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Structural, biochemical, and cellular characterization of MIGA2

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DISCLAIMER

ABSTRACT

Mitoguardin-2, a newly discovered mitochondrial protein at contact with the ER and/or lipid droplets (LDs) was characterized as a lipid transporter and its lipid transfer ability is essential for its cellular function in mitochondria and lipid droplets. The methods for structural determination, biochemical (lipid binding, lipid competition, lipid transfer between liposomes, and between lipid droplets and liposomes), and cellular experiments (mitochondria morphology, triglyceride measurement, lipid droplet quantification) to explore its function in vivo and in vitro were described in this protocol. The methods in this protocol can also be adapted for studying other soluble proteins.

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KEYWORDS

Protein purification and crystallization, lipid transfer, lipid competition, TAG measurement, lipid droplets

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MATERIALS TEXT

Almost all lipids were purchased from Avanti Polar Lipids: DOPC (850375), liver PE (840026), DGS-NTA (Ni) (709404), Liss Rhod PE (810150), Brain PI (4,5) P2 (840046), NBD-PA (810176), NBD-PS (810195), 18:1 NBD-PS (810198), NBD-PC (810133), NBD-PE (810156）, NBD-cholesterol (810250), NBD-sphingomyelin (810219), 16:0 PA (830855), triolein (18:1 TG) (870110). Palmitic acid (P0500) was ordered from Sigma Aldrich. Sodium dithionite was from Sigma Aldrich (157953). Etomoxir was purchased from MedChemExpress (HY-50202).

Structural determination of mitoguardin-2: protein prep, crystallization, MR, and structure determination

1 Expression, Purification of C.elegans MIGA (106-496)

1.1 The pET28-6xhis-sumo-CeMIGA(106-496) construct was expressed in C43 (DE3) *Escherichia coli* cells. Cells were grown at 37°C to an OD_{600} of 0.6–0.8 when protein expression was induced with 0.8 mM IPTG, and then cells were cultured at 18°C for another 6 hours. Cells were harvested and stored at -80°C.

1.2 The cell pellet was resuspended in buffer A (20 mM HEPES, pH 7.4, 200 mM NaCl, 1mM TCEP, and 5% glycerol) containing 1× complete EDTA-free protease inhibitor cocktail (Roche 1187358001) and lysed in an Emulsiflex-C5 cell disruptor (Avestin). Cell lysates were clarified via centrifugation at 27,000 g for 30 min. To collect the protein, the supernatant was incubated with Ni-NTA resin (QIAGEN30210) for **01:00:00** at **4 °C** . Then the resin was washed tandemly with buffer B (buffer A + 0.005% Triton X-100), buffer C (buffer B + 10mM imidazole), buffer D (buffer B + 20mM imidazole), buffer E (buffer B + 40mM imidazole), buffer A to remove extra Triton X-100. Retained protein was eluted from the resin with buffer A supplemented with 250 mM imidazole. Sumo protease was added to digest overnight at 4°C. After removing imidazole by buffer exchange, the sample was bound to the Ni-NTA resin again to remove protease and 6xhis-sumo. The flow-through was collected, concentrated in a 30-kD molecular weight cutoff (MWCO) Amicon centrifugal filtration device, and loaded onto a Superdex 200 16/60 column (GE Healthcare) equilibrated with buffer F (20 mM HEPES, pH 7.4, 200 mM NaCl, 1mM TCEP). Peak fractions containing pure MIGA were recovered and concentrated. The protein concentration was determined by the absorbance at 280nm.

1h

The gel filtration profile looks like below. The expected molecular weight of the construct is 45.109kD. According to the SEC column standards, the CeMIGA(106-496) runs as a dimer.

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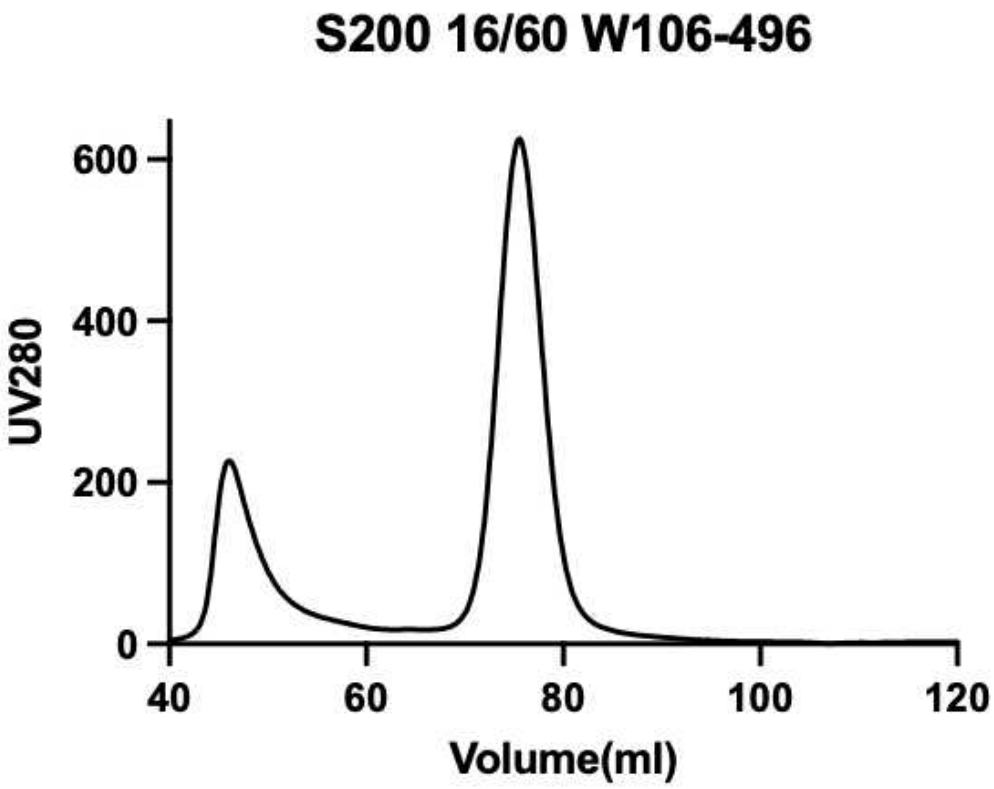

Size exclusion profile of CeMIGA (106-496), the protein peak is around 80ml, which is eluted as a dimer

2 Crystallization and structure determination of CeMIGA (106-496)

2.1 Crystals of *C. elegans* MIGA (106-496) at **6 mg/mL** were grown at 18°C using the sitting-drop vapor-diffusion method. Equal volumes of protein and reservoir solution (0.2 M sodium malonate, pH 7.4, 22% PEG335) were mixed. Crystals, which belonged to spacegroup $P3_121$ (a=91.3, b=91.3, c=366.74 Å), were transferred to solutions that also included cryo-protectants (25% ethylene glycol) and flash-frozen in liquid nitrogen.

