The Role of Lipid Droplets in Autophagy

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Abstract:

Autophagy is a process in which proteins and organelles are engulfed in vesicles and then degraded by lysosomes or vacuoles. Autophagy is a process important in cellular homeostasis and its malfunction has been tied to several diseases. Lipid Droplets (LDs) are organelles that function as storage of neutral lipids and play a central role in cellular lipid metabolism. This review will outline the connections between LDs and (macro)autophagy and the involvement of LDs in autophagy. First an overview will be given of all proteins (discovered so far to be) involved in both lipid metabolism and (macro)autophagy and their functions in both. The consequences will then be discussed. Finally more direct functions of LDs in autophagy will be shown and the implications of these functions discussed.

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Introduction:

Autophagy is an evolutionarily conserved process in which excess or aberrant proteins and organelles are engulfed in a vesicle which then fuses with a lysosome or vacuole leading to the degradation of the contents of the vesicle[1]. Malfunction of autophagy has been revealed to play a role in several diseases, including: Cancer[1], *Salmonella* infections[1,2] and even neurodegenerative diseases[1,3] such as Alzheimer[4] and Huntington[5]. Genes that encode for proteins that are involved in autophagy are called AuTophaGy related genes (or ATGs)[1,6]. Autophagy comes in three main forms Macroautophagy, Microautophagy and Chaperone-Mediated Autophagy (CMA)[7].

Lipid Droplets (LDs) are central players in cellular lipid homeostasis[8]. LDs are metabolically highly dynamic organelles whose primarily function as storage depots of neutral lipids[8]. LDs are composed of TriAcylGlyrols (TAGs) and Steryl Esters (SE) (both neutral lipids) in approximately equal amounts[8]. Most proteins resident in the LDs are involved in lipid metabolism, also protein composition of the LDs is dependent on growth phase and the growth conditions and media composition of the cells[8–10]. In most cases LDs are linked with the Endoplasmatic Reticulum (ER) via junctions. LDs also seem to form junctions with the mitochondria[11,12]. LDs seem to accumulate at the ER-vacuole/lysosome interface forming a tri-organelle junction[11,13]. LDs are broken down by a selective form of autophagy known as lipophagy. This can happen in a manner similar to microautophagy[14] or in one similar to macroautophagy[15].

However there might be more happening between lipid droplets and autophagosomes than only lipophagy. Several recent articles reveal connections between lipid droplets and autophagy, and reveal a possible auxiliary role for lipid droplets in autophagy. In this review the information in these articles is going to be covered and the possible involvement of lipid droplets in the formation of Autophagosomes will be discussed. First the types of autophagy will be explained, as will the biogenesis of the lipid droplets.

Processes of autophagy and lipid droplet biogenesis:

Autophagy:

As previously mentioned, autophagy comes in three main forms Macroautophagy, Microautophagy and Chaperone-Mediated Autophagy (CMA)[7].

Macroautophagy has several steps (figure 1). First is autophagy induction, which is regulated by the TOR (Target Of Rapamycin) protein. TOR is a serine-threonine kinase that is active under nutrient rich conditions[1,16]. When active, TOR hyperphosphorylates the Atg13 protein preventing it from associating the Atg1 protein to form a complex[1,17]. This complex is required for the induction of macroautophagy. When nutrients are scarce TOR becomes inactive, this in turn prevents hyperphosphorylation of Atg13. As a result Atg13 associates with Atg1 forming a complex that initiates the second step; nucleation of autophagy proteins. During this second step Atg1 and Atg13 recruit Atg11, Atg13, Atg14, Atg17, Atg29, Atg31 to a site called the Phagophore Assembly Site (PAS). Once together these proteins will form a cup-shaped vesicle called the phagophore or isolation membrane[1,18]. This leads to the third step; the biogenesis, maturation and completion of the autophagosome. During this step the phagophore will grow enwrap the target, connection its two

ends to form the double membrane autophagosome[18]**.** The key protein during this step is the Atg8 which controls the expansion of the phagophore; also the amount of Atg8 determines the size of the autophagosome[19]. This protein is inserted into phagophore as Atg8-PE (PhospatidylEthanolamine) and remains present in the inner and outer membrane of the autophagosome up until its degradation[20]. Once the autophagosome is completely formed, the fourth step is initiated: fusion of autophagosomes. During this step the autophagosomes fuse with either vacuoles (in yeast)[1] or lysosomes (in higher eukaryotes)[1,18] upon which the fifth step begins; degradation of the autophagic bodies and its contents. During this step the contents of the autophagosome are broken down. In case of yeasts acidification of the vacuoles was shown to be important for degradation[21] after which the final step occurs recycling of degradation products. This means that the nutrients generated by the autophagy will be reused by the cell.

