Supporting Information for

Gemini-based lipoplexes complement the mitochondrial phenotype in MFN1-Knockout mouse embryonic fibroblasts

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Determination of the effective charge of the gemini cationic lipid (GCL) and the MYC-MFN1 plasmid.

The composition of the mixed lipid acting as cationic gene vector is given in terms of its molar fraction (α) 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, GCL, DOPE, and DNA, respectively, or b) the effective charge ratio (ρ_{eff}) expressed as the ratio between the charges of positive GCL/DOPE 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 GCL and DNA respectively; (q^+_{eff,L^+}) and ($q^-_{eff,D}$) are the effective charges of GCL and plasmid DNA (pDNA, MYC-MFN1 in our case) per bp; and M_{L^0}, M_{L^+} and M_D are the molecular weight of the DOPE, GCL and MYC-MFN1 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 ρ_{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 (ζ) of lipoplexes as a function of (m_L/m_D) (see **Figure 1A**). A sign inversion on the charge in the (ζ) sigmoidal plots occurs at $(m_L/m_D)_\Phi$ and this value is related to α 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 (ρ_{eff}). For that, the effective charge of the GCL (q^+_{eff,L^+}) was first determined for both GCL1/DOPE-ctDNA and GCL2/DOPE-ctDNA ($\alpha = 0.2$) 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 MYC-MFN1 ($q^-_{eff,D}$) is then obtained with equation (4) using the effective charge of the GCL (q^+_{eff,L^+}) and the experimental electroneutrality value $(m_L/m_D)_\Phi$ for the GCL/DOPE-pDNA lipoplex containing MYC-MFN1 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 (ρ_{eff}) of the lipoplex at $\alpha = 0.2$ is obtained by substituting (q^+_{eff,L^+}) and the MYC-MFN1 ($q^-_{eff,D}$) in equation (2).

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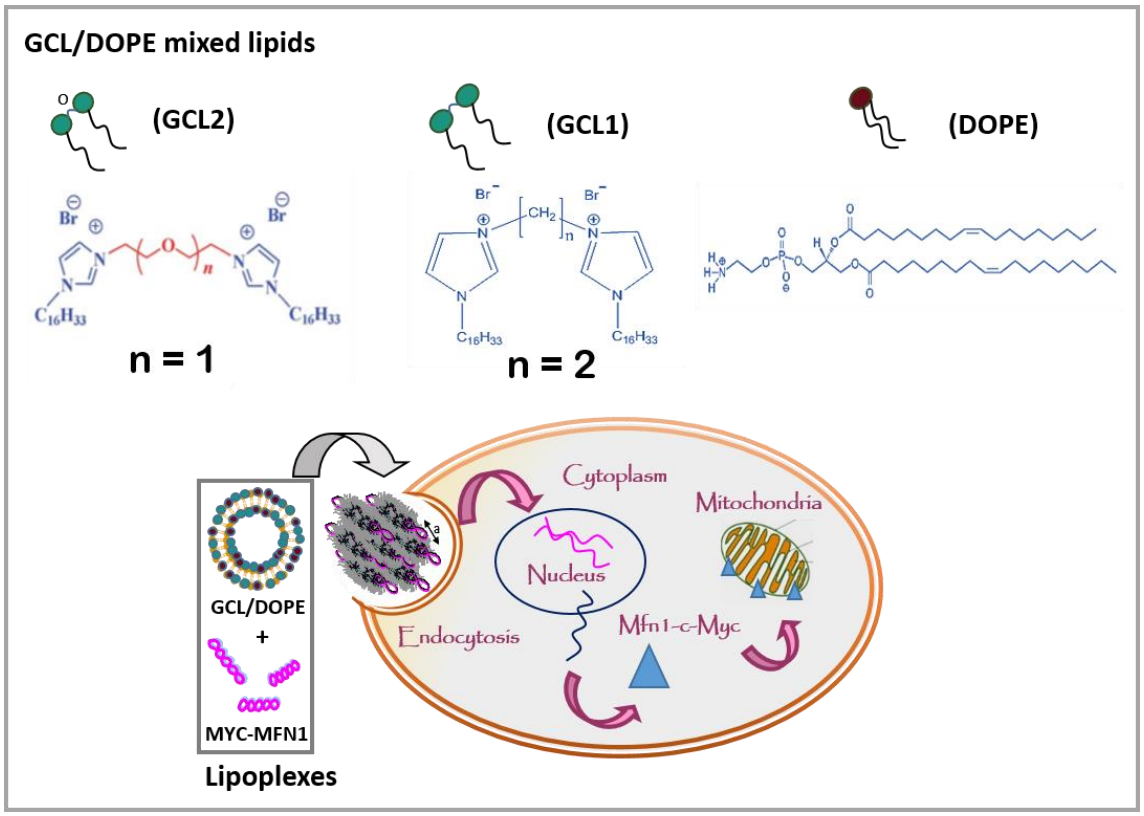

