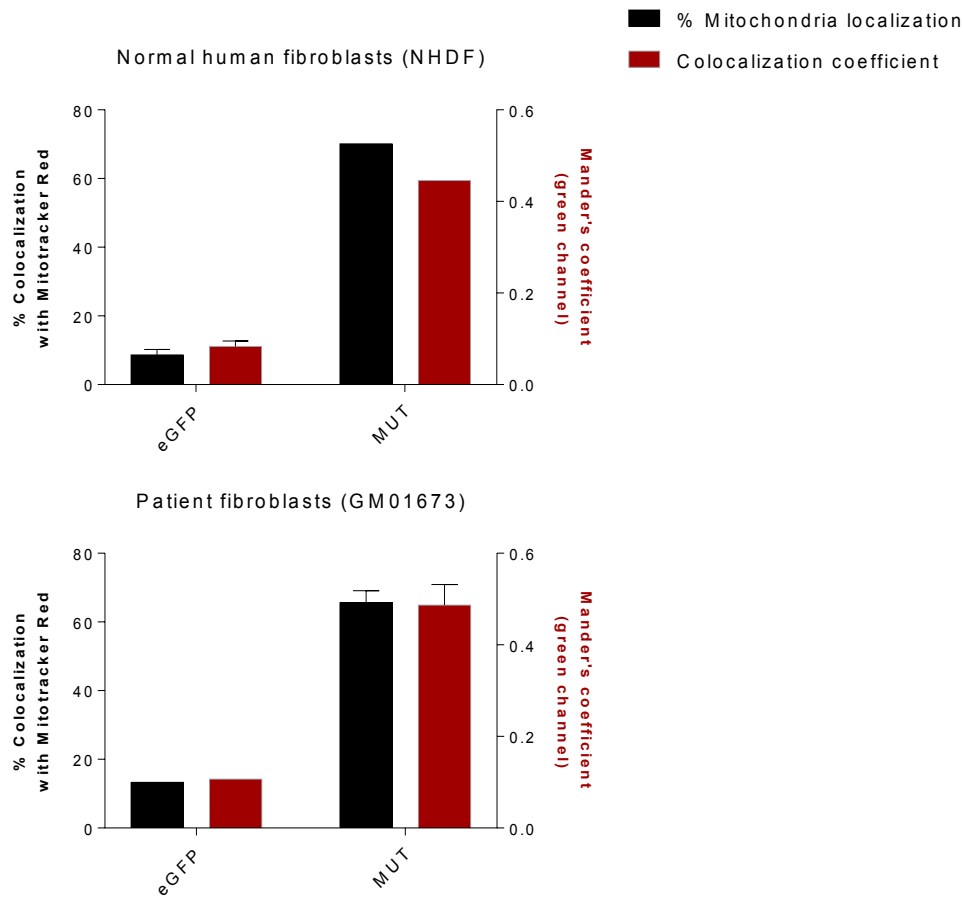


**Supplemental Information**

**Systemic Messenger RNA Therapy  
as a Treatment for Methylmalonic Acidemia**

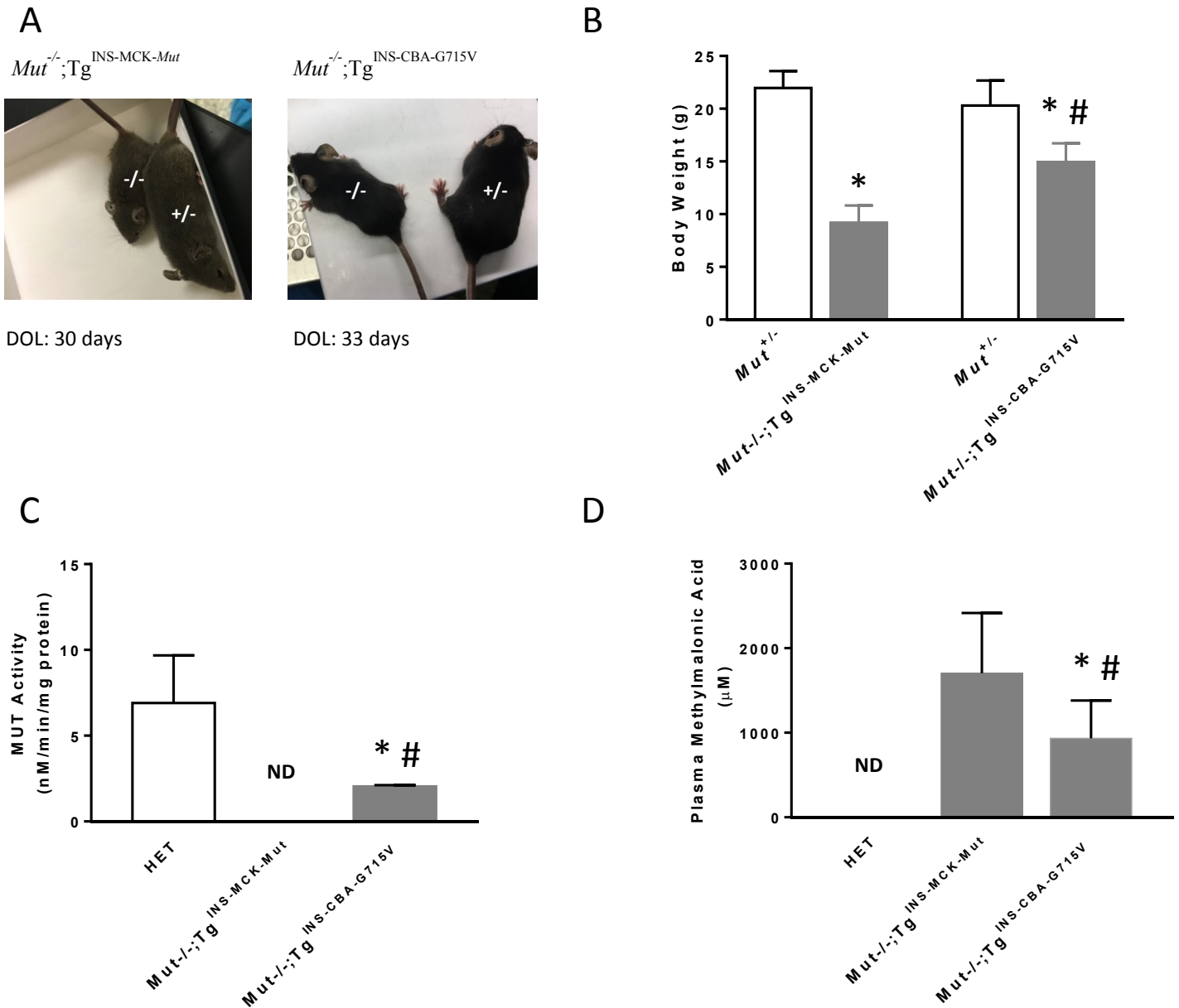
**Ding An, Jessica L. Schneller, Andrea Frassetto, Shi Liang, Xuling Zhu, Ji-Sun Park, Matt Theisen, Sue-Jean Hong, Jenny Zhou, Raj Rajendran, Becca Levy, Rebecca Howell, Gilles Besin, Vladimir