OMTN, Volume *33*

Supplemental information

Lipid nanoparticle-targeted mRNA formulation as a treatment for ornithine-transcarbamylase deficiency model mice

Kazuto Yamazaki, Kenji Kubara, Satoko Ishii, Keita Kondo, Yuta Suzuki, Takayuki Miyazaki, Kaoru Mitsuhashi, Masashi Ito, and Kappei Tsukahara

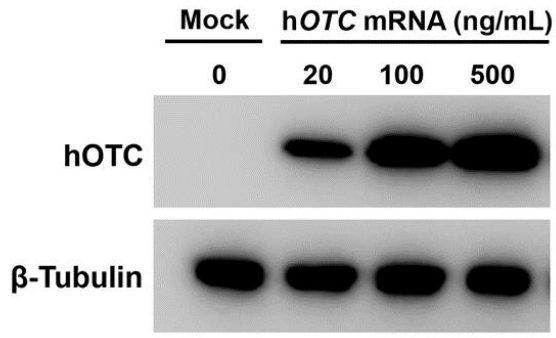

Figure S1: Western blotting analysis of hOTC protein expression in Hep3B cells transfected with codon-optimized h*OTC* mRNA. We used Lipofectamine MessengerMAX mRNA Transfection Reagent. For Mock, only the transfection reagent was added. β-Tubulin protein levels served as an internal control.

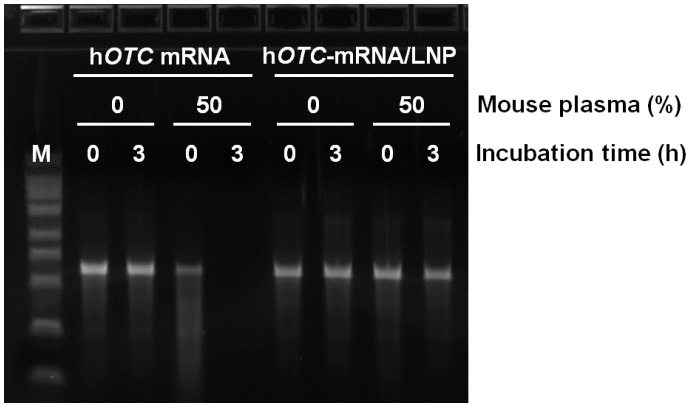

Figure S2: The stability test of mRNA formulated in LNP. h*OTC* mRNA and LNP-formulated h*OTC* mRNA (h*OTC*-mRNA/LNP) were incubated in the absence or presence of 50% mouse plasma from female BALB/c mice at 37 °C for 3 h. The samples were electrophoresed on a 1% agarose gel after immediately mixing with 50% mouse plasma (0 h). M, DynaMarker RNA High for Easy Electrophoresis (BioDynamics Laboratory, Tokyo, Japan).

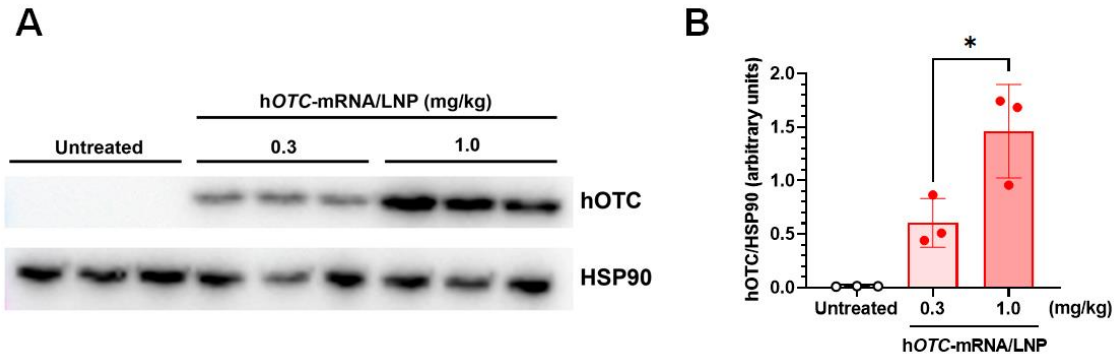

Figure S3: A dose-dependent increase in hOTC protein expression by h*OTC*-mRNA/LNP. Female BALB/c mice were intravenously injected with h*OTC*-mRNA/LNP at 0.3 or 1.0 mg/kg, and liver samples were harvested the next day. (**A**) Hepatic hOTC protein expression was analyzed by western blotting. HSP90 protein levels were used as an internal control. (**B**) The results of **A** were quantified. Values are expressed as means ± SD with individual values (n = 3). * indicates $p < 0.05$ by two-tailed unpaired Student's t-test.

