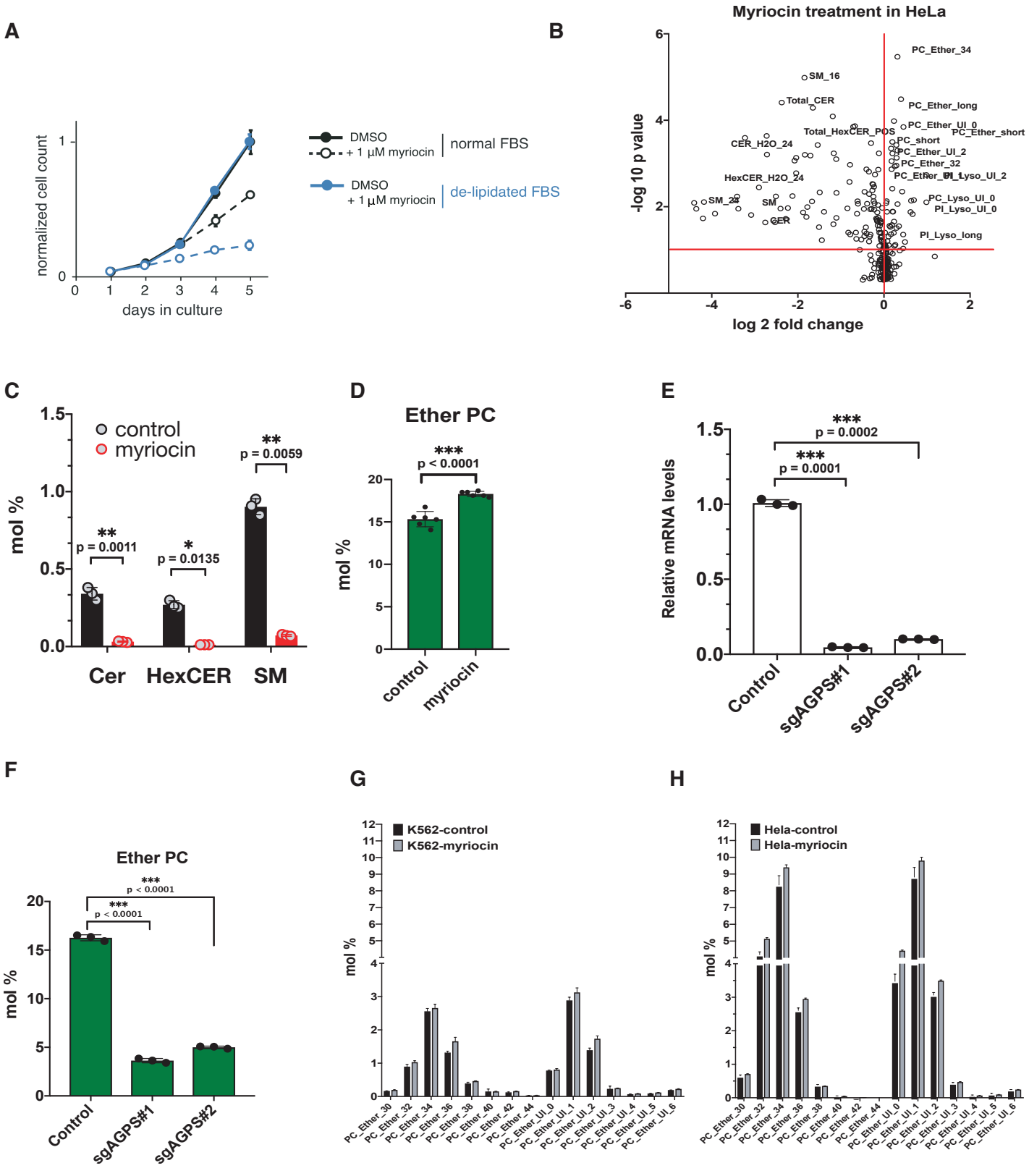

A
normalized cell count
days in culture
DMSO
+ 1 μM myriocin
normal FBS
DMSO
+ 1 μM myriocin
de-lipidated FBS
B
Myriocin treatment in HeLa
-log 10 p value
log 2 fold change
PC_Ether_34
SM_16
Total_CER
PC_Ether_long
PC_Ether_UI_0
Total_HexCER_POS
PC_Ether_short
CER_H2O_24
PC_short
PC_Ether_UI_2
PC_Ether_32
HexCER_H2O_24
PC_Lyso_UI_2
SM_24
SM
PC_Lyso_UI_0
PI_Lyso_UI_0
CER
PI_Lyso_long
C
control
myriocin
mol %
p = 0.0011
p = 0.0135
p = 0.0059
Cer
HexCER
SM
D
Ether PC
p < 0.0001
mol %
control
myriocin
E
p = 0.0002
p = 0.0001
Relative mRNA levels
Control
sgAGPS#1
sgAGPS#2
F
Ether PC
p < 0.0001
p < 0.0001
mol %
Control
sgAGPS#1
sgAGPS#2
G
K562-control
K562-myriocin
mol %
H
Hela-control
Hela-myriocin
mol %

Figure S1. Validation of the phenotypes found in the CRISPRi screening. Related to Figure 1 and Figure 2.

(A) Growth curves are shown for K562 cells grown in media containing normal or de-lipidated fetal bovine serum (FBS), and treated with 1 μM myriocin for the indicated time period. In both normal and delipidated conditions, cell counts are normalized to day 5 counts for respective untreated controls. Each point represents average $\pm$ standard deviation from 3 independent measurements. **(B)** Lipid changes in Hela cells after 3-day inhibition of sphingolipid synthesis with 1.5 μM myriocin. Log 2 of the fold change to control cells in the X-axis and the -log P value in the Y-axis (n=3). **(C)** Relative sphingolipid levels over the total of lipids detected and after 1.5 μM myriocin treatment in Hela cells (n=3). **(D)** Relative ether phosphatidylcholine (PC) levels upon myriocin treatment (n=6). **(E)** Knockdown efficiency of two different sgRNA targeting AGPS and measured by quantitative real-time PCR (qRT-PCR) (n=3). **(F)** Relative ether PC levels over the total of lipids detected in the two different AGPS CRISPRi cell lines (n=3). **(G)** mol% distribution of ether PC species in K562 cells (n=3). **h,** mol% distribution of ether PC species in Hela cells.