Scheme 1. GCL/DOPE mixed lipid formed by $(C_{16}(CH_3)_2Imidazol)_2C_n$ (n = 2) (GCL1) gemini cationic lipid (M_w =772.87 g/mol), $(C_{16}(CH_3)_2Imidazol)_2(C_2O)_n$ (n = 1) (GCL2) gemini cationic lipid (M_w = 816.92 g/mol) and 1,2-Dioleoil-sn-glicero-3-fosfoetanolamine (DOPE) (M_w =744.05 g/mol). Scheme of the lipoplex formation and uptake, plasmid release and protein expression.

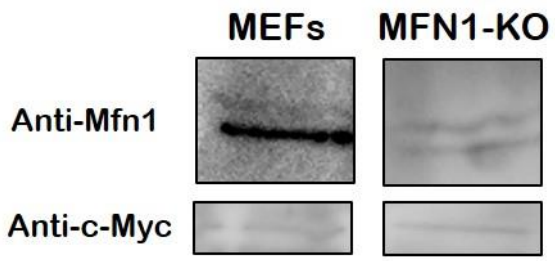

Figure S1. Western Blot images of MEFs wt and MFN1-KO MEFs as revealed with anti-Mfn1 (at 80 KDa, 75 KDa bands) and anti-c-Myc (100 kDa band). The unspecific levels were used to quantify the overexpression of Mfn1 and Mfn1-c-Myc.

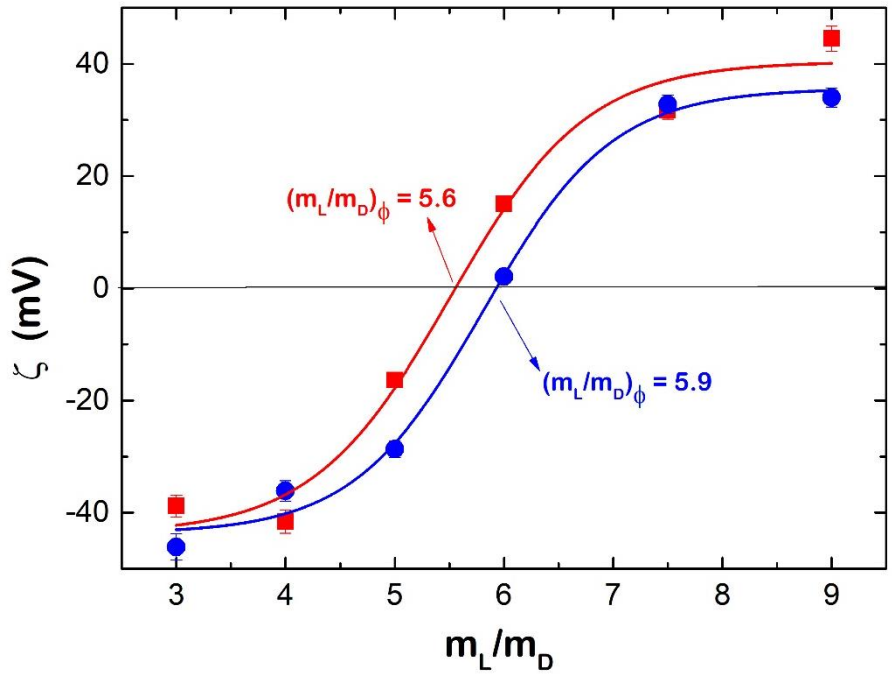

Figure S2. Zeta potential of GCL/DOPE-ctDNA at different Lipid/DNA mass ratio. $(m_L/m_D)_{\Phi,}$ is the electroneutrality value. Red squares for GCL1/DOPE-ctDNA and blue circles for GCL2/DOPE-ctDNA.

