Supplemental Experimental Procedures

Lipids and Miscellaneous Reagents

BODIPY-LacCer was synthesized as described previously (Martin and Pagano, 1994). C8-ceramide, monosialoganglioside GM_3, D-lactosl-β1-1'-*N*-octanol-D-*erythro*-sphingosine (C8-LacCer) were from Matreya, Inc. (State College, PA). *N*-hexanoyl sphingosylphosphorylcholine (C6-sphingomyelin; C6-SM), phosphatidylcholine (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPC), and bovine brain SM were from Avanti Polar Lipids (Alabaster, AL). Fluorescent Alexa Fluor AF594 or AF647 labeled albumin, transferrin (Tfn), and dextran were from Molecular Probes (Eugene, OR); DiI-LDL was from Intracel, Inc. (Frederick, MD). Tyrosine kinase inhibitor PP2 was from CALBIOCHEM (San Diego, CA). Cholesterol, *Clostridium difficile* Toxin B, and FB1 were from Sigma Chemical Co. (St. Louis, MO). NB-DGJ was from Toronto Research Chemicals (Toronto, Canada). PPPP was gift from Dr. J. Shayman, Univ. of Michigan. The IL-2R β chain antibody mik-β3, labeled with phycoerythrin (PE), and anti-PM-Cav1 (C43420) and anti-Golgi-Cav1 (C37120) were from BD Pharmingen, Inc. (San Diego, CA). Plasmids encoding the IL-2R β chain (IL-2R β) (A. Dautry-Varsat, Institut Pasteur, Paris), DN AP180 (H. McMahon, MRC Laboratory of Molec. Biol.), and Dyn2 K44A (M. McNiven, Mayo Foundation, Rochester MN), and WT, CA and DN RhoA and Cdc42 (in pcDNA3.1) (D. Billadeau, Mayo Foundation) were generous gifts as noted. GFP-RhoA Q63L and GFP-Cdc42 Q61L were generated by subcloning into the EGFP-C3 vector (BD Clontech) at the Hind III and Apa I sites. HA-Rho and HA-Cdc42 were from the Univ. of Missouri cDNA resource center (Rolla, MO). Cav1-mRed was generated as described (Sharma et al., 2004).

Lipid analysis

Lipid extraction and analysis were performed as described (Puri et al., 2003). SLs were separated by TLC and identified by comparison to known standards using $CHCl_3/CH_3OH/15$ mM $CaCl_2$ (65:35:8; v/v/v) as the developing solvent. Primulin was used as a detection reagent, and lipids were quantified by scanning densitometry.

RhoA and Cdc42 activity assay

RhoA and Cdc42 activities were determined using commercial kits [RhoA activation kit (Cat. No. BK036) from Cytoskeleton, Denver, CO; Cdc42 activation kit (Cat. No. 17-286) from Upstate Biotechnology, Charlottesville, VA]. For both assays, serum-starved cells were incubated in Ham' F-12 with 10% serum for 10 min, chilled on ice, and washed once with PBS. For RhoA activity, cells were lysed in 500 μl lysis buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycolate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml each of aprotinin and leupeptin, 1 mM PMSF). Lysates were centrifuged, equal volumes were incubated with the GST-Rho binding domain of Rhotekin RBD beads (20 μg protein/sample) for 1 hr at 4°C, washed three times, and eluted with SDS sample buffer. Bound RhoA was analyzed by SDS-PAGE separation on a 12% polyacrylamide gel, followed by immunoblotting with a monoclonal antibody against RhoA (Cat. No. BK036; Cytoskeleton, Denver, CO). For Cdc42 activity, cells were lysed in lysis buffer (23 mM HEPES, pH 7.5, 150 mM NaCl, 1% lgepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, 10 μg/ml each of aprotinin and leupeptin). Cleared lysates were incubated with 20 μg of GST-p21-binding domain of PAK1 (GST-PBD) for 45 min at 4°C, washed three times, and eluted with SDS sample buffer. Bound Cdc42 was analyzed by Western blotting as described above, using a

monoclonal anti-Cdc42 antibody (Cat. No. 17-286; Upstate Biotechnology, Charlottesville, VA).

