ELMOD2 is anchored to lipid droplets by palmitoylation and regulates adipocyte triglyceride lipase recruitment

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Abstract Adipocyte triglyceride lipase (ATGL) is the major enzyme involved in the hydrolysis of triglycerides. The Arf1-coat protein complex I (COPI) machinery is known to be engaged in the recruitment of ATGL to lipid droplets (LDs), but the regulatory mechanism has not been clarified. In the present study, we found that ELMOD2, a putative noncanonical Arf-GTPase activating protein (GAP) localizing in LDs, plays an important role in controlling ATGL transport to LDs. We showed that knockdown of ELMOD2 by RNA interference induced an increase in the amount of ATGL existing in LDs and decreased the total cellular triglycerides. These effects of ELMOD2 knockdown were canceled by transfection of small interfering RNA-resistant cDNA of wild-type ELMOD2 but not by that of mutated ELMOD2 lacking the Arf-GAP activity. ELMOD2 was distributed in the endoplasmic reticulum and mitochondria as well as in LDs, but palmitoylation was required only for distribution to LDs. An ELMOD2 mutant deficient in palmitoylation failed to reconstitute the ATGL transport after the ELMOD2 knockdown, indicating that distribution in LDs is indispensable to the functionality of ELMOD2. These results indicate that ELMOD2 regulates ATGL transport and cellular lipid metabolism by modulating the Arf1-COPI activity in LDs.

Introduction The lipid droplet (LD) is an organelle that exists in most cells and stores lipids mainly as triacylglycerols (TAGs) and sterol esters (Fujimoto and Parton, 2011; Walther and Farese, 2012). Recent studies have revealed that LDs are active organelles engaged in a wide range of functions, but many of these functions are related to the stored lipids, which are utilized for various cellular activities, including β-oxidation, membrane biogenesis, and synthesis of signaling molecules. Thus, to study lipid metabolism, and also to understand LD functions, it is important to clarify the molecular mechanism that regulates the lipid stores.

Hydrolysis of TAG is the first step of lipid utilization and is mediated by three enzymes: adipocyte triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoglyceride lipase, which work in this order to hydrolyze one TAG molecule to generate one glycerol and three free fatty acids (Zechner et al., 2012). Among the three enzymes, HSL has been studied most extensively: HSL exists as a soluble protein in the cytosol in the resting condition, but upon a β-adrenergic stimulus, HSL is phosphorylated by protein kinase A and recruited to LDs by binding to an LD-resident protein, perilipin1, which is also phosphorylated by protein kinase A. Because of this mechanism, fatty acid release after β-adrenergic stimulation is enhanced to 50–100 times that of the resting state (Londos et al., 1999).
In comparison with HSL, the molecular mechanism that recruits ATGL to LDs is less well understood. ATGL partially exists in the cytosol (Villena et al., 2004; Zimmermann et al., 2004), but most ATGL is likely to exist in a membrane-bound form (Sonii et al., 2009). Transport of ATGL to LDs has been thought to require vesicular trafficking, because knockdown of Arf1, coat protein complex I (COPI), or GBF1, an Arf–guanine nucleotide exchange factor, induced a decrease of ATGL in LDs (Beller et al., 2008; Sonii et al., 2009) and/or generation of fewer and larger LDs (Beller et al., 2008; Guo et al., 2008), but the detailed mechanism has not been clarified. The simplest model of this mechanism may be that COPI-coated vesicles originating in the Golgi and/or endoplasmic reticulum–Golgi intermediate compartment (ERGIC) carry ATGL to LDs. Alternatively, the COPI (and/or COPII) coat might create an environment in the ERGIC (and/or ER exit site) that facilitates lateral conveyance of ATGL to adjacent LDs (Sonii et al., 2009). In these proposed model hypotheses, the Arf1-COPI machinery would function in locations other than LDs. The existence of Arf1 and COPI in LDs (Bartz et al., 2007; Sonii et al., 2009), however, suggested another possibility: the Arf1-COPI system functions in LDs themselves.

In the present study, we examined the function of ELMOD2 with regard to the recruitment of ATGL to LDs. ELMOD2 is a member of the ELMO domain–containing protein family, and despite the lack of the signature sequence of the canonical Arf-GTPase-activating proteins (GAPs), the ELMO domain of ELMOD1 has Arf-GAP activity (East et al., 2012), and recombinant ELMOD2 exhibits in vitro GAP activity to Arf1 as well as to Arf2, Arf3, and Arf6 (Bowzard et al., 2007; Ivanova et al., 2014). We found that endogenous ELMOD2 has Arf-GAP activity and that knockdown of ELMOD2 leads to an increase of ATGL in LDs and a decrease of the cellular TAG content. We also found that localization of ELMOD2 in LDs depends on palmitoylation and that both the Arf-GAP activity and palmitoylation are critical for ELMOD2 functionality. The results indicated that ELMOD2 down-regulates the recruitment of ATGL to LDs by suppressing the Arf1-COPI activity in LDs. ELMOD2 is the first protein that has been shown to be targeted to LD by palmitoylation and could be a new drug target for controlling cellular lipid metabolism.

