

RESEARCH ARTICLE

# Interactomic study on interaction between lipid droplets and mitochondria

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## ABSTRACT

**An increasing body of evidence shows that the lipid droplet, a neutral lipid storage organelle, plays a role in lipid metabolism and energy homeostasis through its interaction with mitochondria. However, the cellular functions and molecular mechanisms of the interaction remain ambiguous. Here we present data from transmission electron microscopy, fluorescence imaging, and reconstitution assays, demonstrating that lipid droplets physically contact mitochondria *in vivo* and *in vitro*. Using a bimolecular fluorescence complementation assay in *Saccharomyces cerevisiae*, we generated an interactomic map of protein-protein contacts of lipid droplets with mitochondria and peroxisomes. The lipid droplet proteins Erg6 and Pet10 were found to be involved in 75% of the interactions detected. Interestingly, interactions between 3 pairs of lipid metabolic enzymes were detected. Collectively, these data demonstrate that lipid droplets make physical contacts with mitochondria and peroxisomes, and reveal specific molecular interactions that suggest active participation of lipid droplets in lipid metabolism in yeast.**

**KEYWORDS** peroxisomes, bimolecular fluorescence complementation assay, protein-protein interaction, lipid metabolism, Erg6

## INTRODUCTION

Lipid droplets are spherical organelles existing in many cell types and consisting of a core of neutral lipids and a coating of

a monolayer of phospholipids with peripheral as well as embedded proteins (Murphy, 2001; Tauchi-Sato et al., 2002). It was considered an inert particle for energy storage until recent years when increasing evidence showed that lipid droplets are highly dynamic and are able to interact with other organelles (Martin and Parton, 2006; Goodman, 2008; Murphy et al., 2009; Zhang et al., 2010). In particular, lipid droplets were found to physically contact the ER, mitochondria, endosomes and peroxisomes, although the physiological function of these contacts remains unknown. Studies of lipid droplet dynamics will pave the way for a better understanding of the cellular network for energy and mass transfer (particularly lipids) as well as the pathology of metabolic diseases.

Lipid droplets are frequently seen attached to mitochondria in adipocytes (Novikoff et al., 1980; Blanchette-Mackie and Scow, 1983) and hepatocytes (Kalashnikova and Fadeeva, 2006). In skeletal muscle cells, association of lipid droplets with mitochondria has also been observed (Shaw et al., 2008) and found to become more abundant when energy requirements increase due to physical exercise (Tarnopolsky et al., 2007). Lipases such as hormone-sensitive lipase (HSL) (Egan et al., 1992) and adipose triglyceride lipase (ATGL) (Zimmermann et al., 2004) have been found in lipid droplets, demonstrating a localized capacity for mobilizing energy stores. The product of lipase activity, free fatty acids, is the substrate for  $\beta$ -oxidation in the mitochondrion and/or peroxisome, suggesting that an interaction between these organelles is related to energy utilization. Besides energy transfer, lipid trafficking may be another purpose of an interaction between lipid droplets and mitochondria. In yeast,  $\beta$ -oxidation occurs in peroxisomes, but physical contacts between lipid

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droplets and mitochondria were still seen, and many mitochondrial proteins were found to be localized in lipid droplets (Binns et al., 2006). These findings indicate that the interaction between lipid droplets and mitochondria may be involved in multiple physiological events and may be well regulated. Additionally, it has been reported that lipid droplets can move in a microtubule- and dynein-dependent manner (Guo et al., 2005). The SNARE protein synaptosome associated protein 23 (SNAP23) was reported to mediate the interaction between lipid droplets and mitochondria (Jägerström et al., 2009), although the molecular mechanism remains unknown.

In the present study, we focused on protein-protein interactions to search for mediators of the association between lipid droplets and mitochondria by bimolecular fluorescence complementation (BiFC) assay in *Saccharomyces cerevisiae*. BiFC enables direct visualization of protein-protein interactions in living cells, based on the formation of a fluorescent complex of two non-fluorescent fragments of a fluorescent protein. Interaction between a bait and a prey protein, each fused to one of these fragments, brings the fragments into close contact, resulting in a fluorescent signal. BiFC is an effective and straightforward tool to study protein-protein interactions, avoiding the possibility of non-physiological protein interactions caused by cell lysis and mixing the contents of different cellular compartments. Many protein-protein interactions have been detected successfully in different cell types and organisms using BiFC assays (Hu and Kerppola, 2003; Brasaemle et al., 2004; Blondel et al., 2005). Furthermore, in yeast, proteins being examined by BiFC are expressed under the control of their native promoters (Sung and Huh, 2007). Here, we present data demonstrating the existence of contact between lipid droplets and mitochondria in skeletal muscle tissue and cells by morphology studies and *in vitro* reconstitution assays. Using a BiFC assay we studied the interactomics of lipid droplets and mitochondria and peroxisomes in *S. cerevisiae* and detected 116 protein-protein interactions.

