Supplementary Information (additional figures and tables)

Boosting intracellular delivery of lipid nanoparticle-encapsulated messenger RNA

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Supplementary Figure Legends:

Figure S1. Kinetics and dose of LNP transfection is comparable to commercially available lipoplex. Normalized luciferase expression was measured at 3, 12 or 24 hr time points in HeLa cells transfected with different concentrations of luciferase mRNA packaged with (a) lipoplex or (b) LNP. All experiments were conducted with $n = 3$; mean ± SD.

Figure S2. Lipoplex transfection is mTOR-dependent. (a) Transfection of HAP1-WT cells using mRNA-lipoplexes (50 ng mRNA) in the presence of varying concentrations of Everolimus or Torin 1. (b) Results of electroporation of free mRNA (1 µg) into MEF-TSC2-WT and MEF-TSC2-KO cells using various pulse voltages, pulse widths and pulse numbers. (c) Upon amino acid influx into the lysosome, mTORC1 translocates to the cytoplasmic surface of lysosomes, ultimately triggering ribosomal biogenesis and mRNA translation. TSC1/2 complex negatively regulates mTOR in the absence of amino acids, thereby preventing its downstream signaling. In the case of TSC2 knockouts, the TSC1/2 complex fails to form, leaving mTORC1 in a constitutively active state leading to increased translation of exogenously delivered and protein production. On the other hand, mTORC1 inhibition by Torin 1 and Everolimus results in significantly lowered expression after LNP mediated mRNA delivery. Transfection measured as normalized luciferase expression, all experiments were conducted with $n = 3$; mean ± SD. Statistical analysis of the data was done by student's t-test ($0.05 \geq *p > 0.01$, $0.01 \geq **p > 0.005$, $***p \leq 0.005$).

Figure S3. Screening of previously reported molecules that enhance gene delivery led to minor increase in lipoplex based mRNA transfection. (a) Dose response of mRNA-lipoplexes after 1-hr pre-treatment of cells with bioactive molecules. (b-d) Effects of pre-incubation of bioactive molecules prior to transfection with mRNA lipoplexes (50 ng mRNA) with either lipoplexes or cells for (b) 15 min, (c) 1 hr, or (d) 18 hr. Lipoplex alone serves as control. CQ = Choloroquine (100 µM), MDP = Muramyl Dipeptide with C-18 fatty acid chain (10 µM), P103 = Pluronic® P103 ((a) 1 µM), (b-d) 2 µM), F127 = Pluronic® F127 (1 µM), GBA = Guanabenz Acetate (50 µM), P84 = Pluronic® P84 (2 µM). Lipoplex alone serves as control. Transfection measured as

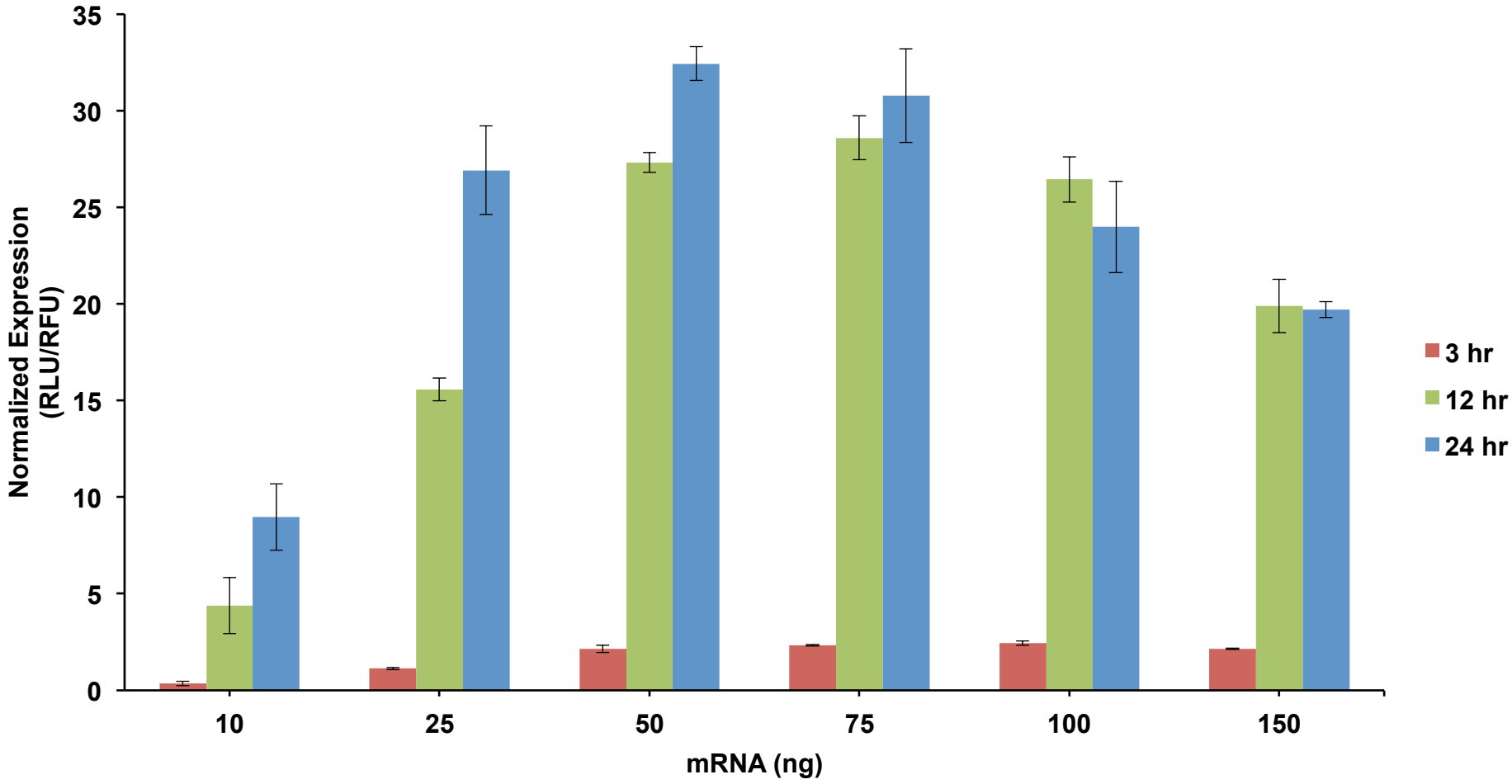
normalized luciferase expression, all experiments were conducted with n = 3; mean ± SD. Statistical analysis of the data was assessed by student's t-test ($0.05 \geq *p > 0.01$, $0.01 \geq **p > 0.005$, $***p \leq 0.005$).