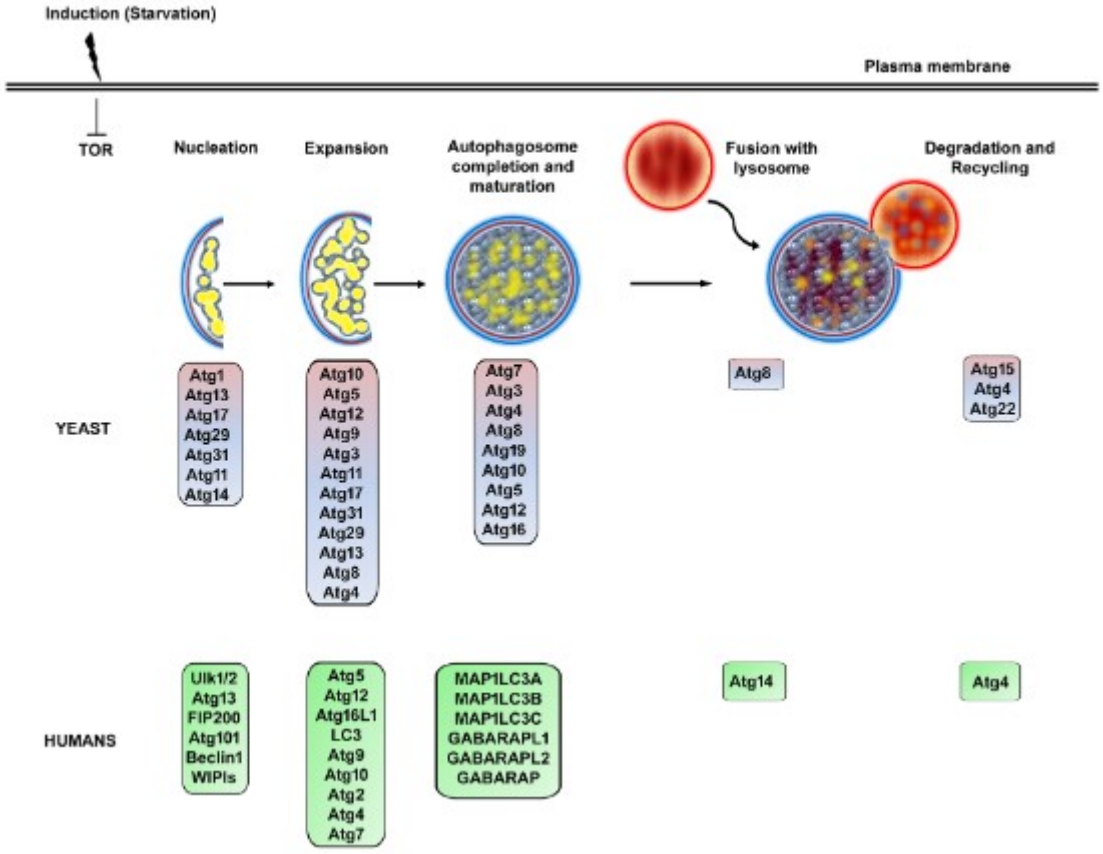

Figure 1: Schematic demonstrating the various steps in the autophagy process. The yeast and human autophagy proteins involved in nucleation, expansion, autophagosome maturation and completion, fusion, and degradation processes are mentioned. (image and description copied from Chinchwadkar *et al.*[1])

In microautophagy pieces of cytoplasmic material (this can be pieces of an organelle) are directly engulfed by either vacuolar or lysosomal membrane, upon which they are degraded. In Chaperone-Mediated Autophagy (CMA) a protein is recognized by the Hsc70 complex which transports it to the lysosome. There the protein is unfolded, transported across the lysosomal membrane and degraded[22]. Macroautophagy and microautophagy both have general and selective forms[1,23], while Chaperone-Mediated Autophagy (CMA) doesn't as it is selective per definition[22]. The CMA pathway is only found in higher eukaryotes[22]. The Cytoplasm-to-Vacuole Targeting (Cvt) pathway is considered a form of selective macroautophagy, despite the fact that the pathway is biosynthetic rather than degradative[24,25]. This pathway constitutively and selectively transports vacuolar hydrolases to the

vacuoles via autophagosomes[24,25]. In contrast to normal autophagy the content of the autophagosomes (the hydrolases) is not degraded[24,25]. (Macro)autophagy can be induced either by putting cells under stress conditions (such as nitrogen starvation)[1] or by Rapamycin which inhibits TOR[26].The autophagosome is formed primarily form the ER membrane, but also from other membranes such as that of the mitochondria[27].

Lipid Droplets:

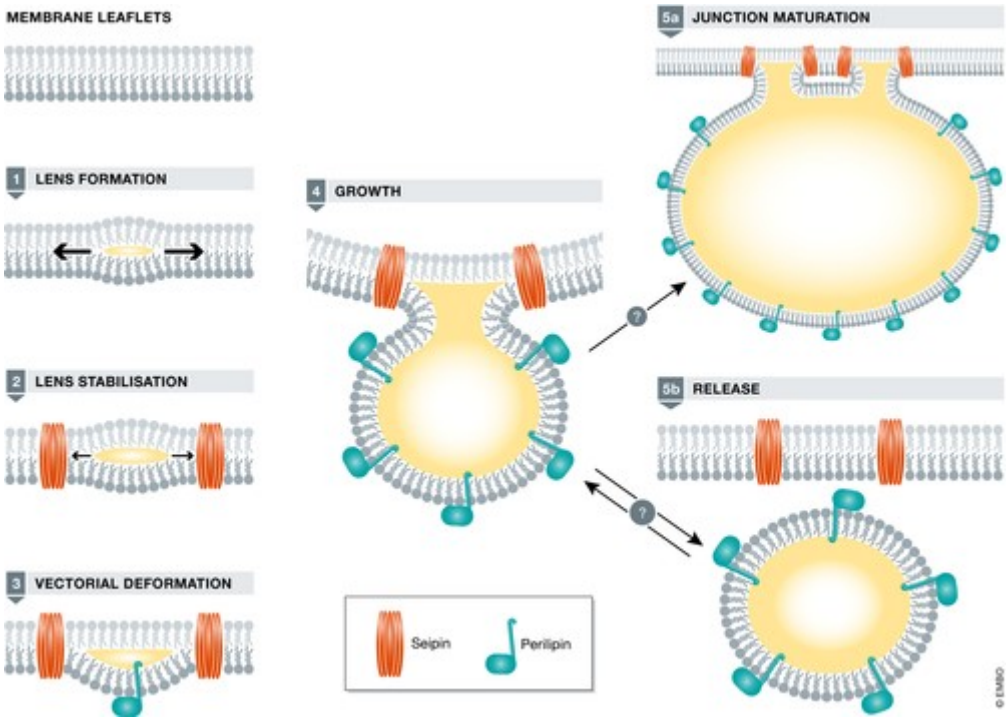

Figure 2: Schematic representation of the steps in Lipid Droplet assembly. See text for details. (image copied from Henne *et al.*[11])

Lipid Droplet biogenesis goes in several steps, the first of which is Lens formation. During this step neutral lipids synthesized in the ER coalesce together by lateral diffusion forming a lens[11]. This occurs because the energy costs of interacting with each other is lower than that of interacting with other lipids[28]. Once the lens is formed the second step lens/nascent droplet stabilization occurs, during which the lenses/nascent droplets are stabilized by seipin[29]. This allows the third step of vectorial membrane deformation to occur. This entails that the lens buds outward towards the cytosol forming a droplet. Perilipins are likely involved in membrane deformation as these proteins are abundantly present in nascent droplets/lenses[11]. Recticulons, REEP proteins and atlastins are also likely to be involved as these proteins promote tubular shapes in the ER[11]. The fourth step is droplet growth during which the nascent droplets grows and becomes surrounded by outer ER leaflet lipids; thereby maturing into LDs[11]. Then there are two possible fifth steps which are mutually exclusive[11]. In the first option the LDs remain attached to the ER via a junction; in the second the LDs detach from the ER. The second option seems to occur much less often than the first[11,30] and seems to occur only in higher eukaryotes, not in yeasts[11,31]. In most cases however LDs remain closely associated with the ER through ER-droplet junctions maintained by seipin (Sei1p in yeast)[11].