Presnyak, Staci Sabnis, Kerry E. Murphy-Benenato, E. Sathyajith Kumarasinghe, Timothy Salerno, Cosmin Mihai, Christine M. Lukacs, Randy J. Chandler, Lin T. Guey, Charles P. Venditti, and Paolo G.V. Martini**

## SUPPLEMENTARY FIGURES AND FIGURE LEGENDS



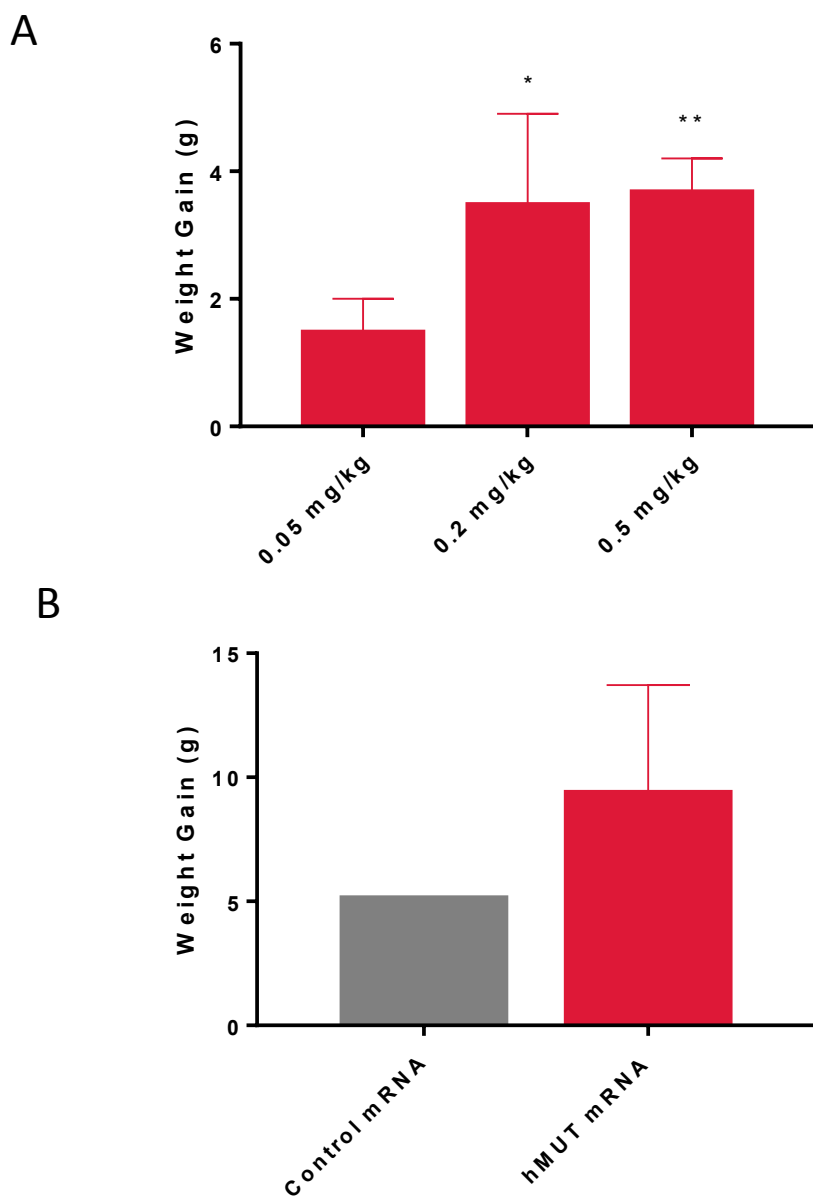
### Suppl. Figure 1. Expressed hMUT protein colocalized with mitochondria, Related to Figure 2.

