

# **Effect of EPA on Intercellular Lipid Droplets Degradation**

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## *Abstract*

Although the beneficial effects of omega-3 fatty acid in reducing the risk of various of human diseases, such as hypertriglyceridemia and nonalcoholic fatty liver disease, have been demonstrated in clinical and pre-clinical studies, the mechanism of its action is poorly understood.

several studies has been reported that Dietary supplementation with fish oil induces many changes in plasma TG profile.

N-3 fatty acid found in fish oil has been reported that reduce plasma triglyceride and VLDL levels. Intercellular lipid droplets is the key regulator of plasma fatty acids and lipoproteins level.

Here we show that n-3 fatty acid supplementation triggers intercellular lipid droplets degradation independent from known fatty acid mobilization pathways namely lipophagy and lipolysis .

ATGL and HSL are considered as two major lipolysis enzymes. SiRNA study of these two lipolysis enzymes did not attenuate lipid droplets degradation.

Lipophagy has been reported as a selective mechanism for degradation of lipid droplets during the starvation condition. Knock down of autophagy (Macroautophagy) related proteins, could not block degradation of intercellular lipids by EPA.

Degradation of lipid droplets is lysosomes dependent and requires lysosomal motility machinery. Lysosomes are interacting directly with lipid droplets during the process that is similar to kiss and run pattern.

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The morphological examination of this process by electron microscopy indicated its resemblance to microautophagy like structure.

Importantly, (over expression) Arl8b which has been shown that play a role in peripheral distribution of lysosomes along with FYCO1, specifically accelerates the effect of EPA on degradation of intercellular lipid droplets independent from its role in engagement of lysosomal plus end distribution.

In particular, Arl8b recruited HOPS protein complex in EPA dependent fashion and silencing of HOPS complex interfered with normal lysosomal degradation of lipid droplets. Thus, this finding reveals new mechanism for intercellular lipid mobilization and offer an explanation for the therapeutic benefits of omega-3 fatty acids.

## Abbreviations

<b>ADRP</b>	<b>Adipose differentiation-related protein</b>
<b>ATGL</b>	<b>adipose triglyceride lipase</b>
<b>cAMP</b>	<b>cyclic adenosine monophosphate</b>
<b>CIDE</b>	<b>Cell death-inducing DFF45-like effector</b>
<b>CGI-58</b>	<b>Comparative Gene Identification-58</b>
<b>DA</b>	<b>Dominant Active</b>
<b>DG</b>	<b>Diacylglycerol</b>
<b>DHA</b>	<b>Docosahexanoic acid</b>
<b>DN</b>	<b>Dominant Negative</b>
<b>EPA</b>	<b>Eicosapentanoic acid</b>
<b>FA</b>	<b>fatty acid/fatty acyl</b>
<b>FFA</b>	<b>free fatty acid</b>
<b>FYCO1</b>	<b>FYVE and coiled coil domain containing 1</b>
<b>FYVE</b>	<b>PtdIns(3)P binding domain</b>
<b>GTP</b>	<b>Guanosine triphosphate</b>
<b>GFP</b>	<b>Green fluorescent protein</b>
<b>HOPS</b>	<b>homotypic fusion and vacuole protein sorting complex</b>
<b>HSL</b>	<b>Hormone sensitive lipase</b>
<b>LAMP</b>	<b>Lysosome-associated membrane protein</b>

<b>LD</b>	<b>Lipid Droplet</b>
<b>MTOC</b>	<b>Microtubule organization center</b>
<b>M6PR</b>	<b>Mannose-6-Phosphate receptor</b>
<b>ORP1L</b>	<b>(oxysterol-binding protein) related protein</b>
<b>PC</b>	<b>Phosphatidylcholine</b>
<b>PE</b>	<b>Phosphatidylethanolamine</b>
<b>PKA</b>	<b>Protein kinase A</b>
<b>PS</b>	<b>Phosphatidylserine</b>
<b>Rab</b>	<b>Ras-related in brain</b>
<b>PM</b>	<b>Plasma membrane</b>
<b>RFP</b>	<b>Red fluorescent protein</b>
<b>RILP</b>	<b>Rab7 interacting lysosomal protein</b>
<b>SNARE</b>	<b>N-ethylmaleimide-sensitive-factor attachment receptor</b>
<b>TG</b>	<b>Triglyceride</b>
<b>TIP47</b>	<b>Tail-interacting protein 47</b>
<b>VPS</b>	<b>Vacuolar protein sorting</b>
<b>VLDL</b>	<b>Very Low density Lipoprotein</b>
<b>WT</b>	<b>Wild Type</b>

## ***Introduction & Background***

Different epidemiological studies support the hypothesis that fish oil consumption reduces plasma triacylglycerol (TG) levels in normal and hypertriglyceridemic individuals, especially in very low density lipoprotein (VLDL) fraction. Even in diabetic patients, consumption of n-3 fatty acid leads to lowered plasma TG (1).

Fish oil are enriched in long-chain polyunsaturated n-3 fatty acids, especially eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) (2). Dietary intake of these fatty acids is effective in lowering plasma TG. Although EPA can be synthesized from alpha linoleic acid, this is insufficient as compared to direct consumption of EPA from fish oil supplement (2). EPA is a precursor of DHA and n-3 ecosanoids.

Long-chain n-3 fatty acids have been shown to be cardioprotective, through a multitude of different pathways. Metabolic experiments in humans and animal models have indicated that these fatty acids inhibit the production of VLDL by liver(3). The mechanism of this effect has been further evaluated by using the cell culture models.

Different mechanisms have been proposed to explain the fish oil action 1) It increases clearance of triglyceride lipoproteins by either lipolysis or lipoprotein lipase 2) It increases LDL receptor activity(3) 3) It inhibits assembly and secretion of newly synthesized apoB as a VLDL particle into the blood(4) 4) It promotes intracellular degradation of VLDL in a post-ER and pre-Golgi compartment(5).

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However, the precise mechanism by which fish oil induces the TG lowering is a matter of debate.

### **Lipid Droplets :**

Lipid droplets are the intracellular sites for storage of neutral lipids. LDs are ubiquitous cellular organelle which accumulate extra amount of lipids for the conditions that food source become limiting(6). Lipid droplets which have been called lipid bodies, fat bodies and oil bodies have been found in many species including yeast, plant, bacteria and mammals. They traditionally considered as a fast storage depot however the new studies specially in adipocytes bring the new notion that they are very active organelle and are involved in metabolism and intercellular trafficking machinery(7).

Dysregulation of cytosolic lipid droplets is associated with different metabolic syndrome such as obesity, diabetes and atherosclerosis. Lipid droplets play an important role in energy balance and steroid hormone biogenesis. Almost every cell line is able to produce and accumulate lipid droplets (7,8).

however the neutral lipids in lipid droplets are different and vary depend on cell type.

The size and number of cytosolic lipid droplets is varied and increase during the excess lipid accumulation inside the cells in order to prevent lipotoxicity(8).

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Cells are using lipid storage pool (LDs) as a source of energy during nutritional scarcity. The lipid that stored in lipid droplets also can be used for other important cellular functions such as signalling and membrane phospholipid biosynthesis (9). Lipid droplets are formed from neutral lipid core which surrounded by phospholipid monolayer and lipid droplets associated proteins family (PAT protein; Perilipin, ADRP and Tip47) (10). Despite organelle membrane biology, lipid droplets are surrounded by a single phospholipid monolayer. The core contains TAG (triglyceride) and CE (cholesterol ester). The content of lipid droplets regarding to their neutral lipids, is different and depends on cells line (11). In macrophage CE is more abundant whereas in adipocyte TAG is the prominent form of neutral lipid in lipid droplets(12). The Phospholipid monolayer around lipid droplets consist of phosphatidylethanolamine (PE), Phosphatidylcholine (PC) and Phosphatidylinositol(PI)(13).

Lipid droplets are coated with an evolutionary conserved family of proteins called PAT proteins. Some of these proteins are exclusively localized to lipid droplets such as ADRP and some are shared by other intercellular trafficking pathways such as Tip47.

### **PAT protein Family :**

PAT proteins family are functioning predominantly in lipid metabolism. They have an important role in regulation of intercellular lipid droplets. Pat family proteins contains highly conserved amino acid region at their N terminus which is defined as a PAT domain. Despite this homology, each of the pat protein family member has a specific and distinct function regarding regulation of intracellular Fat (14).

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### **ADRP:**

ADRP is 50 kDa protein which express ubiquitously in different tissues with high level of expression in adipocytes(15). ADRP expression is very low in undifferentiated adipocytes and it increases rapidly during the differentiation which makes it one of the early marker for monitoring adipocyte differentiation. ADRP expression has been shown that increased in the liver of mice which treated with the Carnitine Palmitoyltransferase I inhibitor, Etomoxir, and resulted in neutral lipid accumulation(16).

This observation suggested the possible role of ADRP in lipid droplets formation and stabilization in hepatocytes.

ADRP localized exclusively to neutral lipid droplets . ADRP level of expression has been used as a marker of lipid droplets accumulation inside the cytosol. Interestingly , the expressions of ADRP and Perilipin, another pat protein family member, are mutually exclusive; ADRP protein is not detected on lipid droplets that also express Perilipin (17).

In cultured cells, it has been shown that the ectopic expression of Perilipin or ADRP increases the capacity of cells to take up long chain fatty acids from the medium and accumulates them as Lipid droplets (18). Overexpression of ADRP increases the number and size of lipid droplets in hepatocytes, while knock down of the Adrp gene decreases hepatic lipid droplets number. It has been reported that ADRP expression is up regulated during hepatic steatosis in both humans and mouse models (19).

A peroxisome proliferators-activated receptor (PPAR) response element has been identified within the promoter region of the human ADRP gene, that mediates the

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upregulation of transcription in response to agonists of PPAR in rat and human hepatocyte cell lines (20,21).

In agreement with high level of ADRP expression in hepatic steatosis, fatty liver formation was markedly prevented in mice whose ADRP expression was nullified by gene knockout or antisense oligonucleotides. These studies indicated the possible role of ADRP in the formation or stabilization of lipid droplets in liver cell line(22).

### **Perilipin:**

Perilipin is representing another member of lipid droplets associated PAT protein. This protein has been detected exclusively on lipid droplets surface. It plays an important role in lipid droplets mobilization by manipulation of lipolysis process(23). Perilipin has three isoforms ( A,B and C) (24).

It has been reported that this protein also is involved in lipid droplet formation and size regulation by controlling the CIDEC protein activity(25).

The stability of the protein inside the cells is depend on the availability of fat and under the low fat condition, it is going to be degraded by proteosomal degradation pathway(26).

Under the basal condition, this ubiquitously expressed PAT protein is decorating lipid droplets and protecting them against the activity of hormone sensitive lipase. In contrast , It is also able to promote lipolysis activity throughout its phosphorylation(27). Perilipin knock down mice showed no repose to lipolysis stimulation and resulted in accumulation of cytosolic lipid droplets in this animal model(28).

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### **Tip47:**

TIP47 (tail-interacting protein of 47 kD) ubiquitously expressed and was initially identified in a yeast two-hybrid screen for proteins that interact with the cytoplasmic tail of the mannose 6-phosphate receptors and act by directing their transport from endosomes to the trans-Golgi network. Tip47 also known as a placental protein, shares sequence homology with two other lipid droplet-associated proteins, ADRP and perilipin ( PAT protein family member )(29).

TIP47 can be recruited to lipid droplets (LDs) by an amino-terminal sequence comprising 11-mer repeats(30).Base on extensive amino acid sequence similarity between Tip47 and other PAT proteins, it has been suggested that it has the same physiological roles in lipid related diseases(29).

However unlike other PAT protein family members, it has been detected in both cytosolic compartment and lipid droplets. TIP47 is expressed in hepatocytes, enterocytes, macrophages, and other tissues and is increased in response to lipid loading condition(31).

Interaction between Tip47 and lipid droplets is dependent upon lipid availability which has been suggested possible role of TIP47 in lipid trafficking or lipid droplet assembly(29).

Interestingly it has been shown that Tip47 has an apolipoprotein like structure(29). Inhibition of TIP47 decreases cytosolic TAG accumulation and formation of lipid droplets.

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### **VLDL and Hypertriglyceridemia**

Hypertriglyceridemia is one of the key features of liver metabolic syndrome and is caused primarily by hepatic overproduction of VLDL(32).

Hepatic VLDL overproduction and impairment in catabolism/clearance of TG-rich lipoproteins from circulation represent the two major contributors to hypertriglyceridemia. Many patients with hypertriglyceridemia represent elevated plasma TG, accumulation of small dense LDL particles and reduced HDL cholesterol particles, all of which are closely associated with cardiovascular diseases(33).

VLDL is a major source of plasma TG. Hepatic VLDL production correlates with apoprotein synthesis and availability of lipid by intracellular lipid synthesis machinery.

Apolipoprotein B is a large amphipathic protein which exists in two forms: apoB100 and apoB48. In humans, apoB100 is expressed in the liver and is present on VLDL, IDL and LDL(34).

The apoB proteins are usually referred according to the percentage of apoB100 that they represent in terms of amino acid sequence.

The apoB has a pentapartite structure consisting of one globular N-terminal structure, two domains of amphipathic b-sheets and two domains of amphipathic a-helices(34,35).

ApoB is essential for the intracellular assembly of VLDL in the liver which this assembly will be happen in the secretory pathway. ApoB acquires its tertiary structure in the ER, by a folding process that depends on chaperon proteins(36).

If the correct tertiary structure is not achieved, the protein is retained in the ER and retracted through the membrane channel and sorted for proteasomal and autophagic

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degradation(36). The N-terminal 1000 amino acid show homology with the lipid-binding pocket and the sequence is an importance for the formation of VLDL because it interacts with the microsomal triglyceride transfer protein (MTP)(37). MTP catalyses the transfer of lipids to apoB during the formation of lipoproteins. MTP is a heterodimer consisting of a 97-kDa lipid-binding and transfer subunit(38). Availability of lipids in the vicinity of MTP is crucial for proper acquisition and transfer of lipids to the site of VLDL assembly within the ER. Lack of sufficient lipids supply compromises VLDL assembly and maturation, resulting in poorly lipidated apoB polypeptides that are prone to co or post-translational degradation(39).

Secretion of apolipoprotein B and lipids is compulsory linked. Cells synthesize apoB constitutively at a rate in excess of which VLDLs are secreted. Most data suggest that the supply of TAG and phospholipid is determines how much of the apoB made moves across the ER membrane and therefor how much VLDL is secreted(36).

Lipid becomes associated with apoB in two steps during the VLDL assembly. At the first step, small and dense apoB containing particle will be formed and in the second step this small and dense apoB will associate with lipid droplet free apoB which containing mainly TG (40).

The restriction of dietary lipids can reduces the hepatic secretion of apoB lipoproteins. Lipid deprivation slows the lipidation of apoB which mediates by MTP and increases proteasomal degradation of apoB (39).

VLDL can be formed in two different forms, TG-poor VLDL (VLDL2) and TG-rich VLDL (VLDL1) (41). VLDL2 can either be secreted (directly) as such or further

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lipidated to form VLDL1 in the secretory pathway before it is secreted. The assembly and secretion of VLDL1 is highly dependent on TG level in liver (hepatocytes) and apoCIII.

Formation of VLDL1 helps to increase secretion of TG from the liver, which is one of the hallmarks of plasma lipid profile in hypertriglyceridemia(42). In this regard most of the studies showed that this increase is correlated with total intracellular TG in hepatocytes.

Hepatic storage pool of TAG in the cytosol is one of the important sources of fatty acids during the VLDL assembly process(43). An interesting feature in this process come from the fact that newly synthesized fatty acids has a minor contribution to apoB lipidation and most of the fatty acids are utilized for the formation of cytosolic lipid droplet before being utilized for lipidation of apoB to form VLDL. Most of the TAG in VLDL is not synthesized de novo but produced from pre-existing cytosolic TAG droplets through a lipolysis-re-esterification cycle (43,44).

So there is a strong relationship between the CLD and VLDL1. In addition Manipulating lipid droplet coat proteins also showed that cytosolic lipid droplets play an important role in formation and secretion of VLDL1. For example, over expression of ADRP, one of the important lipid droplet coat proteins and marker during the lipid accumulation resulted in increased level of intercellular TG and number of lipid droplets along with decreased secretion of VLDL1(45). In that study they also showed that by overexpression of ADRP, the rate of fusion between the lipid droplets increased which result in a decrease in VLDL secretion.

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### **Lipolysis:**

The regulation of intracellular lipolysis process is complex multi factorial process. Maintaining the balance between lipid synthesis and lipolysis is an important task in order to maintain body's energy hemostasis. Dysregulation of these process can be inked to different metabolic disorders such as insulin resistance.

Multiple receptors and hormones are responsible for cellular lipolysis. Among these factors Beta Adornergic agoinst and antilipolytic factor such as insulin play an important role(46). The major and important intracellular pathway for activation of lipolysis is the activation of adenylate cyclase. Activation of this pathways will increase the level of cAMP which leads to phosphorylation of PKA (47). Even though PKA is the major player in lipolysis process other factors have an important role in lipolytic reaction.

Access of lipases to stored lipids is depend upon Perilipin a member of PAT protein family (Perilipin, ADRP and TIP47) which decorates lipid droplets (48).

Perilipin deficient mice and in vitro experiments indicated a dual role for perilipin in lipolysis process . Under the basal condition, perilipin protects lipid droplets from lipolysis enzymes however under the lipolytic stimulation condition , perilipin will be phosphorylated and accelerates lipid droplets degradation by giving the enzymes access to lipid droplets(48). Lipolysis (Lipid mobilization process) is governed by different enzymes. Among the factors which are involved in lipolysis process, ATGL and HSL are the major players which act in order during lipid mobilization process.

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The latest model for lipid mobilization process suggesting that TAG ( lipid droplets ) are hydrolyzed in sequential process. ATGL and HSL are necessary for proper hydrolysis of Tri and diglycerides, respectively. The last step in lipolysis process is performed by Monoglyceride lipase (MGL), which hydrolyzes monoglycerides to form glycerol and free fatty acids(49).

### **ATGL:**

ATGL which also called phospholipase A2 $\xi$  and desnutrin is responsible enzyme for intracellular TG hydrolysis(50,51).

ATGL is expressed almost in every tissue such as cardiac muscle, type I fibers of skeletal muscle, testis, macrophages and with high level of expression in adipocyte(52). The activity of this enzyme is dependent on its specific domain called patatin domain (51).

It has been shown that ATGL is involved in both basal and hormonal lipolysis activity.

ATGL is active specifically toward triglyceride hydrolysis which resulting in formation of free fatty acids and diacylglycerides .This enzyme has high level of specificity toward TAG (10 fold higher than other enzymes and for other substrates) compare to cholesterol easter (CE) or other neutral lipids(53).

Knock down of ATGL in mice resulted in accumulation of TAG and lipid droplets in all tissues(54).

In human, mutation in ATGL were associated with enzyme malfunctioning and accumulation of lipid droplets in almost every tissue of body. The autosomal recessive disorder of this enzyme in human has been named neutral lipid storage disease with

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myopathy (NLSDM)(55). However so far only five cases has been reported that carrying this recessive disorders. Lack of ATGL activity among the human patient resulted in cardiac abnormalities and hepatomegaly(56). Most of the mutations reported so far lead to the expression of truncated versions of ATGL and true null alleles have not been observed. Interestingly compare to mice, patients with lack of ATGL function was not obese. It has been suggested that compensatory feed back mechanism for lipid synthesis in ATGL deficient subjects prevents accumulation of fat in their adipocytes. Importantly, no insulin resistance has been reported among these patients.

During the lipolysis process , ATGL is acting on first step of process by hydrolyzing TAG to DAG . Activation of ATGL, unlike HSL, is not directly depends on PKA phosphorylation.

like other lipases that act on lipid droplets such as lipoprotein lipase or pancreatic lipase which their activity dependent on another coactivator, Activity of ATGL is strongly regulated by an activator protein named  $\alpha/\beta$  hydrolase domain containing protein 5 (ABHD5); also known as a comparative gene identification-58 (CGI-58)(57). it has been reported that in presence of CGI-58 , ATGL activity increased nearly 20 fold.

CGI-58 regulates ATGL throughout the activity of perilipin as one of the lipid droplets coat protein family. In this model, Perilipin sequesters CGI-58 in the basal level, thereby preventing activation of ATGL and blocking basal lipolysis. PKA activation leads to perilipin phosphorylation, release of CGI-58, and subsequent activation of ATGL(58).

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### **HSL:**

Hormone sensitive lipase which has been known as a CE hydrolysis enzyme is encoded by the LIPE gene. It is 84 kDa phosphoprotein which acts toward the CE and TAG. HSL expresses in two different forms, long and short. The long form of HSL mostly is found in organelle which are responsible for steroid hormone production such as testes. In this organelle hormone sensitive lipase act as a CE hydrolysis in order to produce free cholesterol which is required for hormone biosynthesis. The short form of HSL normally found in adipose tissues and help to releases fatty acids from adipocytes as a main source of energy(59,60).

HSL plays an important role in mobilization of fatty acids from adipocytes. SiRNA study of HSL showed drastic reduction in lipogenesis and alteration in adipose metabolism(61). Hormone sensitive lipase is found also in lower level in skeletal muscle, macrophage and liver (62). Overexpression of HSL in pancreatic beta cells resulted in lipotoxicity and glucose intolerance(63). The enzyme has an ability to hydrolyze TG, DAG and MAG ( Monacylglyceride ). Phosphorylation of HSL by protein kinase A, stimulates its translocation to the surface of lipid droplets and initiates the lipolysis process(62).

Hormone sensitive lipase activity is regulated by its phosphorylation and activity of beta adrenergic receptors. Beta adrenergic stimulation can activate PKA and as a consequence of this activation it will activates hormone sensitive lipase. HSL can be phosphorylated on five serine residues (563, 565, 600, 659, 660) in vitro (62).

The model of HSL action during lipolysis developed base on observation that mice lacking HSL ( knock out mice) were not obese and instead of accumulation of TAG in

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tissues mostly accumulated diacylglycerides (61). It has been shown that lipolytic activation of cultured adipocytes is associated with proteins rearrangement on surface of lipid droplets and movement of HSL from cytosol to surface of lipid droplets.

Interestingly, HSL knockout mice showed 60% reduction in triglyceride lipase activities and a twofold increase in white adipose tissue size however they were not obese.

Importantly, HSL knock down mice increased diacylglycerides accumulation which was indicated the important role of HSL in diacylglycerides hydrolysis during lipolysis process (61).