Proteins with dual functions:

Several studies have revealed that some proteins involved in autophagy are also involved in lipid metabolism as well as lipid droplet biogenesis and homeostasis (meaning control of the size and number of lipid Droplets)(Table 1).

mTORC complex:

The TOR protein is the main controller of autophagosome formation[1]. A recent study[32] revealed that Leptin induces lipid droplet formation in IEC-6 cells (epithelial cells of the small intestine of *Rattus norvegicus*[33]). The same study also revealed that Leptin also induces phosphorylation of the main downstream targets (p70S6K and 4EBP1) of the mTORC complex (of which mTOR (mammalian TOR) is a part of) and that inhibition of TOR by rapamycin significantly inhibits formation of LDs[32]. Together these results seem to indicate that TOR is also a controller of LD formation. This is not strange because, as the authors of the article note, besides from controlling autophagy and LD formation mTOR also modulates protein synthesis and ribosome biogenesis[32,34]. Here it must be noted however that it is possible that the decrease of LDs was caused by an increase in autophagy (thus also of lipid droplets) triggered by rapamycin inhibiting mTOR and thus triggering autophagosome formation[1]. However it is also possible that LD formation is controlled by mTOR via another mechanism other than (macro)autophagy. Further research needs to be carried out to see if using rapamycin still inhibits LD formation, if phagophore expansion and/or autophagosome maturation are prevented trough knocking out of genes (preferably Atg8 as this protein is central in phagophore expansion). If this is the case then mTOR does not control LD formation via (macro)autophagy.

Atg2:

Atg2 is a protein required for autophagosome formation and the Cvt pathway in yeast[35,36]. In humans there are two Atg2 proteins Atg2A and Atg2B, both are required for the elongation of the phagophore[37] and the formation of autophagosomes but they are redundant to each other[38].Several studies[37–39] are supportive of Atg2 proteins having a roles in the biogenesis and homeostasis of LDs. A study[38] in HeLa cells[40] with GFP-tagged Atg2A revealed by staining LDs that Atg2A localized to LDs. It was also revealed that this localization happened independently of other Atg proteins and that the Atg2A localization to both the autophagosome and the LDs is dependent on amino acids 1723–1829[38]. The same study revealed by inhibiting Atg2A and Atg2B with siRNA, that Atg2A and Atg2B regulate the size, number and distribution of LDs as the inhibition of these proteins caused an increase in size and number of LDs and also caused LDs to cluster together. The data also indicated this inhibition was independent of autophagy. A study[39] in U2OS[41], G361[42] and HeLa cells corroborated these findings and revealed that the localization of Atg2A to the LDs is independent of the autophagic status (meaning regardless of the amount of nutrients available) of the cells. A study[37] in Mouse Embryonic Fibroblasts (MEFs)[43], HeLa cells and HEK293T[44] cells revealed that amino acids 1723–1829 in Atg2A required for localization to autophagosomes and LDs form an amphipathic helix. By deleting parts of the *ATG2A* gene, the same study revealed that amino acids 1-198 is required for the localization to the phagophore (and thus the autophagosome) while amino acids 1830-1938 are required for the localization to the LDs. Together these studies indicated that human Atg2 proteins; or at least Atg2A; localize to LDs; where they regulate the size, number and distribution of the LDs via a mechanism other than autophagy. Further research is required to determine how the mechanism in question works. Further inquiries are also required to see if the Atg2 proteins in other organisms also display localization to the LDs regardless of autophagic status.

This also needs to be done for human Atg2B. The fact that (human) Atg2 proteins are localized to the LDs regardless of autophagic status may also indicate that LDs are involved in the formation of autophagosomes.

Atg5, Atg7 and Atg12:

Atg5 is a protein that in mice conjugates with Atg12, when Atg12 is activated by Atg7. This complex is required for the maturation of autophagosomes[45–47]. In a study[45] in a MEF (Mouse Embryonic Fibroblasts) an *atg5* knockout strain was generated. This strain displayed a halted growth of LDs during adipogenesis (differentiation of the MEF cells to adipocytes). Another study[48] showed that White Adipose Tissue (WAT) cells in mice lacking Atg7 showed a similar phenotype, displaying halted adipogenesis and multiple smaller LDs instead of one, which occupies nearly the entirety of the cell volume. In both articles[45,48] the authors stated that since the absence of the Atg protein in question (Atg5 in one, Atg7 in the other) caused problems with adipogenesis, autophagy must be important in adipogenesis. However since Atg2A also regulates LD size, number and distribution in a mechanism independent of autophagy, it is possible that the same applies for Atg5 and Atg7. Localization studies similar to those done for Atg2(A) will have to be performed to see if this is possible. It is interesting to note however that a study[49] in mice with *atg5* and *atg12* knocked out in the POMC neurons[50], revealed that in absence of Atg12 but not of in the absence Atg5, POMC neurons are less sensitive to Leptin and the mice displayed increased obesity in comparison to WT mice when on a high fat diet. The authors of this article then state that since conjugation of Atg5 and Atg12 is necessary for autophagy, Atg12 counteracts the excessive weight gain in mice via a mechanism independent of autophagy. This supports the theory that Atg5 and Atg7 (which are closely associated with Atg12) influence adipogenesis and LD growth in a mechanism independent of autophagy. But as mentioned earlier further research is required to substantiate such claims. Since the *atg5* and *atg7* knockouts displayed halted LD formation and growth[45,48], it is possible that the halt in adipogenesis was caused by the halted LD growth. Future studies will be needed to reveal if this is the case. If it is, then the functions (aside from autophagosome formation) of Atg5 and Atg7 might be in LD formation and growth and control adipogenesis through this. Future studies with cultured cells are also needed to see if Atg12 has a more direct influence on LD formation like Atg5 and Atg7 do. Also it is interesting that the absence of Atg12 causes leptin sensitivity to drop, as leptin was established earlier to lipid droplet formation by inducing mTOR.

Atg14L:

Atg14L is a protein that (in humans) is important for the targeting of the PtdInsKC3 complex to the ER which enable the membrane rearrangements necessary for the formation of phagophores and autophagosomes[39]. A study in U2OS, G361 and HeLa cells revealed that the Atg14L is localized to the LDs (being colocalized with Atg2A) and that its localization is independent of the autophagic status (i.e. regardless of the amount of nutrients available) of the cells**.** Since Atg14L is colocalized with Atg2A to the LDs, it is an intriguing possibility that Atg14L regulates LD size, number and distribution independently of autophagy in a manner similar to Atg2A.