Figure S4. Identification of MK-571 as a transfection-enhancing additive. (a) mRNA-lipoplex transfection (50 ng mRNA) in the presence of different concentrations of (i) bioactive lipids L1-L6 and (ii) bioactive lipids L7-L12 **(Supplementary Table 2)** and (b) arachidonic acid. (c) Chemical structure of MK-571, Montelukast, Pranlukast, and Zafirlukast. (d) mRNA-lipoplex transfection (50 ng mRNA) in the presence of different concentrations of MK-571, Montelukast, Pranlukast, or Zafirlukast. Lipoplex alone serves as control. Transfection measured as normalized luciferase expression, all experiments were conducted with n = 3; mean ± SD.

Figure S5. The addition of MK-571 to LNPs enhances transfection without altering particle structure. (a) Table depicting size, PDI and encapsulation efficiency of LNP and LNP-MK571. (b) Agarose gel electrophoresis of free mRNA, LNP and LNP-MK571 in the presence of heparin (5-20 µg) or Triton X-100 (1-10%). (c-e) HAP1-Rab4-KO (c), HAP1-Rab5-KO (d), and HAP1-Rab7-KO (e) cells were transfected with LNP-MK571 at a range of mRNA doses and normalized luciferase expression was compared to wild-type. (f) Luciferase expression of LNP and LNP-MK571 was compared in HAP1-Rab7-KO cells. All experiments were conducted with n = 3; mean ± SD. Statistical analysis of the data was assessed by student's t-test ($0.05 \geq *p > 0.01$, $0.01 \geq **p > 0.005$, $***p \leq 0.005$). (g) Luciferase expression in BALB/c mice 6 hr post intravenous administration of LNP or LNP-MK571 (see **Fig. 2f**, n = 6; mean ± SD). (h) Luciferase expression in corresponding mouse organs at 6 hr post intravenous administration of LNP or LNP-MK571 (see **Fig. 2g**, n = 6; mean ± SD).

Figure S1

a.


Normalized Expression (RLU/RFU)
35
30
25
20
15
10
5
0
10
25
50
75
100
150
mRNA (ng)
3 hr
12 hr
24 hr

b.

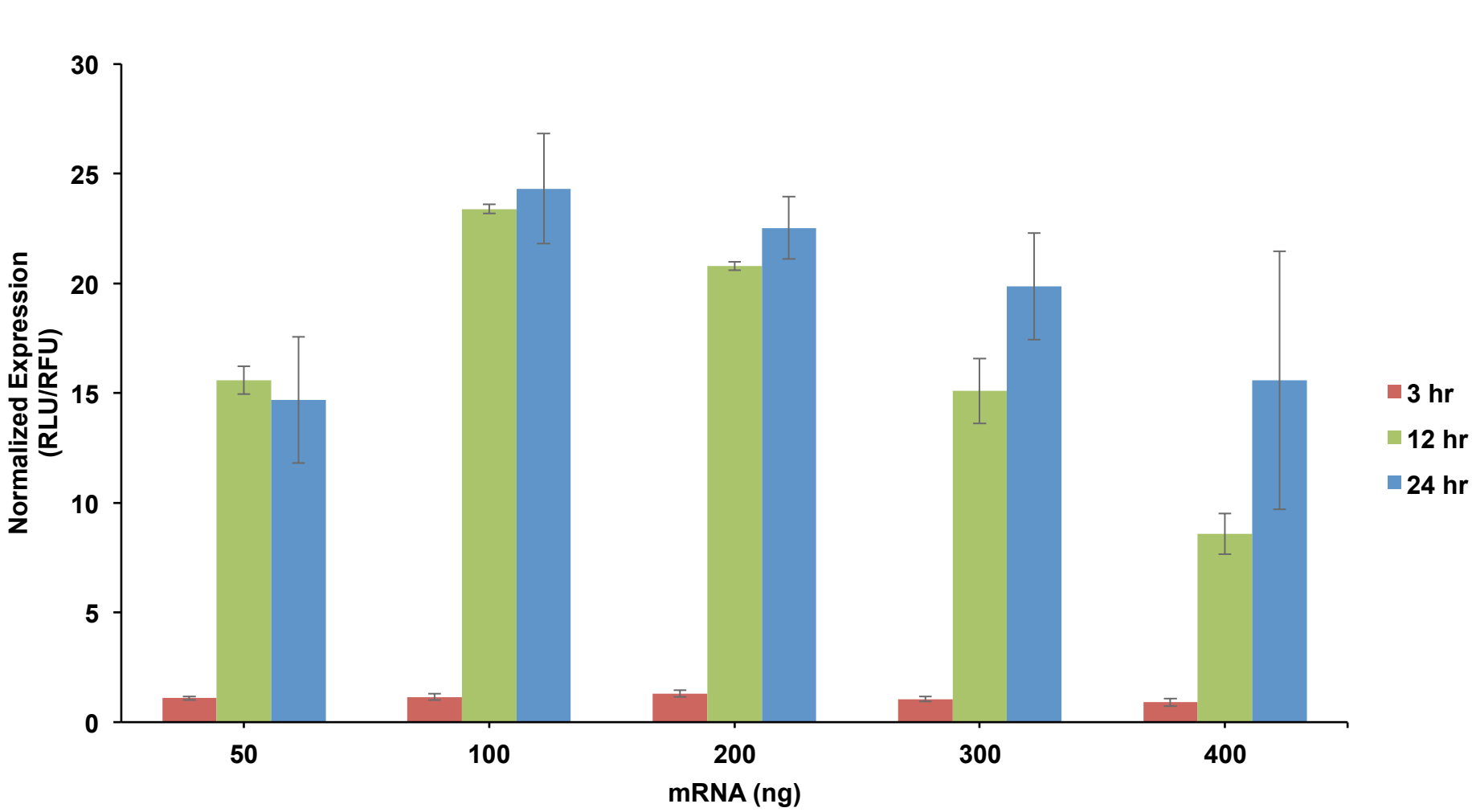
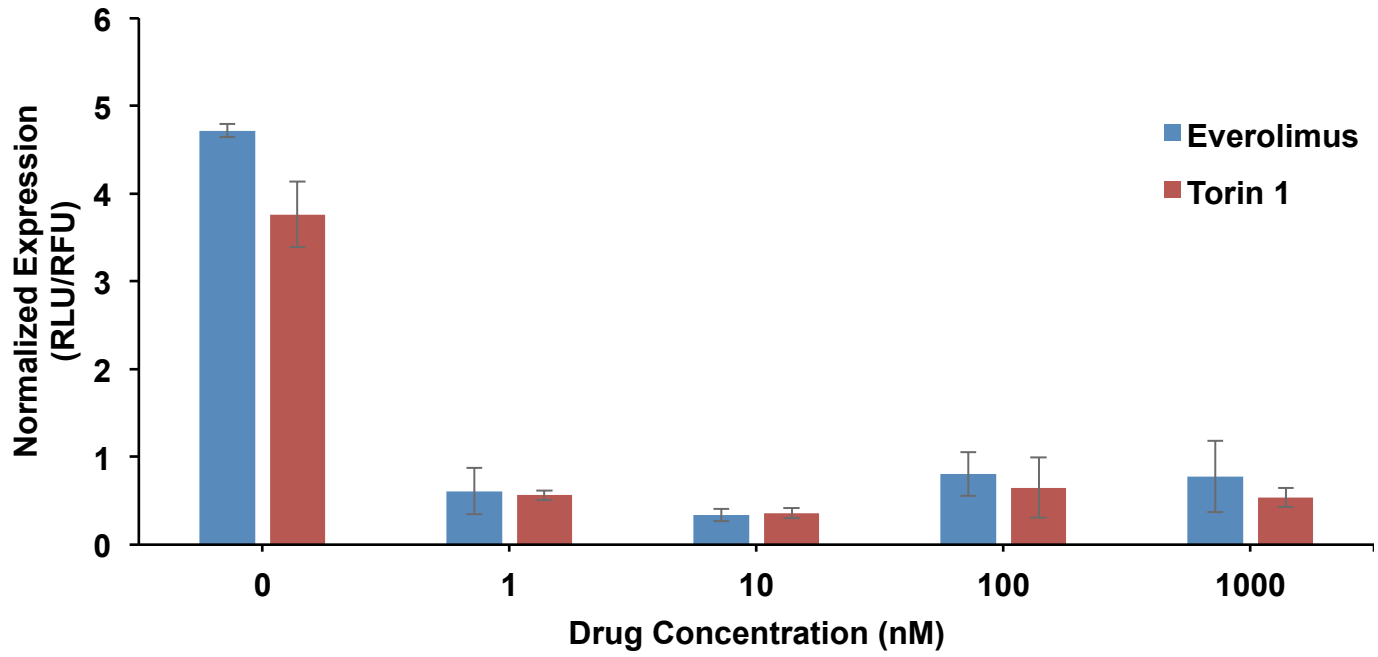

