# Supporting Information

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## SI Methods

**Method for Fig. S1.** NIH 3T3 cells were labeled overnight with 1 mM propargyl-Cho. The labeled cells were washed with PBS, incubated with PBS + 0.1% Triton X-100 for 5 min at room temperature, followed by formaldehyde fixation and staining with 10 $\mu$M Alexa568-azide. Control cells labeled with propargyl-Cho were processed identically, except that the detergent was omitted. The cells were counterstained with Hoechst (to reveal their DNA), and were imaged by fluorescence and DIC microscopy.

**Method for Fig. S9.** To verify that propargyl-Cho labels cellular phospholipids, NIH 3T3 cells were incubated with or without 500 $\mu$M propargyl-Cho overnight, followed by isolation of total lipids, and their separation by HPLC on a normal phase column. Lipids from propargyl-Cho-labeled cells show a prominent HPLC peak not found in control cells, while also containing less PC than the controls (in Fig. S9, compare panels *C* and *B*). To determine which lipid peaks contain Cho and propargyl-Cho, the HPLC-fractionated lipids were subjected to phospholipase D treatment (which hydrolyzes Cho head groups to Cho) followed by LC/MS detection of Cho and propargyl-Cho. This analysis showed that the additional peak seen in propargyl-Cho-labeled lipid samples contains propargyl-Cho but not Cho, that the PC peak contains Cho but not propargyl-Cho, and that Cho and propargyl-Cho were not released by PLD treatment of any of the other peaks. Interestingly, the intensity of the additional lipid peak from propargyl-Cho-labeled cells closely matches the decrease in intensity of the PC peak, suggesting that the new peak likely represents phosphatidyl-propargyl-Cho.

We measured what fraction of Cho is substituted by propargyl-Cho in NIH 3T3 cells labeled for 24 h with 0, 100, 250, or 500 $\mu$M propargyl-Cho added to normal media (which contains 30 $\mu$M Cho). Total lipids extracted from these cells were treated with PLD followed by quantitative LC/MS to measure the amount of Cho and propargyl-Cho present in the head groups of phospholipids. As shown in Fig. S9*D*, the incorporation of propargyl-Cho into phospholipids increases with its concentration in the media; 500 $\mu$M propargyl-Cho will label 48% of the total Cho-containing phospholipids after 24 h. The high level of propargyl-Cho incorporation is consistent with the strong signal observed by microscopy and with the results of total lipid analysis by electrospray ionization-tandem mass spectrometry. We also measured the labeling of 293T cells with propargyl-Cho after 24 h in normal media. Due to the higher cell cycle rate of 293T compared to NIH 3T3 cells, propargyl-Cho incorporation was even higher in 293T cells: at 250 $\mu$M propargyl-Cho, 36% of Cho head groups are replaced with propargyl-Cho, while at 500 $\mu$M propargyl-Cho, the degree of substitution reaches 80%.

Total lipids from NIH 3T3 cells labeled overnight with 500 $\mu$M propargyl-Cho and from untreated controls were isolated by methanol-chloroform extraction (1). The solvent was removed under reduced pressure and equal amounts of the total lipids were separated on an Agilent 1200 HPLC equipped with an evaporative light scattering detector (ELSD), using a 9.5 $\times$ 250 mm, 5 $\mu$m silica gel column, at a flow rate of 3 mL/min, as previously described (1). Lipid standards for 1,2-dioleoyl phosphatidylethanolamine (PE), bovine liver phosphatidylinositol (PI), 1,2-dioleoyl phosphatidylserine (PS), 1,2-dioleoyl phosphatidylcholine (PC), and chicken egg sphingomyelin (SM) were from Avanti Polar Lipids. Under the above conditions, the retention times were: 21.7 min for PE, 25.3 min for PI, 34.1 min for PS, 39.4 min for PC, and 43.5 min for SM. Phosphatidylcholine labeled with propargylcholine was detected as a broad peak with a retention time of 33.0 min.

To identify propargyl-Cho incorporated into phospholipids, HPLC fractions were collected and evaporated under reduced pressure. The dried lipid fractions were resuspended in water by sonication, adjusted to 60 mM sodium phosphate pH 7.4, 10 mM $CaCl_2$ and 10 U/mL phospholipase D (cabbage PLD, Sigma-Aldrich, P8398), and incubated at 37 °C for 1 h to release the phospholipid head groups. The samples were centrifuged at 10,000 rpm for 5 min and the supernatants were analyzed by LC/MS for the presence of Cho and propargyl-Cho ions, on an Agilent 6130 Quadrupole LC/MS system, using a 4.6 $\times$ 100 mm Luna C-18 column.

To measure propargyl-Cho incorporation into phospholipids, total lipids were isolated from NIH 3T3 or 293T cells labeled for 24 h with the indicated concentration of propargyl-Cho added to complete media. The total lipids were treated with PLD, followed by LC/MS analysis as above. Mass-to-charge ratios of 104 (Cho) and 128 (propargyl-Cho) were extracted from the mass spectra and their peaks integrated using Agilent Chemstation software. The peak areas were converted to nanomoles of Cho and propargyl-Cho using linear calibration curves obtained from standards of pure Cho and propargyl-Cho. These values were used to calculate the molar ratio between Cho and propargyl-Cho in the total lipid samples.

**Detailed Methods for Lipid Analysis by Electrospray Ionization-Tandem Mass Spectrometry.** ***ESI-MS/MS Lipid Profiling.*** Lipid profiling was performed by automated electrospray ionization-tandem mass spectrometry. Data acquisition and analysis, and acyl group identification were carried out as described previously (2, 3) with modifications. The samples were dissolved in 1 mL chloroform. An aliquot of 40 $\mu$L extract in chloroform was used. Precise amounts of internal standards, obtained and quantified as previously described (4), were added in the following quantities (with some small variation for different batches of internal standards): 0.60 nmol di12:0-PC, 0.60 nmol di24:1-PC, 0.60 nmol 13:0-lysoPC, 0.60 nmol 19:0-lysoPC, 0.30 nmol di12:0-PE, 0.30 nmol di23:0-PE, 0.30 nmol 14:0-lysoPE, 0.30 nmol 18:0-lysoPE, 0.30 nmol 14:0-lysoPG, 0.30 nmol 18:0-lysoPG, 0.30 nmol di14:0-PA, 0.30 nmol di20:0(phytanoyl)-PA, 0.20 nmol di14:0-PS, 0.20 nmol di20:0(phytanoyl)-PS, 0.23 nmol 16:0–18:0-PI, and 0.16 nmol di18:0-PI. The sample and internal standard mixture was combined with solvents, such that the ratio of chloroform/methanol/300 mM ammonium acetate in water was 300/665/35, and the final volume was 1.2 mL.

