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Supplemental Information

Downregulation of the Arg/N-degron Pathway Sensitizes Cancer Cells to Chemotherapy *In Vivo*

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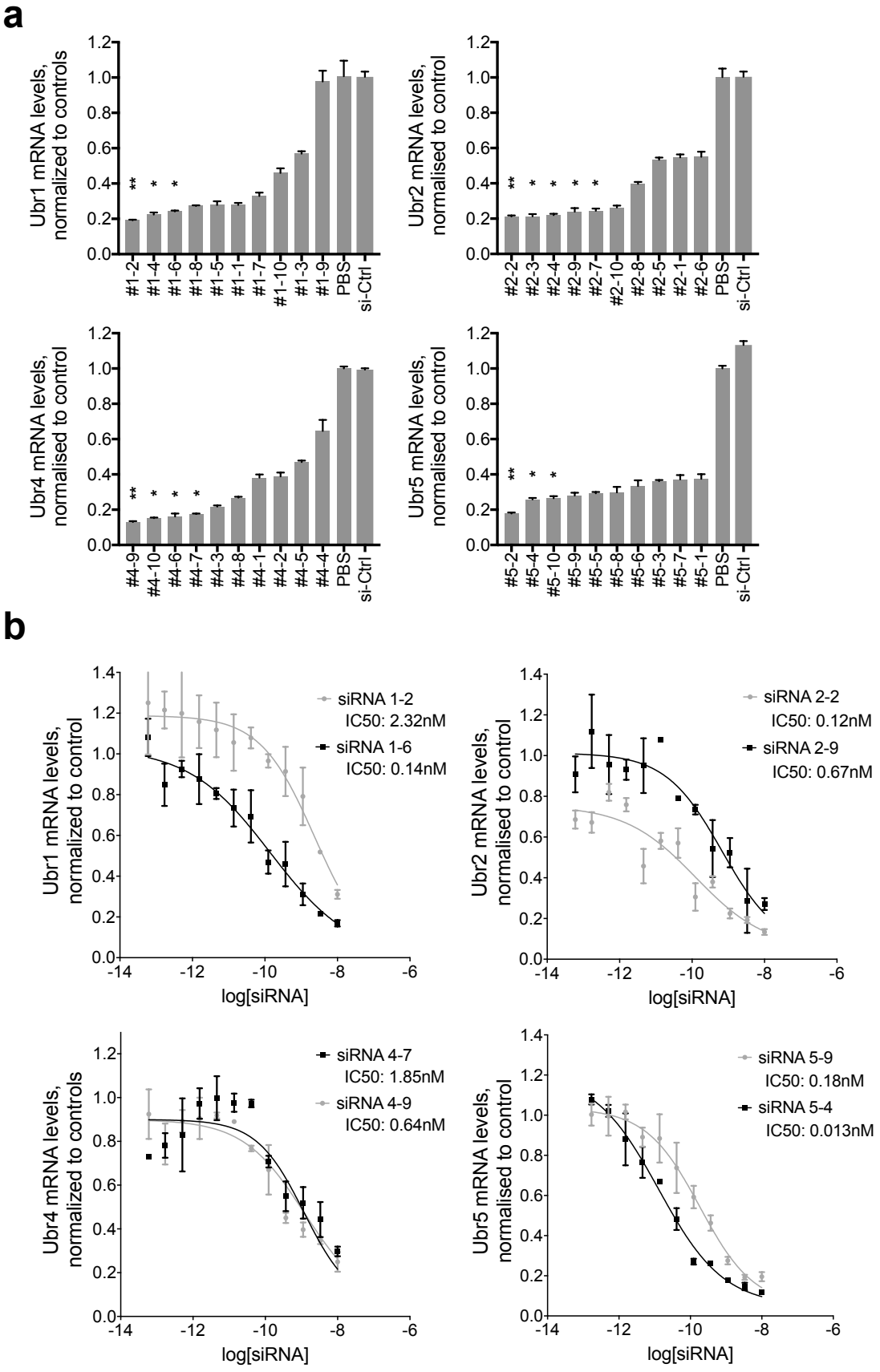

Figure S1a-b. Selection of siRNA, in vitro and in vivo. (a) mRNA downregulation in Hepa 1-6 cells by 1nM of specific siRNA against Ubr1, Ubr2, Ubr4 or Ubr5. mRNA levels were analyzed 24h post transfection. (b) Dose-dependence of mRNA downregulation in Hepa 1-6 cells. mRNA levels were analyzed 24h post transfection (mean ± SD). P values were obtained using a one-way ANOVA, comparing to controls. (*P <0.05, **P< 0.005, ***P< 0.0005, ****P 0.0001)

