CONTENS OF APPENDIX

APPENDIX FIGURES LEGEND

Appendix Figure S1. Generation of Huh7 stable cells with *SIRT5* knockdown

#1 and #2 refer to two different shRNAs against *SIRT5* as described in Material and Methods. The indicated endogenous proteins were detected by western-blotting. Related to Figure 1A.

Appendix Figure S2. *SIRT5* knockdown induces oxidative DNA damage in HepG2 cells

(A) Knockdown of *SIRT5* induces oxidative DNA damage response. In HepG2 cells with stable *SIRT5* knockdown, the indicated classical oxidative DNA damage response markers were determined by western blot analysis. #1 and #2 refer to two different shRNAs against *SIRT5* as described in Material and Methods.

(B) Knockdown of *SIRT5* increases cellular ROS. In HepG2 cells with stable *SIRT5* knockdown, ROS level was determined in cell extracts as described in Material and Methods.

Data information: Shown are average values with standard deviation (S.D.) of triplicated experiments. *denotes the $p < 0.05$, **denotes the $p < 0.01$, and ***denotes the $p < 0.001$ for the indicated comparisons by two-tailed unpaired Student's *t*-test. Related to Figure 1B.

Appendix Figure S3. Identification of the specificity of SIRT5 antibody

In HEK293T cells with stable *SIRT5* knockdown, immunofluorescence staining was performed to detect endogenous SIRT5 using the antibody against SIRT5 as

described in Materials and Methods. Representative immunofluorescence images (original magnification, 40 x; a single focal plane, scale bar, 20 μm) are shown.

Appendix Figure S4. *SIRT5* knockdown does not affect the catalase activity

In HepG2 stable cells with *SIRT5* knockdown, the catalase activity was measured using a commercial kit as described in Material and Methods. Shown are average values with standard deviation (S.D.) of triplicated experiments. n.s.=not significant.

Appendix Figure S5. SIRT5 is co-localized with ACOX1

Immunofluorescence staining was performed in HeLa cells overexpressing HA-SIRT5, and the indicated proteins were detected using the indicated antibodies as described in Materials and Methods. Representative immunofluorescence images (original magnification, 630 x; a single focal plane, scale bar, 5 μm) are shown.

Appendix Figure S6. *SIRT5* knockdown increases lysine succinylation of ACOX1

Flag-ACOX1 was overexpressed in *SIRT5* knockdown HEK293T cells, and was then purified by IP with Flag beads, followed by western blot to detect the indicated post-translational modifications of Flag-ACOX1 as described in Materials and Methods. Related to Figure 2C.

Appendix Figure S7. H158Y mutation in SIRT5 does not affect its interaction

with ACOX1

Flag-ACOX1 was co-expressed with HA-SIRT5 or HA-SIRT5^{H158Y} in HEK293T cells. Proteins were purified by IP with Flag beads, followed by western blot to detect SIRT5 or SIRT5^{H158Y} with an HA antibody. Related to Figure 2E.

Appendix Figure S8. Verification of the methodology for ACOX1 activity assay

In HepG2 stable cells with *ACOX1* knockdown, the ACOX1 activity in whole cell lysate (~1 x 10^5 cells) was measured as described in Material and Methods.

Data information: Shown are average values with standard deviation (S.D.) of triplicated experiments. *denotes the $p < 0.05$, **denotes the $p < 0.01$, and ***denotes the $p < 0.001$ for the indicated comparisons by two-tailed unpaired Student's *t*-test

Appendix Figure S9. *SDHA* knockdown changes the cellular concentrations of succinate and fumarate

(A-B) In HepG2 cells with stable *SDHA* knockdown, metabolites were extracted, and the intracellular levels of fumarate **(A)** and succinate **(B)** were determined by GC-MS analysis as described in Materials and Methods. Shown are average values with standard deviation (S.D.) of triplicated experiments. #1 and #2 refer to two different shRNAs against *SDHA* as described in Material and Methods. Related to Figures 3E-F.