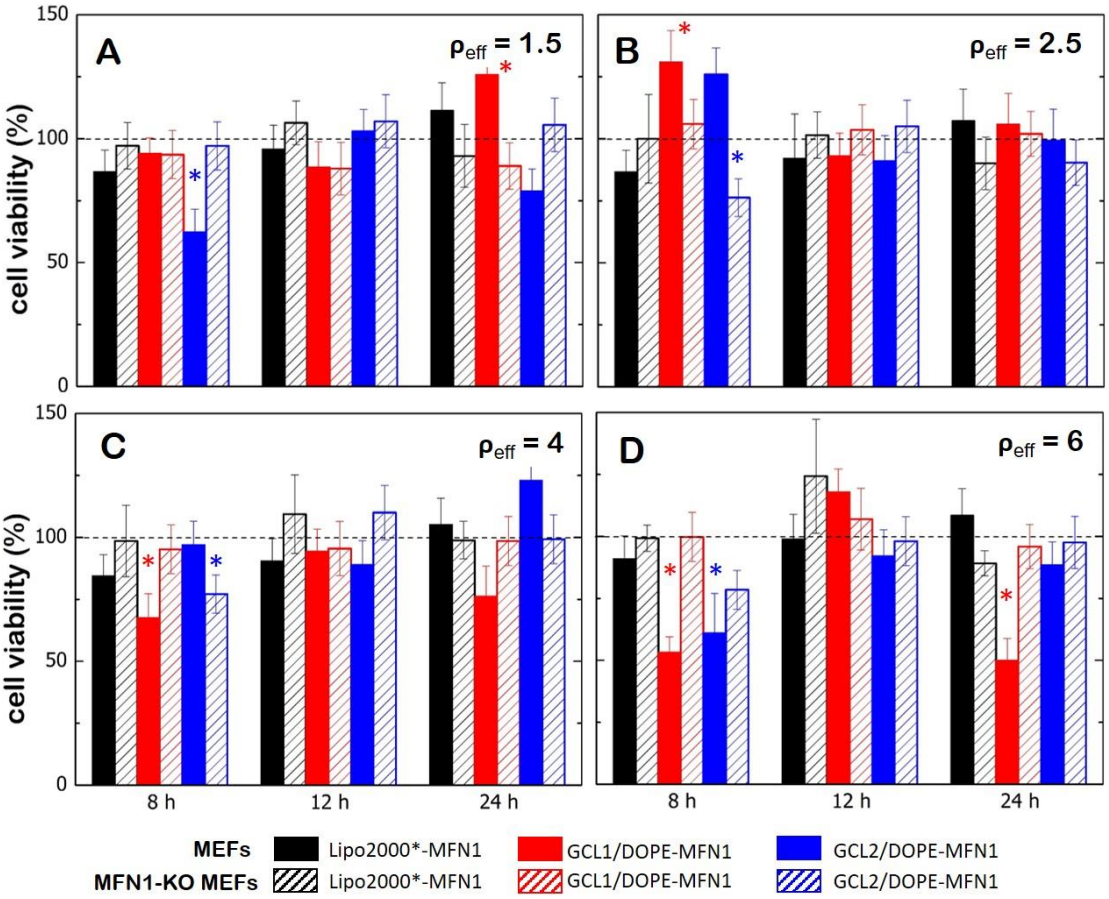

Figure S3. Cell viability of MEFs wt and MFN1-KO MEFs upon incubation with different lipoplexes at **A)** ρ_{eff} = 1.5 **B)** ρ_{eff} = 2.5 **C)** ρ_{eff} = 4 and **D)** ρ_{eff} = 6 after 8, 12 and 24 hrs transfection. The Student's *t* test was performed to measure the significance of statistical difference between the different groups and the negative control (in the absence of treatment). $p < 0.05$ was considered statistically significant.