Figure S1a-b

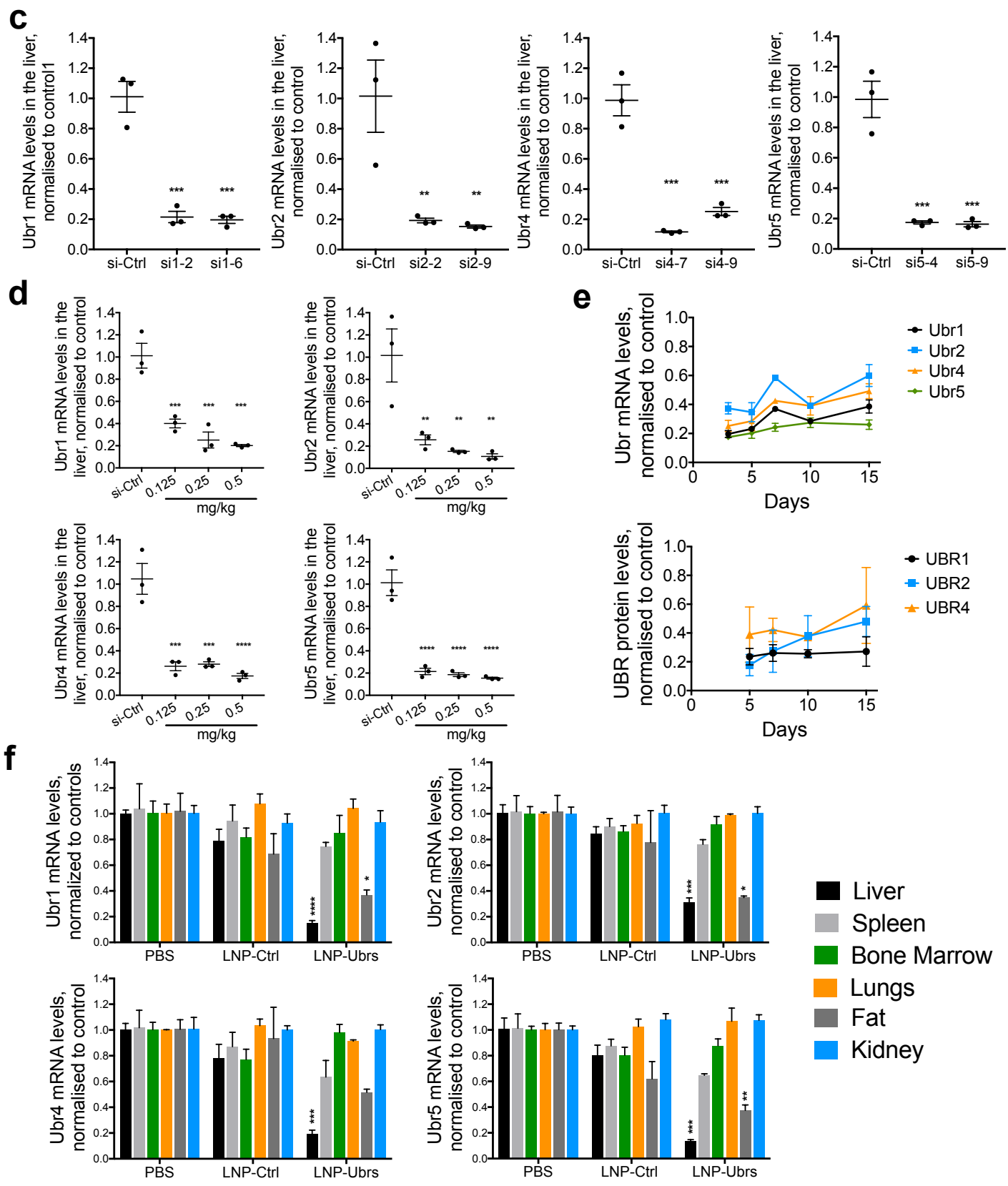

Figure S1c-f. Selection of siRNA, in vitro and in vivo. (c) Ubr mRNA levels in the total liver 3 days post injection of one of two specific LNP-siRNA (d) Dose-dependence downregulation of mRNA levels in total liver analyzed 3 days post injection (e) recovery of Ubr mRNA and protein levels in the liver at 3, 5, 7, 10 or 15 days after single-dose injections of LNP-siRNA (f) Tissue specific activity of systemically delivered siRNA formulated into LNPs analyzed 3 days post injection. For all in vivo experiments, n=3 and results presented as mean ± SEM. P values were obtained using a one-way ANOVA, comparing to controls. (*P <0.05, **P< 0.005, ***P< 0.0005, ****P 0.0001)