## RESULTS

### Contact between lipid droplets and mitochondria

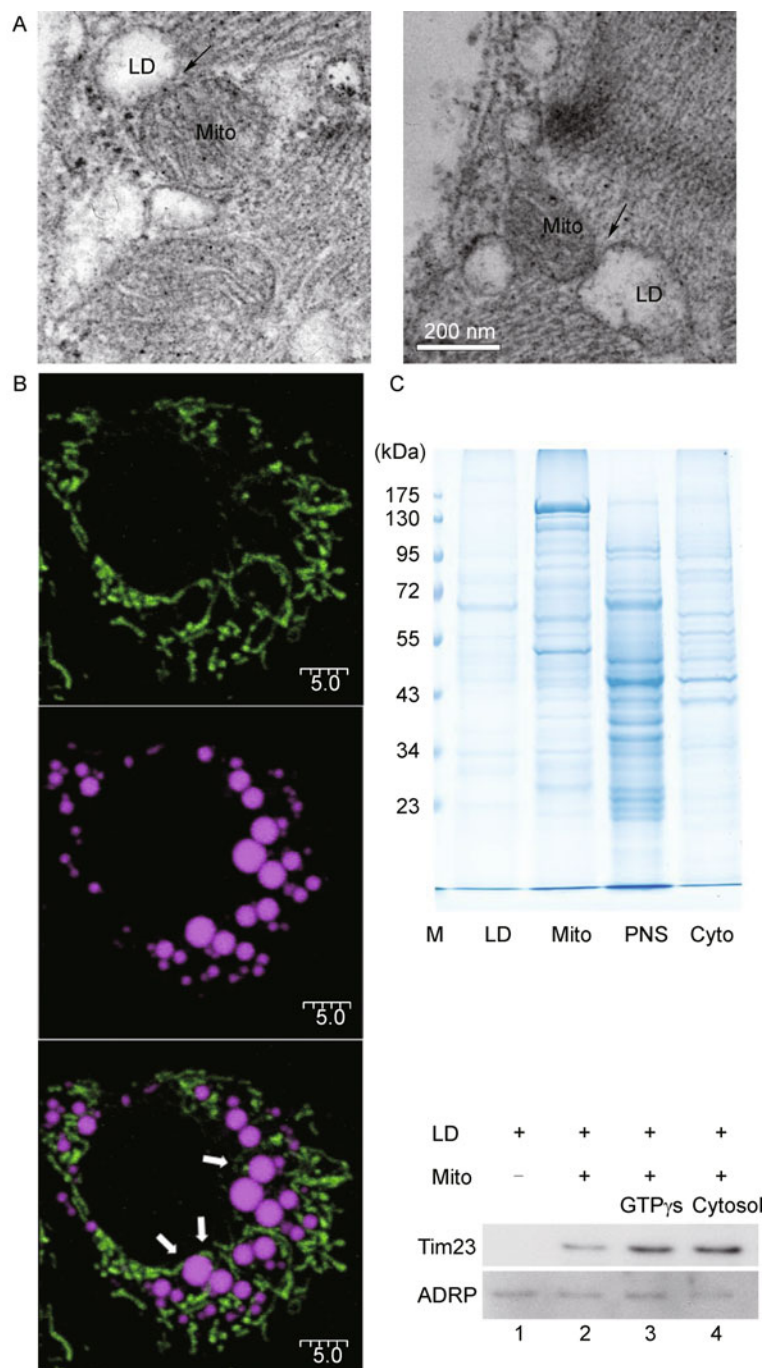
Skeletal muscle cells are one of the major energy consumers in the animal body and the interaction between lipid droplets and mitochondria probably plays a key role in energy homeostasis. Previous morphological studies have revealed that lipid droplets and mitochondria might physically contact each other in muscle tissues (Shaw et al., 2008). Consistent with this, we observed a close association between mitochondria and lipid droplets in rat skeletal muscle tissue by transmission electron microscopy (TEM). Interestingly, some lipid droplets and mitochondria appeared to share portions of the membrane as a boundary (Fig. 1A, left panel, arrow). In

other cases, lipid droplets and mitochondria were closely associated but retained independent membranes (Fig. 1A; right panel, arrow). To verify the interaction in living cells, we treated cells of the rat skeletal muscle cell line L6 with oleate for 3 h to induce the formation of droplets. After washing off the oleate, the cellular positions of mitochondria and lipid droplets were determined by confocal microscopy and 3D reconstruction. Consistent with TEM results, lipid droplet-mitochondrion contact was frequently observed (Fig. 1B; lower panel, arrow), and statistical analysis revealed that the contact rate was correlated with lipid droplet size (data not shown). These data suggest that lipid droplet-mitochondrion interactions are dynamic.