Unfractionated lipid extracts were introduced by continuous infusion into the ESI source on a triple quadrupole MS/MS (API 4000, Applied Biosystems). Samples were introduced using an autosampler (LC Mini PAL, CTC Analytics AG) fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30 $\mu$L/min.

Sequential precursor and neutral loss scans of the extracts produce a series of spectra, with each spectrum revealing a set of lipid species containing a common head group fragment. Lipid species were detected with the following scans: PC, SM, and lysoPC, $[M + H]^+$ ions in positive ion mode with Precursor of 184.1 (Pre 184.1); propargyl-PC (MPC), propargyl-SM (MSM), and propargyl-lysoPC (lysoMPC), $[M + H]^+$ ions in positive ion mode with Precursor of 208.1 (Pre 208.1); PE and lysoPE, $[M + H]^+$ ions in positive ion mode with Neutral Loss

of 141.0 (NL 141.0); PI, $[M + NH4]^+$ in positive ion mode with NL 277.0; PS, $[M + H]^+$ in positive ion mode with NL 185.0; and PA, $[M + NH4]^+$ in positive ion mode with NL 115.0. SM was determined from the same mass spectrum as PC (precursors of *m/z* 184 in positive mode) (5, 6) and by comparison with PC internal standards using a molar response factor for SM (in comparison with PC) determined experimentally to be 0.39. MPC and MSM were determined in comparison to the PC standards, without correction for response factor for MPC as compared to PC and using the same response factor for MSM as for SM. LysoMPC was determined in comparison to lysoPC internal standards. The scan speed was 50 or 100 u per sec. The collision gas pressure was set at 2 (arbitrary units). The collision energies, with nitrogen in the collision cell, were +28 V for PE, +40 V for PC (and SM) and their propargyl derivatives, +25 V for PI, PS and PA. Declustering potentials were +100 V. Entrance potentials were +15 V for PE, +14 V for PC (and SM) and their propargyl derivatives, PI, PA, and PS. Exit potentials were +11 V for PE, +14 V for PC (and SM) and their propargyl derivatives, PI, PA, and PS. The mass analyzers were adjusted to a resolution of 0.7 u full width at half height. For each spectrum, 9 to 150 continuum scans were averaged in multiple channel analyzer (MCA) mode. The source temperature (heated nebulizer) was 100 °C, the interface heater was on, +5.5 kV or -4.5 kV were applied to the electrospray capillary, the curtain gas was set at 20 (arbitrary units), and the two ion source gases were set at 45 (arbitrary units).

The background of each spectrum was subtracted, the data were smoothed, and peak areas integrated using a custom script and Applied Biosystems Analyst software. The lipids in each class were quantified in comparison to the two internal standards of that class. The first and typically every $11^{th}$ set of mass spectra were acquired on the internal standard mixture only. Peaks corresponding to the target lipids in these spectra were identified and molar amounts calculated in comparison to the internal standards on the same lipid class. To correct for chemical or instrumental noise in the samples, the molar amount of each lipid metabolite detected in the "internal standards only" spectra was subtracted from the molar amount of each metabolite calculated in each set of sample spectra. The data from each "internal standards only" set of spectra was used to correct the data from the following 10 samples. Finally, the data were corrected for the fraction of the sample analyzed and normalized to the sample "dry weights" to produce data in the units nmol/mg.


1. Stith BJ, et al. (2000) Quantification of major classes of *Xenopus* phospholipids by high performance liquid chromatography with evaporative light scattering detection. *J Lipid Res* 41:1448–1454.
2. Devaiah SP, et al. (2006) Quantitative profiling of polar glycerolipid species from organs of wild-type *Arabidopsis* and a phospholipase Dalpha1 knockout mutant. *Phytochemistry* 67:1907–1924.
3. Bartz R, et al. (2007) Lipidomics reveals that adiposomes store ether lipids and mediate phospholipid traffic. *J Lipid Res* 48:837–847.
4. Welti R, et al. (2002) Profiling membrane lipids in plant stress response. *J Biol Chem* 277:31994–32002.
5. Brugger B, Erben G, Sandhoff R, Wieland FT, Lehmann WD (1997) Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry. *Proc Natl Acad Sci USA* 94:2339–2344.
6. Liebisch G, Lieser B, Rathenberg J, Drobnik W, Schmitz G (2004) High-throughput quantification of phosphatidylcholine and sphingomyelin by electrospray ionization tandem mass spectrometry coupled with isotope correction algorithm. *Biochim Biophys Acta* 1686:108–117.

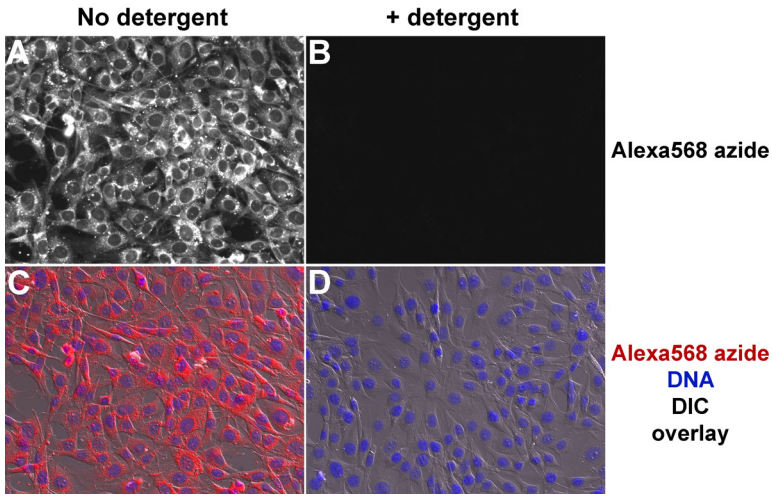


**Fig. S1.** Detergent sensitivity of the propargyl-Cho stain. The intensity of the propargyl-Cho signal (stained with Alexa568-azide) is greatly decreased if cells are permeabilized with detergent before fixation (*B* and *D*), compared to the no detergent control (*A* and *C*); this is consistent with propargyl-Cho incorporation into phospholipids. The propargyl-Cho stain was also greatly diminished if the detergent wash was performed after the azide reaction. The Alexa568 azide stain is shown in monochrome (*A* and *B*) or in red, overlayed with DNA (blue) and DIC micrographs (*C* and *D*).