Figure S1c-f

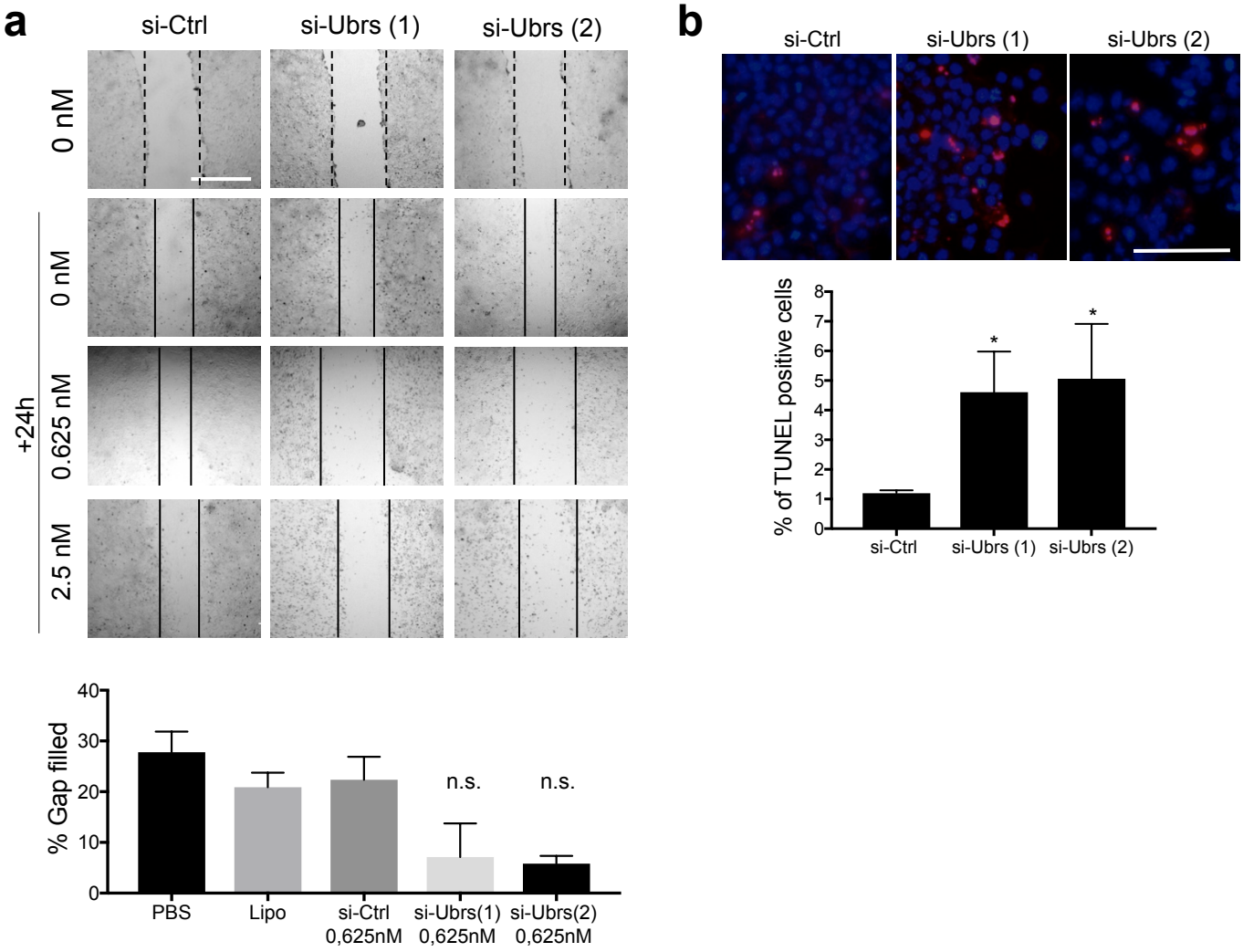

Figure S2. In vitro phenotype observed after Ubr downregulation is not an off target effect of siRNA. (a) Migration assay performed on Hepa 1-6 cells after 72h of exposure to two different sets of siRNA against Ubrs. (b) Analysis of cell death by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) in Hepa 1-6 cells, after 72h of exposure to two different sets of siRNA against Ubrs, at 10nM. Red nuclei represent TUNEL-positive cells. Scale bar, 100 um. Results show mean ± SD. P values were determined by Mann Whitney tests (*P <0.05)

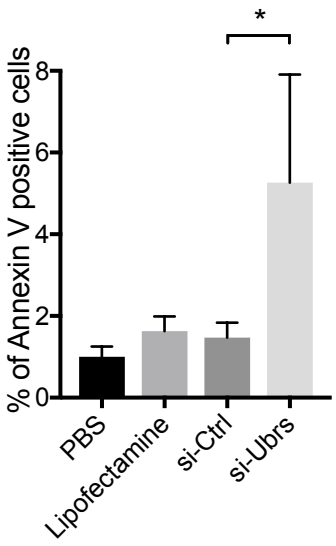

Figure S3. Ubr downregulation causes an increase in apoptosis. Analysis of cell death by Annexin V / 7AAD staining, evaluated by flow cytometry, in Hepa 1-6 cells, after 72h of exposure to siRNA against Ubrs, at 1nM. Results show mean ± SD. P values were determined by Mann Whitney tests (*P <0.05)