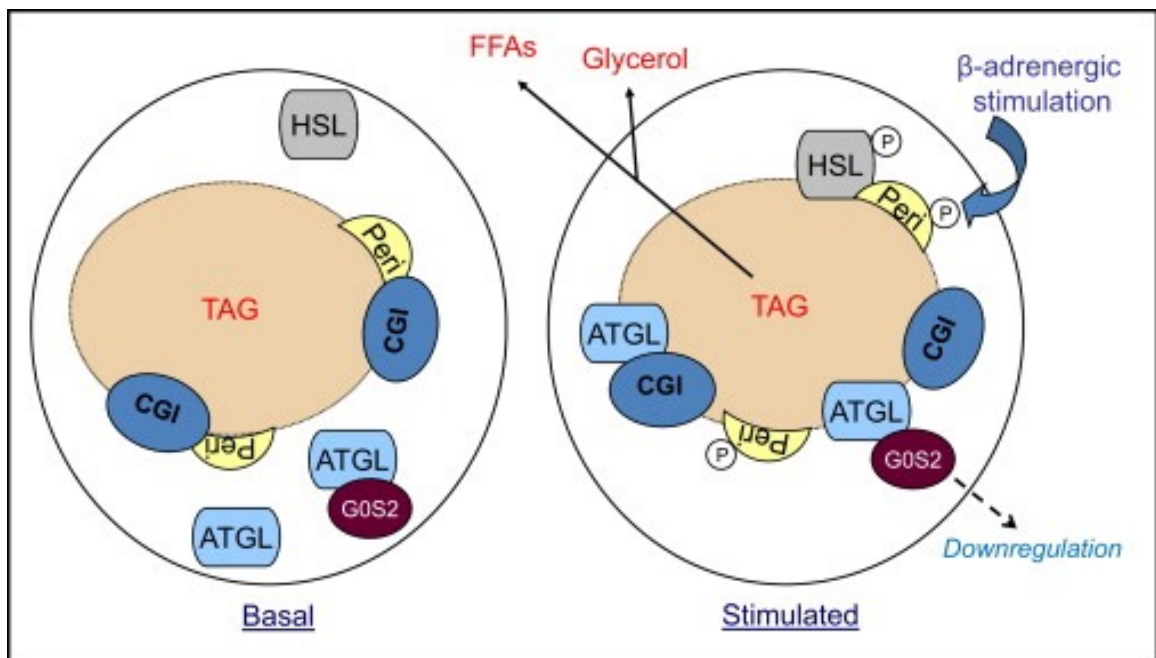
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### **Fig 1. Model for regulation of lipolysis process under basal and stimulated state.**

Under the basal condition perilipin is decorating around lipid droplets and protecting them from HSL and ATGL. Under stimulated condition , perilipin will be phosphorylated and this will facilitate recruitment of HSL and ATGL to lipid droplets surface. (*Xingyuan Yang , Xin Lu , Marc Lombès , Geun Bae Rha , Young-In Chi , Theresa M. Guerin , Eric J. Smart , Jun Liu .Cell Metabolism, Volume 11, Issue 3, 2010, 194 - 205*)

## Introduction & Background

**Fig1**



## ***Introduction & Background***

### **Lysosomes :**

Lysosome is the main organelle for degrading both endogenous and exogenous macromolecules.

Lysosomal lumen contains different form of hydrolysis enzymes for the broad range of substrates. The lysosomal membrane is formed from single phospholipid bilayer which controls the passage of material into and out of lysosome and its ability to fuse with other organelles (64).

Lysosomal membrane contains different form of transporter which help to export and important molecules to the lysosomal lumen. Each of these transporters have high level of specificity for different substrates (65).

### **Lysosomal Fusion :**

lysosomes are constantly fuse with different organelles such as endosomes, plasma membrane and autophagosome(66). They also show exceptional capacity for homotypic fusion between themselves which help them to redistribute their contents throughout the cell's lysosomal Pool. This will help them to keep their cellular complement persistent even with their constant fusion with other organelle (64). In common with other fusion

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machineries, the fusion between lysosomes and their target membrane requires specific molecular machinery and regulatory proteins.

Fusion requires targeting proteins that determine specificity of the membranes which are going to fuse. These proteins are called SNARE and will be required in both donor and acceptor membranes (67).

The process can be divided in to three main steps which are tethering, SNAREs assembly and eventually fusion.

During tethering process, tethering factors will bring the vesicle and target membrane into their close proximity (66). One of the best characterized tethering factor is vacuole protein sorting complex (HOPS) which is recruited by Rab7 to their target membrane.

After establishing tether between lysosomes and their target membrane, trans SNAREs complex need to be formed (68).

There are several different pairs of SNAREs which involved in fusion between lysosome and target membranes. Vamp7, Syntaxin-7, VTI1B (VPS10 tail interactor- 1B) and syntaxin-8, are required both for homotypic and hetrotypic Lysosomal fusion (67).

One of the best studied model for lysosomal fusion is fusion between lysosome and endosomes. There are two mechanism have been proposed for fusion between these two organelles.

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It has been shown that upon interaction between lysosomes and endosomes, their content will be mixed. The interaction and fusion between lysosomes and endosomes can be transient ( kiss and Run ) or permanent (69).

Heterotypic fusion event is required for transfer of lysosomal hydrolysis from the lumen of late endosomes to the lysosomes. The fusion between lysosomes and endosomes can make a hybrid organelle (70).

Lysosomes are also able to fuse with plasma membrane which triggered by increase in intercellular Ca concentration, for their exocytosis as a secretory lysosomes. The process also can help to repair plasma membrane and provide extra membrane for damaged area on the cell surface (71).

Lysosomes are also able to fuse with autophagosomes. The mechanism is similar to homotypic vacuole fusion. Among the fusion proteins machinery's that involve in lysosomal fusion, HOPS proteins complex has been shown to be important in autophagosomes-lysosome ( vacuole ) fusion in *S. cerevisiae* and *Drosophila melanogaster* (72). In mammalian cells, RAB7 has been implicated in the fusion of autophagosomes with lysosomes. Fusion is disturbed in cells that are depleted of LAMP1 and LAMP2, two membrane associated lysosomal proteins, as well as Rab7 GTPase (73).

It has been shown that some of the proteins that are required for endosome-lysosome fusion in mammalian are the same as those required for autophagosome-lysosome fusion (64).

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### **Lysosomal Motility:**

Like many other cytosolic organelles, lysosomes move along the cytosolic microtubule by the action of motor proteins. Intercellular lysosomal movement is essential and important for many cellular events. Microtubule based movements have been demonstrated for lysosomes (74).

Position and motility of lysosomes will change significantly in response to different stimuli such as rapamycin and starvation condition (75). So understanding lysosomal transport and motility help to understand how different signalling and protein work together to control lysosomal distribution inside the cells.

The position and movement (motility) of lysosomes is controlled by microtubule dependent kinesin and dynein motor proteins(76). Actin and myosin base dependent motor proteins also reported that are important for lysosomal motility and location. Microtubule base movement of lysosomes is in bidirectional (stop and go) fashion because of the switching activity of dynein motors for minus end and toward the prenuclear region and kinesin motor for plus end movement toward the peripheral (76,77).

Kinesin moves towards the fast growing end (or plus end), away from the MTOC, and cytoplasmic dynein moves towards the minus end .

It has been shown that overexpression of kinesin motor protein push all of the lysosomes toward the peripheral suggesting the important role of Kinesin motor protein in lysosomal peripheral distribution.

The peripheral ( radial ) distribution and centrifugal extension of tubular lysosomes along microtubules is kinesin dependent process. Rab7 is controlling lysosomal movement and

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position by recruitment of kinesin and dynein-dynactin motors to the lysosomal membrane. Rab7 cycles between GTP( active ) and GDP ( inactive ) state and upon activation is able to recruit specific effectors which are important for regulation of lysosomal motility (78). Two Rab7 effector proteins, FYCO1 and RILP, play a significant role in lysosomal distribution inside the cytosol. They act by modulating the motor proteins activity which interact with lysosomes (79).

RILP is interacting with dynein-dynactin motor protein and regulating minus end movement of lysosomes. FYCO1 on the other hand is interacting with kinesin-1 motor proteins and engaging lysosomal plus end movement(79).

Another protein which is involved in lysosomal plus end movement and peripheral distribution through regulation of kinesin-1 motor protein is Arl8b. This protein has been shown that act as a critical regulator of lysosomal movement during its trafficking and microbial Killing (80).

### **FYCO1:**

Rab7 GTPase plays an important role in autophagic vesicles transport and fusion of these vesicles with lysosomes. FYCO1(FYVE and coiled-coil domain-containing protein 1) has been identified as one of Rab7 effector protein (81).

FYCO1 similar to RUFY ( Run and FYVE containing protein ) and EEA1, contains FYBE/GOLD ( Golgi dynamic ) domain at its C terminal and RUN domain, a long coiled coil domain at its N terminal (82). In this regard, FYVE and coiled-coil domain-contain-

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ing protein (FYCO1) as a Rab7 effector has been shown that binds to LC3(microtubule-associated protein 1 light chain 3) on autophagosomal membrane and mediates microtubule base plus end movement of autophagosomes (81). FYCO1 acts by linking autophagosome to microtubule plus end directed molecular motor, Kinesin superfamily protein.

In agreement with this finding, it has been shown that Under SiRNA FYCO1 autophagosomes are accumulated in prenuclear region of the cytosol . Overexpression of FYCO1 on the other hand redistributed autophagosomes to peripheral (82).

### **RILP:**

RILP( Rab interacting lysosomal protein ) represents another effector for Rab7 GTPase and both proteins act together in order to regulate the trafficking of late endocytic vesicles (83). RILP along with Rab7 plays an important role in maintenance of Lysosomal distribution inside the cytosol. RILP is linking Rab7 containing lysosomes to the motor complex and cytoskeleton (84). Overexpression of RILP can bypass the requirement for Rab7 in minus end movement of lysosomes.

Immunofluorescence study of RILP protein showed high degree of colocalization with Lamp1 and Lamp2 proteins. It recruits dynein-dynactin motor complex to lysosomal Rab7 protein . Overexpression of RILP promotes centripetal movement of lysosomes and their accumulation around microtubular organization centre( MTOC) (83). Expression of truncated mutant of RILP (RILP-C33, lacking the N-terminal half) disperse lysosomes and

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block lysosomal degradation process which was similar to Rab7 dominant negative ( continuously GDP ) overexpression condition (83).

### **Arl8b:**

Arl8b (ADP ribosylation factor like 8b) is a member of the Arf family GTPase proteins. It contains 186 amino acids in length and has molecular weight of 22 kDa (80). As GTPase protein, it switches between GDP (inactive) and GTP (active) form. Arl8b is ubiquitously expressed in tissues and is especially high in brain, heart, skeletal muscle, kidney and liver. Arl8b plays an important role in microtubule dependent motility of lysosomes, chromosome segregation and endocytosis. Arl8b is involved in endosomes to lysosome vesicular trafficking. SiRNA of Arl8b resulted in a delay in delivery of LDL receptor and CD1 cargo to lysosome(85).

Arl8b also is interacting with beta tubulin which suggested to be independent from its guanine nucleotide binding state (86).

Different proteomic studies identified Arl8b as a lysosomal membrane protein. It is the only GTPase protein that has been shown that localized to lysosomal membrane and its Localization is depend on its guanine nucleotide-bound state. Arl8b WT and its constitutive active form (dominant active, GTP bound form) localize to lysosomes which indicates its GTP dependence of localization to lysosomal membrane. Arl8b overexpression resulted in microtubule dependent accumulation of lysosomes in cell peripheral (87).

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Peripheral distribution of lysosome by Arl8b is mediated by its kinesin-interacting protein (SKIP). SKIP was identified as a target of the Salmonella effector protein SifA and showed that it connects Arl8b to Kinesin-1 motor protein (85,88).

Homotypic fusion and vacuole protein sorting ( HOPS ) complex members were also identified as an Arl8b effector protein. In this regard, Arl8b function as regulator of cargo delivery to lysosomes by regulating lysosomal fusion machinery (85).

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### **Autophagy :**

Autophagy is a highly conserved process in mammalian system . It plays an important role in different physiological events such as adaptation to environmental /nutritional stress, cellular remodelling and determination of lifespan (89). Autophagy is one of the main regulatory mechanism for maintaining cellular hemostasis by degradation of intercellular compartments. Autophagy mechanism requires group of proteins which called Atg or autophagy related proteins (90). The molecular pathways regulating autophagy have been best characterized in yeast. 31 different Atg proteins have been identified in yeast and their mammalian orthologues have been described. Autophagy can be induced through different mechanism such as starvation (deprivation of amino acids) and differentiation (91).The process is tightly regulated by mTORC1 signalling pathway(92).

Different forms of autophagy share the core mechanism for degradation of intercellular organelles or compartments.

Autophagy is initiated by formation of cup-shaped isolation membranes that grow and sequester cytoplasmic cargo in order to form double membrane autophagosome. Engulfed region of cytoplasm, organelles or aggregated proteins then will be delivered to lysosomes for degradation(93).

Autophagy is an important adaptive response for cell survival under different physiological condition. Stress specially will up regulate cellular autophagy machinery and process.

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During the lack of sufficient nutrient supply, autophagy becomes activated which during this process intracellular nutrients are used for cell survival (91). Even though autophagy originally explained as a starvation induced adaptation mechanism in order to provide energy for the cells to survive, this mechanism also provides protection against aging and different pathological conditions such as infection, neurodegeneration, diabetes and cancer (94). Autophagy also acts as a housekeeping mechanism by removal of unwanted cellular structures through degradation of excess or damaged interacellular organelles. It has been shown that autophagy also involved in immunity which in this case it can help body to eliminate invading pathogens(95).

Three different forms of autophagy deliver cytoplasmic compartments to lysosomes for degradation. They described as: macroautophagy, microautophagy and chaperon mediated autophagy ( CMA ).

### **Macroautophagy :**

Macroautophagy is an interacellular degradation mechanism which conserved among mammalian. During this process, cytosolic compartment will be sequestered as an autophagosome and delivered to lysosome for degradation (96).

The complexity of autophagosome formation can be divided in to three main steps including vesicle nucleation, elongation and maturation.

Induction of autophagy starting by nucleation process which involves PIK3C3, p150, Ambra1 and Bcl-2-interacting protein (Beclin-1) which together form the core of the

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PIK3C3 complex. This complex later will interact with Atg14L. In this complex Bec-1 will act as main platform for interaction with other Atg proteins (97).

Activity of PIK3C3 will generate PI3P which recruits other Atg proteins which called as an autophagy nucleation process. This process is followed by membrane expansion which involves the Atg2-Atg18 complex, Atg12-Atg5-Atg16 conjugation system and Atg8-phosphatidylethanolamine (Atg8-PE) conjugation process (98 ).

Atg12 is activated by the E1-like enzyme, Atg7. Atg12 is then transferred to Atg10, an E2-like enzyme, and then covalently linked to Atg5. Atg12-Atg5 conjugate then interacts with Atg16L1 to form multimeric complex . Atg16L is active during the membrane elongation and determine the autophagosome membrane curvature(97).

Second important Atg protein during the membrane expansion is Atg8/LC3 ( micro-tubule-associated light chain-3 ) protein. the process of autophagosome membrane elongation will continue by LC3 lipidation process. LC3 will be cleaved by Atg4 protein and following cleavage it will bind to phosphatidylethanolamine (PE) to produce LC3-PE (LC3-II). In contrast to LC3 (LC3-I), LC3-II specifically localizes to the autophagosomal membrane and recognized as an autophagy specific marker.

LC3-II associates with the autophagosomal membrane, where the lipidated protein can mediate membrane elongation and closure. LC3-II is degraded late in the autophagic pathway, after autophagosome fusion with a lysosome (99).

After formation of autophagosome, it will traffic along microtubules by a dynein dependent mechanism to reach prenuclear region which lysosomes mostly are located.( near the MTOC, microtubule organization centre )(100).

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Autophagosomes are able to fuse with endosomes in their endocytic pathway. Fusion of autophagosome with endosomes can form amphisome. Autophagosome/amphisome then fuse with lysosome and form autolysosome(101).

Many factors are regulating autophagosome-lysosome fusion such as NSF, SNARE and Rab7 GTP proteins (102).

Fusion of autophagosome-lysosome allows mixing of their contents and degradation of the cargo by lysosomal acid hydrolases, which has specificity for different substrates. The degraded products are then transported back to the cytosol for recycling (90).

Constitutive levels of autophagy are required for cell survival and function in most organs, including the liver.

Macroautophagy plays an important role in regulation of liver lipid metabolism .Inhibition of autophagy by 3MA ( 3-methyladenine ), inhibitor of class III phosphatidylinositol 3-kinase in cultured primary hepatocytes resulted in accumulation of lipid inside the cells (103). Amino acid deprivation specially with leucine and glutamine showed the same effect in hepatocytes. the most important evidence regarding the importance of autophagy in liver hemostasis came from the study of specific autophagy knockout mice model which achieved by specific knock down of Atg7 gene (104).

Under this condition mice became enlarged (with increase in size of their liver) and intercellular evaluation showed accumulation of polyubiquitinated proteins and alteration in morphology of mitochondria and peroxisome.

New finding regarding regulation of liver lipid content by autophagy showed that under starvation condition lipid droplets can be used by cell throughout autophagy (lipophagy)

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process(105). Inhibition of autophagy increased TGs and LDs number in vitro and in vivo.

### **Microautophagy:**

Microautophagy is a lysosomal degradation process which contains direct engulfment of cargo (cytoplasmic) by lysosomes (106). Microautophagy can be induced by nitrogen starvation or rapamycin via autophagic regulatory signalling pathways. It has been suggested that membrane hemostasis, regulation of organelle size and cell survival under nutritional starvation condition are the main functions of microautophagy(107).

Microautophagy act as a helper of macroautophagy process inside the cells. It has been reported that microautophagy is continues process however starvation and rapamycin can induce and accelerate this process. Microautophagy can help and regulate the lysosome size which is affected by macroautophagy process by consuming abundant membrane from autophagic cargos. In this way, Microautophagy also can act as a compensatory machinery for macroautophagy and degradation of the material under basal condition (108). Formation of vesicle in microautophagy is similar to autophagosome formation in autophagy ( macroautophagy )(106).

Microautophagy-dependent lysosomal/vacuolar degradative process would be either non-selective or selective. The non-exclusive Microautophagy engulfs soluble intracellular substrates by the tubular invaginations (109); however, the selective microautophagy sequesters specific organelles with arm-like structure .

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For example, Micropexophagy is a selective microautophagic pathway that engulfs a cluster of damaged and/or superfluous peroxisomes.

Much of the data concerning microautophagy has gathered from studying the role of microautophagy of the mitochondria named mitophagy. In liver mitophagy is one of the important house keeping mechanism for removing damaged mitochondria in order to liver function properly (110).

If autophagic removal machinery able to remove damaged mitochondria, cell death will be avoided.

It has been shown Mitochondrial damage that induces the MPT(mitochondrial permeability transition) leads to death of hepatocytes and these findings provide a mechanism by which insufficient or impaired microautophagy can promote hepatocyte cell death. Mitophagy might be a mechanism to protect against hepatocyte death, by preventing release of apoptotic factors (111).

Autophagy is highly conserved process in both yeast and mammalian. However the lipid microautophagy so far only in yeast has been reported. In this process specific component of autophagy machinery, vacuolar lipase, Atg15 and cytoskeleton, tubulin, are required for degradation of lipid droplets (112). Interestingly some of core autophagic machinery including Atg8/LC3 is not active during the lipid droplet microautophagy in yeast.

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### **Chaperon mediated autophagy( CMA ) :**

During CMA ( chaperon mediated autophagy ) in contrast to macroautophagy, substrates proteins will be delivered to lysosomes directly through the distinct part of the lysosomal membrane (113). Chaperon mediate autophagy is involved in different physiological and pathological condition however CMA only observed in mammalian system so far. Same as macroautophagy different form of stimuli and stress (signalling) such as oxidative stress and starvation can activate chaperon mediate autophagy (114).

The main function of chaperon mediate autophagy is to deliver non essential proteins for degradation by lysosomes. It also facilitates degradation of damaged proteins when cells are facing stressful environment. In contrast to normal macroautophagy, CMA is able to target and degrade soluble proteins by lysosomes .

Chaperon mediated autophagy is distinctively different from the other two mentioned autophagy machineries . During the chaperon mediate autophagy, autophagosome does not form as what has been observed during the macroautophagy. The process is optimized for detecting specific substrates base on their specific motif in order to deliver them to lysosomes for their degradation.

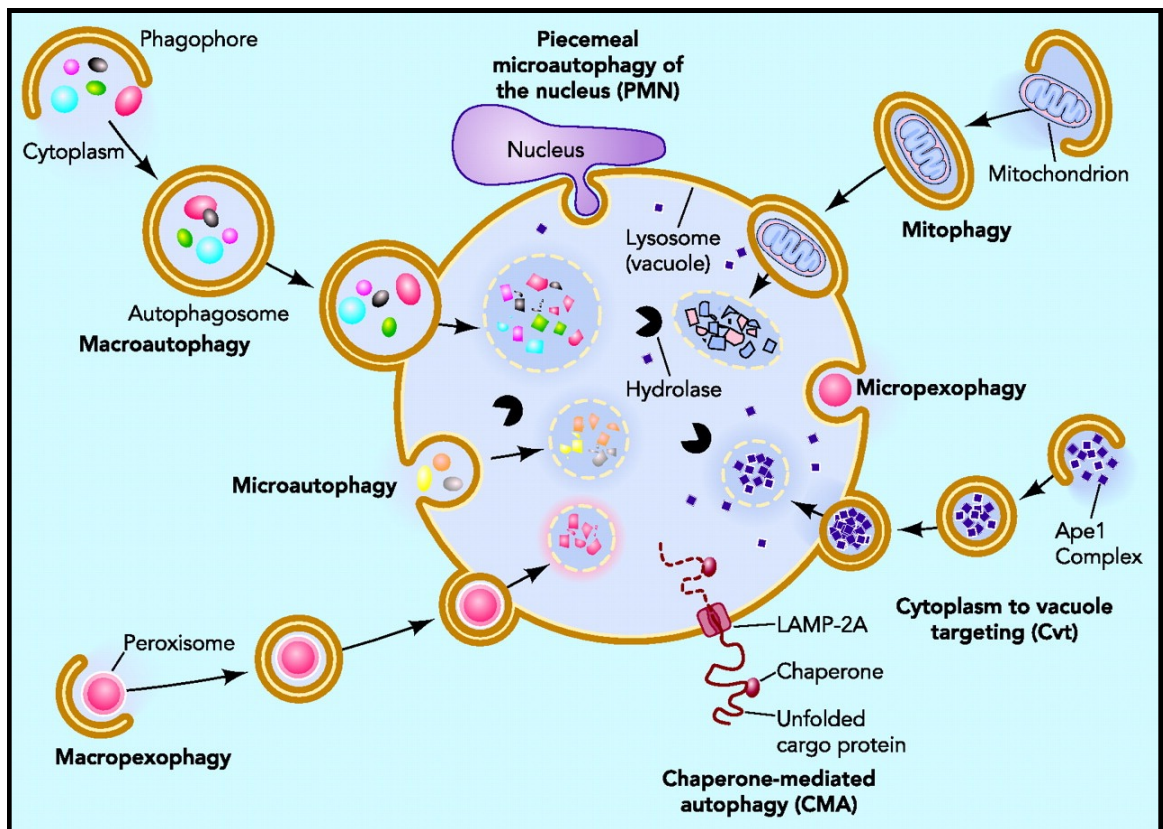
The signalling motif (in substrate protein) in order to recognized by CMA degradation pathway is formed form KFERQ amino acid sequence (115).