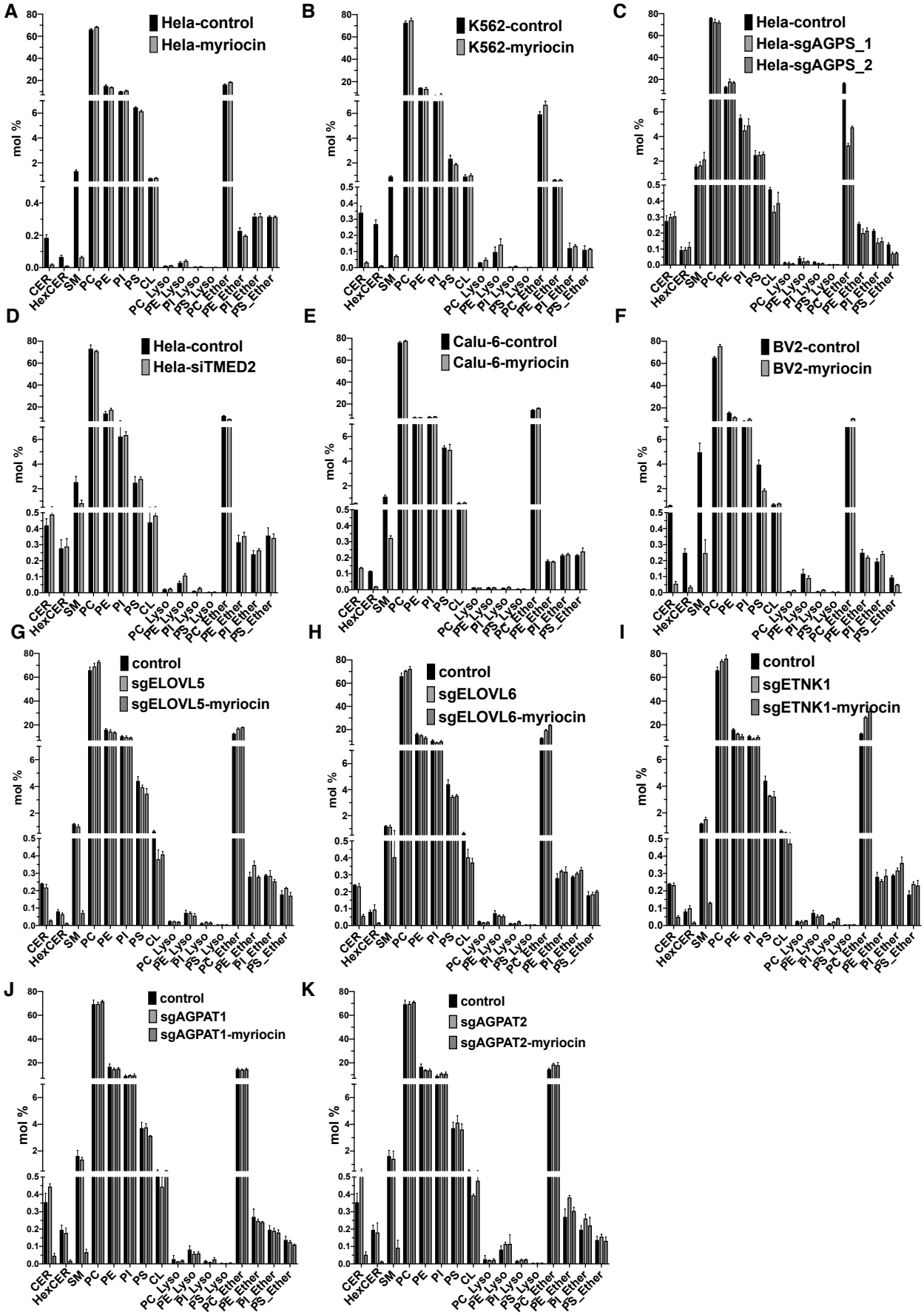

A
Hela-control
Hela-myriocin
B
K562-control
K562-myriocin
C
Hela-control
Hela-sgAGPS_1
Hela-sgAGPS_2
D
Hela-control
Hela-siTMED2
E
Calu-6-control
Calu-6-myriocin
F
BV2-control
BV2-myriocin
G
control
sgELOVL5
sgELOVL5-myriocin
H
control
sgELOVL6
sgELOVL6-myriocin
I
control
sgETNK1
sgETNK1-myriocin
J
control
sgAGPAT1
sgAGPAT1-myriocin
K
control
sgAGPAT2
sgAGPAT2-myriocin
mol %
CER
HexCER
SM
PC
PE
PI
PS
CL
PC_Lyso
PE_Lyso
PI_Lyso
PS_Lyso
PC_Ether
PE_Ether
PI_Ether
PS_Ether

Figure S2. Mol% distribution of major lipid classes. Related to Figure 2 and Figure 3 and Data S3. (A) Hela cells treated with vehicle or 1.5 uM myriocin. **(B)** K562 cells treated with vehicle or myriocin 1 uM. **(C)** AGPS knock-down CRISPRi cell lines (n=3). **(D)** TMED2 knock-down Hela cells (n=3). **(E)** Calu-6 cells treated with vehicle or 1.5 uM myriocin (n=3). **(F)** BV2 cells treated with vehicle or 1.5 uM myriocin. **(G)** ELOVL5 knock-down Hela cells treated with vehicle or 1.5 uM myriocin. **(H)** ELOVL6 knock-down Hela cells treated with vehicle or 1.5 uM myriocin. **(I)** ETNK1 knock-down Hela cells treated with vehicle or 1.5 uM myriocin. **(J)** AGPAT1 knock-down Hela cells treated with vehicle or 1.5 uM myriocin. **(K)** AGPAT2 knock-down Hela cells treated with vehicle or 1.5 uM myriocin. Note that the 100% is calculated summing up CER, HexCER, SM, PC, PE, PI, PS and CL. Please refer to Data S3 to see the different lipid groups.

