# SUPPORTING MATERIAL

# Transgene expression in mice of the Opa1 mitochondrial transmembrane protein through bicontinuous cubic lipoplexes containing gemini imidazolium surfactants

Mónica Muñoz-Úbeda[1,2,*] Martina Semenzato[3], Anais Franco-Romero[3], Elena Junquera[2], Emilio Aicart[2], Luca Scorrano[3] and Iván López-Montero[1,2,*].

*1 Instituto de Investigación Biomédica Hospital 12 de Octubre (imas12), Madrid, Spain*

*2 Dpto. Química Física, Universidad Complutense de Madrid, Spain.*

*3 Fondazione per la Ricerca Biomèdica Avanzata, Venetian Institute of Molecular Medicine (VIMM), Padova, Italy.*

Corresponding author: monicamubeda@quim.ucm.es, ivanlopez@quim.ucm.es

**Determination of the effective charge of the gemini cationic lipid and the MSCV-OPA1 plasmid.** The composition of the mixed lipid acting as cationic gene vector is given in terms of its molar fraction ($\alpha$) of the cationic lipid in the total lipid, while in the lipoplex, the composition may be expressed by: a) the total lipid to DNA mass ratio, defined as ($m_L/m_D = (m_{L^+} + m_{L^0})/m_D$), where $m_L$, $m_{L^+}$, $m_{L^0}$ and $m_D$ are the masses of the total mixed lipid, $(C_{16}Im)_2(C_4O)$, DOPE, DSPE-PEG and DNA, respectively, or b) the effective charge ratio ($\rho_{eff}$) expressed as the ratio between the charges of positive $(C_{16}Im)_2(C_4O)$ mixed lipid and negative DNA phosphate groups. All these quantities are related by the following two equations:

$$\alpha = \frac{m_{L^+} / M_{L^+}}{(m_{L^+} / M_{L^+}) + (m_{L^0} / M_{L^0})} \quad (1)$$

$$\rho_{eff} = \frac{n^+}{n^-} = \frac{q^+_{eff,\,L^+}(m_{L^+} / M_{L^+})}{q^-_{eff,\,D}(m_D / M_D)} \quad (2)$$

where $n^+$ and $n^-$ are the number of moles of positive and negative charges of $(C_{16}Im)_2(C_4O)$ and DNA respectively; $(q^+_{eff,L^+})$ and $(q^-_{eff,D})$ are the effective charges of $(C_{16}Im)_2(C_4O)$ and plasmid DNA (pDNA, MSCV-OPA1 in our case) per bp; and $M_L{}^0$, $M_L{}^+$ and $M_D$ are the molecular weight of the DOPE, $(C_{16}Im)_2(C_4O)$ and MSCV-OPA1 per bp, respectively.

The eletroneutrality ratio of the lipoplex $((m_L{}^+ + m_L{}^0) / m_D)_\Phi$ is reached for a particular formulation at which the positive charges of the mixed lipid and those negative of DNA balance ($\rho_{eff} = 1$). Values of $\rho_{eff}$ higher than the electroneutrality ratio are required for lipoplexes to become a potentially cell transfecting agent as the positively charged lipoplexes allow them crossing the negatively charged cell membranes.[1] The electroneutrality ratio can be accurately determined by measuring the zeta potential ($\zeta$) of lipoplexes as a function of ($m_L/m_D$) (see **Figure 1A**). A sign inversion on the charge in the ($\zeta$) sigmoidal plots occurs at $(m_L/m_D)_\Phi$ and this value is related to $\alpha$ through the equations (1-2) by:

$$\left(\frac{m_L}{m_D}\right)_\Phi = \left(\frac{m_{L^+} + m_{L^0}}{m_D}\right)_\phi = \frac{q^-_D[\alpha M_{L^+} + (1-\alpha)M_{L^0}]}{q^+_L \alpha M_D} \quad (3)$$