Appendix Figure S10. Succinylation of multiple lysine residues affects ACOX1

activity

The indicated K-to-R mutants of Flag-ACOX1 were overexpressed in HEK293T cells, and the ectopically expressed proteins were purified by IP with Flag beads, followed by ACOX1 activity assay as described in Materials and Methods. Shown are average values with standard deviation (S.D.) of triplicated experiments.

Appendix Figure S11. *SIRT5* knockdown does not change the ERK/AKT phosphorylation pathway

In HepG2 stable cells with *SIRT5* knockdown, the indicated proteins were determined by western blot analysis using the indicated antibodies as described in Materials and Methods.

Appendix Figure S12. SIRT5-regulated succinylation activates ACOX1 and promotes anchorage-independent cell growth in HepG2 liver cells

The capability of stable HepG2 cells with single or double knockdown of *SIRT5*/*ACOX1* to exhibit anchorage-independent growth was determined by soft-ager colony formation assay as described in Material and Methods. Related to Figures 5D-E.

Appendix Figure S13. Single or double knockdown of *SDHA*/*ACOX1* does not change the ERK/AKT phosphorylation pathway

In HepG2 stable cells with single or double knockdown of *SDHA/ACOX1*, the

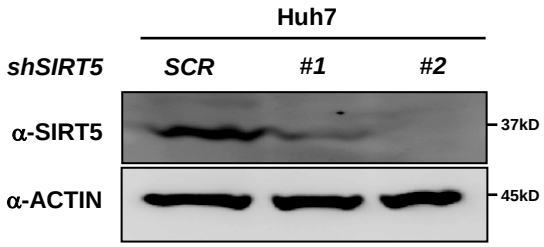
indicated proteins were determined by western blot analysis using the indicated antibodies as described in Material and Methods.

Appendix Figure S14. γH2AX is broadly increased in HCC tumors

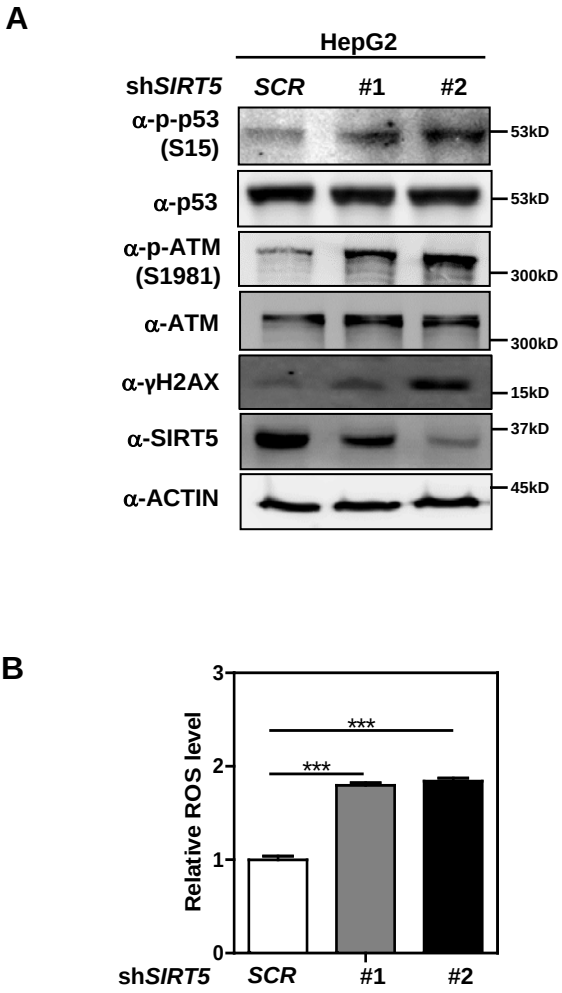
(A-B) In a study cohort consisting of 118 HCC patients, γH2AX protein was detected by IHC staining as described in Material and Methods. Representative images (original magnification, 200 x; scale bar, 50 μm) are shown **(A)**. Quantification of γH2AX IHC analysis is present in **(B)**.

