Appendix:

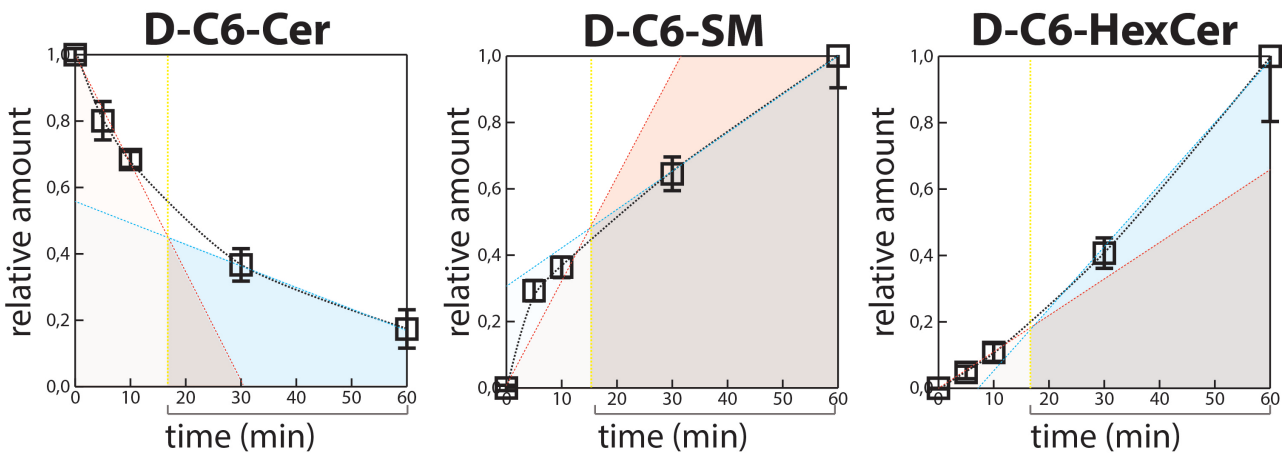
Sphingolipid metabolic flow controls phosphoinositide turnover at the *trans* Golgi network.

Serena Capasso, Lucia Sticco, Riccardo Rizzo, Marinella Pirozzi, Domenico Russo, Nina A. Dathan, Felix Campelo, Josse van Galen, Maarit Hölttä-Vuori, Gabriele Turacchio, Angelika Hausser, Vivek Malhotra, Isabelle Riezman, Howard Riezman, Elina Ikonen, Chiara Luberto, Seetharaman Parashuraman, Alberto Luini, and Giovanni D'Angelo

Contains:

Appendix figures:

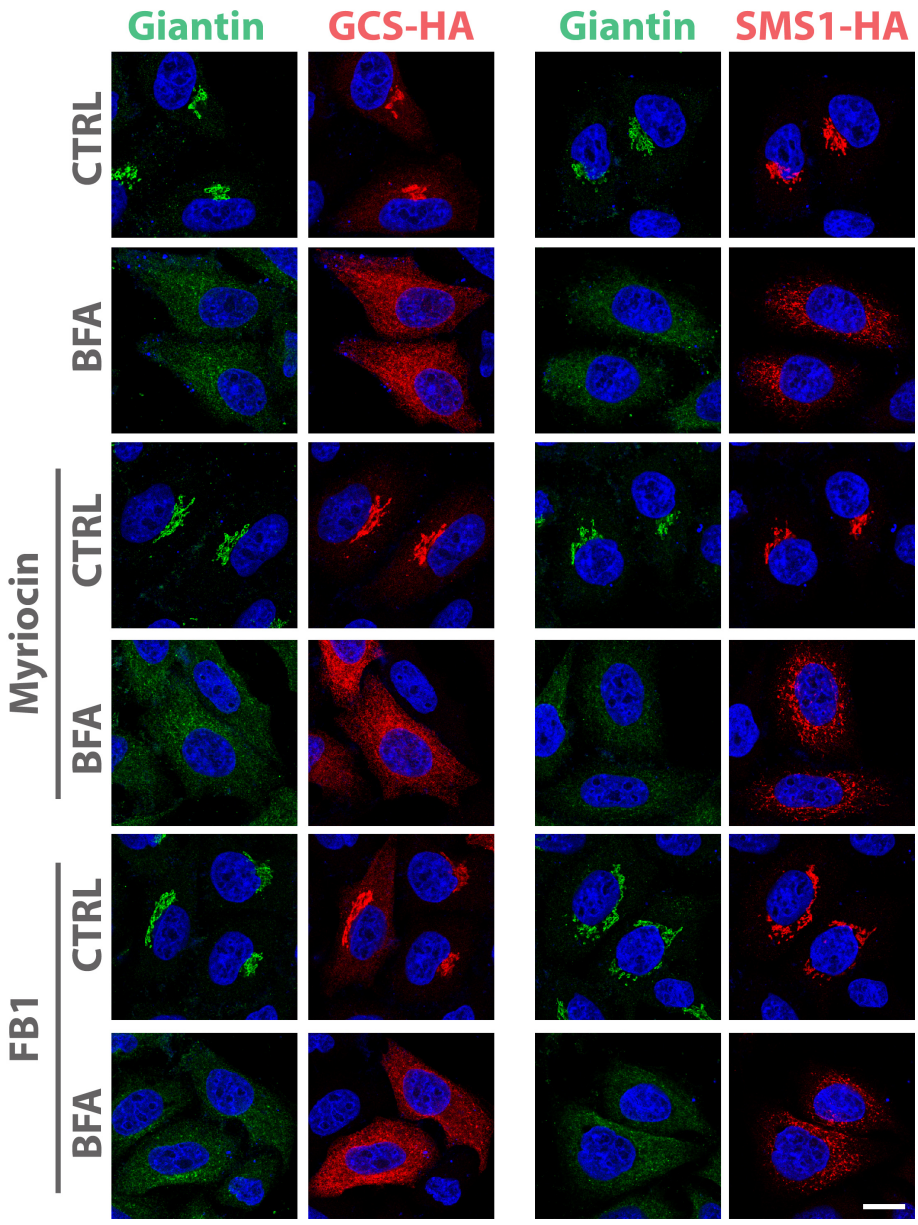
Appendix Figure S1



Appendix figure S1: *Dynamics of cell response to SL flow.*

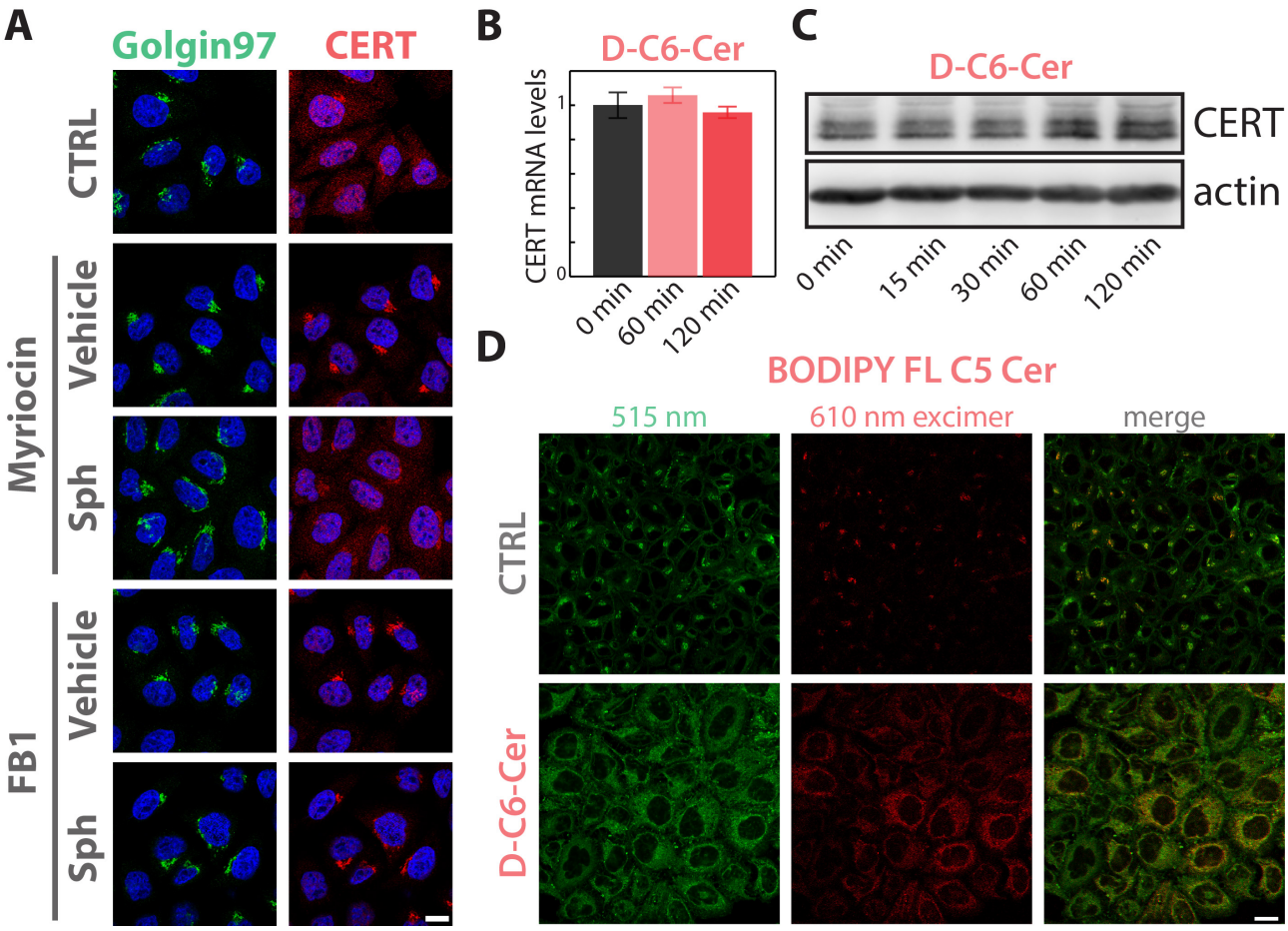
HeLa cells treated with FB1 (50μM) for 24 hours were fed with C6-D-Cer (10μM) for the indicated times. The amounts of C6-D-Cer (left panel), C6-D-SM (middle panel), and C6-D-HexCer (right panel) were measured by Mass Spectrometry following lipid extraction and HPLC separation and are reported as relative amounts over time. Red and cyan dashed lines indicate the early and late conversion/ synthetic rates of the different metabolites. Data are means ± S.D. from 3 independent experiments.