Normalized Expression (RLU/RFU)
30
25
20
15
10
5
0
50
100
200
300
400
mRNA (ng)
3 hr
12 hr
24 hr

Figure S2

a.



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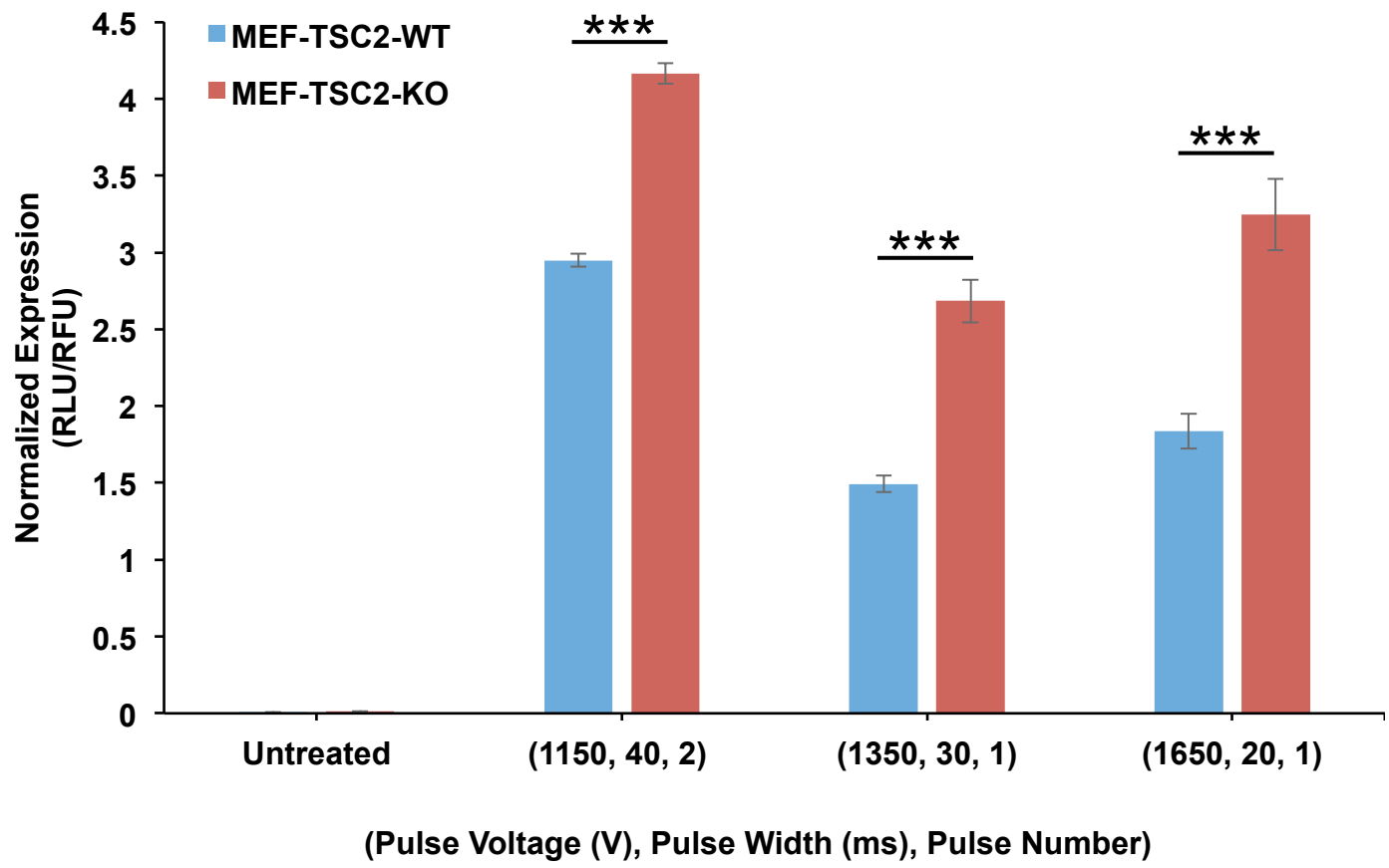

