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Supplemental Information

Membrane Destabilization Induced by Lipid Species Increases Activity of Phosphorothioate-Antisense Oligonucleotides

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SUPPLEMENTARY DATA

Supplemental materials

Chemicals and Antibodies

Cholesterol, ceramide, free fatty acids, and fatty acid free bovine serum albumin were from Sigma. N-Rh-PE was from Avanti Polar Lipids. Vybrant™ Alexa Fluor™ 555 Lipid Raft Labeling Kit, Bodipy 493/503, Bodipy-C16, NBD-cholesterol, and NBD-ceramide were from ThermoFisher. Magic Red substrate was from Immunochemistry Technologies.

Antibodies against ERK (4695), p44/42 MAPK (Erk1/2) (4370), and LAMP1 (9091S) were from Cell Signaling Technology. Anti-EEA1 (610456) was from BD Bioscience. LBPA antibody (6C4, Z-PLBPA) was from Echelon. Anti-rabbit (170-6515) and anti-mouse (170-6516) secondary antibodies conjugated to HRP were from Bio-Rad. Antibodies against EGFR (ab52894), EGFR (phospho Y1092, ab205827), Ezrin (ab4069), and AF647 (ab150079) as well as anti-mouse secondary antibodies conjugated to AF488 (ab150113) and AF647 (ab150115) and anti-rabbit secondary antibodies conjugated to AF488 (ab150077) were purchased from Abcam.

Primer probe sets for qRT-PCR

Drosha:
Forward: 5'- CAAGCTCTGTCCGTATCGATCA-3'
Reverse: 5'- TGGACGATAATCGGAAAAGTAATCA-3'
Probe: 5'-CTGGATCGTGAACAGTTCAACCCCGAT-3'

Malat1:
Forward: 5'-AAAGCAAGGTCTCCCCACAAG-3'
Reverse: 5'-TGAAGGGTCTGTGCTAGATCAAAA-3'
Probe: 5'-TGCCACATCGCCACCCCGT-3'

Oligonucleotides

Oligonucleotides

IONIS ID 446654 targets human *PETN*: 5'-Cy3-C*T*G* C*T*A* G*C*C* T*C*T* G*G*A* T*T*T* G*A-3'. The underlined nucleotides are 2'-O-MOE modified, the * indicates phosphorothioate backbone and 5' end is labeled with Cy3.

IONIS ID 851810 targets human *PETN*: 5'-Cy5-C*T*G* C*T*A* G*C*C* T*C*T* G*G*A* T*T*T* G*A-3'. The underlined nucleotides are 2'-O-MOE modified, the * indicates phosphorothioate backbone and 5' end is labeled with Cy5.

IONIS ID 395254 targets human *Malat1*: 5'-G*G*C* A*T*A* T*G*C* A*G*A* T*A*A* T*G*T* T*C-3'. The underlined nucleotides are 2'-O-MOE modified, and the * indicates phosphorothioate backbone.

IONIS ID 25690 targets *Drosha*: 5´-A*T*C* C*C*T* T*T*C* T*T*C* C*G*C* A*T*G*T*G -3'. The underlined nucleotides are 2'-O-MOE modified, and the * indicates phosphorothioate backbone.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Fatty acids increase PS-ASO activities. A) HeLa or **B)** HEK cells were treated with indicated concentrations of PS-ASOs targeting *Malat1* for 4 h, followed by replacement with medium without PS-ASOs but containing 200 μM palmitic acid. After 20 h, the levels of *Malat1* were determined by qRT-PCR. Percent expression relative to non-PS-ASO treated control is plotted. The error bars represent standard deviations from three independent experiments. P<0.01 for 100 μM versus 0 μM (blue); P<0.01 for 200 μM versus 0 μM (red). P values were computed by two-way ANOVA using Prism. **C)** A431 cells were treated with PS-ASOs targeting *Drosha* or *Malat1* for 4 h. Medium was replaced with medium without PS-ASO but containing 100 μM palmitic acid (16:0), stearic acid (18:0), palmitoleic acid (16:1), oleic acid (16:1), heptadecylic acid (17:0), or nonadecylic acid (19:0), and cells were incubated for another 20 h. The levels of *Drosha* and *Malat1* RNAs were determined by qRT-PCR. Percent expression relative to non-treated control is plotted. The error bars represent standard deviations from three independent experiments. **D)** A431 cells pretreated with 200 μM palmitic acid for 16 h were incubated with Cy3-PS-ASO (IONIS ID 446654) for 2 h. Intracellular fluorescence of Cy3-PS-ASO was quantified by flow cytometry. RFU is plotted versus N-Rh-PE concentration; p<0.01 control versus PA treatment (*). P values were computed by student T-test; **E)** Intracellular RFU of N-Rh-PE at indicated concentrate was quantified by flow cytometry in A431 cells pretreated with 200 μM palmitic acid for 16 h and then N-Rh-PE for 2 h.