Mitochondria colocalization for MUT and eGFP was evaluated in normal human fibroblasts and in patient fibroblasts. The amount of MUT (or eGFP) localized in the mitochondria was measured by computing the fluorescent intensity sum in the respective acquisition channels for the voxels colocalized with Mitotracker Red. The values are reported as percent from total intensity sum of MUT or eGFP positive voxels (left axis). The computed Mander's colocalization coefficients for the MUT and eGFP fluorescent channels are plotted on the right axis (red).



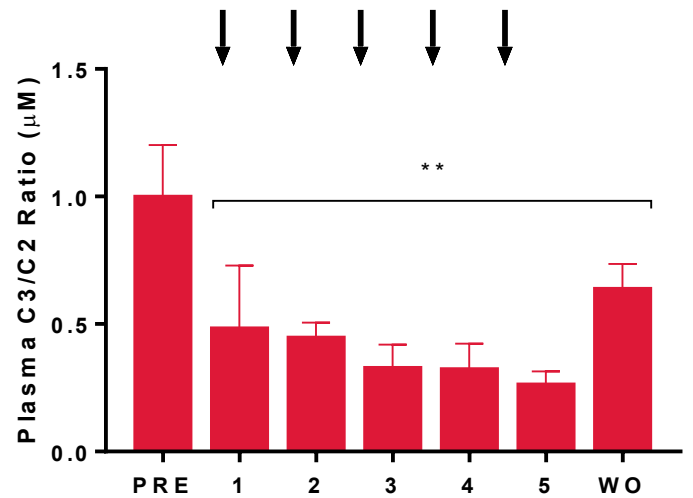
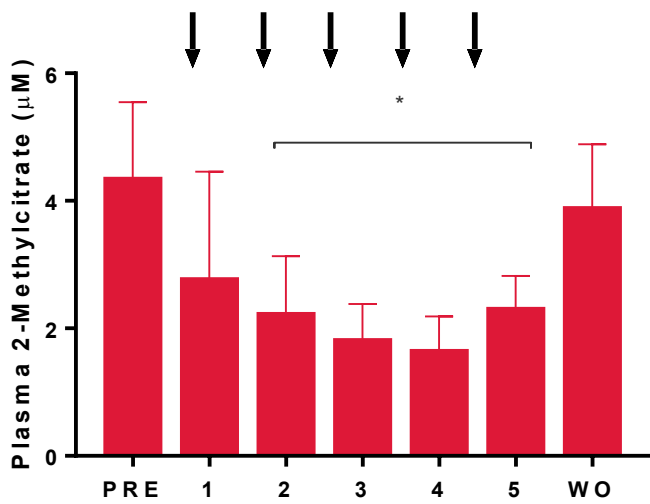
**Suppl. Figure 2. Characterization of  $Mut^{-/-};Tg^{INS-MCK-Mut}$  and  $Mut^{-/-};Tg^{INS-CBA-G715V}$  mice, Related to Figures 4 and 6.**

(A & B) The body weight of 5-week old male  $Mut^{-/-};Tg^{INS-MCK-Mut}$  and  $Mut^{-/-};Tg^{INS-CBA-G715V}$  mice and their heterozygous littermates were measured. Hepatic Mut activity (C) and plasma methylmalonic acid (D) were measured. Data shown as mean  $\pm$  SD.  $p$ -values obtained from unpaired t-tests. \* $p$ <0.05 compared to corresponding heterozygous group, # $p$ <0.05 compared to  $Mut^{-/-};Tg^{INS-MCK-Mut}$  group.  $p$ -values obtained from pairwise Tukey's post-hoc test after two-way ANOVA for (B).  $p$ -values obtained from Tukey's multiple-comparison test after one-way ANOVA for (C) and (D).