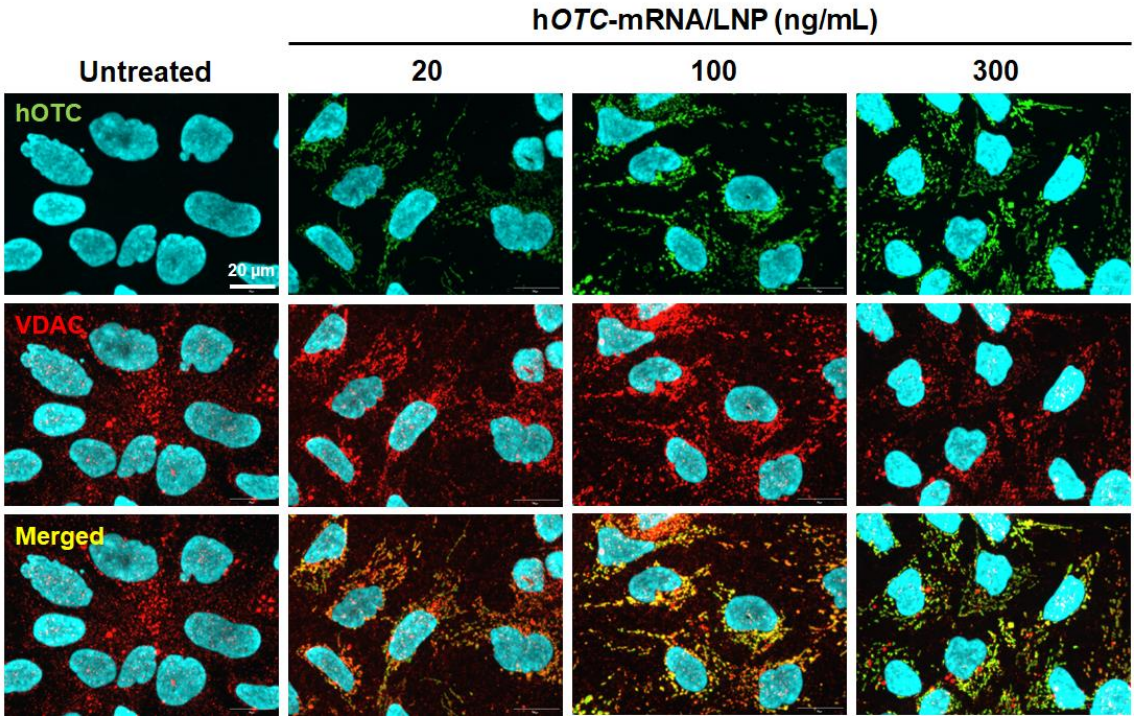

Figure S4: Subcellular localization of hOTC proteins in Hep3B cells treated with h*OTC*-mRNA/LNP at 20, 100, or 300 ng/mL. VDAC, a mitochondrial marker. Scale bars are 20 μm.