Figure S3

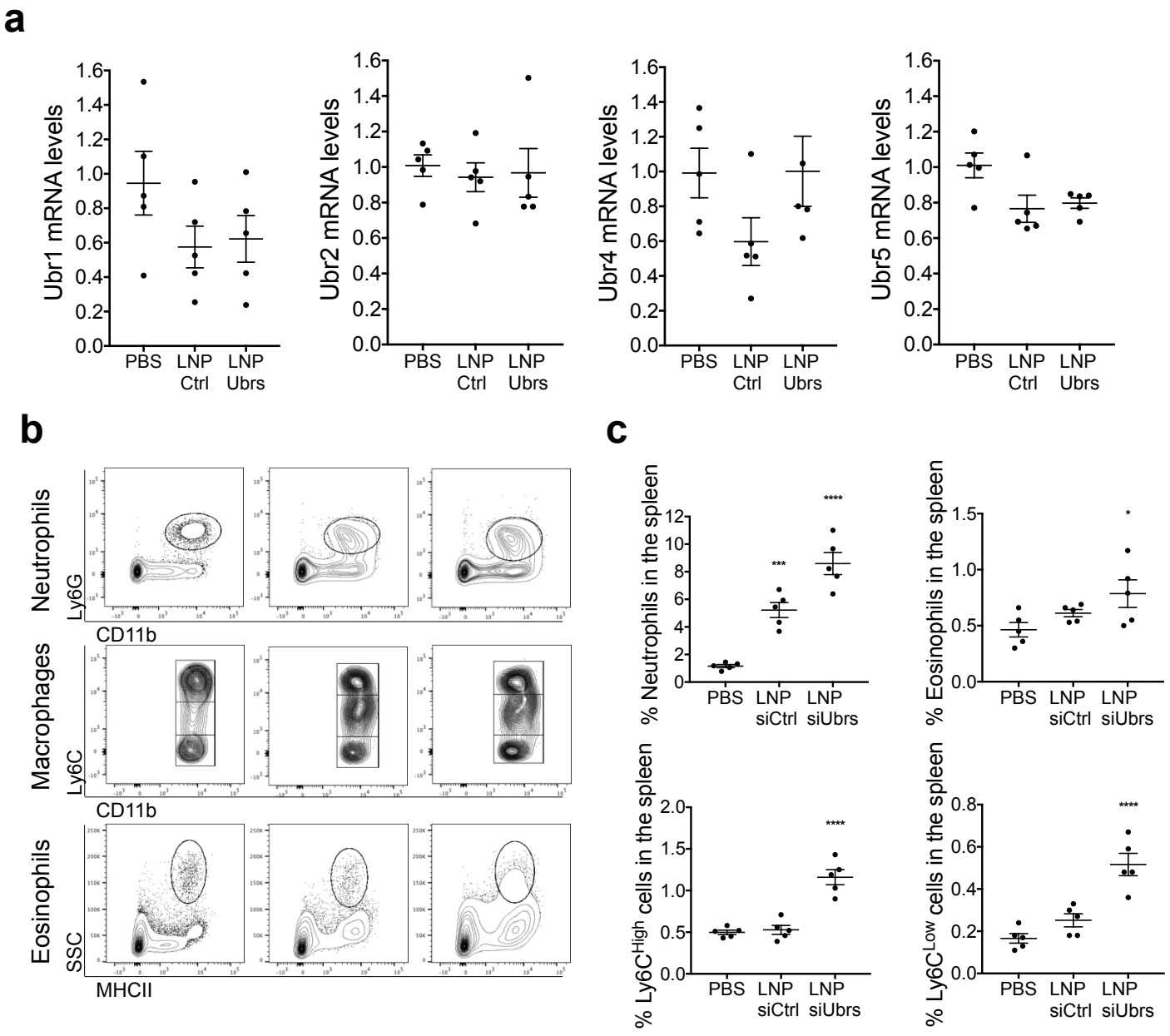

Figure S4. Effect of chronic downregulation of Ubr-Ubiquitin ligases in the spleen. (a) mRNA levels of Ubr1, Ubr2, Ubr4 and Ubr5 in the spleen of LNP-treated mice. (b) Representative flow cytometry analysis of neutrophil, macrophage and eosinophil populations in the spleen of mice treated with LNPs. (c) % of neutrophil, Ly6C^{high} macrophage or eosinophil populations in the spleen of LNP or vehicle treated mice, gated on CD45^{+} cells. Results show mean ± SEM. n=5 mice per group. P values were obtained using a one-way ANOVA, comparing to controls. (*P <0.05, ***P< 0.0005, ****P 0.0001)