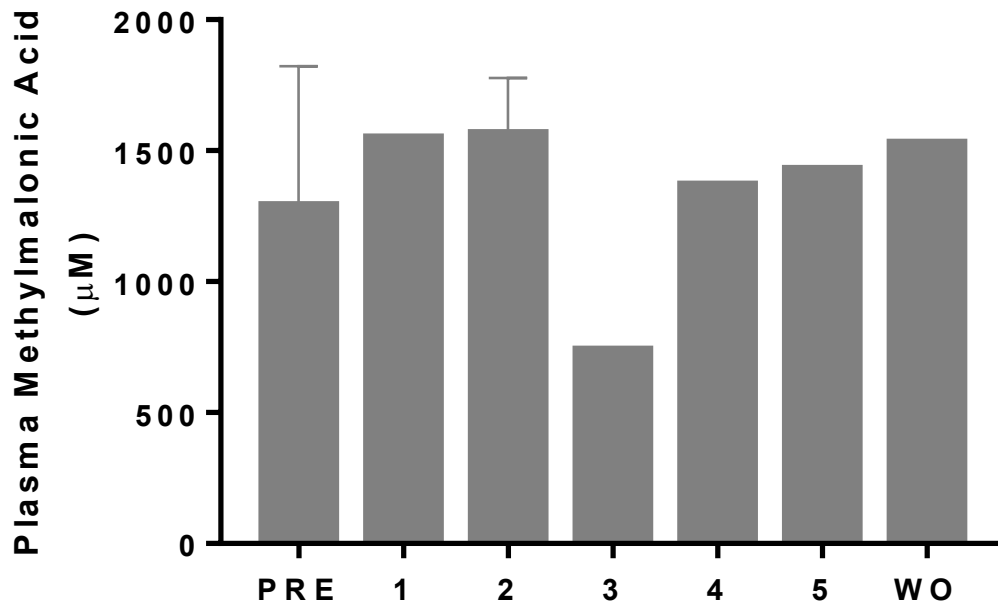
**Suppl. Figure 3. Single and repeat IV administration of *hMUT* mRNA improved weight gain, Related to Figure 5.**

(A) Weight gain in dose groups 2 weeks after a single IV administration. (B) *Mur<sup>-/-</sup>;Tg<sup>INS-MCK-Mut</sup>* mice received weekly IV injections of *hMUT* mRNA or control mRNA for 5 doses at 0.2 mg/kg. Body weights were monitored throughout the study. Only one mouse in the control group survived ( $n = 1$ ). Data shown as mean  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ ,  $p$ -values obtained from unpaired t-tests.



**Suppl. Figure 4. Repeat IV administration of *hMUT* mRNA decreased plasma 2-methylcitrate and C3/C2 carnitine, Related to Figure 5.**

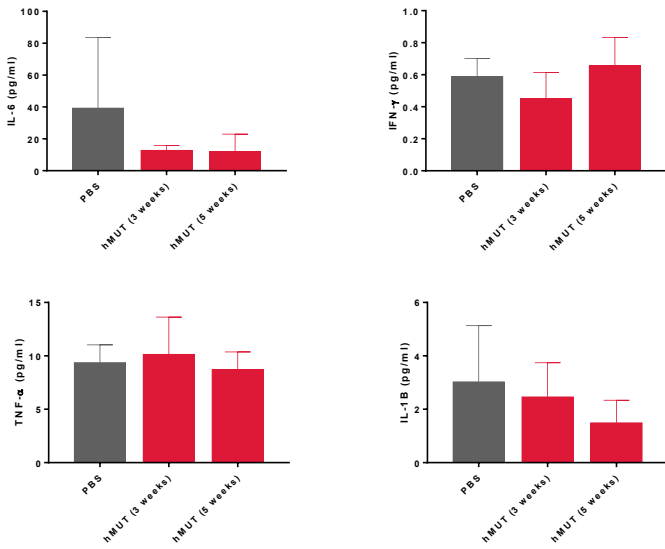
*Mut*<sup>-/-</sup>;Tg<sup>INS-MCK-*Mut*</sup> mice received weekly IV injections of *hMUT* mRNA for 5 doses at 0.2 mg/kg. Plasma 2-methylcitrate and C3/C2 carnitine levels were measured 4 days before the first dose (PRE) and 3 days after each dose in *Mut*<sup>-/-</sup>;Tg<sup>INS-MCK-*Mut*</sup> mice. WO = 10 day washout after the last injection. Data shown as mean ± SD. \**p*<0.05, \*\**p*<0.01, *p*-values obtained from Tukey's multiple-comparison test after one-way ANOVA.