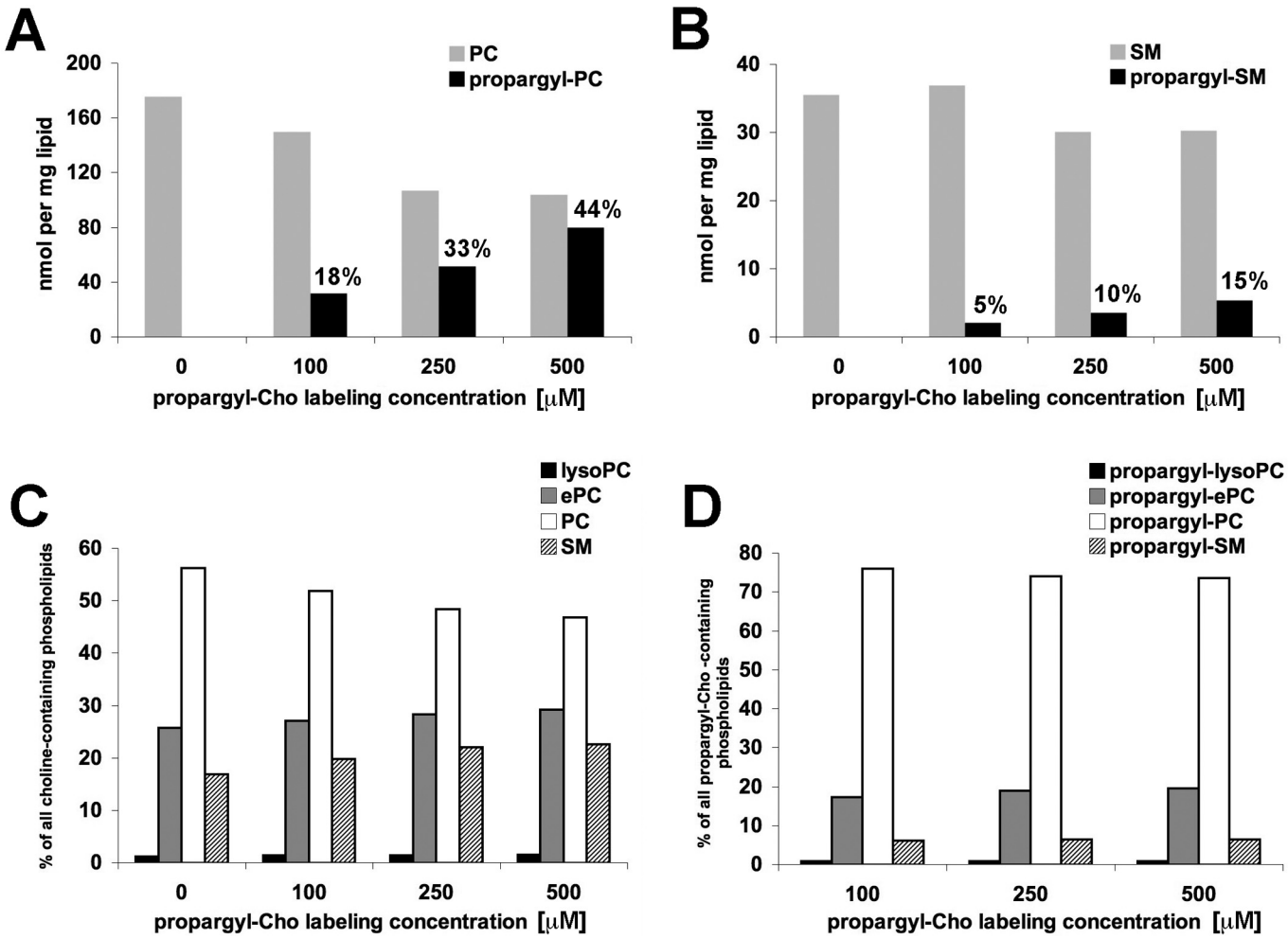


**Fig. S2.** Mass spectrometric quantitation of Cho and propargyl-Cho phospholipids by ESI-MS/MS, as a function of propargyl-Cho labeling concentration (NIH 3T3 cells labeled with 0, 100, 250, or 500 $\mu$M propargyl-Cho). (*A*) Relative amounts of total PC and total propargyl-PC as a function of propargyl-Cho concentration. ''Total PC'' refers to the sum of all PC species, including lysoPC, ePC, and PC. Similarly, ''Total propargyl-PC'' refers to the sum of all propargyl-PC species, including propargyl-lysoPC, propargyl-ePC, and propargyl-PC. Percentages shown indicate the percent of all PC species that contains propargyl-Cho, for a given labeling concentration. (*B*) Relative amounts of SM and propargyl-SM as a function of propargyl-Cho concentration. Percentages shown indicate the percent of SM that contains propargyl-Cho, for a given labeling concentration. (*C*) Distribution of Cho phospholipid classes as a function of propargyl-Cho concentration. (*D*) Distribution of propargyl-Cho phospholipid classes as a function of propargyl-Cho concentration.