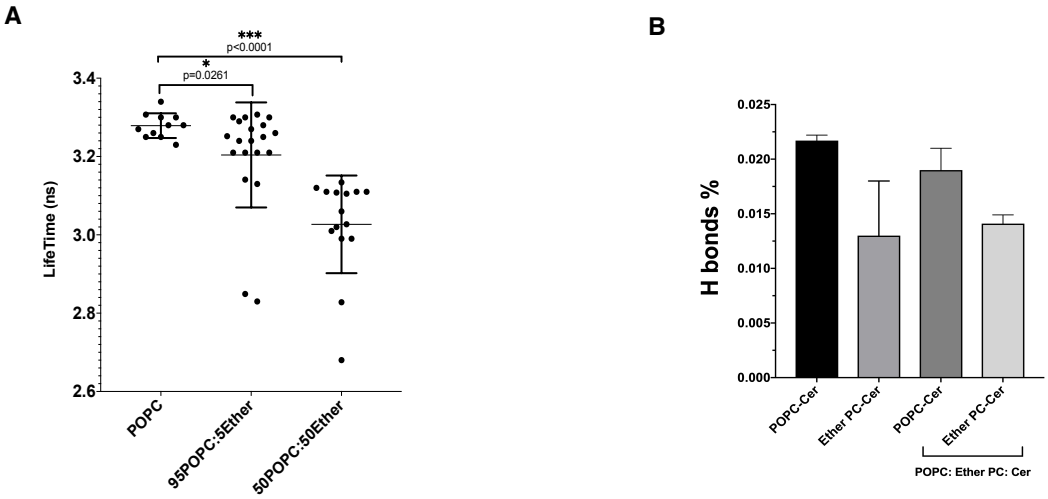


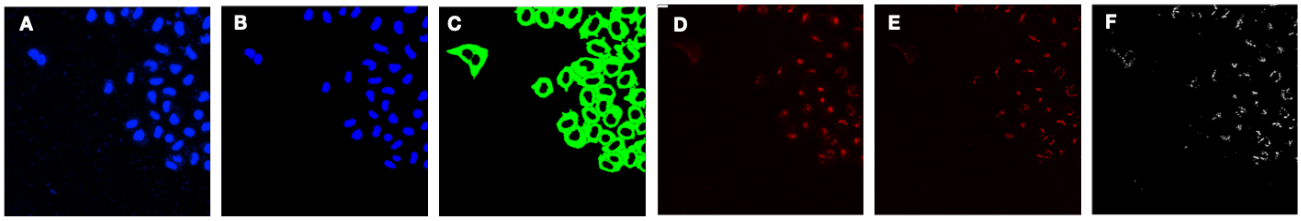
Figure S3. *In vitro* and *in silico* studies of mixtures of ether lipids and sphingolipids. Related to Figure 3.

(A) Fluorescence lifetime values of the FLIPPER-TR® probe as a function of lipid composition in giant unilamellar vesicles. GUVs were composed of a binary mixture of POPC + 5% Ether PC 24:1 as well as an equimolar mixture of POPC and ether PC. **(B)** Percentage of hydrogen bonds between POPC:Cer (80:20) or Ether PC:Cer (80:20) in different systems calculated from MD simulations.

Retention using selective hooks (RUSH): image analysis pipeline

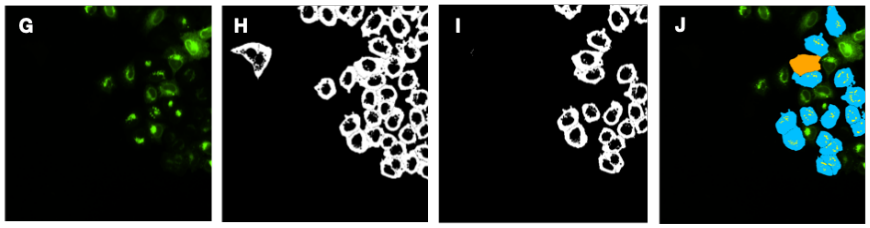
1. Cell body determination: the Hoechst 33342 channel is used to identify individual cells

2. Golgi segmentation: The Golgi is segmented after applying a top hat image modification (GM130, Cy5 channel) to lower the background signal and facilitate the segmentation.



