Supporting Information

Asymmetrical Distribution of Choline Phospholipids Revealed by Click Chemistry and Freeze-Fracture Electron Microscopy

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SUPPLEMENTARY METHODS

Reagents

Propargylcholine bromide used in the experiment was synthesized according to the same protocol and the purity was confirmed by ^{1}H NMR as described previously.[1] Myriocin (Cayman Chemical), Cy3-azide, biotin-azide (baseclick GmBH), mouse anti-biotin antibody (Jackson ImmunoResearch), rabbit anti-biotin antibody (Rockland), colloidal gold (10 nm)-conjugated protein A (PAG10; University of Utrecht Medical Center), and colloidal gold (10 nm)-conjugated goat anti-mouse IgG (GAM10; British BioCell) were purchased from respective suppliers.

Cells

Yeast strains used in this study are listed in Table S1. Deletion mutants were prepared as previously described.[2] Cells were cultured overnight in SC medium or YEPD containing 1 mM propargylcholine bromide and used in log phase.

Huh7 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) containing 10%(w/v) fetal bovine serum and 0.25 mM propargylcholine bromide at 37°C in a 5% CO_2/95% air incubator.

Thin layer chromatography

Yeast was cultured in SC medium supplemented with 1 mM choline or 1 mM propargylcholine for overnight. Total lipids that were extracted from spheroplasts were adsorbed with silica gel in chloroform:methanol (1:1) to remove lipid esters. They then underwent chromatography on HPTLC plates (Silica Gel 60, Merck) using chloroform-methanol-acetic acid-acetone-water (35:25:4:14:2) [3], and were charred using cupric acetate-phosphoric acid.

Lipids developed by thin layer chromatography were blotted to a polyvinylidene difluoride (PVDF) membrane (ATTO) as described.[4] The PVDF membrane was incubated in 0.1 M Tris-HCl buffer containing 1 mM $CuSO_4$, 0.1 M ascorbic acid, and 10 μM biotin-azide for 30 min to conjugate biotin to the propargyl residue. The membrane was blocked with 5% skim milk in PBS containing 0.1%(w/v) Triton X-100 (PBST) for 1 hr and incubated with mouse anti-biotin antibody in PBST for 1 hr followed by horseradish peroxidase-conjugated goat anti-mouse IgG antibody in PBST for 1 hr. The signal was detected by chemiluminescence and captured by Hyperfilm (GE Healthcare).

Fluorescence microscopy

Yeast cells were fixed in 3%(w/v) formaldehyde in 0.1 M PHEM buffer (20 mM PIPES, 50 mM HEPES, 20 mM EGTA, 4 mM $MgCl_2$, pH 6.9) for 1 hr, rinsed, and subjected to the click reaction for 10 min at room temperature. The reaction solution contained 1 mM $CuSO_4$, 0.1 M ascorbic acid, and 50 nM Cy3-azide in 0.1 M Tris-HCl (pH 8.5). After rinsing, the cells were observed using Axiophot2 microscope (Carl Zeiss) with an Apochoromat 63x objective lens.

Quick-freezing and freeze-fracture

A copper EM grid immersed with yeast pellet was sandwiched between a flat aluminum disc (Engineering Office M. Wohlwend) and a copper foil (20 μm thick), and frozen using an HPM 010 high-pressure freezing machine (Leica), according to the manufacturer's instructions. Huh7 cells were cultured on scratched gold foil (20 μm thick) and quick-frozen.[5]

The frozen specimens were transferred to the cold stage of a Balzers BAF 400 apparatus and freeze-fractured at –130°C under a vacuum of approximately 1×10^{-6} mbar. Replicas were made using electron-beam evaporation of carbon (C) (2-5 nm thick), followed by platinum/carbon (Pt/C) (2 nm) and C (20 nm) as previously described.[6]

Thawed replicas were treated with 2.5% SDS in 0.1 M Tris-HCl (pH 7.4) at 60°C overnight. Following SDS treatment, the yeast cell wall was removed using 1 mg ml^{-1} Zymolyase 20T in PBST, 1%(w/v) BSA, and a protease inhibitor cocktail (Nacalai) for 2 hr at 37ºC. After a further treatment in 2.5%(w/v) SDS at 60°C overnight, the replicas were rinsed and stored in buffered 50% glycerol at –20°C.