Appendix Figure S2



Appendix figure S2: *SMS1 and GCS are redistributed to the ER under BFA treatment.* Cells expressing HA- tagged versions of GCS or SMS1 were either incubated with vehicle or with SL inhibitors myriocin (2.5 μM) or FB1 (50 μM) for 24 hours. Subsequently cells were treated with BFA (5μg/mL) for 30 min, fixed and processed for immunofluorescence. Images show the subcellular distribution of GCS-HA and SMS1-HA (red) compared to that of the Golgi marker Giantin (green) under the different treatment conditions. Note that both GCS and SMS1 are predominantly located to the Golgi complex, and that both are redistributed to the ER under BFA administration irrespective of myriocin or FB1 treatments. Bar, 10 μm.

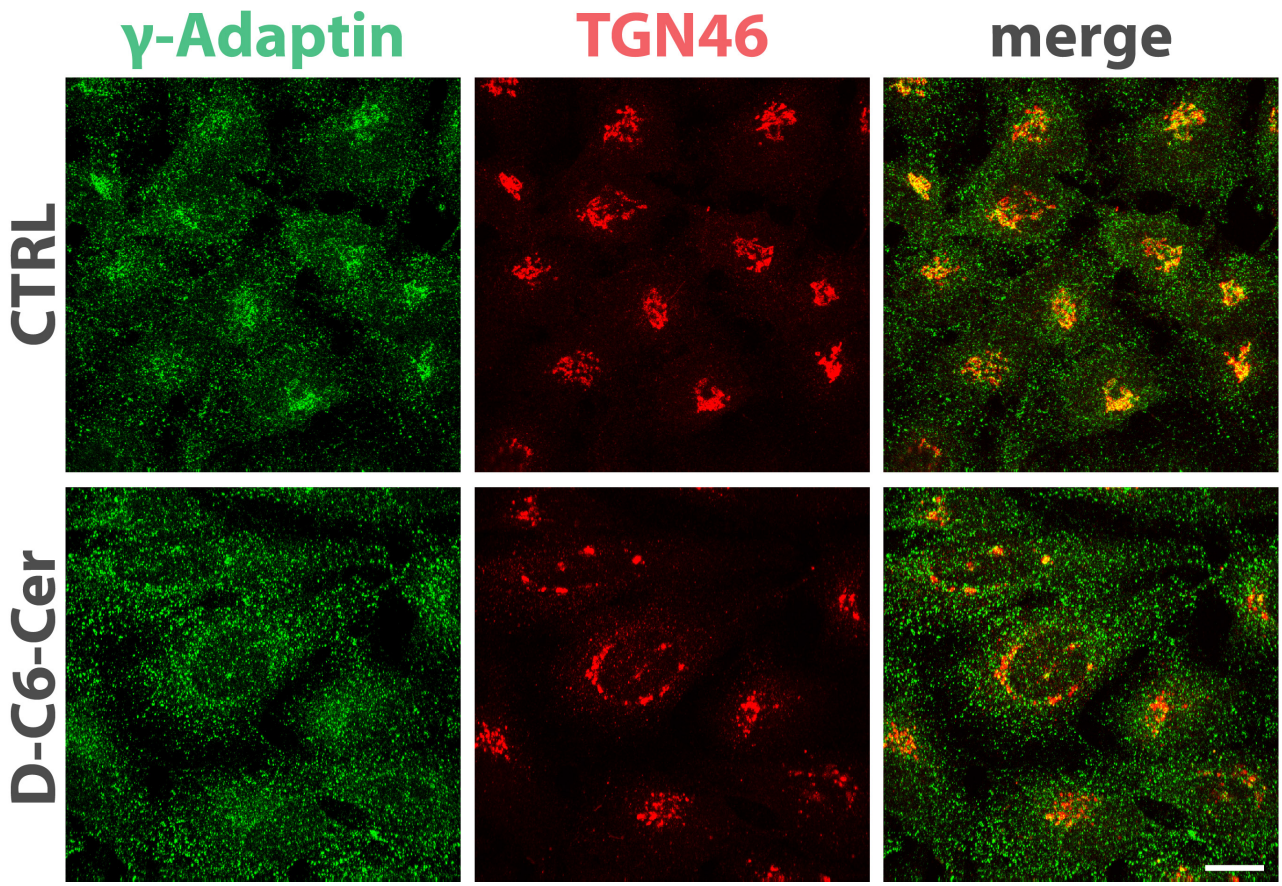
Appendix Figure S3



Appendix figure S3: *CERT association to Golgi membranes depends on SL synthetic flow.*

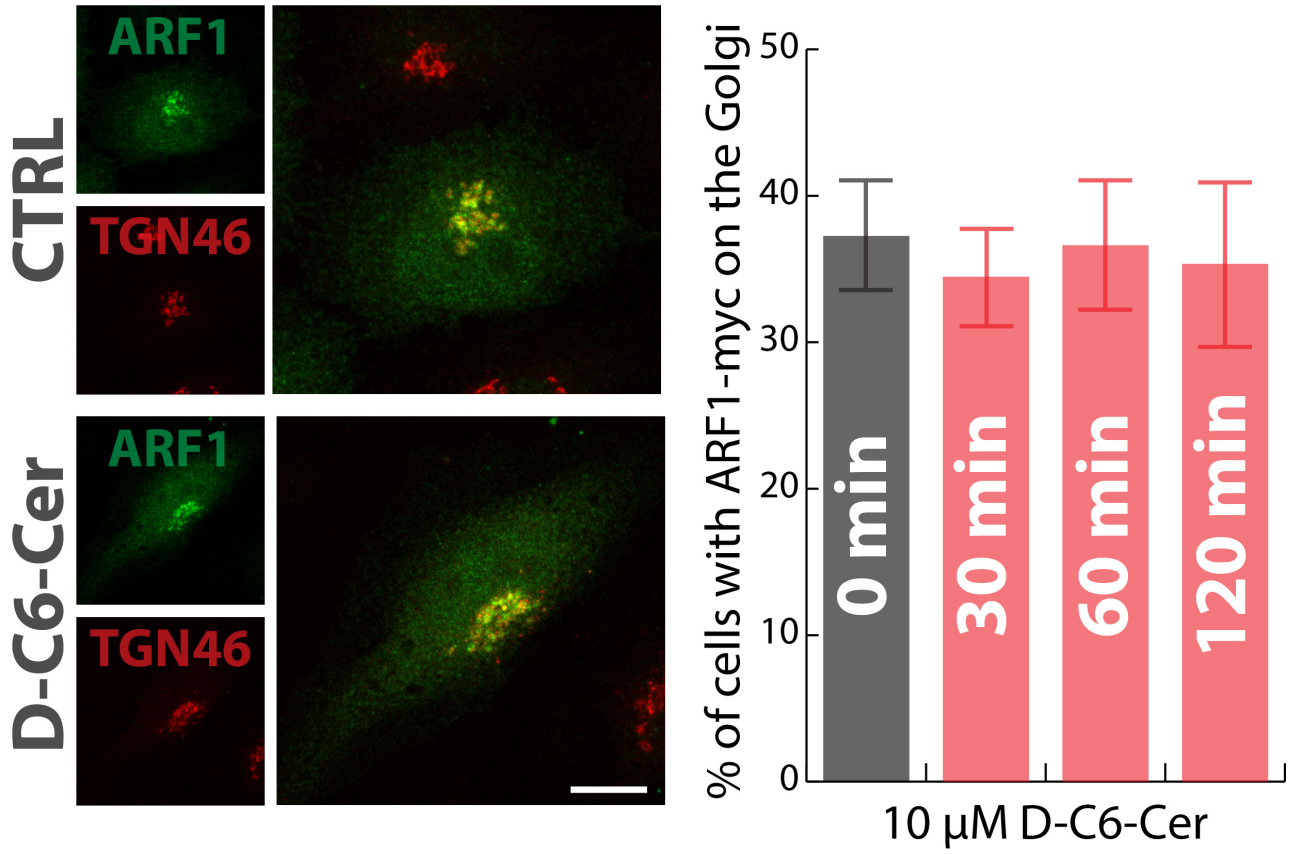
A) CERT localisation in HeLa cells, either non-treated (CTRL), treated with myriocin (2.5 μM for 24 hours) or FB1 (50μM for 24 hours), or treated with 30μM D-Sph for 2 hours after 24 hours treatment with myriocin or FB1. CERT is in red, the TGN marker Golgin 97 is in green, DAPI is in blue. Bar, 10 μm. Note that D-Sph treatment reverts myriocin induced CERT recruitment to the Golgi while it has no effect on FB1 induced recruitment, suggesting that D-Sph conversion to D-Cer is required for CERT displacement. **B)** CERT normalized mRNA levels as assessed by qPCR in HeLa cells treated for the indicated times with C6-D-Cer (10μM). **C)** CERT protein levels as assessed by immunoblotting in HeLa cells treated for the indicated times with C6-D-Cer (10μM). Data are means ± S.E.M. of at least 3 independent experiments. **D)** HeLa cells either non-treated (CTRL) or pre-treated for 2 hours with C6-D-Cer (10μM), were assayed for their capability to concentrate BODIPY-FL-C5-Cer to the perinuclear region. Monomer (515 nm; Green) and excimer (610 nm; Red) emissions are reported. Bar, 10 μm.