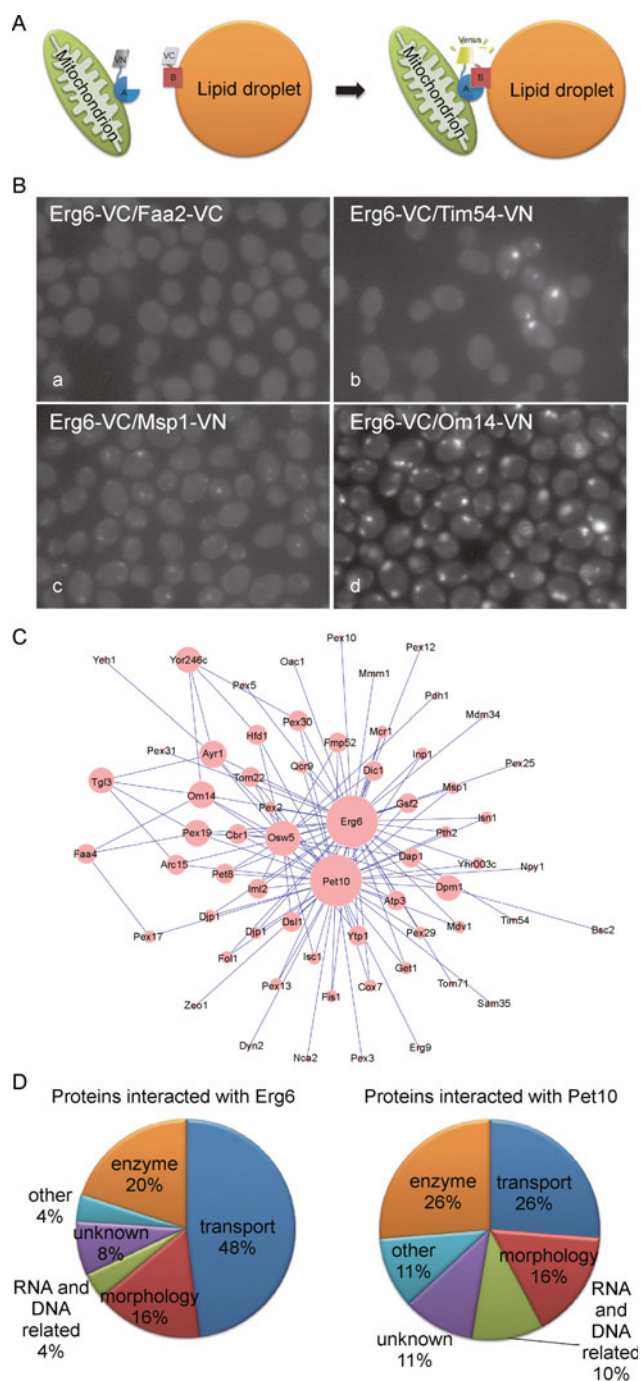
To further verify mitochondrion-lipid droplet interaction, an *in vitro* reconstitution assay using purified lipid droplets and mitochondria was performed as described previously (Liu et al., 2008). SDS-PAGE analysis of the isolated fractions (Fig. 1C; upper panel) demonstrated distinct protein profiles of purified lipid droplets (LD), mitochondria (Mito), postnuclear supernatant (PNS) and cytosol (cyto), suggesting that lipid droplets and mitochondria were highly enriched. After incubation with mitochondria, lipid droplets were isolated from the reaction system, and their surface proteins were analyzed by Western blot. Translocase of the inner mitochondrial membrane 23 (Tim 23), a mitochondrial inner membrane protein, was used as a mitochondrial marker, and adipocyte differentiation-related protein (ADRP) as a lipid droplet protein loading control. While Tim 23 could not be detected (Fig. 1C; lower panel, lane 1) in the proteins obtained from purified lipid droplets, those obtained from lipid droplets after incubation with mitochondria contained Tim 23 (lane 2), suggesting that mitochondria interacted with lipid droplets during incubation. When GTPys or isolated cytosol was added to the reaction system, stronger signals of Tim 23 were detected (lanes 3 and 4), which suggests that GTPys or cytosol stimulated the mitochondrion-lipid droplet interaction. ATP was found to have no effect on the interaction (data not shown). These *in vitro* assays offered further evidence that lipid droplets and mitochondria can interact with each other, and some physiological factors such as GTP and some cytosolic component(s) may regulate their interactions.