Freeze-fracture replica labeling

Freeze-fracture replicas were washed three times with 0.1 M Tris-HCl buffer (pH 8.5) containing 0.001%(w/v) Triton X-100 (TBST), treated with 2%(w/v) cold fish gelatin in TBST for 10 min, and then washed three times with TBST. The click reaction was performed by incubating the replicas in 0.1 M Tris-HCl buffer containing 1 mM $CuSO_4$, 0.1 M ascorbic acid, and 10 μM biotin-azide for 30 min at room temperature with gentle shaking. After washing five times with TBST, the replicas were rinsed once with PBST, blocked with 2%(w/v) cold fish gelatin in PBST for 30 min, and rinsed with PBS. They were incubated with mouse anti-biotin (10 μg ml^{-1}) in 1%(w/v) cold fish gelatin in PBS at 4°C overnight, rinsed five times with 0.1%(w/v) BSA in PBS, treated with GAM10 (1/30) or PAG10 (1/50–1/70) for 30 min at 37°C in 1%(w/v) BSA in PBS, and again rinsed five times with PBST. After brief rinses with distilled water, replicas were picked up on formvar-coated EM grids and observed with a JEM-1011 EM operated at 100 kV. Images were captured using a CCD camera (Gatan) and subjected to further analyses.

Statistical analysis

At least three independent experiments were performed for each analysis. The number of colloidal gold particles was counted manually and the area was measured using ImageJ (NIH). The labeling density in the selected structure was calculated by dividing the number of colloidal gold particles by the area. Because the absolute labeling density varied in different experimental sessions due to uncontrollable factors, such as yeast growth conditions, images from one representative experimental session were used for counting. For each structure, the labeling density for the respective structures was measured in at least 10 different randomly-taken micrographs. Statistical differences between samples were tested using the Mann–Whitney *U* test.

1. Jao, C.Y., Roth, M., Welti, R., Salic, A. (2009) Metabolic labeling and direct imaging of choline phospholipids in vivo, *Proc Natl Acad Sci U S A 106*, 15332-15337.
2. Keiser, C., Michaelis, S., Mitchell, A., *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1994.
3. Xu, G., Waki, H., Kon, K., Ando, S. (1996) Thin-Layer Chromatography of Phospholipids and Their Lyso Forms: Application to Determination of Extracts from Rat Hippocampal CA1 Region, *Microchem J 53*, 29-33.
4. Taki, T., Ishikawa, D. (1997) TLC blotting: application to microscale analysis of lipids and as a new approach to lipid-protein interaction, *Anal Biochem 251*, 135-143.
5. Fujita, A., Fujimoto, T., Ozato-Sakurai, N., Suzuki, H. (2012) A method for efficient observation of intracellular membranes of monolayer culture cells by quick-freeze and freeze-fracture electron microscopy, *J Electron Microsc (Tokyo) 61*, 441-446.
6. Fujita, A., Cheng, J., Fujimoto, T. (2010) Quantitative electron microscopy for the nanoscale analysis of membrane lipid distribution, *Nat Protoc 5*, 661-669.

Supporting Results:

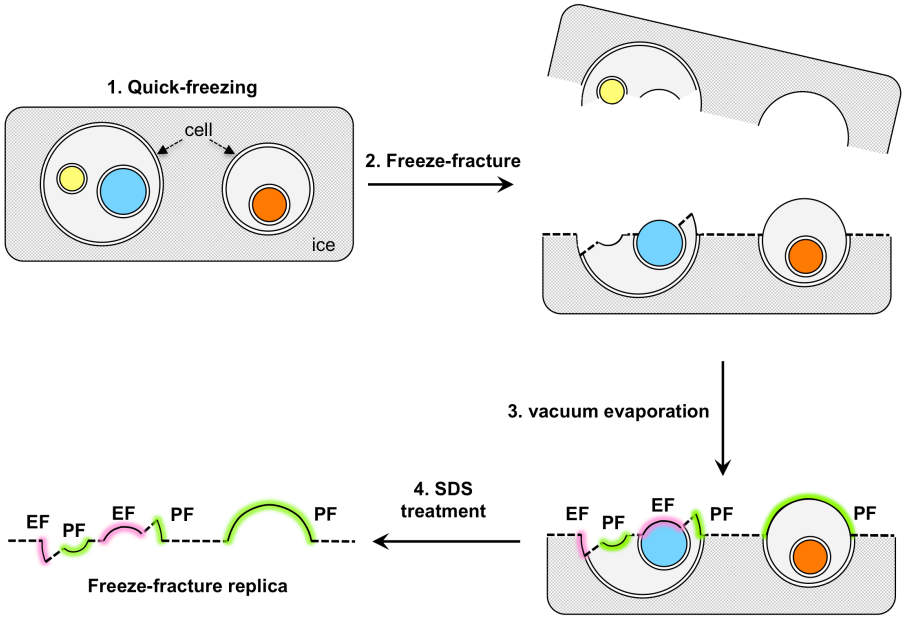

Figure S1. Outline and nomenclature in freeze-fracture EM.