Appendix Figure S4



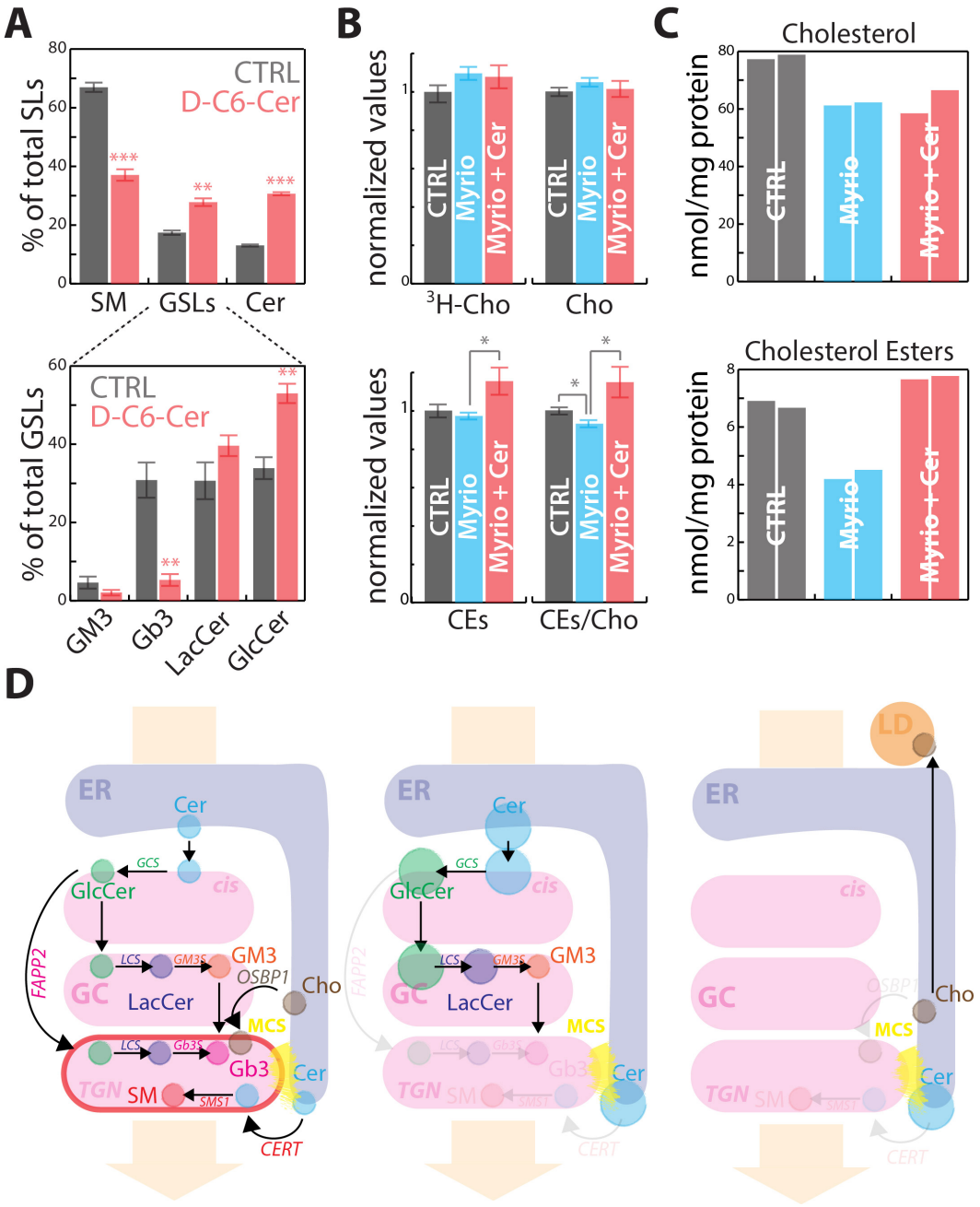
Appendix figure S4: *γ-Adaptin association to the Golgi is sensitive to the SL flow.*
Cells were treated as in **Figure 2A**, fixed and stained for γ-Adaptin (green) and TGN46 (red). Bar, 10 μm.

Appendix Figure S5



Appendix figure S5: *Arf1 localization to the Golgi complex is not affected by SL flow.* Cells expressing a myc-tagged version of ARF1 (ARF1-myc), were treated either with EtOH or D-C6-Cer (10µM) for different times (30, 60, and 120 min). Cells were then fixed and stained with anti-myc antibody (green) and with an anti-TGN46 antibody (red) (left panel). The percentage of transfected cells having ARF1-myc localized to the Golgi is indicated at different time points. Data are means of at least 3 independent experiments ± S.D.. Bar, 10 µm.

Appendix Figure S6

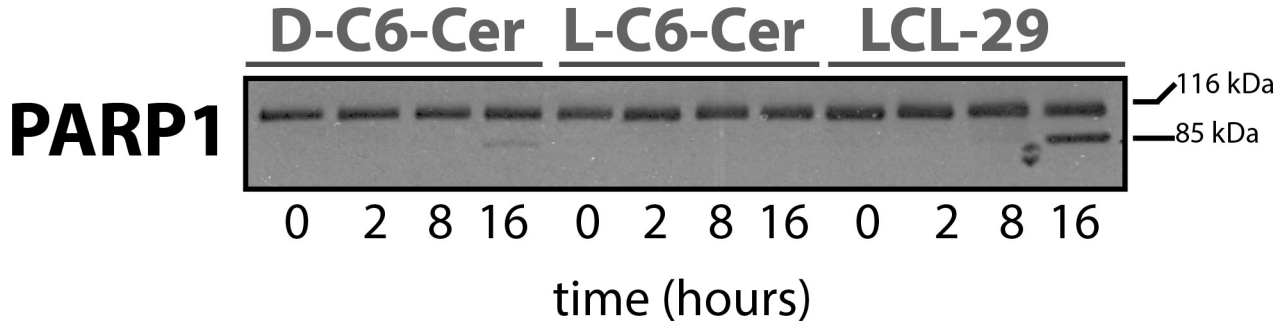


Appendix figure S6: *SL flow controls GSLs and sterol metabolism.*

A) Cells treated either with vehicle (EtOH), D-C6-Cer (10μM, 2 hours) were pulse labelled with ^{3}H-D-Sph for SL and GSL synthesis assessment. The percentage of total radioactivity associated with SM, Cer and GSLs (i.e., GlcCer, LacCer, Gb3, and GM3) in the different conditions was quantitated after lipid extraction and HPTLC separation (upper panel). The percentage of GSLs radioactivity associated with GlcCer, LacCer, Gb3, and GM3 in the different conditions is also reported. Data are means ± S.E.M. of 4 independent experiments. **B)** Cells were treated overnight with vehicle or myriocin (2.5 μM), followed by

2h labelling with ^{3}H-acetate and 4 h chase in the presence or absence of myriocin and D-C6-Cer (10 μM) as indicated. The ^{3}H-radioactivity associated with free cholesterol (^{3}H-Cho), the amount of unlabelled free cholesterol (Cho), and cholesterol esters (CEs) were analyzed after lipid extraction and HPTLC separation. CEs/ Cho ratio in the different conditions is also reported. Data are means ± S.E.M. of at least 7 independent experiments. **C)** Cells were treated overnight with vehicle or myriocin (2.5 μM) followed by 4 h chase in the presence or absence of D-C6-Cer (10 μM) as indicated. The amount of free cholesterol (Cho), and cholesterol esters (CEs) were analyzed by mass spectrometry after lipid extraction and gas chromatography separation. **D)** Schematic representation of the effect of sustained SL flow on CERT, FAPP2, and OSBP1 dependent lipid transport. ER, endoplasmic reticulum; GC, Golgi complex; TGN, trans Golgi Network; LD, lipid droplets.