We examined ~100 crystals. Although most did not diffract to atomic resolution, a small number diffracted to ~4 Å or better. We found a single crystal among them that were cryo-protected in 25% ethylene glycol that diffracted to 3.3 Å, from which we collected the data set used in structure determination

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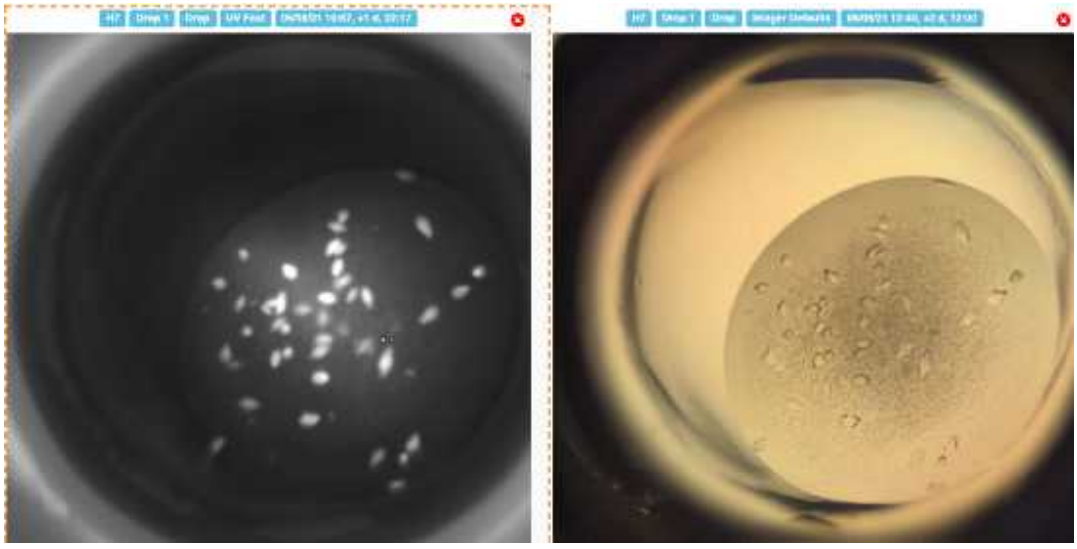
Original protein crystal hits



The crystal hits from initial screening. The left panel is the UV, right panel is the brightfield. The crystals lit up under UV indicates those are protein crystals.

2.2 Diffraction data were collected at the Northeastern Collaborative Access Team (NE-CAT) beamline 24-ID-C at the Advanced Photon Source, using a Dectris EIGER2 X 16M pixel array detector, and processed using XDS.

2.3 For phasing, we used molecular replacement with Phaser MR (McCoy et al., 2007) using a CeMIGA$_C$ model generated byAlphaFold2 Colab (Jumper et al., 2021) preprocessed with phenix.process_predicted_model (Liebschner et al., 2019) to remove the low-confidence loop regions. Four copies of CeMIGA2$_C$ were placed in the asymmetric unit. Initial maps showed clear density for two four-helix bundles, representing dimers of the coiled-coil helices from the CeMIGA linker. The helices were modeled manually in Coot (Emsley et al., 2010). Additional unmodeled density was observed in the Fo-Fc maps within the hydrophobic cavity in two MIGA$_C$domains, which clearly corresponded to bound glycerophospholipids. We built PE 16:0 (chemical id PEF) into these densities and eventually, using non-crystallographic symmetry, placed PE into less well-defined densities in the remaining two monomers. The refinement consisted of cycles of manual rebuilding in Coot and automated refinement in Phenix(Liebschner et al., 2019), including isotropic b-factors, Translation-Libration-Screw refinement, and non-crystallographic symmetry restraints. The coordinates and structure factors have been deposited in the Protein Data Bank (accession no. 8EDV).

Biochemical experiments: lipid binding and competition assay, lipid transfer assay (FRET or end point), aLD preparation, lipid transfer between aLD and Liposomes

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3 Lipid binding and competition assay and mass spectrometry analysis of hMIGA2

The human MIGA2 (170-593) purified from Expi293 cells was used for the lipid binding assay

3.1 18h

Protein Expression: the pCMV10-3xFLAG-Prs-hMIGA2(170-593) plasmid was transfected into Expi293 cells at the density of 2.5~3 million/ml by PEI. The protein expression was enhanced by **3.5 millimolar (mM)** VPA at **18:00:00** after transfection. Cells were harvested and stored at -80°C

3.2 30m

Protein purification: The cell pellet was resuspended in buffer A (20 mM HEPES, pH 7.4, 200 mM NaCl, 1mM TCEP, and 5% glycerol) containing 1× complete EDTA-free protease inhibitor cocktail (Roche 1187358001) and lysed by 5 cycles of freeze-thaw. Cell lysates were clarified via centrifugation at 27,000 g for **00:30:00** and the supernatant was incubated with preequilibrated anti-FLAG M2 affinity resin (Sigma-Aldrich, A2220) for 2 hours at 4°C. The resin was washed with buffer A and incubated overnight with buffer A containing 2.5 mM ATP and 5 mM $MgCl_2$. The protein was eluted with buffer A supplemented with 0.2 mg/ml 3× FLAG peptide (APExBio A6001), concentrated in a 30-kD MWCO Amicon centrifugal filtration device, and loaded onto a Superdex 200 10/30 column (GE Healthcare) equilibrated with buffer A. Peak fractions were pooled and concentrated.