Supp. Table I. Characterization of Albumin uptake in CHO cells

Inhibitor studies

Treatment	Purpose of treatment	Effect on albumin uptake[1]
CPZ	Inhibits clathrin dependent endocytosis	No effect (Fig. 1A)
DN AP180	Inhibits clathrin dependent endocytosis	No effect (Fig. 1A)
Mβ-CD	establishes cholesterol-sensitivity	Inhibits (Fig. 1A)
Genistein	general tyrosine kinase inhibitor	Inhibits (Fig. 1A)
PP2	inhibits *src* kinase & caveolar uptake	No inhibition (Fig. 1A & Supp. Fig. 2)
Toxin B	Inhibits Rho GTPases	Inhibits (Fig. 1B & Supp. Fig. 2)
DN RhoA	Inhibits IL-2R endocytosis	Inhibits (Fig. 1B & Supp. Fig. 4)
DN Cdc42	Inhibits fluid-phase endocytosis	No effect (Fig. 1B & Supp. Fig. 4)
Dyn2 K44A	Inhibits clathrin, caveolar & IL-2R endocytosis	Inhibits (Fig. 1A)

Co-localization studies

Marker	
IL-2R	co-localized with albumin (Fig. 1C)
BODIPY-LacCer	did not co-localize with albumin (Fig. 1C)
Cav1-mRed	did not co-localize with albumin (Supp. Fig. 3)

[1]Endocytosis of AF594-albumin was examined after 5 min at 37°C for inhibitor studies, and endocytosis of AF647- albumin was examined after 1 min at 37°C for co-localizatin studies.

[2](Henley et al., 1997; Pelkmans et al., 2001; Puri et al., 2001; Sabharanjak et al., 2002)

Supp. Table II. Sphingomyelin and GM_3 ganglioside content in SPB-1 and CHO-K1 cells cultured under various conditions.[1]

Temp °C	Medium	Sphingomyelin (nmol/mg protein)		GM_3 (nmol/mg protein)	
		CHO-K1	SPB-1	CHO-K1	SPB-1
33	F12 + 5% FBS	12.1± 2.2	11.0 ± 1.8	2.7± 0.3	2.5 ± 0.1
39	Nutridoma-BO	14.4 ± 1.5	3.7± 0.4	2.0 ± 0.1	0.6 ± 0.1

[1]SPB-1 and CHO-K1 cells were cultured in Nutridoma-BO or F12 medium with 5% FBS at the indicated temperatures for 48 hrs. The amount of sphingomyelin and GM_3 was then quantified relative to cell protein (see "Supplemental Experimental Procedures"). Values are means ± S.D. (n=3).

Supp. Table III. Sphingomyelin and GM_3 ganglioside content in CHO-K1 cells treated with various pharmacological inhibitors[1].

	Sphingomyelin (nmol/mg protein)	**GM_3 (nmol/mg protein)**
None	12.1 ± 2.2	2.7 ± 0.3
FB1	3.5 ± 0.4	0.7 ± 0.1
NB-DGJ	15.0 ± 1.5	1.2 ± 0.2
PPPP	15.6 ± 1.8	0.6 ± 0.1

[1]Cells were cultured in F12 medium with 5% FBS at 33°C in the presence of FB1, NB-DGJ, or PPPP for 48 hrs. The amount of sphingomyelin and GM_3 was then quantified relative to cell protein (see "Supplemental Experimental Procedures"). Values are means ± S.D. (n=3).

Supp. Table IV. Characterization of endocytic mechanisms in CHO cells.