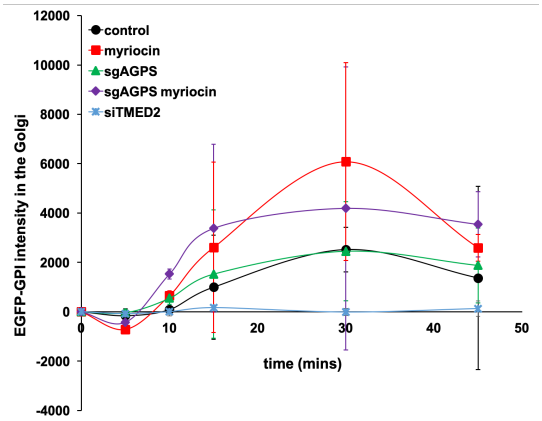
3. Selection of properly transfected cells: a filtering, based on a range of intensities in the GFP channel, is used to positively selected the transfected cells. Those are the only cells kept for further quantification.

4. Quantification of fluorescent cargo intensity in the Golgi per cell: the GFP intensity (average and integrated) in the Golgi mask is extracted for each positive cell. An average over all images in a well is used for the plotting.

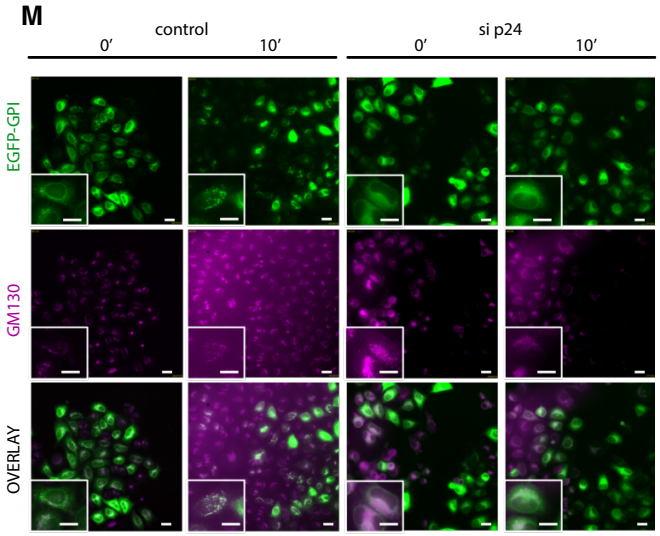


ObjectID	Integrated Intesity	Average Intensity
1	20703076	16419.54
2	2571141	2394.32
3	24884288	14243.64
4	12790525	6848.19