Size exclusion profile

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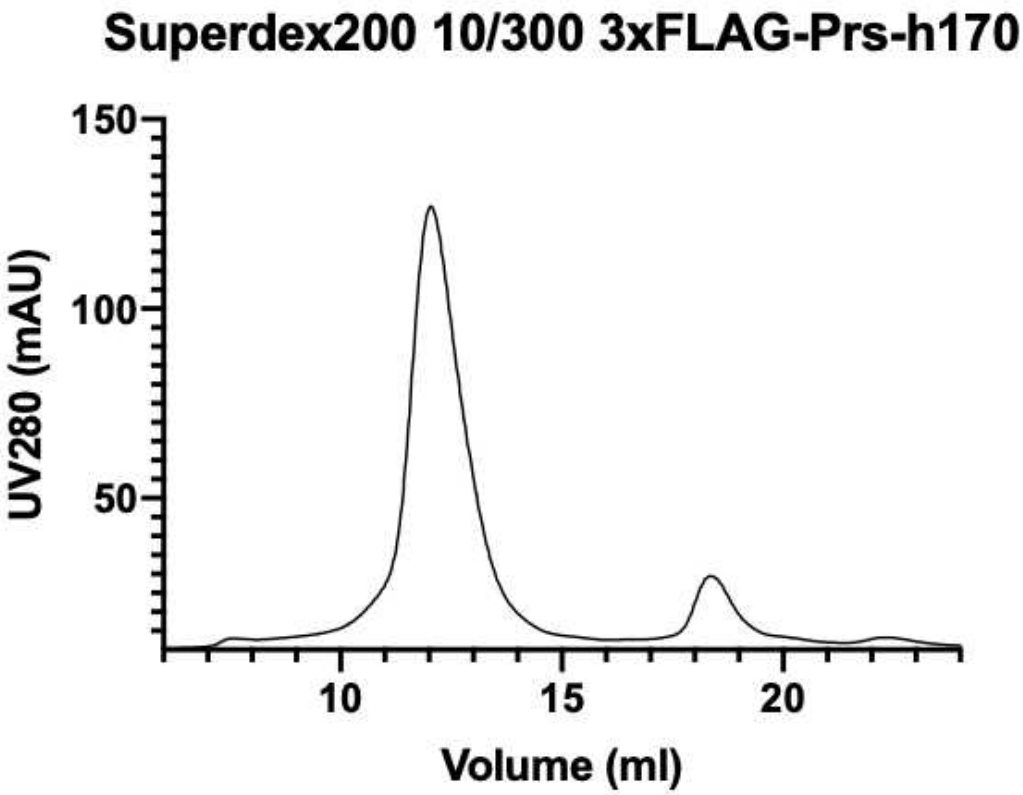

3.3 Lipid binding: Purified hMIGA2 was mixed with 1µl of NBD-labeled lipids (at 1mg/ml) in 20 µl total reaction volumes and incubated on ice for 2 hours. Samples were loaded onto 4–15% Mini-Protean Precast Native gels and run for 90 minutes at 100 V. NBD fluorescence was visualized using an ImageQuant LAS4000 (GE Healthcare). Then gels were stained with Coomassie blue G250 to visualize total protein. Images were analyzed via Fiji (ImageJ).

MIGA2 comigrates with NBD-PA, NBD-PC, NBD-PS, and NBD-PE, but not NBD-cholesterol, and NBD-sphingomyelin.

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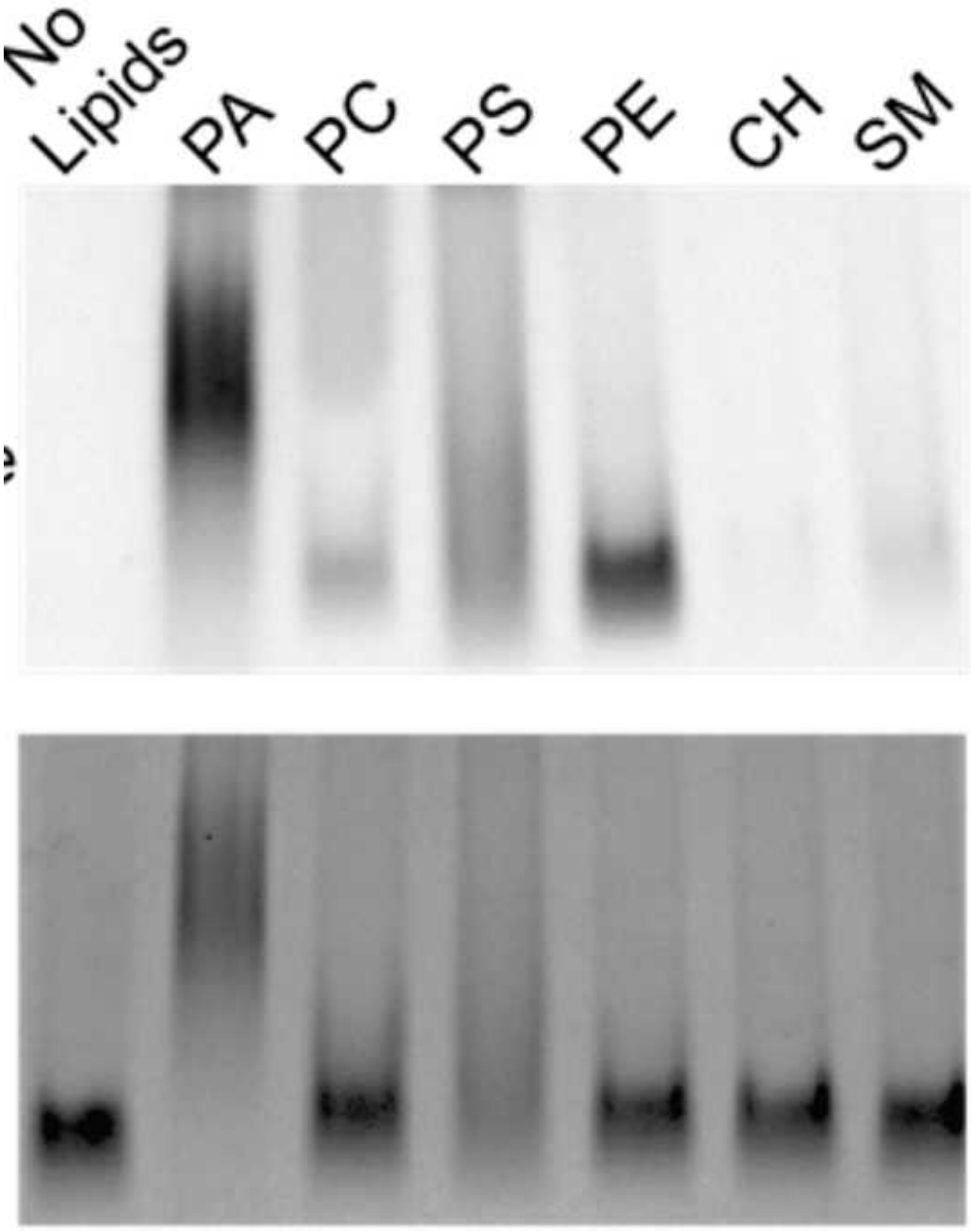