Data information: *denotes the $p < 0.05$, **denotes the $p < 0.01$, and ***denotes the $p < 0.001$ for the indicated comparisons by two-tailed unpaired Student's *t*-test. Related to Figure 6E.

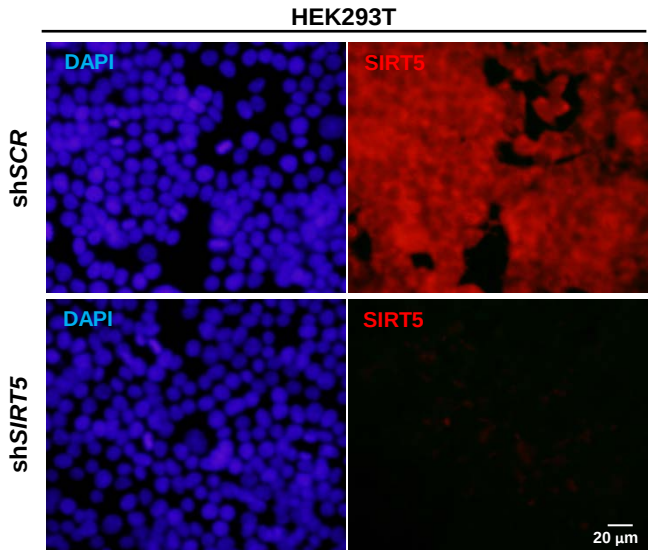
Appendix Figure S1. Generation of Huh7 stable cells with *SIRT5* knockdown



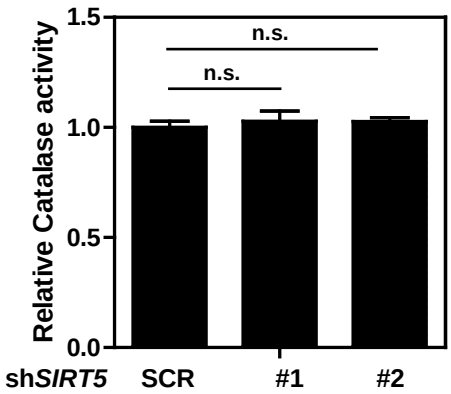
Appendix Figure S2. *SIRT5* knockdown induces oxidative DNA damage in HepG2 cells



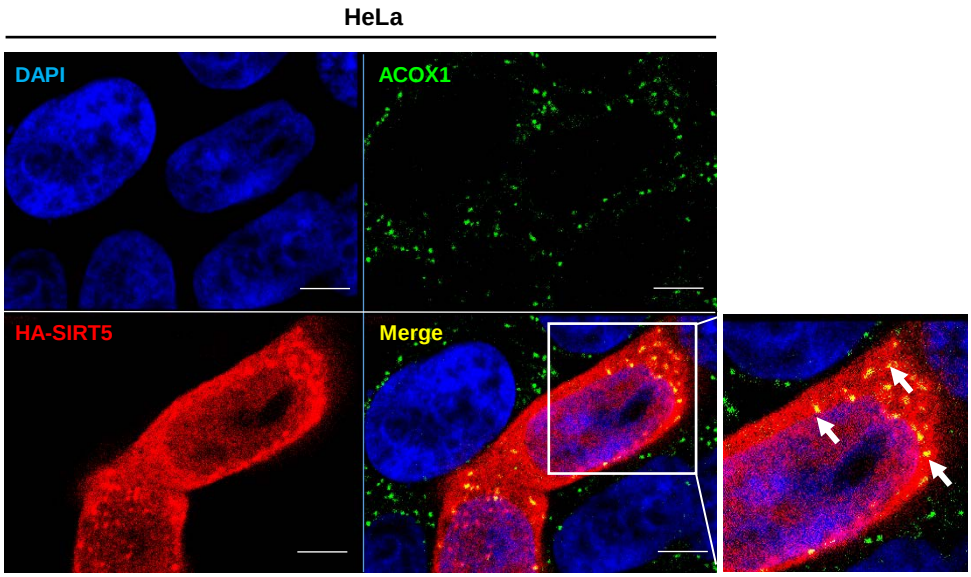
Appendix Figure S3. Identification of the specificity of SIRT5 antibody