	Caveolae	**Cdc42**	**RhoA**	**Clathrin**
Cargo	BODIPY-LacCer	Dextran	IL-2R, Albumin	Tfn, LDL
SL depletion	Red	Red	Red	Green
GSL depletion	Red	Green	Green	Green
PP2	Red	Green	Green	Green
SMase	Green	Red	Red	ND
C8-Ceramide	Green	Red	Red	ND
genistein	Red	Red	Red	Green
mß-CD	Red	Green	Red	Green
DN RhoA	Green	Green	Red	Green
DN Cdc42	Green	Red	Green	Green
Toxin B	Green	Red	Red	Green
DN AP180	Green	Green	Green	Red
CPZ	Green	Green	Green	Red
Dyn 2 K44A	Red	Green	Red	Red

Green, treatments had no effect on internalization of indicated cargo;
Red, treatments inhibited internalization of indicated cargo;
ND, not determined

Cheng et al., Supplemental Figure Legends

Supp. Figure 1. Sphingolipid biosynthesis in mammalian cells. Schematic shows enzymatic steps of SL synthesis that are inhibited by various drugs (FB1, NB-DGJ, or PPPP) or in the temperature sensitive CHO mutant cell line, SPB-1 cells.

Supp. Fig. 2. Effect of PP2 or Toxin B on albumin uptake in CHO-K1 cells. Cells were untreated, or pretreated with PP2 or Toxin B for 1 hr at 37°C, and subsequently co-incubated with BODIPY-LacCer and AF647-albumin for 30 min at 10°C. Samples were then washed, and incubated for 5 min at 37°C prior to back-exchange and observation under the fluorescence microscope. Dashed white lines delineate the cell periphery in each field. Bar, 10 μm.

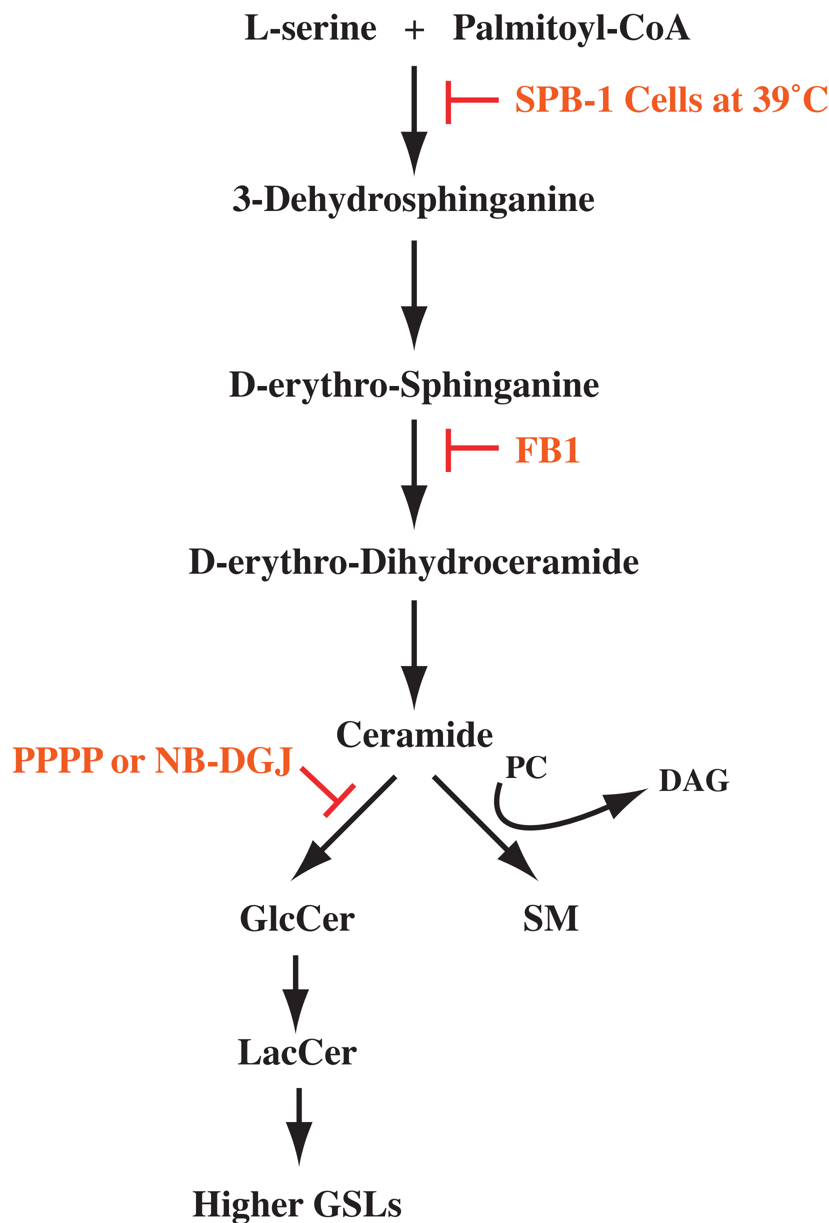
Supp. Fig. 3. Fluorescent LacCer, but not albumin, co-localizes with Cav1-mRed in CHO-K1 cells. Cells transiently transfected with Cav1-mRed were either incubated with AF647-albumin or BODIPY-LacCer for 30 min at 10 °C in HMEM, washed, and warmed for 1 min at 37°C. Samples were then acid-stripped or back exchanged with BSA and observed under the fluorescence microscope at wavelengths appropriate for each fluorophore. Images were rendered in pseudocolor and are presented as overlays (Left panels). Middle and right panels show the boxed region at higher magnification. Bar, 10 μm.