The protein that contains this specific amino acids will be recognized by hsc70 (heat shock protein) and will be delivered to lysosomes for degradation. Lysosomal associated membrane protein 2 (Lamp2) on the lysosomal membrane will facilitate the translocation of chaperon-substrate across the lysosomal membrane (116).

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**Fig 2. Diagram of microautophagy , macroautophagy and chaperon mediated autophagy (Yen W , and Klionsky D J Physiology 2008;23:248-262)**

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### **HOPS protein complex :**

Membrane fusion is required membrane recognition by Rab GTPase and tethering complex which eventually are going to recruits other fusion machineries such as SNARE proteins for complete fusion between two different membranes (117). Vacuole sorting protein complex ( HOPS complex ) can help to organize these machineries on specific membranes. Different studies showed the important role of HOPS protein complex in facilitating fusion between lysosomes and endosomes.

In this regard, vacuole sorting complex ( HOPS ) can act as an effector for Rab proteins and later on recruits SNAREs complex to the target membrane(118).

HOPS protein complex is multi subunit complex which is formed from six different subunits. Subunits of HOPS protein complex are part of the Class C Vps protein complex which has been shown is required for fusion of Golgi originated vesicles with lysosomes. The HOPS hexameric complex is made up from Vps11, Vps16, Vps18 ,Vps33, Vps41 and Vps39. Among these subunits , VPS41 and VPS39 have an ability to bind to Rab proteins, specifically Rab5 and Rab7 GTPases (119).

Electron tomography of HOPS protein complex showed that the complex has a seahorse like structure. In this suggested structure , Vps16-Vps33 along with Vps41 was located in the head and Vps39-Vps11 were formed the tail of this seahorse like structure(119).

Recent finding has been shown that HOPS protein complex is also able to bind to another GTPase protein in its GTP bond state. Arl8b as a one of the Lysosomal movement

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regulator has been shown that binds to Vps41 subunits of HOPS protein complex and plays an important role in T cells and lysosomal function during microbial killing by facilitating the fusion of lytic granules (85).

## *Hypothesis*

Formation of VLDL1 helps to increase secretion of TAG from the liver, which is one of the hallmarks of the plasma lipid profile in hypertriglyceridemia. In this regard, most of the studies showed that this increase is correlated with total intracellular TAG in hepatocytes (15).

So there is a strong relationship between cytologic lipid droplets and VLDL1.

In addition manipulation of lipid droplets coat proteins also showed that accumulation of lipid droplets will be affected upon change in their coat proteins.

From existing data, we hypothesized that EPA is delivering its function and affecting plasma lipid profile by affecting and degrading this intracellular TAG pool.

This hypothesis will be addressed by monitoring intercellular LDs both with live imaging and biochemical assays. Since different mechanisms are responsible for mobilization of intercellular lipid droplets such as autophagy and lipolysis, it is possible that EPA affects the plasma lipid profile by up regulating one of these pathways.

Effect of EPA on these specific pathways will be discussed in great details.

There are some evidence that intercellular LDs are interacting with different organelles however, mostly, function of this interaction is unknown.

Lysosomes consider as a main organelle for degradation of intercellular components.

Beside the Autophagic pathways which delivering substrate to them, they are also able to take up directly and degrade different substates. In recent finding, it has been shown that lysosomes are able to directly uptake both RNA and DNA in an ATP dependent manner (120).

## *Hypothesis*

In parallel to above hypothesis, we hypothesized that EPA treatment can activate and up regulation lysosome function which leads to direct degradation of lipid droplets.

In support of this hypothesis, we will address and test machinery of lysosome function / movement in its molecular level. Involvement of GTPase protein regarding to lysosome activity will be investigated and discussed.

## ***Materials & Methods***

### ***Materials and Methods:***

#### ***Cell Culture and Transfection:***

The A18-C3 cells were cultured in DMEM (Invitrogen) supplemented with fetal bovine serum (20%), penicillin (50 U/mL), streptomycin (50 µg/mL, Invitrogen) and Geneticin/G418 (500 µg/mL, Gibco) at 37 °C in 5% CO<sub>2</sub>. Transfection was conducted when cells were 60%-70% confluence. Appropriate plasmids were mixed with FuGENE HP extreme reagent (Roche Diagnostic), according to manufacturer's protocol, and incubated for 30 min. Twenty-four h after transfection, cells were switched to lipid-rich media containing oleic acid (0.4 mM) for additional 16 h prior to imaging and biochemical analysis. In silencing experiments, appropriate siRNAs were transfected into cells using the FuGENE HP Extreme reagent. Forty-eight h after transfection, cells were switched to lipid-rich media containing oleic acid (0.4 mM) for additional 16 h prior to imaging and biochemical analysis.

#### ***Plasmids and Antibodies:***

GFPAr18b, RFPAr18b, GFPAr18bT34N, RFPAr18bT34N, GFPAr18bQ75L, RFPAr18bQ75L, GFPFYCO1 and GFPRILP are a gift of Roberto Botelho (Ryerson University, Canada), Sean Munro (MRC Laboratory of Molecular Biology, UK), and

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Tuanlao Wang (Xiamen University, China). The following primary antibodies were used in this study: actin (Sigma SAB4200248), ADRP (Abcam 52355), apoB (1D1, University of Ottawa Heart Institute), Arl8b (Fitzgerald 70R-3486), Atg5 (Abcam108327), ATGL (Santa Cruz 365278), FYCO1 (Abcam 126603), HSL (Santa Cruz 25843), LAMP1 (Sigma SAB3500285), Rab7 (Santa Cruz 10767), and RILP (Santa Cruz 98331). Secondary antibodies were goat (sigma A5420), mouse (GE Healthcare UK limited NA931V) and rabbit (GE healthcare UK limited NA934V).

siRNA. All siRNAs were purchased from Ambion (Invitrogen): Arl8b(s179124, 4390771), FYCO1 (s236103, 4390771), RILP (s142632, 4390771), Lamp1 (s129497, 4390771), Rab7 (s131442, 4390771), Atg5 (s172246, 4390771), ATGL (s167782, 4390771), and HSL (s129501, 4390771).

### ***Live Cell Imaging:***

Cells were grown on 35-mm glass bottom dishes (Ibidi GmbH München, Germany) for 16 h in DMEM supplemented with FBS (20%) and oleate (0.4 mM). Cells were imaged using a Zeiss 510 meta confocal microscopes under the 5% CO<sub>2</sub> at 37 °C. Images were captured at the frame-rate of 30 frame/Sec using 60X.1.4 objective and numerical aperture with proper laser according to the dyes specificity. For visualization of lipid

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droplets, lysosomes, mitochondria, and mitochondrial  $\beta$ -oxidation, LipidTOX red (Invitrogen), LysoTracker (Life Technology), Mitotracker (Life Technology), and MitoSox red (Invitrogen), respectively, were added to the media, according to protocols recommended by manufacturers, 15 min before treatment with EPA.

### ***Autophagic flux measurement:***

Cells were cultured with lipid-rich media containing oleic acid (0.4 mM) for 16 h. The lipid-laden cells were incubated with the Cyto-ID green autophagy detection reagent and Hoechst 33342 (Enzo Life Science, UK) for 30 min. The cells were switched to fresh media containing oleic acid or EPA (0.4 mM) and cultured for 1 h. Cells were washed and re-suspended with 1x assay buffer (Enzo Life Science, UK) according to manufacturer's instructions. A control group of cells were treated with rapamycin (500 nM) to induce autophagy.

### ***Quantification of cellular lipid droplet numbers:***

Quantification of lipid droplets was carried out using confocal and differential interface contrast (DIC) microscopy. Lipid droplets were stained with lipidTOX red (Invitrogen) before adding OA or EPA. All images were visualized using a 63X oil immersion objective lens. Z-series stacks were acquired with 0.2  $\mu$ m steps. Before quantification of the number of lipid droplets, 3D images were reconstructed using 3D Image

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reconstruction Plug In. Lipid droplets then counted using Image J Lipid droplet counter Plug In in 50 randomly selected cells.

### ***Measurement of lysosomal residence time on lipid droplet surface:***

Cells were cultured with lipid-rich media containing oleic acid (0.4 mM) for 16 h. The lipid-laden cells were switched to fresh media containing EPA (0.4 mM) for 1 h, and the lysosomal movement towards lipid droplets was recorded by time lapse microscopy. Intensity of lysosomes signal in the vicinity of selected individual lipid droplet was measured using Image J live intensity profiler Plug In over a period of 40 sec. Quantification was carried out for 60 individual lipid droplets from three independent experiments. Constant threshold was used for all of the images in each experiment.

### ***Metabolic labelling of lipids:***

McA-RH7777 cells were metabolically labeled with [3H]glycerol (5  $\mu$ Ci/ml) for 1 and 2 h in DMEM supplemented with 20% FBS and 0.4 mM oleic acid or 0.4 mM EPA. At indicated time points, media were collected and subjected to cumulative rate floatation ultracentrifugation as previously described<sup>22</sup> to separate VLDL1, VLDL2 and IDL/LDL fractions. Lipids were extracted from the fractionated lipoproteins as described before<sup>23</sup> and separated by TLC. The bands containing 3H-TG and 3H-PC were scrapped from TLC plate and radioactivity was quantified by liquid scintillation counting.

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### ***Lipid mass quantification:***

Lipids were extracted from cells using the Bligh/Dyer method<sup>23</sup>, separated by HPTLC in a solvent of hexane:diethylether:acetic acid (105:45:1.5 v/v). TG and CE were stained with Commassie G-25024 and quantified according to known amount of standards spotted on the same HPTLC plates.

### ***In Situ CO-IP:***

Cells were plated in coverslip dishes before any treatment. Cells were transfected with Arl8b mcherry plasmids and followed by treatment ( EPA and OA).

Cells were fixed and permeabilized .

Primary antibodies were diluted in Antibody dilution buffer and then added to the samples.

Cells were incubated for 24 hr in 4 c. Secondary antibodies conjugated with oligonucleotides ,PLAs, Minus and plus were added to the cells .

Upon incubation , Ligase was added to the reaction. Amplification buffer then was added to the cells in order to visualize the close proximity between two proteins. After incubation period , cells were washed and Mountain medium was added to the cells . Interaction was visualized using confocal Microscope.

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### ***Electron Microscopy:***

Cells were cultured in media containing 0.4 mM oleic acid or 0.4 mM EPA for 30-60 min and harvested for morphological investigation. After two washes with ice-cold, 0.2 M sodium cacodylate buffer containing 0.1% calcium chloride, pH 7.4, samples were fixed overnight at 4 oC in 2.5% glutaraldehyde and washed 3X with washing buffer. Pellets were post-fixed with 1% aqueous OsO<sub>4</sub> + 1.5% aqueous potassium ferrocyanide for 1 h, and washed 3X with washing buffer. Cells were dehydrated in a graded alcohol series, infiltrated with graded epon:alcohol and embedded in epon. Sections were polymerized at 58 oC for 48 h. Ultrathin sections (90–100 nm thick) were prepared with a diamond knife using a Reichert Ultracut E-ultramicrotome, placed on 200 mesh copper grids, and stained with 2% uranyl acetate for 6 min and Reynold's lead for 5 min. TEM grids were examined with a Tecnai 12 120 kV TEM equipped with a Gatan 792 Bioscan CCD Camera (Gatan, Inc., Pleasanton, CA).

### ***Immuno-electron Microscopy:***

For immunogold Labeling, pellets of cells cultured in media containing 0.4 mM oleic acid or 0.4 mM EPA were fixed with 0.5% glutaraldehyde and 4% paraformaldehyde in

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0.1M phosphate buffer (pH 7.4) and dehydrated through a series of graded ethanol dilutions, and embedded in LR White acrylic resin (London Resin Company). Ultrathin sections (80 nm) were then placed on Formvar-coated nickel grids. For Post-embedding immunolabeling, the sections were incubated with an anti-Rab7 antibody for 1 h (1:5 dilutions), washed six times in 0.2 M sodium cacodylate buffer containing 0.1% calcium chloride, pH 7.4, incubated with the secondary antibody conjugated with 10-nm gold particles, and washed six times with PBS. To increase contrast, the ultrathin sections were stained with with 2 % uranyl acetate for 6 min and Reynold's lead for 5 min. TEM grids were examined with a Tecnai 12 120 kV TEM equipped with a Gatan 792 Bioscan CCD Camera (Gatan, Inc., Pleasanton, CA, USA).

### ***Electron tomography:***

Samples were prepared as described above. However, thicker sections (~250 nm) were cut for electron tomography and transferred onto carbon coated copper grids. Images were collected on a Titan Krios microscope operated at 300kV using a Gatan Ultrascan 4kx4k CCD camera. For electron tomography (ET), data collection was done at an electron dose of approximately 1500 electrons/Å<sup>2</sup> per tomogram. Focusing was done on an adjacent area in order to minimize electron dose exposure. In total, over 30 tomograms

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were collected at different magnifications ranging between 20k and 50k. Tilt series were taken using the FEI software in the angular range between  $-64^{\circ}$  and  $+64^{\circ}$  with  $2^{\circ}$  increments. For the estimated sample thickness, this would be sufficient for a resolution 2 nm, following the Crowther formula. Reconstruction of 3D volumes was done using the IMOD software suite<sup>25</sup>. The final tomograms were binned 3 times in order to increase the signal to noise ratio. For the determination of the location of metal particles in relation to the carbon substrate, we searched the volumes for high intensity voxels in the reconstructed volume using SPARX. Three-dimensional rendering was done using Chimera<sup>26</sup>.

### ***Statistical Analysis:***

Results of lipid quantification are reported as means  $\pm$  standard deviation. Significance of difference was analyzed using Student's t-test.

## ***Results***

### ***Introduction :***

Quantification of the number of lipid droplets upon treatment with EPA showed a decrease in number of intracellular LDs along with increasing free fatty acid in medium which was similar to increase in circulating free fatty acid during the fasting condition by autophagy.

Macroautophagy is a cellular process that recycles organelles and proteins to maintain cellular homeostasis. Recently, intracellular lipid droplets have been incorporated into the list of autophagic cargo, revealing the role of autophagy in lipid metabolism and regulating intracellular volume of TG.

Autophagy serves as an alternative energy source to sustain cellular function during starvation. By activation of autophagy during the starvation, it will induce mobilization of cytosolic lipid droplets as a source of energy to generate free fatty acids.

To investigate the possible role of autophagy regarding to n-3 fatty acid effect on intracellular lipid droplets, we study trafficking of autophagy machinery after treatment with EPA and oleate using live cell microscopy. We tested the recruitment of LC3-GFP to lipid droplets surface which can be indicative of autophagic pathway activation and autophagy flux.

We also monitored the recruitment of endogenous autophagic protein to lipid droplets surface using immunogold electron microscopy.

In another set of experiments, we monitored the effect of EPA on intercellular lipid droplets after SiRNA of main autophagy machinery proteins including Atg5.

## ***Results***

During lipolysis, triglycerides are broken down through Diglycerides and Monoglycerides to free fatty acids (FFAs) and glycerol. Circulating FFA levels are increased in obesity and type 2 diabetes. High level of FFA impairs glucose and lipid metabolism in liver and adipose tissues. Until recently, hormone-sensitive lipase (HSL) was considered to be the only important regulator of lipolysis. However, animal studies strongly suggest that an additional lipase, adipose triglyceride lipase (ATGL), also plays a role that may be more critical than HSL.

To determine whether or not the effect of EPA on intercellular TG degradation is due to activation of lipolysis pathway and activity of ATGL and HSL enzymes, we monitored the Effect of EPA on intercellular TG degradation after SiRNA ATGL and HSL using confocal microscopy and biochemical assays.

During the investigation of involvement of autophagy in EPA induced lipid droplets degradation, we found that EPA treatment promotes direct interaction between lysosome and lipid droplets which was in the kiss and runs fashion (transient) and was resembling microautophagy like process.

To investigate the role of lysosomal bi directional movement in lipid droplets degradation we monitor lysosomal motility and rate of lipid droplets degradation after transfection with FYCO1 and RILP -GFP proteins.

During the course of investigation regarding to Lysosomal bi directional movement and its effect on Lipid droplets degradation, we identified Arl8b GTPase as a one of the main factor that regulate lysosomal degradation of lipid droplets.

## ***Results***

To understand the role of Arl8b in lysosomal lipid droplets degradation we transfect the cells with Arl8b DN(constant inactive form) and Arl8b DA (constant active form) mCherry proteins. In another experiment, we also measured the effect of Arl8b using SiRNA of Arl8b.

In all of the above conditions lysosomal dynamic and rate of lipid droplets degradation were analyzed using live cell imaging, electron microscopy and biochemical assays.

All of the experiments regarding the effect of EPA on intercellular lipid droplets and VLDL1 assembly/secretion were carried out in apoCIII overexpressing model which is able to produce VLDL1.

## ***Results***

### ***EPA induces Intercellular Lipid Droplets degradation without affecting TAG Secretion***

In order to see the effect of EPA on intercellular lipid droplets, cells were treated with oleate for overnight in order to stimulate synthesizing of intercellular lipids. Overnight treatment with oleate increased the cytosolic fraction TAG and number of lipid droplets as reported perviously which is one of the important hallmarks in fatty liver disease. Accumulation of lipid droplets was confirmed by Lipid Tox red 589/590 staining and electron microscopy. To address whether EPA treatment altered number of lipid droplets, the number of lipid droplets per cells was quantified using the image J(NIH) lipid droplets counter software. The number of lipid droplets decreased dramatically after EPA treatment compared to oleate (Fig3A,B). Both electron microscopy and western blot of ADRP confirmed the decline in amount of cytosolic lipid droplets ( Fig 4A,B ).

As an alternative to lipid droplets quantification by light microscopy, we employed TLC( thin layer chromatography) assay for TAG quantification after treatment with EPA. The TAG concentration of total cell was collected in different time points upon EPA or Oleate treatment. As expected, the amount of TAG was decreased (Fig 5).

One of the possible mechanism in hepatocyte to prevent accumulation of intercellular lipid droplets is up regulation of TAG secretion by accelerating VLDL1 assembly.

In our previous study (41), we demonstrated that apoCIII induced VLDL1 secretion by increasing the rate of apoB lipidation in the presence of exogenous fatty acid.

To address whether EPA induced decrease in intercellular lipid droplets treatment altered VLDL secretion, the rate of VLDL secretion was measured after EPA and oleate treatment.

## ***Results***

Formation of intercellular lipid droplets stimulated by treating cells with oleate for overnight. Cells treated with <sup>3</sup>Hglycerol for 2hr. The lipoproteins secreted into the media were fractionated by floatation ultracentrifugation to separate VLDL1 from other dense lipoproteins. The <sup>3</sup>H-labeled lipids were (fractionated and extracted by TLC) extracted from each fraction and separated by TLC. The effect of EPA on VLDL1 secretion and lipoprotein assembly were compared with oleate. Despite decrease in number of Lipid droplets and intracellular TAG, secretion of VLDL1 from EPA treated cells was attenuated (Fig 6). This results showed that effect of EPA on decreasing intercellular TAG is not by up regulation of VLDL and TG secretion from hepatocytes.

## *Results*

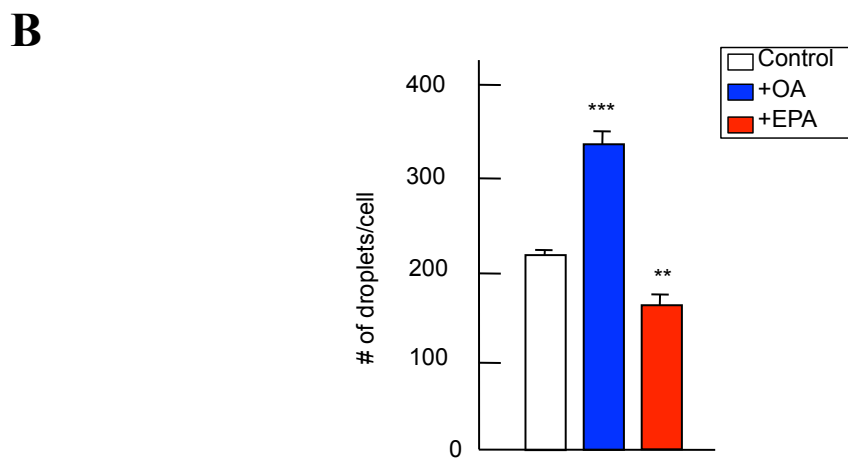
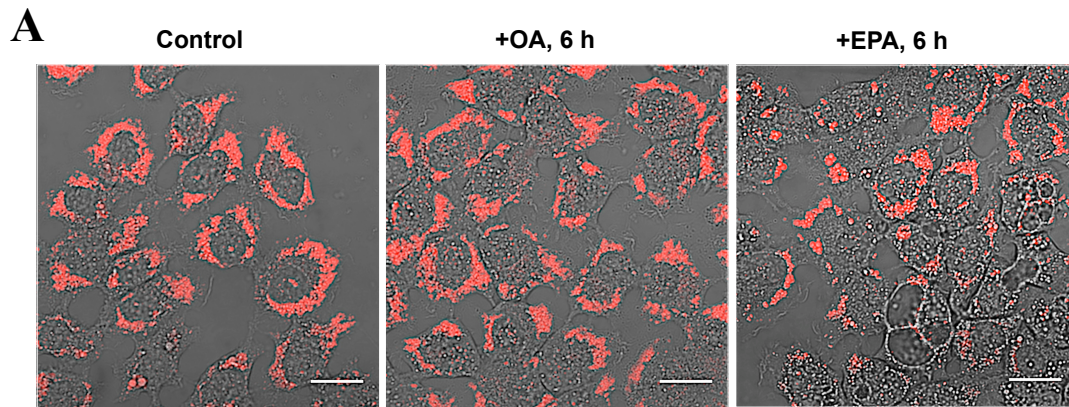
### **Fig 3. EPA treatment decreased intercellular lipid droplets .**

a) imaging analysis of lipid droplets after adding EPA and Oleate .Scale bar, 22  $\mu\text{m}$

b) Quantification of number of lipid droplets 6hr after adding EPA and oleate.Values are means  $\pm$  s.e.m. of three independent experiments (carried out in triplicate). All comparisons are with the control within each treatment condition, \*  $P < 0.05$ , \*\*\*  $P < 0.005$  Student's t-test.