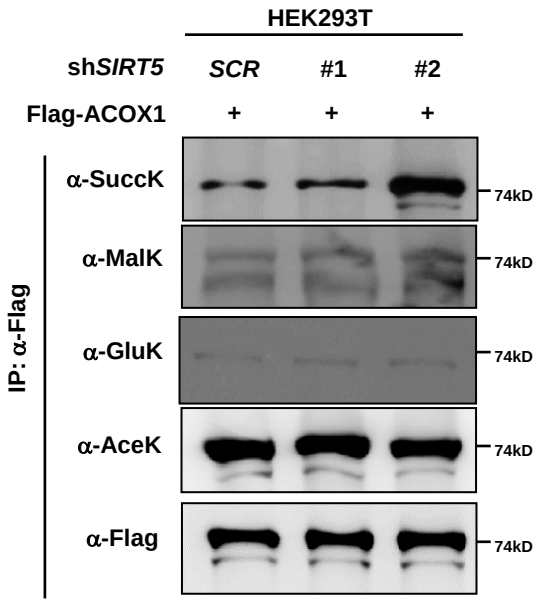
Appendix Figure S4. *SIRT5* knockdown does not affect the catalase activity in HepG2 cells



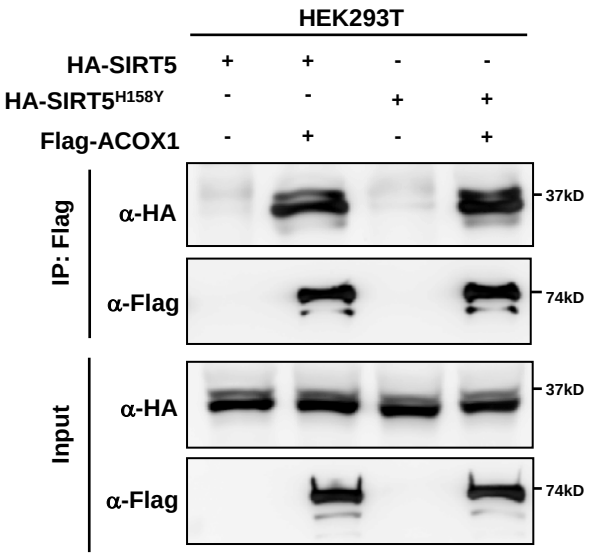
Appendix Figure S5. Co-localization of SIRT5 and ACOX1 in cells



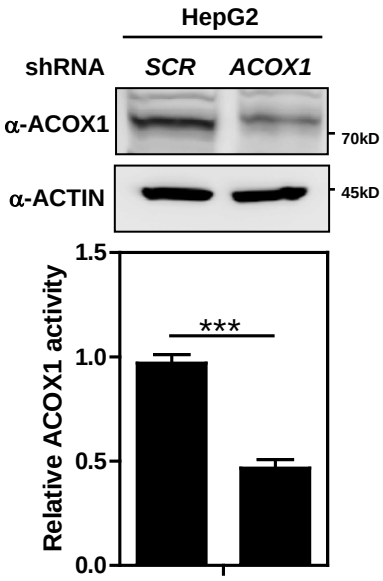
Appendix Figure S6. *SIRT5* knockdown increases the lysine succinylation level of ACOX1