### Screening the proteins involved in the lipid droplet-mitochondrion (or peroxisome) interaction by BiFC assay in *S. cerevisiae*

To explore the proteins involved in the organelle interaction, we took advantage of BiFC assays in *S. cerevisiae* to analyze interactions between proteins found in our organelles of interest *in vivo*. The C-terminus of the fluorescence protein Venus (VC) was fused to the lipid droplet proteins, and the N-terminus (VN) to the mitochondrial proteins. We expected fluorescence signals to be generated by the fusion of VC and VN if the proteins interacted with each other (Fig. 2A). Since,



**Figure 1. Interaction between mitochondria and lipid droplets in skeletal muscle cells.** (A) The ultrastructure of rat skeletal muscle tissue was obtained by transmission electron microscopy (TEM). Mito, mitochondrion; LD, lipid droplet. Bar = 200 nm. (B) L6 cells were treated with 200 μmol/L oleate in the growth medium for 12 h and maintained in the growth medium for 3 h after washing oleate off. Mitochondria (upper panel) and lipid droplets (middle panel) were stained with MitoTracker Green FM and LipidTOX Deep Red, respectively. Fluorescence images were obtained using a confocal microscope and 3D reconstructed by software *FLUOVIEW*. The contact between lipid droplets and mitochondria were determined by 3D reconstruction movie and indicated by arrows (lower panel). Bar = 5 μm. (C) Proteins were extracted from purified lipid droplets (LD), mitochondria (Mito), cytosol (Cyto) and postnuclear supernatant (PNS) from C57 mouse liver tissues and were resolved by 10% SDS-PAGE. The gel was stained with a Colloidal Blue Staining Kit (upper panel). M, molecular weight marker. Purified lipid droplets and mitochondria were subjected to *in vitro* reconstitution assays. GTP<sub>γ</sub>S (lane 3) or isolated cytosol (lane 4) was added to the reaction system. Total proteins of lipid droplets were isolated from the system and were analyzed by immunoblotting with the indicated antibodies (lower panel).



**Figure 2. Proteins involved in the organelle interaction.** (A) The schema of BiFC assay probing lipid droplet and mitochondrion interaction. The interaction between C-terminally VN-tagged mitochondrial protein A and C-terminally VC-tagged lipid droplet protein B brings VC and VN into close proximity resulting in a fluorescent signal. (B) The BiFC assay was performed and signals from fluorescence complementation were monitored by fluorescence microscopy. Diploid yeast cells expressing C-terminally VC-tagged Tgl3 and Faa2 were analyzed for BiFC and used as a negative control (a). The fluorescence images with 3 different signal patterns are shown in b, c and d. The two genes C-terminally tagged with VC or VN are indicated as gene X-VC/gene Y-VN. (cC) The protein-protein interaction network visualized by Cytoscape. The node size was according to node degree. (D) The proteins interacting with Erg6 (upper panel) or Pet10 (lower panel) were catalogued by their function referring to SGD and the percentages of each category are indicated.

unlike mammalian cells, yeasts carry out  $\beta$ -oxidation in peroxisomes, we included peroxisomal proteins as well in our BiFC assays. We selected 22 lipid droplet proteins as baits and 225 mitochondrial and peroxisomal proteins as preys for our screen. We chose 22 lipid droplet proteins, 81 mitochondrial proteins, and 21 peroxisomal proteins based on their localizations as reported in the Yeast GFP Fusion Localization database (Huh et al., 2003). An additional 103 proteins of the outer membrane of mitochondria and 20 peroxisomal proteins were chosen from the *Saccharomyces* genome database (SGD).

In yeast cells, the number and size of lipid droplets vary depending on cell growth. To obtain robust BiFC signals, the yeast cells were cultured in YPD medium and inoculated into SC medium for fluorescence detection when growing in the logarithmic phase. Compared with cells grown in the YPD medium, cells grown in SC medium displayed reduced autofluorescence (Fig. S1A; right panel vs left panel) and increased lipid droplet formation (Fig. S1B; right panel vs left panel).

We found 116 protein pairs that yielded positive signals in our BiFC assays. They included 8 lipid droplet proteins, 37 mitochondrial proteins and 19 peroxisomal proteins (Tables 1 and 2). There are 52 pairs of protein-protein interactions corresponding to interactions between lipid droplets and mitochondria (or peroxisomes) listed in Table 1. Table 2 displays interactions detected between proteins for which there is conflicting or ambiguous localization information in the two databases. In some cases, this may be due to the fact that the proteins have multiple localizations. In our experiments, the BiFC signals appeared in a punctate pattern, consistent with localization to lipid droplets. However, the signal intensity, number of puncta and localization patterns varied (Fig. 2Bb–d). This hints at a complex interplay between these organelles. Two VC-tagged strains were mated as a negative control and no fluorescence was observed in these cells (Fig. 2Ba).