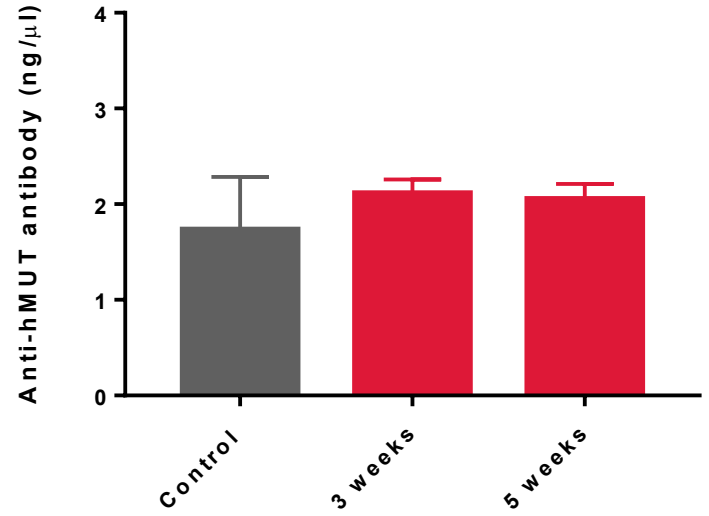
Suppl. Figure 5. Repeat IV administration of control mRNA showed no metabolic response, Related to Figure 5.

*Mut*<sup>-/-</sup>;Tg<sup>INS-MCK-*Mut*</sup> mice received weekly IV injections of *hMUT* mRNA, or a control mRNA for 5 doses at 0.2 mg/kg. Plasma methylmalonic acid levels were measured 4 days before the first dose and 3 days after each dose in *Mut*<sup>-/-</sup>;Tg<sup>INS-MCK-*Mut*</sup> mice. WO = 10-day washout after the single dose.

A



B



**Suppl. Figure 6. Repeated dosing of *hMUT* mRNA did not increase inflammation markers or anti-*hMUT* antibodies, Related to Figure 5.**

*Mut*<sup>+/-</sup>;Tg<sup>INS-CBA-G715V</sup> mice received weekly IV injections of PBS or *hMUT* mRNA for 3 or 5 doses at 0.2 mg/kg.

Serum was collected 24 hours after the 3<sup>rd</sup> or 5<sup>th</sup> weekly IV dose. Serum proinflammatory cytokines levels (A) and anti-*hMUT* antibody (B) were measured. Data presented as mean  $\pm$  SD. *p*-values obtained from unpaired t-tests.

**Supplemental Table 1. Full codon-optimized *hMUT* mRNA sequence, Related to Figure 1.**

N7mGpppGmGGAAAU AAGAGAGAAAAGAAGAGUAAGAAGAAAUAUAAGAGCCACCAUGCUGCGGGC  
CAAGAACCAGCUGU UCCUGCUGAGCCUCACUACCUGCGGCAGGUGAAGGAGAGCAGCGGCAGCCG  
GCUGAUCCAGCAGCGGCUGCUGCACCAGCAGCAGCCCCUGCACCCCGAGUGGGCCGCCUGGCCAA  
GAAGCAGCUGAAGGGCAAGAACCCCGAGGACCUGAUCUGGCACACGCCCGAGGGCAUCAGCAUCAA  
GCCCCUGUACAGCAAGCGGGACACCAUGGACCUGCCCGAGGAGCUGCCCGGCUGAAGCCCUUCAC  
CCGGGGCCCCUACCCACCAUGUACACCUUCCGGCCCUGGACCAUCCGGCAGUACGCCGGCUUCAGC  
ACCGUGGAGGAGAGCAACAAGUUCUACAAGGACAACAUCAAGGCCGGCCAGCAGGGCCUGAGCGUG  
GCCUUCGACCUGGCCACCCACCGGGGCUACGACAGCGACAACCCACGGGUGCGGGGCGACGUGGGC  
AUGGCCGGCGUGGCCAUCGACACCGUGGAGGACACCAAGA UCCUGUUCGACGGCAUCCCUUGGAG  
AAGAUGAGCGUGAGCAUGACCAUGAACGGCGCCGUGAUCCCGUGCUGGCCAACUUCAUCGUGACC  
GGCGAGGAGCAGGGCGUGCCCAAGGAGAAGCUGACCCGGCACCAUCCAGAACGACA UCCUGAAGGAG  
UUCAUGGUGCGGAACACCUACAUCUCCCCUCCGAGCCAGCAUGAAGAUAUCGCCGACAUCUUC  
GAGUACACCGCCAAGCACAUGCCCAAGUUCAACAGCAUCAGCAUCAGCGGCUACCACAUGCAGGAG  
GCCGGCGCCGACGCCAUCCUGGAGCUGGCCUACACCCUGGCCGACGGCCUGGAGUACAGCCGGACC  
GGCCUGCAGGCCGGCCUGACCAUCGACGAGUUCGCGCCCCGGCUGAGCUUCUUCUGGGGCAUCGGC  
AUGAACUUCUACAUGGAGAU CGCCAAGAUGCGGGCCGGCCGGCGGCUGUGGGCCCACCGAUUCGAG  
AAGAUGUUC CAGCCCAAGAACAGCAAGAGCCUGCUGCUGCGGGCCACUGCCAGACCAGCGGCUGG  
AGCCUGACCGAGCAGGACCCCUACAACAACAUCGUGCGGACCGCCAUCGAGGCCAUGGCCGCCGUG  
UUCGGCGGCACCCAGAGCCUGCACACCAACAGCUUCGACGAGGCCUGGGCCUGCCCACCGUGAAG  
AGCGCCCGGAUCGCCCGGAACACCCAGAUAUCAUCCAGGAGGAGAGCGGCAUCCCCAAGGUGGCC  
GACCCCUGGGGCGGCAGCUACAUGAUGGAGUGCCUGACCAACGACGUGUACGACGCCGCCUGAAG  
CUGAUCAACGAGAU CGAGGAGAUGGGCGGCAUGGCCAAGGCCGUGGCCGAGGGCAUCCCCAAGCUG  
CGGAUCGAGGAGUGCGCCGCCGGCGGCAGGCCCGGAUCGACAGCGGCAGCGAGGUGAUCGUGGGC  
GUGAACAAAGUACCAGCUGGAGAAGGAGGACGCCGUGGAGGUGCUGGCCAUCGACAACACCAGCGUG  
CGGAACCGGCAGAU CGAGAAGCUGAAGAAGAUAAGAGCAGCCGGGACCAGGCCCUUGGCCGAGCGG  
UGCCUGGCCGCCUGACCGAGUGCGCCGCCAGCGGCGACGGCAACAUCUGGCCCUUGGCCGUGGAC  
GCCAGCCGGGCCCGGUGCACCGUGGGCGAGAUCACCGACGCCUGAAGAAGGUGUUCGGCGAGCAC