Figure S2. Free fatty acids induce lipid droplets within 4 h. Immunofluorescent staining for lipid droplets in cells that were treated with 200 μM palmitic acid for indicated times. The nuclei were stained with DAPI (blue). Scale bars: 2 μm.

Figure S3. Prolonged treatment of cells with ceramide decreases PS-ASO uptake but increases membrane fusion rates. A) A431 cells were pretreated with 10 μM ceramide for 16 h or were not treated (control). Intracellular fluorescence of Cy3-PS-ASO (IONIS ID 446654) was quantified by flow cytometry. RFU is plotted versus Cy3-PS-ASO concentration. **B)** Intracellular fluorescence of N-Rh-PE in A431 cells pretreated with 10 μM ceramide for 16 h and then incubated with N-Rh-PE for 2 h. RFU is plotted versus N-Rh-PE concentration. $p<0.01$ control versus ceramide treatment (*). P values were computed by student T-test. **C)** HeLa or **D)** HEK cells were treated with indicated concentrations of PS-ASOs targeting *Malat1* for 4 h, followed by replacement with medium without PS-ASOs but containing 10 μM ceramide. After 20 h, the levels of *Malat1* were determined by qRT-PCR. Percent expression relative to non-PS-ASO treated control is plotted. The error bars represent standard deviations from three independent experiments. $P<0.01$ for 100 μM versus 0 μM (blue); $P<0.01$ for 10 μM versus 0 μM (red). P values were computed by two-way ANOVA using Prism.

Figure S4. **Prolonged treatment of cells with cholesterol decreases PS-ASO uptake but increases membrane fusion rates. A**) Intracellular fluorescence of Cy3-PS-ASO (IONIS ID 446654) was quantified by flow cytometry in A431 cells pretreated with 50 μM MCD-complexed cholesterol or untreated (control) in the presence of 1 μM Sandoz 58-035 for 16 h. $p<0.01$ control versus cholesterol treatment (*). P values were computed by student T-test. **B**)

Intracellular RFU due to N-Rh-PE was quantified by flow cytometry in A431 cells pretreated with 50 μM MCD-complexed cholesterol in the presence of 1 μM Sandoz 58-035 for 16 h and then incubated with N-Rh-PE for 2 h. RFU is plotted versus N-Rh-PE concentration. **C)** HeLa or **D)** HEK cells were treated with indicated concentrations of PS-ASOs targeting *Malat1* for 4 h, followed by replacement with medium without PS-ASOs but containing 50 μM MCD-complexed cholesterol. After 20 h, the levels of *Malat1* were determined by qRT-PCR. Percent expression relative to non-PS-ASO treated control is plotted. The error bars represent standard deviations from three independent experiments. $P<0.01$ for 100 μM versus 0 μM (blue); $P<0.01$ for 10 μM versus 0 μM (red). P values were computed by two-way ANOVA using Prism.