Supp. Fig. 4. Effect of DN RhoA and Cdc42 on the uptake of fluorescent albumin and dextran in CHO-K1 cells. Cells were co-transfected with CFP-Nuc and RhoA T19N or Cdc42 T17N constructs. The internalization of AF594 albumin and dextran (5 min at 37°C), relative to untransfected cells, was observed by fluorescence microscopy. The nuclei of transfected cells fluoresce green due to the expression of CFP-Nuc. Albumin and dextran fluorescence is shown in red. Dashed white lines delineate the cell peripheries in each field. Note the inhibition of albumin uptake in the RhoA T19N-transfected cell relative to an adjacent, non-transfected cell (upper left panel) and the inhibition of dextran uptake in the Cdc42 T17N-transfected cell. Bar, 10 μm. Dashed white lines delineate the cell periphery in each field. Bar, 10 μm.

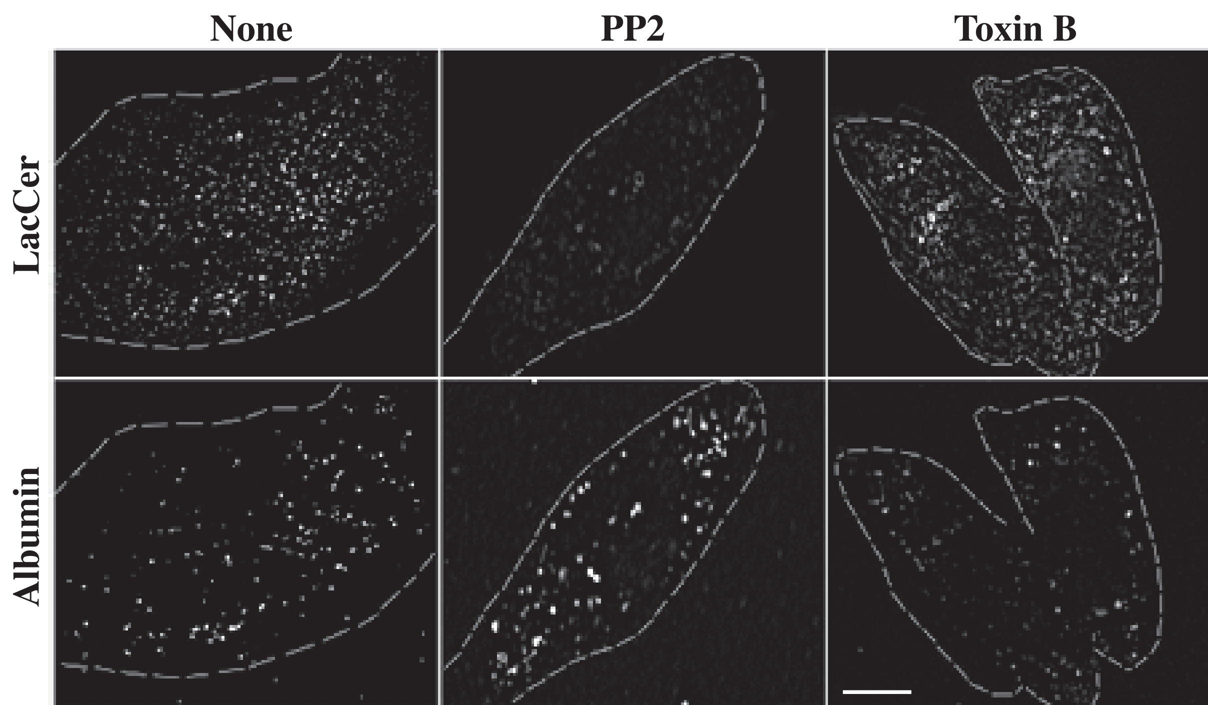
Supp. Figure 5. SL levels and endocytosis of markers in SPB-1 cells following shift to non-permissive culture conditions. SPB-1 cells were cultured under non-permissive conditions for various times (A,C), or for 48 hrs (B). (A) The intracellular SM and GM_3 content at each time point was determined by lipid extraction and analysis (see "Supplemental Experimental Procedures"). Values are expressed as percent of initial levels found for each lipid prior to shifting to the non-permissive temperature, and are the mean ± SD of 3 independent measurements. (B) CHO-K1 and SPB-1 cells were cultured under non-permissive conditions for 48 hrs. Cells were then incubated with BODIPY-LacCer for 30 min at 10°C, washed, and further incubated at 37°C for the indicated time, followed by back-exchange with BSA (see "Methods"). Samples were observed under the fluorescence microscope and internalized was quantified by image analysis. Values are mean ± SD from three independent experiments (n = 30 cells per data point). (C)