Figure S4

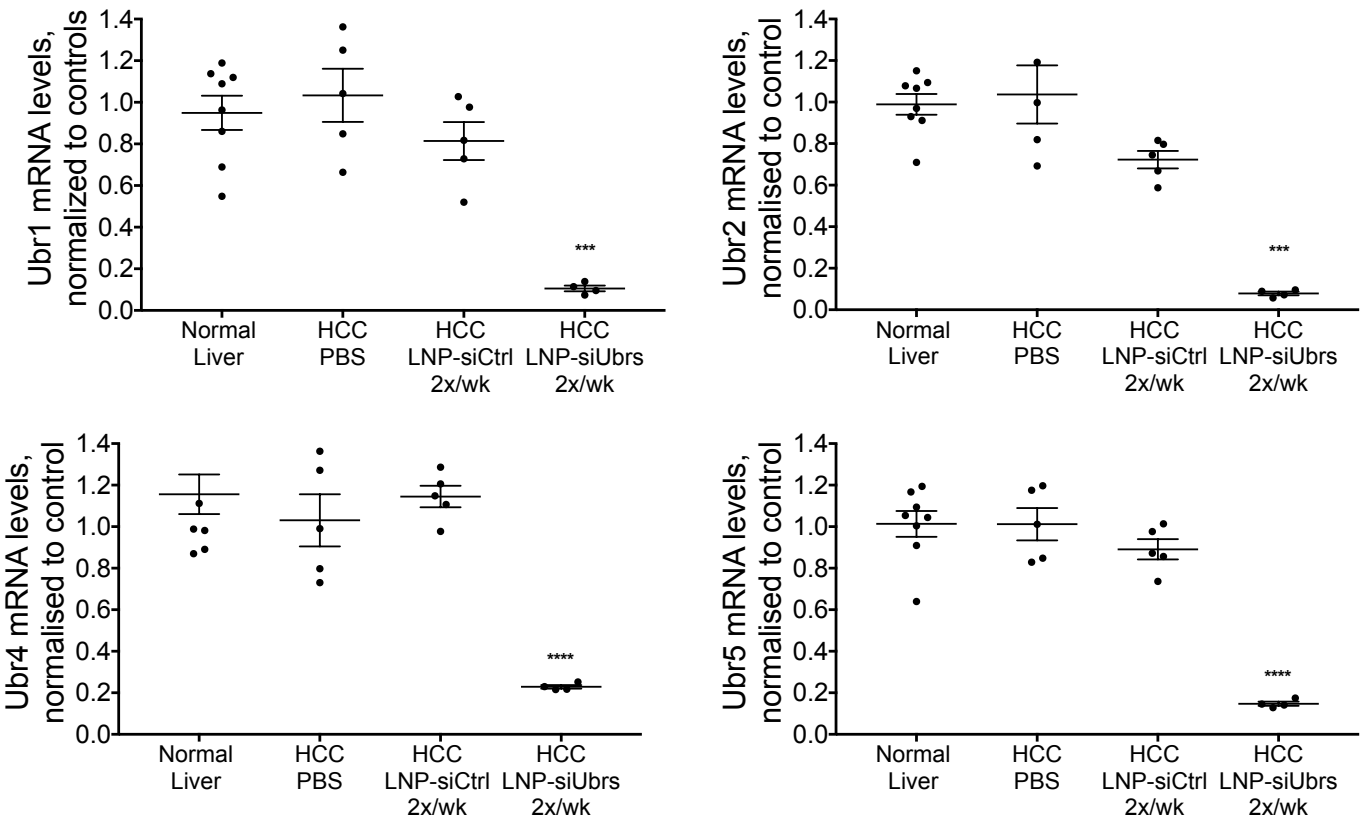

Figure S5. Expression and downregulation of Ubr-Ubiquitin ligases in the liver of HCC mice. mRNA levels of Ubr1, Ubr2, Ubr4 and Ubr5 in the liver of healthy and HCC mice was measured after 5 weeks of bi-weekly i.v. injections of a total of 1.4mg/kg of LNPs. (***P< 0.0005, ****P 0.0001)

Figure S5

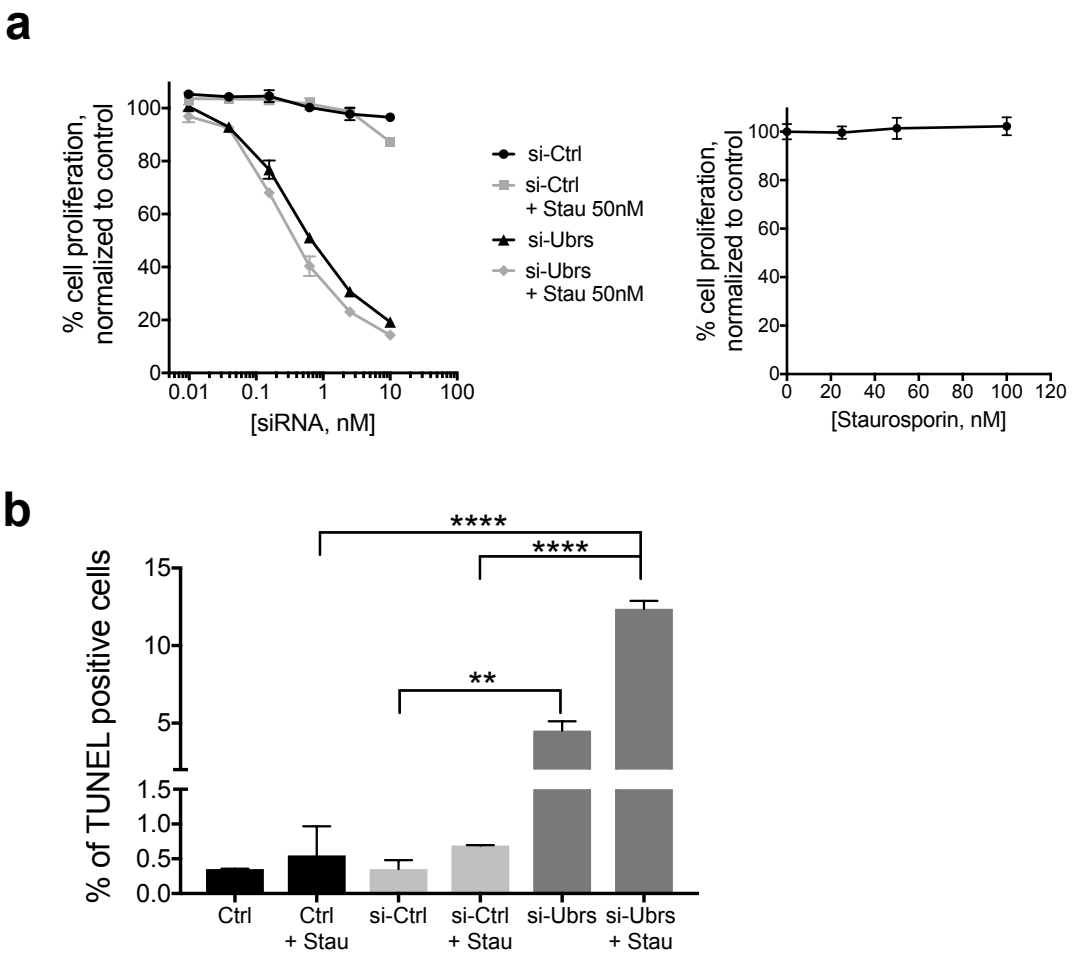

Figure S6. Knockdown of Ubr-Ubiquitin ligases of the N-end rule potentiates the action of apoptosis inducing drugs in vitro. (a) Proliferation of Hepa 1-6 cells after exposure to siRNA and Staurosporine (left) or Staurosporine alone (right). (b) Analysis of cell death by TUNEL, in Hepa 1-6 cells after 72h of exposure to 1nM siRNA against Ubrs and an additional 24h to Staurosporine at 50nM. Results show mean ± SD. P values were determined by a one way Anova (**P 0.001, ****P <0.0001)