### The interaction between lipid droplet and mitochondrion (or peroxisome) may be important for lipid metabolism

Among the 22 lipid droplet proteins examined, 8 were found to interact with prey proteins. Of these 8 proteins, Erg6 and Pet10 were the most active, interacting with many mitochondrial and peroxisomal proteins and generating 75% of the total positive signals. The network of the interactions was visualized by Cytoscape (Shannon et al., 2003), wherein node degree was mapped to node size (Fig. 2C). Furthermore, besides mitochondrial and peroxisomal proteins, Erg6 interacted with 10 other lipid droplet proteins including 2 enzymes of sterol biosynthesis, 2 enzymes of triglyceride metabolism and 6 with unknown function (Table 3). Interestingly, Pet10 is one of these 10 lipid droplet proteins that interact with Erg6. Therefore, it is possible that Erg6 and

**Table 1** Protein-protein interactions identified by BiFC assay in *S. cerevisiae*

Gene	Lipid droplet proteins					
	Erg6	Tgl3	Faa4	Pet10	Yor246c	Osw5
Pdh1						+
Fol1	+ <sup>a</sup>			+		
Nca2				+		
Yhr003c	+			+		
Sam35				+		
Mcr1	+			+		
Pth2	+			+		
Om14	+	+	+	+	+	+
Mdv1	+			+		
Msp1	+			+		
Atp3	+			+		+
Pet8	+			+		+
Qcr9	+			+		
Cox7	+			+		
Mdm34	+					
Tom71	+					
Mmm1	+					
Tim54	+					
Oac1	+					
Pex30	+			+	+	
Inp1	+			+		
Pex3				+		
Pex13	+			+		
Pex2	+			+		
Pex17			+	+		
Pex5	+					
Pex25	+					
Pex12	+					
Pex10	+					
Total	24	1	2	19	2	4

<sup>a</sup> BiFC signal positive; upper panel, mitochondrial proteins; lower panel, peroxisomal proteins.

Pet10 constitute a “reaction core” in lipid droplets. We categorized the mitochondrial and peroxisomal proteins that interact with Erg6 or Pet10 according to their functions. The pie charts in Fig. 2D show that most of the proteins that interact with Erg6 (upper panel) or Pet10 (lower panel) are either enzymes or proteins involved in transport, implying that lipid droplets might function primarily in metabolism and trafficking.

Three of the several pairs of interacting proteins identified in our assays are involved in lipid metabolism: Erg6/Mcr1, Tgl3/Ayr1, and Tgl3/Pex11 (Table 4). Erg6 is an enzyme involved in ergosterol synthesis and converts zymosterol to fecosterol by methylating at position C-24. It is interesting that

**Table 2** Protein-protein interactions identified by BiFC assay in *S. cerevisiae* (supplement)

Gene	Lipid droplet proteins								Localization	
	Erg6	Tgl3	Yeh1	Faa4	Pet10	Bsc2	Yor246c	Osw5	SGD	GFP
Arc15	+ <sup>a</sup>	+			+				mito <sup>b</sup>	Actin
Jsn1	+				+				mito	Ambiguous
Tom22	+				+			+	mito	Ambiguous
Iml2	+				+			+	mito	cyto <sup>c</sup> nu <sup>d</sup>
Hfd1					+		+	+	mito	endo <sup>e</sup> LD <sup>f</sup>
Cbr1	+				+				mito	ER
Ayr1	+	+	+		+		+	+	mito	ER
Fmp52	+				+			+	mito	ER
Erg9					+				mito	ER
Gsf2	+				+			+	mito	ER
Dpm1	+				+	+		+	mito	ER
Dic1	+				+			+	mito	ER
Get1	+				+				mito	ER
Zeo1					+				mito	ER, peri <sup>g</sup>
Dap1	+				+			+	mito	ER, cyto
Isc1					+				mito	No <sup>h</sup>
Ytp1	+				+			+	mito	No
Fis1	+				+				mito/pero <sup>i</sup>	Ambiguous
Pex31	+								pero	Ambiguous
Pex32	+								pero	Ambiguous
Pex29	+				+				pero	Ambiguous, cyto, nu
Npy1					+				pero	cyto, nu
Pex19	+	+		+	+			+	pero	cyto
Dsl1	+				+			+	pero	ER
Dyn2					+				pero	ER
Djp1	+				+				pero	ER
Total	20	3	1	1	24	1	2	12		

<sup>a</sup>BiFC signal positive; <sup>b</sup>mitochondrion; <sup>c</sup>cytosol; <sup>d</sup>nucleus; <sup>e</sup>endosome; <sup>f</sup>lipid droplet; <sup>g</sup>cell periphery; <sup>h</sup>no localization scored for ORF; <sup>i</sup>peroxisome.

