

## Lipid droplet growth: Regulation of a Dynamic Organelle

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### Abstract

Intracellular lipid droplets (LDs) are remarkably dynamic and complex organelles that enact regulated storage and release of lipids to fulfil their fundamental roles in energy metabolism, membrane synthesis and provision of lipid-derived signaling molecules. Although small LDs are observed in all types of eukaryotic cells, it is adipocytes that present the widest range of sizes up to the massive unilocular droplet of a white adipocyte. Our knowledge of the proteins and associated processes that control LD dynamics is improving. The dynamic expression of LD-associated proteins is vital for controlling LD biology and is most apparent during adipocyte differentiation. Recent findings on the molecular mechanisms of lipid droplet enlargement reveal the importance of distinct functional groups of proteins and phospholipids.

**Highlights** (max 5 points 85 characters each)

Lipid droplet fusion involves major changes in the LD membrane components.

Phosphatidic Acid is a key regulator of LD dynamics.

Adipocyte differentiation invokes profound regulation of lipid droplet proteins.

Transitions between white and BRITE adipocytes require lipid droplet remodelling.

### Introduction

Survival in constantly fluctuating environments that expose organisms to times of both feast and famine have selected for the ability of cells to efficiently store and release energy. As excessive free fatty acids are toxic for the cell [1], their esterification into triacylglycerol (TAG) and accumulation within intracellular lipid droplets (LDs) provides a controllable mechanism to buffer and store releasable energy in an inert

form [2]. Once considered passive accumulators of fat, LDs are now emerging as dynamic organelles playing a central role in the regulation of lipid metabolism [3]. LDs are composed of a neutral lipid core surrounded by a phospholipid monolayer embedding a diverse set of proteins that facilitate the modulation of key parameters such as their size, stability, interactions and regulatable lipid storage.

As LD diameter can range from 0.1-100 $\mu$ m they can be considered the most morphologically dynamic organelle. LD growth can take place by the fusion of preexisting LDs, in situ lipid biosynthesis, or by the transfer of lipids from adjacent organelles, including the endoplasmic reticulum (ER) and other LDs. In this review we summarize the latest discoveries in relation to the mechanism of LD growth, with special attention to their dynamics in adipocytes.

### **The ER in LD biogenesis and growth**

The first steps in forming LDs are considered to take place in the ER where neutral lipids are synthesised and packaged in lens-like structures that grow and bud from the ER membrane (reviewed in [4, 5]). Recent findings indicate that the budding of LDs from the ER to the cytosol is facilitated by fat-storage-inducing transmembrane (FIT) proteins [6] which could be mediated by the direct binding [7] and partitioning of de novo TAG. Unexpectedly, a further study suggests that LDs are simultaneously accessible by proteins expressed in the cytosol and in the ER lumen [8]. Two models compatible with this unique topology are suggested, both involving the presence of ER membranes surrounding the LDs, with LDs emerging from either the cytosolic or luminal leaflet of the ER. The luminal model would imply that cytosolic proteins such as PLINs and ATGL would not bind the LD monolayer but a TAG-rich ER bilayer surrounding the LD. Adding further complexity to LD cell biology, a recent investigation by Ohsaki et al. has convincingly confirmed the presence of intranuclear LDs in hepatocyte cell lines [9] and revealed Promyelocytic Leukemia Protein Isoform II (PML-II) as a critical factor in their formation. Nuclear LDs potentially provide a source of fatty acid-derived ligands for nuclear receptors such as peroxisome proliferator-activated receptors (PPARs). In addition, PML has been found to be a potent activator of PPAR signalling and fatty acid oxidation [10, 11].

The ER protein SEIPIN has emerged as a key component of the protein machinery involved in LD biogenesis/maturation [12-14]. SEIPIN acts in ER-LD

contact sites to enable nascent LDs to acquire more lipids from the ER and grow to form mature LDs [12, 15]. SEIPIN also regulates the metabolism of phosphatidic acid (PA) at the LD-ER contact, acting as a scaffolding protein recruiting PA-metabolism enzymes such as LIPIN1 and AGPAT2 [16, 17] as well as controlling PA levels by inhibiting GPAT [18]. In addition to being the biosynthetic precursor of phospholipids and TAGs, PA is a cone-shaped phospholipid that facilitates LD fusion [19, 20] and could be accommodated in the regions of negative membrane curvature associated to LD budding and LD-ER connections [21]. The downregulation of the ER enzymes CDS1 or CDS2, which consume PA to form CDP-DAG, increases PA levels in the ER but also in LDs, an effect accompanied by LD enlargement and also observed with the downregulation of LIPIN1 [22]. The importance of PA in LD dynamics is highlighted by the localization of AGPAT3 and LIPIN1 $\gamma$  in LDs [23, 24], suggesting an in situ mechanism to control PA levels through its synthesis/degradation by these enzymes.