In general, linear DNAs, such as calf thymus DNA (ctDNA), have its negative charge totally available for the cationic lipid, i.e., $q_{linear\ D} = -2$ per base pair. However, plasmid DNA remains in a supercoiled conformation[2-7] rendering a much less negative charge than its nominal one ($q^-_{eff,D} \ll -2/bp$). The determination of the effective charge of both, the cationic lipid $(q^+_{eff,L^+})$and the pDNA $(q^-_{eff,D})$ is required then to quantitatively formulate lipoplexes with different effective charge ratios ($\rho_{eff}$). For that, the effective charge of the $(C_{16}Im)_2(C_4O)$ $(q^+_{eff,L^+})$ was first determined using equation (3) and the experimental value of $(m_L/m_D)_\Phi$ measured from zeta potential (**Figure S2**), and assuming $q_{linear\ D} = -2$/bp. The effective charge of MSCV-OPA1 $(q^-_{eff,D})$ is then obtained with equation (4) using the effective charge of the $(C_{16}Im)_2(C_4O)$ $(q^+_{eff,L^+})$ and the experimental electroneutrality value $(m_L/m_D)_\Phi$ for the $(C_{16}Im)_2(C_4O)$/DOPE/DSPE-PEG lipoplex containing MSCV-OPA1 plasmid DNA (**Figure 1**):

$$q^{-}_{eff,D} = \left(\frac{m_L}{m_D}\right)_{\Phi} \left(\frac{q^{+}_{L^+}\alpha M_D}{\alpha M_{L^+} + (1-\alpha)M_{L^0}}\right) \quad (4)$$

The effective charge ratio ($\rho_{eff}$) of the lipoplex at $\alpha = 0.2$ is obtained by substituting ($q^{+}_{eff,L^+}$) and the MSCV-OPA1 ($q^{-}_{eff,D}$) in equation (2).

## REFERENCES


(1) Dias, R. S.; Lindman, B. DNA Interaction with Polymers and Surfactants; *Wiley & Sons: Hoboken, NJ* **2008**.

(2) Muñoz-Úbeda, M.; Misra, S. K.; Barrán-Berdón, A. L.; Aicart-Ramos, C.; Sierra, M. B.; Biswas, J.; Kondaiah, P.; Junquera, E.; Bhattacharya, S.; Aicart, E. Why is less cationic lipid required to prepare lipoplexes from plasmid DNA than linear DNA in gene therapy? *J. Am. Chem. Soc*. **2011**, *133*, 18014-18017.

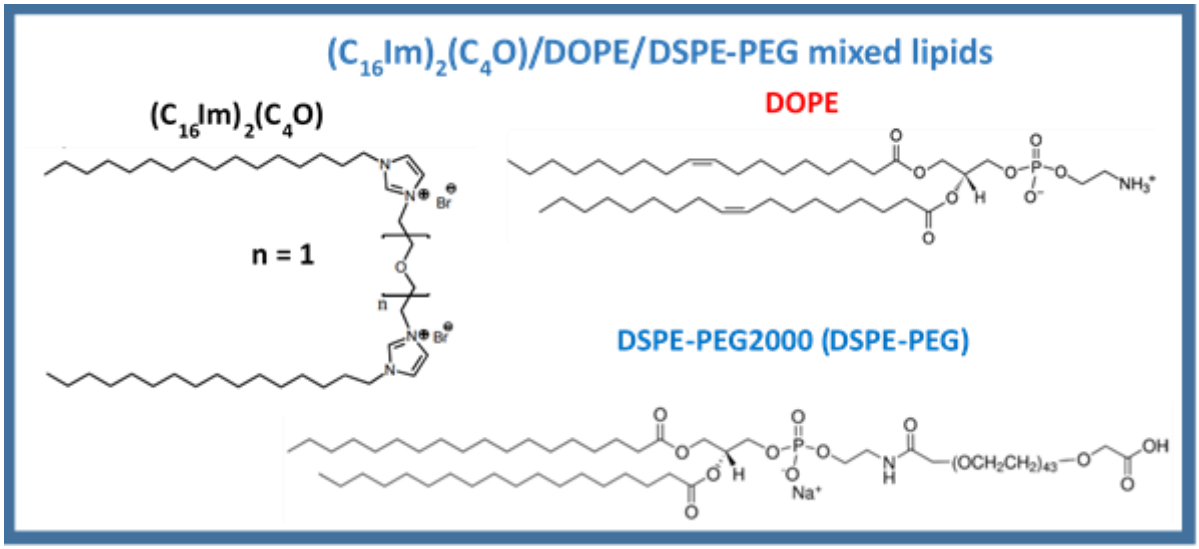
(3) Barrán-Berdón, A. L.; Misra, S. K.; Datta, S.; Muñoz-Úbeda, M.; Kondaiah, P.; Junquera, E.; Bhattacharya, S.; Aicart, E. Cationic gemini lipids containing polyoxyethylene spacers as improved transfecting agents of plasmid DNA in cancer cells. *J. Mater. Chem. B* **2014**, *2*, 4640-4652.