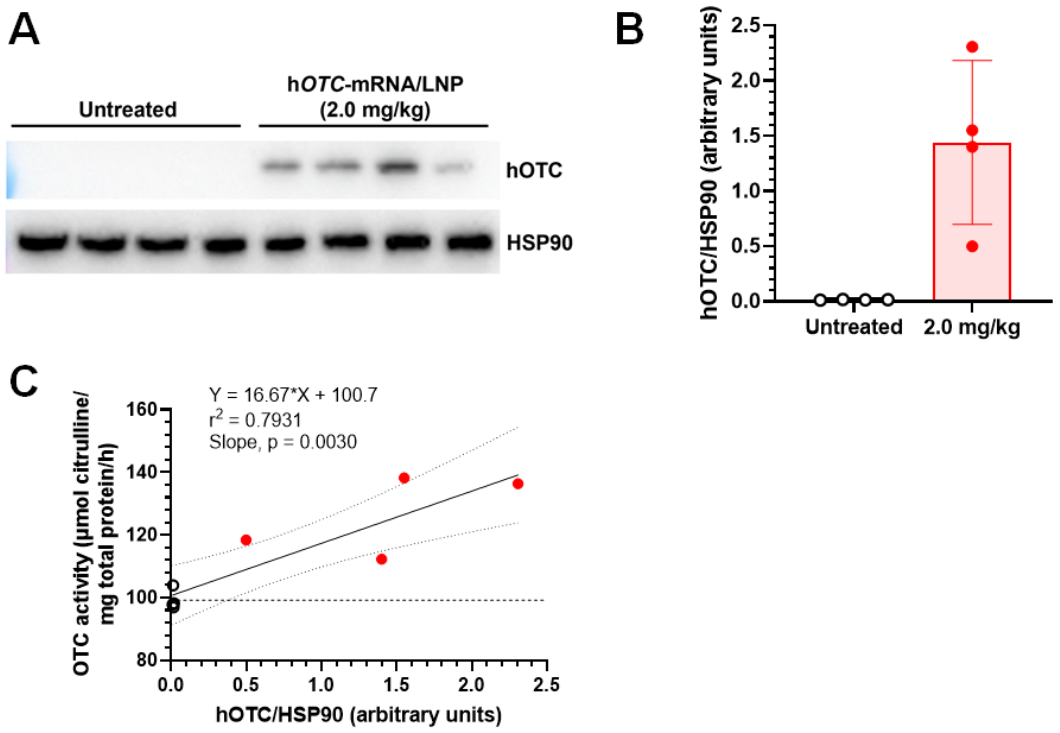

Figure S5: Relationship between hOTC protein expression and OTC activity in the liver of female BALB/c mice intravenously injected with h*OTC*-mRNA/LNP at 2.0 mg/kg. Liver specimens were harvested three days after the treatment. Untreated mice were used as a control. (**A**) Western blots of hepatic hOTC protein in untreated and h*OTC*-mRNA/LNP-treated mice. Heat shock protein 90 (HSP90) was used as an internal control. (**B**) Quantification of hOTC protein levels revealed by western blots in **A** are expressed relative to HSP90 protein levels. Values are expressed as means ± SD with individual values. (**C**) Regression line between hepatic hOTC protein levels and OTC activity. Untreated, open white circles; h*OTC*-mRNA/LNP-treated, red-filled circles. The dotted lines indicate 95% confidence. The dashed line shows the mean of OTC activity of the untreated group. Untreated, n = 4; treated, n = 4.

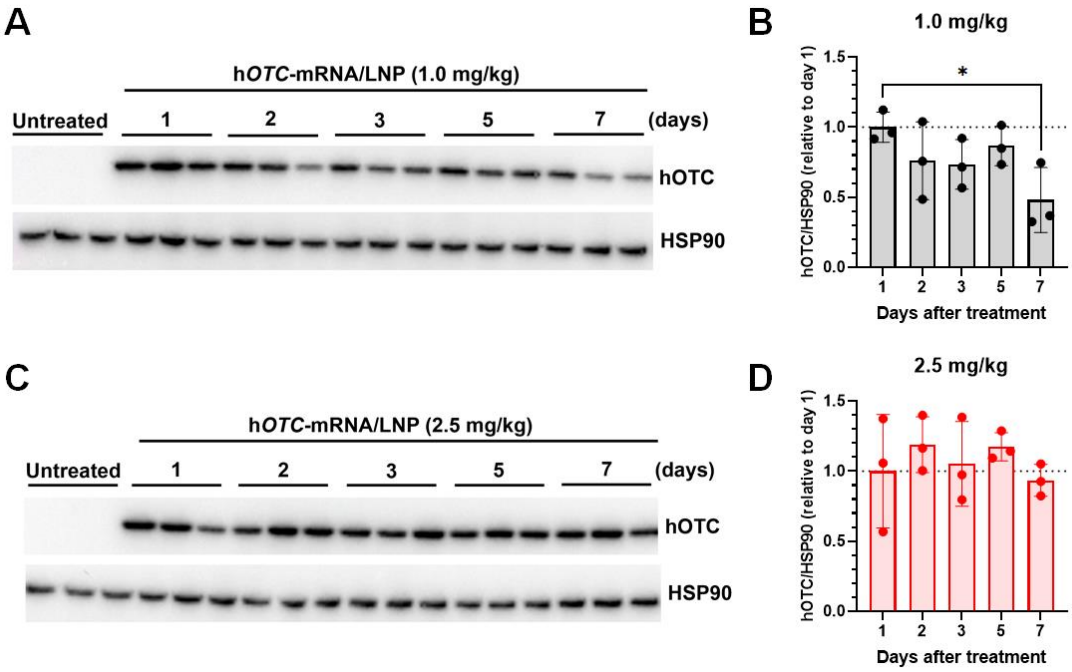

Figure S6: Time-course changes of hepatic hOTC protein expression induced by h*OTC*-mRNA/LNP treatment. Female BALB/c mice were intravenously injected with h*OTC*-mRNA/LNP at 1.0 or 2.5 mg/kg. Liver samples were collected 1, 2, 3, 5, and 7 days after the treatment. Untreated (UT) mice were used as a negative control for hOTC expression. n = 3 per each time. Western blot analysis of hepatic hOTC protein expression of mice treated with h*OTC*-mRNA/LNP at 1.0 mg/kg (**A** and **B**) and 2.5 mg/kg (**C** and **D**). HSP90 protein levels were used as an internal control. Values are expressed as means ± SD with individual values relative to those on day 1. Tukey's multiple comparisons test compared time-course changes of hOTC protein expression. * indicates $p < 0.05$.