LDs and ER could be directly connected by a continuity in their membranes [24, 25], which would allow the transfer of lipids from the ER to support LD growth. In addition, these LD-ER connections could promote LD growth by facilitating the diffusion of lipogenic enzymes from the ER to increase in situ lipid synthesis [24, 26]. A selection mechanism for hairpin proteins diffusing to LDs from the ER may be mediated by the ER-associated degradation (ERAD) complex, with the ubiquitin ligase Doa10 facilitating the selective degradation of the ER pool of these proteins [27]. In addition, ADP-ribosylation factor 1 (Arf1)/COPI complex has been found to have a key role in the triggering the formation of membrane bridges linking the ER and LDs [28, 29]. However, freeze-fracture electron microscopy showed adjacent LDs and ER with independent membranes [30], and ER proteins such as GPAT4 are only observed in a subset of LDs, indicating that such connections could be transient or not present in some LDs.

## **LD-LD Fusion**

LD fusion occurs through two major mechanisms which produce substantially different outcomes (Fig. 1). LD-LD coalescence involves the formation of a continuity between the phospholipid monolayers of both LDs to rapidly merge their core contents. Although LD-LD coalescence is rarely observed in physiological conditions [31], it

occurs spontaneously as a result of altered phospholipid composition in the LD monolayers, either by deficient levels of LD-stabilizing PC [32] or by accumulation of the fusogenic PA [20]. This fusion will result in a decrease in the total LD surface/volume ratio, but the fate of the spare membrane components is not well understood.

A distinct LD fusion process emerged from the study of CIDE proteins, which are required for the formation of supersized LDs in adipocytes [33]. Both CIDEA and CIDEc catalyse a slow fusion mechanism in which a donor LD transfers its content to a larger LD in a process driven by their internal pressure gradient [34]. CIDE proteins stabilize LD pairs by the formation of trans homodimers at the LD-LD contact site [19, 34, 35], where they facilitate the transport of neutral lipids through the phospholipid monolayer. The lipid transfer step requires the presence of a cationic amphipathic helix, which by interacting with PA could interfere with the phospholipid barrier to increase its permeability to TAG [19]. CIDEc activity is enhanced and regulated by accessory proteins such as PLIN1 and Rab8a [35, 36]. However, CIDE proteins alone are probably sufficient to fulfil the full process as the expression of murine CIDEA in yeast produces LD enlargement despite these organisms lacking CIDE homologues and therefore are unlikely to present functional interactors [19].

A remaining question in CIDE-triggered LD enlargement is the fate of the phospholipids and proteins of the donor LD, which may be degraded or transferred to the expanding LD or other membranes. LDs have a limited capacity for proteins and their shrinking during lipolysis provokes the displacement of the proteins with lower affinity for the monolayer due to macromolecular crowding [37]. During CIDE activity, protein crowding could selectively influence the protein composition of large LDs (Fig. 1B). Similarly, the mechanisms to enlarge the membrane of the acceptor LD are not known and could involve the activation of PC synthesis through translocation of phosphocholine cytidyltransferase to the phospholipid-deficient expanding LDs [32], or in situ synthesis of PC from diffusing LysoPC [38] by LD-bound lysophosphatidylcholine acyltransferases [39]. Alternatively, the phospholipid deficiency during LD expansion could facilitate coalescence with small LDs, which would supply the phospholipid excess resulting from each fusion.

### **LD enlargement in brown, white and BRITE adipocytes**

There are two types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). Although both white and brown adipocytes accumulate large amounts of fat, their LDs differ in size, number and protein content [40]. White adipocytes optimize fat storage by accumulating lipids in a single giant (unilocular) LD occupying most of the cytoplasm. In contrast, brown adipocytes are filled with a many LDs of relatively smaller size (multilocular) which are very tightly associated with mitochondria [41]. This conformation increases the surface accessible to lipases facilitating the rapid release of fatty acids in response to cold-activated signals to fuel the mitochondria for the generation of heat by the UCP1-dependent uncoupling of oxidative phosphorylation [42].