In freeze-fracture, a phospholipid bilayer membrane is split into two phospholipid-monolayers, or half-membranes, exposing the hydrophobic interface. In a high vacuum better than 1 x 10^{-6} mbar, thin layers of carbon and platinum are deposited onto the fractured specimen by evaporation. The specimen (i.e., half-membranes backed up with carbon-platinum layers from the hydrophobic side) is taken to the atmosphere and treated with an SDS solution. This treatment removes extramembranous materials adhering to the hydrophilic surface (i.e., the true surface) of the membrane, making phospholipid head groups accessible to probes. In freeze-fracture EM, areas representing the exoplasmic (luminal) and cytoplasmic phospholipid leaflets are called the exoplasmic face (E face, EF) and the protoplasmic face (P face, PF), respectively.

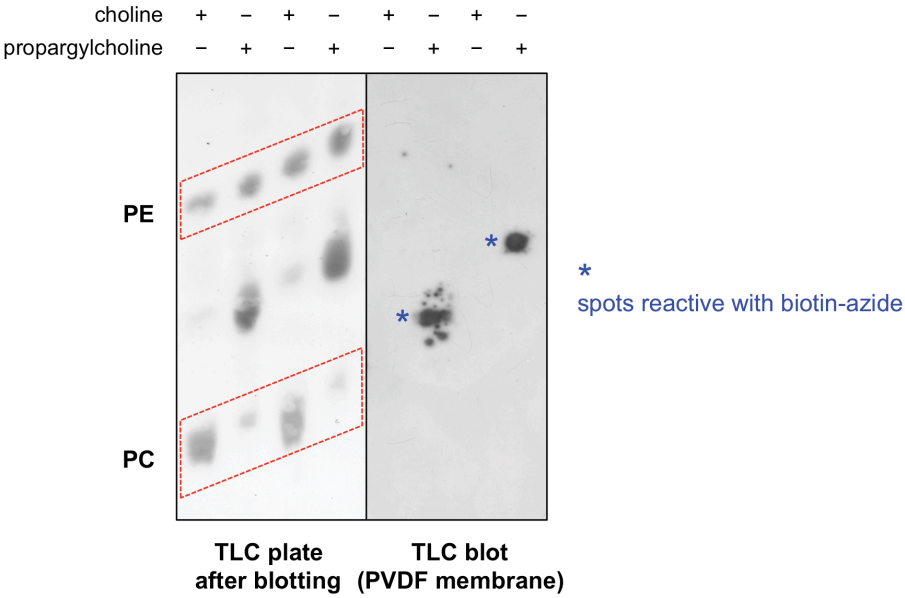

Figure S2. TLC blotting.

Total lipids of wild-type yeast cultured with 1 mM choline or 1 mM propargylcholine were developed by thin layer chromatography and transferred to a PVDF membrane. The membrane was reacted with biotin-azide to detect the propargyl residue. The positive reaction in the PVDF membrane (right) clearly corresponded to the upper spot that was observed only in the sample incubated with propargylcholine. The HPTLC plate after blotting was charred to show the position of phospholipids (left).