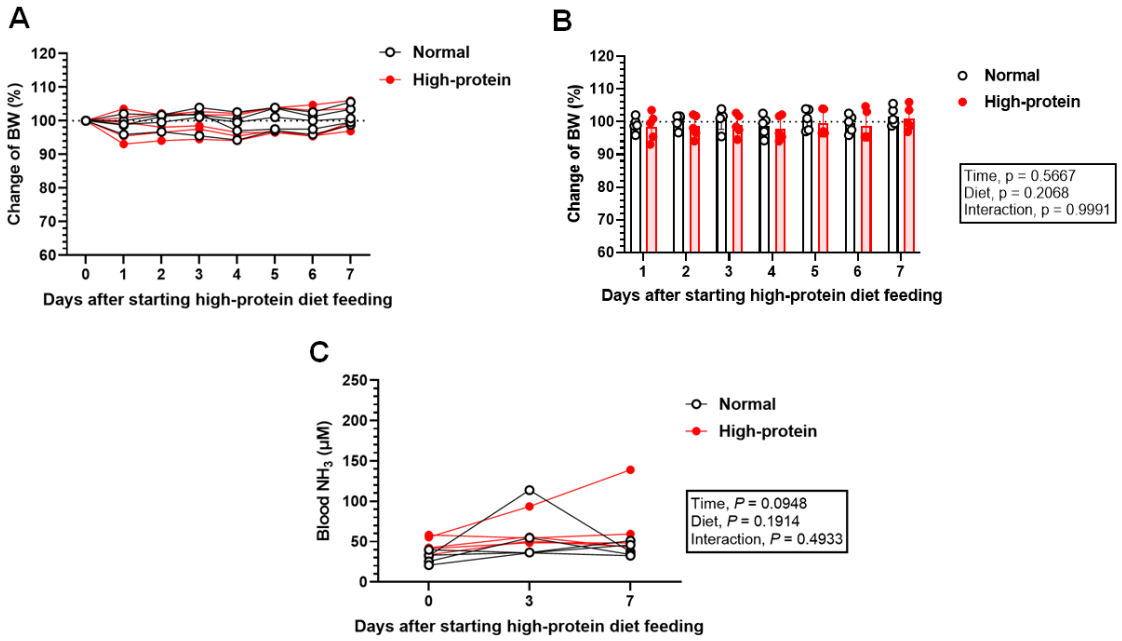

Figure S7: Effects of a high-protein diet feeding on body weight and blood NH_3 concentrations of wild-type male B6 mice. We monitored body weight (BW) and blood NH_3 concentrations of mice fed the high-protein diet (40%/kcal) or normal MF diet *ad libitum* from days 0 to 7. Blood was collected from the cheek vein to determine NH_3 concentrations on days 0 (before the feeding of the high-protein diet), 3, and 7. (**A**) BW changes of individual mice (% of those on day 0). The dotted line indicates 100%. (**B**) Comparison of BW changes between normal and high-protein diet feeding from days 1 to 7. Data were analyzed by two-way ANOVA ($F_{6,56}$ = 0.8099, p = 0.5667 for time; $F_{1,56}$ = 1.631, p = 0.2068 for diet; $F_{6,56}$ = 0.05883, p = 0.9991 for interaction). Values are expressed as means ± SD with individual values. The dotted line indicates 100%. (**C**) Changes in blood NH_3 levels of individual mice were analyzed by two-way repeated measures ANOVA ($F_{1.885,15.08}$ = 2.800, p = 0.0948 for time; $F_{1,8}$ = 2.037, p = 0.1914 for diet; $F_{2,16}$ = 0.7387, p = 0.4933 for interaction). n = 5 per group.