Despite its functional relevance, the mechanisms leading to unilocular or multilocular LDs remain unclear. Although culture models of WAT and BAT adipocytes have been established [43, 44], unilocular adipocytes are rarely seen in 2D cultures, highlighting the need for innovative 3D culture systems to study the differential biology of white and brown adipocytes. Pre-adipocyte differentiation involves a progressive accumulation of fat in supersized LDs in parallel with induction of adipogenic genes. Examination of a 3T3-L1 adipocyte differentiation gene expression profile dataset [45] reveals dynamic changes in the levels of genes encoding LD-associated proteins (Fig. 2). Genes associated with de novo lipogenesis (*Lpin1*, *Dgat2*, *Acs11*, *Fasn*) show a rapid induction in expression from day 0 to day 4-6 followed by a decline to around 50% of maximal expression by day 18. *Cidec* follows the same expression profile in fitting with its role in LD enlargement. For most genes encoding LD proteins associated with lipolysis the trend was to rapidly increase expression between day 0 and day 6 with the high level of expression maintained up to day 18 (*G0s2*, *Lipe*, *Lpl*, *Bscl2/Seipin* and *Ces3*). However, *Pnpla2* (ATGL) and *Abhd5* (CGI-58) showed profiles more similar to the lipogenesis genes. Of the perilipins, *Plin1* showed a pattern fitting with its role in regulating lipolysis with an early increase that was maintained to day 18. These data indicate that during differentiation there is a peak in lipogenesis around day 4-6 when TAG is being generated and stored in expanding LDs whereas the lipolysis machinery is maintained to facilitate energy release in the mature adipocyte.

Time-lapse studies revealed that the formation of large LDs during adipogenesis involves LD fusion by lipid transfer characteristic of CIDE proteins [19, 46]. CIDEA and CIDEc appear as the key elements controlling the formation of

supersized LDs in adipocytes, as CIDEA/CIDEC double-deficient mice showed LDs of circa 0.6  $\mu\text{m}$ , which could represent the diameter achieved by CIDE-independent enlargement mechanisms [47]. Whereas, unlimited CIDE-triggered LD-LD fusion would lead to the unilocular phenotype characteristic of white adipocytes, this process must be halted at the multilocular stage in brown adipocytes. The differential expression of CIDE proteins in WAT and BAT could be important in cell-specific LD morphologies as in mice CIDEC is highly expressed in WAT and BAT and CIDEA is largely restricted to BAT [48]. However, the presence of CIDEA is not enough to induce a multilocular phenotype as in humans it is also expressed in WAT [49] and the white adipocytes of transgenic mice expressing human CIDEA in WAT maintained their unilocular structure [50]. In contrast, CIDEC is essential for the formation of unilocular LDs, as multilocular LDs are found in WAT of CIDEC-null mice, where the induction of CIDEA can partially sustain LD enlargement but is not sufficient to form unilocular LDs [47]. PLIN1 could facilitate the formation of unilocular droplets as it selectively interacts with CIDEC [51], but not CIDEA, increasing its TAG transfer efficiency [19]. Interestingly, the third member of the CIDE family, CIDEB, can also produce LD-LD TAG transfer in hepatocytes, but only the subset of hepatocytes expressing CIDEC or CIDEA will produce supersized LDs [52].

Profound remodelling of LDs is also observed in mature adipocytes when responding to signal transduction pathways. Study of the murine BAT LD proteome using mass spectrometry revealed increased levels of ADRP, PLIN1, HSL, ATGL and CIDEA following cold exposure [41], suggesting high rates of lipolysis as well as LD enlargement. Under conditions of prolonged lipolysis adipocytes experience a LD remodelling cycle, with progressive reduction on the LD size, followed by the formation of new LDs, which are subjected to an enlargement process [48], likely to be CIDE-triggered. This futile cycle of TAG degradation and resynthesis implies the release of substantial amounts of heat that will constitute a UCP1-independent thermogenic system.

An additional adipocyte termed BRITE (brown-in-white or beige) is a multilocular brown-like adipocyte found in WAT. The number of BRITE adipocytes is highly increased following cold exposure through beta adrenergic signalling. Importantly, lineage tracing studies show that white adipocytes within inguinal WAT can reversibly convert to BRITE adipocytes [53]. The transdifferentiation events are

apparent at both the gene expression and morphological level including transitions between the unilocular and multilocular LD appearance. The remodelling of WAT LDs, in response to cold acclimatisation, is consistent with direct control by LD-associated proteins accompanied by potentiation of the lipolytic machinery due to increased expression of ATGL and CGI-58 and decreased G0s2 [48]. Although mRNA expression of most LD proteins was increased in WAT of cold exposed mice, the greatest change was the enhancement of CIDEA. The coincident induction of CIDEA indicates that LD-LD fat transfer is an important process during WAT browning.