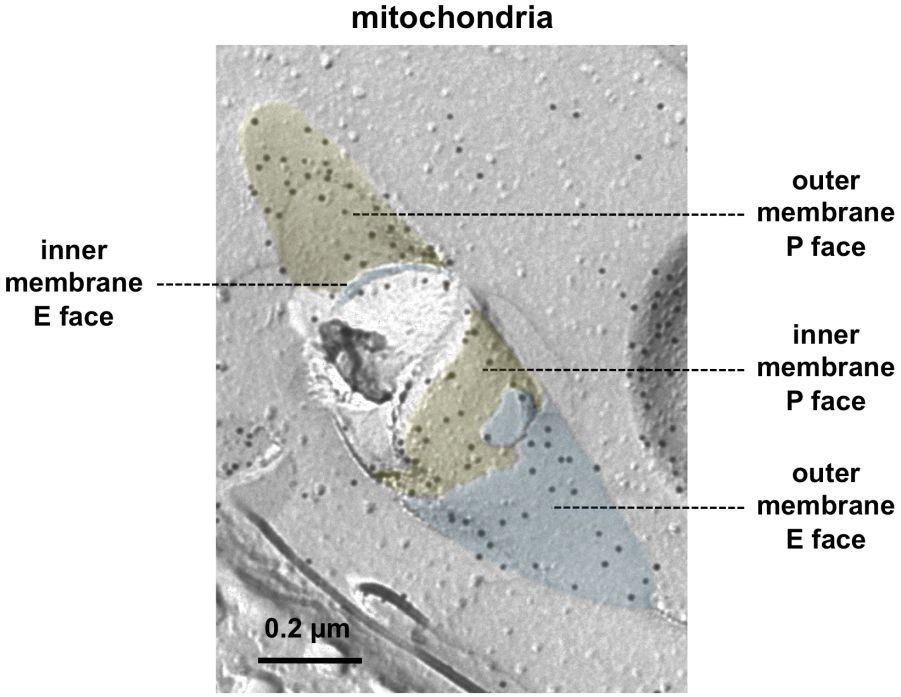

Figure S3. Cho-PL labeling in the yeast mitochondrial membrane.

The mitochondrion shown in the square bracket of Figure 2C is magnified. Representative samples of all four fracture faces, i.e., the P and E faces in the outer and the inner membranes, are presented.

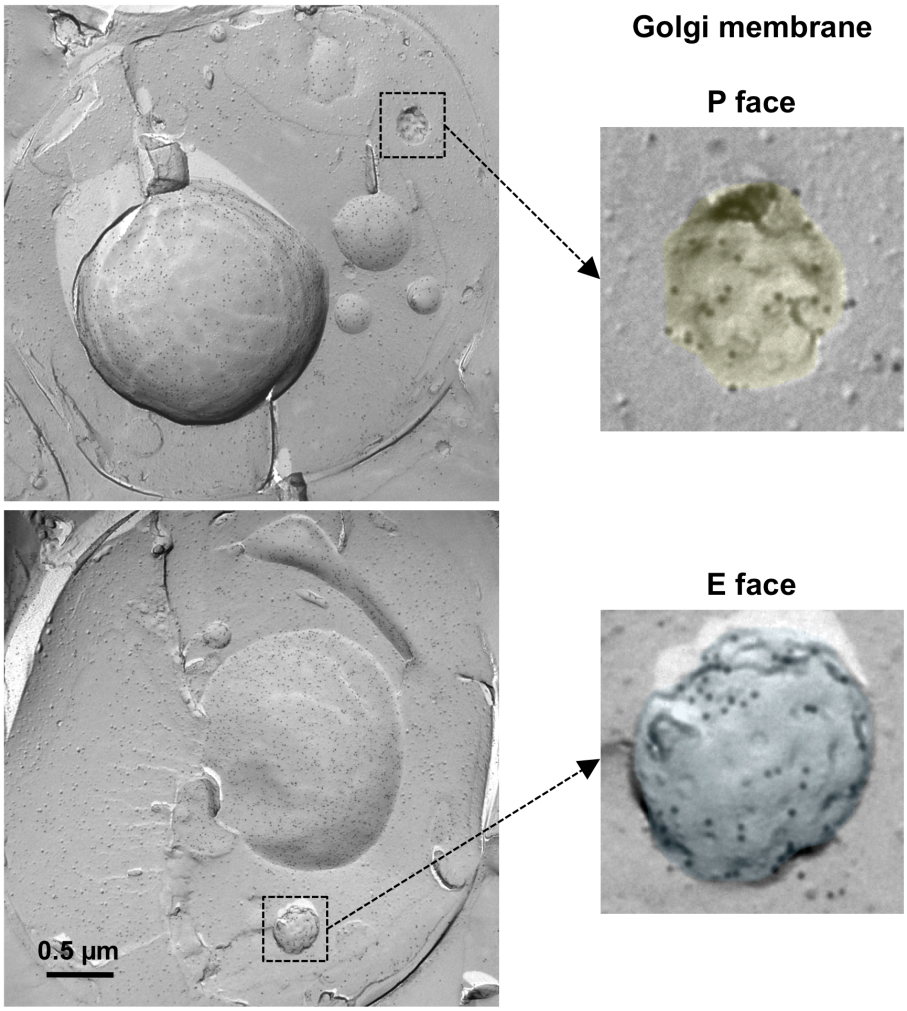

Figure S4. Intracellular disposition of the Golgi.

The Golgi structures in the dot squares are shown in a higher magnification in Figure 2D.