(4) Martinez-Negro, M.; Guerrero-Martinez, A.; Garcia-Rio, L.; Domenech, O.; Aicart, E.; de Ilarduya, C. T.; Junquera, E. Multidisciplinary approach to the transfection of plasmid DNA by a nonviral nanocarrier based on a gemini-bolaamphiphilic hybrid lipid. *ACS Omega* **2018**, *3*, 208-217.

(5) Misra, S. K.; Muñoz-Úbeda, M.; Datta, S.; Barrán-Berdón, A. L.; Aicart-Ramos, C.; Castro-Hartmann, P.; Kondaiah, P.; Junquera, E.; Bhattacharya, S.; Aicart, E. Effects of a delocalizable cation on the headgroup of gemini lipids on the lipoplex-type nano-aggregates directly formed from plasmid DNA. *Biomacromolecules* **2013**, *14*, 3951-3963.

(6) Lyubchenko, Y. L.; Shlyakhtenko, L. S. Visualization of supercoiled DNA with atomic force microscopy in situ. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 496-501.

(7) Foldvari, M.; Badea, I.; Wettig, S.; Verrall, R.; Bagonluri, M. Structural characterization of novel gemini non-viral DNA delivery systems for cutaneous gene therapy. *J. Exp. Nanosci*. **2006**, *1*, 165-176.




**Scheme S1:** Structures of $(C_{16}Im)_2(C_4O)$ gemini cationic lipid, the zwitterionic phospholipid DOPE and the polyethylene-glycol DSPE-PEG2000 (DSPE-PEG).