The transition from BRITE back to white adipocyte involves the acquisition of the unilocular phenotype and is coincident with the downregulation of LD-related genes, including Cidea, Cideb, Lpl, Dgat2, Plin3, Plin5 [54]. Overall these changes point to brown/BRITE adipocytes having a higher capacity to generate and turn over LDs and thus indicate a more dynamic nature than those in white adipocytes.

## **Conclusion**

The biosynthesis and expansion of LDs is driven by complex and integrated mechanisms involving interactions with other organelles and recruitment of enzymes required for expansion of the lipid core and modulating the phospholipid monolayer composition. The remodelling of LDs, which is essential for developmental processes and responses to physiological metabolic requirements, is dependent on LD-associated proteins. Further understanding of the molecules and mechanisms that mediate LD dynamics will provide important insights into the many metabolic diseases and other pathologies that are intrinsically linked with LD biology.

## **Figure Legends**

### **Figure 1. LD membrane remodelling in LD fusion.**

A) LD-LD coalescence is a rapid process favoured by a phospholipid deficiency in the monolayer (LD1) or an accumulation of fusogenic phospholipids such as PA (LD2). The resulting reduction in LD surface/volume implies an increase in phospholipid packing which may selectively influence protein binding. B) CIDE proteins form trans

complexes and interact with PA to promote slow LD fusion by the transfer of TAGs from small to large LDs. Phospholipid packing will increase in the shrinking LD and decrease in the expanding LD. Low affinity LD-proteins could be expelled from the shrinking LD due to protein crowding. In contrast, the expanding LD will increase its protein binding capacity, and could be selectively filled by the low-affinity proteins released from the donor LDs as a result of a local increase in the cytosolic concentration of these proteins and the loose phospholipid packing in the enlarged LD. C) Models coupling LD growth with membrane expansion. Translocation of CCT $\alpha$  to the expanding LD could activate PC synthesis, which should be supplied to the LD from the ER (left panel). Alternatively, the presence of ACSL3 and LPCAT1/2 in LDs could favour the in situ generation of PC from LysoPC, which could be released from the ER or the shrinking LD by PLA<sub>2</sub> (right panel). Abbreviations: Lipid droplet (LD), endoplasmic reticulum (ER), phosphatidylcholine (PC), phosphatidic acid (PA), cell death-inducing DFF45-like effector protein (CIDE), CTP:phosphocholine cytidyltransferase A (CCT $\alpha$ ), phospholipase A2 (PLA<sub>2</sub>), long-chain acyl-CoA synthetase 3 (ACSL3), lysophosphatidylcholine acyltransferase (LPCAT), diacylglycerol cholinephosphotransferase (CPT).

**Figure 2. Profile of genes encoding LD-associated proteins during adipogenesis.**

Expression of genes encoding LD proteins and markers adipogenesis (Pparg, Dlk1, Fabp4) and were analysed for 8 time points (days 0, 2, 4, 6, 8, 10, 14, and 18) of 3T3-L1 cell differentiation, in triplicate, that were submitted to the GEO public repository database (identifier: GSE34150) [45, 55]. Upper panel shows schematic illustration of 3T3-L1 cell differentiation to adipocyte over time course. For lower panel, normalised expression values were obtained from GEO2R (NCBI online gene expression tool) and data plotted are mean  $\pm$  Standard Deviation. The Perilipin genes (Plin 1-4) are grouped, as are genes associated with lipogenesis, lipolysis (Pnpla2/ATGL, Abhd5/CGI-58, Bslc2/Seipin, carboxylesterase 3 (Ces3)) or other functions (Mettl7b, caveolins Cav1 and Cav2, and carbonic anhydrase 3 (Car3)). The validity of the dataset was confirmed by the induction Fabp4 and Pparg over differentiation along with reduction in the pre-adipocyte marker gene Dlk1.



## References

(●) special interest, (●●) outstanding interest.