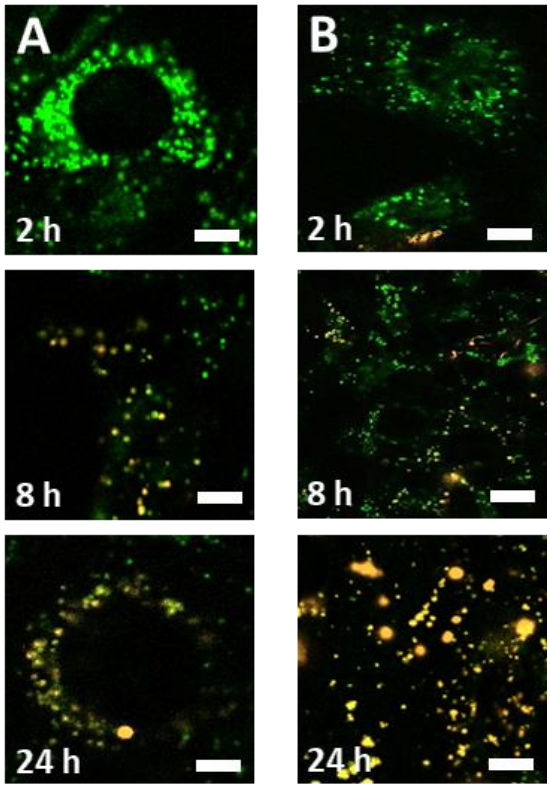

Figure S4. Uptake of **A)** Lipo2000*-MFN1 and **B)** GCL1/DOPE-MFN1 mixed lipid at ρ_{eff} = 4 (labeled with the fluorescent dye RhPE, red channel) into MEFs wt (labeled with Lysotracker, green channel) at different incubation times. Scale bars are 10 µm.