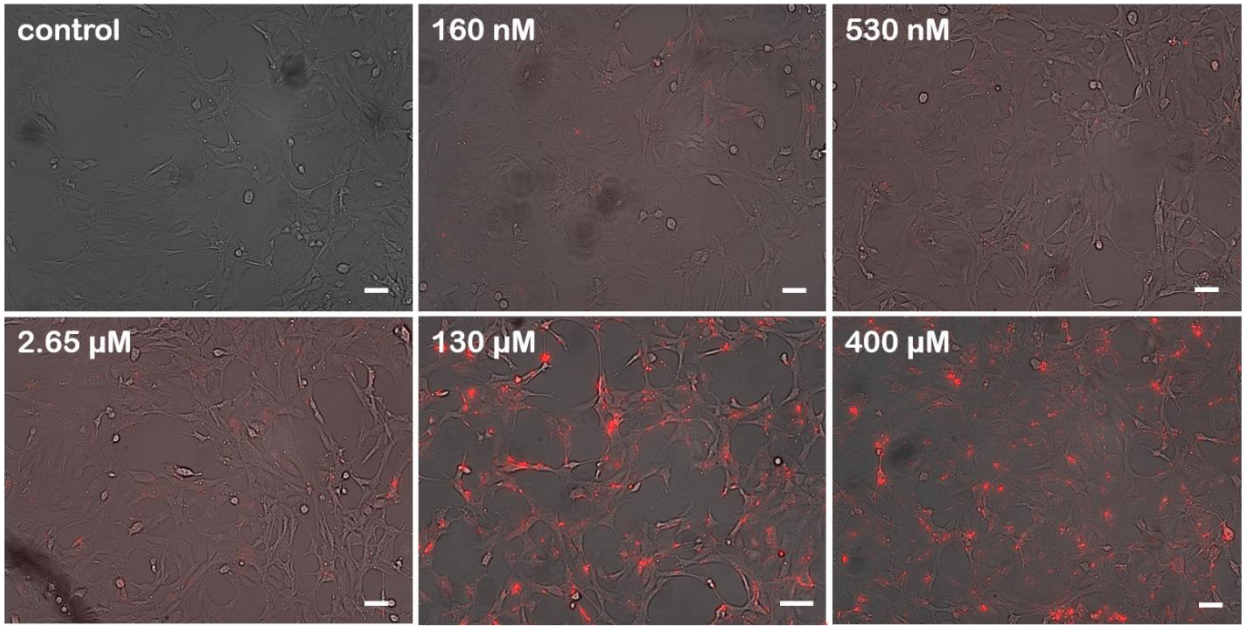



**Figure S1**. Uptake of $(C_{16}Im)_2(C_4O)$/DOPE/DSPE-PEG/OPA1 lipoplexes labeled with the fluorescent dye DiR' (red channel) into MEFs wt after 24 h incubation and at different concentration. Scale bars are 20 µm.

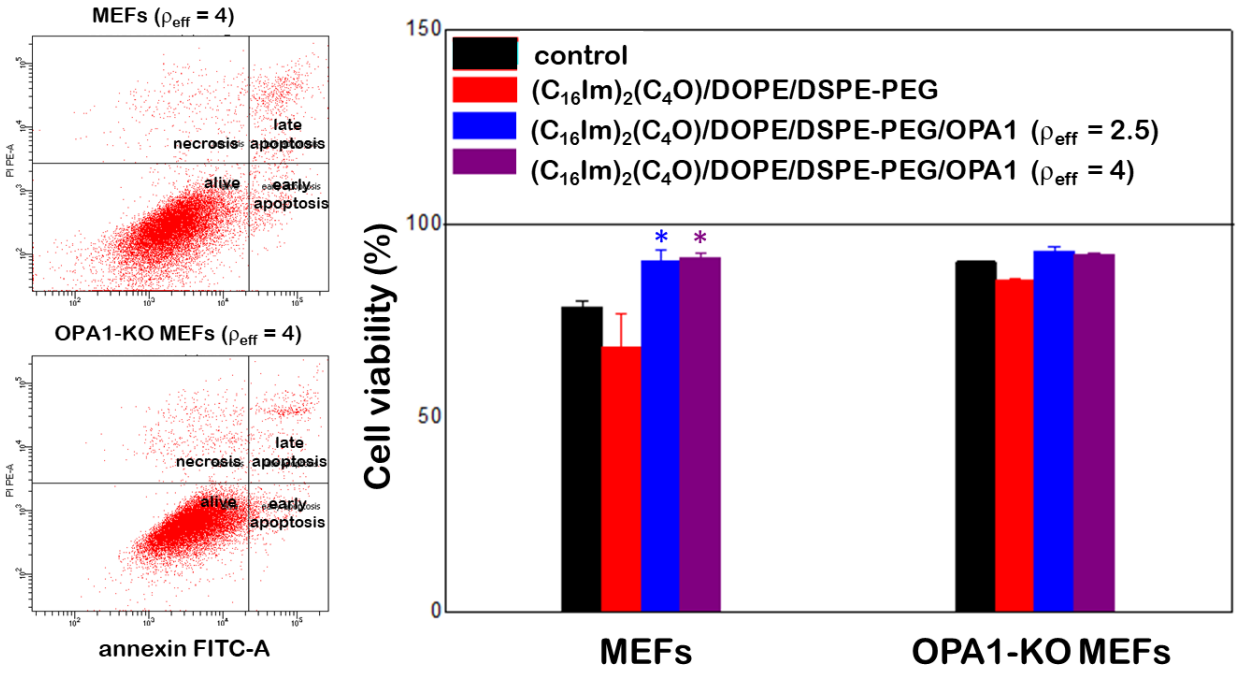



**Figure S2**. Apoptosis after 24h of treatment was measured by flow cytometry through Annexin V-FITC and PI staining. Representative plots for MEFs wt ($\rho_{eff}$ = 4) and OPA1-KO MEFs ($\rho_{eff}$ = 4) are shown in the left panel. The percentage of alive cells is indicated in the right panel. The data are presented as the means ± the SD from three separate experiments (* $p < 0.05$).