Mcr1, which is also an enzyme involved in ergosterol biosynthesis, is localized to the mitochondria and was found in our assay to interact with Erg6. It is possible that lipid droplets and mitochondria separately complete parts of the ergosterol synthetic process, and then deliver or exchange metabolites between them via physical interaction to complete ergosterol biosynthesis. Similarly, fatty acids released from triglycerides might be hydrolyzed by Tgl3 in lipid droplets and feed into Ayr1-involved phosphatidic acid synthesis in mitochondria or Pex11-involved  $\beta$ -oxidation in peroxisomes. These protein-protein interactions suggest that lipid droplets might both offer substrates to or accept substrates from lipid metabolic processes occurring in mitochondria or peroxisomes and complete the metabolism via physical interactions with them. It would be economical for cells to generate lipids by using metabolic intermediates delivered by lipid droplets

rather than by *de novo* synthesis, and our data provide support for this model.

## DISCUSSION

In mammalian cells, fatty acid oxidation mainly occurs in mitochondria, but the majority of cellular fatty acids come from the lipolysis of triglycerides in lipid droplets. It is possible that fatty acids might be transferred from lipid droplets to mitochondria via direct physical interactions between these organelles. Lipid droplet proteomic studies carried out in several species, tissues, and cell lines such as yeast (Binns et al., 2006), plant cells (Katavic et al., 2006), *Drosophila* (Beller et al., 2006; Cermelli et al., 2006), liver (Turr ot et al., 2006), 3T3-L1 cells (Brasaemle et al., 2004), and CHO cells (Liu et al., 2004) have identified hundreds of proteins

**Table 3** Lipid droplet proteins interacting with Erg6

Name	Molecular function	Biological process
Erg7	Lanosterol synthase activity	Ergosterol biosynthetic process
Tgl4	Sterol esterase activity	Triglyceride catabolism
	Triglyceride lipase activity	Triglyceride mobilization
Yeh1	Sterol esterase activity	Sterol metabolism
Tgl3	Triglyceride lipase activity	Triglyceride catabolic process; Cellular lipid metabolic process
Coy1	Unknown	Golgi vesicle transport
Yor246c	Oxidoreductase activity	Unknown
Pet10	Unknown	Aerobic respiration
Rrt8	Unknown	Unknown
Bsc2	Unknown	Unknown
Ymr148w	Unknown	Unknown

**Table 4** Protein-protein interactions involved in lipid metabolism between lipid droplets and mitochondria or peroxisomes

Lipid droplet protein (bait)			Mitochondrial or peroxisomal protein (prey)		
Name	Molecular function	Biological process	Name	Molecular function	Biological process
Erg6	Sterol	Ergosterol	Mcr1	Cytochrome b5 reductase	Ergosterol biosynthetic process
	24-C-methyltransferase	biosynthetic process			
Tgl3	Triglyceride lipase activity	Triglyceride catabolic process	Ayr1	Acylglycerone-phosphate reductase	Phosphatidic acid biosynthetic
		Cellular lipid metabolic process			
			Pex11	Unknown	Fatty acid oxidation

localized in lipid droplets. Interestingly, a variety of mitochondrial proteins were frequently identified in isolated lipid droplets in proteomic studies (Table S1), suggesting either dual localization or physical association of these mitochondrial proteins with those of lipid droplets. For instance, the mitochondrial protein NADH-cytochrome b5 reductase 3 was found in isolated lipid droplets in several species (Table S1). In our study, this protein was found to interact with lipid droplet proteins Erg6 and Pet10 (Table 2; Cbr1). This and other interactions identified in our study provide a mechanistic explanation for the presence of mitochondrial proteins in lipid droplets reported in previous studies.

Almost every cellular event is mediated by networks of protein-protein interactions. Interactions of lipid droplet proteins with each other or with those of other cellular compartments are likely essential for lipid droplet functions. Additionally, interactions between specific protein pairs might subserve organelle-to-organelle contact. Unfortunately, the BiFC assay cannot distinguish between interactions involved in organelle contact from those related to other cellular functions. However, the properties of the proteins can provide us some important clues.

Previous studies have reported that ergosterols function as regulators of membrane permeability and fluidity (Gaber et al., 1989). Additionally, increased sterol concentrations promote

membrane fusion (Tedrick et al., 2004). Based on these results from previous studies and our results, we can speculate that lipid droplets move along the cell skeleton to contact mitochondria, leading to physical and functional coupling mediated by lipid droplet and mitochondrial proteins. Erg6, acting along with other enzymes in lipid droplets, and mitochondrial enzymes together, completes ergosterol synthesis. Increased ergosterol concentrations could alter the properties of the membrane and consequently make the membrane microenvironment suitable for protein movement. This could then allow exchange of proteins between the organelles.