Figure S6

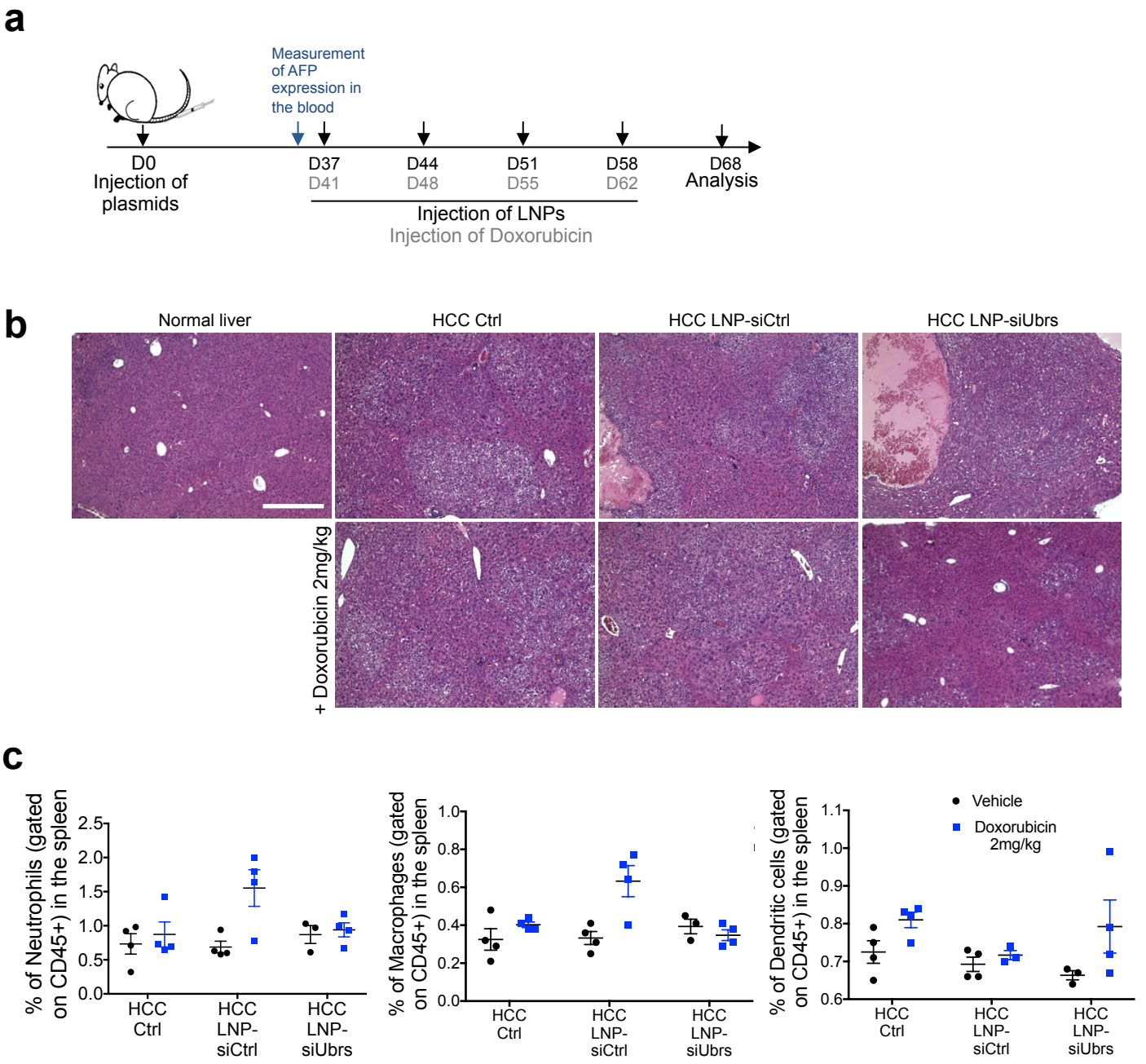

Figure S7. Chemotherapy in HCC is more efficient together with downregulation of the N-end rule pathway. (a) Schematic representation of the experiment: timeline of tumor induction (injection of oncogene-encoding plasmids) and repeated injections of LNP-formulated siRNA (black) and Doxorubicin (green). Tissues were collected for analysis on day 68 after tumor induction. (b) H&E staining of liver sections from HCC mice treated with LNPs and Doxorubicin. Scale bar = 100um. (c) Analysis by flow cytometry of Neutrophils, Macrophages and Dendritic cells in the spleen of HCC mice treated with LNPs and Doxorubicin.