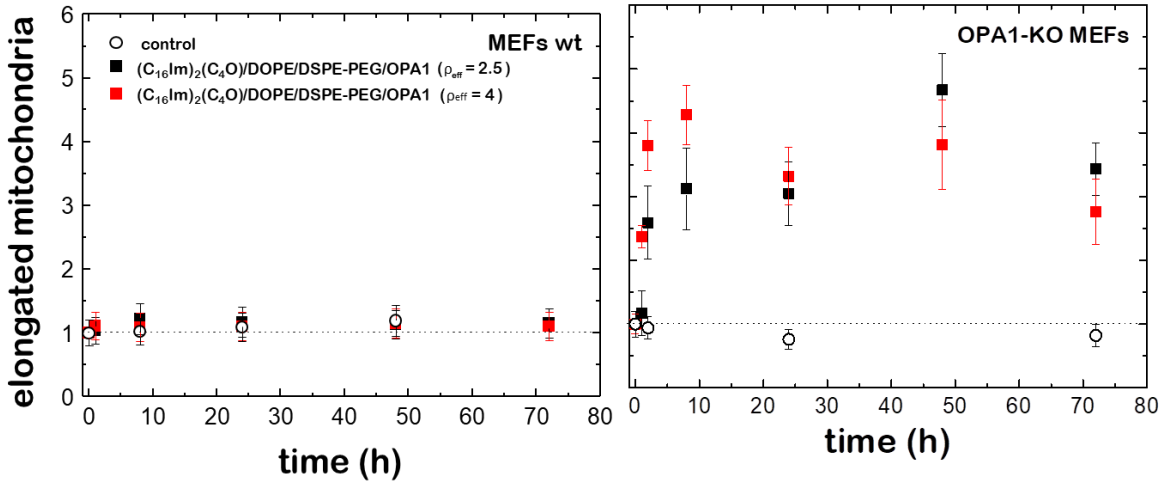



**Figure S3.** Time evolution of the elongated mitochondria ratio with respect to initial elongated mitochondria for MEFs wt (left panel) and OPA1-KO MEFs (right panel) after transfection with $(C_{16}Im)_2(C_4O)$/DOPE/DSPE-PEG/OPA1 lipoplexes at $\rho_{eff}$ = 2.5 (black squares) and 4 (red squares). As a control, the mitochondrial phenotype was also imaged in the absence of treatment (hollow symbols). See Methods section for details. Student's t-test was performed to measure the significance of statistical difference between the different groups and the negative control (in the absence of treatment). $p < 0.05$ was considered statistically significant.