*Results*

**Fig 3**



## *Results*

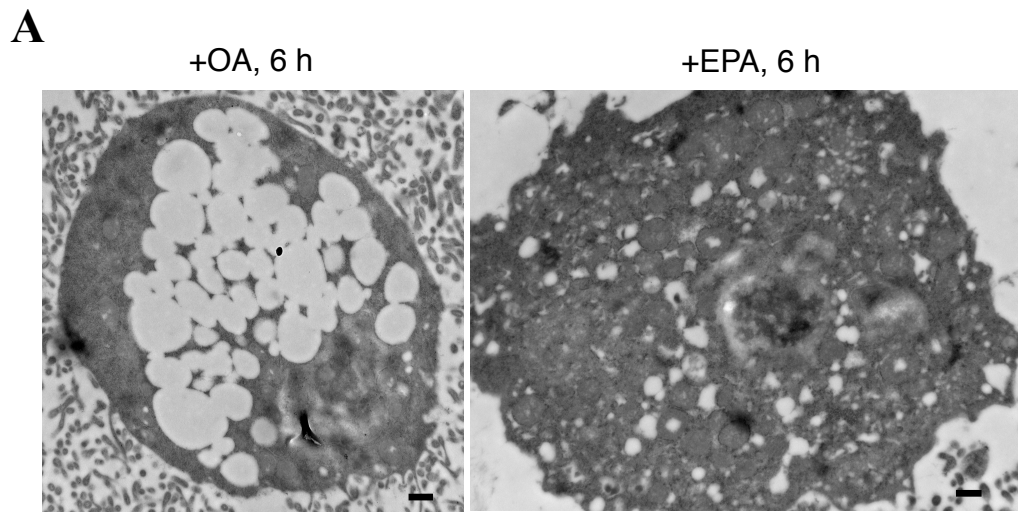
### **Fig 4. EM analysis of intercellular lipid droplets degradation by EPA**

a) Transmission EM showing cells treated with EPA have diminished cytosolic lipid droplets as compared with that in OA-treated cells. Scale bar, 500 nm.

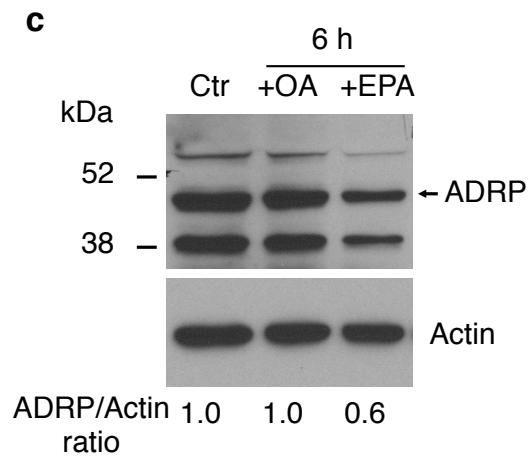
b) western blot of ADRP shows decrease in ADRP level under EPA treatment condition .

*Results*

**Fig4**



**B**



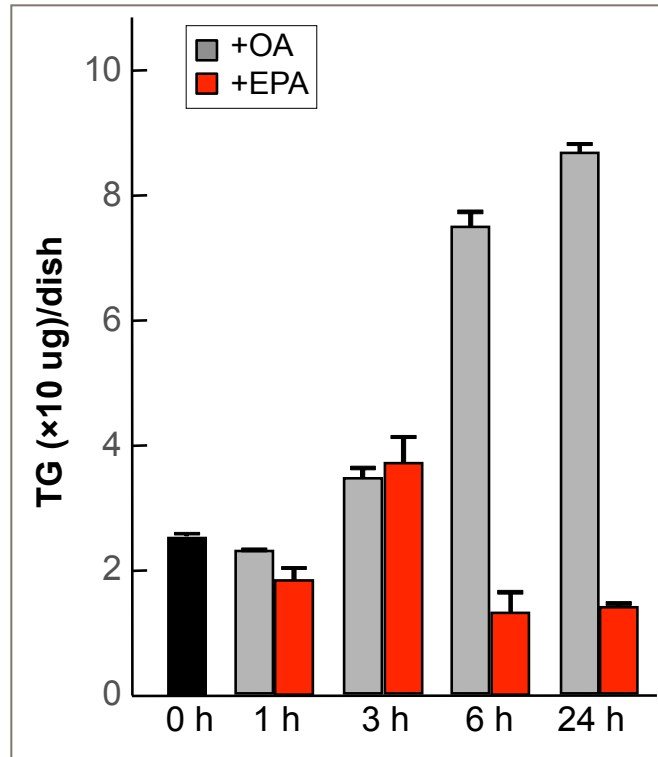
## ***Results***

### **Fig 5. Intercellular TAG mass measurement .**

Cells were treated with oleate for overnight in order to let accumulate cytosolic lipid droplets.

TAG connection of total cell was collected in different time points upon adding EPA or oleate. Amount of TAG was decreased in EPA treated cells

Fig5



## *Results*

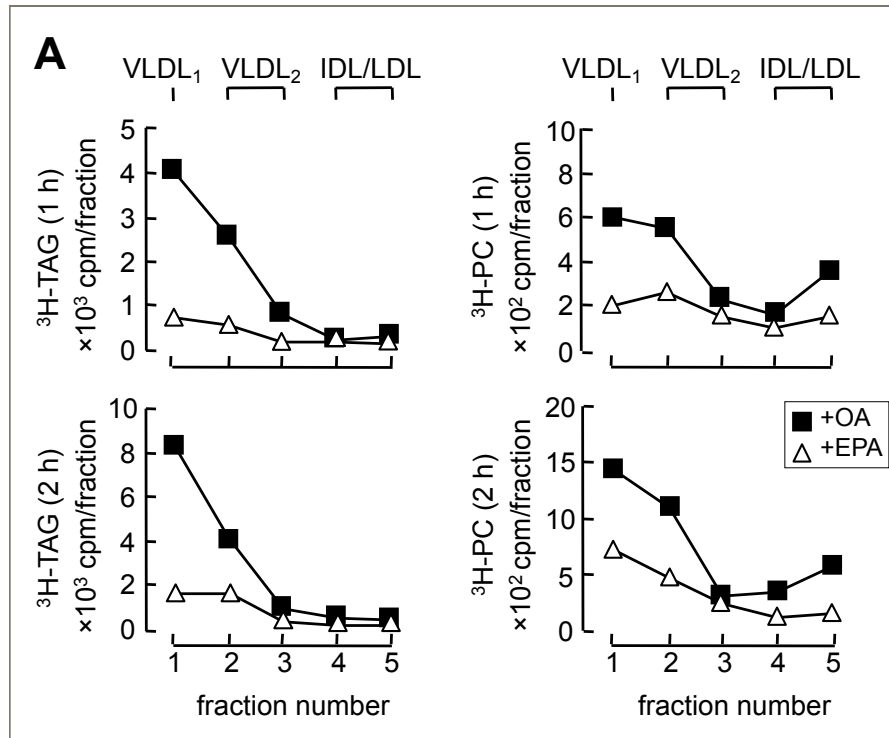
**Fig 6. Secretion of metabolically labeled TG associated with VLDL1, VLDL2, and intermediate density lipoproteins (IDL)/low density lipoproteins (LDL).**

TAG , secretion of VLDL1 from EPA treated cells was attenuated.

EPA induces intercellular lipid droplets degradation independent from VLDL secretion

.

Fig 6



### ***Role of conventional autophagy and lipolysis pathways in EPA induced Intracellular lipid droplets degradation***

Cytosolic Lipid droplets can be sequestered inside autophagosomes and delivered to lysosomes for degradation by the lysosomal lipases (105). Subsequent different studies in other cell types have revealed that this is not a process exclusive for hepatocytes but also it is detectable in almost every cell type. To determine whether autophagy is responsible for degradation of lipid droplets after treatment with EPA, we used SiRNA ATG5 in order to block the autophagic pathway and autophagic membrane formation.

The number of lipid droplets between SiRNA Atg5 transfected and control cells after overnight treatment with oleate was comparable (Fig7).

We next examined whether the number of lipid droplets were modified in SiRNA ATG5 cells after treatment with EPA. In parallel, we confirmed that the SiRNAs specifically reduced accumulation of the target protein by western blot (Fig 8c). The number of lipid droplets reduced in comparable level to non SiRNA ATG5 cells (Fig7). Lipid mass measurement and western blot of ADRP under Atg5 SiRNA confirmed the above result (Fig 8).

We also confirmed the minor role of conventional macroautophagic pathway in lipid droplets degradation after treatment with EPA by monitoring the trafficking of LC3-GFP ( Fig ). LC3-GFP is a protein that commonly used for detection of autophagic activity as LC3 II associates with autophagosomal membrane upon induction of autophagy. Overnight treatment of LC3-GFP transfected cells with oleate showed slightly decrease in the number of lipid droplets which is in agreement to the role of autophagy in lipid

droplets degradation as a part of lipolysis machinery upon lipid loading condition. After adding EPA to medium we did not observe recruitment of LC3-GFP to LDs in our experimental system, arguing against this possibility(Fig 9A). Because lipid Tox red staining of lipid droplets is an indirect measurement of lipid droplets number, we also used electron microscopy (EM) to visualize and quantify autophagic events in different time points after treatment with EPA. We could not see any of indicated autophagic markers, Atg5 or Atg8 on lipid droplets surface. Measuring autophagic flux after adding EPA did not show any increase in autophagy activity (Fig 9 B).

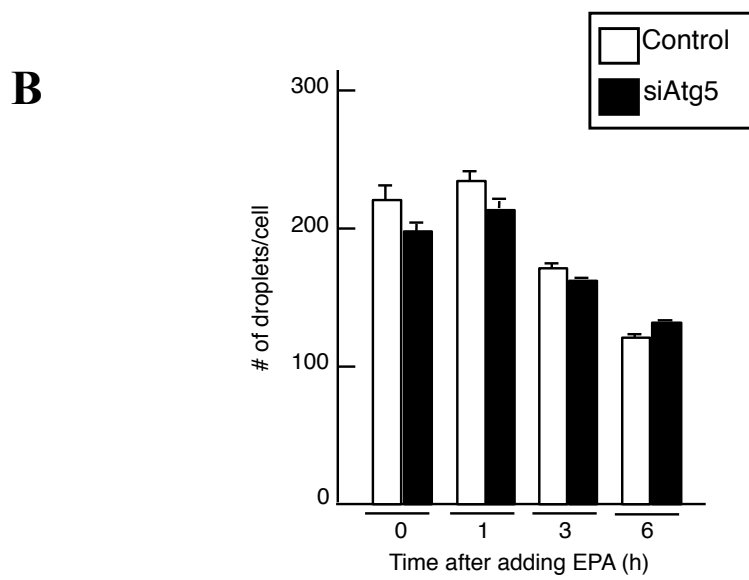
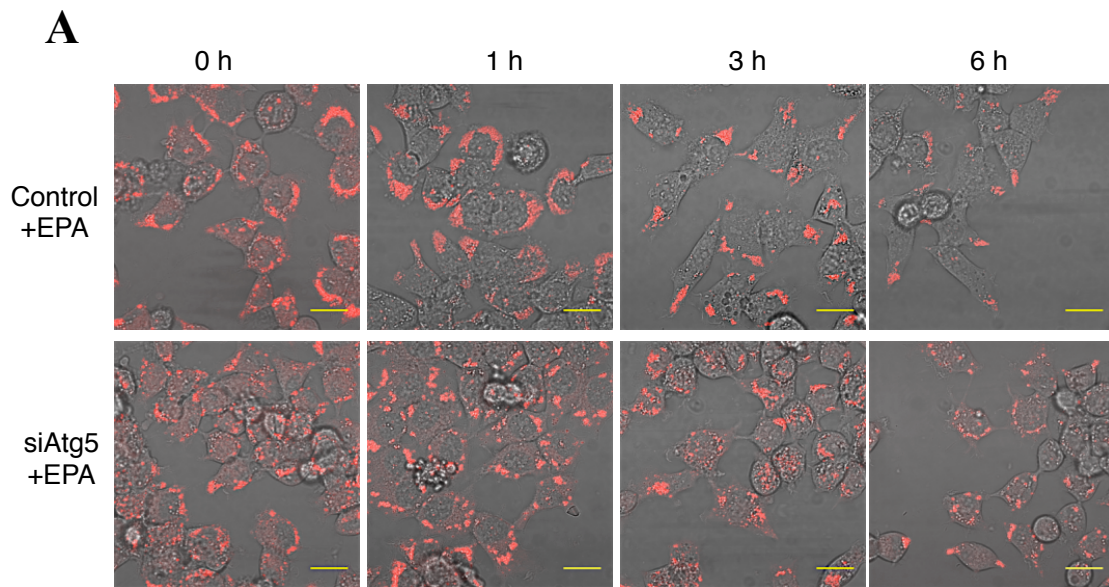
In persuading to understand the mechanism for EPA induced lipid droplets degradation we start to monitor lipolysis machinery upon treatment with EPA.

Lipolysis is the main process for turning over of intercellular lipids which stored in cytosol (as lipid droplets). This process controlled by number of enzymes. ATGL and HSL are the key enzymes for degradation of intercellular lipid droplets . Using SiRNA ATGL and HSL did not block degradation of intercellular lipids upon treatment with EPA as indicated by quantification of number of lipid droplets (Fig 10).

**Fig 7. EPA-triggered lipid degradation does not require Atg5-dependent autophagy.**

- a) Imaging analysis of lipid droplets (stained with lipidTOX Red) in cells transfected with Atg5-specific siRNA (siATG5) or scrambled siRNA (Control). Scale bars, 22  $\mu$ m
- b) Quantification of number of lipid droplets in different time points after adding EPA.

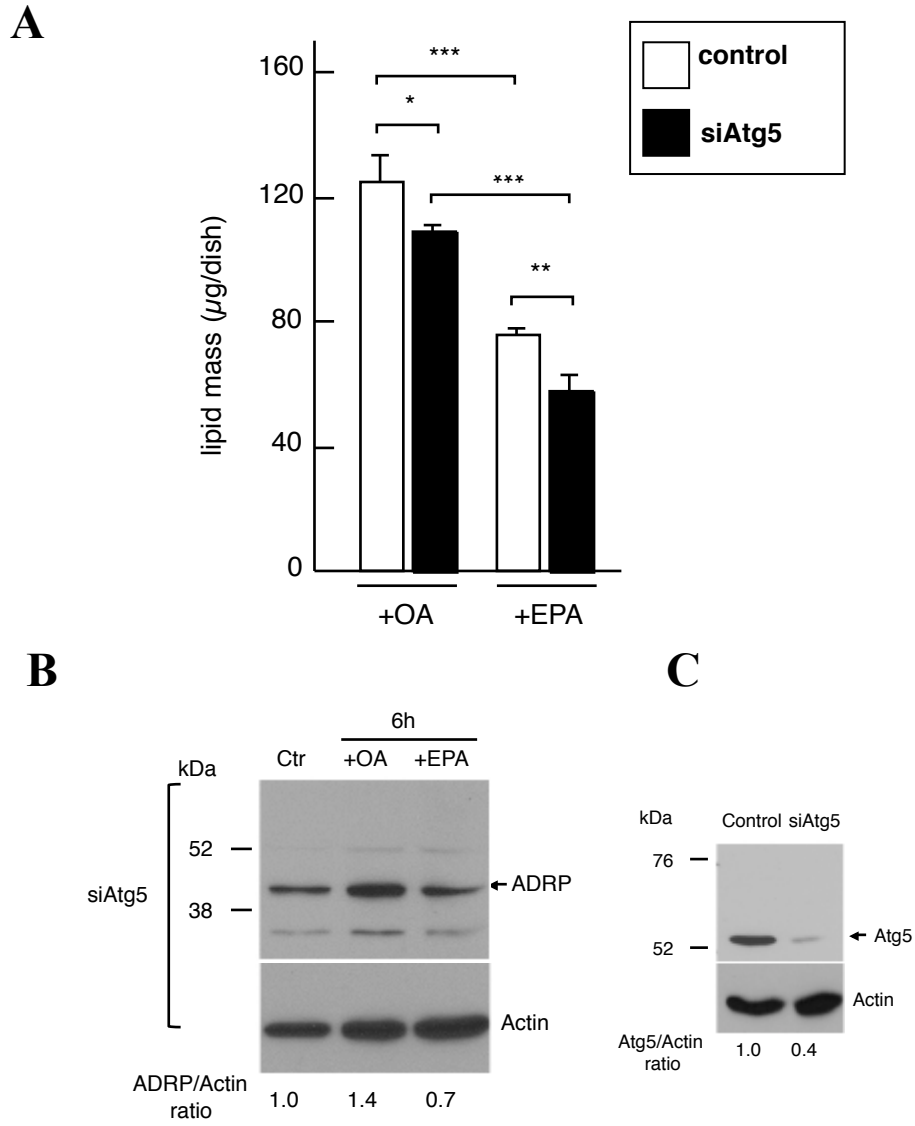
**Fig 7**



**Fig 8. Lipid mass measurement of intercellular TAG under SiRNA ATG5**

- a) Knock down of Atg5 ( autophagy ) did not affect EPA induced intercellular lipid droplets degradation.
- b) western blot of ADRP shows decrease in ADRP level even under the ATG5 knock down.
- c) Western blot of Atg5 showing the efficiency of silencing.

**Fig 8**



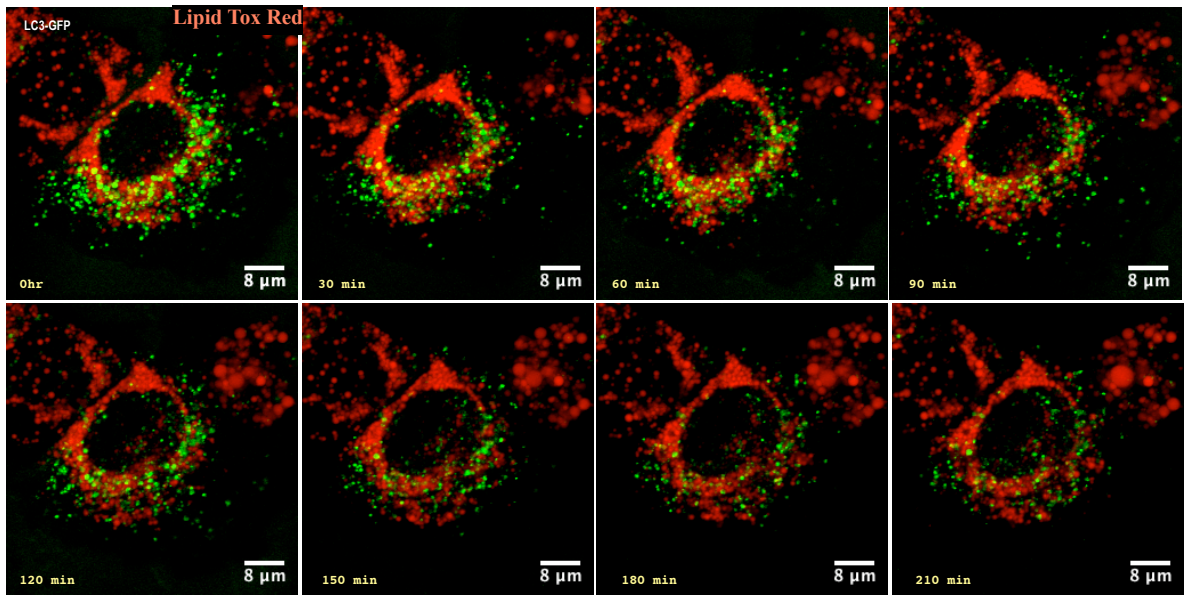
**Fig 9. Treatment of the cells with EPA did not induce recruitment of LC3-GFP to the lipid droplets surface and did not induce autophagic flux.**

cells were transfected with LC3-GFP. 24 hr after transfection, cells were treated with oleate for overnight. cells were treated with or without EPA (0.4mM).lipid droplets were stained with lipid Tox red. images were acquired in different time points after adding EPA to medium.

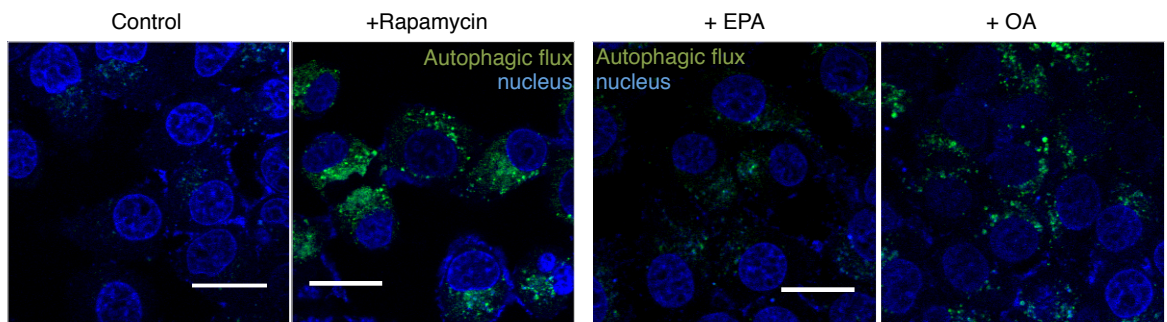
b) Cyto-ID assay shows no induction of autophagy flux after adding EPA to medium .Rapamycin was used for positive control. Note enhanced autophagic flux (Green) in the perinuclear region (nucleus stained blue). Scale bar, 16  $\mu$ m. g, EPA treatment, as compared with OA, does not induce autophagic flux. Scale bar, 16  $\mu$ m

**Fig 9**

**A**



**B**



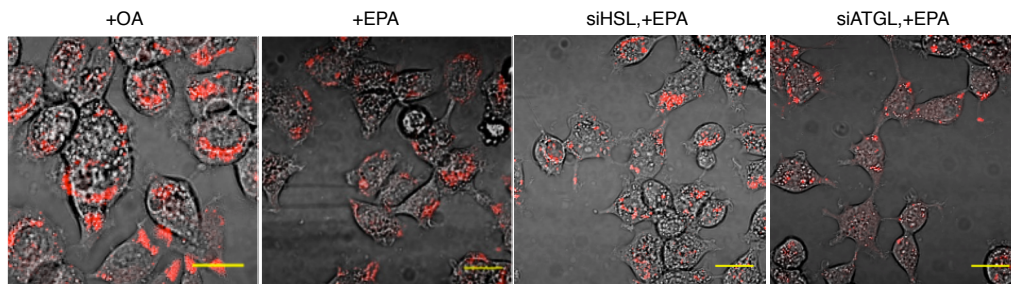
**Fig 10. EPA-triggered lipid degradation does not require cytosolic lipase ATGL or HSL.**

Cells treated with SiRNA or scrambled SiRNA for control.