Lipid binding results: the upper panel is the NBD-fluorescence channel, the bottom panel is the protein signal. Because samples migrate on native gel based on their mass/charge ratio and MIGA2 dimer binds different amounts of lipids, the PA and PS signals are smeared.

4 Lipid competition assay

The human MIGA2 (170-593) purified from bacteria was used for the lipid competition assay

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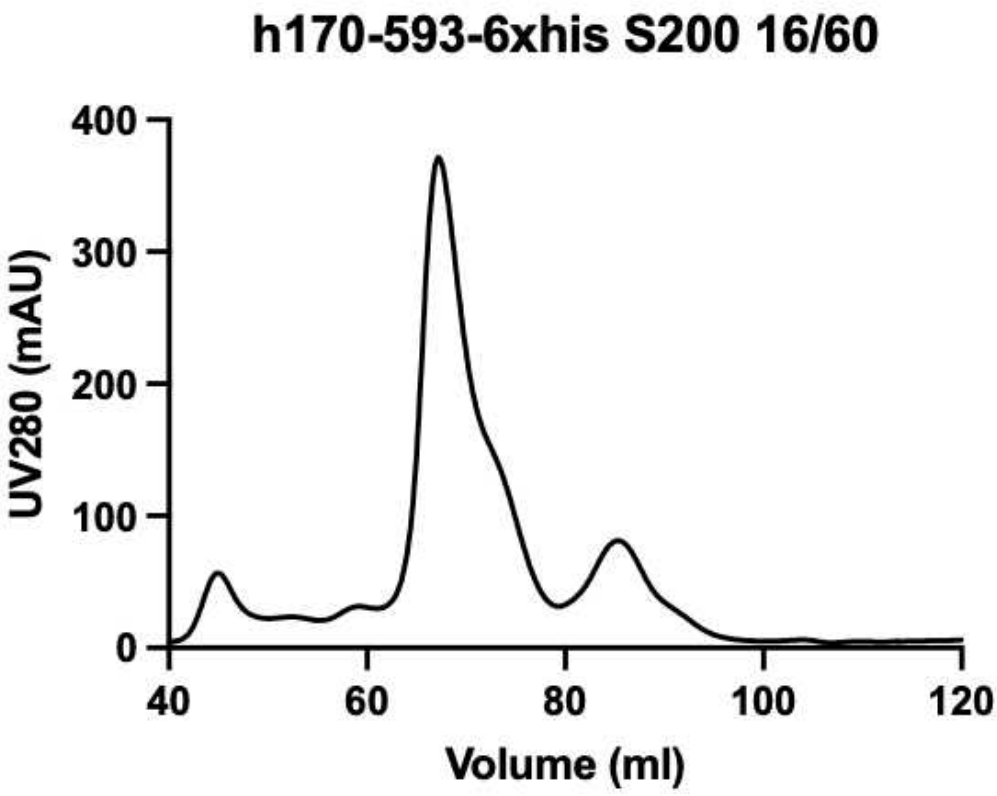
4.1 Protein expression and purification: MIGA2(170-593)-6xhis were expressed in BL21 (DE3) *Escherichia coli* cells. The expression and purification of these constructs were the same as that for CeMIGA(106-496) except that no detergent was added during washes, and no sumo digestion and rebinding, instead loading onto Superdex 200 16/60 column directly after elution and concentration.



Size exclusion profile

4.2 Lipid competition: NBD–PC and another non-labeled lipids at a 1:1 molar ratio (1.13 mM each) were mixed with hMIGA2(170-593)-6xhis in 40 µl reaction volumes and incubated on ice for 2 hours. Samples were loaded onto 4–15% Mini-Protean Precast Native gels and run for 90 minutes at 100 V. NBD fluorescence was visualized using an ImageQuant LAS4000 (GE Healthcare).

Non-labeled PC, PA, FA, or TAG can compete with the NBD-PC for MIGA2 binding.