SPB-1 cells were cultured under non-permissive conditions for the indicated times as in (A). Internalization of the indicated marker was then measured after 5 min at 37°C as in Fig. 2 Results are expressed relative to initial levels of internalization seen for each marker prior to shifting cells to the non-permissive temperature. Values are mean ± SD from three independent experiments (n = 30 cells per data point).

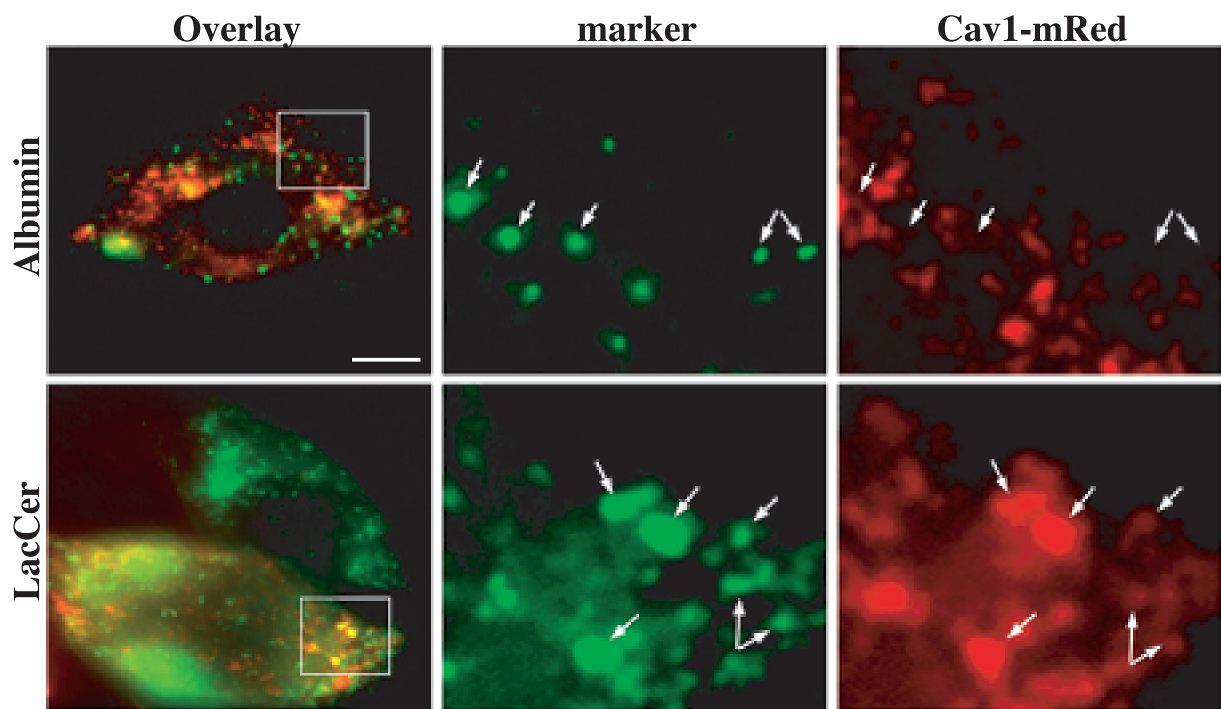
Supp. Fig. 6. GTP-loading of RhoA and Cdc42 in cells treated with inhibitors. Activated RhoA and Cdc42 were isolated by binding to specific substrates using commercially available assay kits (See Supplemental Experimental Procedures). Total RhoA or Cdc42 in cell lysates was determined in parallel by Western blotting using specific antibodies against RhoA or Cdc42.