**RESULTS**

**Subcellular distribution of ELMOD2**

We identified ELMOD2 as an LD protein via proteomic analysis of an LD preparation obtained from human hepatocellular carcinoma cell line Huh7 cells (Suzuki et al., 2012). Another proteomic study using Caco-2/TC7 cells also identified ELMOD2 as an LD-associated protein (Bouchoux et al., 2011). To study ELMOD2, we first generated a polyclonal antibody against a synthetic peptide corresponding to amino acids 191–202 of the ELMOD2 protein. In Western blotting when ELMOD2 was knocked down with RNA interference (RNAi); see Figure 5A later in this article.

The anti-ELMOD2 antibody was used to examine subcellular fractions of Huh7 cells by Western blotting. Consistent with the result of the proteomic analysis, ELMOD2 was detected in the LD fraction obtained either by sucrose density–gradient ultracentrifugation or by differential ultracentrifugation (Figure 1B). ELMOD2 was also found in the microsome and mitochondrial fractions obtained by differential ultracentrifugation (Figure 1B).

In comparison with Huh7 cells, HeLa cells harbor a far smaller number of LDs under normal culture conditions. Thus, when HeLa cells were fractionated by OptiPrep density–gradient ultracentrifugation, ELMOD2 was found largely in the membrane fraction (Figure 1C). But after cells were cultured with 0.4 mM oleic acid (OA) for 12 h to increase LDs, ELMOD2 was observed in the LD fraction as well as in the membrane fraction (Figure 1C), indicating that ELMOD2 in HeLa cells also distributes in LDs.

Because the anti-ELMOD2 antibody did not work in immunofluorescence labeling, we observed the distribution of GFP-ELMOD2 and ELMOD2-V5. Consistent with the results of Western blotting for endogenous ELMOD2, both GFP-ELMOD2 and ELMOD2-V5 showed prominent accumulation around LDs (Figure 1D). A similar distribution was also reported for ELMOD2-hemagglutinin (HA; East et al., 2012).

In addition to their dense distribution around LDs, the tagged ELMOD2 proteins were also found to distribute in a reticular pattern. To identify the location of this non-LD distribution, we observed GFP-ELMOD2 correlatively with various organelle markers in HeLa cells. The results showed that the reticular distribution of GFP-ELMOD2 frequently overlaps with that of SERCA2 (ER) and Tom20 (mitochondria) (Figure 1E) but not with that of ERGIC53 (ER–Golgi intermediate compartment), GM130 (Golgi), EEA1 (early endosome), or tubulin (microtubule) (Supplementary Figure S1).

Preembedding immunoelectron microscopy using anti-GFP antibody confirmed that GFP-ELMOD2 distributed on the LD surface (Figure 1F). Consistent with the Western blotting and immunofluorescence microscopic results, the labeling for GFP-ELMOD2 was also observed in the ER and mitochondria (Figure 1F).

**ELMOD2 is released from LDs and membranes only by detergent treatment**

Proteins are targeted to LDs by several different mechanisms (Walther and Farese, 2012), but the amino acid sequence of ELMOD2 does not imply any known mechanism. To study how ELMOD2 is bound to LDs and membranes, we treated subcellular fractions with a nonionic detergent (1% Triton X-100), an alkali (0.1 M Na2CO3, pH 11), or a high-salt solution (1 M NaCl) and then examined them to determine whether ELMOD2 was released to a soluble fraction.

ELMOD2 in the LD fraction was scarcely solubilized when treated with either the alkali or the high-salt solution, but it was completely solubilized by Triton X-100 (Figure 2A). This behavior was similar to that of ATGL, whereas adipose differentiation-related protein (ADRP), which is bound to LDs via several hydrophobic segments (McManaman et al., 2003; Nakamura and Fujimoto, 2003; Targett-Adams et al., 2003), was solubilized to a certain degree by the alkali or high-salt treatment.