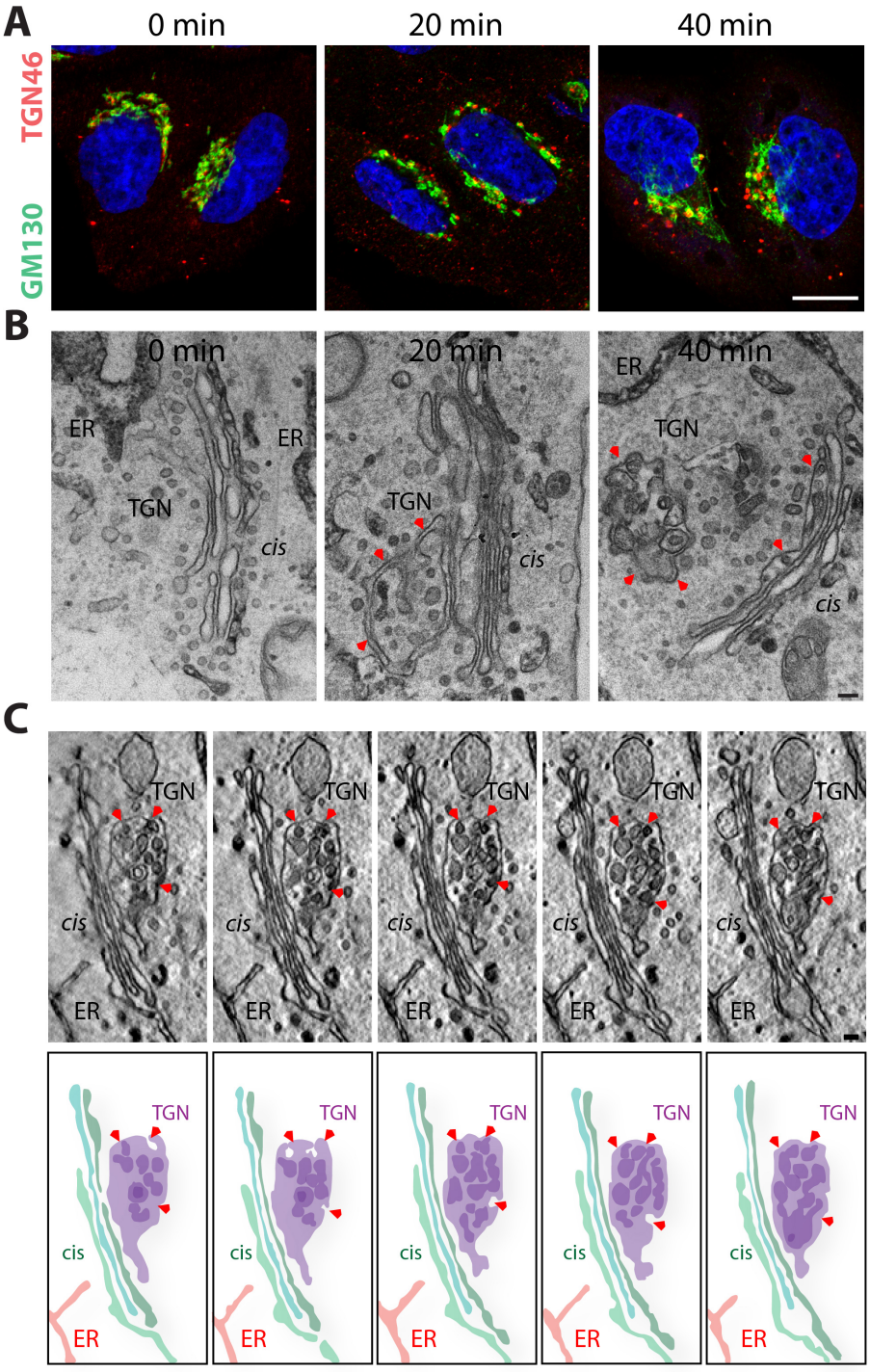
Appendix Figure S7



Appendix figure S7: *Sustained SL flow controls PtdIns(4)P at the TGN independently from pro-apoptotic signalling.*

HeLa Cells treated either with D-C6-Cer, L-C6-Cer, or LCL-29 (10μM) for the indicated times were lysed and lysates subjected to SDS-PAGE and immunoblotting with anti PARP1 antibody to evaluate caspase-dependent PARP1 cleavage (appearance of the 85 kDa PARP1 fragment).

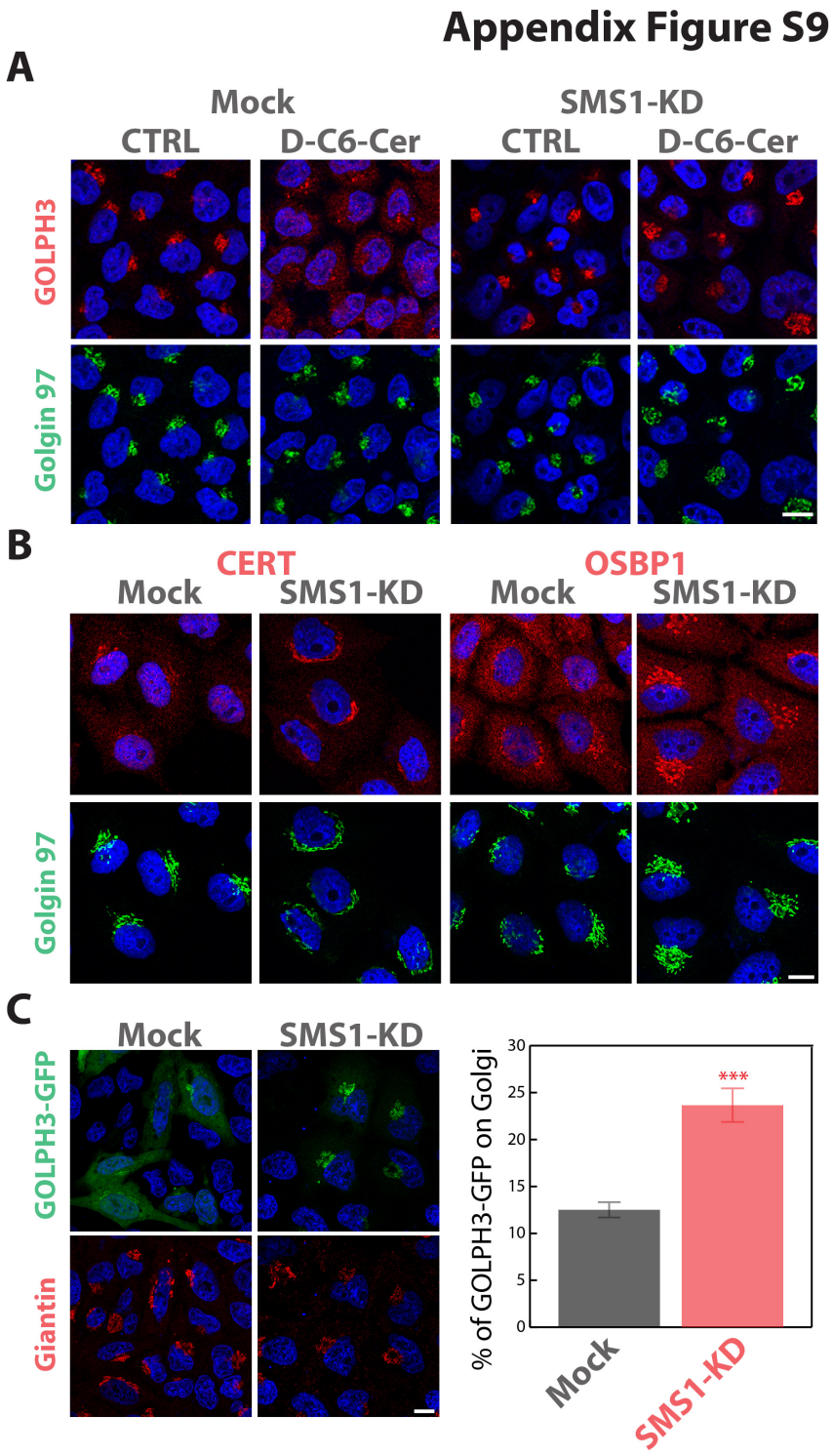
Appendix Figure S8



Appendix figure S8: *Sustained SL flow specifically affects TGN structure.*

A) Cells treated either with vehicle (EtOH), or D-C6-Cer (10μM) for the indicated times were fixed and stained with antibodies directed against *cis*-Golgi (GM130, green) and TGN (TGN46, red) markers. Bar, 10μm. **B)** Cells transfected with HRP-KDEL (to stain ER and *cis*-Golgi membranes by DAB reaction) were treated as in **(A)** fixed and processed for

electron microscopy as detailed in **Methods**. Red arrowheads indicate cisternal curling (middle panel) and multi-vesiculated structures at the *trans* Golgi pole and at the TGN (left panel). Bar, 100nm. **C)** Electron tomographic frames (upper panels) and graphic representation (lower panels) of a Golgi stack from a cell treated for 30 min with D-C6-Cer (10μM). Red arrowheads indicate inward budding events. Bar, 100nm.