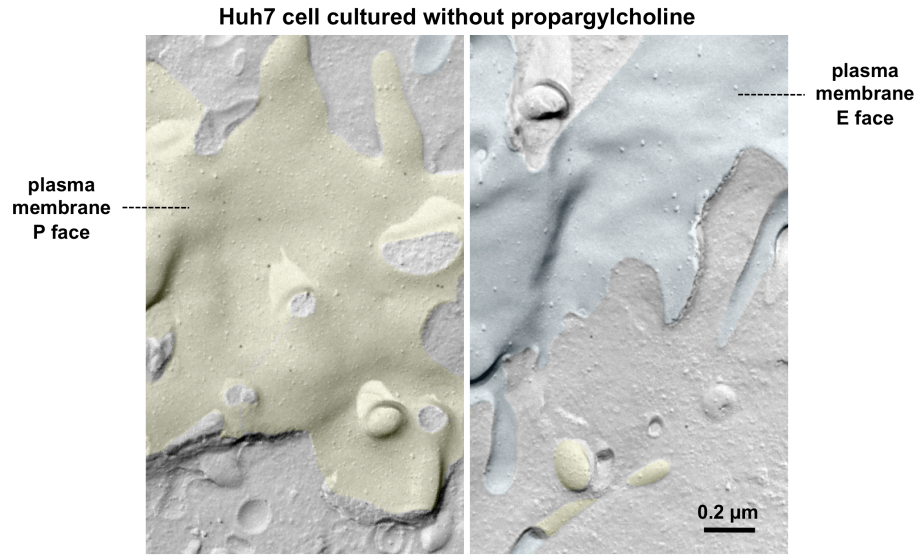

Figure S5. Control experiment in mammalian cells.

Huh7 cells cultured without propargylcholine were processed for the Cho-PL labeling in the freeze-fracture replica using the same procedure as that shown in Figure 3B. Labeling was virtually absent in this control sample, which verifies the specificity of the reaction.

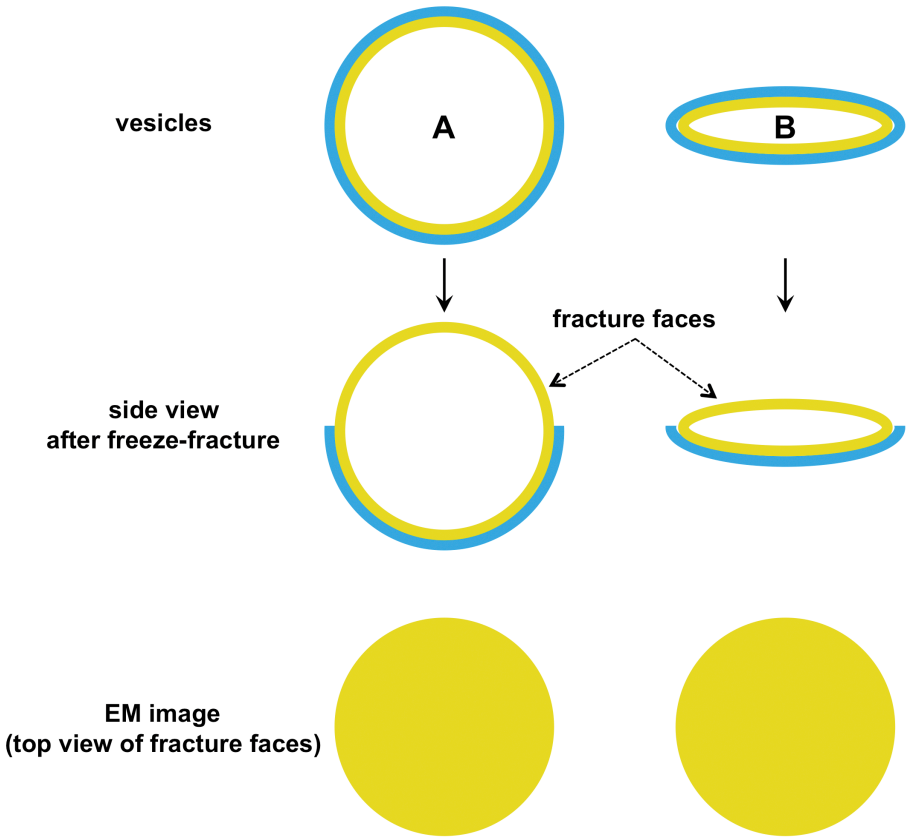

Figure S6. A possible bias in the measurement of membrane areas in EM images.
The area of fracture faces in the two vesicles (A, B) may appear the same in the two-dimensional EM image (top view of fracture faces), but the real area is larger for vesicle A than for vesicle B due to the difference in the curvature (side view).

Table S1. A list of yeast strains used in the present study

Name	Genotype	Reference/origin
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson et al., 1988
YT114	SEY6210; *cho2::kanMX6 opi3::hphNT1*	This study
YT113	SEY6210; *cki1::hphNT1 eki1::kanMX6*	This study
YT604	SEY6210; *sur1::hphNT1 csh1::kanMX6*	This study