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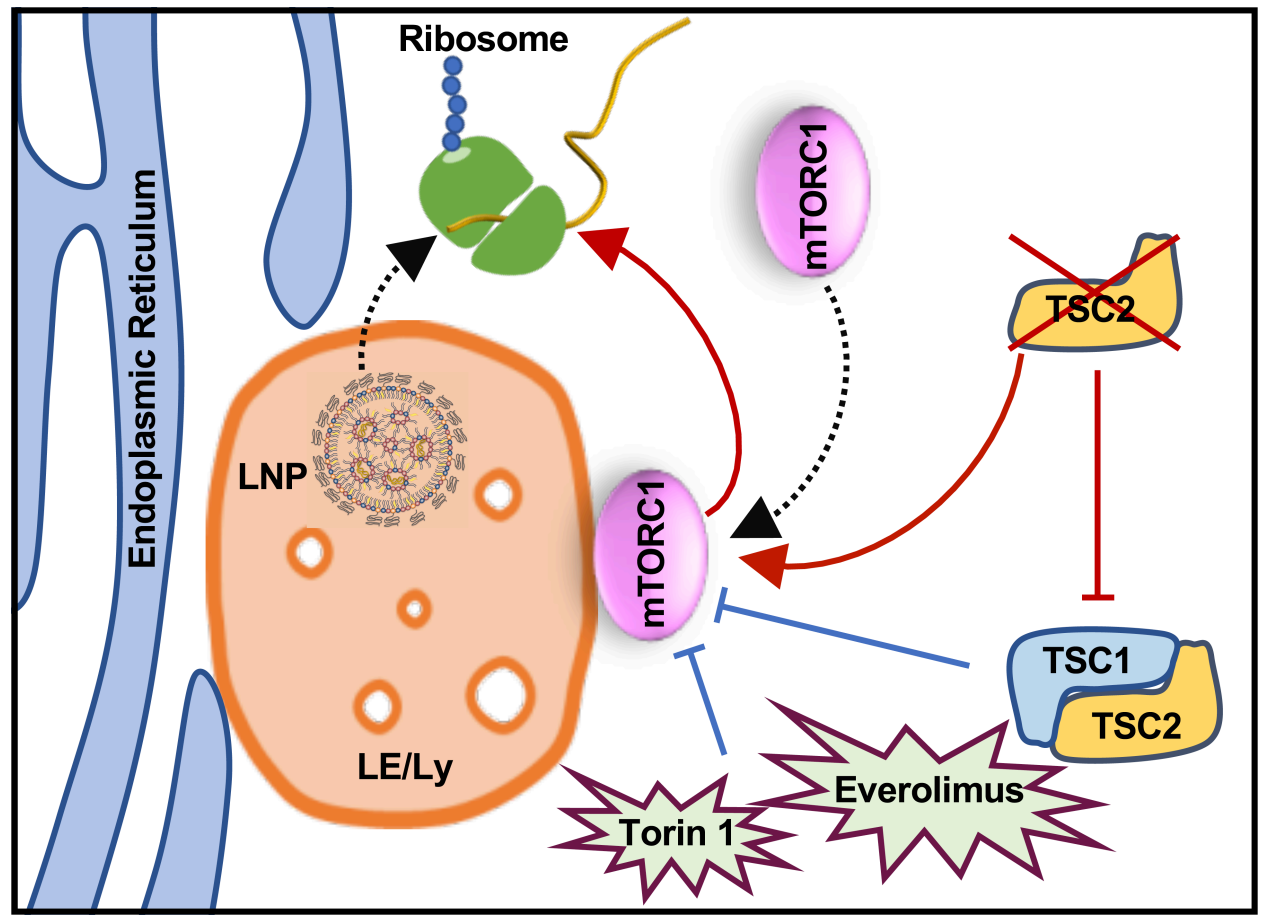


Figure S3

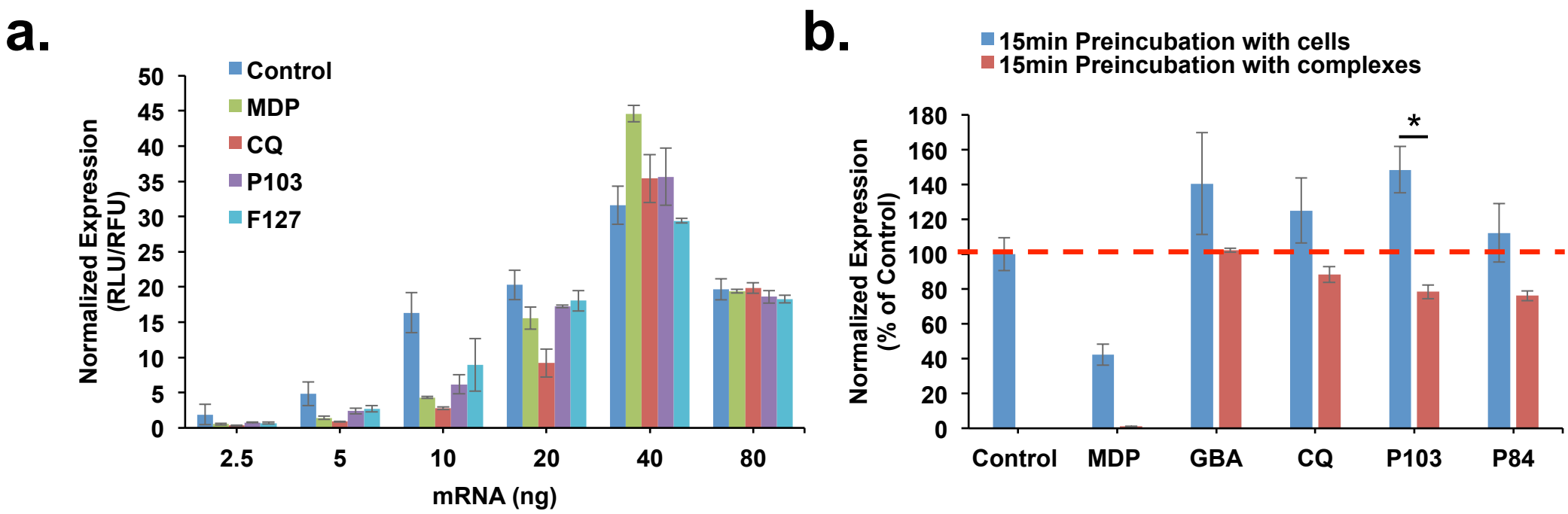
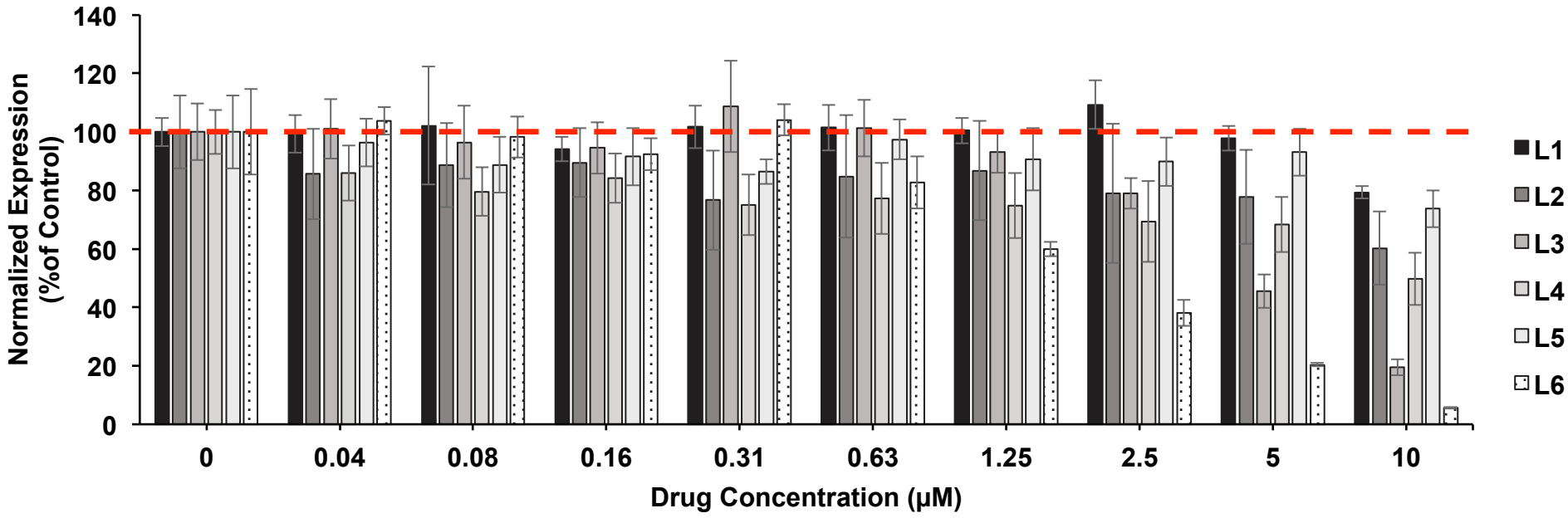