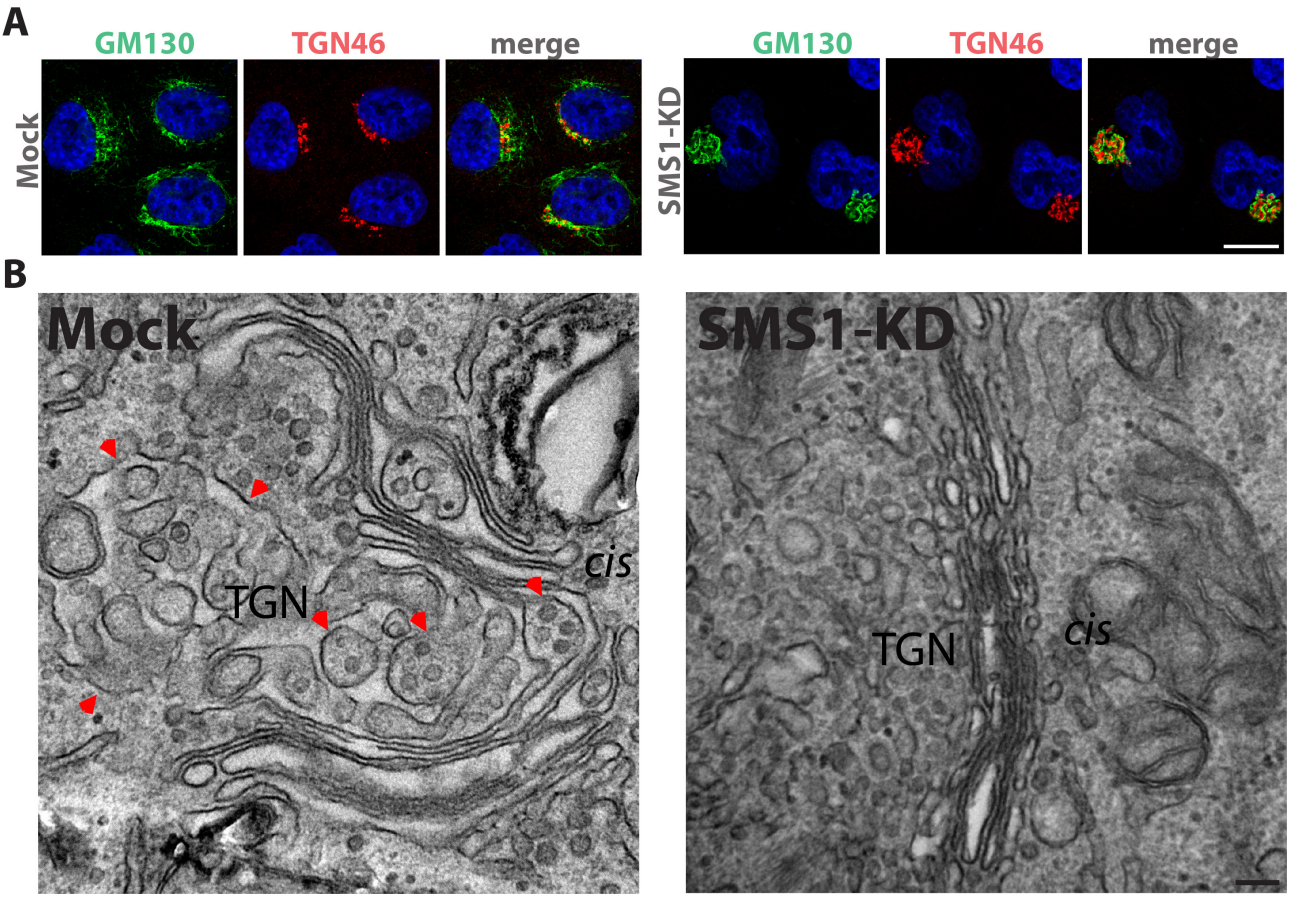


Appendix figure S9: *SM synthesis controls PtdIns(4)P binding proteins association to the TGN*

A) SMS1-KD cells were treated with either with EtOH (CTRL), or D-C6-Cer (10μM) for 2 hours, fixed, and stained with DAPI (blue), an anti-Golgin97 antibody (green) and anti-GOLPH3 antibody (red). **B)** Cells either mock treated or SMS1-KD were fixed, and stained with DAPI (blue), an anti-Golgin97 antibody (green) and either anti CERT or anti OSBP1

antibodies (red). **C)** SMS1-KD cells were transfected with GFP- tagged GOLPH3 fixed, and stained with DAPI (blue), and anti-Giantin antibody (red). The percentage of GOLPH3-GFP associated with the Golgi is reported in mock interfered (n=27) and SMS1-KD cells (n=27). Data are means ± S.E.M.

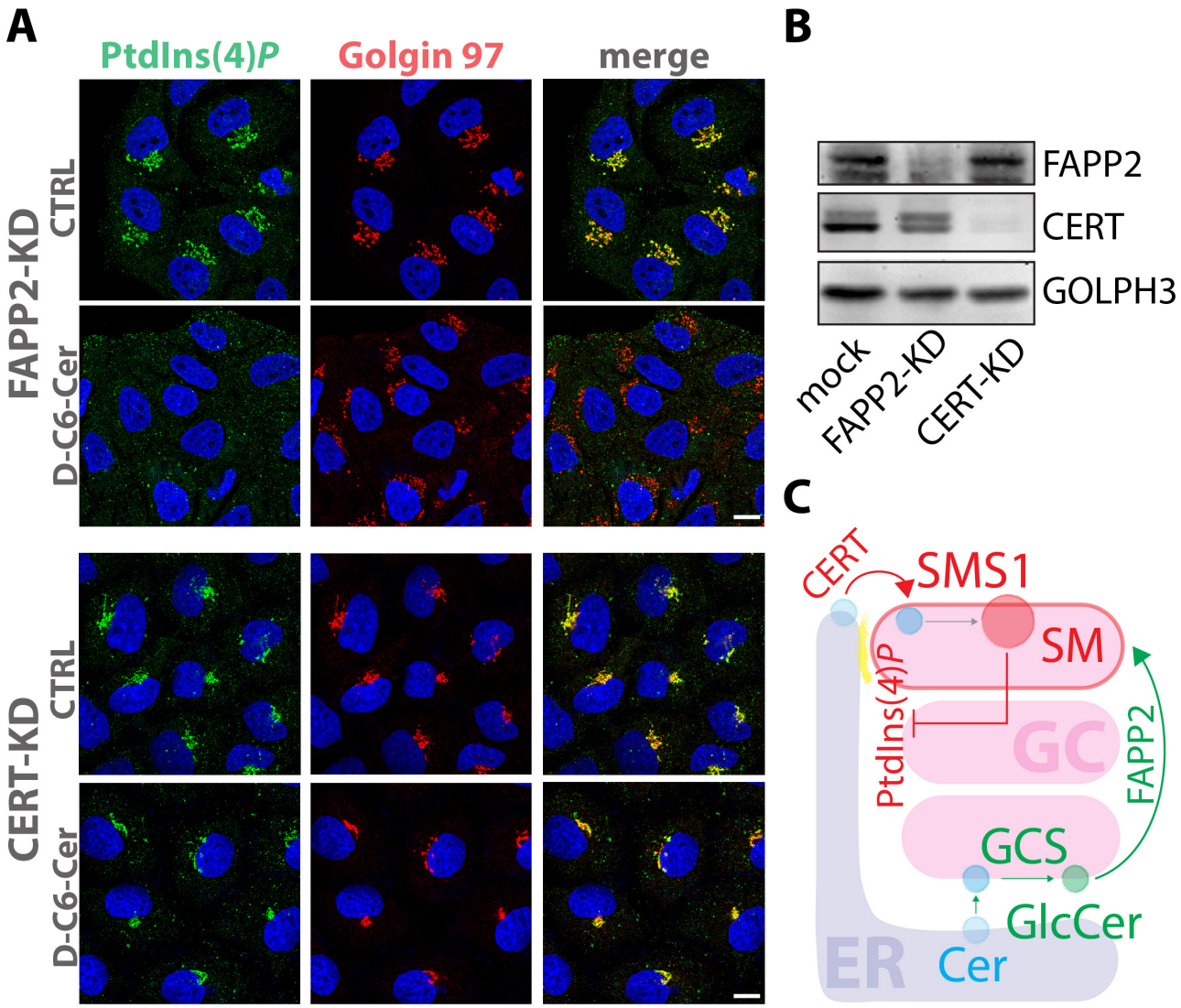
Appendix Figure S10



Appendix figure S10: *Cer to SM conversion affects TGN structure.*

A) Cells mock treated or SMS1-KD were treated with D-C6-Cer (10μM) for 2 hours, fixed, and stained with antibodies directed against *cis*-Golgi (GM130, green) and TGN (TGN46, red) markers. Bar, 10μm. **B)** Cells mock treated or SMS1-KD were treated as in **(A)** fixed and processed for electron microscopy as detailed in **Methods**. Red arrowheads indicate cisternal curling and multi-vesiculated structures at the *trans* Golgi pole and at the TGN characteristic of D-C6-Cer treated cells. Bar, 100nm.