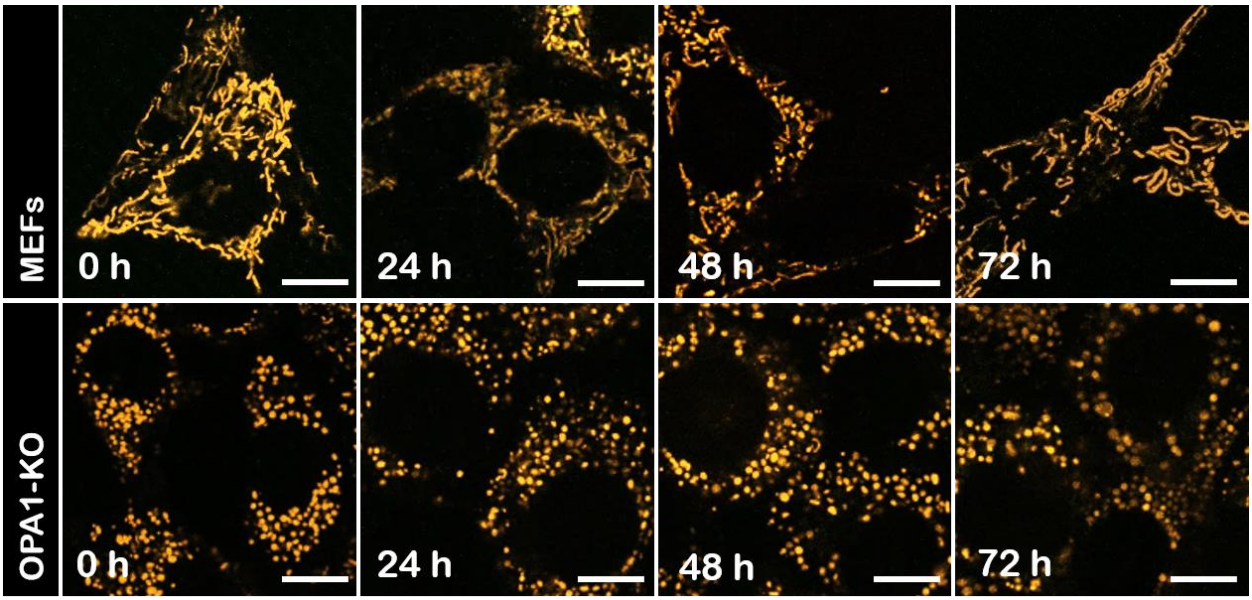



**FIGURE S4.** Confocal fluorescence microscopy images MEFs wt (upper row) and OPA1-KO MEFs (lower row) at different time intervals in the absence of treatment. The mitochondrial network was labeled with TMRM (1 µM). Scale bars are 10 µm.