Cheng et al., Supp. Fig. 1



Cheng et al., Supp. Fig. 2



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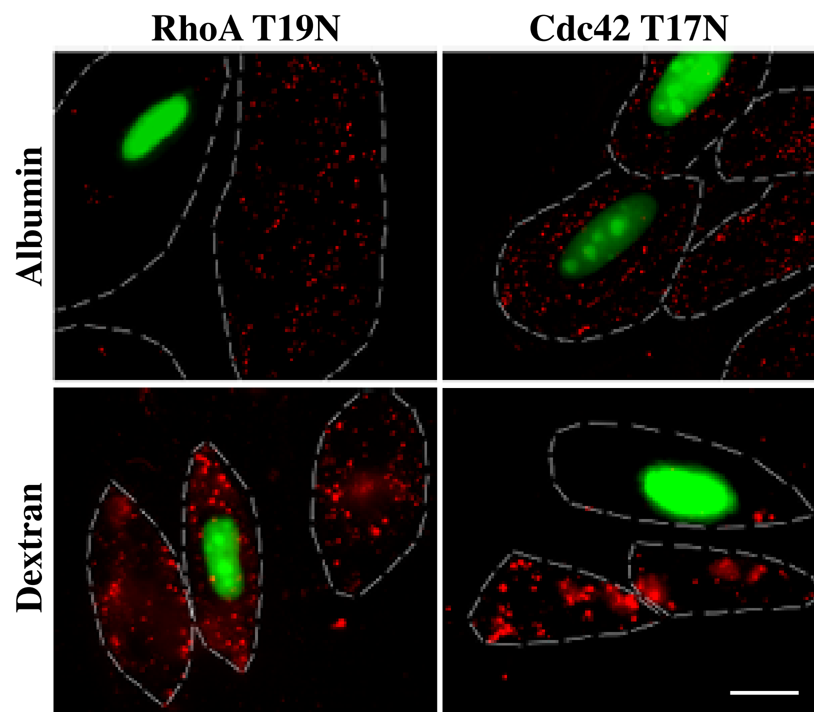