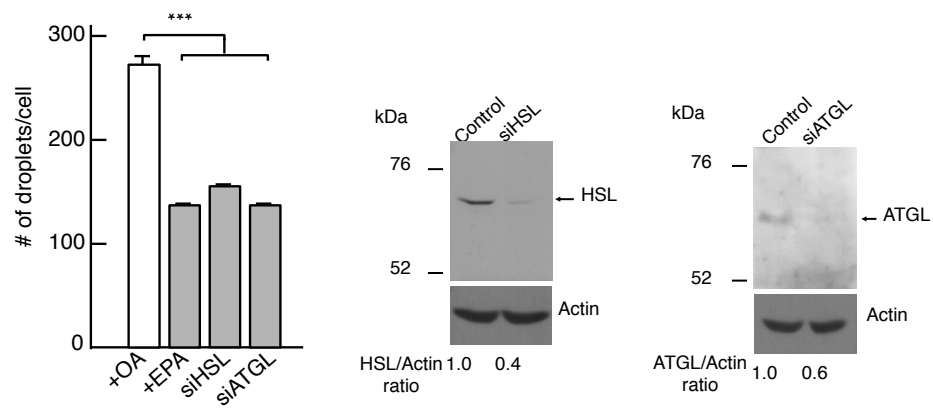
a, Imaging analysis of lipid droplets (stained with lipidTOX Red) in cells transfected with ATGL- or HSL-specific siRNA. Scale bars, 22  $\mu$ m. b, Quantification of number of lipid droplets 6 hr after adding EPA to medium. In all cases degradation of intercellular LDs was comparable to control. c) Western blots of HSL or ATGL showing the efficacy of silencing. Control, cells transfected with scrambled siRNA.

**Fig 10**

**A**



**B**



## ***Results***

### ***EPA induces lysosomal cytosolic distribution and promoting transient Interaction between lysosomes and lipid droplets.***

Nutrition availability is one the factors that correlates the cytosolic position of lysosomes and accumulation of lysosomes in prenuclear region was coordinated with autophagosome formation and autophagic flux (75).

To further evaluate the role of Autophagy and lysosomes in intracellular lipid droplets degradation, we monitored the position of lysosomes before and after adding EPA to medium.

When we tested for lysosomal distribution after treatment with EPA , we noticed that the lysosomes were dramatically scattered only in EPA treated cytosol which was in striking contrast to that observed in classic autophagic events (75)(Fig 11A).

Interestingly, change in position of lysosomes from clustering in prenuclear to scattering around the cytosol was accompanied with a decline in the number of lipid droplets.

It has been shown that Cytosolic pHi is able to regulate the position of lysosomes as well as mTORC1 activity (75). Measuring cytosolic PH using pHrodo Green revealed that EPA treatment dramatically decreased the cytosolic PH compared to control and oleate treated cells (Fig 11B).

To better understand the interconnection between these two phenotype, we further blocked the cytosolic distribution of lysosomes by knocking down, KIF1B, a member of kinesin superfamily proteins which has been shown that is able to promote lysosomal peripheral redistribution (77). Using SiRNA for this kinesin superfamily member, we could confirm the correlation between lysosomal cytosolic scattering and lipid droplets degra-

## ***Results***

ation which was evident by accumulation of lipid droplets even after treatment with EPA (Fig 12 A,B).

To ascertain that EPA-triggered lipid turnover is indeed a lysosome-dependent process, we depleted the lysosome-associated proteins LAMP1 (73) or Rab7 (79) that are known to be important for the functionality of lysosomes by using small interfering RNAs (SiRNA). Silencing LAMP1 or Rab7 completely abolished EPA-triggered lipid turnover, and the extent of inhibition was identical to that induced by alkalinisation of lysosomes using NH<sub>4</sub>Cl (Fig 13 A,B & Fig 14 B).

This results uncovered that EPA can stimulate lysosomal degradation of cytosolic lipid droplets by triggering lysosomal redistribution from prenuclear region toward the lipid droplets.

Lysosomes have an ability to fuse and transiently interact with different organelles and cellular compartments such as late endosomes and autophagosomes and plasma membrane.

Using live cells imaging, we were able to demonstrate transient interaction between the lysosomes and lipid droplets upon treatment with EPA (Fig 14).

Interplay between the lipid droplets and lysosomes was mimicking kiss and run pattern which has been reported for some other organelle such mitochondria as well as lysosome itself (69).

Ultrastructural analysis using transmission EM (Fig 15 A,B) showed direct contact between lipid droplets and lysosomes in EPA-treated cells. Under no circumstances were autophagosome-like structures ( double membrane ) encasing lipid droplets observed.

## ***Results***

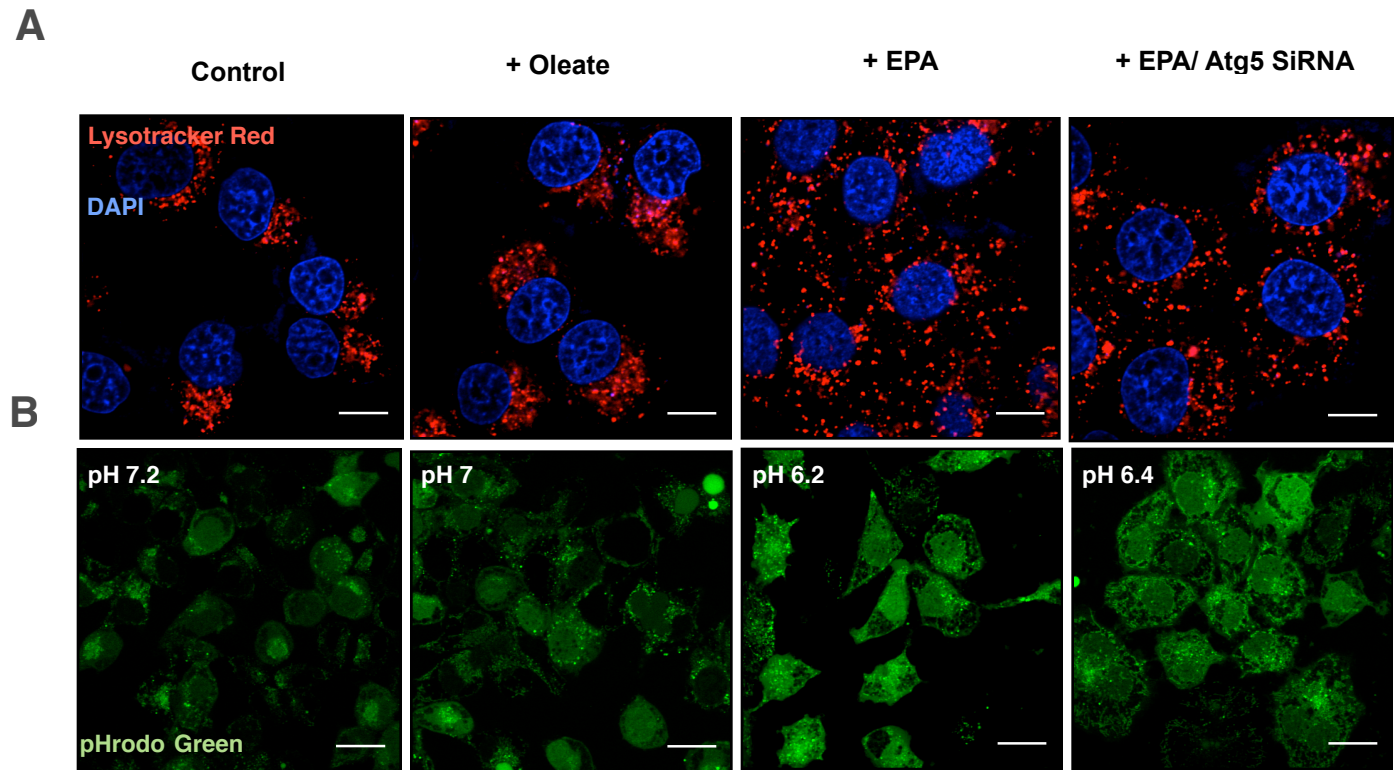
Even with transient nature of interaction between lysosome and lipid droplets ,we were able to capture the direct fusion between the lysosomes and lipid droplets. A portion of the lipid droplet often seemed to be engulfed by the lysosomes at the contact site. This type of structure also can be same as microautophagy like structure .

## *Results*

### **Fig 11 .Change in lysosomal position and cytosolic pH in response to EPA treatment**

McA-RH7777 cells either not transfected or were transfected with different SiRNAs.- For accuracy regarding the SiRNA transfection , all of the SiRNAs were labelled with SiRNA tracker. Cells were treated with oleate for overnight. Images were taken 1 hr upon adding EPA to medium.a)Note that change in lysosomal position after EPA treatment under all condition.b)change in cytosolic pH ; 1 hr after treatment with EPA cells were washed with LCIS medium and resuspended in LCIS 2X concentration plus pHrodo green. Cells were incubated at 37c for 30 min . Cytosolic pH measured using scanning confocal microscope with 490Ex/510Em rate.Note the pH drop upon adding EPA and its correlation with position of lysosomes.

Fig 11



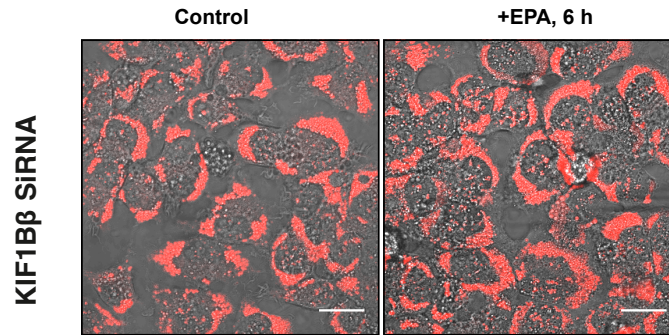
## *Results*

### **Fig 12. Effect of KIF1B knock down on lipid lipid droplets degradation**

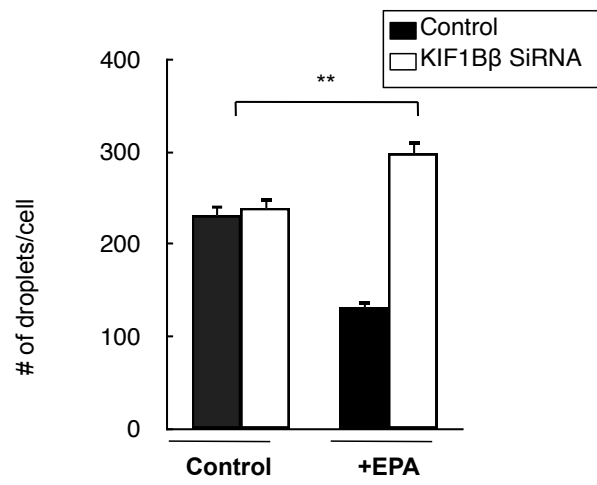
McA-RH7777 were transfected with SiRNA specific for Kif1beta or control. Cells were treated with oleate for overnight. SiRNA transfection did not have an effect on lipid droplets accumulation as it was comparable to control. Cells were treated with EPA and number of lipid droplets were quantified 6 hr after adding EPA to medium. Values are means  $\pm$  s.e.m. of three independent experiments (carried out in triplicate). All comparisons are with the control within each treatment condition, \*  $P < 0.05$ , \*\*\*  $P < 0.005$  Student's t-test.

Fig 12

A



B



## *Results*

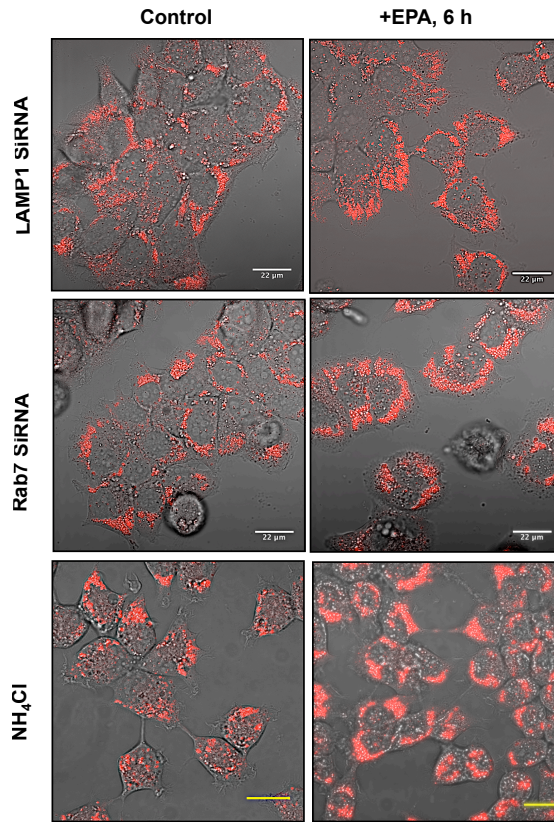
### **Fig 13. EPA-triggered lipid degradation requires functional lysosomes and their intact molecular machinery**

a,b) Treatment with NH<sub>4</sub>Cl or silencing LAMP1 or Rab7 blocked EPA-triggered lipid degradation.

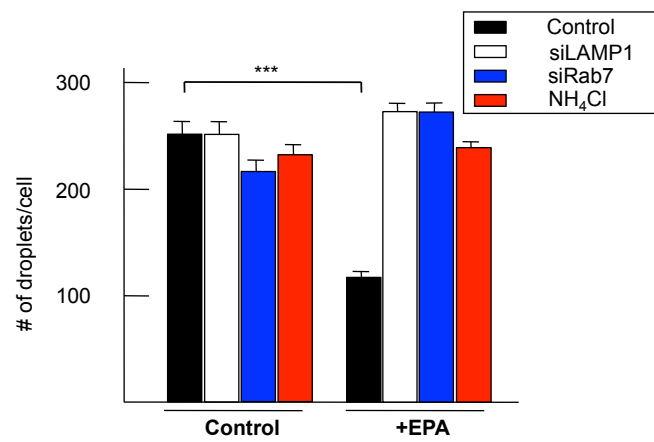
McA-RH7777 were transfected with LAMP1, Rab7 or control SiRNAs. cells were treated with oleate for overnight. 6 hr after adding EPA to medium lipid droplets were stained and images were taken. b) alternatively, cells treated with NH<sub>4</sub>Cl 30 min before adding EPA to medium. Values are means  $\pm$  s.e.m. of three independent experiments. Scale bar, 22  $\mu$ m

Fig 13

A



B



## *Results*

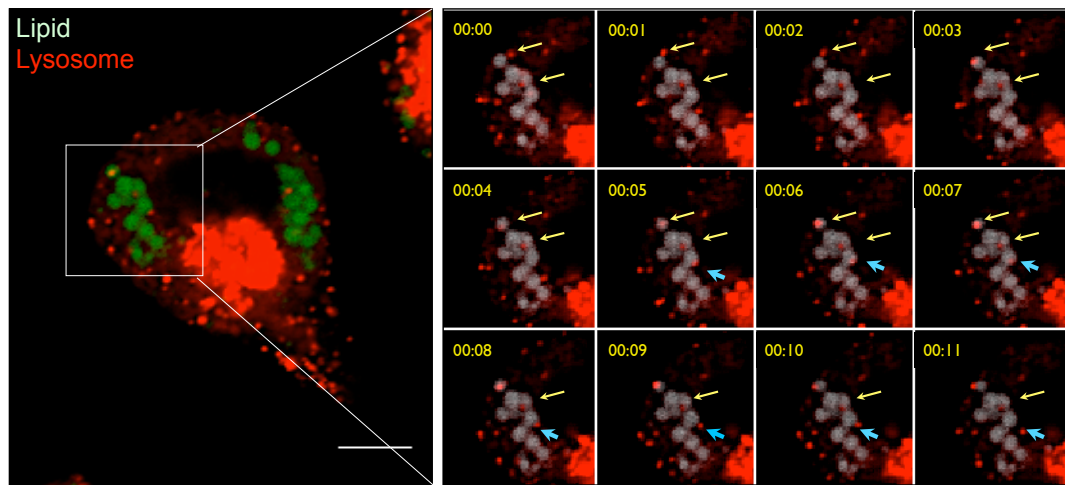
### **Fig 14. Interaction of lysosomes with Lipid droplets after adding EPA to medium.**

Recruitment of lysosomes to lipid droplets after treatment with EPA. Cells were plated in MetTeck coverslip for live cell microscopy. Cells were treated with oleate (0.4mM) for overnight. Lysosomes were stained with lyso tracker red. Images were taken after adding EPA (0.4mM) to the medium. Images were acquired at the frame rate of 11 frame/Sec. A) selected area is repressing the interaction between the lysosomes with lipid droplets( yellow arrow) and kiss and run event between the lipid droplets and lysosomes (blue arrow). Movie is available in supplemental Data.b) Effect of ammonium chloride on blocking the recruitment of lysosome to the lipid droplets. Cells were treated with ammonium chloride 10nM before adding EPA.

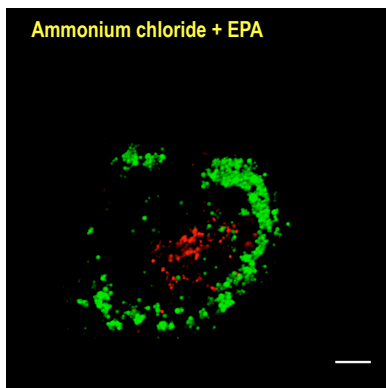
*Results*

**Fig 14**

**A**



**B**



## *Results*

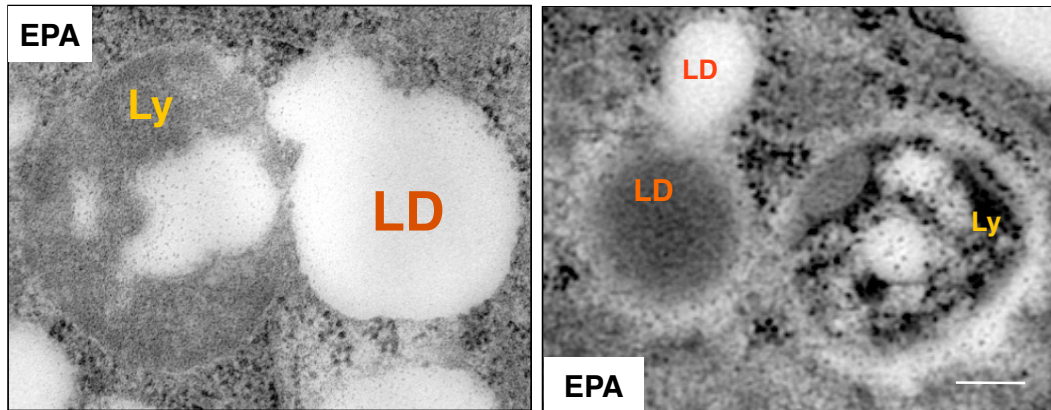
### **Fig 15. Electron micrographs of lipid droplet interaction with lysosome after adding EPA**

Cells were treated with oleate overnight and fixed upon addition of EPA (0.4mM). Time for fixation was correlated with live cell imaging of interaction between the lysosomes and lipid droplets. Bar is 100 nm.

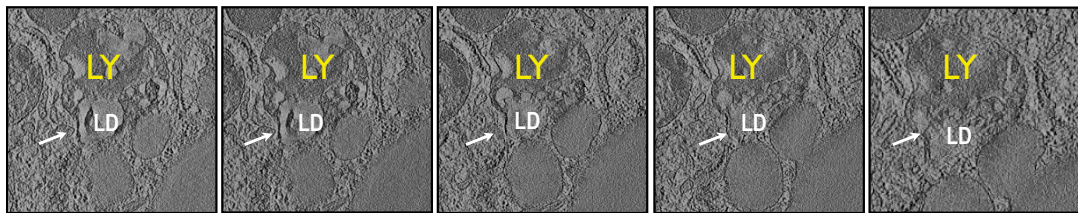
electron tomography of lysosome lipid droplets interaction .b)Close up sequential images of interaction between lysosome and lipid droplets. Note engulfment of piece of lipid droplets by lysosome ( white arrow ).

Fig 15

A



B



## ***Results***

### ***Bidirectional movement of lysosomes is required for its proper function:***

Efficient lysosomal function requires its bi-directional movement(75).In order to understand the mechanism behind the lysosomes-lipid droplets interaction,

We examined the machinery that involves in lysosomal peripheral distribution regarding EPA treatment.

Among the proteins that are important for lysosomal bi Directional movement ORPL1, FYCO1, RILP and Arl8b has been shown that play an important role during the process. Except for ORPL1 which its over expression showed no discernible effect on lysosome-Lipid droplets interaction(upon adding EPA)(Unpublished Data), overexpressing of FYCO1 and RILP affected EPA-triggered lipid degradation (Fig 16 A,B). Overexpression of GFP RILP blocked EPA-triggered lipid turnover, as a result of clustering of lysosomes around MTOC(microtubule organizing centre) (Fig 17).

Overexpression of GFP FYCO1 also blocked EPA-triggered lipid turnover while forcing the lysosomes towards the cell periphery (Fig 18).

SiRNA of FYCO1 and RILP confirmed the effect of these proteins overexpression on lipid droplets degradation by EPA(Fig 19). Under the FYCO1 silencing condition , all of the lysosome were accumulated in prenuclear region and no movement of lysosomes toward the lipid droplets was observed. In contrast under the RILP SirRNA all of the lysosomes were scattered and easily observable in peripheral of the cytosol.

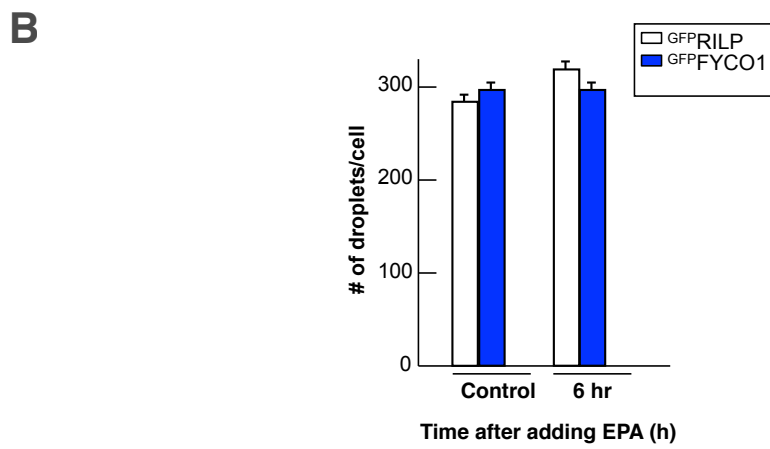
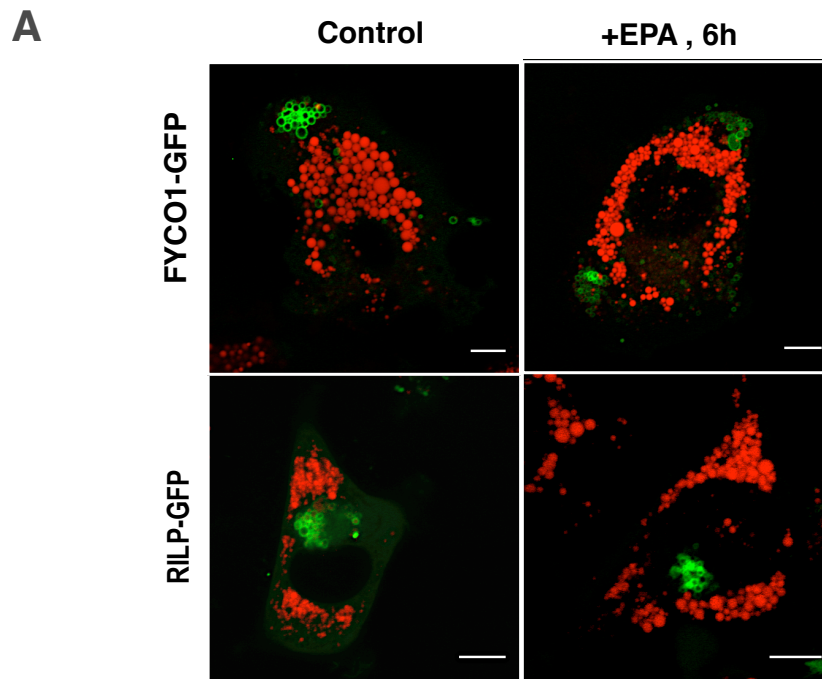
Quantification of lipid droplets long with measuring ADRP expression level showed gradual increase in intracellular lipid droplets upon treatment with EPA (Fig 20).