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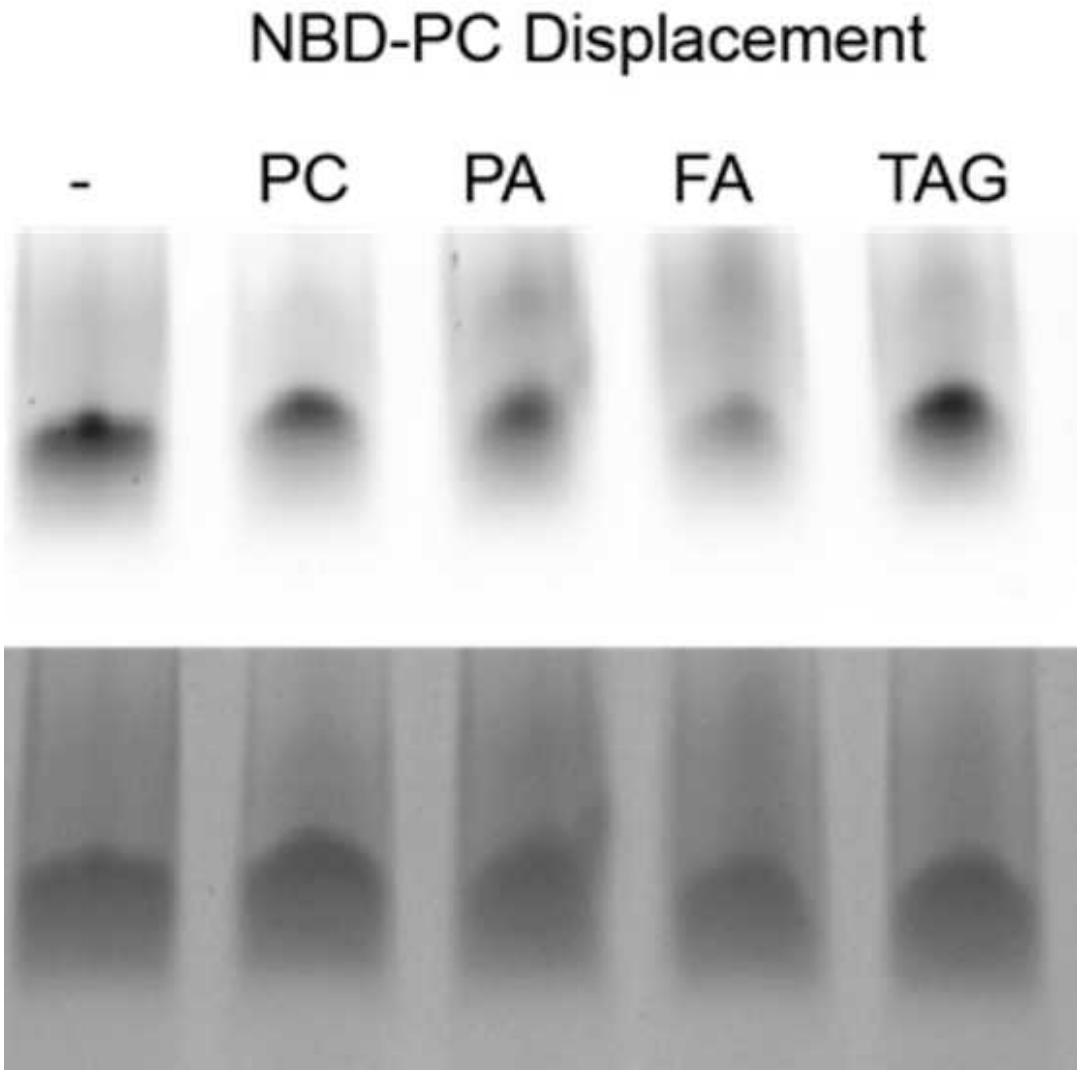

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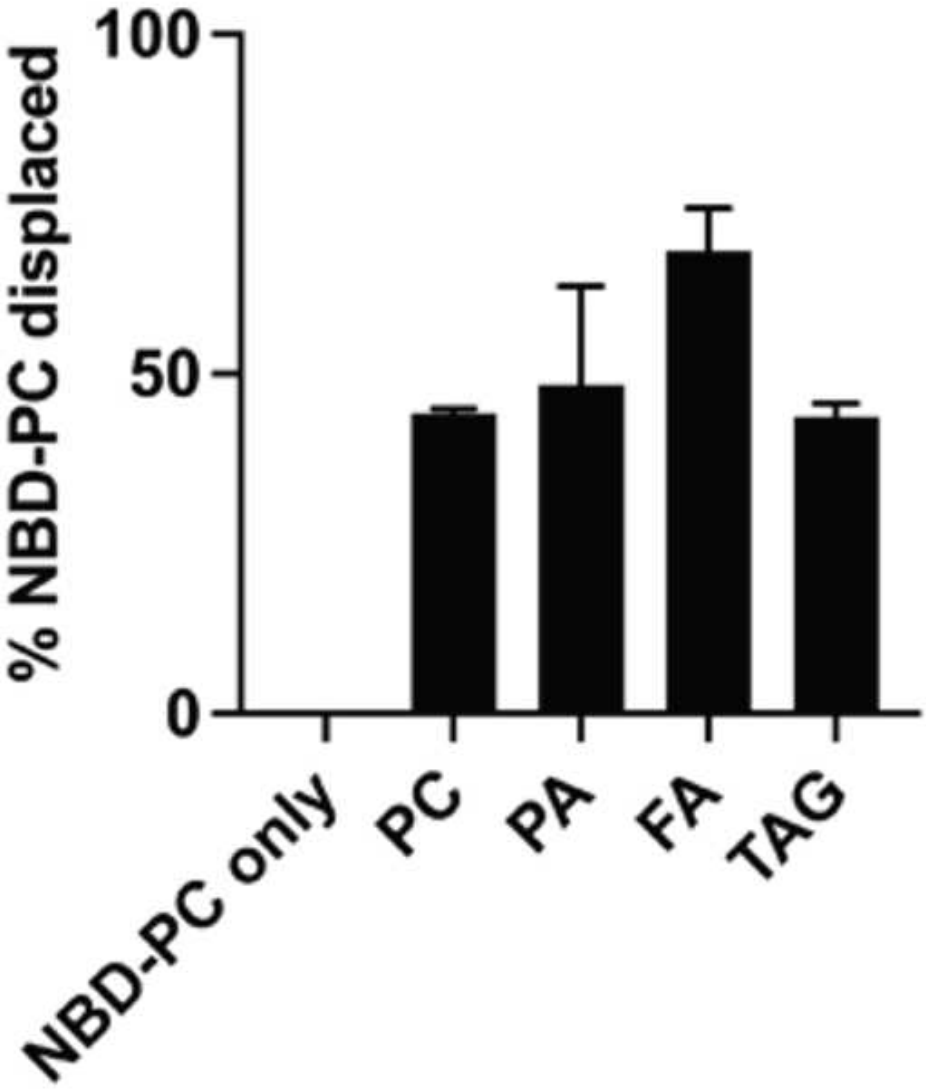

hMIGA2(170-593)-6xhis was incubated with a 1:1 molar ratio of NBD-PC_{acyl} and unlabeled lipid and examined by native PAGE

5 Lipidomics and native MS

The human MIGA2 (170-593) purified from Expi293 cells was used for lipidomics. The human MIGA2 (170-575) purified from bacteria was used for the Native mass spectrum.

5.1 Protein expression and purification: the expression and purification of the proteins were the same as in steps 3 and 4.