Atg15:

In yeast (specifically *Saccharomyces cerevisiae*) Atg15 is responsible for the lysis of macroautophagic bodies in the vacuole, allowing for the degradation of the content of these bodies[26,51]. Atg15 is also required for the degradation of Cvt bodies and multivesicular bodies[51], and is also required for (micro)lipophagy[14]. A study in a *S. cerevisiae atg15* knockout strain revealed primarily through

fluorescence microscopy with strained LDs that the number of LDs dropped during the stationary phase (specifically after diauxic shift) in the knockout strain. The study also revealed, by mutating the putative lipase motif of Atg15 and by altering the localization of Atg15, that the putative lipase motif and localization to the vacuole is required for Atg15 to be able to maintain the LD number after the diauxic shift. Furthermore the study revealed that knocking out *tgl3* and *tlg4* in the *atg15* knockout strain prevented the drop in LD number after diauxic shift. This means that Atg15 prevents the drop in LD number by controlling and suppressing lipolysis enzymes Tgl3 and Tgl4. This was confirmed by quantifying phospholipids in both Wild-Type (WT) and *atg15* knockout cells. Finally the study compared the viabilities strains lacking *ATG1*, *ATG15* and/or *TGL3* with the WT strain. This revealed that *atg15* knockout caused a stronger loss of viability than the WT or *atg1* knockout strain which could partially be alleviated if Tgl3 was also absent, further confirming that enhanced lipolysis was responsible for the drop in viability in those cells. Considering that other Atg proteins have been revealed to have side function which is independent of autophagy and the fact that Atg15 is involved in breakdown of lipid membranes in autophagy[26,51], it is likely that Atg15 also suppresses lipolysis by Tgl3 and Tgl4 via mechanism independent of autophagy.

LC3:

LC3 is a protein involved in autophagosome formation in higher eukaryotes[1] and it is an homolog of the Atg8 protein in yeast[52]. When autophagy is induced LC3 is converted from its soluble LC3-I form to its autophagosomal membrane bound form LC3-II[53,54]. This is required for phagohore expansion[1]. LC3-II is considered a good marker for autophagy[55]. A study showed that under starvation conditions LC3 (specifically LC3-II) is localized to LDs aside from the autophagosomes[56]. A study[57] performed in HeLa, HepG2[58] and 3T3-L1 cells[59] used siRNA to inhibit production of LC3 and then observed the cells under a fluorescence microscope. The cells displayed a strong reduction in LD formation. The same study did the siRNA inhibition of LC3 in 3T3-L1 cells which were differentiating into adipocytes showing that also these cells displayed a reduction in LD formation. Together these results show that LC3-II is required for the formation of LDs when under starvation conditions. LC3 most likely influences LD biogenesis in a mechanism independent of autophagy as autophagy cannot construct organelles; only degrade them. Further research will be required to elucidate the mechanism trough which LC3 regulate LD biogenesis.

TMEM41B:

TMEM41B is an ER transmembrane protein required for the creation of closed and mature autophagosomes. A study[60] in H4(*Homo sapiens* neuroglioma cells)[61] and HeLa cells revealed this trough LC3-II immunostaining. The same study revealed trough Electron Microscopy (EM) that in absence of TMEM41B the number of LDs increases and the size of the LDs also increases. This study also measured the flux of Fatty Acids (FAs) from the LDs to the mitochondria revealing that FA utilization was significantly lower in absence of TMEM41B. Together these findings indicate that TMEM41B Is involved in the mobilization and β-oxidation of Lipids/FAs of the LDs. It is important to note that in contrast to the other proteins covered here, the role of TMEM41B in both autophagy and lipid mobilization was discovered in the same study[60]. This means that for TMEM41B a secondary function in lipid mobilization wasn't really discovered. Therefore it is unclear which function is primary and which is secondary, if there even is a hierarchy between the two functions. This might be something to investigate in a future study. It might also be possible that the mobilization of lipids

is required for the formation of autophagosome, meaning LDs are involved in autophagosome formation. This was suggested earlier when Atg2 was discussed.

Table 1: Proteins involved in both (macro)autophagy and lipid metabolism/Lipid Droplet (LD) formation or maintenance

Protein	Function in autophagy	Function in Lipid metabolism	Organism of origin of gene	Expressed in: organism (cell type)
mTOR	Controls autophagy trough mTORC pathway	Controls pathway for lipid droplet formation	*Rattus norvegicus*	*Rattus norvegicus* (IEC-6[32])
Atg2A	Phagophore expansion	Regulates LD size and distribution, involved in LD biogenesis (localization to LD)	*Homo sapiens*	*Homo sapiens* (HeLa[37–39], U2OS[39], G361[39], HEK293T[37]) and *Mus musculus* (MEF[37])
Atg2B	Phagophore expansion	Regulates LD size and distribution, involved in LD biogenesis.	*Homo sapiens*	*Homo sapiens* (HeLa[38])
Atg5	Phagophore expansion	Adipogenesis (absence impairs adipogenesis and LD formation)	*Mus musculus*	*Mus musculus* (MEF[45])
Atg7	Phagophore expansion	Adipogenesis (absence impairs adipogenesis and LD formation)	*Mus musculus*	*Mus musculus* (MEF[48])
Atg12	Phagophore expansion	Controls lipid Levels in POMC cells (absence cause obesity)	*Mus musculus*	*Mus musculus* (POMC neurons[49])
Atg14	Phagophore formation	Function unknown (localized to LD in same manner as Atg2A thus possibly same function)	*Homo sapiens*	*Homo sapiens* (HeLa[39], U2OS[39], G361[39])
Atg15	Degradation of autophagic bodies	Regulates LD number (absence causes decrease in LD number under nutrient starvation)	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*[51]
LC3	Phagophore expansion	LD biogenesis (absence reduces LD formation)	*Homo sapiens* and *Mus musculus*	*Homo sapiens* (HeLa[57], HepG2[57]) and *Mus musculus* (3T3-L1[57])
TMEM41B	Autophagosome maturation	Lipid mobilization (absence causes enlarged LDs)	*Homo sapiens*	*Homo sapiens* (HeLa[60], H4[60])
PNPLA5	Required for optimal autophagy (absence causes reduction in all forms of macroautophagy)	Phospholipase capable of metabolizing TriGlyceride (TG)	*Homo sapiens*	*Homo sapiens* (HeLa[62])

PNPLA5:

A study[62] in HeLa cells revealed that the cells showed an increase in both (macro)autophagy (using LC3-GFP as a marker for autophagosome formation) and LD number when the cells were grown on Oleic Acid (OA). The study also showed a close association between LDs and autophagosomes when grown on OA. The study then screened the PNPLA 1-4 (part of the PNPLA 1-5 protein family of Phosolipases[63]),which can metabolize TriGlycerids (TG), by knocking them out. This revealed that out of the five PNPLA proteins only PNPLA5 knockdown inhibited LC3-II conversion and therefore autophagy. The study also revealed that this knockdown affected various types of (macro)autophagy including mitophagy (autophagy of mitochondria), xenophagy (autophagy of bacteria) and bulk autophagy of the cytosol. Together this strongly indicates that PNPLA5 is important for the formation of autophagosomes. This makes PNPLA5 unique amongst the proteins discussed here as it is primarily involved in lipid metabolism and has its secondary function in autophagy instead of the other way around. It is possible, as noted by the authors of the article, that the lipase activity of PNPLA5 is important for the generation of the phospholipids of the autophagosomal membrane. A future study will have to look into this. Also further research is required to see if PNPLA5 localizes to the autophagosome as this might indicate that its role in autophagy might not (solely) be supplying lipids for autophagosomes. It is important to note is that the authors used LC3-II as a marker for autophagy which, according to a study[64], might not be as reliable as an indicator of autophagy as commonly thought.

Summary and general discussion of proteins with a dual function:

In this paragraph proteins that have a function in both autophagosomes and lipid Droplets/lipid metabolism were discussed. Most of these proteins have their function in autophagosome biogenesis and the secondary function in lipid Droplets. The exceptions to this rule were TMEM41B, for which both functions were discovered simultaneously, and PNPLA5 whose primary function is in lipid metabolism. Important to note is that almost all the proteins discussed were from mammals (mice and humans). The one exception to this rule is Atg15 which came from *S. cerevisiae*. This might indicate that the yeast homologues of the other proteins discussed in this paragraph may display a similar dual function. This dual function has been ruled out for the yeast homologues for LC3; Atg8. As a study[14] ruled out the requirement of Atg8 for the formation of lipid Droplets. It must be noted however that the same study also stated that the knockout of Atg15 had no effect on LD formation. But as discussed earlier knocking out Atg15 only has an effect on LDs after the Diauxic shift occurred, which was not performed in the study[14] that ruled out Atg8 and Atg15. This may mean that under more specific conditions knocking out Atg8 may have a noticeable effect on LD biogenesis or homeostasis. Regardless this needs to be researched in a future study because, if the yeast homologues of the protein discussed here also display a dual function, than this might mean that the connection between LDs and autophagosomes is more important than previously assumed.

Direct functions of lipid droplets in autophagy:

Recently several interactions between LDs and autophagosomes have been indicated or suggested. The interaction and corresponding theories will be discussed to get an insight in the implication of these interactions and theories.

Lipid droplets as deposits of excess fatty acids:

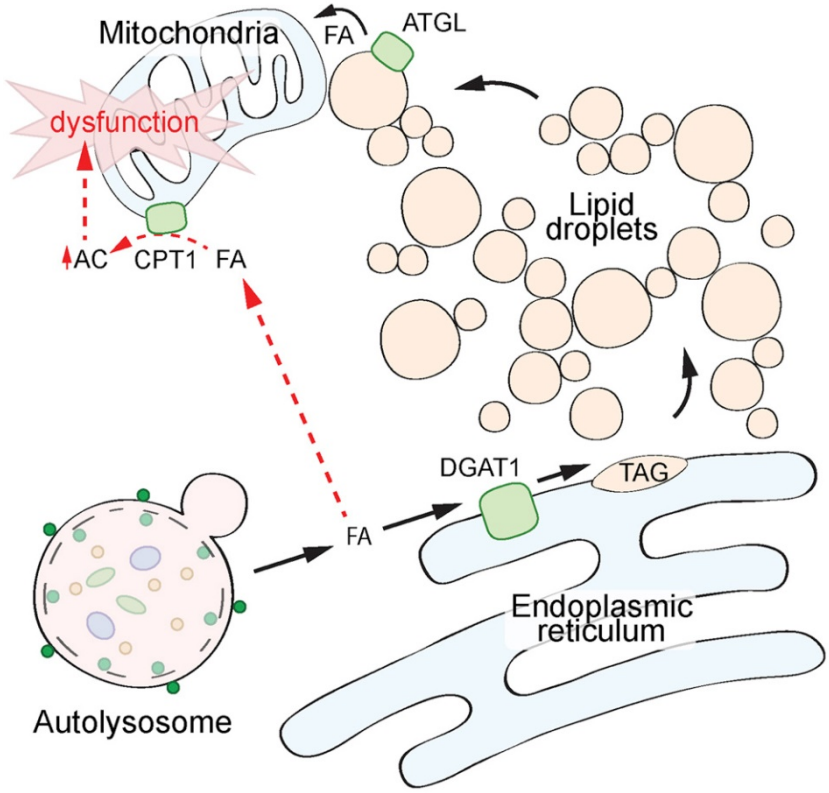

Figure 3: Schematic representation of the formation of LDs in response to autophagy and how the absence of LDs causes mitochondria dysfunction. See text for details. (image copied from Nguyen *et al.* 2017[65])

A study revealed a class of smaller LDs which were detached from the ER[66]. The authors of this article theorized that these LDs may funnel away excess TG from the ER to prevent the ER from accumulating toxic intermediates. A study[67] in which MEF cells revealed that when grown on Hank's Balanced Salt Solution (HBSS)(which lacks amino acids), there was an increase in the number of LDs in comparison to when grown on a richer medium. The study also used a FA pulse-chase assay and a colocalization study to show that the lipids of the extra LDs originate from hydrolyzed phospholipids. Together this indicates that the increase of LDs upon starvation is caused by the autophagy of other organelles, the lipids released during this process ending up in the LDs. The study also revealed that LDs protected the mitochondria from excess FA when the mitochondria are incapable of metabolizing FAs. A study[65] revealed that the autophagy dependent LD biogenesis occurred in several human cells (including HeLa, Huh7[68] and U2OS cells)not only when grown on HBSS but also when grown on other media lacking amino acids. The study also revealed that inhibition of the mTORC complex also caused the increase in LD number even when amino acids were present. Together with the fact that the lipids of the extra LDs originate from hydrolyzed phospholipids, this means that the theory that the lipids of the extra LDs originate from the membranes of organelles degraded by autophagy is most likely true. This same study inhibited DGAT1 and DGAT2 in MEF cells both when grown on nutrient rich oleate containing medium and on nutrient poor HBSS medium. This revealed that inhibition of DGAT1 or DGAT2 only partially inhibited LD formation/biogenesis when the cells were grown on rich medium, requiring inhibition of both proteins in order to completely inhibit LD formation. This experiment also revealed that when cells were grown on

nutrient poor medium inhibition of DGAT1 would largely inhibit LD formation, while inhibition of DGAT2 had no effect. The study also revealed that inhibiting DGAT1 in MEF cells caused an increase acylcarnitines. Since acycarnitines are potentially toxic lipids[65,69–71], this may mean that LD store the acylcarnitines to protect the rest of the cells from its potentially toxic effects. Together the data of all these studies seem to indicate that LDs protect the cell from lipotoxicity by capturing potentially toxic lipids, keeping these lipids from harming the other parts of the cells (figure 3). This also applies to the Lipids generated during the breakdown of organelles by autophagy, meaning that LDs work together with autophagosomes by handling the lipid waste generated by macroautophagy (figure 3).