What proteins might initiate the junction between organelles? Several studies have reported that proteins that regulate membrane trafficking such as Rabs and SNAREs mediate the interactions between lipid droplets and other organelles (Zehmer et al., 2009). Rab proteins are generally known as GTPase switches controlling membrane trafficking among intracellular compartments. Multiple Rab proteins were identified in lipid droplets in a proteomic study by Liu et al. (Liu et al., 2004), and Liu and his coworkers later reported that Rabs are capable of regulating the interaction between lipid droplets and early endosomes (Liu et al., 2007). Like other GTPases, Rabs have two conformations: an inactive form bound to GDP and an active form bound to

GTP. Our results show that GTP can enhance interactions between lipid droplets and mitochondria (Fig. 1C, lower panel), suggesting that GTPases may be involved in the interaction and Rabs may be the mediators. In our BiFC assay, the Rab5 yeast homolog Vsp21 generated a positive signal with Erg6, but C-terminally VN-tagged Vsp21 was also able to interact with cytosolic free VC fragment, so the possibility of false positive could not be excluded. The resolution of this issue awaits future work.

Among the proteins we assayed by BiFC, there were 4 membrane trafficking proteins in lipid droplets—Use1, Snx41, Coy1 and Sso1, and 3 proteins in mitochondrial outer membrane—Mdm10, Mmm1 and Vsp21. However, these proteins were not found to interact with each other, and only Mmm1 was found to interact with Erg6. Although this result was surprising, a likely explanation is that any interactions among these proteins, if present, could be transient in nature. Besides Erg6 and Pet10, another lipid droplet protein Osw5 was also found to interact with many mitochondrial and peroxisomal proteins (Tables 1 and 2), accounting for about 14% of the total positive signals. As SNARE proteins, which mediate membrane fusion, contain cytosolic alpha helices that drive membrane interaction, interestingly, Osw5 is predicted to contain an N-terminal transmembrane domain (Fig. S1C; upper panel) and a C-terminal  $\alpha$  helix (Fig. S1C; lower panel). It is plausible that this  $\alpha$  helix is involved in membrane interaction in a manner similar to those of SNARE proteins, binding to partners present in mitochondria or peroxisomes. Its partner in mitochondrion side might be Tom22, a mitochondrial outer membrane protein. Similar to Osw5, Tom22 is predicted to contain a transmembrane segment (Fig. S1D; upper panel) and a complex cytosolic domain (Fig. S1D; lower panel) that is thought to act as a receptor for precursor proteins. Therefore, Osw5 and Tom22 might be the pair of proteins linking lipid droplets and mitochondria by protein-protein interaction. Another mitochondrial protein, Om14, was also found to be very active in our assay, interacting with 6 lipid droplet proteins including 3 enzymes and 3 proteins of unknown function (Table 1). Om14 is an integral mitochondrial outer membrane protein with 3 helical transmembrane segments. Its interaction with Erg6 (Fig. 2B, panel d) yielded a very strong BiFC signal and, as such, Om14 is another candidate for further investigation.

## MATERIALS AND METHODS

### Materials

Oleate, tricine, GTP $\gamma$ s and concanavalin A were purchased from Sigma-Aldrich (St. Louis, MO). MitoTracker Green FM, LipidTOX Deep Red, Colloidal Blue Staining Kit,  $\alpha$ -MEM and FBS were from Invitrogen (Carlsbad, CA). Yeast extract, peptone, yeast nitrogen base, agar and antibodies against Tim 23 were from BD Biosciences (San Jose, CA). Uranyl acetate, 25% glutaraldehyde solution (EM grade) and lead citrate were all from Electron Microscopy Sciences

(Pennsylvania, USA). Osmium tetroxide (EM grade) was purchased from Nakalai Tesque Co. (Kyoto, Japan). Quetol 812 was purchased from Nisshin EM Co., Ltd. (Tokyo, Japan).

### Cell culture

L6 cells were maintained in  $\alpha$ -minimal essential medium ( $\alpha$ -MEM) supplemented with 10% (v/v) FBS, 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C with 5% CO<sub>2</sub>.