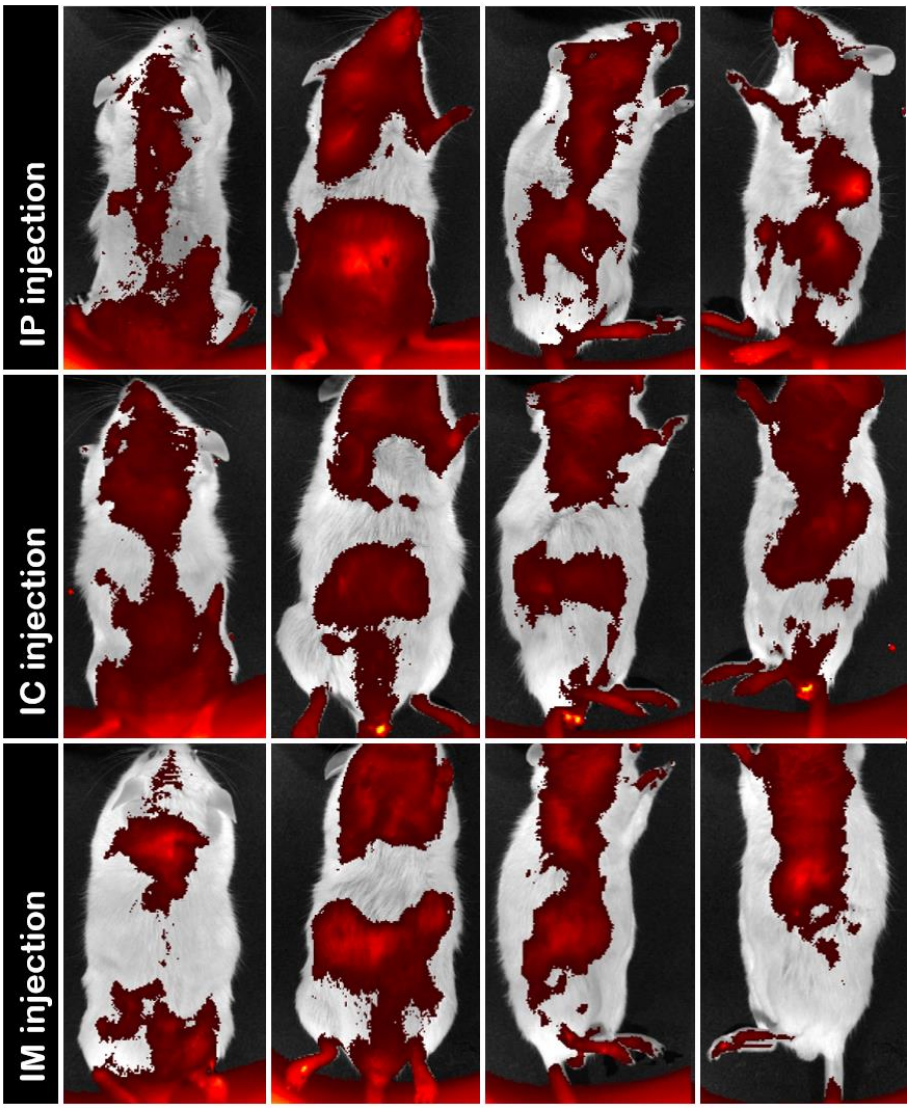



**Figure S5**. *In vivo* biodistribution of DiR'-labeled $(C_{16}Im)_2(C_4O)$/DOPE/DSPE-PEG/OPA1 lipoplexes in CD-1 mice after 4 weeks with treatment via intraperitoneal (IP), intracardiac (IC) and intramuscular (IM) administration.

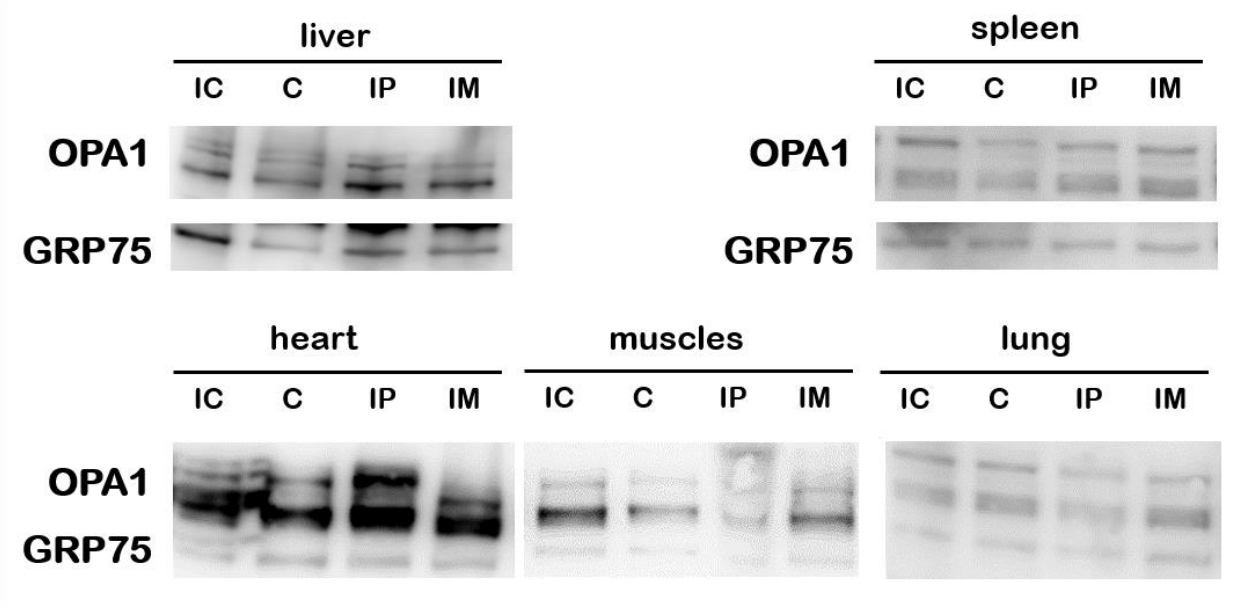



**Figure S6**. Western blot of Opa1 and GRP75 protein levels in liver, spleen, heart, muscles and lung from CD-1 mice after 48 h of transfection with $(C_{16}Im)_2(C_4O)$/DOPE/DSPE-PEG/OPA1 lipoplexes ($\rho_{eff}$ = 4). Different lanes correspond to the groups without treatment (C) and with treatment via intraperitoneal (IP), intracardiac (IC) and intramuscular (IM) administration.