Figure S5. **Lipids incorporate into cells within 4 h and do not significantly change lipid raft staining patterns. A)** Representative immunofluorescent images of indicated cells incubated with 50 μM BODIPY-C16, 50 μM NBD-cholesterol, or 5 μM NBD-ceramide. The nuclei were stained with DAPI (blue). Scale bars, 2 μm. **B)** Representative immunofluorescent images of A431 cells treated with 200 μM palmitic acid (PA), 10 μM ceramide (Ceramide) or 50 μM MCD-complexed cholesterol (Cholesterol), for 16 h and stained for lipid rafts. The nuclei were stained with DAPI (blue). Scale bars, 2 μm.

Fig. S1.

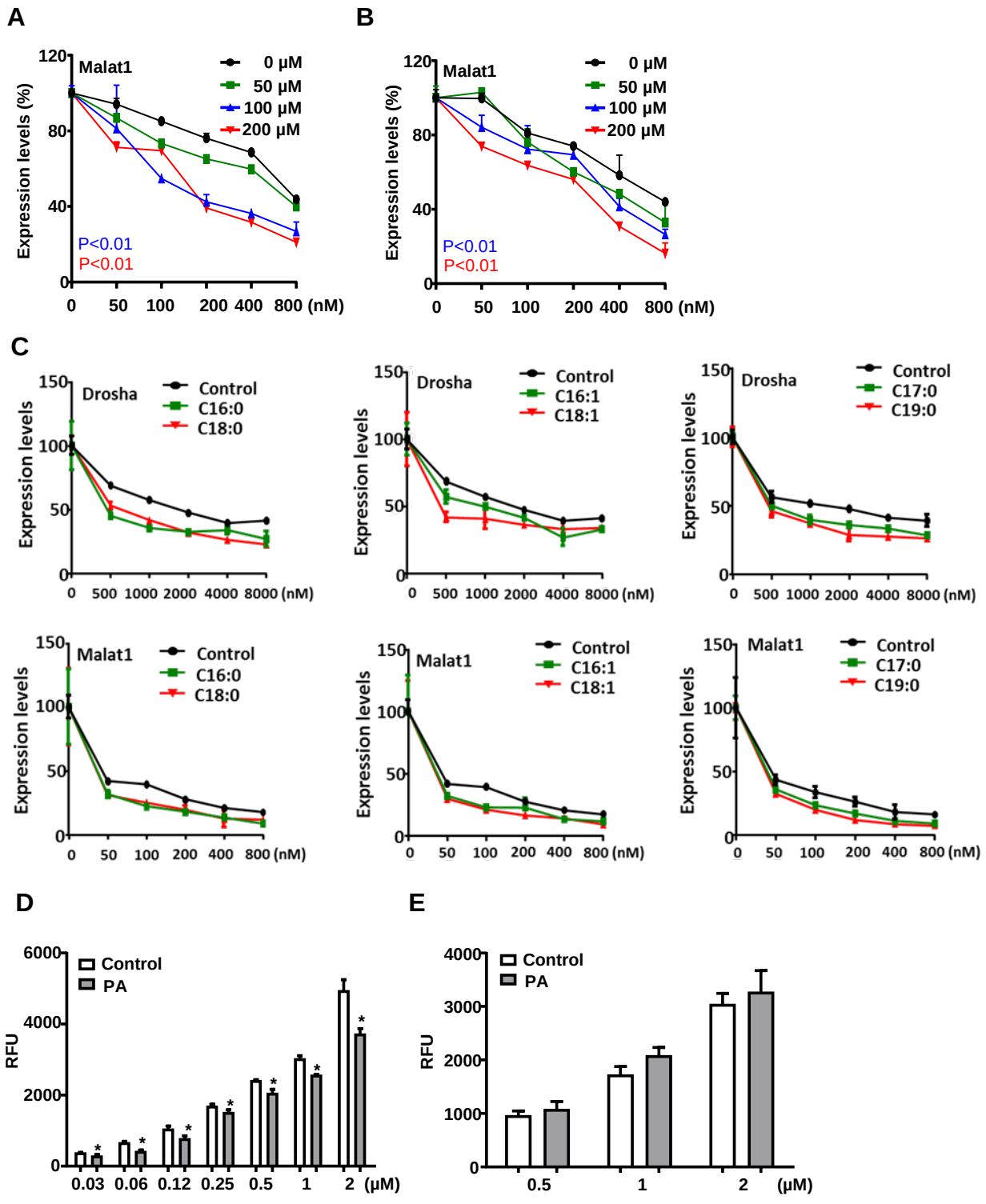

A
Malat1
0 μM
50 μM
100 μM
200 μM
Expression levels (%)
P<0.01
P<0.01
0 50 100 200 400 800 (nM)
B
Malat1