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*Two distinct types of hepatocytes were identified with different LD morphologies; one with small LDs and one with large LDs. The majority of hepatocytes express Cidea and Cidec which promoted lipid exchange and LD fusion in hepatocytes with large LD-containing. In contrast, Cideb was localized to both small and large LD-containing hepatocytes where it promoted LD fusion. Fasting induced Cidea/Cidec and increased the percentage of large LD-containing hepatocytes. Thus, Cideb promotes lipid storage under normal diet conditions, whereas Cidea and Cidec are responsible for liver steatosis under fasting and obese conditions.*

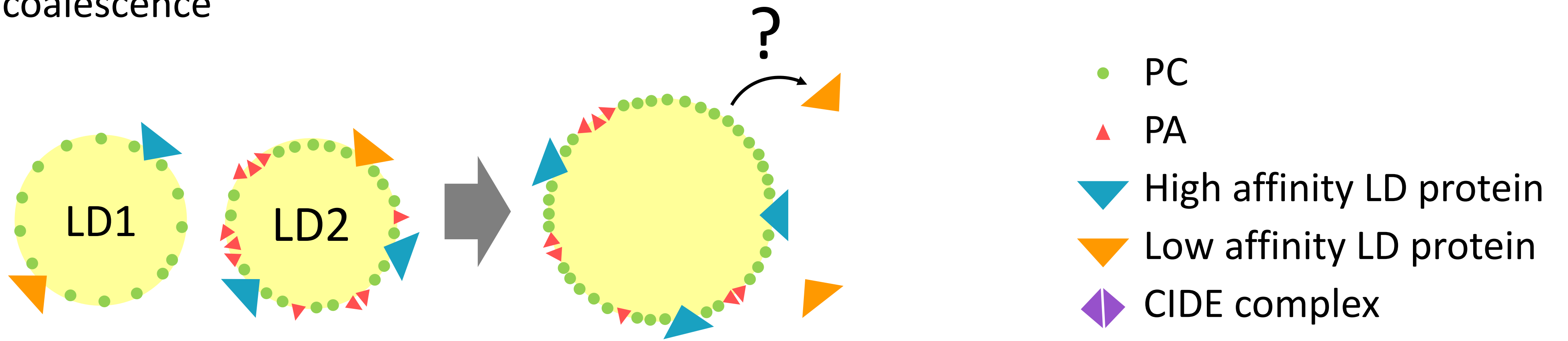
[53] Rosenwald M, Perdikari A, Rulicke T, Wolfrum C. Bi-directional interconversion of brite and white adipocytes. *Nat Cell Biol* 2013; 15:659-667.

• [54] Altshuler-Keylin S, Shinoda K, Hasegawa Y *et al.* Beige Adipocyte Maintenance Is Regulated by Autophagy-Induced Mitochondrial Clearance. *Cell Metab* 2016; 24:402-419.

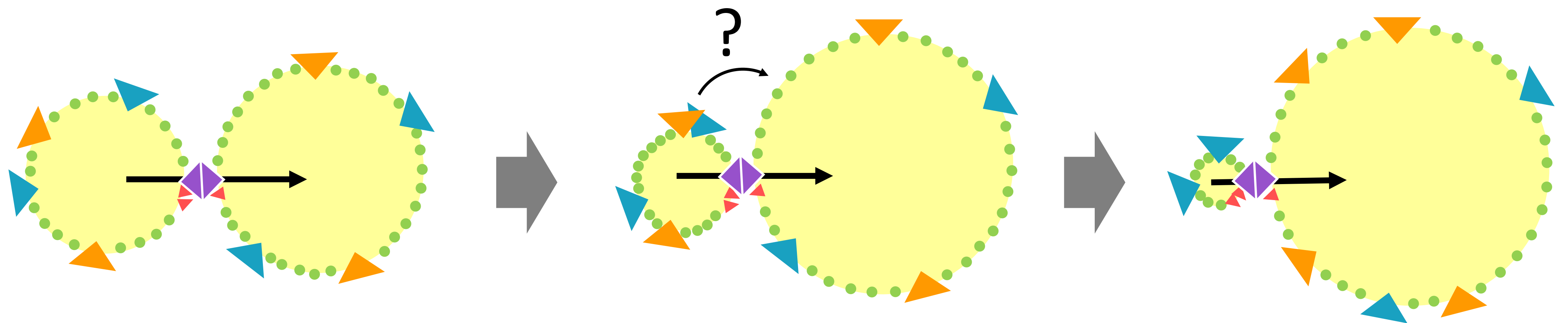
*The transition from beige-to-white adipocyte is tightly coupled to a decrease in mitochondria, increase in autophagy, and activation of Mit/TFE transcription factor-mediated lysosome biogenesis. During the transition, the autophagy pathway is required for mitochondrial clearance with inhibition of autophagy preventing beige adipocyte loss, maintaining high thermogenic capacity and protecting against diet-induced obesity and insulin resistance.*

[55] Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic acids research* 2002; 30:207-210.

### A) LD coalescence



### B) LD-LD lipid transfer



### C) PC supply during expansion