a.
Control
MDP
CQ
P103
F127
Normalized Expression (RLU/RFU)
50
45
40
35
30
25
20
15
10
5
0
2.5
5
10
20
40
80
mRNA (ng)
b.
15min Preincubation with cells
15min Preincubation with complexes
Normalized Expression (% of Control)
180
160
140
120
100
80
60
40
20
0
*
Control
MDP
GBA
CQ
P103
P84
c.
1hr Preincubation with cells
1hr Preincubation with complexes
Normalized Expression (% of Control)
200
180
160
140
120
100
80
60
40
20
0
*
*
Control
MDP
GBA
CQ
P103
P84
d.
18hr Preincubation with cells
18hr Preincubation with complexes
Normalized Expression (% of Control)
140
120
100
80
60
40
20
0
Control
MDP
GBA
CQ
P103
P84

Figure S4

a. i.



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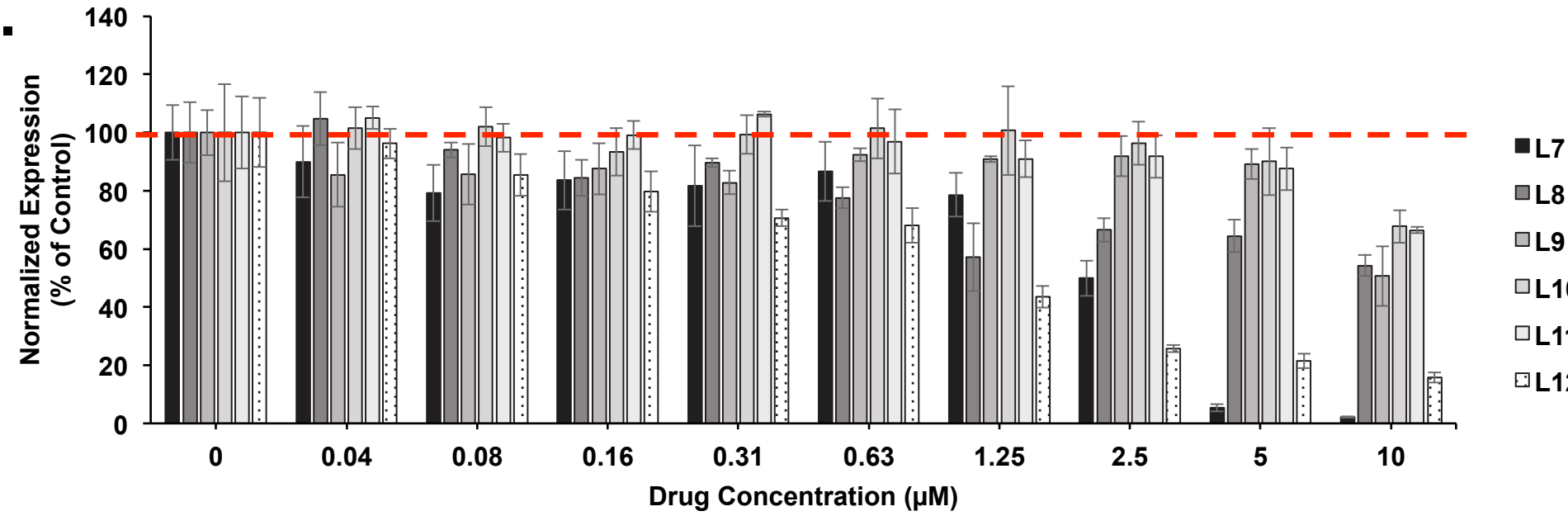

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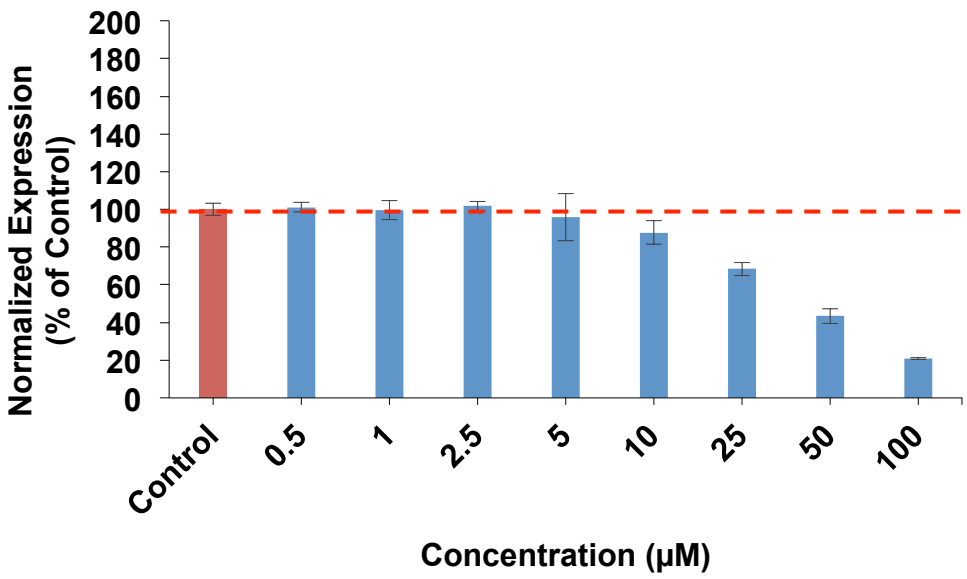