0 μM
50 μM
100 μM
200 μM
Expression levels (%)
P<0.01
P<0.01
0 50 100 200 400 800 (nM)
C
Drosha
Control
C16:0
C18:0
Expression levels
0 500 1000 2000 4000 8000 (nM)
Drosha
Control
C16:1
C18:1
Expression levels
0 500 1000 2000 4000 8000 (nM)
Drosha
Control
C17:0
C19:0
Expression levels
0 500 1000 2000 4000 8000 (nM)
Malat1
Control
C16:0
C18:0
Expression levels
0 50 100 200 400 800 (nM)
Malat1
Control
C16:1
C18:1
Expression levels
0 50 100 200 400 800 (nM)
Malat1
Control
C17:0
C19:0
Expression levels
0 50 100 200 400 800 (nM)
D
Control
PA
RFU
0.03 0.06 0.12 0.25 0.5 1 2 (μM)
E
Control
PA
RFU
0.5 1 2 (μM)

Fig. S2.

A

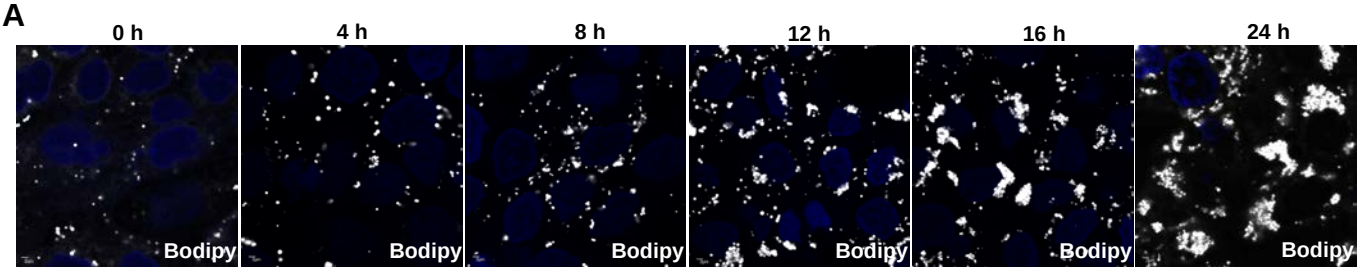

0 h
4 h
8 h
12 h
16 h
24 h
Bodipy
Bodipy
Bodipy
Bodipy
Bodipy
Bodipy

Fig. S3.