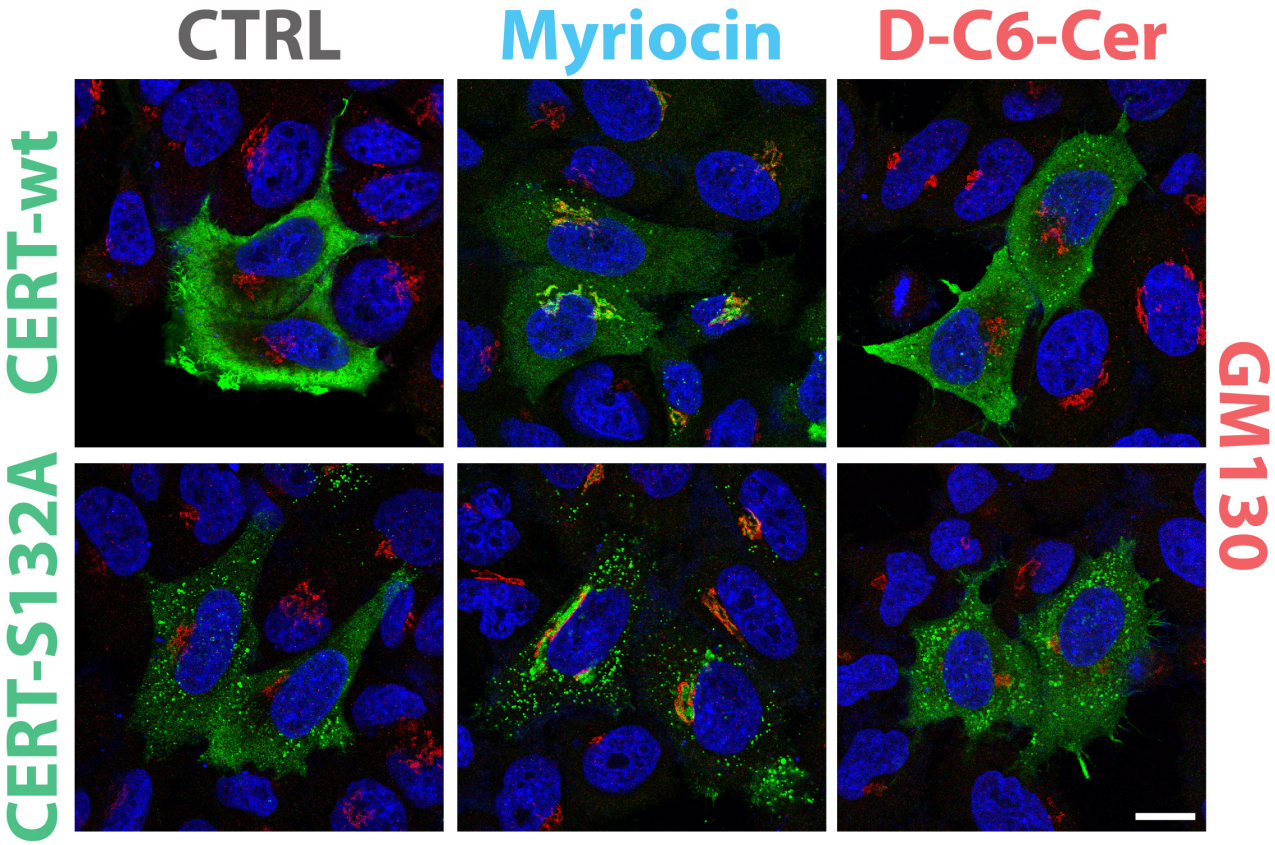
Appendix Figure S11



Appendix figure S11: *The CERT-SMS1 axis controls PtdIns(4)P levels at the TGN*

A) Cells treated with FAPP2 or CERT directed siRNAs were treated either with EtOH, or D-C6-Cer (10μM) for 30 min, fixed, permeabilized and stained with DAPI (blue), an anti-Golgin97 antibody (red) and a specific anti PtdIns(4)*P*-antibody (green; left panels). Bars, 10 μm. **B)** FAPP2 and CERT KD efficiencies were evaluated by Western Blotting. **C)** Schematic representation of CERT-SMS1 axis control on TGN PtdIns(4)*P*.

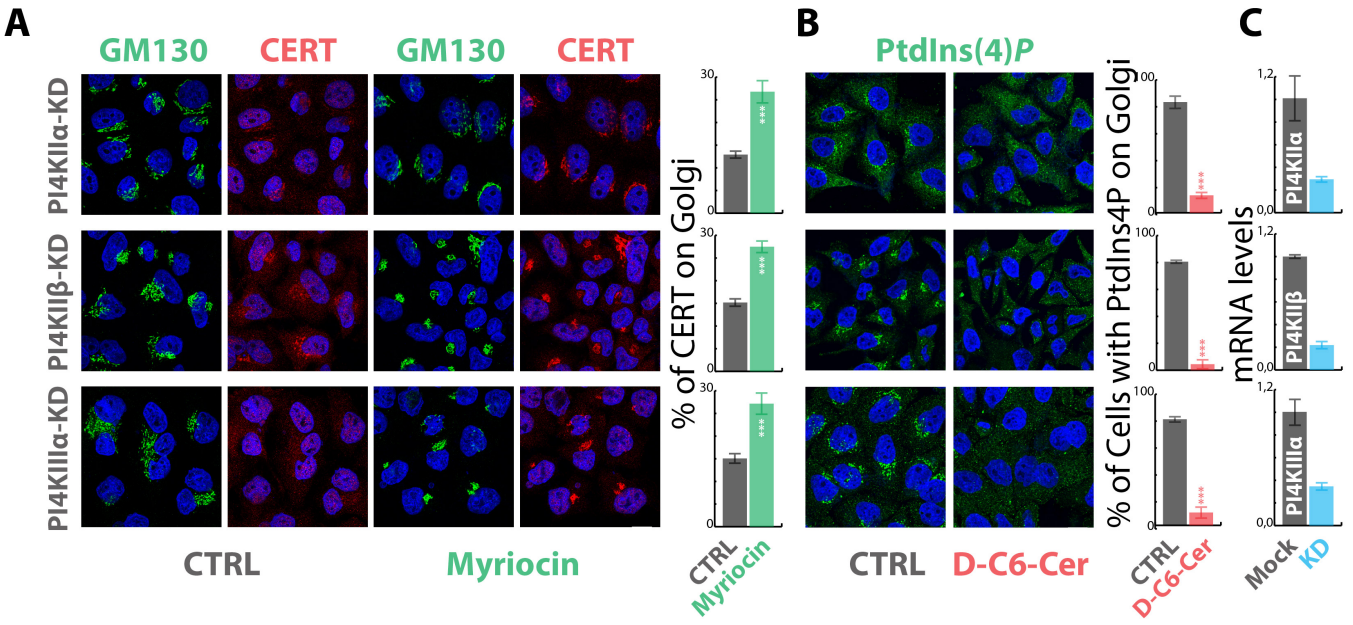
Appendix Figure S12



Appendix figure S12: *PKD phosphorylation on CERT minimally influences CERT localization in response to sustained SL flow.*

Cells expressing Flag-tagged CERT-wt or CERT-S132A were either non-treated (CTRL, grey), treated with myriocin (2.5 μM, 24 hours) (myriocin, cyan), or pre-treated with myriocin (2.5 μM, 24 hours), and then treated with D-C6-Cer (10μM) for 2 hours (D-C6-Cer; red). After fixation cells were stained with DAPI (blue) anti-Flag (green), and anti-GM130 (red) antibodies. Bar, 10 μm.

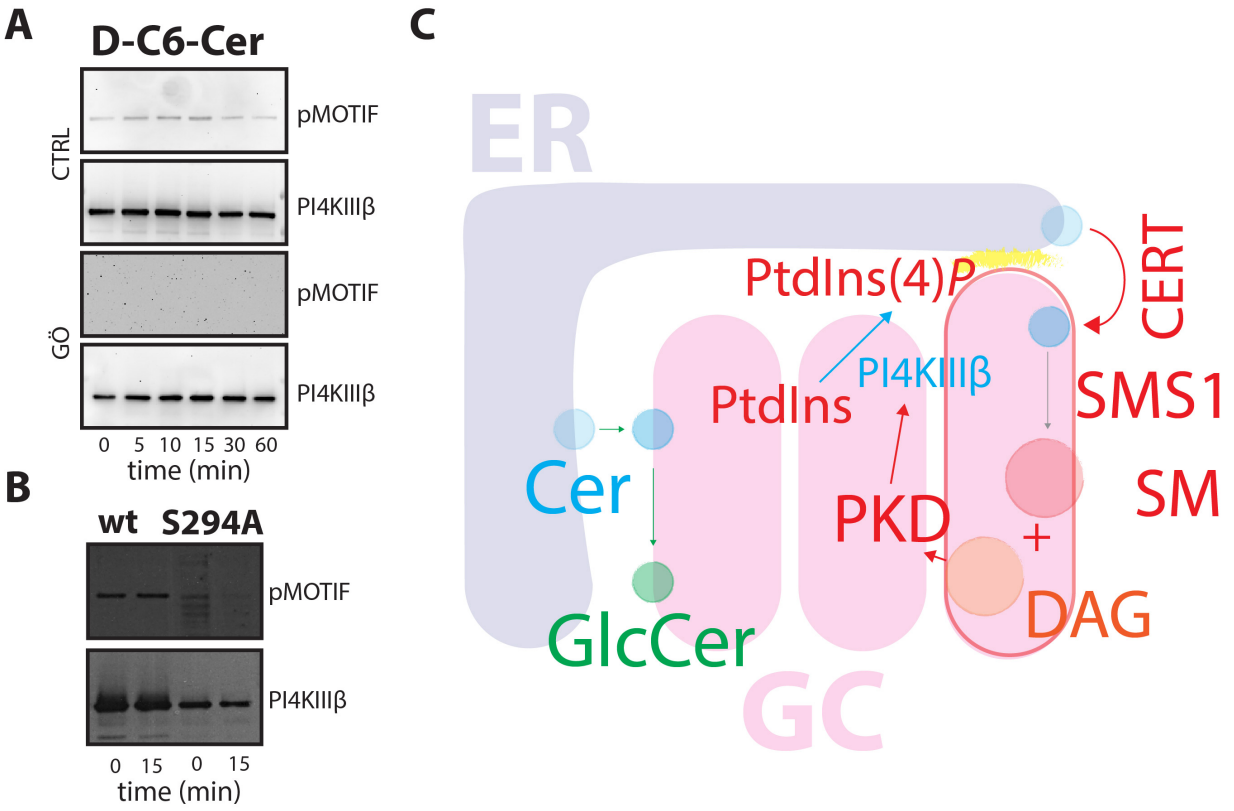
Appendix Figure S13



Appendix figure S13: *Other PI4Ks do not control the Golgi PtdIns4P pool in response to SL flow*

Cells silenced for the expression of PI4KIIα, PI4KIIβ, or PI4KIIIα were treated with **A)** myriocin (2,5 µM; 24 hours) or **B)** with D-C6-Cer (10µM; 60 min). In **(A)** CERT recruitment to the Golgi region was evaluated by immunofluorescence and quantitated as in **Figure 1C**. In **(B)** PtdIns(4)*P* consumption under D-C6-Cer (10µM, 1 hour) was evaluated as in **Figure 4A**. **C)** The KD efficiency for PI4KIIα, PI4KIIβ, and PI4KIIIα is assessed by RT qPCR (right histograms). Bars, 10 µm. Data are means ± S.E.M, of at least 3 independent experiments.