Role of lipid droplets in autophagosome formation:

Recently, the complex pathway of LD involvement in autophagosome formation has partially been elucidated. First a study[72] in *S. cerevisiae* revealed that even when exposed to nitrogen starvation the total amount of proteins in the cells remains the same as during exponential growth, while the amount of TAG and LDs increased in comparison to the exponential growth phase. The study then generated *dga1*, *lro1*, *are1* and *are2* knockout strains. Dga1 and Lro1 are enzymes necessary for synthesis of TAG[73–75], while Are1 and Are2 are required for synthesis of SE[76,77]. The knockout strain was then tested with a Pho8Δ60 assay[78,79] and a GFP-Atg8 cleavage assay[78,80]. This revealing that knocking out *dga1* and *lro1* significantly reduced (macro)autophagic activity while knockout of *are1* and *are2* only had a minor effect, knocking out all four proteins had an even stronger effect. Together this means that TAGs and, to a lesser extent, SEs are required for the formation of LDs. The Cvt pathway appeared to be unaffected by the absence of the four proteins, meaning that the Cvt pathway most likely can go without the synthesis of TAG and SE. In addition to this knockout experiment also revealed that WT and *are1 are2* knockout strain had a similar amount of Atg8-GFP spots (indicative of autophagosomal membrane). On the other hand the *dga1 lro1* knockout strain showed a lower amount of spots while the strain lacking all four proteins showed more spots than the WT strain. Furthermore the knockout revealed that TAG synthesis is required for the recruitment of downstream Atg proteins. Finally the same study revealed that LDs transiently interact (so called Kiss-and-run interactions) with membranes containing Atg8-PE (such as autophagosomes). Another study[62] also revealed these same interactions in mammalian cells (specifically HeLa cells). It is noted by the authors of the study[72] in the discussion that the LD formation was also impaired in the knockout strains thus it is possible that the formation of LDs instead of TAG synthesis is required for formation of autophagosomes.

Several studies[62,81,82] looked further into this. Of these studies Shpilka *et al.*[81] and Velazquez *et al.*[82] were most noteworthy as these focused extensively on the role the LD has in autophagosome biogenesis in *S. cerevisiae* and yielded similar data, yet the two studies arrived at two (seemingly) contradictory conclusions.

Shpilka *et al.* started off by using cerulenin to block Fatty Acid Synthase (FAS), the enzyme responsible for the synthesis of all C16 and C18 FAs[81,83,84], thereby blocking the synthesis of fatty acids. These cells along with mock-treated cells were subjected to nitrogen starvation and then tested using a GFP-Atg8 cleavage assay (Shpilka *et al.* always used GFP-Atg8 cleavage assay to test for autophagy). This revealed that mock treated cells delivered GFP-Atg8 to the vacuole and cleaved it but cells treated with cerulenin did not, causing GFP-Atg8 to form spot/puncta outside of the vacuole. Using radio-labeled Ape1 (one of the proteins transported to the vacuole by the Cvt pathway[25]) it was also revealed that cerulenin also inhibited the processing and maturation of Ape1,

indicating that FAS activity is necessary for the Cvt pathway to function. *fas1* and *fas2* (Fas1 and Fas2 are the components of FAS) knockout strains were made and tested for autophagy. This revealed that, when grown on medium with fatty acids, the knockout strains displayed normal (macro)autophagy. But when fatty acids were absent, autophagy was inhibited. Also a Pho8Δ60 assay revealed that Pho8Δ60 activity, and therefore (macro)autophagic activity, decreased along with the preincubation time with cerulenin. Together these results indicate that depletion of fatty acids inhibits autophagy.

Shpilka *et al.* then revealed trough fluorescence microscopy that the decrease in autophagic activity after cerulenin treatment correlated with the decrease in the number of LDs. It was also revealed that when cells were treated with cerulenin, the localization of LD stains shifted to the ER and that LD accumulation was inhibited. To look further into the necessity of LDs for autophagosome formation, a quadruple knockout strain (*dga1 lro1 are1 are2*) was generated. Cells of this strain were then observed using fluorescence microscopy revealing that these cells were lacking in LDs. This revealed that (macro)autophagy induction by nitrogen starvation was inhibited in absence of LDs while the Cvt pathway was not. This is in line with a previous study[72]. The DGA1 and ARE2 genes were inserted into the quadruple knockout strain under a galactose inducible promoter, revealing that when grown on galactose LDs were formed. When subjected to nitrogen starvation (in a galactose containing medium) (macro)autophagy occurred. It was also revealed addition of exogenous FAs could not make (macro)autophagy occur under nitrogen starvation in the quadruple knockout strain. In addition the quadruple knockout strain displayed a morphology similar to the WT (with the exception of lacking LDs) when grown on "rich" YPD medium, But under nitrogen starvation conditions autophagic bodies were hard to detect while these were easily visible in the WT strain. Together this seems indicate that LDs are required for starvation induced autophagy. Shpilka *et al*. also generated two strains lacking in either SE (*are1 are2*) or TAG (*dga1* lro1). A GFP-Atg8 cleavage assay revealed defective autophagic processing in both strains. Also observations under a fluorescence microscope revealed that the double (*are1 are2* and *dga1 lro1*) and quadruple (*are1 are2 dga1 lro1*) knockout strains had no GFP-Atg8 in their vacuoles indicating a lack of (macro)autophagy. Atg8 accumulated in multiple spots in the *are1 are2* and quadruple knockout strains, but there was no accumulation in the *dga1 lro1* strain. Atg8 accumulated in its unlipidated form in the *dga1 lro1* strain, but in its lipidated form in the *are1 are2* and quadruple knockout strains. Indicating these spots are related to autophagosomes. Also in the double and quadruple knockout strains the starvation induced degradation of long-lived proteins was inhibited. Knockout strains of several TAG lipases (*tgl3*, *tgl4*, *tgl5* and *ayr1*), several SE hydrolases (*tgl1*, *yeh1* and *yeh2*) and *ldh1* (Ldh1 protein is involved in both) were made. Fluorescence microscopy revealed that only the *ayr1*, *yeh1* and *ldh1* knockout strains showed inhibition of (macro)autophagy; the *ayr1 ldh1* strain showed significant inhibition of (macro)autophagy. Together these results indicate that both TAG and SE are required for the formation of autophagosomes.