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5.2 Lipidomics analysis: Gel filtrated 3xFLAG-hMIGA2(170-593) was sent to Michigan State University's Collaborative Mass spectrometry Core for untargeted lipidomics analysis. The sample was spiked with internal standards and a calibration mixture. Lipids were extracted with MTBE twice, and after drying, resuspended in isopropanol containing 0.01% BHT. The sample was resolved by a Shimadzu Prominance HPLC and lipid species were detected by a Thermo Scientific LTQ-Orbitrap Velos mass spectrometer in both positive and negative ionization modes. Lipid species were quantified based on internal standards and summed by lipid class.

5.3 Native MS: The hMIGA2 sample was buffer exchanged to 200 mM ammonium acetate (MP Biomedicals), 2 mM DTT with Zeba Spin Desalting Columns (Thermo-Fisher Scientific). The protein concentration in the analyzed sample was in the range between 1 µM and 5 µM. Native-MS was performed on Q Exactive UHMR (Thermo-Fisher Scientific) using in-house nano-emitter capillaries. The tips in the capillaries were formed by pulling borosilicate glass capillaries (OD: 1.2 mm, ID: 0.69 mm, length: 10 cm, Sutter Instruments) using a Flaming/Brown micropipette puller (ModelP-1000, Sutter Instruments). Then the nano-emitters were coated with gold using rotary pumped coater Q150R Plus (Quorum Technologies). To perform the measurement the emitter filled with the sample was installed into Nanospray Flex Ion Source (Thermo-Fisher Scientific). MS parameters for the analysis of the proteins or protein complexes include spray voltage 1.1–1.3 kV, capillary temperature 275°C, resolving power 6250 at m/z of 400, ultrahigh vacuum pressure 4.6e-10–8.18e-10, in-source trapping between −100 V and -200 V.

6 FRET-based lipid transfer assay

6.1 Protein expression and purification: hMIGA2(170-593)-6xhis and hMIGA2(306-593)-6xhis were used for the transfer assay. The expression and purification were the same as in step 4.

6.2 Liposome preparation: Lipids in chloroform were mixed (donor liposomes: 61% DOPC, 30% liver PE, 2% NBD-labeled lipids(all NBD-labeled lipids are 18:1-12:0 with NBD incorporated into the fatty acyl chain), 2% Rh-PE, and 5% DGS-NTA (Ni); acceptor liposomes: 65% DOPC, 30% liver PE, and 5% PI(4,5)P_2) and dried to thin films and vacuumed for 30 minutes. Lipids were subsequently dissolved in buffer F at a total lipid concentration of 1 mM and incubated at 37°C for 1 hour, vortexing every 10-15 min. Liposomes were subjected to 10 freeze-thaw cycles alternating between liquid nitrogen and room temperature water bath with vortexing every three cycles. Crude liposomes were then extruded through a polycarbonate filter with 100 nm pore size a total of 11 times via a mini extruder (Avanti Polar Lipids) and used within 24 hours.

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6.3 Lipid-transfer experiments were set up at 30°C in 96-well plates, with 100 µl reaction volumes containing 200 µM lipids in donor liposomes and 200 µM lipids in acceptor liposomes. Proteins (0.25 µM each of tether and lipid transport protein) were added to start the reaction, and after excitation at 460 nm, NBD emission (538 nm) was monitored for 30 minutes using a Synergy H1 Multi-Mode Microplate Reader (Agilent).Triton X-100 was added to terminate the reaction and get the maximum reading.

6.4 Dithionite control assay: Lipid transfer assays were performed as above, except for the addition of freshly prepared dithionite (to 5 mM final concentration) after the last time point, and NBD fluorescence was monitored for an additional 5 minutes.

7 FRET-based lipid transfer from artificial lipid droplets to liposomes (liposomes were prepared as in step 6)

7.1 ⚠

Artificial lipid droplets preparation: Artificial LDs were prepared according to (Wang et al., 2016). Briefly, 2 mg of total phospholipids (81% DOPC, 10% DOPE, 2% 2% NBD-labeled lipids, 2% Rh-PE, and 5% DGS-NTA (Ni) by molar ratio) were mixed and dried to thin films and vacuumed for 30 minutes. 5 mg of TAG (Sigma-Aldrich, T7140) was added to the phospholipid film. The lipids were resuspended in 100µl buffer F. The sample was vortexed for 4 minutes, with 10 seconds on, and 10 seconds off. The milky lipid mixture was centrifuged at 20,000 g for 5 minutes. The fraction containing artificial LDs formed a floating white band at the top of the tube. The underlying solution and pellet were removed. This process was repeated until no pellet formed upon centrifugation. The white band was resuspended in 100µl buffer F and centrifuged at 1000g for 5 minutes. The solution underneath the floating white band was collected. The low-speed centrifuge was repeated until there is no white band formation after the centrifuge. The concentration of lipids in the final solution was determined by NBD fluorescence compared with the liposomes containing the same ratio of NBD- and Rh- lipids. The LDs were stable at 4°C for a week.

7.2 Positive staining of the artificial lipid droplets or liposomes: The positive staining was done as described before (Wang et al., 2016), Briefly, 8µl of purified artificial LDs or liposomes were loaded onto the glow-discharged carbon film coated grids (Electron Microscopy Sciences, CF400-CU) for 1min followed by blotting with filter paper to remove the extra sample. The sample was then fixed with 1% osmium tetroxide for 10 min and washed three times with deionized water. The sample was stained with 0.1% tannic acid for 5 min and 2% uranyl acetate for 5 min and washed with deionized water. The micrographs were recorded on a Talos microscope at 110kV with 36,000

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magnification at Yale Science Hill EM facility.

7.3 The transfer experiment was done as in step 6, except that 80μM lipids in donor LDs and 80μM lipids in acceptor liposomes, and 0.25μM proteins were used.