Figure S7

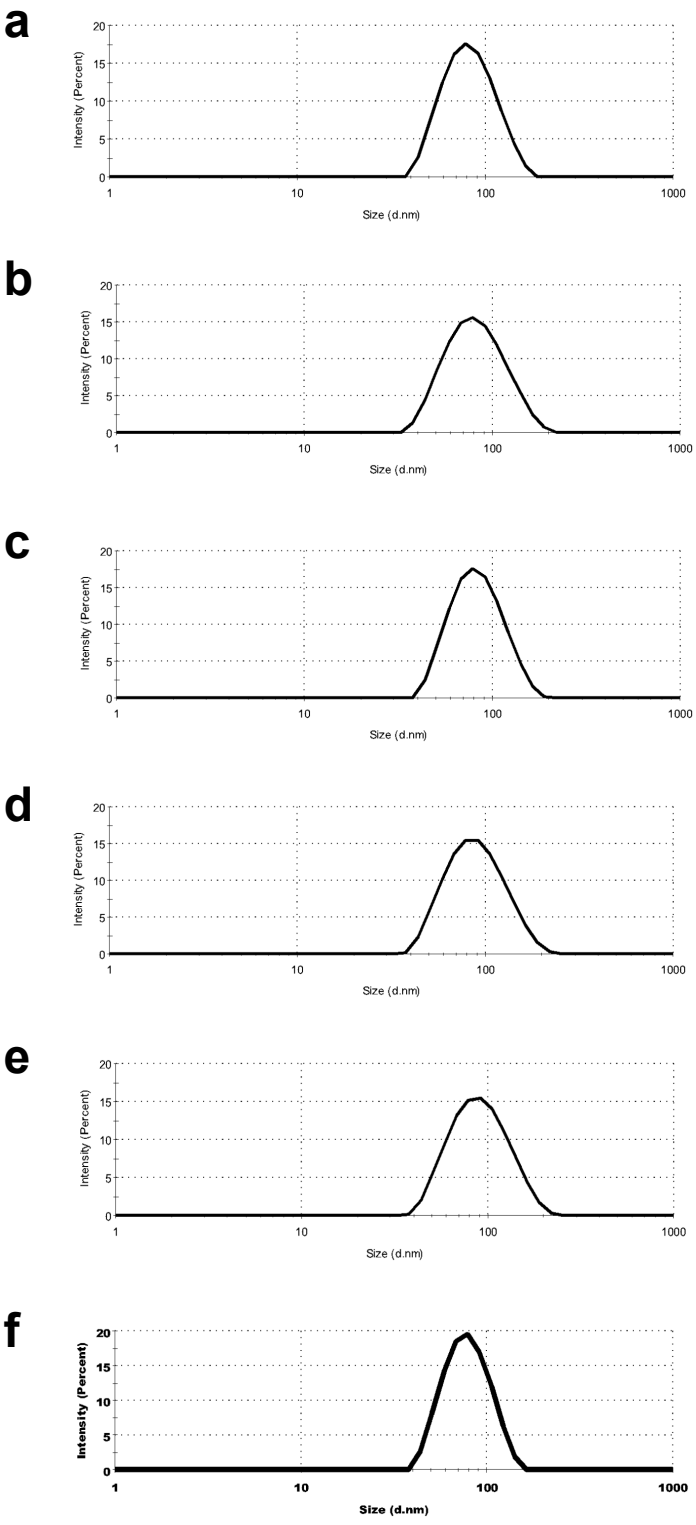

Figure S8. Size distribution by intensity for lipid nanoparticles used in this study. (a) LNP with siRNA against Ubr1. (b) LNP with siRNA against Ubr2. (c) LNP with siRNA against Ubr4. (d) LNP with siRNA against Ubr5. (e) LNP with siRNA against all Ubrs. (f) LNP with siRNA against Luciferase (ctrl-siRNA).

Table S1: Selected siRNA used in the study

		Sense	Anti-sense
si-Ctrl	si-LUC	cuuAcGcuGAGuAcuucGATsT	UCGAAGuACUcAGCGuAAGTsT
si-Ubr1	si-1-1	caAAGuAGuGuucauAAAAdTsdT	UUUuAUGAAcACuACUUUGdTsdT
	si-1-2	ccuucGGuGAuAAaAuAcAdTsdT	UGuAUUUuAUcACCGAAGGdTsdT
	si-1-3	gguuAuGGcucAccAGAAAdTsdT	UUUCUGGUGAGCcAuAACCdTsdT
	si-1-4	gacuuuAGAcAGAuAuuuudTsdT	AAAAuAUCUGUCuAAAGUCdTsdT
	si-1-5	gacAGGAAcAAuAaAuucAdTsdT	UGAAUUuAUUGUUCCUGUCdTsdT
	si-1-6	**caGAcuAGGuGcuauuucAdTsdT**	**UGAAAuAGcACCuAGUCUGdTsdT**
	si-1-7	gauuAAAcAGuAuaAuAcAdTsdT	UGuAUuAuACUGUUuAAUCdTsdT
	si-1-8	gcuuAGAGAAuGucAuAAAdTsdT	UUuAUGAcAUUCUCuAAGCdTsdT
	si-1-9	ggcccGGcuGuuAcuGAAAdTsdT	UUUcAGuAAcAGCCGGGCCdTsdT
	si-1-10	caGAAuAucGGGuuAuAAudTsdT	AUuAuAACCCGAuAUUCUGdTsdT
si-Ubr2	si-2-1	gauGGuGAAcAGccAAucAdTsdT	UGAUUGGCUGUUcACcAUCdTsdT
	si-2-2	ccAAGAAAAAGuuaGcAuudTsdT	AAUGCuAACUUUUUCUUGGdTsdT
	si-2-3	auuGuuAAGcAAAaGuGAAdTsdT	UUcACUUUUGCUuAAcAAUdTsdT
	si-2-4	aaGuGAAGuGGcAuAuAAAdTsdT	UUuAuAUGCcACUUcACUUdTsdT
	si-2-5	aguGAAGuGGcAuauAAAudTsdT	AUUuAuAUGCcACUUcACUdTsdT
	si-2-6	aguGGcAuAuAAAuuuccAdTsdT	UGGAAAUUuAuAUGCcACUdTsdT
	si-2-7	gcuccuAccucuAaGuGAAdTsdT	UUcACUuAGAGGuAGGAGCdTsdT
	si-2-8	gauAGAAcAuccucuuAGAdTsdT	UCuAAGAGGAUGUUCuAUCdTsdT
	si-2-9	**ggcGAGAGAuGuucGAcAAdTsdT**	**UUGUCGAAcAUCUCUCGCCdTsdT**
	si-2-10	gacuAuGGGAAGAgAuucAdTsdT	UGAAUCUCUUCCcAuAGUCdTsdT
si-Ubr4	si-4-1	caAAGAAGAuGAcuAcGAAdTsdT	UUCGuAGUcAUCUUCUUUGdTsdT
	si-4-2	gcAAGuGuAGuucaGuGAAdTsdT	UUcACUGAACuAcACUUGCdTsdT
	si-4-3	gaAccuAGGGuuuccGAAAdTsdT	UUUCGGAAACCCuAGGUUCdTsdT
	si-4-4	gcAuuuGGcuGuuaGccAudTsdT	AUGGCuAAcAGCcAAAUGCdTsdT
	si-4-5	cgAucAAccuGuAcuAcAAdTsdT	UUGuAGuAcAGGUUGAUCGdTsdT
	si-4-6	cacGGAGcAuuGuauuAcAdTsdT	UGuAAuAcAAUGCUCCGUGdTsdT
	si-4-7	**ggAcAuGAccAcAgGuAcAdTsdT**	**UGuACCUGUGGUcAUGUCCdTsdT**
	si-4-8	gccGGuAucAAGAacAAcAdTsdT	UGUUGUUCUUGAuACCGGCdTsdT
	si-4-9	ccAuGGAAAuGAGauuGAAdTsdT	UUcAAUCUcAUUUCcAUGGdTsdT
	si-4-10	gcuuGAGuGuGuAcAucuudTsdT	AAGAUGuAcAcACUcAAGCdTsdT
si-Ubr5	si-5-1	agcuGAAcAAGuAcAAuuudTsdT	AAAUUGuACUUGUUcAGCUdTsdT
	si-5-2	ggAGcAGGcuAcuauuAAAdTsdT	UUuAAuAGuAGCCUGCUCCdTsdT
	si-5-3	ggcAcAAGuuGuucuAcAAdTsdT	UUGuAGAAcAACUUGUGCCdTsdT
	si-5-4	**ugAuAAGGAuGGAacAAAAdTsdT**	**UUUUGUUCcAUCCUuAUcAdTsdT**
	si-5-5	guAGcuAcuGAAAauAAcAdTsdT	UGUuAUUUUcAGuAGCuACdTsdT
	si-5-6	ccGAGAAGAcuGAaAuAcudTsdT	AGuAUUUcAGUCUUCUCGGdTsdT
	si-5-7	gcGcucAGAAAGAaAuAcAdTsdT	UGuAUUUCUUUCUGAGCGCdTsdT
	si-5-8	gaAucAGGGAGGAucGcAAdTsdT	UUGCGAUCCUCCCUGAUUCdTsdT
	si-5-9	ggcuucGuccAAAaAGAAAdTsdT	UUUCUUUUUGGACGAAGCCdTsdT
	si-5-10	caGccAAuuGGAAaAuGcAdTsdT	UGcAUUUUCcAAUUGGCUGdTsdT