ELMOD2 in the membrane fraction showed a similar behavior to ELMOD2 in LDs, in that it was solubilized only with the Triton X-100 treatment. On the other hand, ATGL in the membrane fraction showed resistance to extraction with Triton X-100, but it was solubilized at least partially with alkali or high-salt treatments (Figure 2B; Sonii et al., 2009). Calnexin and GM130, a transmembrane protein in the ER and a peripheral membrane protein in the Golgi, respectively, were examined as controls to validate the rationale of the experiment; as expected, calnexin was solubilized only by Triton X-100, whereas GM130 was largely solubilized by either alkali or high salt (Figure 2B). These results suggested that ELMOD2 is bound to both LDs and membranes through hydrophobic
interactions. They also implied that the anchorage of ATGL to LDs and membranes may be mediated by different mechanisms.

**ELMOD2 is anchored to LDs with palmitoylation**

The above results were intriguing, considering that ELMOD2 lacks hydrophobic sequences that are likely to mediate direct membrane anchorage (Bowzard et al., 2007). Actually, ELMOD2 mutants lacking either the C-terminal ELMO domain (aa 1–125) or the N-terminal non-ELMO domain region (aa 126–293) or a mutant made of the central portion alone (aa 70–200) were not distributed in LDs (Supplemental Figure S2). Although these results may have been caused by a gross change in protein conformation, they were consistent with the supposition that no particular hydrophobic portion anchors ELMOD2 to LDs.

**FIGURE 1:** Subcellular distribution of ELMOD2. (A) Validation of the rabbit anti-ELMOD2 antibody by Western blotting. The antibody recognized endogenous ELMOD2 in Huh7 cells. The positive reaction was observed at the mobility expected from the molecular mass of 35 kDa (arrowhead). ELMOD2-V5 and GFP-ELMOD2 expressed by cDNA transfection were also recognized by the anti-ELMOD2 antibody as extra bands corresponding to the size of the tagged protein, which was confirmed using anti-V5 and anti-GFP antibodies, respectively. A nonspecific reaction is shown by an asterisk (*). (B) Endogenous ELMOD2 in Huh7 cells was enriched in the LD fraction. Subcellular fractions of Huh7 cells were obtained either by sucrose density–gradient ultracentrifugation or by differential centrifugation. With either method, LDs were concentrated in the fraction of the lowest density, which was confirmed by the enrichment of ADRP (perilipin2). Note that ELMOD2 was also detected in the microsomal and mitochondrial fractions obtained by differential ultracentrifugation. (C) Endogenous ELMOD2 in HeLa cells was also detected in the LD fraction. Fractions enriched with LDs (L), membranes (M), and the cytosol (C) were obtained by OptiPrep density–gradient centrifugation and subjected to Western blotting with anti-ELMOD2 antibody. HeLa cells harbor few LDs in the normal culture condition (−OA), and the reaction in the LD fraction was apparent only when cells were cultured with 0.4 mM OA for 12 h (+OA). The cytosol fraction was overloaded to show a paucity of soluble ELMOD2. (D) ELMOD2, either conjugated with GFP at the N-terminus (a, green) or tagged with V5/His at the C-terminus (b, red), showed concentration around LDs in Huh7 cells. LDs were stained with BODIPY558/568-C12 (a, red) or BODIPY493/503 (b, green). (E) GFP-ELMOD2 (green) showed colocalization with SERCA2 and Tom20 (red), indicating distribution in the ER and mitochondria, respectively. HeLa cells were cultured with 0.4 mM OA for 3 h to induce LD formation. (F) Preembedding immunoelectron microscopy of GFP-ELMOD2 in HeLa cells cultured with 0.4 mM OA for 3 h. The results confirmed the presence of GFP-ELMOD2 in LDs (red arrowhead), the ER (yellow arrowheads), and mitochondria (green arrowheads).
We hypothesized that anchorage of ELMOD2 to LDs and/or membranes may occur through lipid modification. As ELMOD2 lacks typical consensus sequences for N-terminal myristoylation and C-terminal prenylation, we examined whether ELMOD2 is palmitoylated. HEK293T cells were cotransfected with cDNAs of GFP-ELMOD2 and DHHC3, a palmitoyltransferase, and incorporation of palmitate to GFP-ELMOD2 was examined by metabolic labeling with [3H]palmitate (Fukata et al., 2004). GFP-ELMOD2 was found to incorporate [3H]palmitate, although the signal intensity was less than that of a representative DHHC3 substrate, PSD95 (Figure 3A). The result indicated that ELMOD2 is palmitoylated in cells.

Palmitoylation of ELMOD2 was further confirmed by replacing all five cysteines with alanine (GFP-ELMOD2 [−5Cys]): that is, the incorporation of [3H]palmitate was significantly reduced in GFP-ELMOD2 [−5Cys] in comparison with that in GFP-ELMOD (wild-type [WT]) (Figure 3B). Replacement of two cysteine residues at the 94th and 98th positions with alanine (GFP-ELMOD2 [−2Cys]) decreased the labeling to some extent but not to the basal level, suggesting that ELMOD2 may be palmitoylated in other cysteine residues as well (Figure 3B).

