 are then encapsulated into double mem-  
38 brane vesicles (autophagosomes) that  
39 fuse with endolysosomes for degradation  
40 [1,2]. On the autophagosome surface,  
41 ATG8 proteins are removed via the  
42 deconjugation activity of ATG4 cysteine  
43 proteases. ATG8 proteins are also found  
44 on single-membrane compartments of  
45 the endolysosomal pathway where they  
46 fulfill nonautophagic functions, a phenom-  
47 enon named noncanonical autophagy. The  
48 best-studied example is LC3-associated

phagocytosis (LAP) where phagosomes  
recruit lipidated LC3 (accordingly named  
LAPosomes) in order to optimize tissue  
homeostasis and immune response regu-  
lation [3,4].

During a liquid chromatography-tandem  
mass spectrometry analysis of lipidated  
human LC3A, Durgan *et al.* observed  
that autophagy activation led to the  
exclusive conjugation of LC3A to the  
aminoglycerophospholipid phosphatidyl-  
ethanolamine (PE) [5]. By contrast, cells  
manipulated for LC3 recruitment to  
endolysosomal compartments displayed  
a mix of LC3A species: besides PE, a  
sizable fraction of LC3A was conjugated  
to phosphatidylserine (PS), an amino-  
glycerophospholipid that only differs from  
PE by the presence of a carboxyl group.  
Such a hybrid lipidation status also applied  
to LC3B/C and GABARAP members, with  
PS-conjugation ranging from 10 to 30%  
of the lipidated ATG8 pool, indicating that PS  
conjugation is not restricted to a particular  
ATG8 protein. ATG8-PS could be de-  
tected on ATG8-positive endolysosomal  
membranes induced by various stimuli,  
including infection with the influenza A  
virus. In addition, ATG8-PS was present on  
other endocytic/endolysosomal membranes  
including macropinosomes, phagosomes,  
lysosomes, and entotic vesicles. Mecha-  
nistically, ATG8-PS conjugation was de-  
pendent on the so-called WD40 domain  
of ATG16L1, a domain that does not regu-  
late the role of ATG16L1 in ATG8-PE con-  
jugation [6]. Moreover, the authors found  
that while most ATG4s could delipidate  
LC3B-PE and GABARAP-PE, only ATG4B  
and ATG4D delipidated LC3B-PS and  
GABARAP-PS [5]. In the case of liposomes  
harboring both LC3B-PE and LC3B-PS  
species, ATG4D also mediated the stron-  
gest PS deconjugation effect. In cells  
treated to promote ATG8 recruitment to  
endocytic membranes, LC3B-PS was also  
targeted by both ATG4D and ATG4B for  
effective delipidation. Under conditions of  
LAPosome induction in a macrophage-like

cell line, LC3A-PS persisted for a longer  
period than LC3-PE, indicating a differential  
kinetic of delipidation. Possibly, the pres-  
ence of the carboxyl group and its associ-  
ated negative charge could impact the  
activity of ATG4 proteases and contribute  
to slow down ATG8-PS delipidation. Over-  
all, the data identify an alternate mode of  
ATG8 lipidation with specific recruitment to  
single-membrane compartments. 58

The issue of ATG8 protein deconjugation  
from single-membrane compartments  
was also a topic of interest for Ligeon  
*et al.* [7]. Besides confirming that the  
ATG12-ATG5-ATG16L1 conjugation com-  
plex is recruited to LAPosomes in human  
monocyte-derived macrophages exposed  
to the yeast cell wall derivative zymosan,  
the authors observed that components of  
the so-called class III PI3K autophagy com-  
plex, such as VPS34 and ATG14, can be  
recruited at the level and contribute to the  
formation of such single-membrane LC3  
positive vesicles. As zymosan-containing  
LAPosomes appeared to mature rather  
slowly, Ligeon *et al.* wondered what could  
be the basis of LAPosome stabilization  
and considered a possible role for the  
NADPH oxidase 2 (NOX2) complex, as  
it can modulate the formation of LC3-  
positive phagosomes [8,9]. Several com-  
ponents of an active NOX2 complex could  
be detected on the surface of zymosan-  
containing LAPosomes, including gp91,  
p22-phox and p40-phox [7]. Through pro-  
duction of reactive oxygen species (ROS),  
NOX2 generated an oxidative microenvi-  
ronment within LAPosomes. The level of  
oxidation detected within LAPosomes  
was clearly higher, and significantly more  
stable, than that observed within phago-  
somes devoid of LC3. Consistent with an  
important role for NOX2 in the formation  
and functionality of LAPosomes, the pre-  
treatment of macrophages with NOX  
inhibitors lowered the capacity to form  
LAPosomes and reduced the associated  
oxidation potential. Because ROS-mediated  
oxidation can inhibit the delipidation activity 97