## *Results*

**Fig 16. Over expression of GFP FYCO1 and GFP RILP, Plus and minus end Lysosomal associated motor proteins, blocked intercellular lipid droplets degradation by EPA.**

Cells were transfected with GFP FYCO1 and RILP before treating with oleate for overnight. 6 hr after adding EPA to medium, lipid droplets were stained using lipid Tox red and images were taken in z stack for quantification of number of lipid droplets. Values are means  $\pm$  s.e.m. of three independent experiments (carried out in triplicate). All comparisons are with the control within each treatment condition.

Fig 16



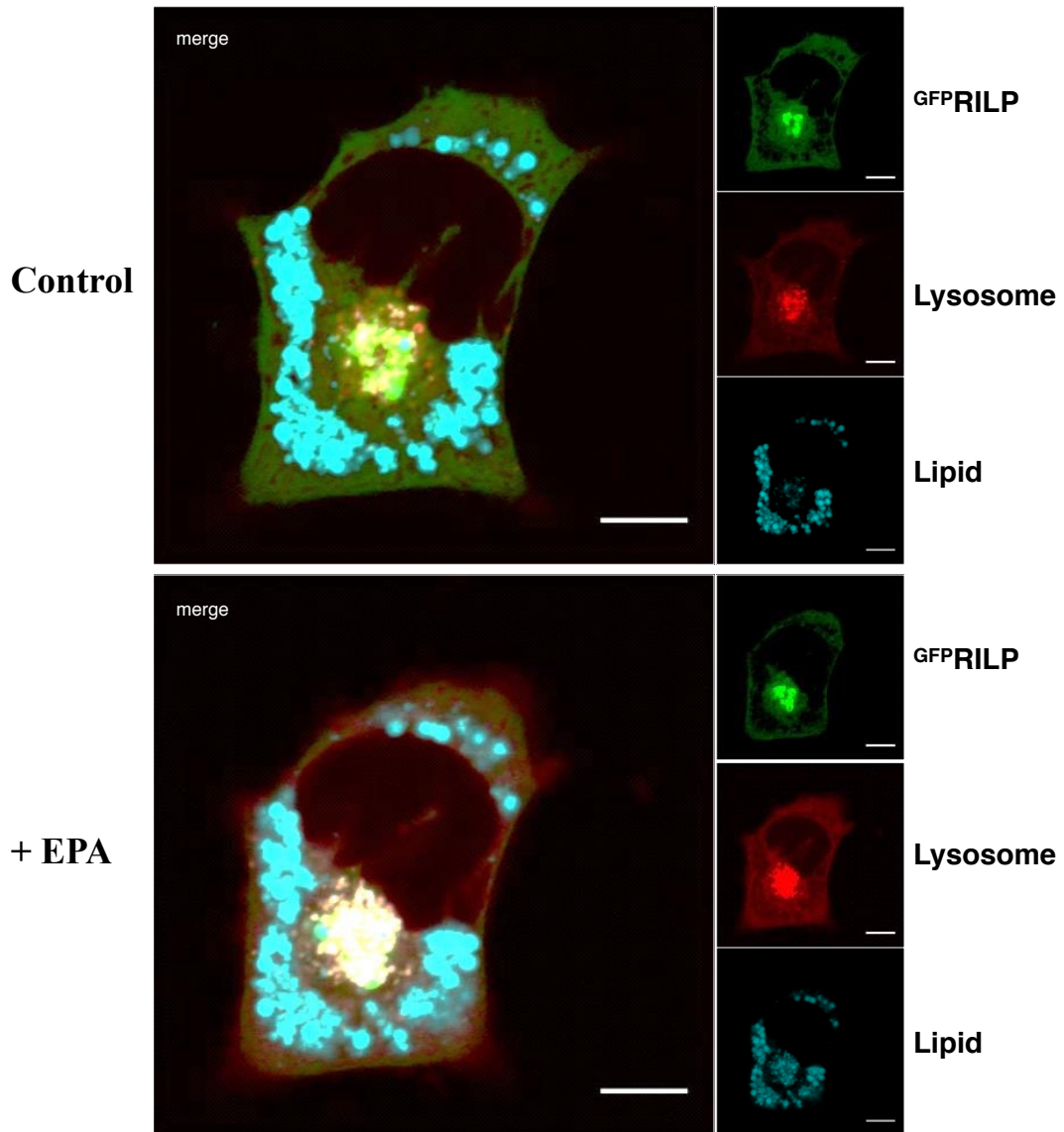
## *Results*

**Fig 17. Overexpression of GFP -RILP protein resulted in accumulation of lysosome in prenuclear region and close to MTOC area.**

Cells were transfected with GFP-RILP proteins before treatment with oleate and EPA. 1hr after adding EPA , lipid tox red( Cy5 ) and LysoTracker red were added for visualization of lipid droplets and lysosomes respectively. Motility/distribution of lysosomes were analyzed using live cell imaging.

*Results*

**Fig 17**

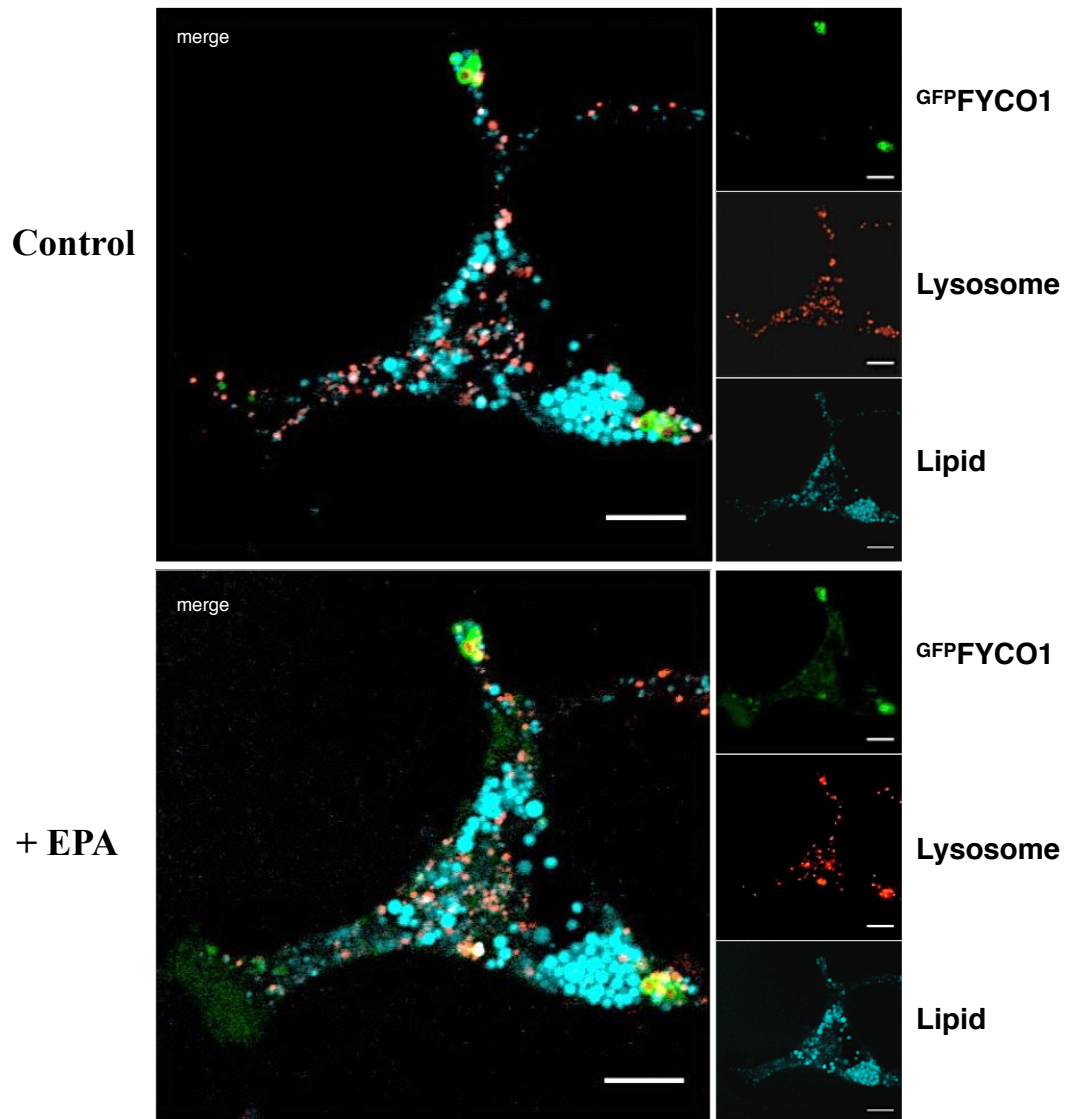


## *Results*

### **Fig 18. Overexpression of GFP-FYCO1 protein resulted in accumulation of lysosome in peripheral of cytosol.**

Cells are transfected with GFP-FYCO1 protein before treatment with oleate and EPA. 1hr after adding EPA , lipid tox red and LysoTracker red were added for visualization of lipid droplets and lysosomes respectively. Motility/distribution of lysosomes were analyzed using live cell imaging. (Movie is available in supplemental movie ), Note that even with peripheral distribution of lysosomes almost zero interaction between lysosome and lipid droplets were detected.

**Fig 18**

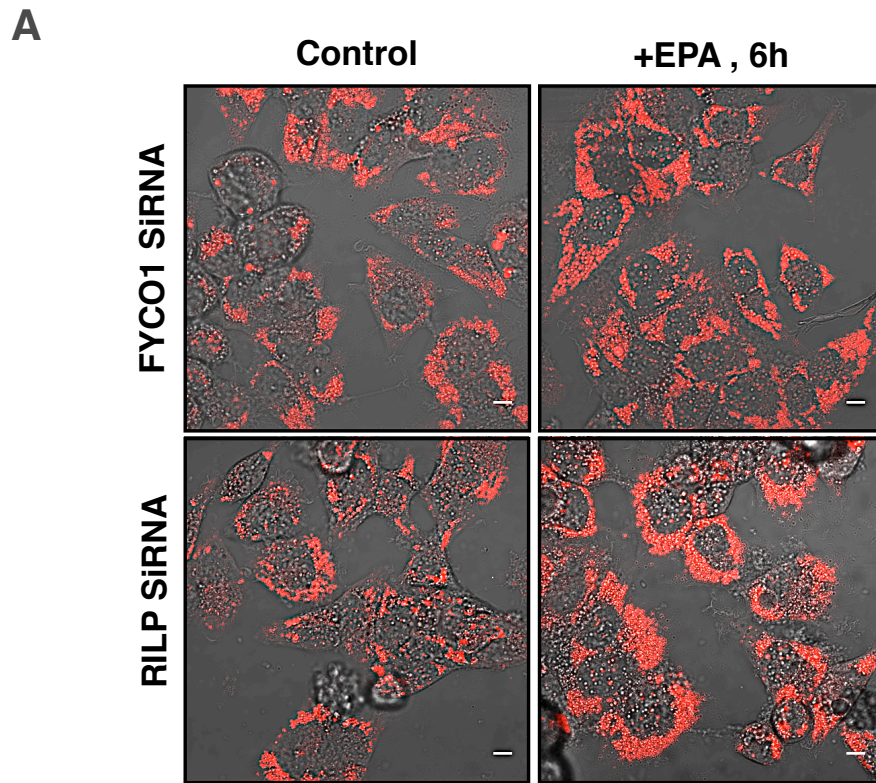


## *Results*

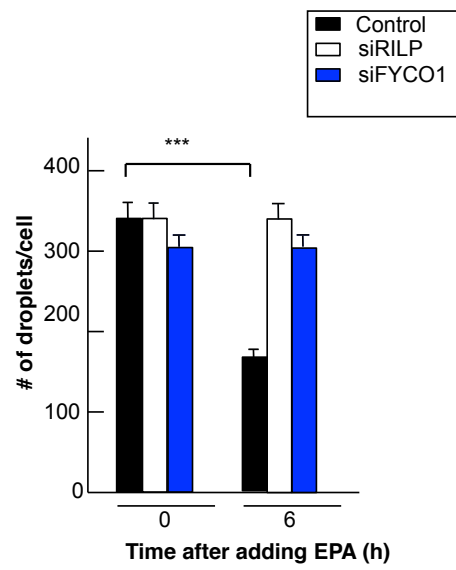
### **Fig 19. Silencing FYCO1, RILP blocked EPA-triggered lipid degradation.**

Cells were transfected with either SiRNA FYCO1 , RILP or control 24 hr before treatment with oleate for overnight. 6 Hr after adding EPA ( 0.4mM) to medium , images were taken in Z stacks for quantification of lipid droplets. Values are means  $\pm$  s.e.m. of three independent experiments (carried out in triplicate). All comparisons are with the control within each treatment condition, \*  $P < 0.05$ , \*\*\*  $P < 0.005$  Student's t-test. Scale bars, 22  $\mu\text{m}$ ..

Fig 19



**B**

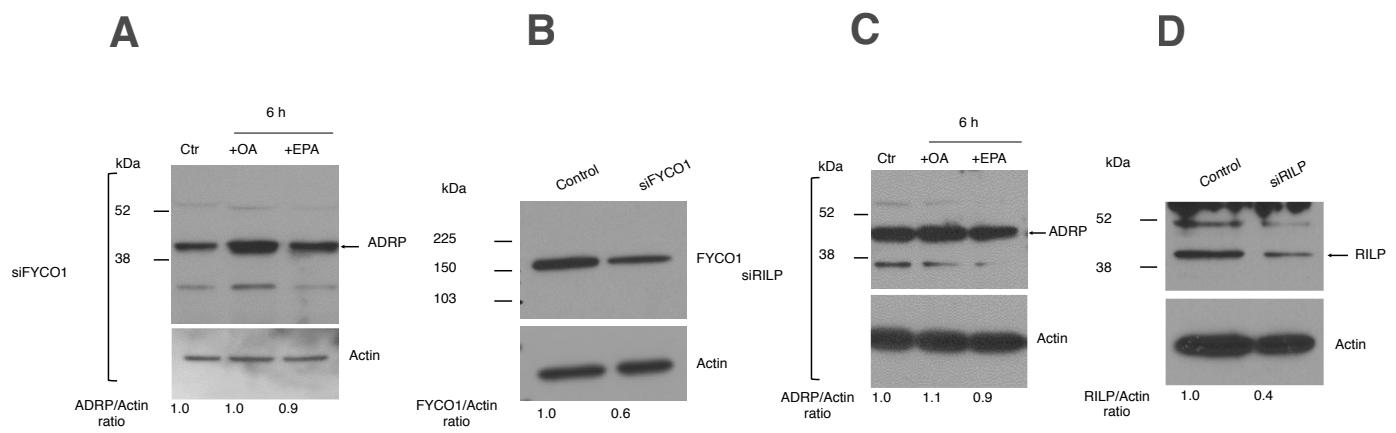


## *Results*

### **Fig 20. Western blots of ADRP in FYCO1 and RILP silenced cells**

a,c ) blockage of lipid droplets degradation upon adding EPA to medium as it is represented by accumulation of ADRP. b,d), Western blots of FYCO1 and RILP proteins showing the efficacy of silencing. Control, cells transfected with scrambled siRNA.

Fig 20



## ***Results***

### ***Arl8b accelerates degradation rate of intracellular Lipid droplets upon treatment with EPA.***

Unexpectedly, in contrast to FYCO1, overexpression of GFP Arl8b which normally induces centrifugal movement of lysosomes resulted in remarkably rapid degradation of lipid droplets(Fig 21).

Quantification of number of lipid droplets showed slight acceleration in the rate of lipid droplets degradation compare to non transfected cells upon adding EPA to medium (Fig 22).

Overexpression of GFP Arl8b, unlike that of FYCO1, did not promote spontaneous centrifugal distribution of lysosomes towards the cell periphery. Rather, lysosomes remained in the perinuclear region in GFP Arl8b overexpressing cells, and lysosomal interaction with lipid droplets occurred only after EPA treatment (Fig 23). Throughout the whole process, GFP Arl8b was intimately associated with lysosomes, and close contact of Arl8b-containing lysosomes with lipid droplets was seen in both live imaging and immuno-EM (Fig23B). Moreover, the accelerated lipid degradation in Arl8b-overexpressing cells was observed only after EPA treatment.

To define mechanisms whereby EPA-triggered lipid degradation is altered by Arl8b, we examined the spatiotemporal relationship between lysosomes and lipid droplets following Arl8b silencing.

Depletion of Arl8b, like that of FYCO1 and RILP, completely abolished EPA-triggered lipid turnover (Fig 24A,B,C).

## ***Results***

Although silencing Arl8b blocked EPA-triggered lipid degradation, it did not appear to affect lysosomal distribution or motility toward the lipid droplets and interaction between lysosome and lipid droplets still detectable upon EPA treatment (Fig 25).

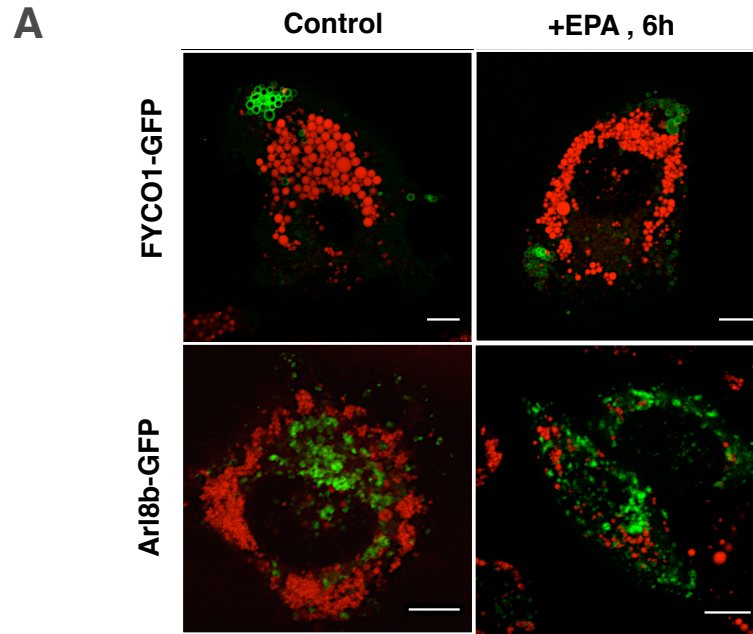
These results together suggest a model whereby anterograde and retrograde motility of lysosomes, mediated by FYCO1 and RILP, respectively, are essential but insufficient for EPA-triggered lipid turnover, and lipid degradation additionally requires Arl8b independent from its role in Lysosomal peripheral movement.

## *Results*

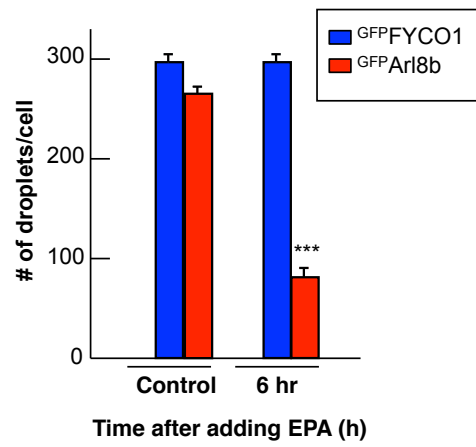
### **Fig 21. Overexpression of Arl8b assisting lipid droplets degradation compare to FYCO1 , another Lysosomal plus end motor associated protein.**

Cells were transfected with FYCO1 and Arl8b GFP. Cells were treated with oleate for overnight . 6 hr after adding EPA to medium lipid droplets were stained by lipidTox red and quantified using Image J lipid droplets quantification plug in .a) Images of lipid droplets under FYCO1 and Arl8b GFP overexpression. b) Quantification of lipid droplets from there independent experiments . Scale bar 8  $\mu$ m.

Fig 21



**B**

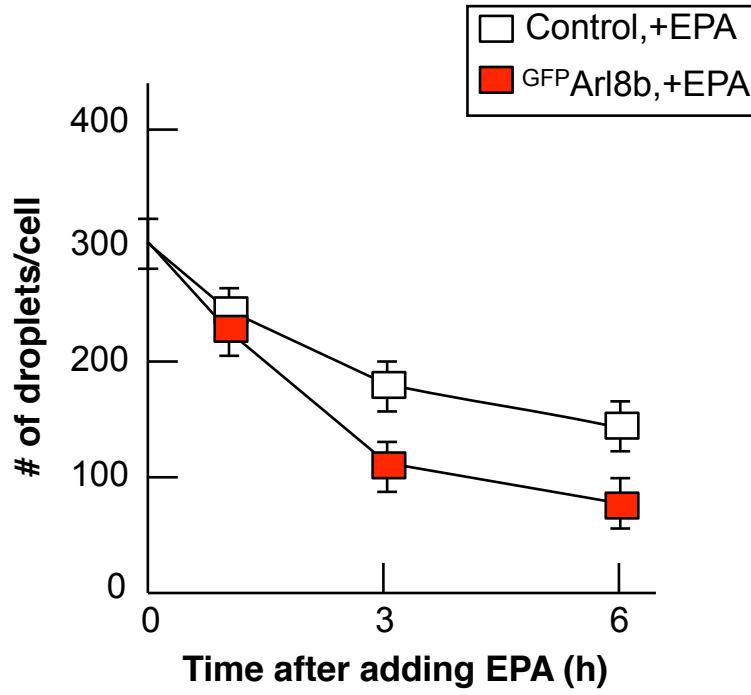


## *Results*

**Fig 22. Arl8b GFP overexpression is accelerating the rate of lipid droplets degradation after adding EPA to medium compare to control.**

Cells were transfected with and without Arl8b GFP plasmid. Cells were treated with oleate for overnight. Images were taken in different time points after adding EPA to medium. Number of lipid droplets were quantified in different time points using image J quantification software. Quantification was carried out in three different independent experiments.