To examine whether ELMOD2 is palmitoylated without overexpression of DHHC3, we analyzed Huh7 cells expressing GFP-ELMOD2 (WT) and GFP-ELMOD2 [−5Cys], using the acyl-biotinyl exchange (ABE) method (Nottake et al., 2009). The results clearly indicated that GFP-ELMOD2 (WT) but not GFP-ELMOD2 [−5Cys] was palmitoylated in Huh7 cells (Figure 3C).

To examine whether the targeting of ELMOD2 to LDs is dependent on palmitoylation, we observed distribution of GFP-ELMOD2 with or without replacement of cysteine residues. In this experiment, to exclude the possibility that endogenous ELMOD2 makes complexes with the GFP-tagged ELMOD2 mutants, we took advantage of the molecular replacement approach. HeLa cells were first treated with ELMOD2 small interfering RNA (siRNA) and then transfected with siRNA-resistant ELMOD2 cDNAs. Replacement of one or two of the five cysteine residues did not cause an obvious change in distribution of GFP-ELMOD2 (Supplemental Figure S3). In contrast, accumulation around LDs was significantly reduced for GFP-ELMOD2 [−5Cys] (Figure 4A). It was notable, however, that GFP-ELMOD2 [−5Cys] was still distributed in the ER and mitochondria (Figure 4B). These results indicated that palmitoylation is specifically required for targeting to LDs. Consistently, treatment with 2-bromopalmitate, which inhibits protein palmitoylation, abolished distribution of GFP-ELMOD2 in LDs, whereas it did not influence the distribution in the ER and mitochondria (Supplemental Figure S4).

Distribution of GFP-ELMOD2 [−5Cys] was also examined by Western blotting of subcellular fractions. In comparison with GFP-ELMOD2 (WT), a much smaller proportion of GFP-ELMOD2 [−5Cys] was partitioned to the LD fraction (Figure 4C). In contrast, the proportion of GFP-ELMOD2 [−5Cys] in the membrane and cytosolic fractions was equivalent and larger than that of GFP-ELMOD2 (WT), respectively (Figure 4C). These results confirmed the microscopic observations, in that ELMOD2 in LDs was decreased significantly by the lack of palmitoylation, whereas ELMOD2 in the ER and mitochondria was not.

**Knockdown of ELMOD2 increases ATGL in LDs and reduces TAG**

Previous studies showed that down-regulation of the Arf1-COPI machinery decreases ATGL in LDs and/or promotes LD enlargement (Beller et al., 2008; Guo et al., 2008; Soni et al., 2009). Considering that ELMOD2 was shown to have Arf1-GAP activity in vitro (Bowzard et al., 2007; Ivanova et al., 2014), we hypothesized that ELMOD2 is related to the regulation of Arf1-COPI machinery in LDs. We thus examined whether endogenous ELMOD2 has Arf1-GAP activity using a pull down with GST-GGA3, which binds to the GTP form but not to the GDP form of Arf1 (Sany and Casanova, 2001). The amount of Arf1-HA that was precipitated with GST-GGA3 was significantly greater in cells treated with ELMOD2 siRNA than in cells treated with control siRNA (Figure 5A), indicating that endogenous ELMOD2 in HeLa cells may function as GAP to Arf1.

If ELMOD2 exerts Arf1-GAP activity, a decrease of ELMOD2 should activate Arf1 and increase ATGL in LDs, thereby leading to a decrease of TAG. To test this hypothesis, we examined the effect of ELMOD2 knockdown on the amount of ATGL in LDs and TAG in the total cell in HeLa cells. Through the knockdown of ELMOD2, the protein expression level of ATGL was not changed, but the relative proportion of ATGL in the LD fraction was increased (Figure 5B). The increase of ATGL in LDs after ELMOD2 depletion was also observed by immunofluorescence labeling. In this experiment, LD-resident TIP47 (perilipin3) was taken as a reference, and the labeling intensity of ATGL relative to that of TIP47 was measured. The relative labeling...
For verification of the specificity of the above experiment, siRNA-resistant ELMOD2 cDNA was transfected to determine whether the effect of ELMOD2 knockdown on the ATGL distribution can be canceled. We discovered that the increase of ATGL in LDs caused by ELMOD2 RNAi was suppressed significantly in cells expressing GFP-ELMOD2, thus excluding the possibility of off-target effects of siRNA (Figure 6A).