98 of ATG4A and ATG4B [10], Ligeon *et al.*  
 99 studied the impact of NOX2-generated  
 100 ROS on ATG4B delipidation activity dur-  
 101 ing LAP [7]. When macrophages were  
 102 engineered to express a mutant form of  
 103 ATG4B that is insensitive to oxidation,  
 104 the level of lipidated LC3B and the fre-  
 105 quency of LAPosomes detected upon  
 106 phagocytosis induction remained low rela-  
 107 tive to macrophages expressing a wild  
 108 type version of ATG4B. Thus, through the  
 109 negative regulation of ATG4B delipidation  
 110 activity, ROS modulate the stability of LC3  
 111 on phagosomes during LAP. In terms of  
 112 mechanism, oxidation triggers ATG4B  
 113 aggregation, a state that appears to inhibit

its delipidating activity. More precisely, the  
 oxidation of a cysteine residue (Cys-78)  
 located close to, yet distinct of, the active  
 site cysteine (Cys-74) is instrumental for  
 ATG4B inhibition. With respect to the func-  
 tional consequences of stabilizing lipidated  
 LC3 on LAPosomes, the monitoring of  
*Candida albicans* antigen presentation by  
 human MHC class II molecules revealed  
 that, relative to wild type ATG4B, oxidation-  
 insensitive ATG4B reduced the capacity of  
 macrophages to present *C. albicans*  
 antigens to CD4 T cells. Hence, the  
 oxidation-dependent neutralization of  
 ATG4B delipidation activity that stabilizes  
 LAPosomes contributes to a productive

antigen presentation by MHC class II  
 molecules, presumably through an opti-  
 mized antigen processing/loading.

Together, the studies by Durgan *et al.* and  
 Ligeon *et al.* provide us with novel insights  
 into the biology of lipidated ATG8 factors  
 recruited to single-membrane compart-  
 ments. They reveal an alternative mode of  
 lipidation and identify important parameters  
 involved in their maintenance (Figure 1).  
 As PE/PS hybrid lipidation may repre-  
 sent a hallmark of ATG8-positive single-  
 membrane compartments, additional  
 studies are needed to document whether  
 it occurs in distinct cell types and organ-  
 isms. Whether the conditions that promote  
 ATG8 association to single membranes  
 modulate the activity of PS synthases  
 and/or decarboxylases is another open  
 question. Along the same line, further  
 investigation should examine whether ox-  
 idation uniformly or differentially regulates  
 proteases of the ATG4 family and whether  
 the functional consequences on antigen  
 presentation apply to other antigens and  
 other antigen-presenting cells.

**Declaration of interests**  
 No interests are declared.

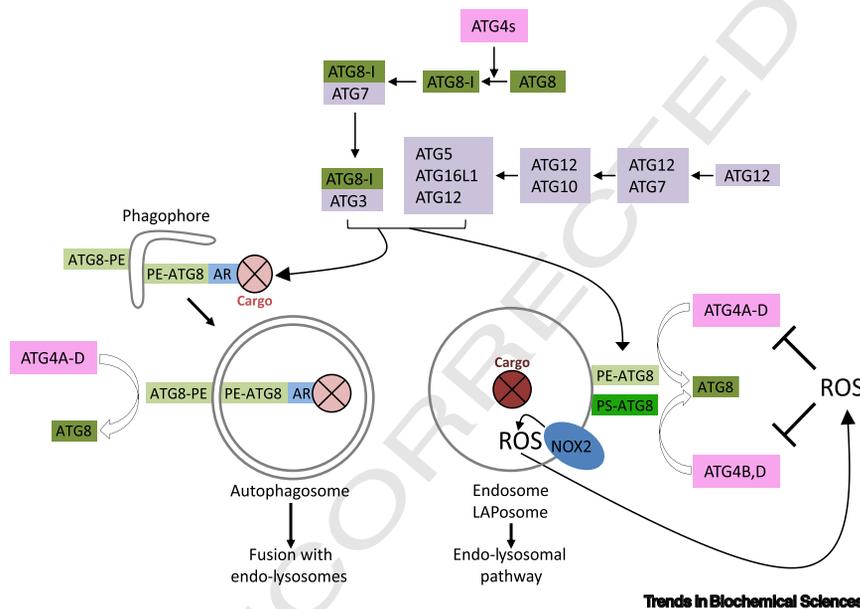
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**Q1** Figure 1. ATG8 factors recruited to single-membrane endolysosomal compartments vary in their lipidation mode and are stabilized by oxidation-dependent inhibition of ATG4 proteases. ATG8 factors become lipidated under both autophagic and endocytic/phagocytic situations. ATG12 and ATG5 conjugate with ATG16L1 in the presence of ATG7/ATG10, while ATG4 cysteine proteases (ATG4A-D) promote the processing of native ATG8s to generate ATG8-I, and ATG7/ATG3 factors render them available for phosphatidylethanolamine (PE) or phosphatidylserine (PS) lipidation (ATG8-II) and anchoring into membranes. Whereas only ATG8-PE gets recruited to phagophores that evolve into double-membrane autophagosomes where encapsulation of autophagy receptor (AR)-associated cargoes occurs, both ATG8-PE and ATG8-PS are recruited to single-membrane endolysosomes such as LC3-associated phagosomes (LAPosomes). The level of vesicle-associated ATG8s is regulated by ATG4s that mediate their delipidation: ATG4A-D can deconjugate PE from ATG8, whereas only ATG4B and ATG4D deconjugate PS. In macrophages, LAPosome induction is accompanied by the assembly of NADPH oxidase 2 (NOX2)-generating reactive oxygen species (ROS), which may diffuse in the immediate cytosolic milieu via transmembrane channels. The oxidation of ATG4B impairs its delipidating activity and LAPosome-anchored ATG8s get stabilized. The figure speculates that ATG4s other than ATG4B may also be regulated by oxidation, a point that remains to be documented.

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