Fig 22



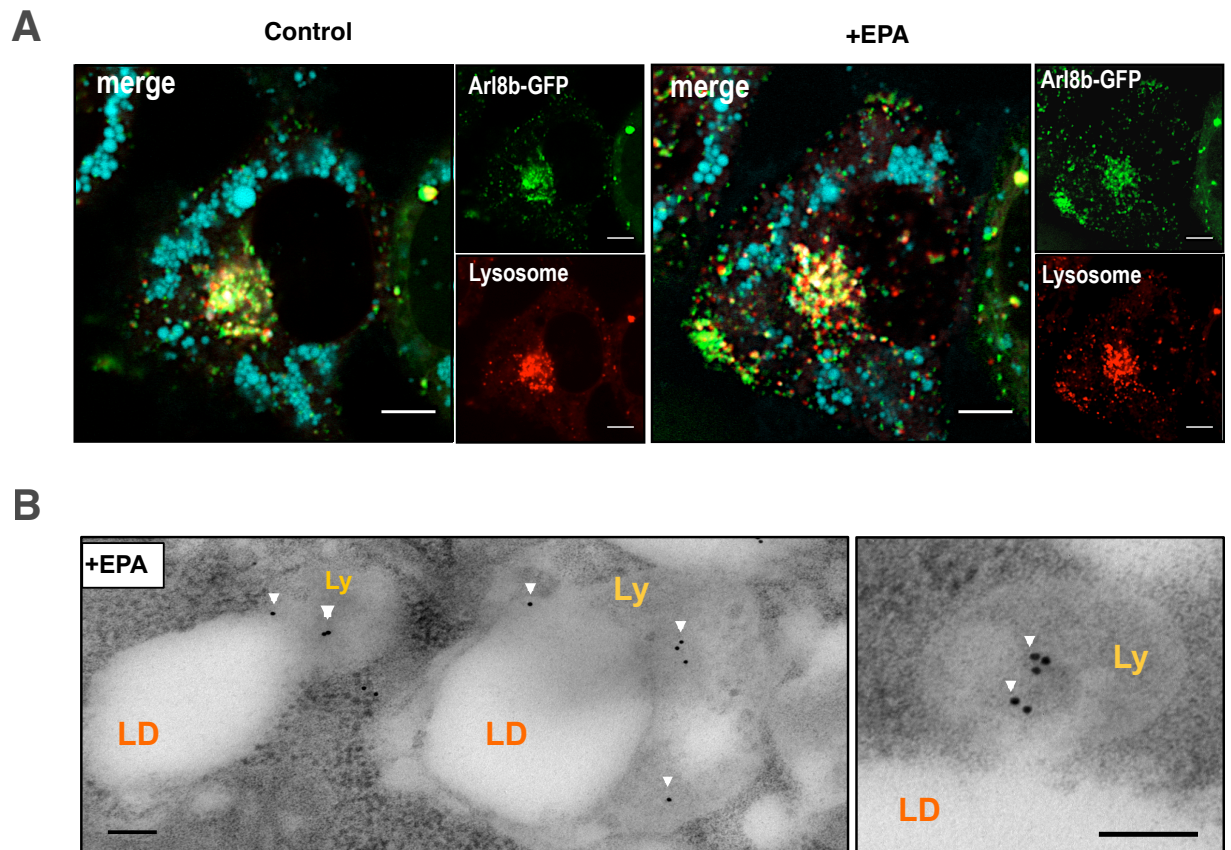
## *Results*

### **Fig 23. Arl8b and Lysosomes are interacting and moving together toward lipid droplets after adding EPA to medium .**

a) Cells were transfected with Arl8b GFP and treated with oleate for overnight. 30 min after adding EPA to medium, localization of Arl8b and lysosome was monitored. Note that Arl8b lysosomes are interacting with lipid droplets as a one particle. Scale bars, 8  $\mu$ m.

b) immunoGold EM of Arl8b. Under all conditions Arl8b was detected on lysosomes and in close vicinity of lipid droplets after adding EPA. Scale bar is 500nm.

Fig 23



## *Results*

### **Fig 24. Silencing of Arl8b blocked EPA induced lipid droplets degradation.**

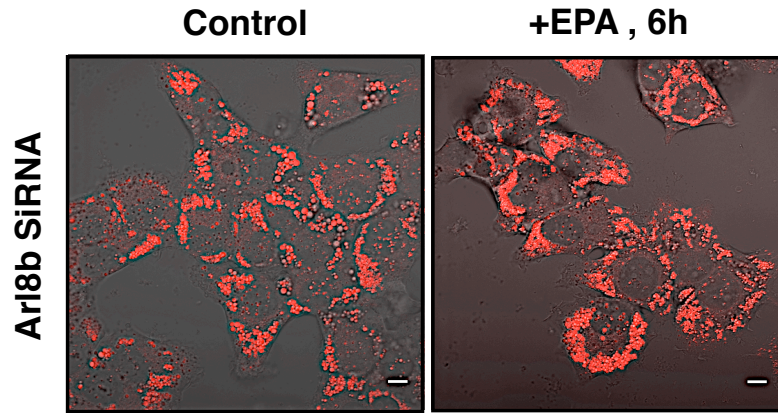
a) Cells were transfected with SiRNA Arl8b or control 24hr before adding oleate for overnight. 6hr after adding EPA to medium , images were taken in Z stacks for quantification of lipid droplets .

b) Quantification of number of lipid droplets.\* P < 0.05, \*\*\* P < 0.005 Student's t-test.

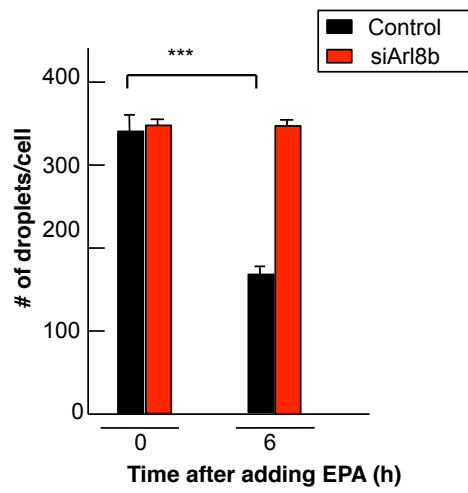
c) western blot of ADRP shows blockage of lipid droplets degradation upon adding EPA to medium.

Fig 24

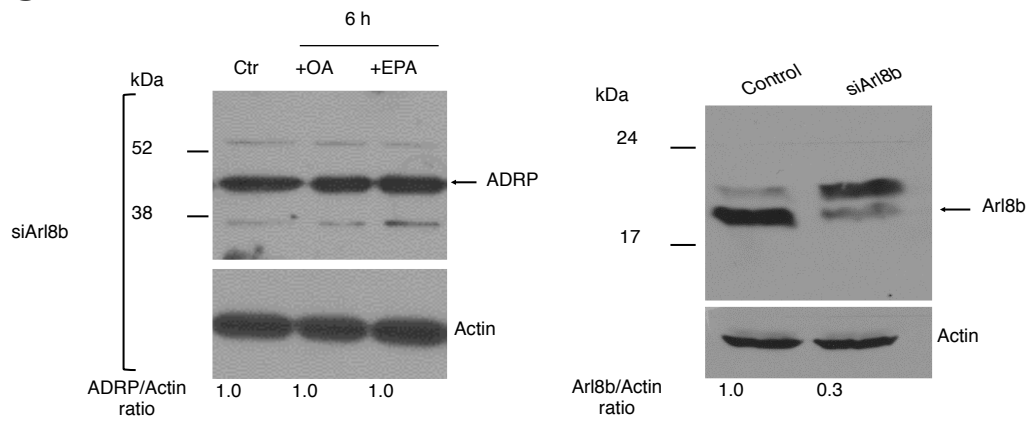
A



B



C



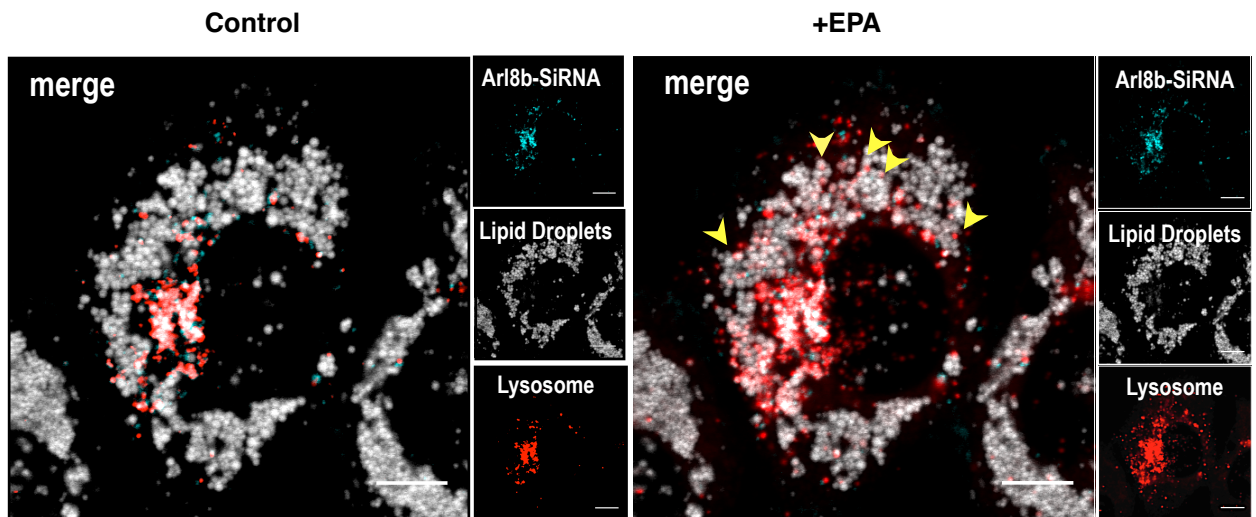
## *Results*

**Fig 25. Silencing of Arl8b does not affect lysosomal interaction with lipid droplets after adding EPA to medium.**

EPA-triggered lysosomal interaction with lipid droplets (the “on” phase) was not affected by Arl8b silencing. Cells were transfected with Arl8b siRNA were identified using siRNA-Tracker. Yellow arrows show lysosomal interaction with lipid droplets. Scale bars, 8  $\mu\text{m}$ .

*Results*

**Fig 25**



## ***Results***

### ***Arl8b affecting the rate of lipid droplets degradation by modulating the lysosomal interaction with lipid droplets.***

This Data suggests that Arl8b probably plays a role beyond merely promoting lysosomal motility or interacting with lipid droplets. To gain further insights into Arl8b action, we took advantage of Arl8b DN ( constitutive GDP form ) and DA (constitutive GTP form ) in order to further examine the role of Arl8b on lipid droplets degradation.

In contrast to general function of GTPase proteins , over expression of DN form of Arl8b accelerated the rate of lipid droplets degradation further than Arl8b WT. In contrast over-expression of Arl8b DA promotes accumulation of lipid droplets over different time points upon adding EPA to medium (Fig 26A,B).

To understand the mechanism behind the effect of constitutive active and inactive form of Arl8b, we used live cell imaging to monitor the lysosomal movement under these conditions . Even though under the both conditions lysosomes moved toward the lipid droplets and interact with them, but it was an obvious difference in lysosomal minus end movement and its disassociation rate after its interaction with lipid droplets.

Analysis of association/dissociation dynamics between lysosomes and lipid droplets revealed distinct features associated with various forms of Arl8b.

In wild-type Arl8b expressing cells, association between lysosome and lipid droplets (the “on” phase), followed by dissociation (the “off” phase) from lipid droplets which the process happened totally in 20 seconds (Fig 27 and Fig30).

Overexpression of GFP Arl8bQ75L, that putative GTP-form of Arl8b, resulted in continues lysosomal interaction with lipid droplets and thus resulting in attenuated lipid degra-

## ***Results***

dation. Live imaging shows prolonged lysosomal adherence to the droplet surface in GFP Arl8bQ75L-expressing cells with no follow up with off phase. (Fig 28 and Fig 30)

In contrast, expression of the constitutive-inactive form of Arl8bT34N showed rapid disassociation of lysosomes from lipid droplets, indicting Arl8bT34N involves in off phase of this interaction (Fig 29 and Fig 30). These results suggest that Arl8b possibly govern the kinetics of EPA triggered lipid degradation by controlling the tethering between lysosome and lipid droplets and as a result association/disassociation rate of lysosome from its target membrane.

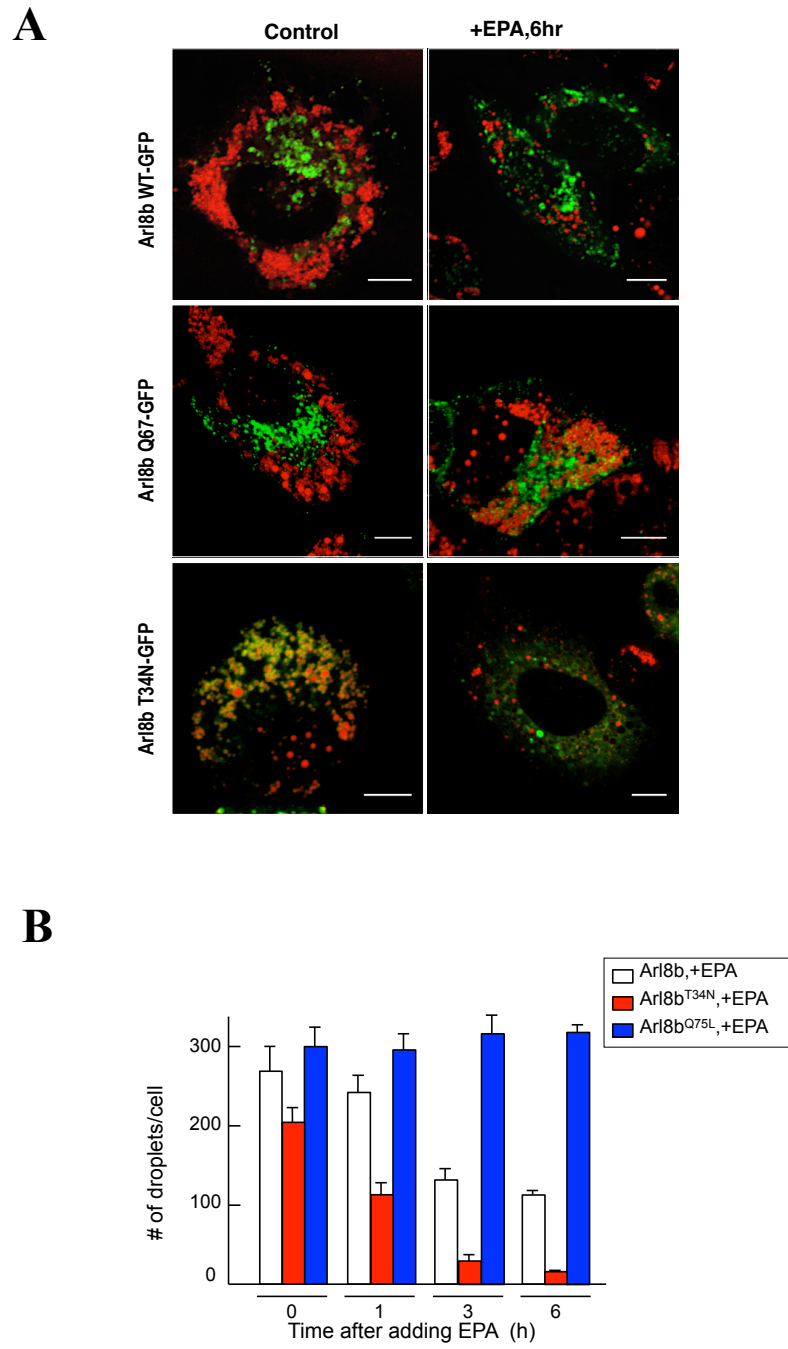
## *Results*

### **Fig 26. Effect of overexpression of different Arl8b constructs on lipid droplets degradation after adding EPA to medium.**

Overexpression of Arl8b DA blocked lipid droplets degradation. In contrast overexpression of Arl8b DN accelerate the rate of lipid droplets degradation compare to Arl8b WT.

a) Effect of Arl8b constructs on lipid droplets degradation b) Quantification of number of lipid droplets in different time points after adding EPA to medium.

Fig 26



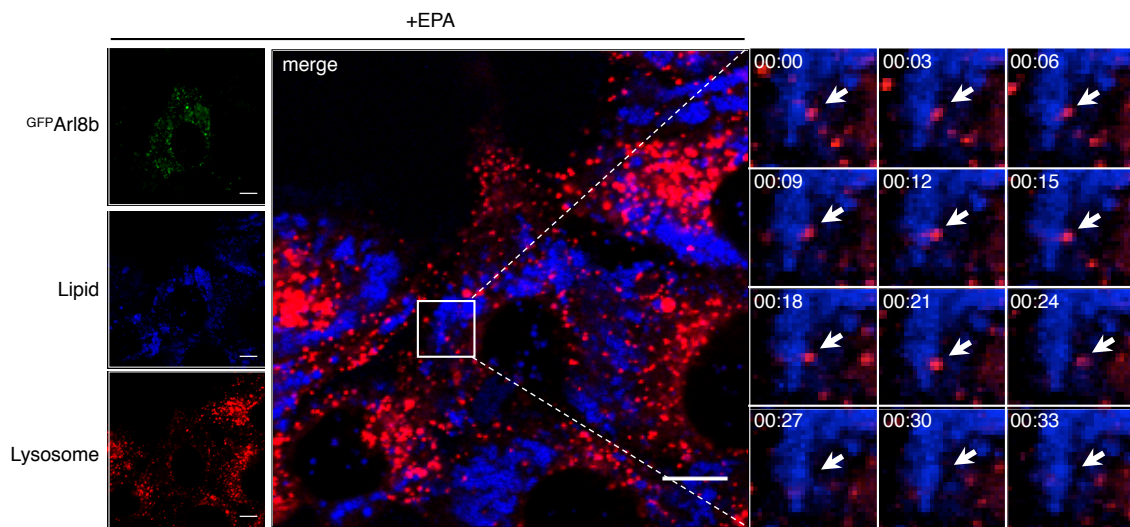
## *Results*

### **Fig 27. Normal on/off motion of lysosomal interaction with lipids occurs in GF-PArl8b-expressing cells.**

Throughout the process, Arl8b GFP is associated with lysosomes. In merged images, signals from Arl8b GFP were removed for clarity. Images are extracted from Supplementary Movie .Note that interaction between Lysosome and lipid droplets last for  $\approx 20$  sec. Scale bars, 8  $\mu\text{m}$ .

*Results*

**Fig 27**



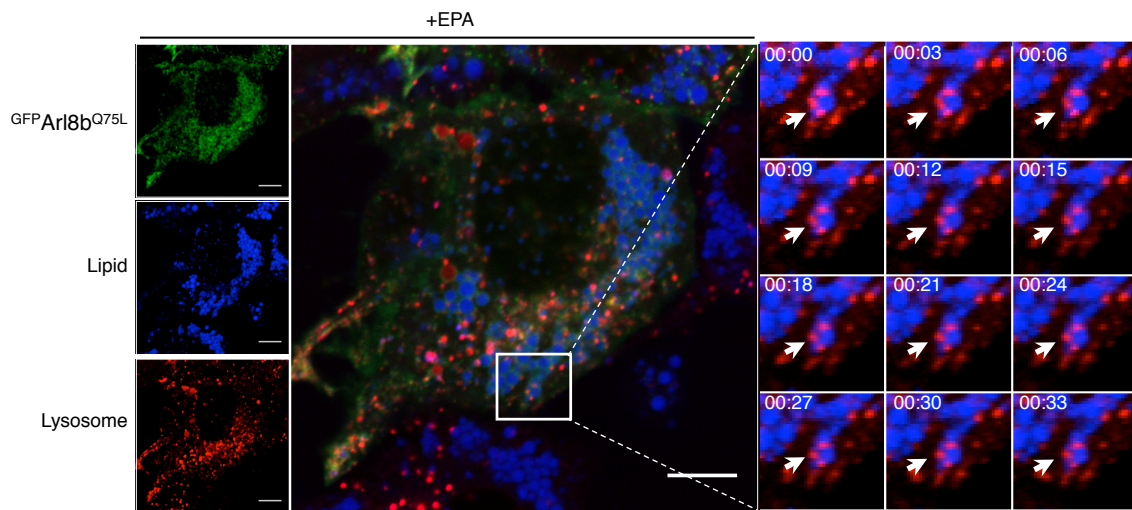
## *Results*

**Fig 28. Overexpression of Arl8b DA resulted in attachment of lysosomes to lipid droplets after adding EPA to medium.**

Arl8b DA eliminate dissociation of lysosome from lipid droplets ( Off phase). Images were extracted from supplemental movies. Note that even after 30 sec lysosomes still are interacting with lipid droplets. Scale bars, 8  $\mu\text{m}$ .

*Results*

**Fig 28**



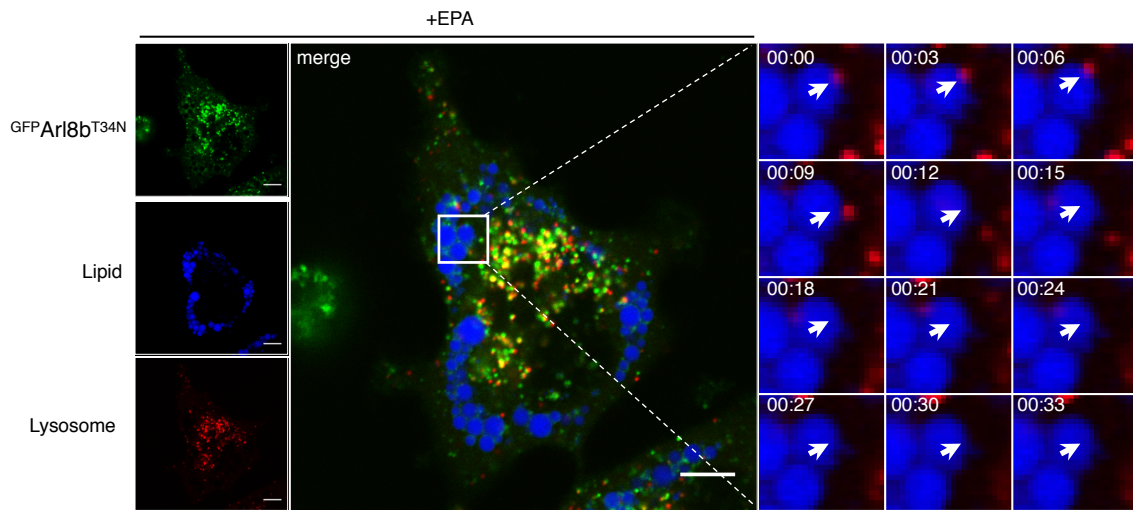
## *Results*

### **Fig 29. Overexpressing of Arl8bT34N ( DN ) accelerates lysosomal dissociation from lipid droplets.**

Arl8b DN promotes lysosomal disassociation from lipid droplets by accelerating the off phase .Scale bars, 8  $\mu$ m.