Uppercase letters: ribonucleotides
Lowercase letters: 2'-O-Methyl nucleotides
s: phosphorothioate

Table S2: Primers used in this study

Name	Sequence, 5'➔3'
mGAPDH dir	AGGTCGGTGTGAACGGATTTG
mGAPDH rev	TGTAGACCATGTAGTTGAGGTCA
mUbr1up	CCCAGCAGTTCCTGTCTTGT
mUbr1lo	ATCAGGAGGCACTTTCAGGC
mUbr2up	AGAGTTTTCAGTCGCAGACCT
mUbr2lo	TGATCGGGTCCATTCCCTGC
mUbr4up	GCAGGGAGGGGTACAAGTTC
mUbr4lo	GGCCTCTAGCCAACCTGAC
mUbr5up	AGAACCATTACCACCACGGC
mUbr5lo	CCACCTCAACCTCTTCCACG

Table S3: Effect of long-term knockdown of Ubr-ubiquitin ligases of the N-End rule in mouse liver on parameters of serum chemistry.

	PBS	LNP-siCtrl	LNP-siUbrs	ANOVA P-Levels
ALP (U/L)	102 ± 11	88 ± 10	212 ± 54§,¶	0.0001
AST (U/L)	102 ± 34	106 ± 35	207 ± 59§,¶	0.0038
ALT (U/L)	19 ± 4	23 ± 3	62 ± 21§,¶	0.0006
BUN (mg/dL)	32 ± 5	31 ± 2	27 ± 4	0.1830
Albumin (g/dL)	2.9 ± 0.1	2.9 ± 0.1	2.6 ± 0.2§,¶	0.0007
Total bilirubin (mg/dL)	0.16 ± 0.05	0.10 ± 0.00	0.20 ± 0.07¶	0.0302
Total protein (g/dL)	4.7 ± 0.1	5.0 ± 0.1$^{❖}$	4.3 ± 0.3§,¶	0.0001
Globulin (g/dL)	1.7 ± 0.1	2.1 ± 0.1$^{❖}$	1.7 ± 0.1¶	<0.0001
Cholesterol (mg/dL)	75 ± 4	86 ± 10	32 ± 3§,¶	<0.0001

ALP: Alkaline Phosphatase ; AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase ; BUN: Blood Urea Nitrogen

§: Tukey post hoc comparison of PBS vs LNP-siUbrs, P< 0.01, P< 0.001
¶: Tukey post hoc comparison of LNP-siCtrl vs LNP-siUbrs, P< 0.01, P< 0.001
❖: Tukey post hoc comparison of PBS vs LNP-siCtrl, P< 0.01

Table S4: Characteristics of lipid nanoparticles used in our study.

LNP-siRNA	Particle size, nm	Polydispersity index, PdI	Zeta potential
LNP si-Ubr1	84,5 ± 1,1	0,093	3,2 ± 1,7
LNP si-Ubr2	85,9 ± 0,6	0,1	3,7 ± 1,7
LNP si-Ubr4	85,4 ± 0,7	0,094	3,4 ± 1,4
LNP si-Ubr5	92,1 ± 2,0	0,1	3,2 ± 1,2
LNP si-Ubrs	94,8 ± 3,7	0,13	2,9 ± 1,5
LNP si-Ctrl	80.0 ± 0.8	0.062	3.9 ± 1.5