K



M



L

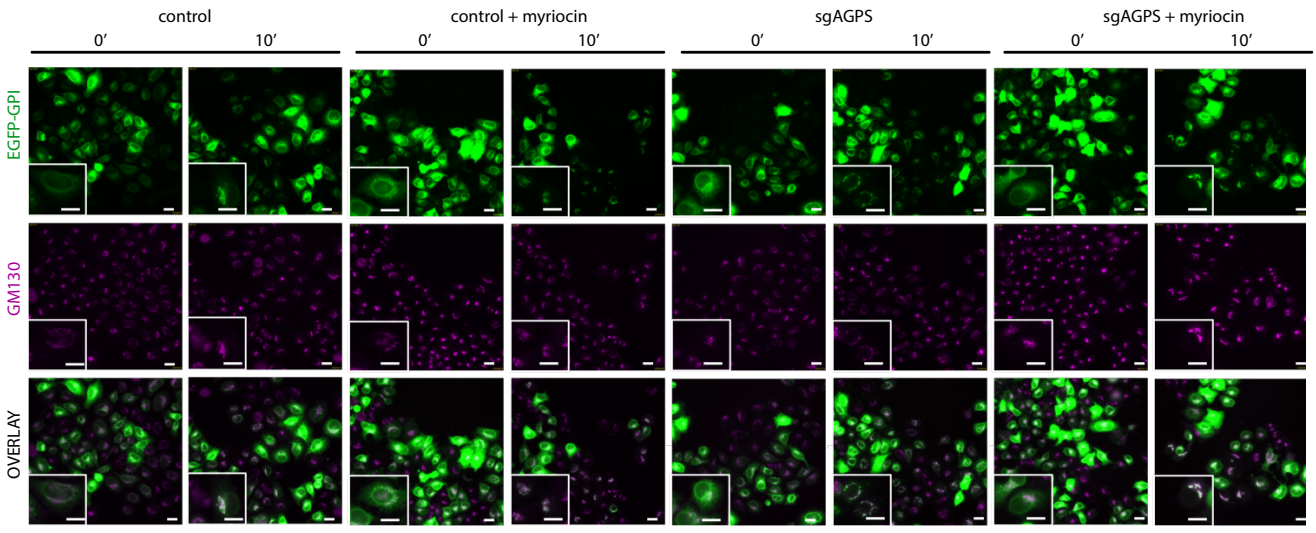


Figure S4. Image analysis pipeline for the RUSH data (retention using selective hooks). Related to Figure 5.

(A) Original image, Hoechst channel. **(B)** Nuclei mask. **(C)** Cell mask. **(D)** Original image, Cy5 channel (Golgi). **(E)** Image after applying a top hat modification to remove the background, Cy5 channel. **(F)** Golgi mask, Cy5 channel. **(G)** Original image, GFP channel (EGFP-GPI). **(H)** Cytoplasm mask. **(I)** Cytoplasm mask of selected cells for further analysis**. (J)** Original image, GFP channel, with the representative mask and a table with intensity values per cell in the image. **(K)** Kinetics of the export of GPI-anchored proteins over 45 minutes after biotin addition. **(L)** Representative microscopy images of the RUSH experiment in control Hela cells, cells where AGPS was knocked-down, cells treated with myriocin, and cells where both treatments are combined. 0'=before biotin addition; 10'= 10 minutes after biotin addition). **(M)** Representative microscopy images of the RUSH experiment in control Hela cells and cells where TMED2 was knocked-down. 0'=before biotin addition; 10'= 10 minutes after biotin addition).