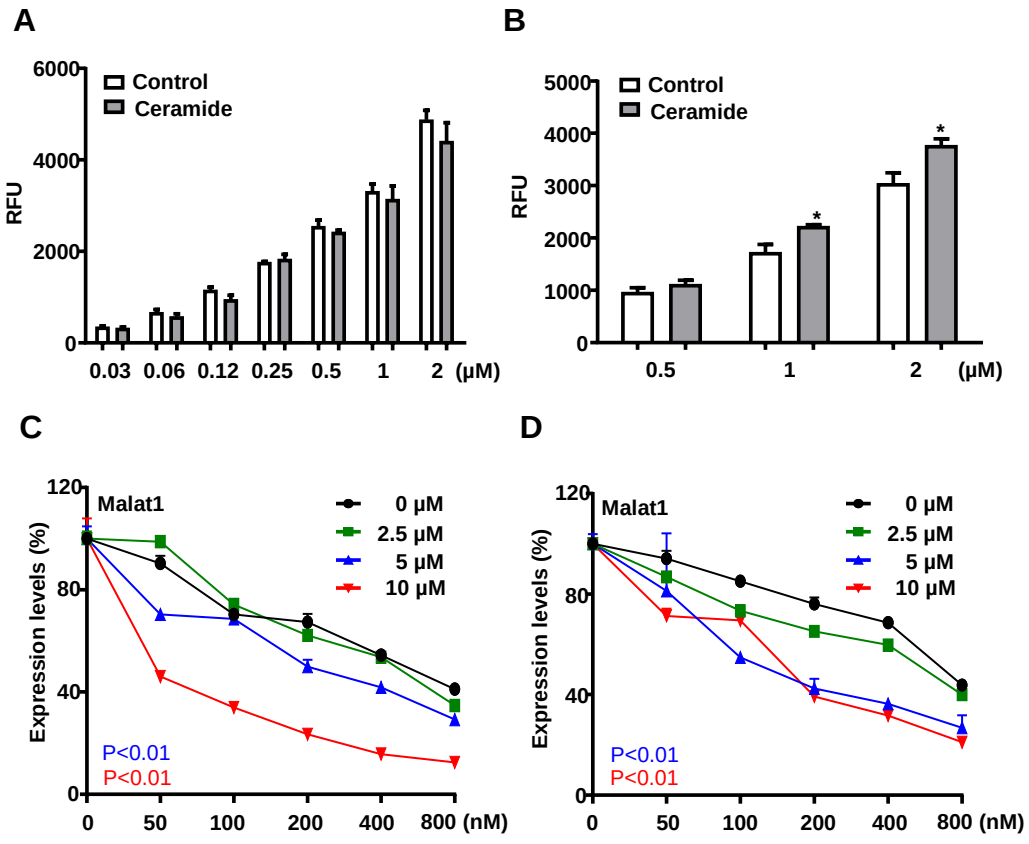

A
B
Control
Ceramide
RFU
6000
4000
2000
0
0.03
0.06
0.12
0.25
0.5
1
2
(μM)
5000
4000
3000
2000
1000
0
0.5
1
2
(μM)
*
C
D
Malat1
0 μM
2.5 μM
5 μM
10 μM
Expression levels (%)
120
80
40
0
P<0.01
P<0.01
0
50
100
200
400
800 (nM)

Fig. S4.