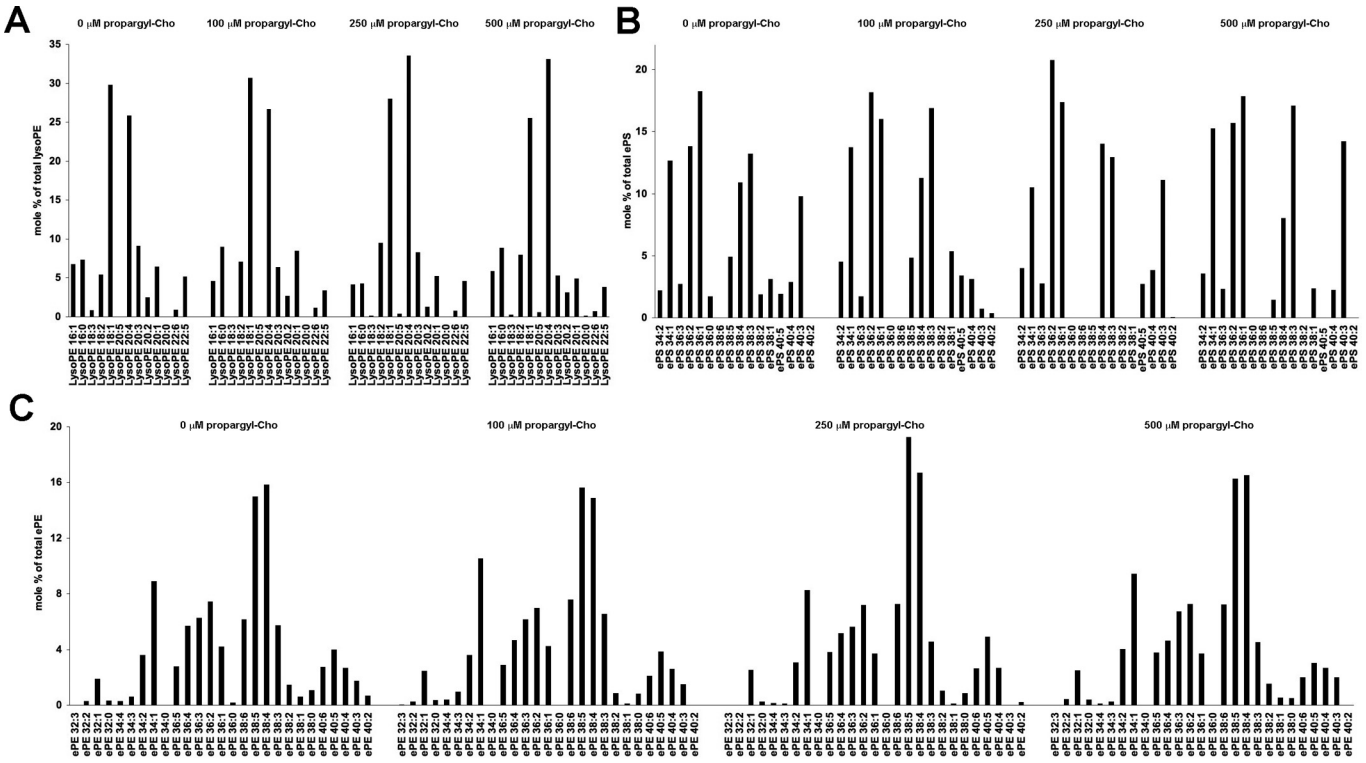


**Fig. S3.** Fatty acid composition of phospholipid species from NIH 3T3 cells labeled or not with propargyl-Cho. (*A*) Graph showing the fatty acid composition of lyso-phosphatidylethanolamine (lysoPE) species in NIH 3T3 cells labeled or not for 24 h with varying concentrations of propargyl-Cho in complete media. Each species is identified by two numbers: the first is the number of acyl carbons, and the second is the number of double bonds present in the fatty acid residue of the respective lyso-PE species. Propargyl-Cho labeling does not significantly change the natural fatty acid composition of lyso-PE. (*B*) Graph showing the fatty acid composition of ether-linked phosphatidylserine (ePS) species in NIH 3T3 cells labeled or not for 24 h with varying concentrations of propargyl-Cho in complete media. Each species is identified by two numbers: the first is the sum of carbons in the fatty acid and fatty alcohol side chains and the second is the sum of double bonds present in the two fatty acid and alcohol residues of the respective ePS species. Propargyl-Cho labeling does not significantly change the natural fatty acid composition of ePS. (*C*) Graph showing the fatty acid and fatty alcohol composition of ether-linked phosphatidylethanolamine (ePE) species in NIH 3T3 cells labeled or not for 24 h with varying concentrations of propargyl-Cho in complete media. Each species is identified by two numbers: the first is the sum of carbons in the fatty acid and fatty alcohol side chains and the second is the sum of double bonds present in the two fatty acid and alcohol residues of the respective ePE species. Propargyl-Cho labeling does not significantly change the natural fatty acid and fatty alcohol composition of ePE.