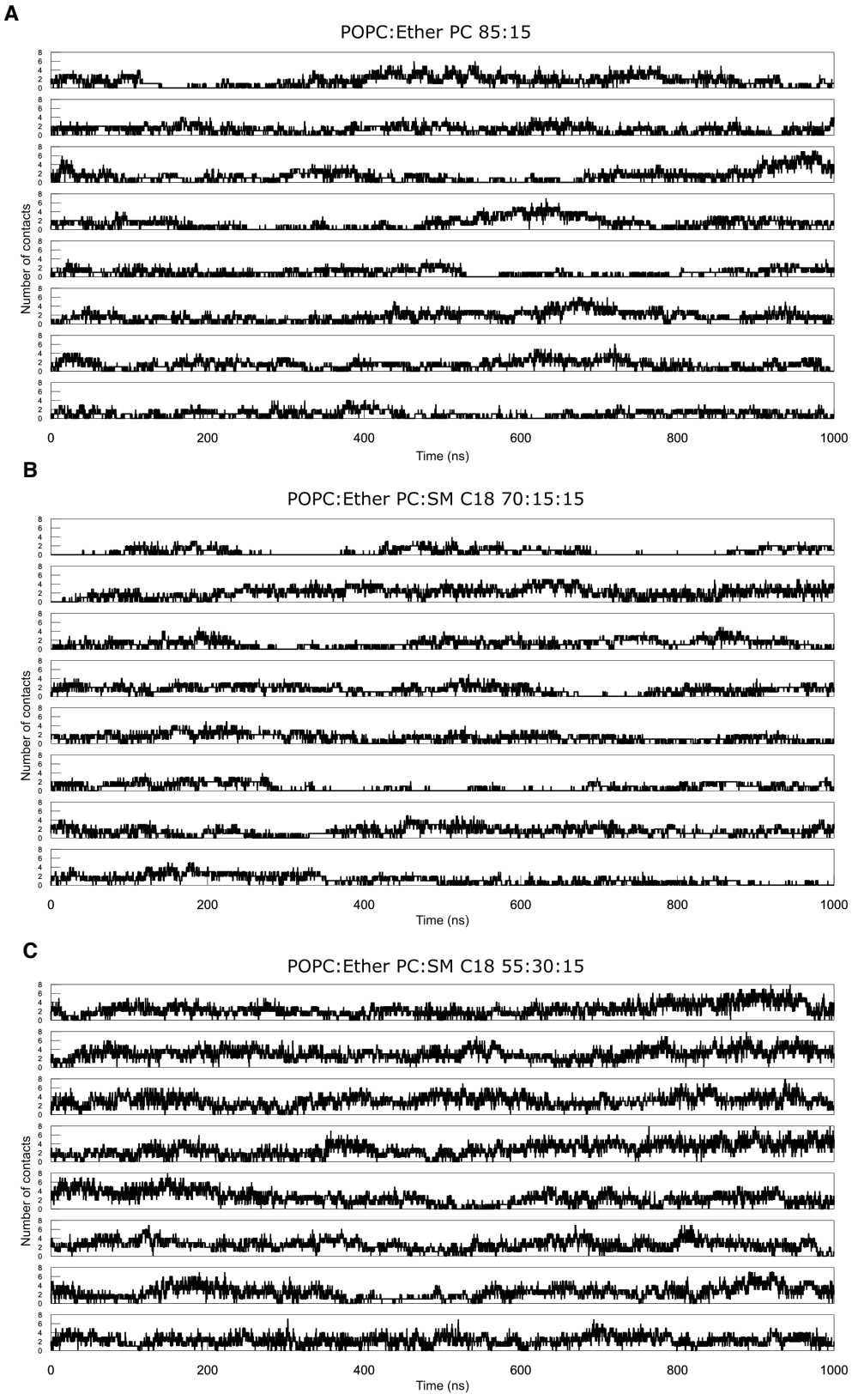

A
POPC:Ether PC 85:15
Number of contacts
0
200
400
600
800
1000
Time (ns)
B
POPC:Ether PC:SM C18 70:15:15
Number of contacts
0
200
400
600
800
1000
Time (ns)
C
POPC:Ether PC:SM C18 55:30:15
Number of contacts
0
200
400
600
800
1000
Time (ns)

Figure S5. Contacts between p24 TMD and EtherPC do not show phase transition behavior and are stable during the entire length of the simulations. Related to Figure 6. (A), (B) and (C) Number of contacts between phosphate atoms and the TMD of p24, using a cutoff of 0.7 nm, in POPC:EtherPC 85:15 or POPC:EtherPC:SMC18 70:15:15 or in POPC:EtherPC:SMC18 55:30:15 systems. Each row represents a different replica.

Lipid Class	Standard	Polarity	Mode	m/z ion	Collision Energy
Phosphatidylcholine $[M+H]^+$	DLPC	+	Product ion	184.07	30
Phosphatidylethanolamine $[M+H]^+$	PE31:1	+	Neutral ion loss	141.02	20
Phosphatidylinositol $[M-H]^-$	PI31:1	-	Product ion	241.01	44
Phosphatidyserine $[M-H]^-$	PS31:1	-	Neutral ion loss	87.03	23
Cardiolipin $[M-2H]^{2-}$	CL56:0	-	Product ion	acyl chain	32
Ceramide	C17Cer	+	Product ion	264.34	25
Dihydroceramide	C17DHCer	+	Product ion	266.40	25
Hexacylceramide	C8GC	+	Product ion	264.34	30
Hexacyldihydroceramide	C8GC	+	Product ion	266.40	30
Sphingomyelin	C12SM	+	Product ion	184.07	26

Table S1: Detection of Lipids by MS/MS (Related to Lipidome Analyses). Related to STAR Methods.

Composition	Ratio	Number of replicas	Length (ns)
POPC: Cer	80:20	2	200
ether PC: Cer	80:20	2	200
POPC: ether PC:Cer	40:40:20	2	200
POPC:ether PC + p24	85:15	8	1000
POPC:ether PC:SM C18+ p24	70:15:15	8	1000
POPC:ether PC:SM C18+ p24	55:30:15	8	1000

Table S2: List of all the MD setups, with bilayer composition, number of replicas and length of simulations. Related to Figure 6 and Figure S5.