Velazquez *et al.*[82] generated the same double (*are1 are2* and *dga1 lro1*) and quadruple (*dga1 lro1 are1 are2*) knockout strains and subjected them to GFP-Atg8 cleavage assay and cytRosella assay[85]. This revealed that when exposed to nitrogen starvation the cells of all strain displayed defective autophagy, but autophagy was on the level of WT cells when induced by rapamycin. It was also revealed through a western blot that these differences were not a result of differential activation of TORC1. Under the microscope the double and quadruple knockout strains all showed WT numbers of autophagosomes when induced with rapamycin, but not when induced with nitrogen starvation. The

quadruple knockout strain showed also showed Atg8 spots localized to the ER Exit Sites (ERES), which might explain the observation of Shpilka *et al*. that Atg8 is localized in multiple spots in the quadruple knockout strain while the *are1 are2* strain showed no such spots. Velazquez *et al.* thus revealed that LDs are required for starvation induced autophagy but not Rapamycin induced autophagy.

Velazquez *et al*. tested the Unfolded Protein Response (UPR) of the quadruple knockout strain revealing, that while it was four times higher than the WT strain, UPR did not interfere with autophagy as autophagic flux was unaffected in both WT and quadruple knockout strains when the UPR was inhibited. Shpilka *et al.* also revealed that when under nitrogen starvation conditions the quadruple knockout strain showed expansion of both the ER and the mitochondria. In addition the mitochondria showed strangely shaped cristae. Velazquez *et al.* made a similar observation stating that interconnected ER network collapsed into a simplified network in the quadruple knockout strain under nitrogen starvation conditions. It was noted by the authors of both articles that the expansion of the ER[81] and the collapse of the ER network[82] most likely occurs due to inability of the ER of storing its excess FAs in the LDs. Velazquez *et al.* also revealed that there were no structural changes to the ER when autophagy was induced by rapamycin. Velazquez *et al.* also used 10 µg/ml cerulenin to inhibit FA synthesis in the quadruple knockout strain under starvation conditions revealing that this restored a WT-like morphology and showed an improved cell viability. This seems in direct contradiction with Shpilka *et al.* who stated that using cerulenin to inhibit FA synthesis in the quadruple knockout strain under starvation conditions caused "a rapid and complete loss of viability". Shpilka *et al.* used 50 µM Cerulenin which is the same as 10 µg/ml (molecular weight: 223,272 g/mol[86]), ruling out different concentrations as the cause of the different conclusions. It must be noted that even in Shpilka *et al* the quadruple knockout strain still had some viable cells after being treated with cerulenin, but this does not explain the discrepancy between the two articles. There was no explanation given by Shpilka *et al.* for the expansion of the mitochondria but, since LDs protect mitochondria from excess FAs[67], it might be possible that in absence of LDs mitochondria swell due to excess FAs like in the ER. Together this indicates that LDs work as buffers of FAs, which is required for autophagy to function normally.

Velazquez *et al.* revealed that the PhosophoLipid (PL) composition was altered in the quadruple knockout. PhosphatidylInositol (PI) content increased while Phosphatidic Acid (PA) and PhosphatidylGlycerol (PG) content decreased. PI is synthesized out of inositol, this reaction also involves a derivative of PA, causing PA to be consumed in the synthesis process[87]. When the quadruple knockout strain was grown on a nitrogen starvation medium without inositol, PI and PA content was similar to that of the WT strain. This indicates alleviation as the differences in PL composition were reduced. In addition autophagic flux improved significantly although WT levels were not reached. This indicates that the altered PL composition interfered with autophagosome formation. The quadruple knockout strain was also more sensitive/less resistant to FAs when inositol was absent, indicating that PI are used to buffer the excess FAs. This is further supported by the fact that the quadruple knockout strain showed an increase in Ino1 (enzyme for synthesis of inositol[88]). Velazquez *et al.* then knocked out *OPI1* in both the WT and quadruple knockout strains generating two new strains (*opi1* and *dga1 lro1 are1 are2 opi1*). Opi1 is an inhibitor of the transcription PL synthesis genesis genes; when OPI1 is deleted ER membrane expansion occurs. This revealed that under nitrogen starvation conditions the *dga1 lro1 are1 are2 opi1* strain showed improved autophagic flux and FA resistance in comparison to the quadruple knockout strain. This effect was even stronger when inositol was absent, to the point of the flux being close to the WT level. In

presence of inositol PI content still increased and PA content decreased, but as noted before, starvation induced autophagy still occurred. Cerulenin treatment in absence of inositol caused the quadruple knockout strain to display the same level autophagic flux as the *dga1 lro1 are1 are2 opi1* strain, indicating that cerulenin and *opi1* knockout affect the same pathway. Aberrant localization of Atg8 (localization to ERES) still occurred under nitrogen starvation conditions in absence of inositol in both quadruple knockout and *dga1 lro1 are1 are2 opi1* strains. On the other hand cerulenin treatment in absence of inositol prevented the formation of aberrant Atg8 spots under nitrogen starvation conditions, indicating that puncta formation is highly sensitive to excess FA; even under improved FA resistance in *dga1 lro1 are1 are2 opi1* cells. Together this reveals the system that LDs buffer excess FAs and that without LDs the cells produce more PI to buffer FAs. But the increase in PI disrupts autophagosome formation. However as pointed out in the discussion of Velazquez *et al.*, this does not rule out the possibilities that LDs also contribute FAs to autophagosomes.

Considering the aberrant localization of Atg8 in the absence of LDs the previous theory (that Atg8 is important for autophagosome biogenesis under nitrogen starvation conditions) might be wrong. If the theory is correct then that means that LDs and Atg8 are influencing each other, i.e. for one to function properly the other is required. Further research will be required in order to determine if this is the case.