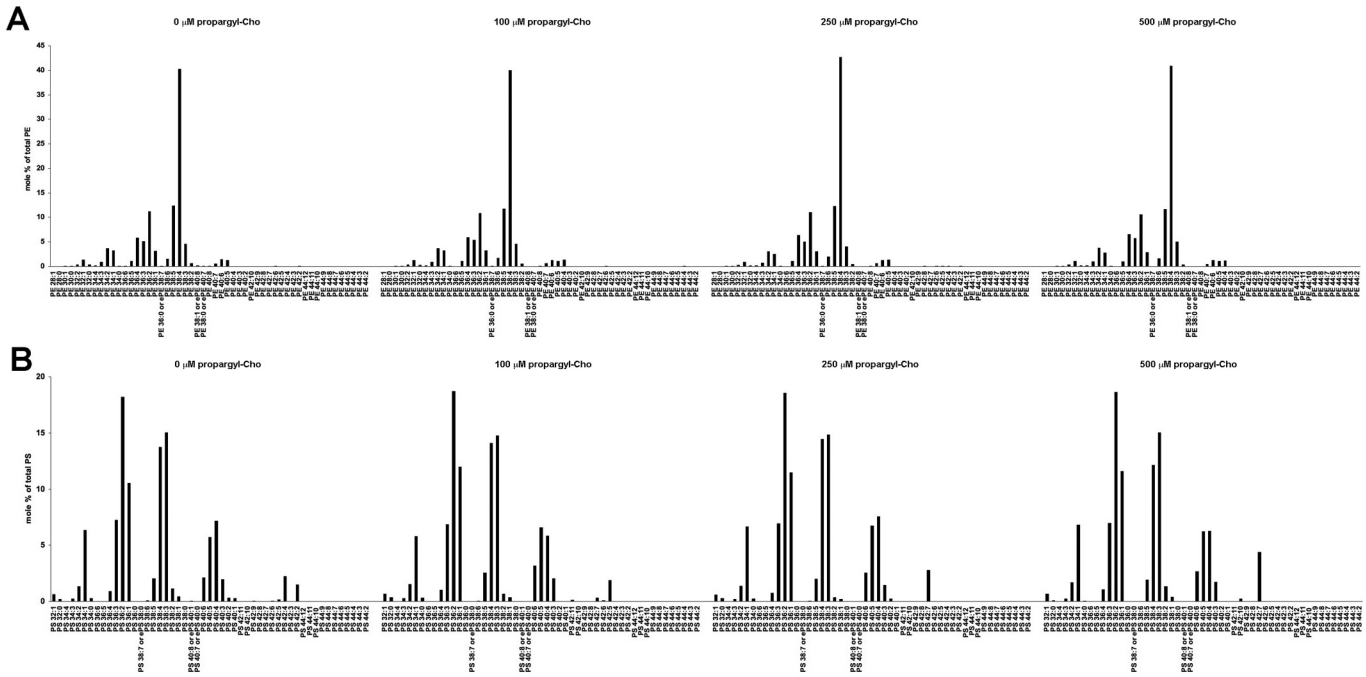


**Fig. S4.** Fatty acid composition of phospholipid species from NIH 3T3 cells labeled or not with propargyl-Cho. (*A*) Graph showing the fatty acid composition of phosphatidylethanolamine (PE) species in NIH 3T3 cells labeled or not for 24 h with varying concentrations of propargyl-Cho in complete media. Each species is identified by two numbers: the first is the sum of acyl carbons, and the second is the sum of double bonds present in the two fatty acid residues of the respective PE species. In some cases the mass spectrometric method used could not unambiguously distinguish between PE or ePE, due to identical *m/z* values. Propargyl-Cho labeling does not significantly change the natural fatty acid composition of PE. (*B*) Graph showing the fatty acid composition of phosphatidylserine (PS) species in NIH 3T3 cells labeled or not for 24 h with varying concentrations of propargyl-Cho in complete media. Each species is identified by two numbers: the first is the sum of acyl carbons, and the second is the sum of double bonds present in the two fatty acid residues of the respective PS species. In some cases the mass spectrometric method used could not unambiguously distinguish between PS or ePS, due to identical *m/z* values. Propargyl-Cho labeling does not significantly change the natural fatty acid composition of PS.

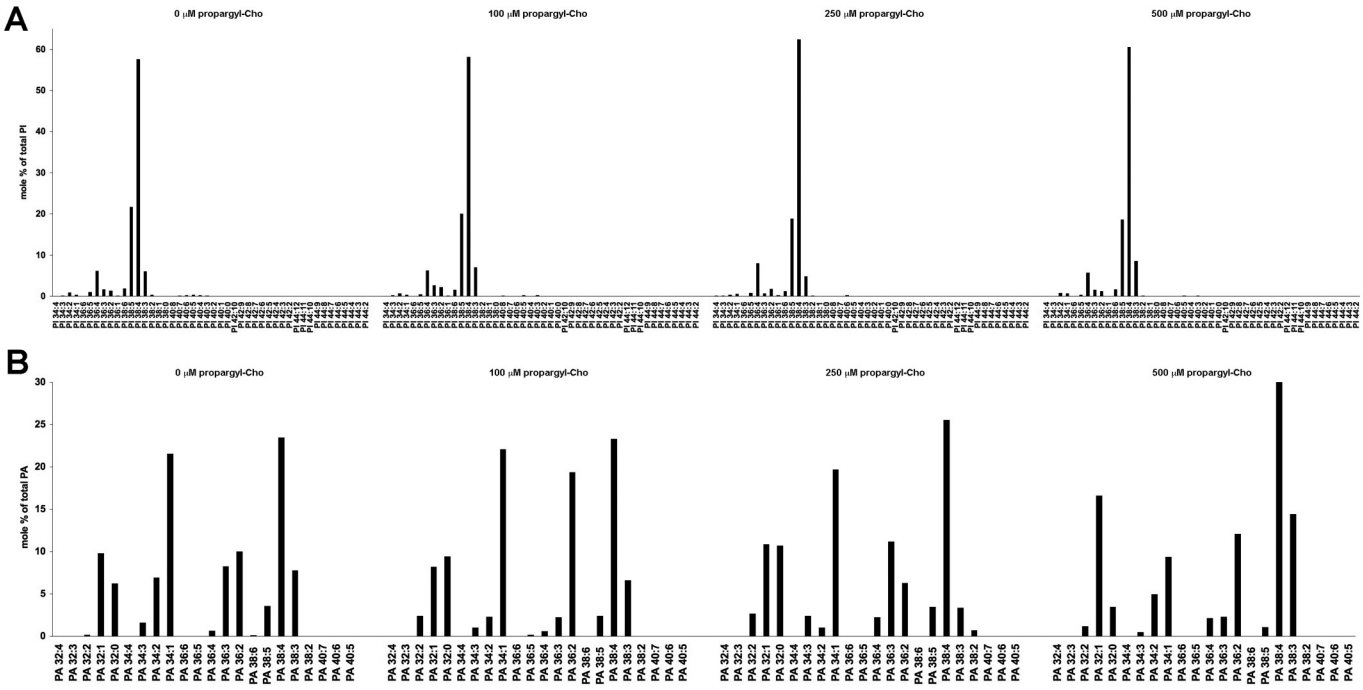


**Fig. S5.** Fatty acid composition of phospholipid species from NIH 3T3 cells labeled or not with propargyl-Cho. (*A*) Graph showing the fatty acid composition of phosphatidylinositol (PI) species in NIH 3T3 cells labeled or not for 24 h with varying concentrations of propargyl-Cho in complete media. Each species is identified by two numbers: the first is the sum of acyl carbons, and the second is the sum of double bonds present in the two fatty acid residues of the respective PI species. Propargyl-Cho labeling does not significantly change the natural fatty acid composition of PI. (*B*) Graph showing the fatty acid composition of phosphatidic acid (PA) species in NIH 3T3 cells labeled or not for 24 h with varying concentrations of propargyl-Cho in complete media. Each species is identified by two numbers: the first is the sum of acyl carbons, and the second is the sum of double bonds present in the two fatty acid residues of the respective PA species. Propargyl-Cho labeling does not significantly change the natural fatty acid composition of PA.

