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► **To cite this version:**

Mohyeddine Omrane, Thomas Melia, Abdou Rachid Thiam. LC3 conjugation to lipid droplets. Autophagy, 2023, pp.1-3. 10.1080/15548627.2023.2249390 . hal-04234779

**HAL Id: hal-04234779**

**<https://hal.science/hal-04234779v1>**

Submitted on 10 Oct 2023

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# *LC3 conjugation to lipid droplets*

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## Abstract.

Autophagy and lipid droplet (LD) biology are intricately linked, with autophagosomes breaking down LDs in response to different signals. LDs play crucial roles in forming autophagosomes possibly by providing essential lipids and serving as a supportive autophagosome assembly platform at the ER-LD interface. LDs and autophagosomes share common autophagic proteins, such as VPS13, Atg2, DFCP1, and Atg14, but their dual functions remain poorly understood. In our recent study, we found that prolonged starvation leads to Atg3 localizing to large LDs and lipidating LC3B, revealing a non-canonical autophagic role on LDs. In vitro, Atg3 associated with purified and artificial LDs, and conjugated Atg8 proteins. In long-term starved cells, only LC3B was found on the specific large LDs, positioned near LC3B-positive membranes that undergo lysosome-mediated acidification. This implies that LD-lipidated LC3B acts as a tethering factor, connecting autophagosomes to LDs and promoting degradation. Our data also support the notion that certain LD surfaces may function as lipidation stations for LC3B, which may move to nearby sites of autophagosome formation. Overall, our study unveils an unknown non-canonical implication of LDs in autophagy processes.

## Keywords:

Lipid droplets, noncanonical autophagy, LC3B, ATG3, prolonged starvation, organelle biology, membrane contact sites.

## Abbreviation:

LD: lipid droplet, ER: endoplasmic reticulum, ATG: autophagy-related protein, LC3B: microtubule-associated protein 1 light chain 3 beta, LIR motif: LC3B-interacting region, P62: Sequestosome-1, PLIN1: Perilipin-1, ATGL: Adipose Triglyceride Lipase, VSP13: Variable small protein 13, DFCP1: Double FYVE-containing protein 1, PE: Phosphatidylethanolamine, ATP: Adenosine triphosphate, E2 enzyme: Ubiquitin-conjugating enzyme.

Autophagosomes are double-membrane vesicles that enclose and deliver micrometer-range cargos to lysosomes for degradation. A crucial reaction in the formation of autophagosomes is the conjugation of Atg8 proteins (LC3s or GABARAPs) to PE phospholipids of the membrane. This process is mediated by the E2 enzyme Atg3, which is recruited to the membrane by the ATG5-ATG12—ATG16L1 complex. Atg8 is the major ligand on autophagosomes that binds to cargo receptors having an LIR motif.

Autophagosomes degrade lipid droplets (LDs), the major cellular lipid storage organelles, through a process known as lipophagy. Lipophagy-targeted LDs are typically in the  $\mu\text{m}$  size range or smaller. Recently, we investigated the fate of larger LDs during starvation<sup>1</sup>. We utilized 3T3-L1 differentiated adipocytes and Huh7 liver cells, which can contain large LDs, e.g.  $\gg 1\mu\text{m}$  in size. To allow sufficient time for LD consumption and explore various regimes of autophagy, the cells were starved for extended periods, up to 72hrs.

After approximately 6hrs of starvation, only LC3B and no other Atg8 proteins localized to a few large LDs. LD proteins like Plin1 and ATGL, which have LIR motifs, were not responsible for LC3B localization to the LDs. Similarly, P62, a major adaptor of LC3B that could bind to ubiquitinated LD proteins, was not responsible for the LD localization of LC3B. LC3B presence on LDs depended on Beclin1 and Atg5. Based on our overall observations, we developed two hypotheses: LC3B on LDs was from closely apposed autophagic membranes, or conjugation to the LD phospholipid monolayer. We ruled out the first possibility e.g. by employing a hypotonic medium-based organelle swelling approach, which demonstrated that the LC3B around LDs was not associated with a bilayer. Hence, the reliance of this phenomenon on Atg5/Beclin1 could be attributed to 1) the involvement of the proteins in the direct recruitment of conjugation machinery to LDs or 2) an interplay with classical autophagy to develop appropriate LD substrates. The latter model would involve the action of autophagy during the initial stages of starvation to release cues that trigger other autophagic mechanisms upon prolonged fasting. Hence, inhibiting the autophagic machinery would impede the release of these cues and prevent LC3B lipidation to LDs.