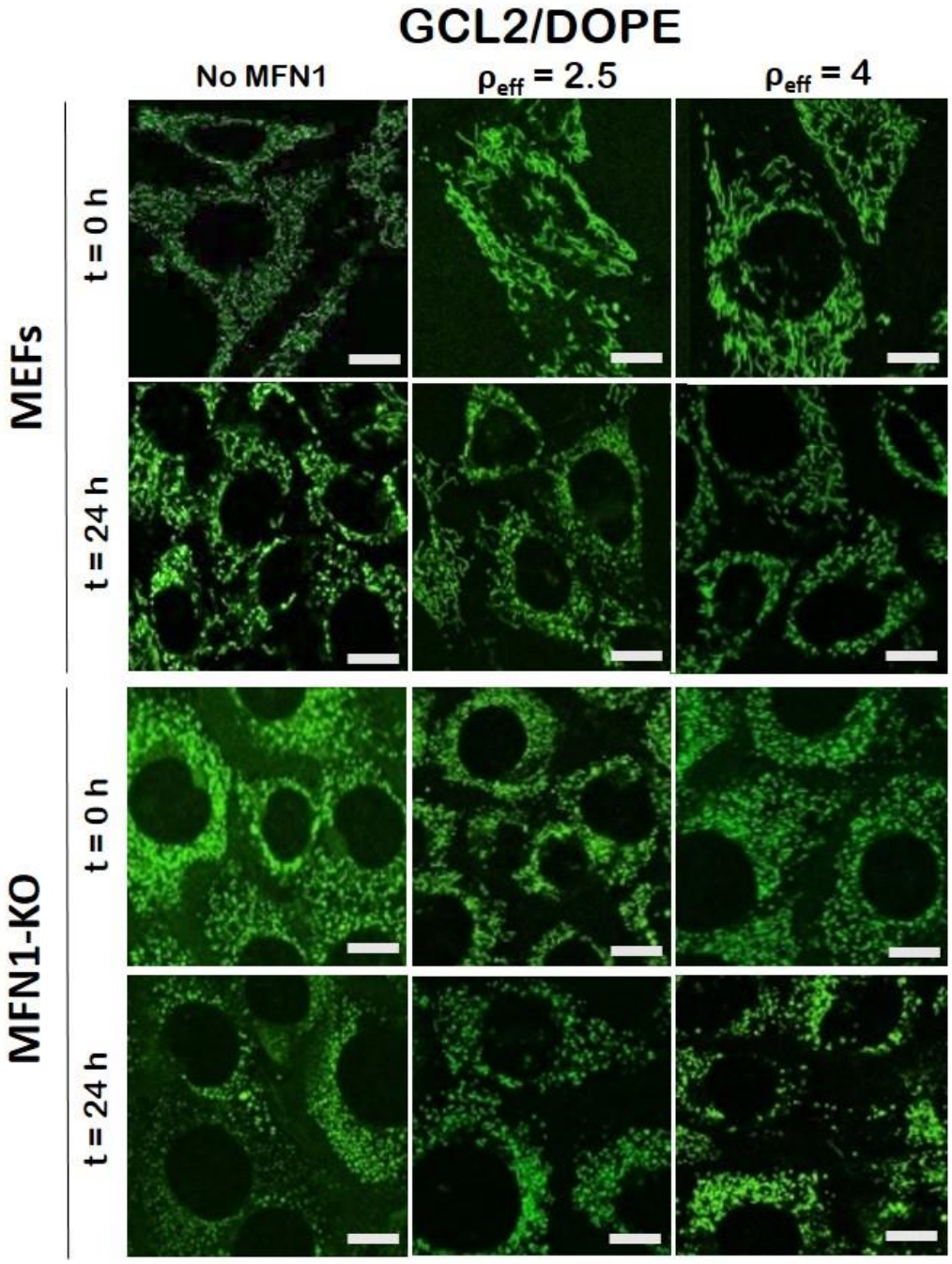

Figure S5. Confocal images of MEFs wt and MFN1-KO MEFs upon incubation with GCL2/DOPE mixed lipid ([GCL] = 0.5 μM) or GCL2/DOPE-MFN1 lipoplexes at ρ_{eff} = 2.5 and 4, and at 0 and 24 hrs after incubation. The mitochondrial network is visualized with Rho123. Scale bars are 10 μm.