Theories about possible functions of LDs in autophagy:

Ice2 and Ldb16 are two proteins that reside on the contact sites of the ER and LDs[89,90]. Ice2 couples TAG utilization to lipid synthesis in the ER[89], while Ldb16 is important for phospholipid metabolism and LD size[90]. In Shpilka *et al.* the *ice2* and *ldb16* knockout strains revealed that the absence of Ice2 cause an excess number of LDs (due to inability to properly utilize them), while absence of Ldb16 caused supersized LDs. The authors note in the discussion that lipids might be funneled from the LDs to the ER and this process might be essential for the formation of autophagosomes (figure 4). While Velazquez *et al.* has ruled out that this is essential for the formation of autophagosomes, it is still possible that this lipid flow still occurs. It might in fact be possible that the flow of lipid from LDs to ER does not directly contribute to the formation of autophagosomes, but instead replaces the lipids and FAs the ER uses to form the autophagosome. This hypothetical process could potentially occur both before and after autophagosome formation. Considering that such a process would continuously occur under starvation conditions, it might not be possible to distinguish in which order this takes place. It be must be noted that the distinction between these two possibilities may not be absolute. In a future study autophagosome biogenesis needs to be induced while flow of FAs from LDs to ER is blocked. If the ER volume decreases under these conditions then that indicates that LDs help replace the FAs and Lipids the ER contributed to autophagosome biogenesis.

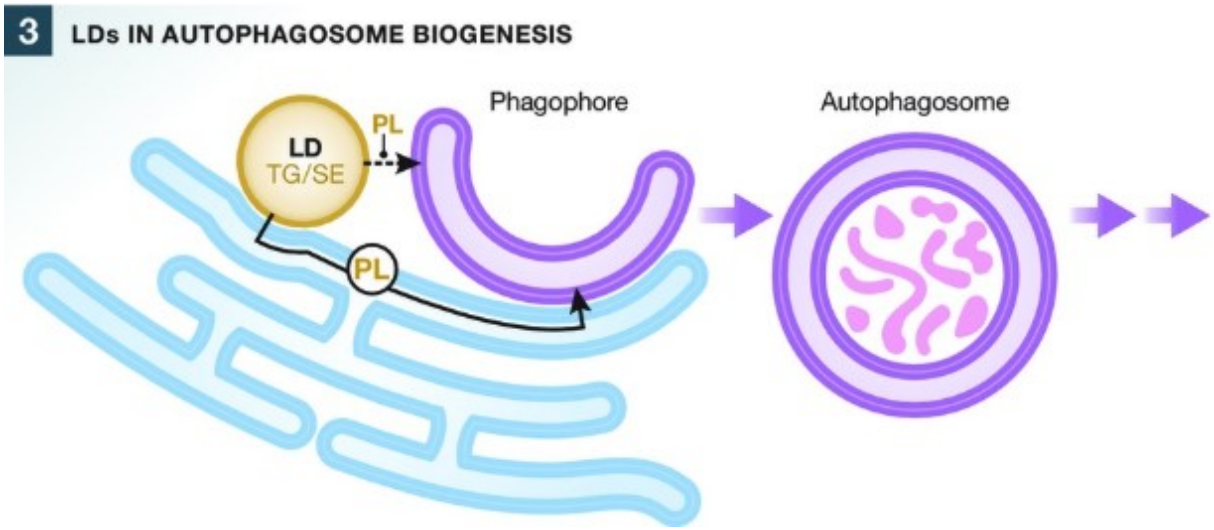

Figure 4: Schematic representation of the theory that LDs supplies lipids to the ER and via the ER to the Phagophore and thus the autophagosome (theory proposed by Shpilka *et al.*). (image copied from Deretic 2015[91])

A recent study has revealed that contact sites between mitochondria and ER are important for autophagosome formation[92]. Another study revealed that mitochondria are physically and metabolically linked by Perilipin5 in mice[12]. LDs seem to accumulate at the ER-vacuole/lysosome interface forming a tri-organelle junction[11,13]. Considering these facts, along with the conclusions reached earlier in this paragraph, it might be possible that the tri-organelle junction is involved in and important for autophagosome biogenesis.

It is also possible that the LDs do directly contribute Lipids and FAs for the formation of autophagosomes, as the direct origin of all the lipids of the autophagosome is still unknown[27]. But that the contributed lipids are for the optimization of the autophagosomes. This theory holds some merit as the quadruple knockout strain (lacking in LDs) in Velazquez *et al*. always had a lower autophagic flux than the WT strain regardless of the methods used to alleviate the drop in autophagic flux (under some conditions the knockout strain came very close to WT levels but never matched it). This might indicate that the LDs are necessary for the optimization of LD formation.

Interactions between the LDs and the "final destination" of autophagosomes, the vacuole, were also investigated. This was done to see if LDs have any influence on (macro)autophagy by influencing the vacuole. A study[93] in *S. cerevisiae* revealed that LDs are consumed in a manner independent of autophagy during growth resumption, and that the lipids released this way are channeled to the vacuole. This way the LDs are involved in vacuole homeostasis. The authors noted in the discussion that a similar interaction between LDs and lysosomes in higher eukaryotes. Considering that LDs are consumed for vacuole homeostasis, it is also possible that there also other interaction between the LDs and the vacuoles including one possibly important for autophagy. Another study[13] in *S. cerevisiae* revealed that ER-Vacuole contact sites (NVJs) are involved in the biogenesis of starvation and therefore autophagy induced[65,67] LDs. The biogenesis of these LDs occurs at the NVJs This might explain how the FAs generated during (macro)autophagic degradation of organelles ends up in the LDs, as they are transferred to the LDs via the LD contact sites with the vacuole (LD formation at NVJs means automatic contact sites with both ER and vacuoles).

Summary of direct functions of Lipid droplets:

This paragraph revealed an important role of the lipid droplets in autophagy. Primarily by handling excess fatty acids whether generated during the autophagy of an organelle or simply present in the environment. By handling the fatty acids the lipid droplets protect both the ER and the mitochondria damage. This also prevents an altered lipid composition from forming in response to the excess fatty acids and thereby prevents the altered composition from disruption autophagosome biogenesis. This reveals a more general importance for lipid droplets in relation to autophagy.

Closing remarks:

In this review several proteins involved in both lipid metabolism and (macro)autophagy were shown, most of which were discovered in mammalian cells. Also shown here is that lipid droplets protect organelles (specifically ER and mitochondria) from excess fatty acids and in doing so, also prevent disruption of (macro)autophagy. Together this indicates that the interactions between lipid droplets/lipid metabolism and autophagosomes/(macro)autophagy are more extensive than previously thought. There are still many gaps in knowledge as to how these two organelles and the corresponding processes are connected. Therefore theories as to how the unknown parts may function were given. Here proposals are given for future research to close these gaps in knowledge. It is most important for the studies done in mammals to be performed in yeast and vice versa to see if the conclusions reached by the discussed articles hold true in general. Despite this, the role of lipid droplets/lipid metabolism in (macro)autophagy is still likely to be more important than previously thought.

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