8 End-point lipid transfer assay

8.1 Protein expression and purification: hMIGA2(170-593)-6xhis and hMIGA2(306-593)-6xhis were used for the transfer assay. The expression and purification were the same as in step 4. The PS-specific probe (GST-$C2_{Lact}$) was purified as described before(Horenkamp et al., 2018; Moser von Filseck et al., 2015)

8.2 Liposome preparation: Light donor liposomes (63% DOPC, 30% liver PE, 2% fluorescently labeled lipids, and 5% DGS-NTA (Ni)) were prepared similarly as above except that either NBD-lipids or Rhodamine-PE, but not both, were included. The donor liposomes for the $C2_{Lact}$-PS-Probe-based assay were composed of 60% DOPC, 30% liver PE, 5% PS, and 5% DGS-NTA (Ni). For heavy acceptor liposomes, lipids in chloroform were mixed, dried, and vacuumed as above. The lipid film was dissolved in buffer F containing 0.75 M sucrose at a total lipid concentration of 1 mM and incubated at 37°C for 1 hour, vortexing every 10-15 min. Liposomes were subjected to 10 freeze-thaw cycles, and then mixed with 2 volumes of buffer F and pelleted for 40 min at 18,500 g. The resulting liposome pellet was resuspended in buffer F and pelleted again for 30 min. The second pellet was resuspended to 1 mM total lipid concentration in buffer F and used immediately.

8.3 Lipid transfer reactions were performed in **100 μL** volumes. The reaction was initiated by adding **250 micromolar (μM)** lipids each in light donor and in heavy acceptor liposomes into **0.32 micromolar (μM)** protein (tether and $hMIGA2_{long}$ or $hMIGA2_{C}$) and terminated by the addition of **10 μL** terminate cocktail (2 **2 mg/mL** proteinase K, **1.8 Molarity (M)** imidazole, **50 millimolar (mM)** EDTA). The complete digestion of the proteins was confirmed by SDS-PAGE gel. Light and heavy liposomes were then separated by centrifugation at 18,500 g for **00:15:00** and heavy liposomes recovered in the pellets were resuspended in **100 μL** buffer F. Fluorescence signal (NBD: ex 440 nm, em 514 nm; Rhodamine: ex 550 nm, em 590 nm) present in the pellet was determined using a Synergy H1 Multi-Mode Microplate Reader (Agilent). A separate standard curve of fluorescence intensity to the amount of fluorescent lipids was made for NBD-PS (both acyl or head-group modified) and Rhodamine-PE, and the amount of fluorescent lipids being transferred in the assay was

15m

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determined by the standard curve. For the C2lactadherin PS-probe-based transfer assay, after the reaction, 10x terminating cocktail (1.8M imidazole, 100mM EDTA) was added before the donor and acceptor liposomes were separated. Donor and acceptor fractions were analyzed by SDS-PAGE gel to quantify the PS probe in each of the fractions.

Cellular experiments: TAG measurement, imaging 1h 0m 30s

9 Lipid extraction and TAG measurement:

9.1 Cells in 10 cm dishes were treated with [M]**100 micromolar (µM)** OA for 30 minutes or 24 hours.

9.2 Cells were washed twice with PBS and collected in PBS with cell scrapers; 1/10 of the sample was set aside for protein quantification.

9.3 The protein amount was determined by Bradford (Bio-Rad) assay at 595nm absorbance:

- Resuspend the cell pellet in 50µl 0.1% Triton X-100. Freeze-thaw 3 times and incubate for 10min for sufficient lysing.
- Add 50µl PBS to dilute Triton X-100 to 0.05% to avoid interference. Spin down at 13k for 2 min. Take out the supernatant to a new tube.
- Add 140µl 20% Bradford solution into the well of the96-well plate (transparent). Prepare the standard BSA (2mg/ml, 1mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml, 0mg/ml with PBS).
- Add 10µl clear lysate to 140µl Bradford solution, and incubate at room temperature for 5min. Do a series. dilution of your sample if needed
- Read the absorbance at 595nm using a plate reader.

The standard curve of BSA protein concentration with the reading.

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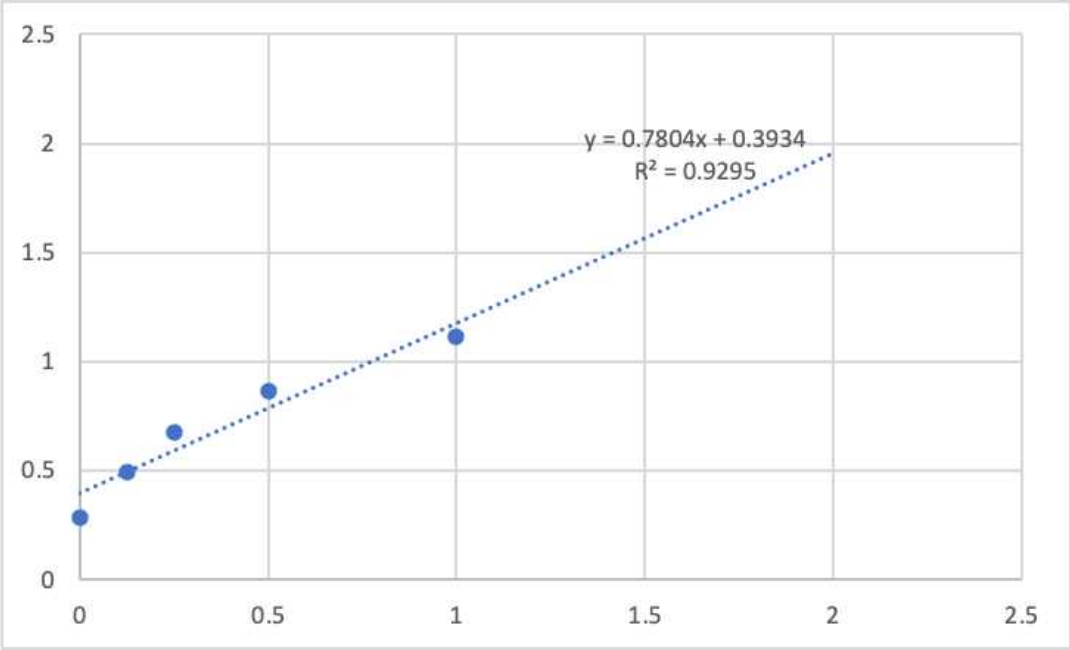

The X-axis is the BSA standard concentration at mg/ml. The Y-axis is the reading from the plate reader.