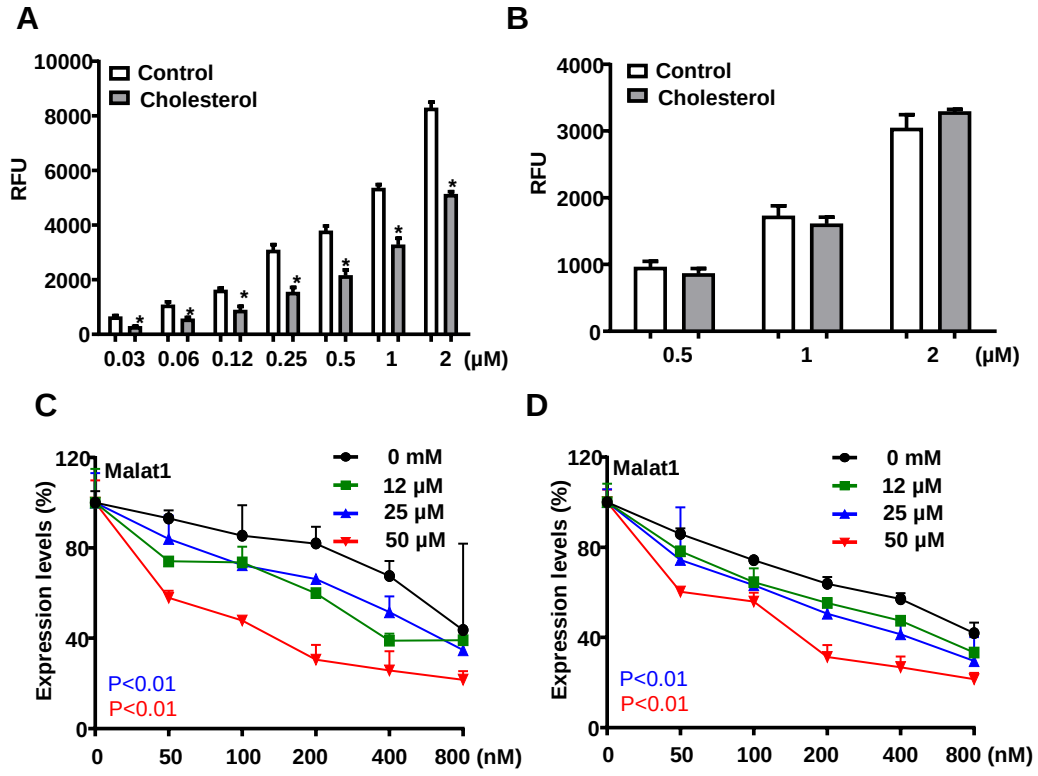
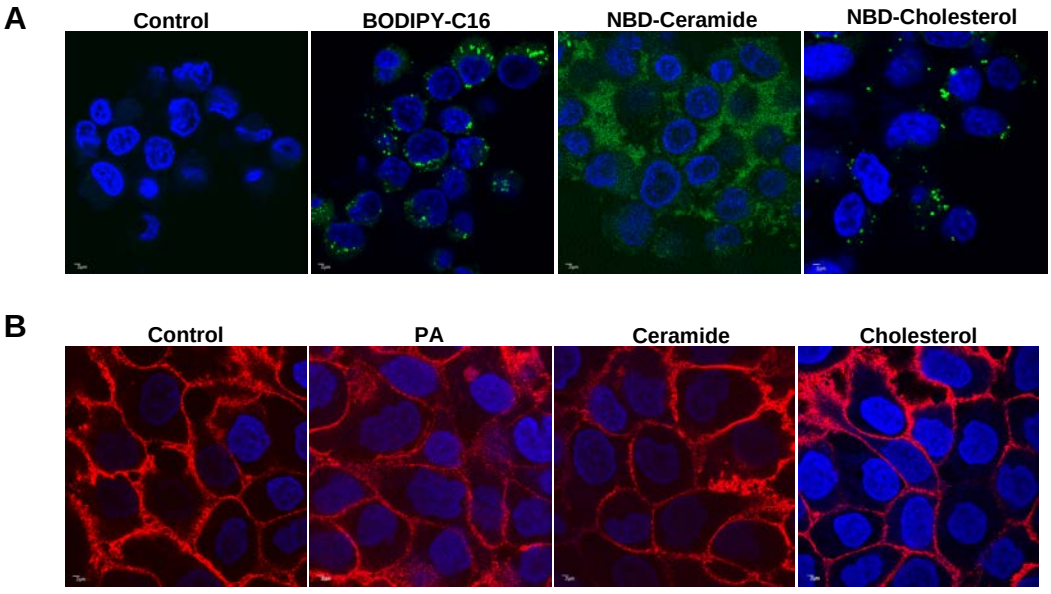

A
Control
Cholesterol
RFU
10000
8000
6000
4000
2000
0
0.03 0.06 0.12 0.25 0.5 1 2 (μM)
B
Control
Cholesterol
RFU
4000
3000
2000
1000
0
0.5 1 2 (μM)
C
Malat1
0 mM
12 μM
25 μM
50 μM
Expression levels (%)
120
80
40
0
P<0.01
P<0.01
0 50 100 200 400 800 (nM)
D
Malat1
0 mM
12 μM
25 μM
50 μM
Expression levels (%)
120
80
40
0
P<0.01
P<0.01
0 50 100 200 400 800 (nM)

Fig. S5.


A
Control
BODIPY-C16
NBD-Ceramide
NBD-Cholesterol
B
Control
PA
Ceramide
Cholesterol