The experiment was extended to test whether the GAP activity of ELMOD2 is necessary for functionality. Expression of a mutant ELMOD2 that lacks the GAP activity (R167K; East et al., 2012; Ivanova et al., 2014) failed to decrease the amount of ATGL in LDs (Figure 6A), indicating the Arf-GAP activity is essential. Likewise, expression of the palmitoylation-deficient GFP-ELMOD2 (∼5Cys) was also unable to decrease the amount of ATGL in LDs (Figure 6B). These results showed that both the Arf-GAP activity and distribution to LDs by palmitoylation are critical for ELMOD2 to recruit ATGL to LDs.

**DISCUSSION**

**ELMOD2 and the Arf1-COPI machinery in LDs**

In the present study, ELMOD2, a putative Arf-GAP protein, was found to be present in LDs. In conjunction with the presence of Arf-GEF (GBF1) in LDs (Soni et al., 2009), the results showed that LDs harbor both positive and negative regulators of Arf1, indicating that the Arf1-COPI machinery may be regulated locally in LDs.

Recent studies showed that Arf1 and COPI induce budding of small-sized LDs (nanodroplets) from artificial LDs (Thiam et al., 2013) and isolated natural LDs (Wilfling et al., 2014). The budding from LDs should entail the reduction of phospholipid packing and the increase of the surface tension in the LD surface; this increased surface tension is thought to induce membrane bridges between LDs and the ER membrane through which membrane-bound ER proteins like ATGL may laterally diffuse to LDs (Wilfling et al., 2014). The present results were consistent with this hypothesis: downregulation of ELMOD2 probably increased the nanodroplet budding by activating the Arf1-COPI machinery in LDs, thus increasing the ATGL recruitment (Figure 7).

In relation to the transport of ATGL from the ER to LDs, it was notable that ATGL in LDs and ATGL in membranes exhibited different behaviors to the alkali and high-salt treatments (Figure 2). This difference may be correlated with two mechanisms of ATGL anchorage, that is, one by hydrophobic binding via a C-terminal domain (Lu et al., 2010; Murugesan et al., 2013) and the other by a protein–protein interaction with an LD protein G0/G1 switch gene-2 (G0S2; Lu et al., 2010; Cornaciu et al., 2011). The interaction with G0S2 that intensity of ATGL to TIP47 increased significantly in cells transfected with ELMOD2 siRNA but not in cells transfected with control siRNA (Figure 5C).

Consistent with the increase of ATGL in LDs, knockdown of ELMOD2 caused a significant decrease of the cellular TAG content (Figure 5D). Both the number and size of LDs were also decreased significantly by the treatment (Figure 5E). These results suggested that ATGL recruited to LDs as a result of ELMOD2 knockdown was functional and that a physiological function of ELMOD2 in the resting condition is to regulate the amount of ATGL in LDs and to maintain TAG storage.
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occur only in LDs is likely to change the conformation of ATGL so that the C-terminal domain binds more tightly to LDs than to membranes. This may help transport ATGL from membranes to LDs unidirectionally and more efficiently than other proteins.

**ELMOD2 is anchored to LDs by palmitoylation**

The present study showed that palmitoylation is necessary for ELMOD2 to localize in LDs. Palmitoylation is known to play active roles in the sorting and trafficking of many proteins (Linder and Deschenes, 2007; Fukata and Fukata, 2010), but, to our knowledge, this is the first example of protein distribution in LDs that hinges on palmitoylation. Although the mechanism by which palmitoylation dictates LD localization of ELMOD2 is not clear, palmitoylation may induce partitioning of ELMOD2 into a raft-like microdomain in the ER membrane (Levental et al., 2010), which may then lead to distribution to LDs as observed for caveolins (Fujimoto et al., 2001). Alternatively, palmitoylation may cause a change in the molecular orientation of ELMOD2 on the membrane (Hayashi et al., 2005), which may induce a new set of molecular interactions to favor LD distribution.

Detailed mechanisms aside, it is important that palmitoylation and depalmitoylation are reversible and regulatable processes that may occur quickly. In this respect, palmitoylation differs from hydrophobic peptide sequences that are likely to conduct targeting of many LD proteins constitutively. Thus it is possible that palmitoylation/depalmitoylation cycles regulate distribution of ELMOD2 dynamically according to the cellular condition. Most of the ~23 S-palmitoyl transferases in mammals are thought to exist in the ER and/or the Golgi (Ohno et al., 2006). Considering the critical role of palmitoylation in dictating the distribution of ELMOD2, it would be important to identify an enzyme(s) engaged in palmitoylation of ELMOD2 and to examine whether and how the enzymatic activity is regulated with regard to lipid metabolism.
It is not likely, however, that palmitoylation/depalmitoylation is the only mechanism that regulates the function of ELMOD2. In fact, it was reported that the GAP activity of recombinant ELMOD2 was inhibited by binding to sigma-1 receptor (Ivanova et al., 2014). Sigma-1 receptor, a transmembrane protein in the mitochondria-associated membrane (Hayashi and Su, 2007), may not bind directly to ELMOD2 in LDs, but other binding partners, including lipids, might modulate the LD-resident ELMOD2 function.