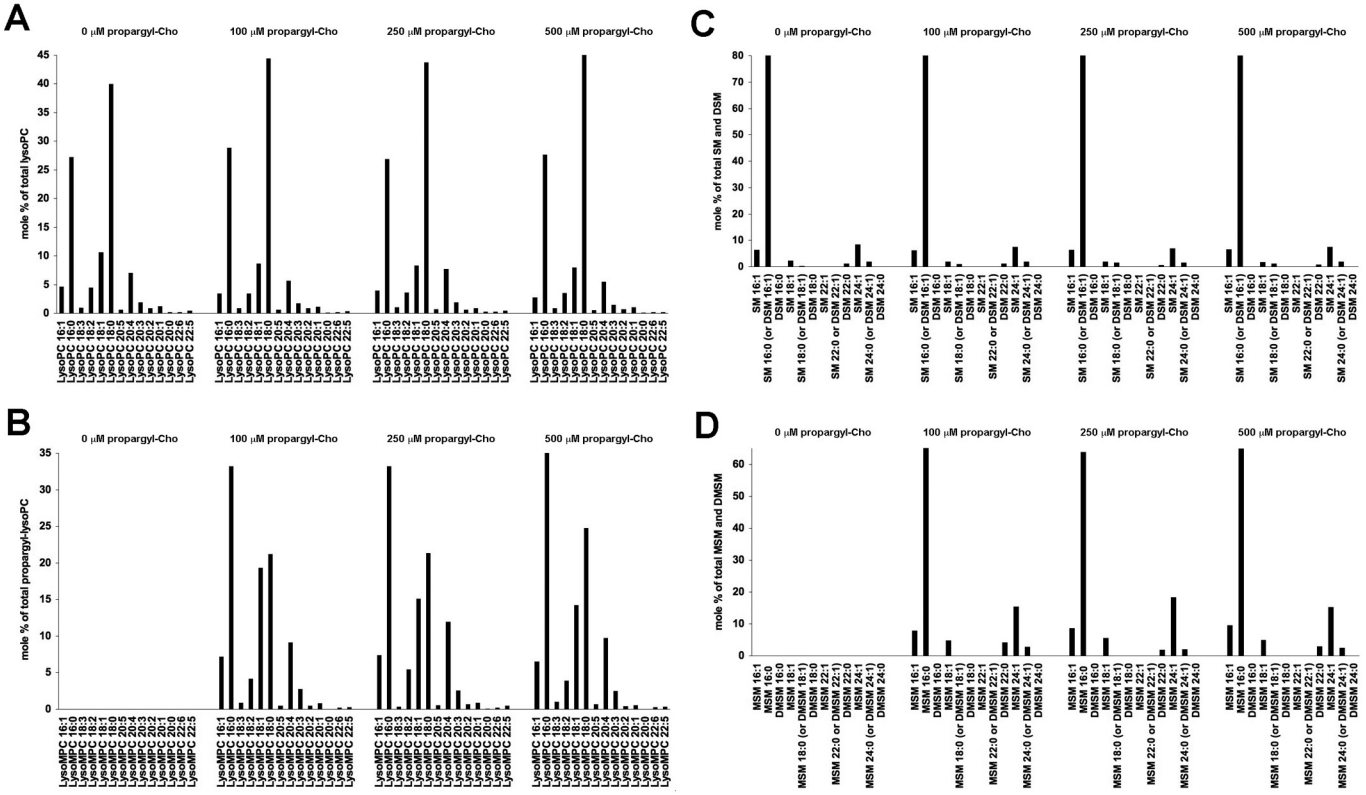


**Fig. S6.** Fatty acid composition of phospholipid species from NIH 3T3 cells labeled or not with propargyl-Cho. (*A*) Graph showing the fatty acid composition of lyso-phosphatidylcholine (lysoPC) species in NIH 3T3 cells labeled or not for 24 h with varying concentrations of propargyl-Cho in complete media. Each species is identified by two numbers: the first is the number of acyl carbons, and the second is the number of double bonds present in the fatty acid residue of the respective lysoPC species. Propargyl-Cho labeling does not significantly change the natural fatty acid composition of lysoPC. (*B*) Graph showing the fatty acid composition of propargyl lyso-phosphatidylcholine (propargyl-lysoPC) species in NIH 3T3 cells labeled for 24 h with varying concentrations of propargyl-Cho in complete media. Each species is identified by two numbers: the first is the number of acyl carbons, and the second is the number of double bonds present in the fatty acid residue of the respective propargyl-lysoPC species. The amount of each propargyl-lysoPC species is proportional to the concentration of propargyl-Cho used for labeling. The fatty acid composition of propargyl-lysoPC is very similar to that of lysoPC. (*C*) Graph showing the fatty acid composition of sphingomyelin (SM) or dihydrosphingomyelin (DSM) species in NIH 3T3 cells labeled or not for 24 h with varying concentrations of propargyl-Cho in complete media. Each species is identified by two numbers: the first is the number of acyl carbons, and the second is the number of double bonds present in the fatty acid residue of the respective SM species. Several species cannot be unambiguously identified as either SM or DSM, due to an inability to distinguish whether the double bond is on the fatty acid residue or the sphingosine. Propargyl-Cho labeling does not significantly change the natural fatty acid composition of SM. (*D*) Graph showing the fatty acid composition of propargyl sphingomyelin (propargyl-SM, or MSM) or propargyl dihydrosphingomyelin (propargyl-DSM, or DMSM) species in NIH 3T3 cells labeled for 24 h with varying concentrations of propargyl-Cho in complete media. Each species is identified by two numbers: the first is the number of acyl carbons, and the second is the number of double bonds present in the fatty acid residue of the respective SM species. Several species cannot be unambiguously identified as either propargyl-SM or propargyl-DSM, due to an inability to distinguish whether the double bond is on the fatty acid residue or the sphingosine. The amount of each propargyl-SM species is proportional to the concentration of propargyl-Cho used for labeling. The fatty acid composition of propargyl-SM is very similar to that of SM.