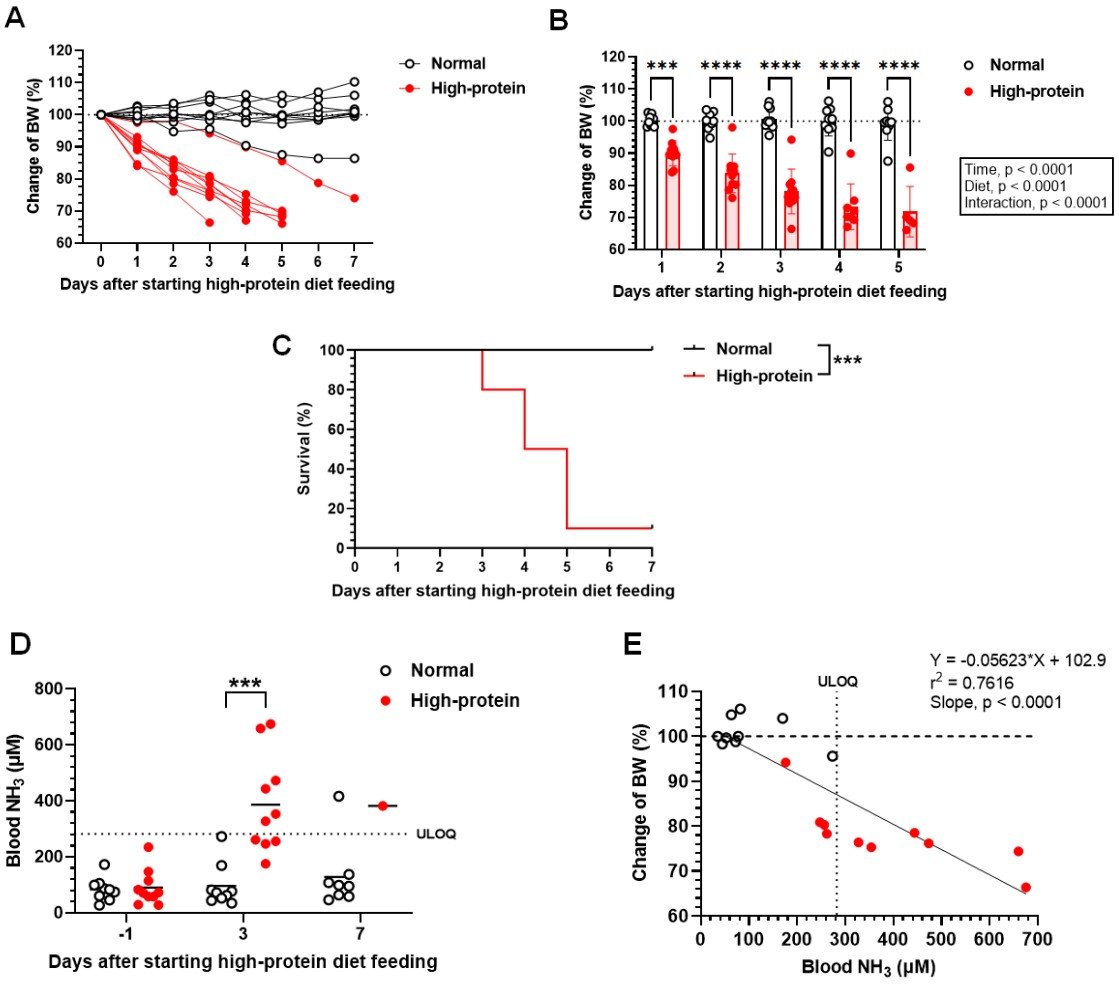

Figure S8: Effects of a high-protein diet feeding on body weight, blood NH_3 concentrations, and survival of male *Otc*$^{spf\text{-}ash}$ mice. We measured body weight (BW) and blood NH_3 concentrations of male *Otc*$^{spf\text{-}ash}$ mice fed the high-protein (40%/kcal) or normal diet *ad libitum* from day 0. Blood samples were drawn from the cheek vein to determine NH_3 concentrations on days −1, 3, and 7. (**A**) Time-course changes of BW of individual mice (% of those on day 0). The dotted line indicates 100%. (**B**) Comparison of BW changes between normal and high-protein diet feeding from days 1 to 5. Two-way ANOVA analyzed data ($F_{4,78} = 9.684$, $p < 0.0001$ for time; $F_{1,78} = 341.5$, $p < 0.0001$ for diet; $F_{4,78} = 8.732$, $p < 0.0001$ for interaction), followed by Šídák's multiple comparisons tests. *** and **** indicate $p < 0.001$ and $p < 0.0001$, respectively. Values are expressed as means ± SD with individual values. Dotted line indicates 100%. (**C**) Kaplan–Meyer survival curves of the normal and high-protein diet-fed groups. *** indicates $p < 0.001$ by log-rank test. (**D**) Comparison of blood NH_3 levels between normal and high-protein diet-fed mutant mice on days −1, 3, and 7. Note that there are values of ULOQ (upper limit of quantification) (282 μM) (blood NH_3 levels were

measured by diluting blood five times, but the values of 6 of 10 mice on day 3 exceeded the upper limit of measurement of the kit and then these values were for reference). *** indicates $p < 0.001$ by Mann–Whitney's U-test. Values are expressed medians with individual values. (**E**) Relationship between blood NH_3 concentrations and body weight changes on day 3. The dotted lines in D and E indicate the value of ULOQ. The dashed line in E shows 100%. n = 9–10 per group.