ELMOD2 and human idiopathic pulmonary fibrosis
ELMOD2 is a candidate gene for susceptibility to idiopathic pulmonary fibrosis (Hodgson et al., 2006), and herpesvirus saimiri infection was shown to be linked with the etiology of the disease (Folcik et al., 2014), but the manner in which ELMOD2 is related to the disease is not known (East et al., 2012). The present results indicated that down-regulation of ELMOD2 causes a decrease of LDs. This finding led us to note that association with LDs is functionally important for viperin, an antiviral protein that is induced by interferon and inhibits a broad range of viruses (Seo et al., 2011). We speculate that reduction of ELMOD2 may compromise viperin’s antiviral activity by reducing LDs. Although this does not explain why the disease occurs specifically in the lung,
we hope that further studies on ELMOD2 may help clarify the pathogenesis of this fatal human disease.

MATERIALS AND METHODS

Cell culture
Huh7 and HeLa cells were obtained from the Japanese Collection of Research Resources Cell Bank and cultured in DMEM and the original Eagle’s minimum essential medium, respectively, supplemented with 10% fetal calf serum and antibiotics at 37°C in a humidified atmosphere containing 5% CO2. OA (Sigma-Aldrich, St. Louis, MO) was treated with 0.4 mM OA for 3 h and doubly labeled for ATGL (green) and TIP47 (red). In comparison with cells expressing GFP alone, the expression of GFP-ELMOD2 (WT) significantly decreased the relative labeling intensity of ATGL to TIP47 in LDs, whereas the expression of GFP-ELMOD2 (R167K) did not (mean ± SD; Student’s t test; *p < 0.05). The results indicated that the Arf-GAP activity is indispensable for the effect of ELMOD2 on ATGL. The experiment was repeated three times, and 50 LDs chosen randomly from five micrographs were examined. (B) The same experiment as in A was performed to examine the effect of siRNA-resistant GFP-ELMOD2 (∼5Cys). The expression of GFP-ELMOD2 (∼5Cys) did not affect the relative labeling intensity of ATGL to TIP47, indicating that palmitoylation is critical for the functionality of ELMOD2 (mean ± SD; Student’s t test; *p < 0.05). The experiment was repeated three times, and 100 LDs chosen randomly from 10 micrographs were examined.

Antibodies
Rabbit anti-ELMOD2 antibody was raised against a peptide (ILSRSNHPKLGY) corresponding to amino acids 191–202 of the human ELMOD2 protein and affinity purified with the antigen peptide. Rabbit anti-TIP47 was obtained as previously described (Ohsaki et al., 2006). Rabbit anti-ERGIC53 and rabbit anti-GM130 antibodies were kindly provided by Nobuhiro Nakamura (Kyoto Sangyo University). Mouse anti-ADRP, guinea pig anti-TIP47 (Progen, Heidelberg, Germany), rabbit anti-ATGL (Cell Signaling Technology, Danvers, MA), rabbit anti-actin, mouse anti-α-tubulin (Sigma-Aldrich), mouse anti-calnexin (BD Biosciences, San Jose, CA), mouse anti-EEA1 (BD Biosciences), rabbit anti-GFP (Frontier Institute, Sapporo, Japan), mouse anti-HA (Nacalai Tesque, Kyoto, Japan), mouse anti-SERCA2 (Affinity Bioreagents, Rockford, IL), rabbit anti-Tom20 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-PD-95 (Thermo Scientific, Waltham, MA), mouse anti-V5 (Invitrogen, Waltham, MA), and fluoronanogold-conjugated goat anti-rabbit immunoglobulin G (IgG; Nanoprobes, Yaphank, NY) antibodies were obtained from the respective suppliers.

Transfection
A plasmid to transduce HA-tagged Arf1 was kindly provided by Kazuhisa Nakayama (Kyoto University). Plasmids encoding GFP-ELMOD2 and ELMOD2-V5 cDNAs were prepared via a conventional method. Control and ELMOD2 siRNA were obtained from Dharmacon. Plasmids and siRNA were transfected using Lipofectamine 2000, Lipofectamine RNAiMAX (Invitrogen), or X-TREMEGENE HP (Roche, Basel, Switzerland) according to the manufacturers’ instructions.