**Supplemental Table 2. Serum chemistry of mice that received LNP-mRNA treatment, Related to Figure 5.**

	Control	Treated (3 weeks)	Treated (5 weeks)	<i>p</i>
ALP (U/L)	86.0 ± 38.1	54.3 ± 10.3	37.0 ± 12.2	NS
AST (U/L)	1048.0 ± 1076.0	803.7 ± 614.0	328.7 ± 231.9	NS
ALT (U/L)	668.3 ± 691.6	789.0 ± 815.5	120.3 ± 58.5	NS
GGT (U/L)	0 ± 0	0 ± 0	0 ± 0	NS
Albumin (g/dL)	2.93 ± 0.40	2.93 ± 0.15	2.93 ± 0.23	NS
Total Bilirubin (mg/dL)	0.33 ± 0.15	0.27 ± 0.15	0.30 ± 0.10	NS
Total Protein (g/dL)	5.47 ± 0.59	5.27 ± 0.15	5.30 ± 0.10	NS
Bilirubin - Conjugated (mg/dL)	0.07 ± 0.06	0.03 ± 0.06	0.03 ± 0.06	NS
Bilirubin - Unconjugated (mg/dL)	0.27 ± 0.15	0.23 ± 0.12	0.27 ± 0.06	NS

Data shown as mean ± SD. One way ANOVA analysis was performed to compare treated vs. control group. No statistical difference was observed.

**Supplemental Table 3. Characterization of formulations, Related to Figures 3, 4, 5 and 6.**

Diameter (nm)	PD Index	Endotoxins (EU/ml)	OSMO (mOsm/kg)	pH	Encapsulation (%)
78 ± 3.5	0.15 ± 0.03	1.25 ± 0.44	313 ± 13.9	7.18 ± 0.05	95.8 ± 0.5

Data combined 4 different batches of formulations and are shown as mean ± SD.

## Supplemental Experimental Procedures

### Volumetric colocalization analysis

Volumetric colocalization analysis was performed in Imaris (Bitplane AG, Zurich, Switzerland). The amount of MUT (or eGFP) localized in the mitochondria was measured by computing the fluorescent intensity sum in the respective acquisition channels for the voxels colocalized with Mitotracker Red. The values are reported as percent from total intensity sum of MUT or eGFP positive voxels. Alternatively, we report the Mander's colocalization coefficients for the MUT and eGFP fluorescent channels as a global colocalization metric.

### Murine models of methylmalonic acidemia

*Mut*<sup>-/-</sup> mice harbor a deletion of exon 3 which encodes the putative substrate-binding pocket in the Mut enzyme. This *Mut* allele is null and unable to produce mature RNA or protein (Chandler et al., 2007b; Chandler et al., 2009). *Mut*<sup>-/-</sup>;Tg<sup>INS-MCK-Mut</sup> mice express the *Mut* gene under the control of a muscle-specific creatine kinase promoter which results in the rescue of mice from neonatal lethality; however, these mice display severe metabolic perturbations, growth retardation, and a hepatorenal mitochondriopathy (Manoli et al., 2011). Plasma methylmalonic acid levels are elevated by 50-200-fold compared to wild type littermates and similar to severe MMA patients (Manoli et al., 2011). *Mut*<sup>-/-</sup>;Tg<sup>INS-CBA-G715V</sup> mice express the mouse homologue of the human *mut* mutation p.G717V under the control of an enhanced chicken β-actin (*CBA*) promoter. These mice have decreased Mut activity in all tissues and moderately increased plasma methylmalonic acid concentrations in the blood, similar to MMA patients harboring the G717V mutation (Senac et al., 2013). *Mut*<sup>+/-</sup> mice were used as controls in current study given that their biochemical parameters are identical to *Mut*<sup>+/+</sup> mice (Chandler et al., 2007a). All mice used in these studies were typically 1.5-3 months of age. IV injections were administered via the tail vein or retro-orbital vein, survival blood collection was via submandibular bleed and terminal blood collection was by cardiac puncture. To harvest tissues, mice were perfused prior to tissue collection to avoid blood contamination.