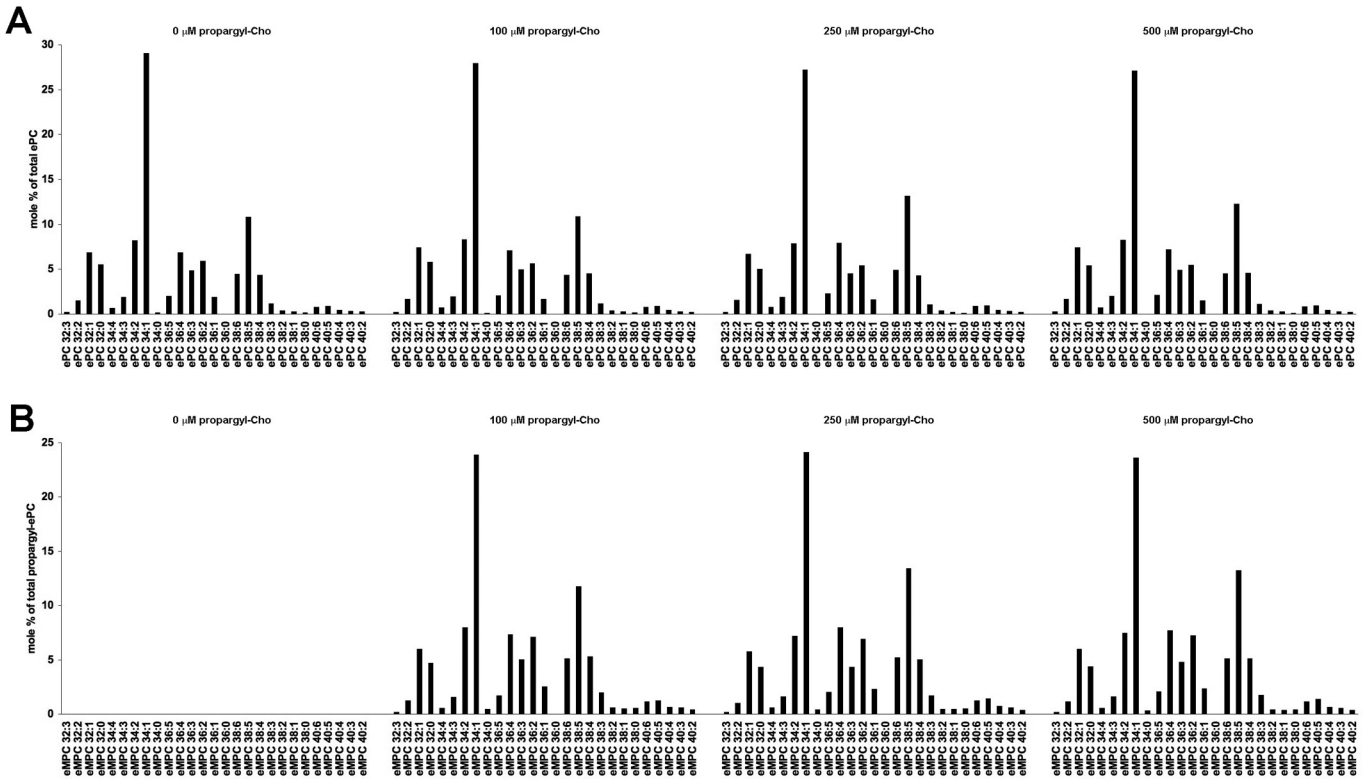


**Fig. S7.** Fatty acid composition of phospholipid species from NIH 3T3 cells labeled or not with propargyl-Cho. (*A*) Graph showing the fatty acid and fatty alcohol composition of ether-linked phosphatidylcholine (ePC) species in NIH 3T3 cells labeled or not for 24 h with varying concentrations of propargyl-Cho in complete media. Each species is identified by two numbers: the first is the sum of carbons in the fatty acid and fatty alcohol side chains and the second is the sum of double bonds present in the two fatty acid and alcohol residues of the respective ePC species. Propargyl-Cho labeling does not significantly change the natural fatty acid and fatty alcohol composition of ePC. (*B*) Graph showing the fatty acid composition of propargyl ether-linked phosphatidylcholine (propargyl-ePC) species in NIH 3T3 cells labeled or not for 24 h with varying concentrations of propargyl-Cho in complete media. Each species is identified by two numbers: the first is the sum of carbons in the fatty acid and fatty alcohol side chains and the second is the sum of double bonds present in the two fatty acid and alcohol residues of the respective propargyl-ePC species. The amount of each propargyl-ePC species is proportional to the concentration of propargyl-Cho used for labeling. The fatty acid composition of propargyl-ePC is very similar to that of ePC.