Subcellular fractionation
Subcellular fractions of Huh7 cells cultured with OA for 12 h were obtained by two different methods. In the method using a sucrose density–gradient ultracentrifugation (Figure 1B), the cells were disrupted by nitrogen cavitation, and the postnuclear supernatant adjusted to 0.27 M sucrose was overlaid with 0.135 M sucrose in a discontinuous sucrose density–gradient ultracentrifugation medium. 8 Fractions were obtained from the top of the sucrose gradient. In the method using differential centrifugation (Figures 1B and 4C), the cells were disrupted by homogenization buffer (30 mM Tris-HCl, pH 7.4, 225 mM mannitol, 75 mM sucrose, 0.1 mM ethylene glycoltetraacetic acid, and protease inhibitor) by being passed through a 26-G needle repeatedly. The mitochondrial fraction was obtained as a pellet by centrifuging the postnuclear supernatant for 10 min at 8000 × g. The supernatant of the centrifugation was overlaid with the homogenization buffer, omitting sucrose, and centrifuged at 166,000 × g for 5 h in a TLS-55 rotor (Beckman). The LD fraction (floating on the top surface), the cytosol fraction (collected using a Centritube Slicer [Beckman]), and the microsome fraction (pellet) were obtained. For analysis of the behavior of ELMOD2 in response to various treatments (Figure 2), LDs and membranes were collected from the top-floating layer and the pellet of the sucrose density–gradient centrifugation. They were treated for 1 h at 4°C with buffer alone, 1% Triton X-100 in buffer, 1 M NaCl in buffer, or 0.1 M Na2CO3 (pH 11). After incubation, LDs were separated from solubilized proteins using the same sucrose-density centrifugation described above, whereas membranes were separated from solubilized proteins by centrifugation at 166,000 × g for 1 h.

FIGURE 6: ELMOD2 functionality and ATGL transport. (A) The effect of ELMOD2 expression on the amount of ATGL in LDs. After knockdown of endogenous ELMOD2 with RNAi, GFP cDNA or siRNA-resistant cDNA of either GFP-ELMOD2 (WT) or GFP-ELMOD2 (R167K) was transfected to HeLa cells. The cells were treated with 0.4 mM OA for 3 h and doubly labeled for ATGL (green) and TIP47 (red). In comparison with cells expressing GFP alone, the expression of GFP-ELMOD2 (WT) significantly decreased the relative labeling intensity of ATGL to TIP47 in LDs, whereas the expression of GFP-ELMOD2 (R167K) did not (mean ± SD; Student’s t test; *p < 0.05). The results indicated that the Arf-GAP activity is indispensable for the effect of ELMOD2 on ATGL. The experiment was repeated three times, and 50 LDs chosen randomly from five micrographs were examined. (B) The same experiment as in A was performed to examine the effect of siRNA-resistant GFP-ELMOD2 (∼5Cys). The expression of GFP-ELMOD2 (∼5Cys) did not affect the relative labeling intensity of ATGL to TIP47, indicating that palmitoylation is critical for the functionality of ELMOD2 (mean ± SD; Student’s t test; *p < 0.05). The experiment was repeated three times, and 100 LDs chosen randomly from 10 micrographs were examined.
inactivating Arf1 in LDs. Arf-GAP activity of ELMOD2 likely down-regulates these processes by membrane bridges through which ATGL is recruited to LDs. The tension of the LD surface. This likely induces the formation of ER–LD to facilitate budding of nanodroplets, thereby increasing the surface on ATGL transport. The active Arf1-COPI complex in LDs is thought

**FIGURE 7:** Schematic diagram that illustrates the ELMOD2 function on ATGL transport. The active Arf1-COPI complex in LDs is thought to facilitate budding of nanodroplets, thereby increasing the surface tension of the LD surface. This likely induces the formation of ER–LD membrane bridges through which ATGL is recruited to LDs. The Arf-GAP activity of ELMOD2 likely down-regulates these processes by inactivating Arf1 in LDs.

For subcellular fractionation of HeLa cells (Figure 1C), the cells were disrupted in homogenization buffer by being passed through a 26-G needle. The postnuclear supernatant, adjusted to 30% OptiPrep (Axys Shield, Dundee, Scotland), was overlaid with 20% OptiPrep and then 10% OptiPrep in a homogenization buffer and the buffer alone and subsequently centrifuged at 166,000 × g for 5 h at 4°C in a TLS-55 rotor (Beckman). Fractions enriched with LDs (floating on the top surface), membranes (collected at the 10%/20% OptiPrep interface), and the cytosol (the 30% OptiPrep layer) were collected using a Centritube Slicer.