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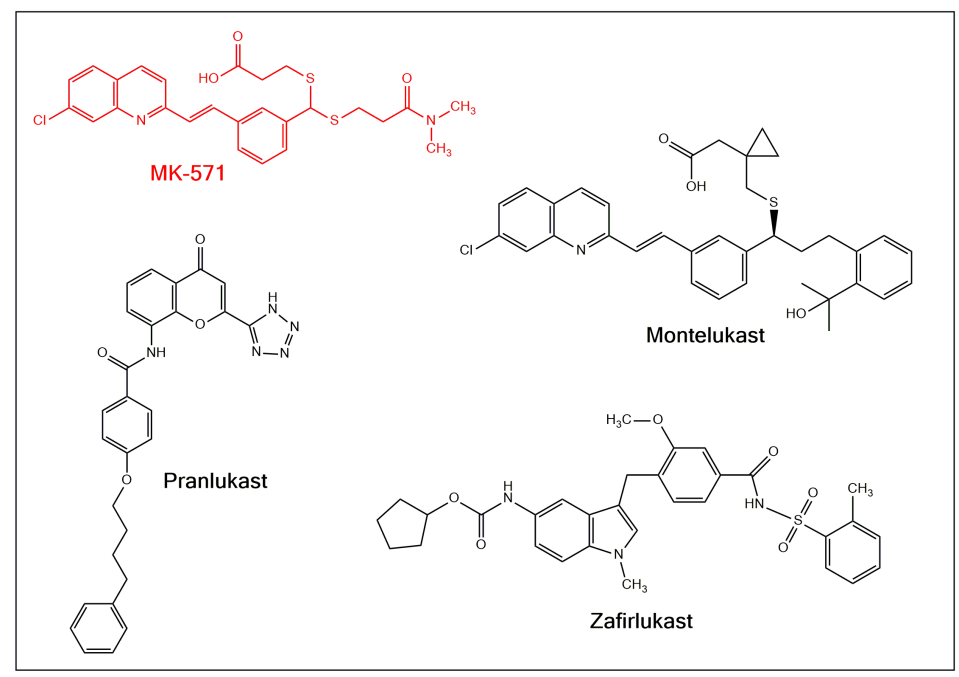

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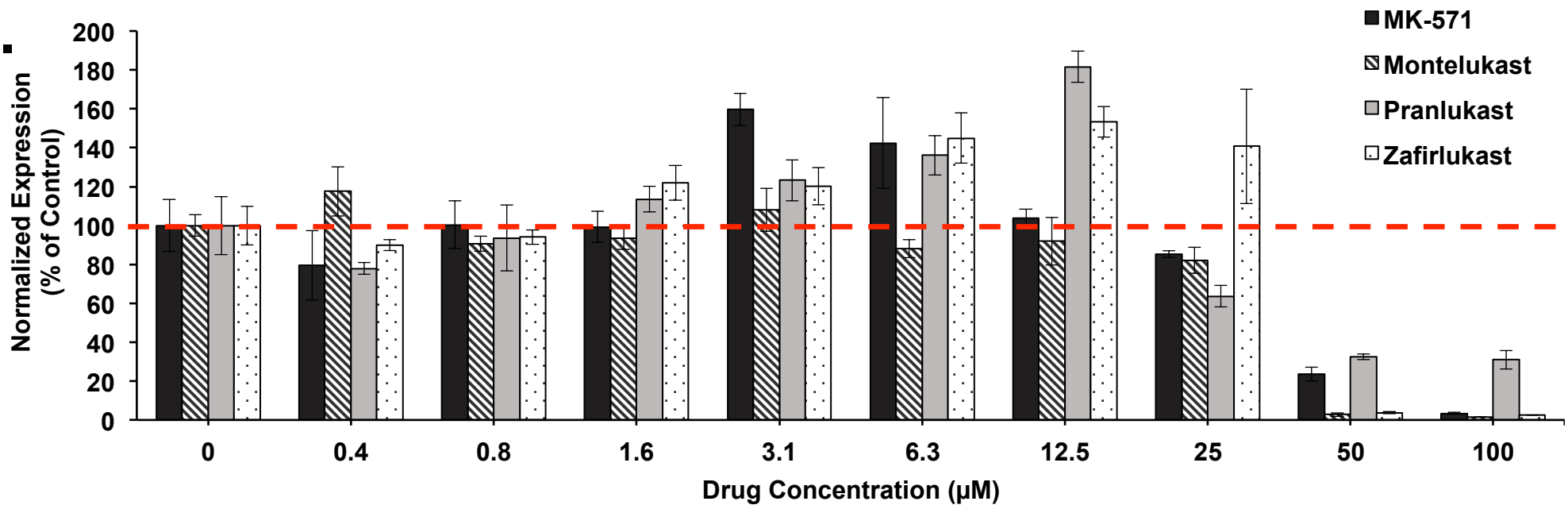
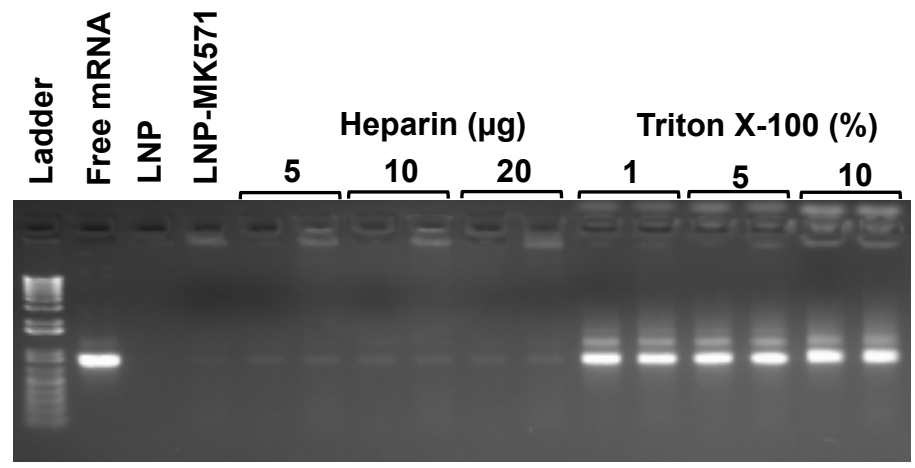


Figure S5

a.