### Transmission electron microscopy

The ultrastructure of rat skeletal muscles was examined by ultra-thin sectioning and transmission electron microscopy (TEM). After collection, male Sprague-Dawley rat muscle tissues were cut into small pieces and then prefixed in 2.5% (w/v) glutaraldehyde in PBS (pH 7.4) at 4°C for 2 days and postfixed in 2% (w/v) osmium tetroxide at room temperature for 1.5 h. The fixed samples were dehydrated by incubation in an ascending concentration series of ethanol (35%, 50%, 70%, 90%, 95% and 100%). After embedding in Quetol 812, samples were prepared as 90 nm sections with Leica EM UC6 Ultramicrotome (Leica company). Each section was stained with 2% (w/v) uranyl acetate for 15 min and then with lead citrate for 5 min. The stained sections were examined under a FEI Tecnai20 (FEI Company) electron microscope.

### Cell fluorescence imaging

L6 cells were treated with 200  $\mu$ mol/L oleate in the growth medium for 12 h, then washed with pre-warmed medium 3 times, maintained in the growth medium for 3 h, and incubated in the growth medium containing 25 nmol/L MitoTracker Green FM and LipidTOX Deep Red (11000 diluted) at 37°C for 30 min. Fluorescence signals were captured by z-axis scanning with a confocal microscope at both the 488/525 nm and 630/660 nm excitation/emission wavelength channels. Three-dimensional (3D) reconstruction and animation was completed using the FLUOVIEW software.

### In vitro reconstitution assay

Lipid droplet purification was performed as previously described (Liu et al., 2008). The pellet from lipid droplet purification was washed twice and resuspended in 1 mL buffer B (20 mmol/L HEPES, pH 7.4, 100 mmol/L KCl, 2 mmol/L MgCl<sub>2</sub>) for mitochondrion purification. The pellet-resuspended mixture was loaded on the top of a Percoll step gradient (2 mL 80%, 4.5 mL 52%, and 4.5 mL 26%) and centrifuged at 40,000 *g* for 45 min at 4°C. The visible interface between 26% and 52% Percoll was collected and washed 3 times with buffer B to remove Percoll, yielding purified mitochondria.

The cytosol was isolated from the postnuclear supernatant (PNS) fraction in the lipid droplet purification steps. The PNS was centrifuged at 200,000 *g* for 45 min at 4°C, and the cytosol was collected between lipid droplets on the top and the membrane pellet on the bottom.

Purified lipid droplets and mitochondria were mixed with or without 1 mmol/L GTP $\gamma$ s or cytosol and incubated at 37°C for 30 min and mixed every 5 min. After incubation, lipid droplets and mitochondria were separated by centrifugation at 20,000 *g* for 3 min at 4°C.

Mitochondria (pellet) and the buffer were removed, and lipid droplets were resuspended with cold buffer B. This step was repeated twice. Lipid droplet proteins were extracted by acetone and dissolved in 2 × sample buffer for Western blot analysis using the indicated antibodies. All animal experiments were approved by the Institutional Animal Care and Use Committee and followed the *Animal Use Guidelines* of the Institute of Biophysics, Chinese Academy of Sciences, Beijing.

### BiFC assay

A single-step, PCR-based approach was applied to tag the C-termini of yeast proteins with yellow fluorescent protein fragments for the BiFC assay (Sung and Huh, 2007). Yeast strains BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4742 (*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) were maintained at 30°C in the YPD medium. The PCR product of N- (VN) or C-terminal fragment (VC) of Venus, a variant of yellow fluorescent protein, was obtained using pFA6a-VN-His3MX6 or pFA6a-VC-kanMX6 as template. Yeast transformation was performed using the lithium acetate method. The strains of VC-tagged lipid droplet proteins derived from BY4742 were mated with the strains of VN-tagged mitochondrial proteins derived from BY4741. The resulting diploid yeast cells were cultured in the synthetic complete (SC) medium to mid-logarithmic phase, transferred to 96-well glass-bottomed microplates coated with concanavalin A and analyzed by fluorescence microscopy. The BiFC signals were observed using a Zeiss Axiovert 200M inverted microscope with a Plan-NeoFluar 100×/1.3 NA oil immersion objective. Fluorescence images were taken using a standard fluorescein isothiocyanate filter set (excitation band pass filter, 450–490 nm; beam splitter, 510 nm; emission band pass filter, 515–565 nm).

### Protein databases

Protein localization information was derived from the following databases:

Yeast GFP Fusion Localization database, <http://yeastgfp.yeast-genome.org>

*Saccharomyces* genome database, <http://www.yeastgenome.org/>

### ACKNOWLEDGEMENTS

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### ABBREVIATIONS

ADRP, adipocyte differentiation-related protein; BiFC, Bimolecular Fluorescence Complementation; PNS, postnuclear supernatant; TEM, transmission electron microscopy; Tim 23, inner mitochondrial membrane 23; VC, C-terminal fragment of Venus; VN, N-terminal fragment of Venus

**Supplementary material** is available in the online version of this article at <http://dx.doi.org/10.1007/s13238-011-1061-y> and is accessible for authorized authors.

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