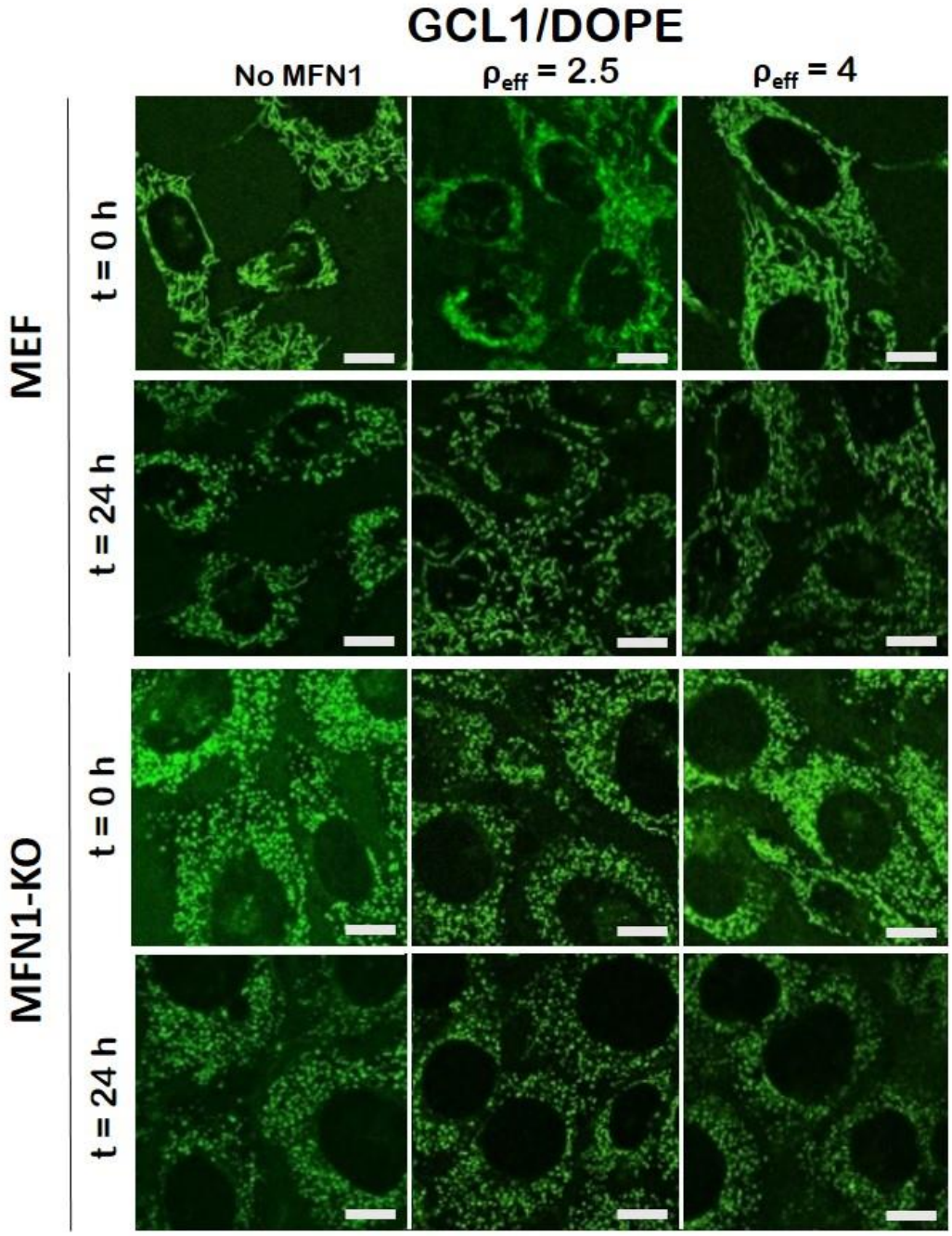

Figure S6. Confocal images of MEFs wt and MFN1-KO MEFs upon incubation with GCL1/DOPE mixed lipid ([GCL] = 0.5 μM) or GCL1/DOPE-MFN1 lipoplexes at ρ_{eff} = 2.5 and 4, and at 0 and 24 hrs after incubation. The mitochondrial network is visualized with Rho123. Scale bars are 10 μm.