Sample	Size (d.nm)	PDI	Encapsulation Efficiency (%)
LNP	116.5 ± 2.5	0.108	92
LNP-MK571	117.0 ± 0.2	0.075	91

b.



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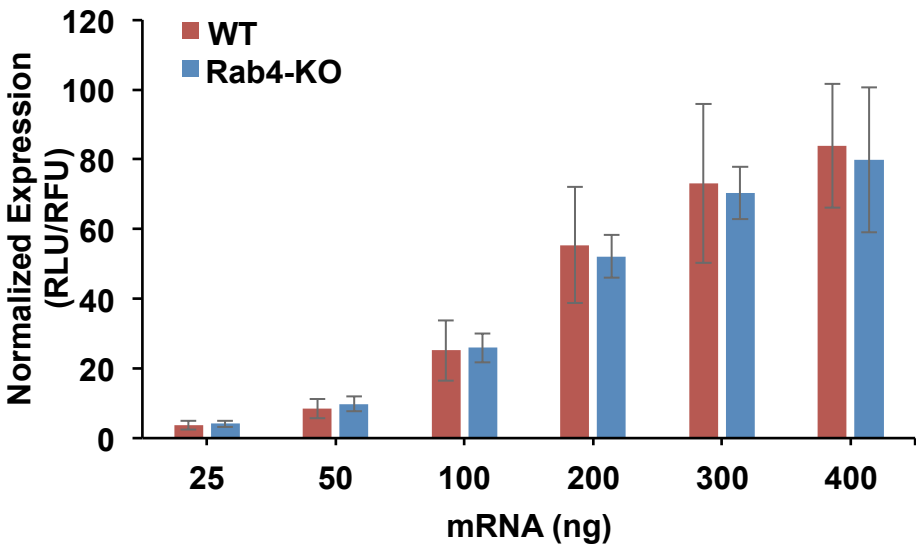

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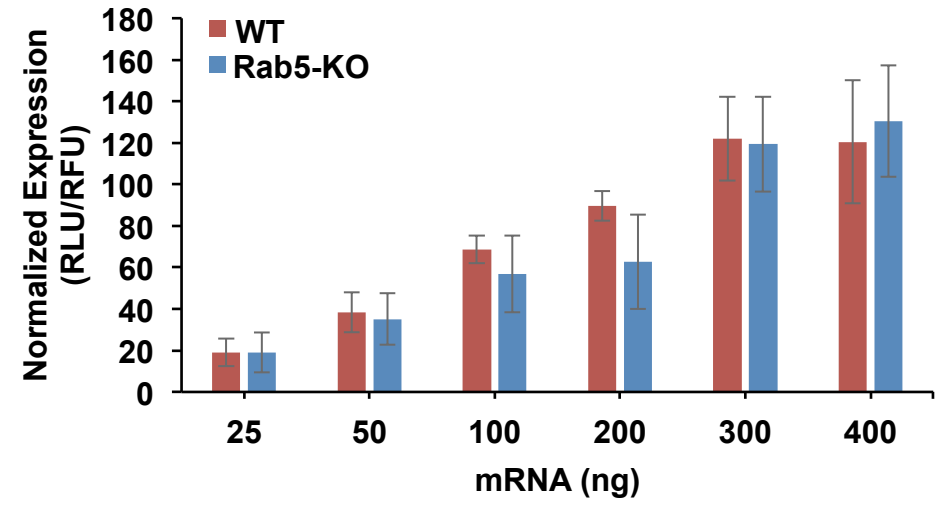

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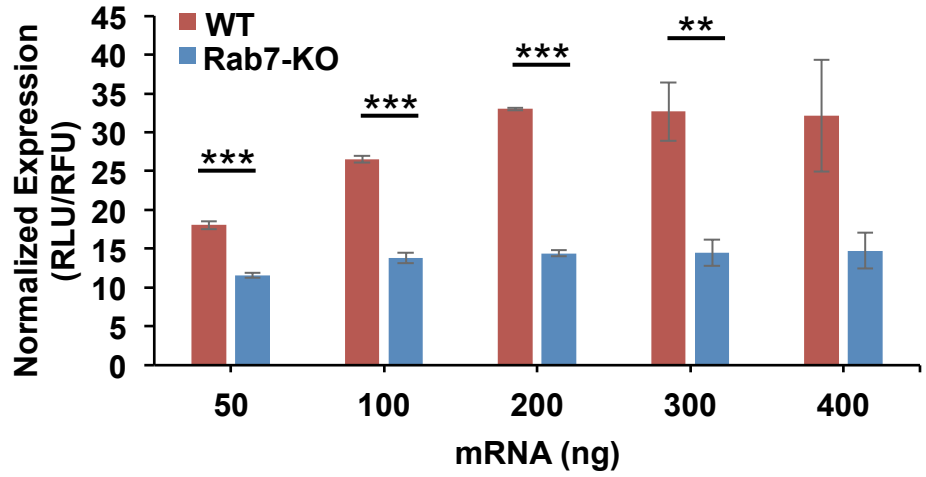

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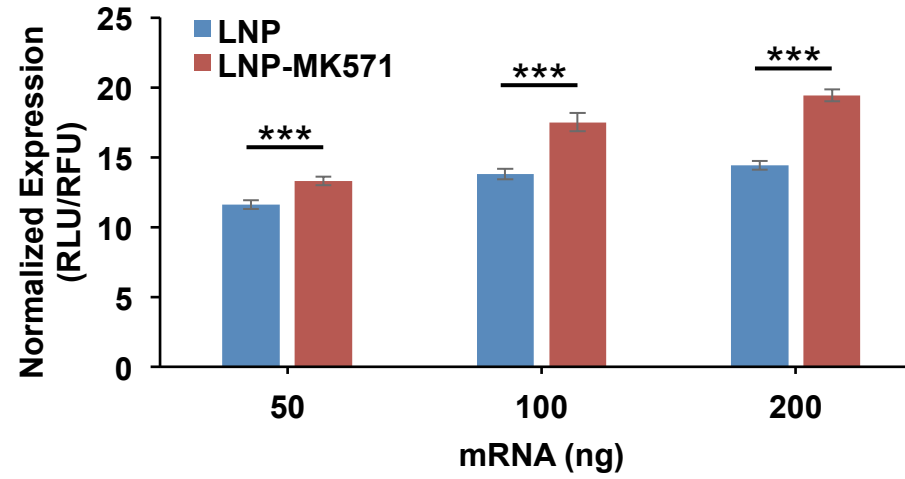