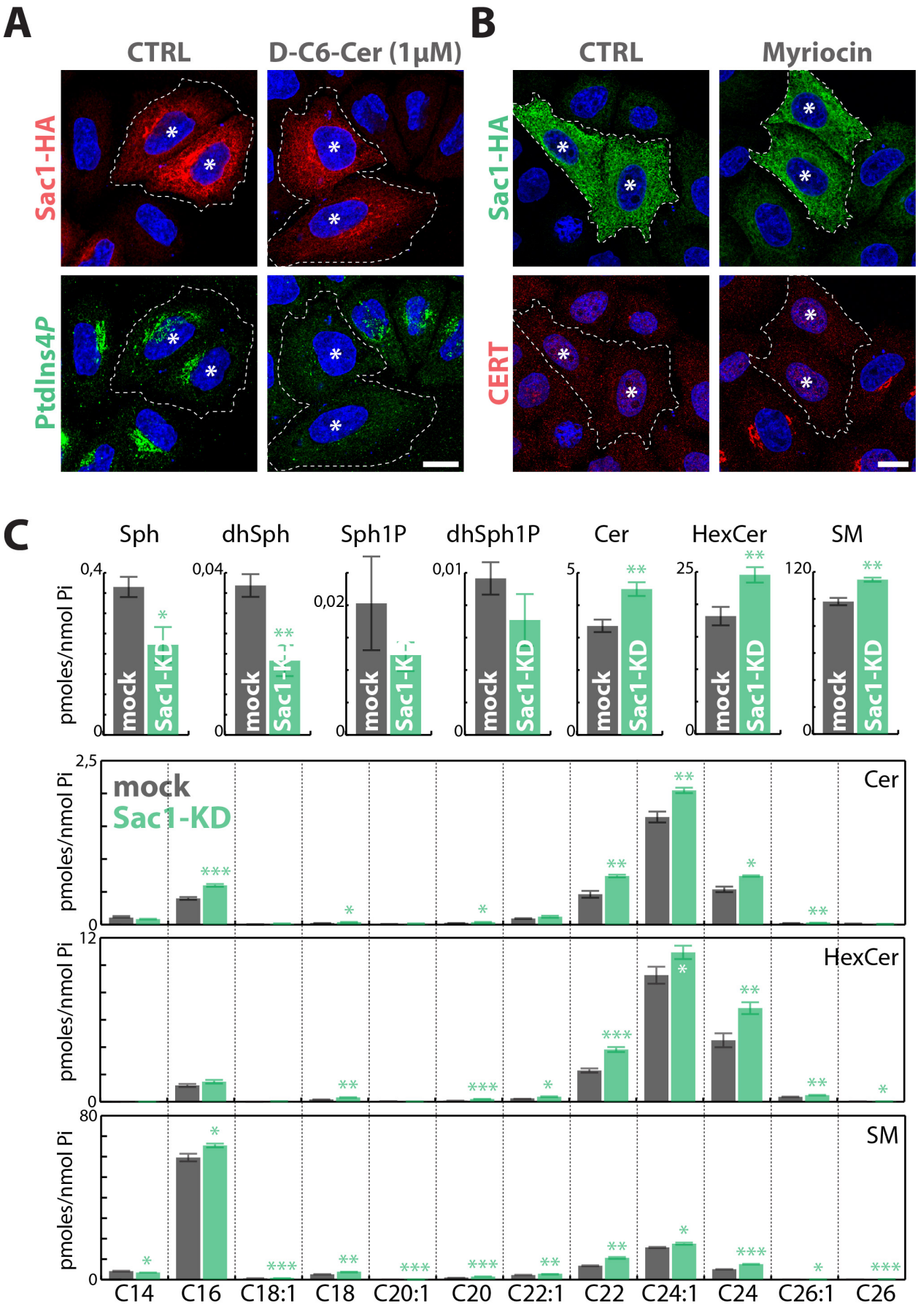
Appendix Figure S14



Appendix figure S14: *PI4KIIIβ response to SL flow*

A) Cells expressing PI4KIIIβ-GFP were treated for the indicated times with D-C6-Cer (10μM) lysed, and protein lysates were subjected to immunoprecipitation by the use of anti-GFP antibody, SDS-PAGE and Western Blotting. PKD dependent phosphorylation of PI4KIIIβ was revealed by pMOTIF antibody and compared to the amount of PI4KIIIβ (detected by anti-GFP antibody) immunoprecipitated at each time point. The PKD inhibitor Gö 6976 (10 μM, pre treatment 1 hour) was used as a specificity control. **B)** Cells expressing PI4KIIIβ-GFP-wt (wt) or PI4KIIIβ-GFP-S294A mutant (S294A) were treated for the indicated times with D-C6-Cer (10μM) lysed, and protein lysates were subjected to immunoprecipitation by the use of anti-GFP antibody, SDS-PAGE and Western Blotting. PKD dependent phosphorylation of PI4KIIIβ was revealed by pMOTIF antibody and compared to the amount of PI4KIIIβ (detected by anti-GFP antibody) immunoprecipitated at each time point. **C)** Schematic representation of SL flow mediated phosphorylation of PI4KIIIβ.

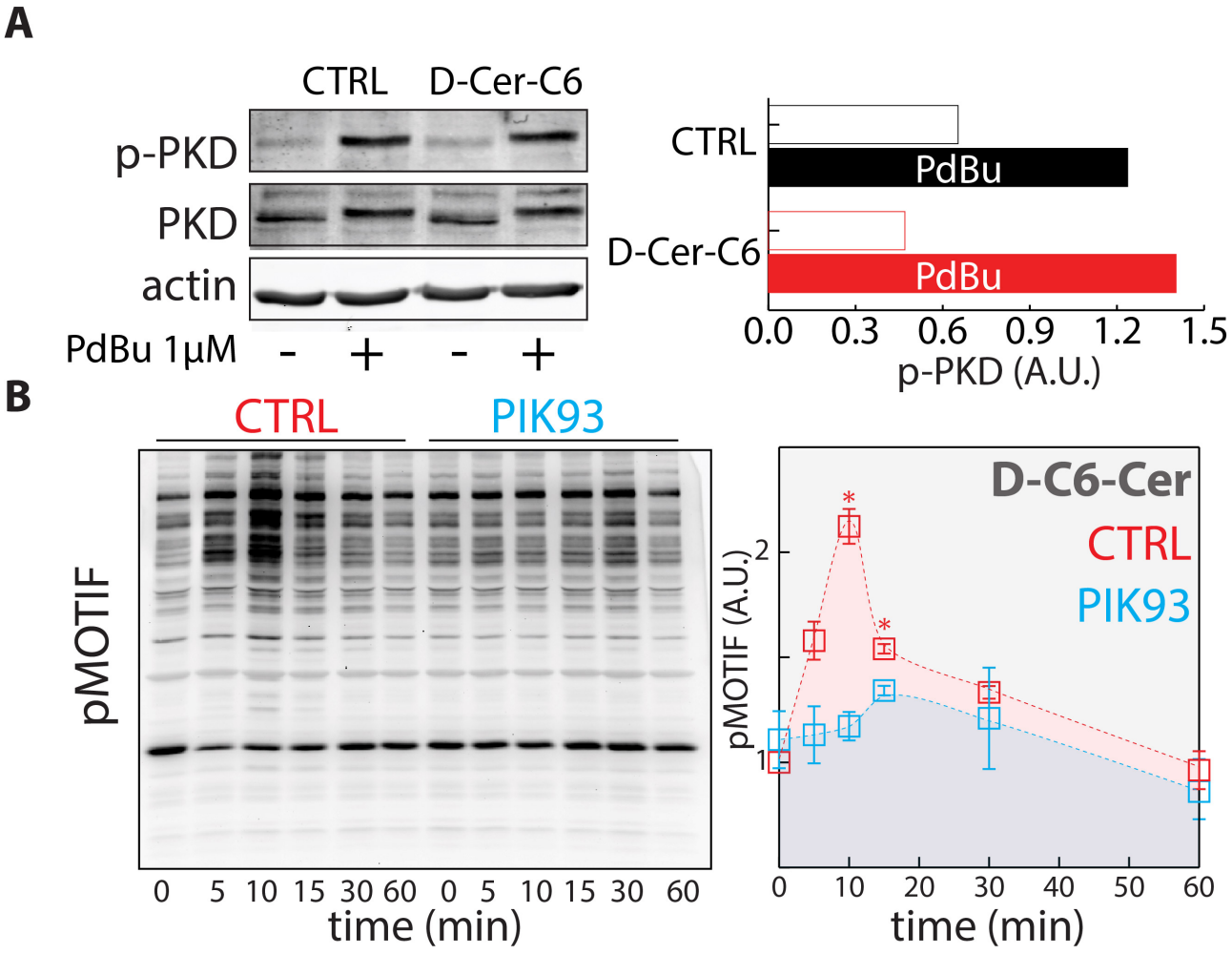
Appendix Figure S15



Appendix figure S15: *Sac1 consumes PtdIns(4)P in response to sustained SL flow*

A) Cells expressing Sac1-HA (red) were treated with D-C6-Cer (1μM, 1 hour) and the PtdIns(4)*P* (green) levels at the TGN were evaluated. **B)** Cells expressing Sac1-HA (green) were treated with myriocin (2,5μM, 24 hours) and CERT (red) recruitment at the TGN was evaluated. Data are representative of at least three independent experiments. Bars, 10 μm. **C)** HPLC-mass spectrometry based SL analysis of Sac1-KD cells. HeLa cells silenced for Sac1 expression were subjected to lipid extraction, HPLC separation and SL analysis as detailed in **Methods**. Sph, sphingosine; dhSph, sphinganine; Sph1P, sphingosine-1-phosphate; dhSPh1P, sphinganine-1-phosphate; HexCer, hexosylceramide. Data are means ± S.E.M. of 3 independent experiments.