9.4 Lipid extraction: was done as in (Du and Yang, 2020). Briefly, the cell pellet was resuspended in hexane-isopropanol (3:2) solvent, then agitated at room temperature for **00:30:00** to extract lipids. The organic solvent was transferred into glass tubes and dry overnight in a chemical hood. 30m

9.5 ⚠ 30m 30s

Measure TAG content:

- if the organic solvent didn't evaporate completely overnight, dry with theN2 stream and put in the hood for ~minutes.
- Add 200µl methanol/methanol mixture (2:1) to redissolve the lipids. Vortex for **00:00:30** , put on ice.
- Transfer 50µl of the resuspended lipid solution into a 96-well plate. (add to the bottom). Dry the solvent by placing the plate on a 55°C heat block for **00:30:00** .
- Add 20µl isopropanol to redissolve the lipid film in the plate wells. Add 150µl enzyme mixture (cayman TAG assay kit). Mix. Add 1 well: 20µl isopropanol+ 150µl enzyme mixture. Incubate at **37 °C** for 30min. Protect from light. I saw a color change when adding the enzyme mixture.
- Measure the absorbance at 540nm using a plate reader.
- For quantification, the final value is µg of TAG per mg protein.

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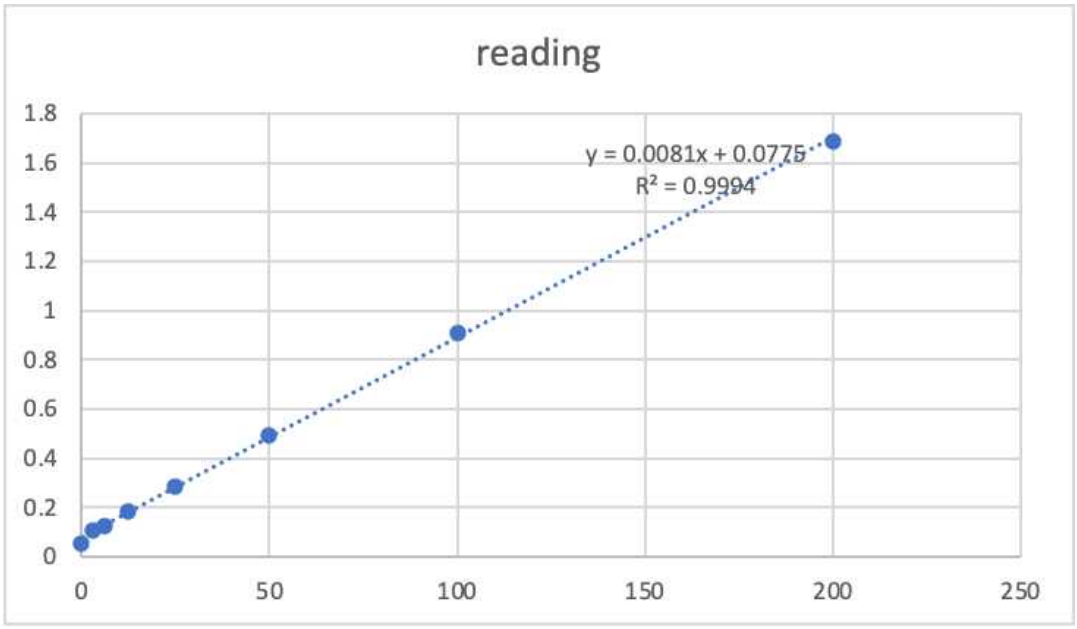
Standard curve



The standard curve of reading and the concentration of standard TAG. The X-axis is the concentration of TAG at mg/dl, the Y-axis is the reading.

10 Live cell imaging:

10.1 Cells were plated on the glass-bottomed 35mm Mattek dishes at the density of 40 k/dish.

Hela cells were cultured in DMEM (Thermo Fisher Scientific 11965092) supplemented with 10% FBS (Thermo Fisher Scientific 10438062) and 1% penicillin-streptomycin (Thermo Fisher Scientific 15140122) at 37°C in a 5% CO2 incubator. Cells were used in experiments before passage 5. DNA transfection was performed using Lipofectamine 3000 (Thermo Fisher Scientific, L3000015) according to the manufacturer's instructions.

10.2 Cells were transfected the next day of plating using Lipofectamine 3000.

10.3 After 1 day, cells were treated with 100 µM OA (CAYMAN, 29557) or the same

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amount of BSA control (CAYMAN, 29556) for 18-24 hours if imaging LDs.

10.4 48 hours after transfection, Cells were incubated with BODIPY 493/503 (Thermo Fisher Scientific #D3922) at 1µg/ml or MitoTracker Green (Thermo Fisher Scientific M7514) at 75-100 nM for 30 minutes, washed with PBS, and subjected to further live-cell imaging in 1x live cell imaging solution (Thermo Fisher Scientific, A14291DJ). For the 30-minute OA treatment, the cells were treated with 100µM OA for 30 minutes before staining and imaging.

10.5 For high throughput imaging of LDs, confocal microscopy was performed on Nikon Ti2-E inverted microscope with a 63x oil-immersion objective, CSUX1 camera Photometrics Prime 95B, Agilent laser 488 and 561 nm, and a temperature setting at 37 C by the Oko Lab control system. Z-stack images were taken. Images were acquired using Nikon Elements and analyzed in Fiji (ImageJ).

10.6 At the Center for Cellular and Molecular Imaging Facility at Yale, imaging was performed with a 63× oil-immersion objective on an inverted Zeiss LSM 880 laser scanning confocal microscope with AiryScan, using Zen Black acquisition software. It has a 63× oil-immersion objective lens with NA = 1.4. Z-stack images were taken. Images obtained with AiryScan were first processed using Airyscan Processing in Zen Black, and all images were analyzed in Fiji (ImageJ).

10.7 Different samples were imaged in one session with the same settings. All data categorizing mitochondrial morphology were scored blindly. For LDs analysis, The Z-stack was maximum projected and the images were set at the same threshold. LDs were analyzed after converting to binary.

10.8 Lipid droplet quantification: in Fiji (ImageJ) maximum project your z-stack images. In the images, the individual cells were identified based on the background, selected, and duplicated. The color was adjusted to only see the lipid droplets, but not any other backgrounds (the setting should be the same for all cells). The image is switched to 8-bit type and converted to mask. Then run analyze particles with "display summarize". The results will be shown in the new dialogue.

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