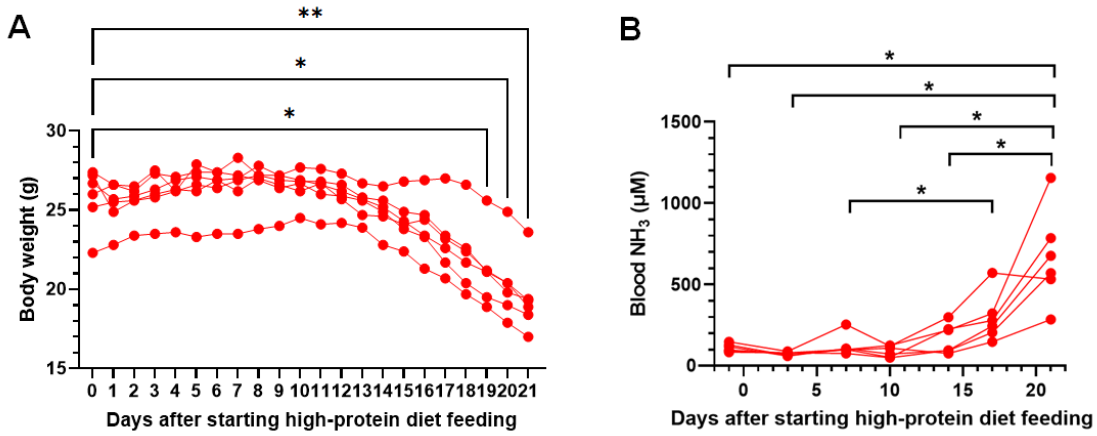

Figure S9: *In vivo* efficacy of single dosing of h*OTC*-mRNA/LNP in OTC deficiency model mice. We monitored body weight and blood NH_3 concentrations of male *Otc*$^{spf\text{-}ash}$ mice treated with h*OTC*-mRNA/LNP. Mice were administered an intravenous bolus of h*OTC*-mRNA/LNP at 3.0 mg/kg on day 0. After the injection, mice were provided a high-protein (40%/kcal) diet *ad libitum*. Blood was sampled from the cheek vein for NH_3 determination on days −1, 3, 7, 10, 14, 17, and 21. (**A**) Comparison of body weight change from day 0 in the h*OTC*-mRNA/LNP-treated group. Data were analyzed by one-way repeated measures ANOVA ($p = 0.0004$), followed by Dunnett's multiple comparisons tests. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively. (**B**) Time-course changes of blood NH_3 levels of h*OTC*-mRNA/LNP-treated mice. Data were analyzed by one-way repeated measures ANOVA ($p = 0.0022$), followed by Tukey's multiple comparisons tests. * indicates $p < 0.05$. $n = 6$ per group.