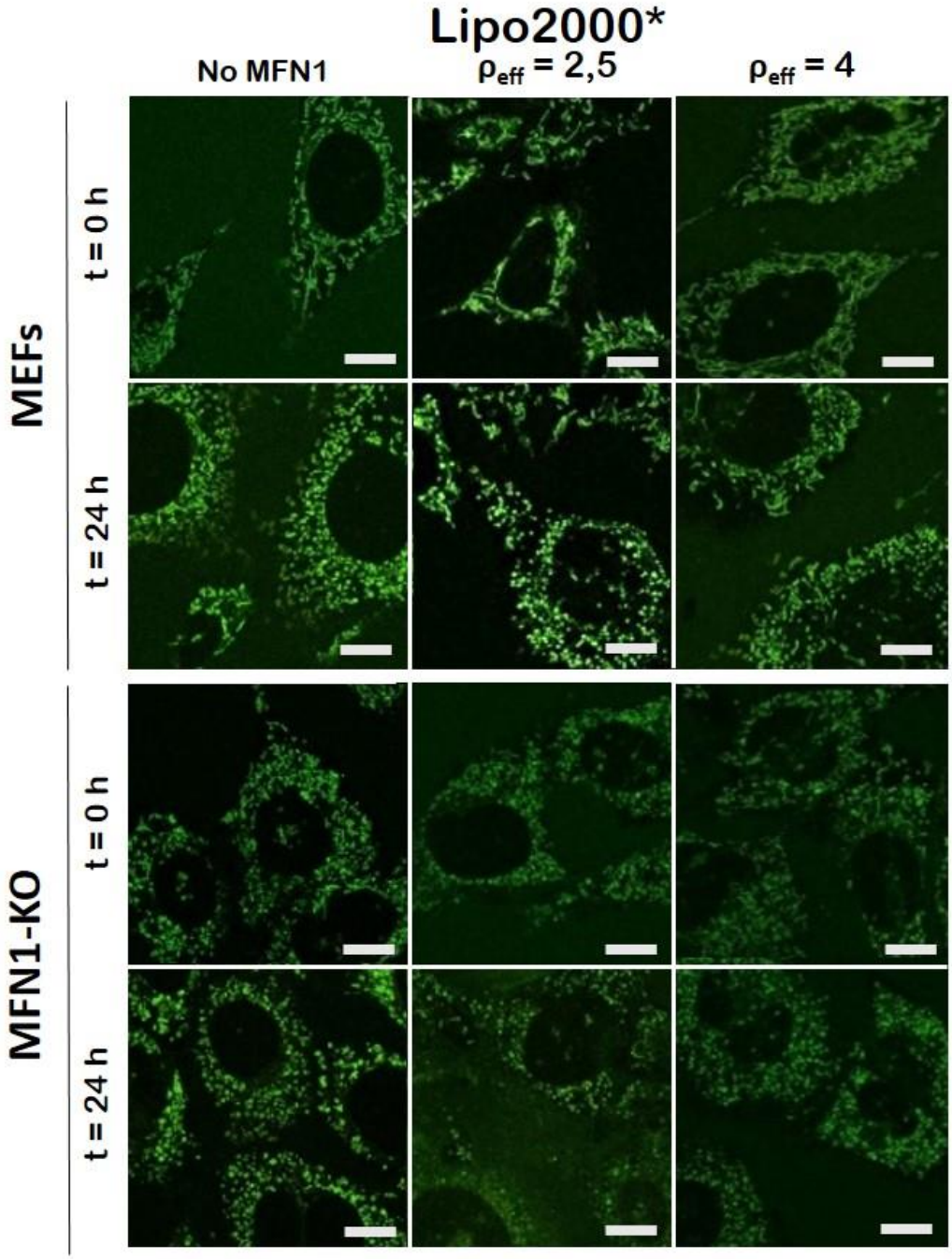

Figure S7. Confocal images of MEFs wt and MFN1-KO MEFs upon incubation with with Lipo2000* liposomes ([Lipo2000*] = 0.5 μM) or Lipo2000*-MFN1 lipoplexes at ρ_{eff} = 2.5 and 4, and at 0 and 24 hrs after incubation. The mitochondrial network is visualized with Rho123. Scale bars are 10 μm.