We investigated the possibility of LC3B being conjugated to LDs (Figure 1a). During prolonged starvation, we found that Atg3 was associated with specific larger LDs, supporting that it acted on LDs. When we deleted Atg3 or introduced a mutation (V15K) to reduce its binding efficiency, the localization of LC3B to LDs was hindered. Conversely, a mutant (K11W) that enhanced binding significantly increased LC3B localization to LDs. These results suggested that Atg3 may bind and conjugate LC3B to the LD phospholipids. To test this model, we adopted in vitro experiments using purified or artificial LDs. Atg3 bound and lipidated LC3B to these LDs; it was also capable of lipidating GABARAPs. Lipidation always occurred, independent of artificial LD size, as long as PE was present. Hence, the preference for larger LDs was likely due to the interfacial phospholipid and proteome composition rather than the size of the LDs.

Atg3 demonstrated binding affinity to model LDs on its own, whereas it did not bind to giant unilamellar vesicles of similar lipid composition. This suggests that the physical recruitment of ATG3 to LDs in cells might be independent or differentially reliant on the Atg5-12—16 complex compared to bilayer membranes. The preference for the LD surface

likely stems from the greater occurrence of phospholipid packing defects on the LD monolayer in comparison to the bilayer.

The LDs that were lipidated with LC3B were consistently in close proximity to LC3B-membrane structures, likely autophagosomes, which appeared as more intense regions in Figure 1b. Through live imaging, we observed that LC3B-positive LDs were specifically recognized by LC3B-membrane structures. To demonstrate the affinity of this interaction, we conducted experiments using purified LC3B-positive vesicles, which were brought into contact with artificial LDs that either were conjugated or not to LC3B. Only LC3B-lipidated LDs developed contacts with the LC3B-vesicle.

The docked autophagosome-like structure became positive for lysosomal markers, indicating the degradation of compounds on or near the LDs, Figure 1c. Accordingly, the few LC3B-positive LDs often lacked PLIN1, suggesting that either PLIN1 was removed prior to LC3B lipidation or it was degraded following lipidation. Further investigations are needed to fully understand the fate of these LDs and PLIN1 in this process. Nonetheless, our findings support the idea that LC3B on the surface of LDs acts as a cofactor facilitating the recruitment of autophagosomes. Given that LC3B can undergo trans homodimerization, it is possible that LC3B-LC3B interactions mediated the interaction between LDs and autophagosomes.

Our findings also agree with the model that LD-lipidated LC3B is transferred to autophagosomes in close proximity, Figure 1c. This transfer could happen directly from the LDs to the autophagosomes via possible physical connections between LDs and autophagosomes, or via the ER. Notably, the trans-homodimerization of LC3B induces hemifusion of bilayers, meaning that, in principle, a contiguous LD-autophagosome leaflet could form. Such a pathway would be particularly activated by prolonged starvation, as a mechanism to alleviate the classical autophagosome biogenesis steps. Intriguingly here, Atg3 might associate with LDs alone and mediate LC3B lipidation, without the physical involvement of the upstream autophagic components. In this viewpoint, a constant supply of PE to the LDs would be necessary for LC3B continuous lipidation. PE supply for LD lipidation could occur through PE diffusion from the ER to LDs or transfer by lipid transfer proteins at ER-LD contact sites. Further investigations are required to explore these aspects and shed light on this possible pathway.

In summary, the localization of Atg3 and the subsequent lipidation of LC3B to LDs introduces a new perspective on the dynamic interplay between autophagy and LDs. By expanding the list of proteins common between autophagosome and LDs, VPS13, Atg2, DFCP1, Atg14, and now Atg3 and Atg8, we emphasize the intricate relationship between these organelles, which is important for their dynamics. It would be unsurprising to discover additional autophagy-related proteins localizing to LDs, depending on specific cues.

1. Omrane M, M'Barek KB, Santinho A, Nguyen N, Nag S, Melia TJ, Thiam AR. LC3B is lipidated to large lipid droplets during prolonged starvation for noncanonical autophagy. *Developmental Cell* 2023;

Acknowledgment. This work was supported by the Fondation pour la Recherche Médicale (FRM: EQU202103012564) and the Programme Emergence de la Ville de Paris (DDEEES 165).

Figure legend:

Figure 1

- a) During prolonged starvation, ATG3 binds alone to LDs and lipidates LC3B, delivered by ATG7. Plin1 level on the LD is decreased on the LD that is lipidated.
- b) Confocal imaging of EGFP-LC3B (green) and LDs (LipidTox, magenta) in differentiated adipocytes (3T3-L1) expressing EGFP-LC3B and incubated in EBSS for 48 hours. The inset displays a 3D reconstructed image from z-stacks of the imaged LD. An LC3B-positive membrane, possibly autophagosomes, is in close apposition to the LC3B-lipidated LDs.
- c) Possible mechanisms. Lipidated LC3B on LD surfaces serves as a tethering factor for autophagosome recruitment. The recruited autophagosomes fuse with lysosomes and undergo acidification, without the LD being fully engulfment. LDs may function as a platform for LC3B lipidation, with lipidated LC3B potentially being transferred to recruited/nascent autophagosomes close to the LD surface, directly or via the ER membrane.