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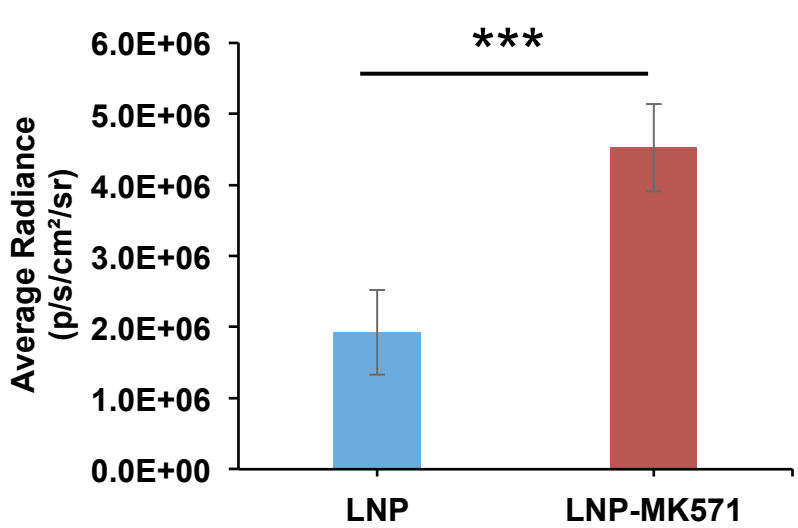

h.

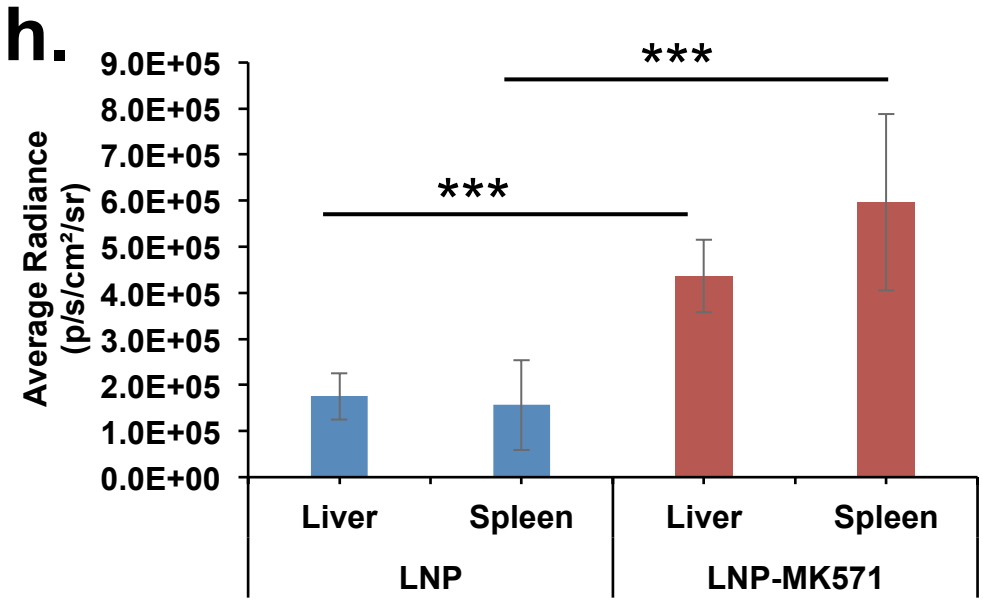

Table S1. Enhancers of Intracellular Delivery

Bioactive Molecule	Proposed Action	Reference
L-18-Muramyl dipeptide (MDP)	Uptake and delivery of bacteria to cytosol	Nakamura, N.; Lill, J. R.; Phung, Q.; Jiang, Z.; Bakalarski, C.; de Mazière, A.; Klumperman, J.; Schlatter, M.; Delamarre, L.; Mellman, I. *Nature* **2014**, *509* (7499), 240–244.
Guanabenz Acetate (GBA)	Enhances siRNA delivery	Osborn, M. F.; Alterman, J. F.; Nikan, M.; Cao, H.; Didiot, M. C.; Hassler, M. R.; Coles, A. H.; Khvorova, A. *Nucleic Acids Res* **2015**, *43* (18), 8664–8672.
Chloroquine (CQ)	Prevent endosomal acidification, enhances nucleic acid delivery	Cordier, C.; Boutimah, F.; Bourdeloux, M.; Dupuy, F.; Met, E.; Alberti, P.; Loll, F.; Chassaing, G.; Burlina, F.; Saison-Behmoaras, T. E. *PLOS ONE* **2014**, *9* (8), e104999.
		Bhattarai, S. R.; Muthuswamy, E.; Wani, A.; Brichacek, M.; Castañeda, A. L.; Brock, S. L.; Oupicky, D. *Pharm Res* **2010**, *27* (12), 2556–2568.
Pluronic® P103 (P103)	Shown to improve DNA delivery	Kabanov, A.; Zhu, J.; Alakhov, V. Genetics, B.-A. in, Ed.; Non-Viral Vectors for Gene Therapy, Second Edition: Part 1; Academic Press, 2005; Vol. 53, pp 231–261.
Pluronic® P84 (P84)		
Pluronic® F127 (F127)		

Table S2. Compounds from Initial Lipid Library Screen with Potential Transfection Enhancing Ability

Compound ID	Compound Name
L1	5S-hydroxy-6E,8Z,11Z,14Z,17Z-eicosapentaenoic acid [5(S)-HEPE]
L2	(±)5,6-dihydroxy-8Z,11Z,14Z-eicosatrienoic acid, 1,5-lactone [(±) 5, 6 – DHET lactone]
L3	(±)12(13)epoxy-9Z-octadecenoic acid [(±) 12(13) – EpOME]
L4	D-myo-Inositol-1,3,4,5-tetraphosphate
L5	D-myo-Inositol-1,2,3,6-tetraphosphate
L6	Lactacystin
L7	Hyperforin
L8	2,3-dinor Thromboxane B1
L9	Prostaglandin D2-biotin
L10	1-(1,2-dihexadecanoylphosphatidyl)inositol-3-phosphate, diammonium salt [PtdIns-(3)-P_1 (1,2-dioctanoyl)]
L11	9α-hydroxy-11,15-dioxo-2,3,4,5-tetranor-prostan-1,20-dioic acid-17,17,18,18,19,19-d_6 [Tetranor-PGDM-d_6]
L12	N1-hydroxy-N8-phenyl-octanediamide [SAHA]