### Cells transfection and co-localization

Isolated human fibroblasts from a healthy normal subject (Control), designated as control in the manuscript, and 2 with vitamin B12 non-responsive MMA (GM50 and GM1673) were purchased from Coriell Institute. These cell

lines have been previously characterized as *mut*<sup>0</sup> and further, with low *MUT* mRNA phenotypes (Ledley et al., 1990; Qureshi et al., 1994). Cells were maintained in MEM with 10% FBS following instructions from Coriell Institute. Human fibroblasts (1 x 10<sup>6</sup>) were transfected with either 1 µg *eGFP* or *hMUT* mRNA by electroporation using Neon Transfection System following the manufacturer's instructions. Cells were lysed 24 hours after transfection, protein was extracted and hMUT expression and activity were measured. To examine the localization of mRNA-encoded hMUT in mitochondria, 1 x 10<sup>6</sup> control and *mut*<sup>0</sup> fibroblasts (GM01673) were transfected with 1 µg of *hMUT* mRNA. 24 hours after transfection, cells were incubated with 200 nM MitoTracker Red CMXRos (M7512, ThermoFisher Scientific) for 30 min to mark mitochondria; after that the cells were fixed in 4% PFA and processed for immunofluorescent staining with anti-MUT mouse mAb (TA506873, Origene) to examine the cellular localization. The cells were counterstained with DAPI for nuclei visualization. To account for non-specific colocalization, similar samples were prepared by transfecting the cells with mRNA encoding eGFP fluorescent protein.

Image acquisition was done on a super-resolution Zeiss Elyra SP1 microscope (Zeiss, Thornwood, NY) in Structured Illumination imaging mode using a 40x magnification (NA = 1.46) oil-immersion objective and 405, 488 and 561 nm laser excitation. Emission bandpass filters were 420-480, 495-550, 570-620 nm. The excitation/emission channels were recorded consecutively, within each channel the raw data contained 3 rotations, 5 phases and 0.1 µm spaced z stack images. The super-resolution images were then reconstructed from raw images using ZEISS SIM processing software and a z-section from the middle of the stack was used for the images in the manuscript.

#### **Quantification of *hMUT* mRNA using a bDNA assay**

*hMUT* mRNA in livers was quantified by branched-chain DNA (bDNA) assay. Briefly, 10-20 mg snap-frozen liver samples were homogenized in a homogenizing solution and mRNA in the liver homogenates was quantified using QuantiGene 2.0 Assay Kit (QS0009, Affymetrix) following the manufacturer's instructions. To make a standard curve, *hMUT* mRNA was added to control liver extracts, ranging from 0.0008 – 0.8 µg/µl. Samples were incubated with probes in a plate for 16-20 hours at 55 °C. Subsequently the plate was washed 3X with wash buffer and incubated with PreAmplifier for 1 hr at 55 °C followed by 3X washes and then incubated with Amplifier for 1 hr at

55 °C. After 3X washes, plate was hybridized with labeled probe for 1 hr at 50 °C. Finally, the plate was incubated with substrate at room temperature for 5 min and luminescence signal was captured by a plate-reader within 15 minutes.

### **Quantification of hMUT by LC-MS/MS**

Mouse livers were homogenized in 100 mM ammonium bicarbonate buffer with 8 M urea. MUT isotopically labeled signature peptides (IIADIFEYTAK\*, natural C and N atoms on either arginine or lysine are fully replaced by <sup>13</sup>C and <sup>15</sup>N isotopes, respectively; Thermo Pierce) were used as internal standards and spiked into each sample. Liver proteins were denatured, followed by reduction using 5 mM TCEP at 37°C for one hour, alkylation by 10 mM iodoacetamide at 25 °C in the dark, then digestion at 37°C for 15 hours with trypsin (trypsin:protein = 1:50 w/w; Catalog # V5280, Promega Inc.). Trypsin digestion reaction was stopped by adding formic acid. Samples were desalted on the SOLA plate, dried, and resuspended in water with 2% acetonitrile and 0.1% formic acid. 0.25 µg total protein was loaded onto the column (75µm\*15cm column packed with Waters Acquity BEH resin 1.7µm\*130A) and subjected to LCMS (Thermo Easy 1000 nano-UPLC, Orbitrap Fusion Mass Spectrometer) analysis. Water (A) and acetonitrile (B) with 0.1% formic acid were used for LC separation. The flow rate was 300 nL/min. The gradient was as follows: 2% B-7% B in 5 min; 7% -35% B in 45 min; 35%-80% B in 5 min; 80%-95%B in 2 min; 95%-95% B in 5 min. MS ran at continuous PRM mode: spray voltage 1900V; S-lens RF: 60%; isolation width: 1.4 m/z; HCD: 30% collision energy; detector: orbitrap, resolution: 60,000; scan range: 100-1800 m/z; AGC: 5E4; max injection time: 200ms.