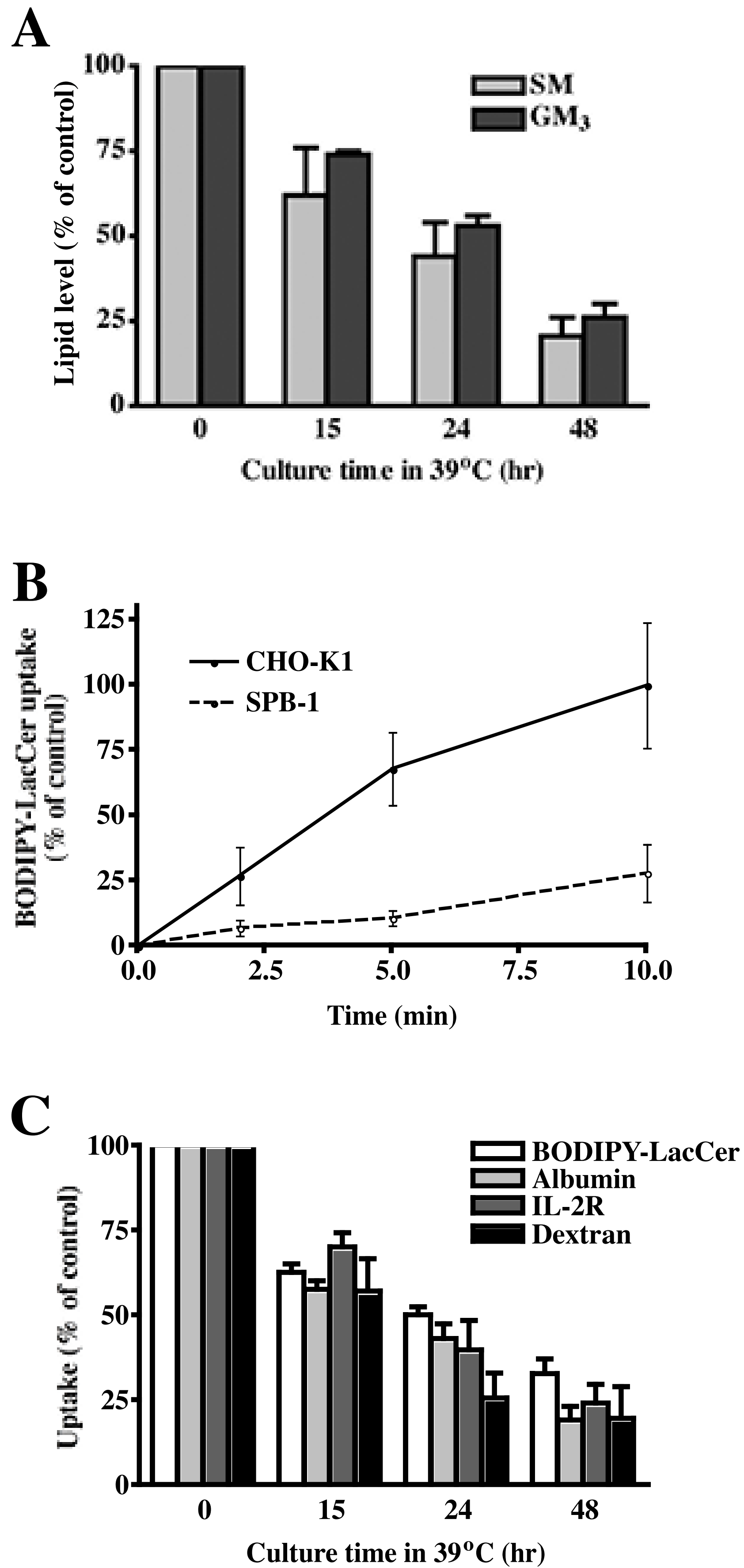
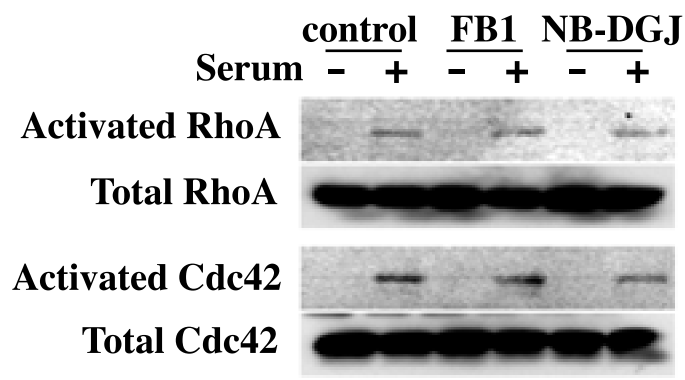


Cheng et al., Supp. Fig. 4



Cheng et al. Supp. Fig. 5



Cheng et al. Supp. Fig. 6