Appendix Figure S16



Appendix figure S16: *PtdIns(4)P consumption terminates PKD signalling*

A) Cells pre-treated with vehicle (EtOH) or 10µM D-C6-Cer (10 µM) for 30 min were treated for 15 min with PdBu (1µM) lysed and lysates were processed for SDS-PAGE and Western Blotting. PKD activation expressed as normalized p-PKD signal is reported. **B)** HeLa cells pre-treated with vehicle (EtOH) or PIK93 (250nM) for 30 min were subjected to D-C6-Cer (10 µM) treatment for the indicated times, lysed and lysates were processed for SDS-PAGE and Western Blotting. pMOTIF antibody was used to monitor PKD activation (left panel). PKD activation in both control (CTRL; red) and PIK93 (cyan) treated cells was evaluated by quantitating phosphorylation of PKD substrates during D-C6-Cer (10µM) administration. Data are means of at least 3 independent experiments ± S.D..

Appendix Table S1: List of the antibodies used in this study

Antibody Name	Catalog number or PMID	Clone	Company names	Dilution	
anti-GFP	ab1218	9F9.F9	ABCAM	1:1000	
HA.11		16B12	COVANCE	1:1000	
Phospho-PKD (Ser916)	2051		CELL SIGNALING	1:1000	II
PKD	2052		CELL SIGNALING	1:1000	II
Anti-(Ser/Thr) PKD substrate (pMOTIF)	4381		CELL SIGNALING	1:1000	
GST	PMID: 27398910			1:5000	
GM130 (rabbit polyclonal)		EP892Y	Abcam	1:500	
GM130 (mouse monoclonal)		35/GM130	DB Bioscienze	1:100	
GOLGI97	A-21270		Molecular Probe	1:100	
TGN46	PMID:9084986		BIO-RAD	1:100	
FAPP2	PMID:17687330			1:200	
PtdIns(4)P	PMID:19508231		Echelon	1:200	
OSBP	HPA039227		Sigma-Aldrich	1:100	II
CERT (COL4A3BP)	HPA035645		Sigma-Aldrich	1:100	II
M6PR	PMID:22983091		Novus Biologicals	1:100	
EEA1		1G11	Abcam	1:100	
LAMP1	ab24170		Abcam	1:1000	
PDI		RL90	Abcam	1:100	
Sec31A		32/Sec31A	DB Bioscienze	1:100	
P115	ab184014		Abcam	1:100	
GRASP65	ab30315		Abcam	1:100	
Beta-COPI	PA1-061		Thermo Fisher	1:100	
Giantin		9B6	Abcam	1:100	
gamma-Adaptin		100/3	Sigma-Aldrich	1:100	
Flag		M2	Sigma-Aldrich	1:100	
myc		4A6	Sigma-Aldrich	1:1000	
PARP1	9542		CELL SIGNALING	1:1000	
GOLPH3 (rabbit polyclonal)	ab98023		Abcam	1:100	
GOLPH3 (mouse monoclonal)	ab69171		Abcam	1:100	II

Antibody Name	Catalog number or PMID	Clone	Company names	Dilution
Transferrin From Human Serum, Alexa Fluor® 568 Conjugate	T23365		Life Technologies	1/400
Donkey anti-Rabbit IgG (H+L) Alexa Fluor® 568 conjugate	A10042		Life Technologies	1/400
Donkey anti-Sheep IgG (H+L) Alexa Fluor® 488 conjugate	A11015		Life Technologies	1/400
Donkey anti-Mouse IgG (H+L) Alexa Fluor® 488 conjugate	A21202		Life Technologies	1/400
Goat anti-Mouse IgM lexa Fluor® 488 conjugate	A21042		Life Technologies	1/400
Goat anti-Mouse IgM(H+L) Alexa Fluor® 568 conjugate	A21043		Life Technologies	1/400
Goat Anti-Rabbit IgG, H & L Chain Specific Peroxidase Conjugate	401315		MerckMillipore	1/10000
Goat Anti-Mouse IgG, H&L Chain Specific Peroxidase Conjugate	401215		MerckMillipore	1/10000

Appendix Table S2: Sequences of siRNAs used in this study

siRNA	siRNA sequence
SMS1_1	CUACACUCCCAGUACCUGG
SMS1_2	CACACUAUGGCCAAUCAGCAA
GCS_1	GAUAUGAAGUUGCAAAGUA
GCS_2	GCGAAUCCAUGACAAUAUA
GCS_3	GGACCAAACUACGAAUUAA
GCS_4	GAUGCUAGAUUGUUUAUAG
CERT_1	GAAGAUGACUUUCCUACAA
CERT_2	GAAGUUGGCUGAAAUGGAA
CERT_3	GCGAGAGUAUCCUAAAUUU
CERT_4	UCAAAGGGAUAAAGUGGUA
PKD1_1	GAAGAGAUGUAGCUAUUAAUU
PKD1_2	GAAAGAGUGUUUGUUGUUAUU
PKD1_3	GAAUGCAGCUUUCAUGUAUUU
PKD1_4	GGAAGGAAAUAUCUCAUGAUU
PKD2_1	UGAGACACCUUCACUUCAUU
PKD2_2	CAAGAACAUUGUCCACUGUUU
PKD2_3	GGAAGAUGGGAGAGCGAUAUU
PKD3_1	GAACGAGUCUUUGUAGUAA
PKD3_2	GAAAGUUCCACACACAUUU
PKD3_3	AAAUAACUGUAGUGGAGUA
OSBP1_1	UACUGGGAGUGUAAAGAAATT
OSBP1_2	CGAACGAGCCACACUCUUUAG
OSBP1_3	GCGAAAUUGAUAUUGUGAAUC
PI4KIIα_1	GUAACUAUCUUGAAGGUUUUU
PI4KIIα_2	AGAAAGGGAGUGAGGACUAUU
PI4KIIα_3	GUGCAUAGCUCUCUGUAGAUU
PI4KIIα_4	GAGACGAGCCCACUAGUGUUU
PI4KIIIα_1	TCCGGACGCCATCCTCTTCTA
PI4KIIIα_2	CAAGAAATCAGGCATGTCTAA
PI4KIIIα_3	CCCGGAGCAACCTGGACATAA
PI4KIIβ_1	ACCATTATTAGGGACCAGAAA
PI4KIIβ_2	CAGAGTACTGGCCTTGTTCAA

PI4KIIβ_3	ATGAACTTTGTGCAAGATTTA
PI4KIIIβ_1	GGACGUGGGUGAUGCCAUUUU
PI4KIIIβ_2	GGAAUGACCUUCGGCAAGAUU
PI4KIIIβ_3	GAGAUCCGUUGCCUAGAUGUU
PI4KIIIβ_4	GCACCGAGAGUAUUGAUAAUU
SACM1L(SacI)_1	AAGGACCAACUUAAACGUUAA
SACM1L(SacI)_2	AACGGUAUGUAAGGUAGUAUA
SACM1L(SacI)_3	CAGGUAGUUAUAGUACUCCAA
PLEKHA8(FAPP2)_1	GAGAUAGACUGCAGCAUAUUU
PLEKHA8(FAPP2)_2	GAAUUGAUGUGGGAACUUUUU
PLEKHA8(FAPP2)_3	GAAAUCAACCUGUAAUACUUU
PLEKHA8(FAPP2)_4	CCUAAGAAAUCCAACAGAAUU

Appendix Table S3: Sequences of primers used for Real Time q-PCR in this study

Primer	Sequence
HPRT1 -For-	5'- AGCTTGCTGGTGAAAAGGAC -3'
HPRT1 -For-	5'- GTCAAGGGCATATCCAACAAC -3'
PI4K2α -For-	5'- CCACTGAAGCATCCTGACTC -3'
PI4K2α -Rev-	5'- GGGTCCGATATCTTTGGAAGG -3'
PI4K2β -For-	5'- GGGTGCCTATCTTGTGGAC -3'
PI4K2β -Rev-	5'- GCCTCTTGATTTTGCACGG -3'
PI4K3α -For-	5'-ACTCCACTGCCTTTGACTAC -3'
PI4K3α -Rev-	5'- GATGCTTATGTCTTCACGCAG -3'
PI4K3β -For-	5'- CTCGGAAACACATGGACAAG -3'
PI4K3β -Rev-	5'- TTTGAGGTTTCGAATGGTGC -3'
Sac1 -For-	5'- GCCATGCAGCTAACTTTGTAG -3'
Sac1 -Rev-	5'- CTTGAGGTTTGGTCTTTGGG -3'
SMS1-For-	5'- GCATTTCAACTGTTCTCCGAAG -3'
Sac1 -Rev-	5'- GATAGACAAGCCACCTCCAG -3'
UGCG-For-	5'- TTCGGGTTCGTCCTCTTC -3'
UGCG-Rev-	5'- GCTTGCTATAAGGCTGTTTGTC-3'