### **Anti-hMUT antibody ELISA**

To determine if *hMUT* mRNA treatment induces anti-hMUT antibody, *Mut*<sup>+/-</sup>;Tg<sup>INS-CBA-G715V</sup> mice were injected with PBS or *hMUT* mRNA at 0.2 mg/kg for 3 and 5 weeks. To quantify antibodies against hMUT protein, Nunc Immuno Maxisorp plates (Thermo Scientific, #442404) were coated with 20 ng human MUT protein (Abcam, #ab115834) in 50mM Na<sub>2</sub>CO<sub>3</sub> and blocked overnight at 4°C. Mouse serum at a 1:20 dilution was incubated at room temperature for one hour, with rabbit anti-human MUT IgG (Novus Biologicals, #NBP1-87423) at 0-50 ng, and used to generate a standard curve. Goat anti-rabbit IgG-HRP (Abcam, #ab6721) and goat anti-mouse IgG H+L-HRP (Fitzgerald Laboratories, #43-GM30) secondary antibodies, diluted 1:100,000, were incubated at room temperature for one hour, prior to development with TMB substrate (Cell Signaling Technology, #7004S).

### **Proinflammatory cytokine analysis**

To determine if *hMUT* mRNA treatment induced inflammation, *Mut*<sup>+/-</sup>;Tg<sup>INS-CBA-G715V</sup> mice were injected with PBS or *hMUT* mRNA at 0.2 mg/kg for 3 and 5 weeks. Proinflammatory cytokines levels were measured in plasma using MesoScale Diagnostics proinflammatory panel 1 mouse kit (MesoScale Diagnostics, #K15048D) following the manufacturer's instructions. Briefly, samples and standards were incubated on plates precoated with capture antibodies for 2 hours at room temperature. After washing and incubation with secondary antibodies for 2 hours, absorbance was read using the QuickPlex SQ 120 after addition of 150µL 2X MSD Read Buffer. Samples cytokine concentrations were calculated using standard curves.

### **Liver specific chemistry panel**

To determine if mRNA injection induced hepatic toxicity, *Mut*<sup>+/-</sup>;Tg<sup>INS-CBA-G715V</sup> mice were injected with PBS or *hMUT* mRNA at 0.2 mg/kg for 3 and 5 weeks. Blood was collected and plasma was prepared and sent to IDEXX (3 Centennial Dr, North Grafton, MA 01536). A liver specific chemistry panel was measured by chemistry analyzer as described by IDEXX.

## Supplemental References

Chandler, R.J., Sloan, J., Fu, H., Tsai, M., Stabler, S., Allen, R., Kaestner, K.H., Kazazian, H.H., and Venditti, C.P. (2007a). Metabolic phenotype of methylmalonic acidemia in mice and humans: the role of skeletal muscle. *BMC Med Genet* *8*, 64.

Chandler, R.J., Tsai, M.S., Dorko, K., Sloan, J., Korson, M., Freeman, R., Strom, S., and Venditti, C.P. (2007b). Adenoviral-mediated correction of methylmalonyl-CoA mutase deficiency in murine fibroblasts and human hepatocytes. *BMC Med Genet* *8*, 24.

Chandler, R.J., Zervas, P.M., Shanske, S., Sloan, J., Hoffmann, V., DiMauro, S., and Venditti, C.P. (2009). Mitochondrial dysfunction in mut methylmalonic acidemia. *FASEB J* *23*, 1252-1261.

Ledley, F.D., Crane, A.M., and Lumetta, M. (1990). Heterogeneous alleles and expression of methylmalonyl CoA mutase in mut methylmalonic acidemia. *Am J Hum Genet* *46*, 539-547.

Manoli, I., Sysol, J., Li, L., Chandler, R., Senac, J., Hoffmann, V., Zervas, P., Schnermann, J., and Venditti, C. (2011). Muscle targeted transgene expression rescues the lethal phenotype of Mut knockout mice. *Molecular Genetics and Metabolism* *102*, 248.

Qureshi, A.A., Crane, A.M., Matiaszuk, N.V., Rezvani, I., Ledley, F.D., and Rosenblatt, D.S. (1994). Cloning and expression of mutations demonstrating intragenic complementation in mut0 methylmalonic aciduria. *J Clin Invest* *93*, 1812-1819.

Senac, J.S., Aswani, V.H., Sysol, J.R., Manoli, I., and C.P., V. (2013). Partial Deficiency Model of MUT Methylmalonic Acidemia (MMA) Displays Diet Inducible Disease and Sensitivity to Acetaminophen (APAP). *Molecular Therapy* *21*, S107.