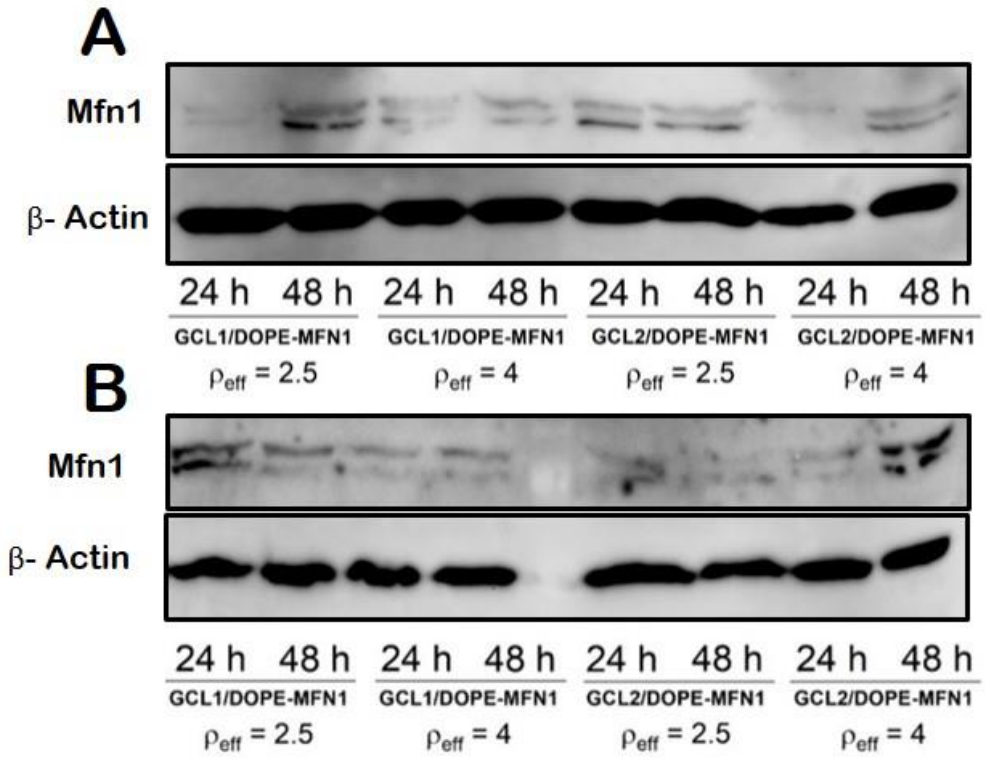

Figure S8. Western blot analysis of endogenous Mfn1 protein levels in **A)** MEFs wt and **B)** MFN1-KO MEFs after transfection with different GCL/DOPE-MFN1 lipoplexes at ρ_{eff} = 2.5 and 4 at different incubation times.

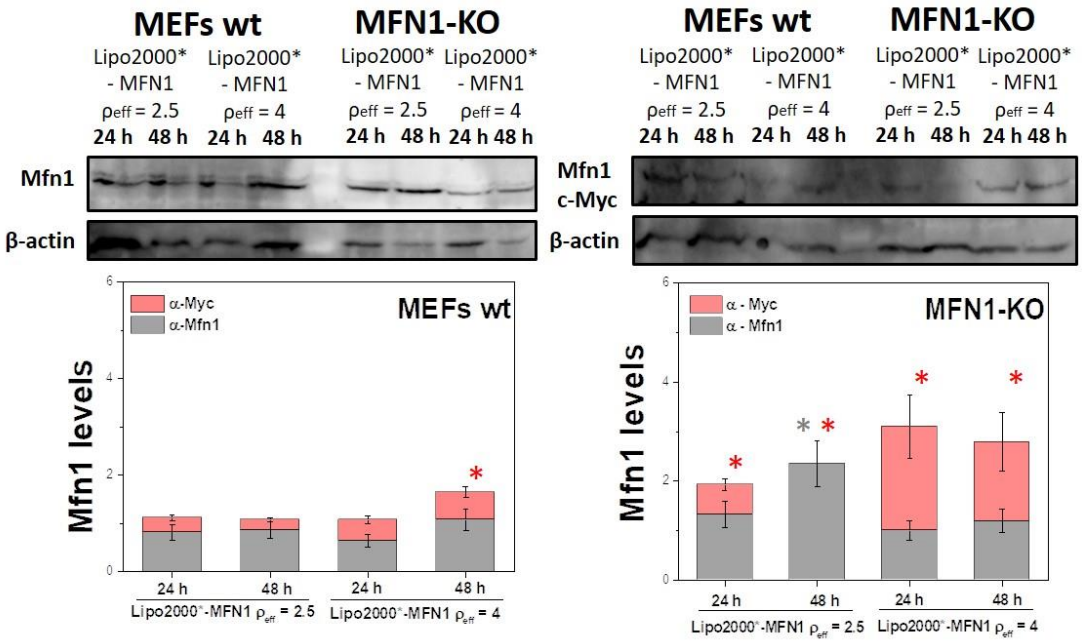

Figure S9. Western blot analysis of endogenous Mfn1 protein levels in MEFs wt and MFN1-KO MEFs after transfection with Lipo2000*-MFN1 complexes at ρ_{eff} = 2.5 and 4 at different incubation times. The Student's *t* test was performed to measure the significance of statistical difference between the different groups and the negative control (in the absence of treatment) from 3 independent experiments. $p < 0.05$ was considered statistically significant.