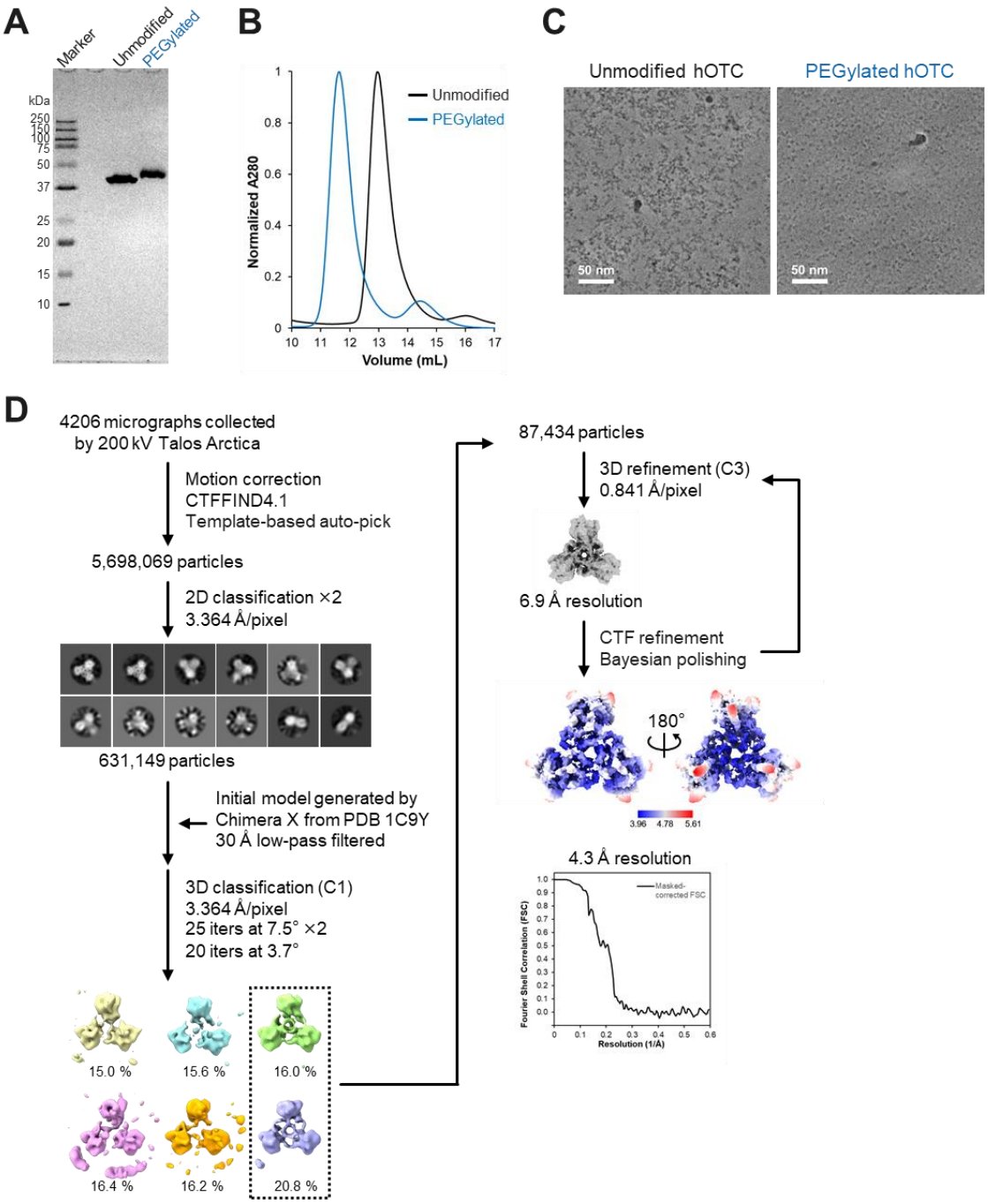

Figure S10: Cryo-EM sample preparation and data processing of hOTC proteins. To improve the quality of verified cryo-EM samples, purified hOTC proteins were PEGylated with PEG8. The modification was confirmed by SDS-PAGE (**A**) and size-exclusion chromatography (**B**). (**C**) Comparison of the particle qualities of unmodified and PEGylated hOTC. (**D**) The workflow of cryo-EM data processing.

Table S1: Physical properties of mRNA-formulating LNPs used in this study.

Formulated mRNA	Z-average (nm)	Polydispersity index	mRNA encapsulation efficiency (%)
Firefly luciferase (*Fluc*)	114	0.02	94%
human ornithine transcarbamylase (h*OTC*)	125	0.02	94%

Table S2: Plasma biochemistry of mice repeatedly dosed with LNP-formulated mRNA.