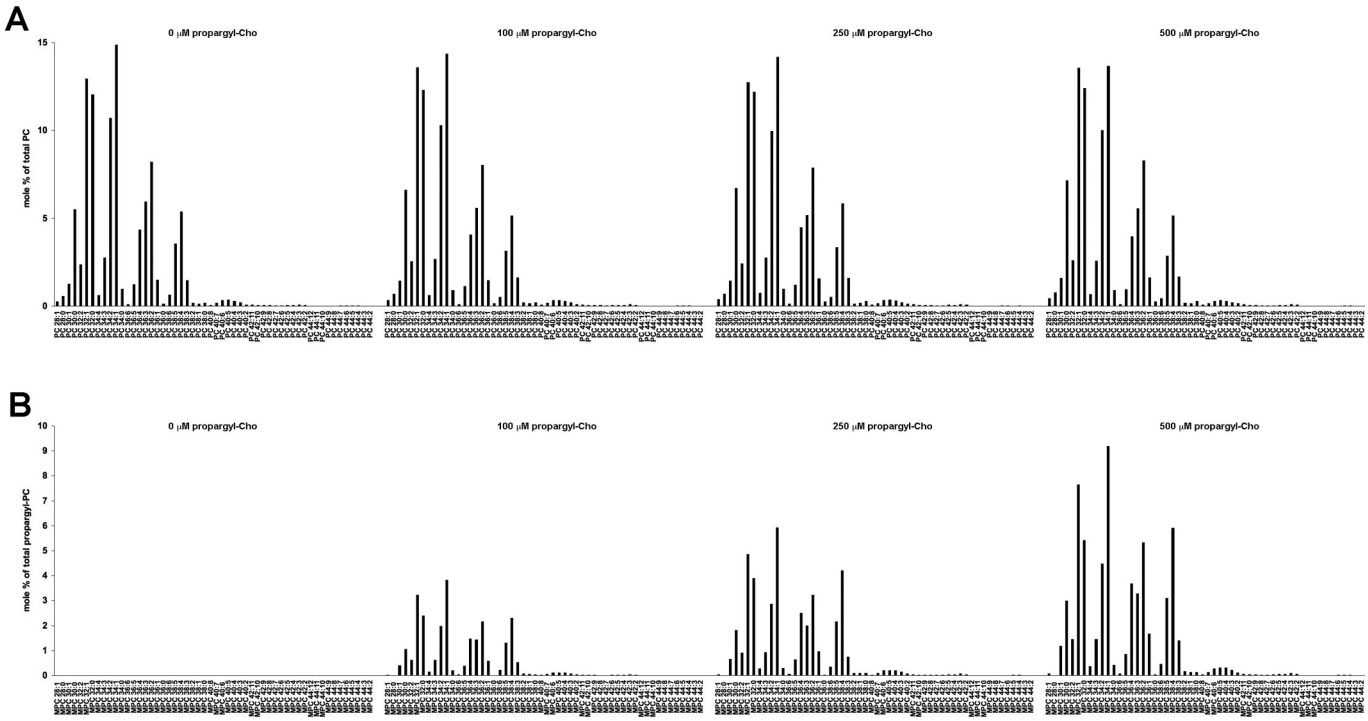


**Fig. S8.** Fatty acid composition of phospholipid species from NIH 3T3 cells labeled or not with propargyl-Cho. (*A*) Graph showing the fatty acid composition of phosphatidylcholine (PC) species in NIH 3T3 cells labeled or not for 24 h with varying concentrations of propargyl-Cho in complete media. Each species is identified by two numbers: the first is the sum of acyl carbons, and the second is the sum of double bonds present in the two fatty acid residues of the respective PC species. Propargyl-Cho labeling does not significantly change the natural fatty acid composition of PC. (*B*) Graph showing the fatty acid composition of propargyl phosphatidylcholine (propargyl-PC, or MPC) species in NIH 3T3 cells labeled or not for 24 h with varying concentrations of propargyl-Cho in complete media. Each species is identified by two numbers: the first is the sum of acyl carbons, and the second is the sum of double bonds present in the two fatty acid residues of the respective propargyl-PC species. The amount of each propargyl-PC species is proportional to the concentration of propargyl-Cho used for labeling. The fatty acid composition of propargyl-PC is very similar to that of PC.

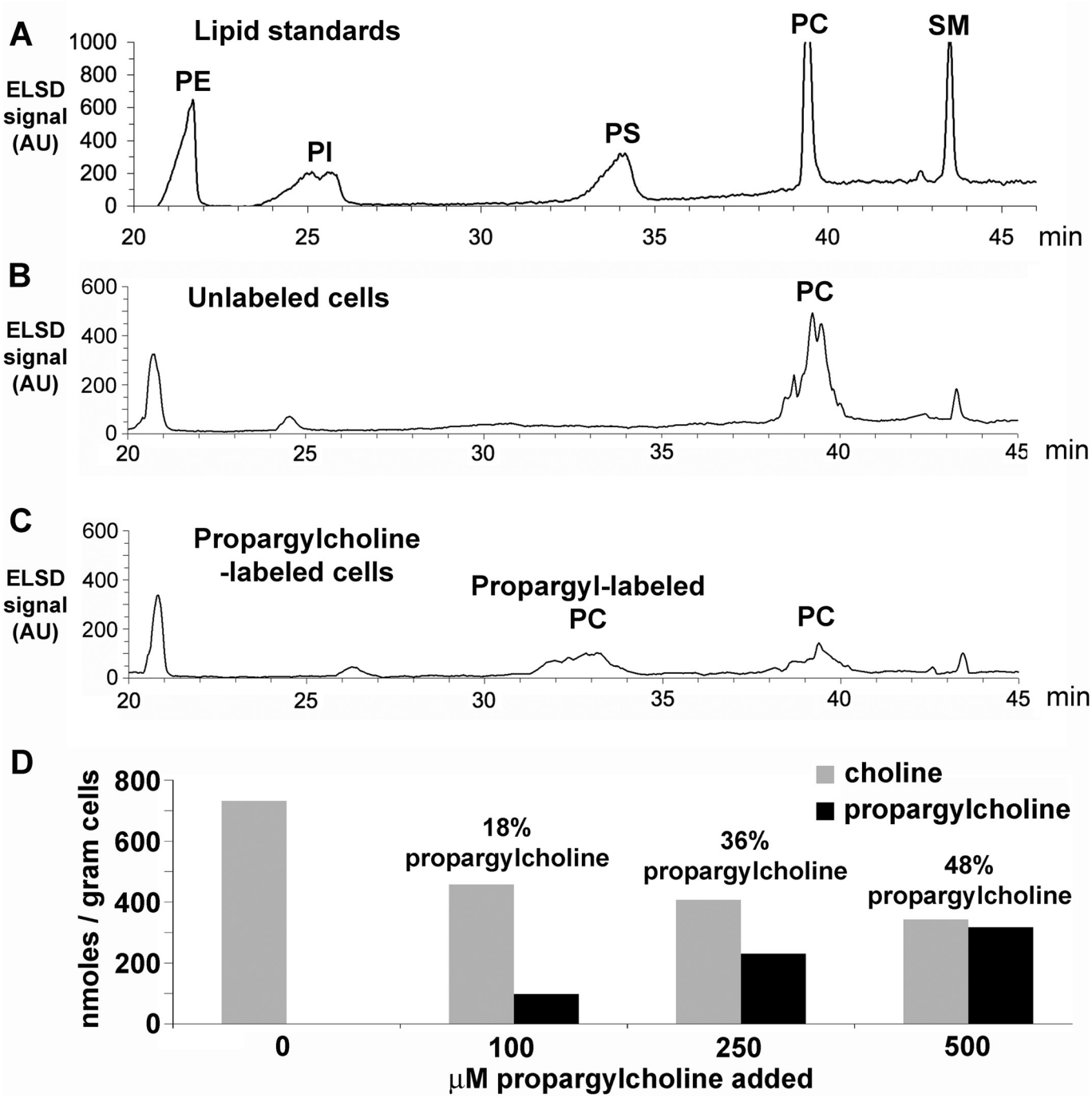


**Fig. S9.** Analysis of propargyl-Cho incorporation into cellular lipids by HPLC, phospholipase D digestion and liquid chromatography/mass spectrometry (LC/MS). (*A–C*) Lipid standards and total lipids isolated from cultured NIH 3T3 cells were separated by HPLC on a silica column. The signal from the evaporative light scattering detector (ELSD, in arbitrary units) is plotted against elution time (in minutes). (*A*) Separation of a mix of phospholipid standards: 1,2-dioleoyl phosphatidylethanolamine (PE), bovine liver phosphatidylinositol (PI), 1,2-dioleoyl phosphatidylserine (PS), 1,2-dioleoyl phosphatidylcholine (PC), and chicken egg sphingomyelin (SM). (*B*) Total lipids from unlabeled cells and (*C*) an equal amount of total lipids from NIH 3T3 cells labeled overnight with 500 $\mu$M propargyl-Cho, separated by HPLC. Note the decrease in the intensity of the PC peak in *C* compared to *B*, and the appearance in *C* of a new peak corresponding to propargyl-Cho-labeled PC. The presence of Cho and propargyl-Cho head groups was determined by treating the isolated peaks with PLD and the mass spectrometric identification of the released Cho and propargyl-Cho. (*D*) Graph showing that the degree of Cho substitution in the total phospholipids of NIH 3T3 cells increases with the concentration of propargyl-Cho in the media. The amount of Cho and propargyl-Cho in phospholipids was determined by phospholipase D digestion of total lipids and quantitative LC/MS analysis of released Cho and propargyl-Cho head groups.