**Pull-down analysis**

Huh7 cells transfected with control or ELMOD2 siRNA were transduced with Arf1-HA cDNA. The cells were lysed in a pull-down buffer (25 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM MgCl_{2}, 1% NP-40, 5% glycerol, and protease inhibitor), and the cleared lysate was reacted with GST-GGA3 coupled with glutathione-Sepharose 4B (GE Healthcare, Little Chalfont, UK) for 1 h at 4°C. After washing, the bound proteins were released by boiling in an SDS sample buffer.

**Western blotting**

Each sample was boiled with SDS sample buffer (50 mM Tris- HCl, pH 6.8, 2% SDS, 10% glycerol). SDS–PAGE and Western transfer were performed using a Mini-PROTEAN and a Trans- BLOT apparatus (Bio-Rad, Hercules, CA), respectively. The Western blot signal was detected by chemiluminescence and captured either by Hyperfilm (GE Healthcare) or by a Light- Capture II imager (ATTO, Tokyo, Japan). The band intensity was measured using CS Analyzer 3 software (ATTO).

**Metabolic labeling with palmitate**

The labeling with [3H]palmitic acid was performed as described previously (Fukata et al., 2013). Briefly, HEK293T cells were transfected with HA-DHHC3 cDNA together with cDNA of either PSD-95-GFP or GFP-ELMOD2. The cells were labeled with 0.25 mCi/ml [3H]palmitic acid for 4 h, and the cell lysate was resolved by SDS–PAGE followed by fluorography and Western blotting.

**ABE method**

The ABE method (Noritake et al., 2009) was modified as follows. Transfected Huh7 cells were solubilized with buffer containing 4% SDS, diluted with the Triton X-100–containing buffer (final concentration: 1% SDS and 0.15% Triton X-100), and reduced with 10 mM Tris-(2-carboxyethyl) phosphine (TCEP) for 30 min. Free cysteine residues were then blocked with 40 mM N-ethylmaleimide (NEM). Excess TCEP and NEM were removed with a chloroform/methanol precipitation (CM ppt). Protein pellets were resuspended with buffer containing 4% SDS and incubated either in buffer H (1 M hydroxyamine, pH 7.0, 1 mM biotin–HPDP [N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide]) to cleave thioester bonds and to biotinylate newly exposed cysteines or in buffer T (1 M Tris-HCl, pH 7.0, 1 mM biotin–HPDP) as a negative control for 1 h. Excess hydroxyamine and biotin–HPDP were removed with a CM ppt. Protein pellets were resuspended with buffer containing 2% SDS and diluted with the Triton X-100-containing buffer. The resultant samples were incubated with 30 μl of NeutrAvidin-agarose (Pierce, Rockford, IL) for 1 h at 4°C. After the beads were washed, bound proteins were suspended in SDS–PAGE sample buffer and boiled at 100°C for 5 min. Samples were subjected to SDS–PAGE and Western blotting with GFP antibody.

**Immunofluorescence microscopy**

Cells were fixed with 3% formaldehyde with or without 0.015% glutaraldehyde in 0.1 M phosphate buffer for 15 min and permeabilized with either 0.01% digitonin in phosphate-buffered saline (PBS) for 30 min or with 0.1% Triton X-100 in PBS for 5 min before blocking and incubation with antibodies. LDs were stained with either BODIPY493/503 or BODIPY 558/568-HPDP as a negative control for 1 h. Excess hydroxyamine and biotin–HPDP were removed with a CM ppt. Protein pellets were resuspended with buffer containing 2% SDS and diluted with the Triton X-100-containing buffer. The resultant samples were incubated with 30 μl of NeutrAvidin-agarose (Pierce, Rockford, IL) for 1 h at 4°C. After the beads were washed, bound proteins were suspended in SDS–PAGE sample buffer and boiled at 100°C for 5 min. Samples were subjected to SDS–PAGE and Western blotting with GFP antibody.

**Immunoelectron microscopy**

HeLa cells transfected with GFP-ELMOD2 cDNA were fixed with 3% formaldehyde and 0.05% glutaraldehyde in 0.1 M HEPES for 30 min, permeabilized with 0.2% saponin for 30 min, and labeled with rabbit anti-GFP antibody followed by Fluorolink gold-conjugated goat anti-rabbit IgG antibody. The specimen was treated with
GoldEnhance (Nanoprobes) to precipitate metallic gold and then postsmacicated, dehydrated, and embedded for electron microcopy. Cells transfected with GFP cDNA were treated in the same manner as a control. Ultrathin sections were observed in a JEOL JEM1011 electron microscope.

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