	Saline (n = 4)	*Fluc*-mRNA/LNP (mg/kg)		h*OTC*-mRNA/LNP (mg/kg)	
		0.3 (n = 4)	1.0 (n = 4)	0.3 (n = 4)	1.0 (n = 4)
AST (mU/mL)	97 ± 32	140 ± 59	127 ± 41	126 ± 53	170 ± 91
ALT (mU/mL)	42 ± 12	65 ± 23	74± 12	46 ± 7	41 ± 3
ALP (mU/mL)	140 ± 6	130 ± 23	146± 17	152 ± 24	137 ± 11
T-Bil (mg/dL)	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
UN (mg/dL)	26.1 ± 1.4	25.9 ± 1.5	29.0 ± 3.8	21.3 ± 2.5	23.0 ± 5.6
CRNN (mg/dL)	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.02
T-P (g/dL)	4.5 ± 0.1	4.6 ± 0.2	4.7 ± 0.2	4.6 ± 0.1	4.8 ± 0.2
Alb (g/dL)	3.1 ± 0.1	3.0 ± 0.1	3.1± 0.2	3.1 ± 0.1	3.2 ± 0.1
Glo (g/dL)	1.4 ± 0.0	1.5 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.7 ± 0.1*
A/G	2.27 ± 0.08	1.99 ± 0.10	1.96 ± 0.19	2.11 ± 0.17	1.93 ± 0.13*
Glu (mg/dL)	242 ± 16	270 ± 23	247 ± 32	251 ± 31	228 ± 34
T-Cho (mg/dL)	77 ± 2	87 ± 6	102 ± 7*	83 ± 11	90 ± 10
TG (mg/dL)	142 ± 31	199 ± 29	144 ± 44	136 ± 53	75 ± 16
Ca (mg/dL)	8.6 ± 0.1	8.9 ± 0.5	9.1 ± 0.3	9.2 ± 0.2	9.4 ± 0.3*
Na (mEq/L)	149 ± 2	148 ± 1	148 ± 1	149 ± 1	151 ± 2
K (mEq/L)	5.5± 0.3	5.1 ± 0.3	5.5 ± 0.5	5.0 ± 0.5	5.3 ± 0.8
Cl (mEq/L)	112 ± 1	111 ± 2	111 ± 01	114 ± 2	115 ± 1

Female BALB/c mice were injected intravenously with LNP-formulated *Fluc* mRNA, h*OTC* mRNA (0.3 or 1.0 mg/kg), or saline on days 0, 7 and 14. Blood was collected by cardiac puncture from fed mice on day 15. AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; T-Bil, total bilirubin; UN, urea nitrogen; CRNN, creatinine; T-P, total protein; Alb, albumin; Glo, globulin; A/G, albumin-globulin ratio; Glu, Glucose; T-Cho, total cholesterol; TG, triglyceride; Ca, calcium; Na, sodium; K, potassium; Cl, chloride. Data were analyzed by the Kruskal–Wallis test, followed by Dunn's multiple comparisons tests. Values are expressed as means ± SD. * indicates $p < 0.05$ compared with the saline-treated group.

Table S3: List of antibodies and reagents used for immunocytochemical and immunohistochemical studies.

Reagents	Cat#	Clone#	Vendor (city/town, state, country)	Dilution rate
Immunocytochemistry				
Mouse anti-human OTC monoclonal antibody	TA802590	OTI2G6	OriGene (Rockville, MD, USA)	×200
Rabbit anti-VDAC1 and VDAC2 antibody	ab154856		Abcam (Cambridge, UK)	×1000
Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L)	A11029		Thermo Fisher Scientific (Waltham, MA, USA)	×1000
Alexa Fluor 555-conjugated goat anti-rabbit IgG (H+L)	A21429		Thermo Fisher Scientific	×1000
Immunohistochemistry				
Mouse anti-human OTC monoclonal antibody	TA802590	OTI2G6	OriGene	×100
Rabbit anti-prohibitin polyclonal antibody	70R-5543		Fitzgerald Industries International (Acton, MA, USA)	×200
Biotinylated goat anti-rabbit IgG	BA-1000		Vector Laboratory (Newark, CA, USA)	×400
Alexa Fluor 647-conjugated streptavidin	S21374		Thermo Fisher Scientific	×400
Alexa Fluor 594-conjugated goat anti-mouse IgG (H+L)	A32742		Thermo Fisher Scientific	×400
Biotin conjugated mouse anti-human OTC monoclonal antibody	TA802590AM	OTI2G6	OriGene	×100
Alexa Fluor 594-conjugated streptavidin	S11227		Thermo Fisher Scientific	×400
Goat anti-mouse serum albumin polyclonal antibody	ab19194		Abcam	×400
Alexa Fluor 647-conjugated chicken anti-goat IgG (H+L)	A21469		Thermo Fisher Scientific	×400
4',6-diamidino-2-phenylindole (DAPI)	40043		Biotium (Fremont, CA, USA)	×10000

Table S4: Main ingredients of normal and high-protein diets.

Nutritional content	Normal (MF)	High-protein diet (40%/kcal)
Water (g)	7.9	9.0
Crude protein (g)	23.1	36.6
Crude fat (g)	5.1	7.1
Crude ash (g)	5.8	3.2
Crude fiber (g)	2.8	5.0
Nitrogen free extract (NFE) (g)	55.3	39.1
Total (g)	100.0	100.0
Calorie (kcal)	359.5	366.6
Protein calorie ratio (%/kcal)	25.7	39.9
Fat calorie ratio (%/kcal)	12.8	17.4
NFE calorie ratio (%/kcal)	61.5	42.7
	Per 100 g diet	