*Results*

**Fig 29**

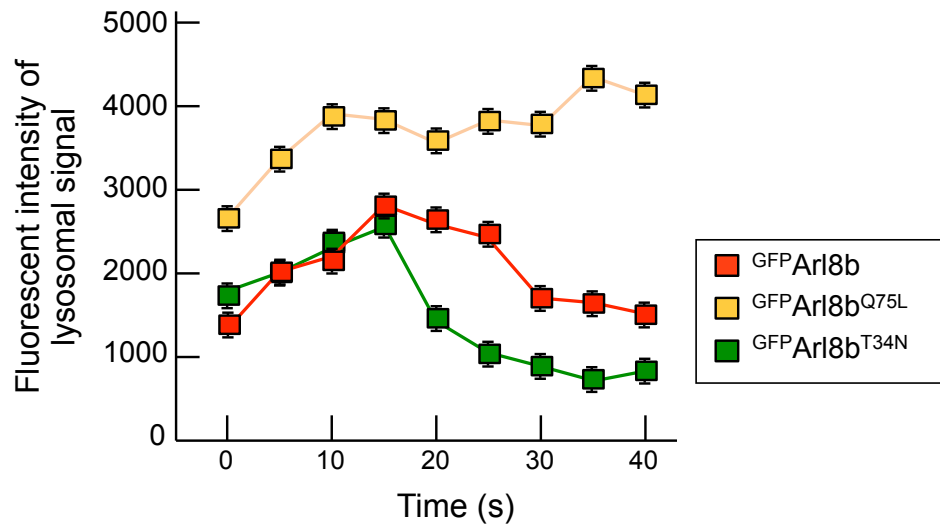


## *Results*

### **Fig 30. Quantification of dynamic of lysosomal interaction with lipid droplets under wild-type or mutant Arl8b overexpressing conditions.**

Cells were transfected with different Arl8b constructs . Cells treated with oleate for overnight. 1hr after adding EPA to medium , lysosomal motility was monitored using time laps microscopy. Intensity of Lysosomal signal in vicinity of lipid droplets was measured in 60 individual lipid droplets from three independent experiments.

Fig 30



## ***Results***

### ***Arl8b is facilitating lysosomal lipid droplets degradation through its interaction with HOPS protein complex.***

Previous report has shown that Arl8b interacts with HOPS protein complex and this complex can act as one of the effector of Arl8b GTPase. In this regard Arl8b is able to regulate microbial killing by controlling endo-lysosome trafficking, cargo delivery, and its fusion to lysosomes (85).

To test the effect of HOPS proteins and their possible role in intercellular lipid degradation, we monitored the effect of Vps41, Vps39 and Vps11 knock down before and after treatment with EPA. The efficiency of knock down was assessed by western blot(Fig32). Quantification of number of lipid droplets before treatment with oleate showed comparable accumulation of lipid droplets to non SiRNA transfected cells. EPA treatment did not promote degradation of intercellular Lipid droplets. Quantification of lipid droplets number showed accumulation of lipid droplets 6 h after adding EPA to medium. SiRNA Vps41, Vps39 and Vps11 resulted in accumulation of lipid droplets compare to control indicating their important role in degradation of lipid droplets(Fig31).

In order to see if accumulation of lipid droplets under the HOPS knock down condition is due to decrease in lysosomal redistribution and interaction with lipid droplets or not, we monitored motility and relocation of lysosomes under the each of HOPS subunits SiRNA condition. No defect in lysosomal movement or its interaction with lipid droplets were observed in cells upon EPA treatment, indicating that SiRNA HOPS complex affecting intercellular lipid droplets degradation independent from lysosomal movement machinery.

## ***Results***

The inhibitory effect of HOPS subunit knockdown on EPA-triggered lipid degradation phenocopies that of Arl8b silencing (Fig32). We hypothesized that Arl8b possibly plays a role by interacting with HOPS protein complex. To investigate this possibility, Cells were transfected with Arl8b mCherry and incubated with oleate for overnight. Using anti RFP and anti VPS11, VPS39 and Vps41 for in situ CO-IP, we did not see any interaction between these HOPS subunits and Arl8bwt under the control condition (oleate overnight). Interestingly, 1 h after adding EPA to medium, we could see clear interaction between Arl8b and two of the mentioned HOPS subunits. Arl8bwt favorably interacted with Vps41 which was in agreement with previous report and to lesser extent with Vps39(Fig33). We did not see any interaction between Arl8bwt and Vps11 even after adding EPA to medium.

Together these data indicate that Arl8b is acting in lysosomal degradation of lipid droplets by recruiting HOPS protein complex and facilitating lysosome-lipid droplets interaction.

## *Results*

### **Fig 31. Effect of HOPS complex SiRNA on EPA induced lipid droplets degradation.**

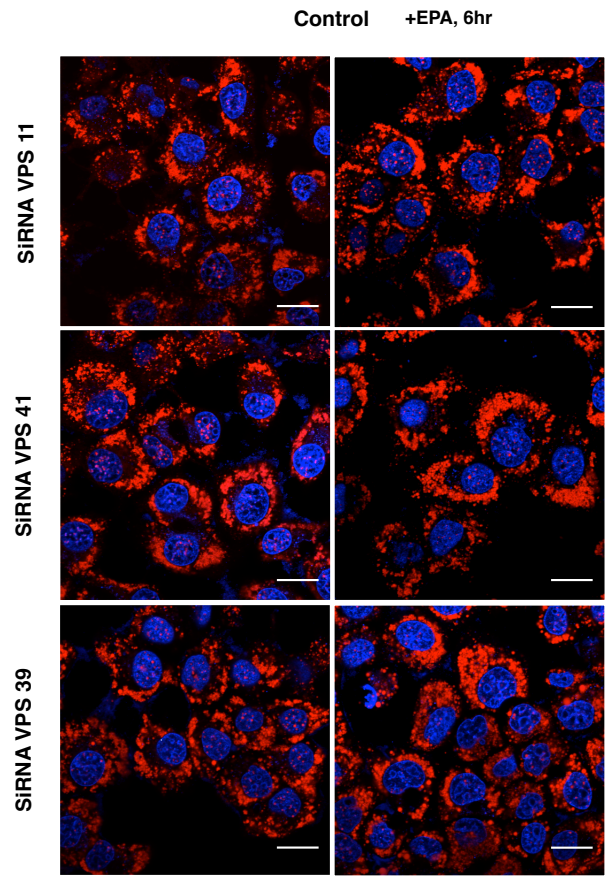
SiRNA of Vps11 , Vps39 and Vps41 is blocked degradation of intercellular lipid droplets after adding EPA to medium . Cells were transfected with SiRNA of HOPS complex or control .

cells were treated with oleate for overnight.

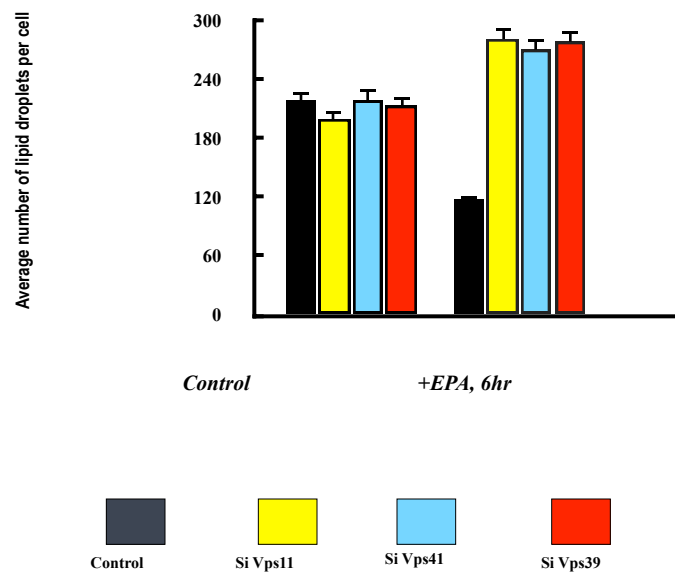
6 hr after adding EPA to medium , lipid droplets were stained by lipid tox red and quantified using image J lipid droplet counter plug in .a) effect of of HOPS SiRNA on intercellular lipid droplets accumulation after adding EPA. Scale bars, 22  $\mu$ m. b)Quantification of number of lipid droplets after adding EPA to medium under the HOPS subunits SiRNA.

Fig 31

A



B



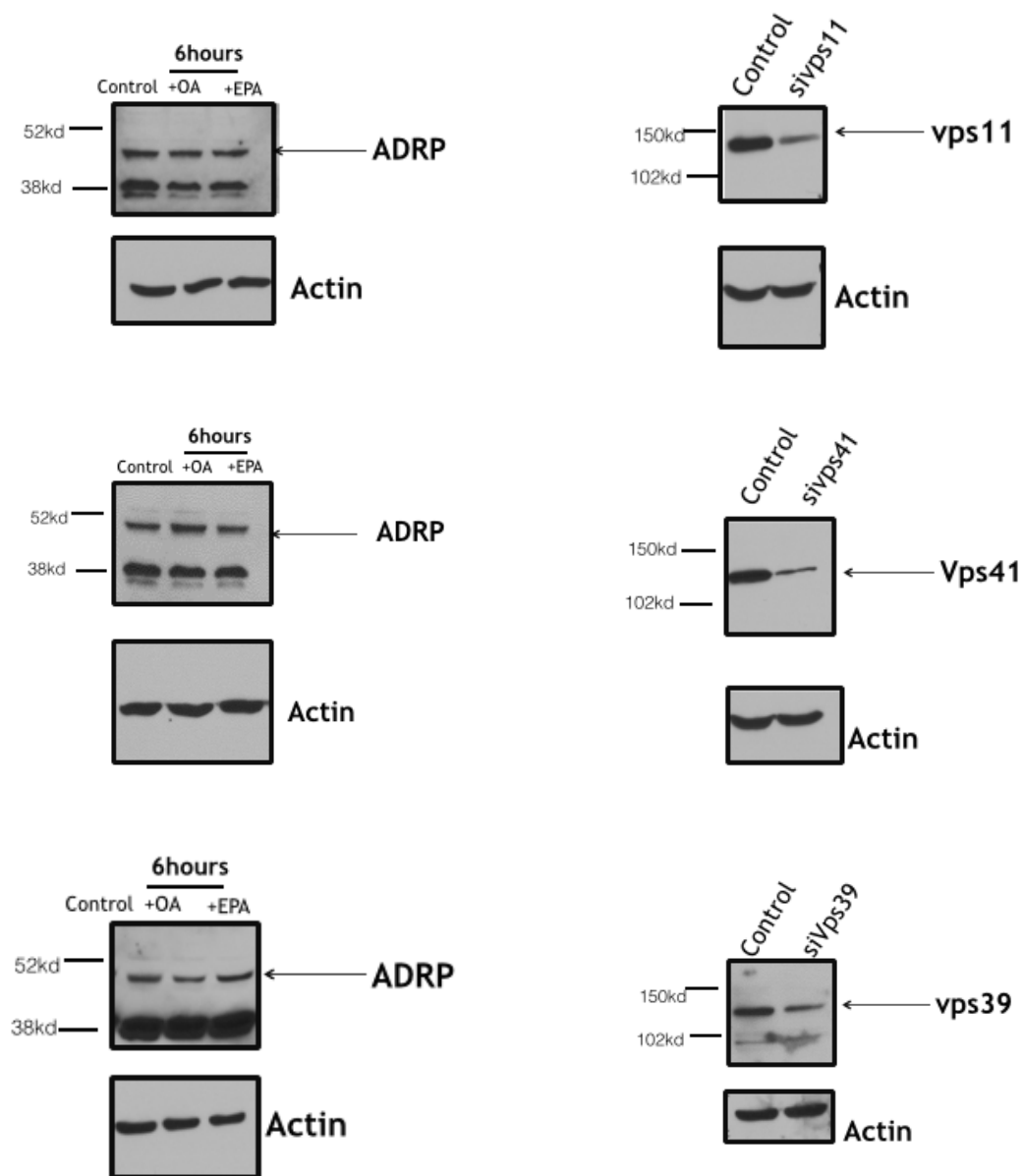
## *Results*

### **Fig 32. Western blot of ADRP protein under SiRNA of HOPS complex.**

Western blot of ADRP shows blockage of lipid droplets degradation under SiRNA of HOPS subunits 6 hr after adding EPA.

Western blot of Vps11, Vps41 and Vps39 proteins showing the efficiency of silencing. Control cells were transfected with scrambled siRNA.

Fig 32



## *Results*

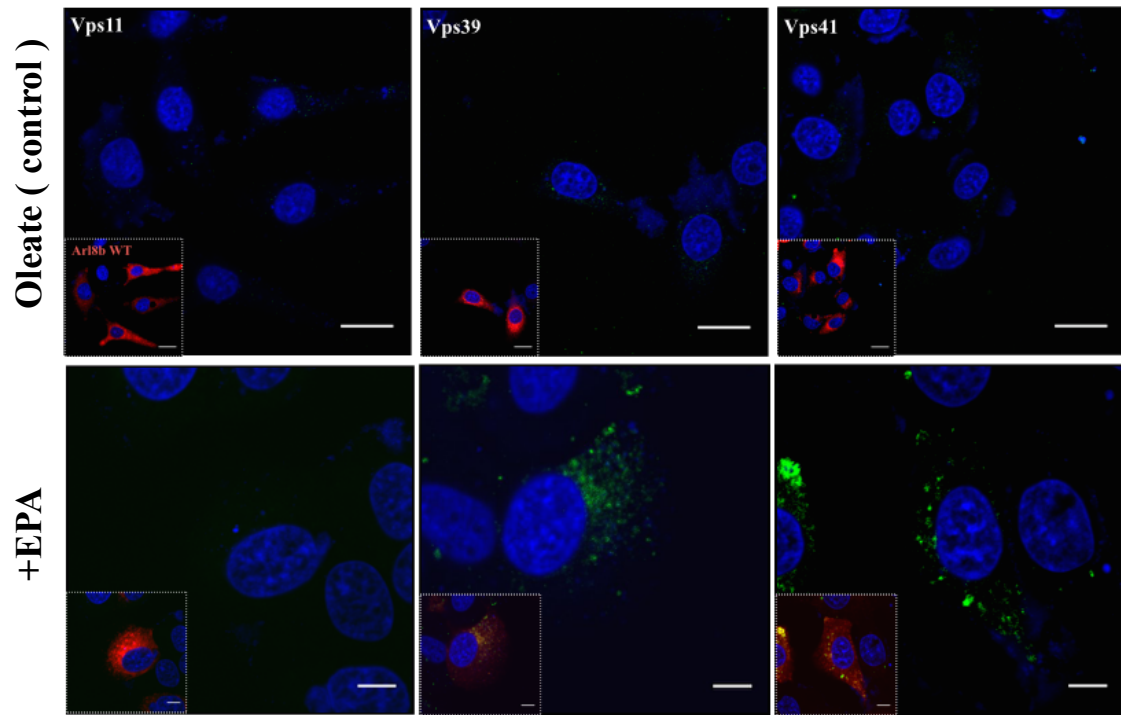
### **Fig 33. Arl8b is interacting with Vps39 and Vps41 after adding EPA to medium.**

In SiTu CO-IP of Arl8b wt -mcherry with HOPS complex subunits , Vps11, Vps39 and Vps41.

Cells were transfected with Arl8b WT mcherry before adding oleate. 1hr after adding EPA cells were fixed and prepared for In Situ CO-IP. For detection, Anti RFP and HOPS subunits were used as a primary antibodies.

Arl8b WT is interacting with Vps39 and Vps41 only after adding EPA to medium. control scale bar is 16 $\mu$ m. For EPA images , scale bar is 8 $\mu$ m.

**Fig 33**



## *Discussion*

It is well documented that supplementation of N-3 fatty acids ( fish oil ) can reduce the development of fatty liver and diabetes. Fish oil ( EPA and DHA ) supplementation widely used for reducing inflammation, liver related and cardiovascular disease. Unfortunately The major question until this study was remained regarding the detail mechanism of EPA action. Cytosolic lipid droplets are playing an important role in inter and extracellular lipid profile. Recent clinical studies regarding the effect of EPA on different subjects showed that fish oil supplementation ( n-3 fatty acid ) is one of the most effective way in preventing development of fatty liver .

In this study we revealed the detail mechanism of action delivered by n-3 fatty acid. Previously it has been reported that EPA supplementation decrease VLDL secretion by inhibition of TAG synthesis (4).

In support of previous report, we observed EPA supplementation impaired VLDL1 secretion However the phenotype was due to ( it was done by ) accelerating intercellular lipid droplet degradation and limiting availability of fatty acid for assembly of VLDL and TAG secretion. Thus the metabolic benefit of consuming fish oil ( n-3 fatty acid ) fundamentally is coming from its effect on intercellular TG and lipid droplets.

Recent finding has shown the involvement of autophagy in hepatic lipid metabolism. In cholesterol-laden macrophages, Atg-dependent autophagic degradation of cholesteryl esters by lysosomes dictates the rate of cholesterol efflux (120).

In contrast to other reports, we found that EPA induced lysosomal degradation of lipid droplets does not involve macroautophagy (regular autophagy machinery). During classical autophagy induced by starvation, lysosome is positioned in perinuclear region to fa-

## *Discussion*

cilitate fusion between lysosomes and upcoming autophagosomes toward MTOC (microtubule organization centre).

Position of lysosomes has been shown is sensitive to change in cytosolic pH.

We found that EPA treatment is engaging lysosomal peripheral distribution and their interaction with lipid droplets In pH dependent fashion. To our knowledge this is the first report which indicates that fatty acid can induce change in cytosolic pH.

We noticed the interaction between lysosome and lipid droplets is transient and is in kiss and Run fashion . This type of lysosomal movement has been reported for phagosome-endosomal interaction and was shown that depends on Rab5 GTPase function(69).

However, upon the interaction we could see engulfment of piece of lipid droplets inside the lysosomes which was followed by their minus end movement toward the prenuclear region. The phenotype and morphology of this interaction was resembling microautophagy.

During microautophagy cargo (vesicles) directly is engulfed by lysosomes(106).

Microautophagy is best characterized in the methylotrophic yeast *Pichia pastoris* . Changing from methanol to glucose environment induced selective microautophagic degradation of peroxisomes(122).

In recent study the degradation of lipid droplets by microautophagy in yeast has been reported Indicating the existence of this pathway for lipid metabolism and degradation in yeast however no such mechanism and pathway observed and reported in the mammalian system.

## *Discussion*

Using live cells imaging we identified that degradation of intercellular lipid droplets by EPA treatment requires lysosomal bidirectional movement .In the current study, We demonstrated that EPA induces intercellular lipid droplets degradation by the mechanism which was closely mimicking microautophagy.

An interesting finding regarding to our results is This study for the first time showed the mechanism of microautophagy is exist in the mammalian system and n-3 fatty acid is delivering its effect by taking advantage of this-until now- unknown pathway.

Interestingly the process of degradation of intercellular LDs by microautophagy type of mechanism was only responsive to EPA treatment and other known lipid droplets autophagy stimuli such as oleate and starvation condition did not promote microautophagy of lipid droplets.

How EPA treatment induces lysosomal redistribution toward the lipid droplets ?

Since treatment with EPA like other types of fatty acid induces the formation of intercellular LDs, We propose that the formation of new lipid droplets upon EPA treatment provides signalling hub for engaging lysosomal redistribution toward the lipid droplets.

Since movement of lysosome is closely regulated by activity of mTORC1 signalling pathway, this newly formed lipid droplets can provide a platform for cross talk between lysosomes and mTORC1 pathway.

In accord to this theory, it has been reported that lipid droplets can act as signalling platform (123).

## *Discussion*

Consistent with our idea, Triacsin C treatment before adding EPA did block movement of lysosomes toward lipid droplets and accumulation of lipid droplets was observed (unpublished data).

In this study, we find that Arl8b is required for lysosomal degradation of lipid droplets however Arl8b did not deliver this function by manipulating lysosomal motility.

In contrast to Arl8b model of action, under our experimental model Arl8b was constantly associated with lysosomes and its silencing did not have any effect on lysosomal movement toward and interaction with lipid droplets. However it is impaired the degradation of lipid droplets by lysosomes.

Importantly recent study identified HOPS protein complex as one of the Arl8b GTPase interaction partner. In this study, Arl8b facilitated cargo delivery to lysosomes throughout interaction with HOPS protein complex.

Knock down of HOPS protein complex totally blocked degradation of lipid droplets similar to what we observed with Arl8b SiRNA.

HOPS protein complex has been shown that involve in vacuolar fusion. It has been shown that it is associated with tethering machinery and is required for vesicle fusion.

We found that Arl8b is recruiting HOPS protein complex to lysosomal membrane and this process is EPA treatment dependent as no interaction was observed under the oleate (control) condition.

We propose recruitment of HOPS protein complex upon adding EPA to the medium is a prerequisite for fusion between lysosomes and lipid droplets. In this regard, this complex

## *Discussion*

is providing special domain on the membranes for assembly of SNAREs proteins and formation of SNAREpins.

In accord with this model, different reports and proteomics studies indicated the role of SNAREs proteins in lipid droplets biogenesis and fusion(124).

Ultimately, what would be the physiological and pathological importance of regarding this understanding?

More recently, it was shown that some of the pathogens such as Hepatitis C virus and Mycobacterium are targeting to intercellular lipid droplet during their cellular invasion and using it for their replication and nutritional purpose.

In addition, different studies indicated that many pathogens use autophagy for their survival and in order to replicate and hide from cellular defence mechanism. To reach this aim, they manipulate the host autophagy machinery for their benefit (125).

Given the role of -until now unknown mechanism - microautophagy like mechanism for degradation of intercellular lipid droplets, it would be interesting to see if any of those pathogens which dependent on intercellular LDs for their survival using/manipulating this new lipid degradation pathway.

Different GTPase protein has been shown that play a role in development of diverse diseases.

Our understanding regarding Arl8b GTPase action and its role in lysosomal lipid droplets degradation shed light on specific lipid mobilization pathway. So It will be important to examine in future the mechanism of these GTPase protein in an animal model.

## *Discussion*

GTPase knock down animal models previously have been generated and showed its usefulness for understanding metabolic diseases (125).

So it would be interesting to see the effect of EPA(n-3 fatty acid in general) on Arl8b specifically knock down an animal model and its effect on development of fatty liver in this model.

Understanding that will give us an advantage regarding development of novel drug targets and strategies in order to overcome some of the lipid related pathological diseases.

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