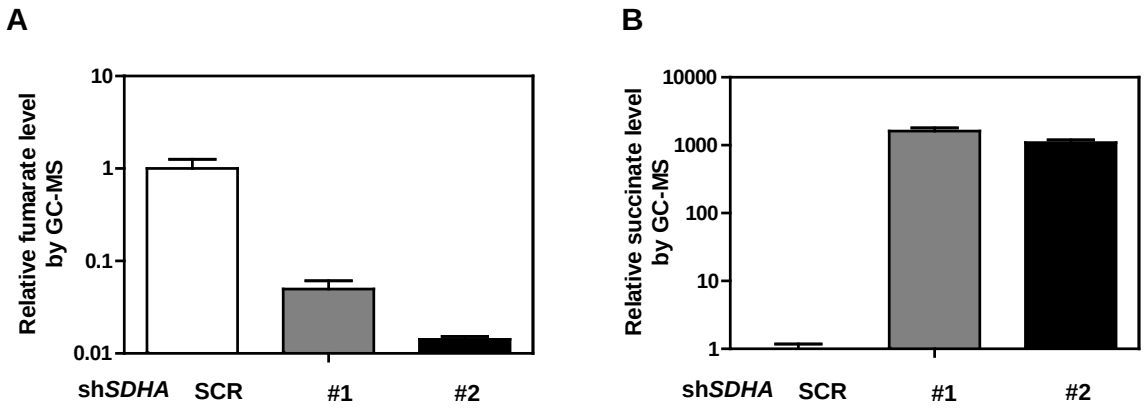
Appendix Figure S7. H158Y mutation in SIRT5 does not affect its interaction with ACOX1



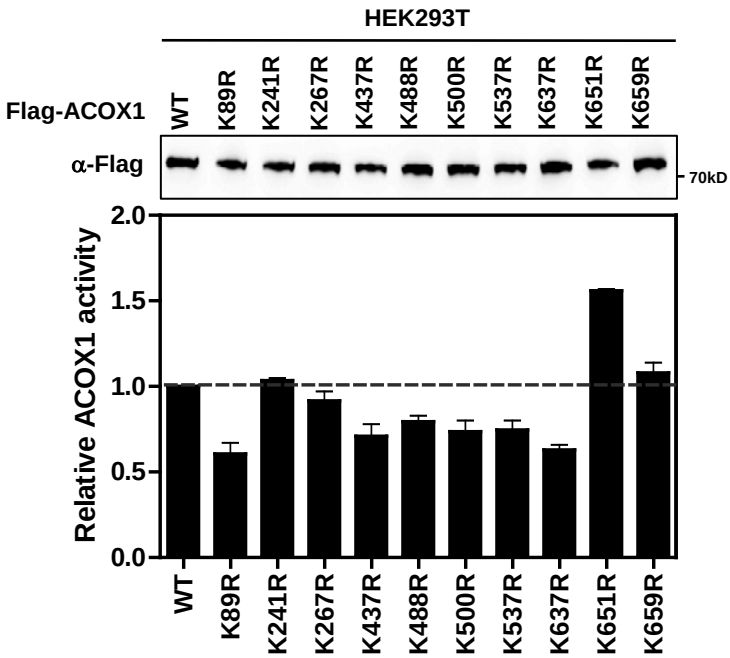
Appendix Figure S8. Verification of the methodology for ACOX1 activity assay



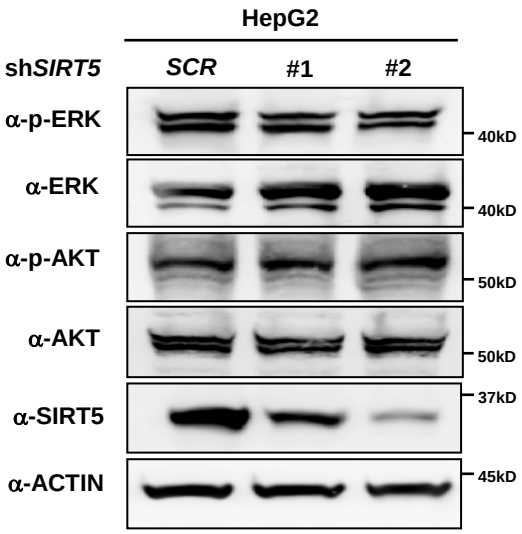
Appendix Figure S9. *SDHA* knockdown changes the cellular concentrations of succinate and fumarate in HepG2 liver cells



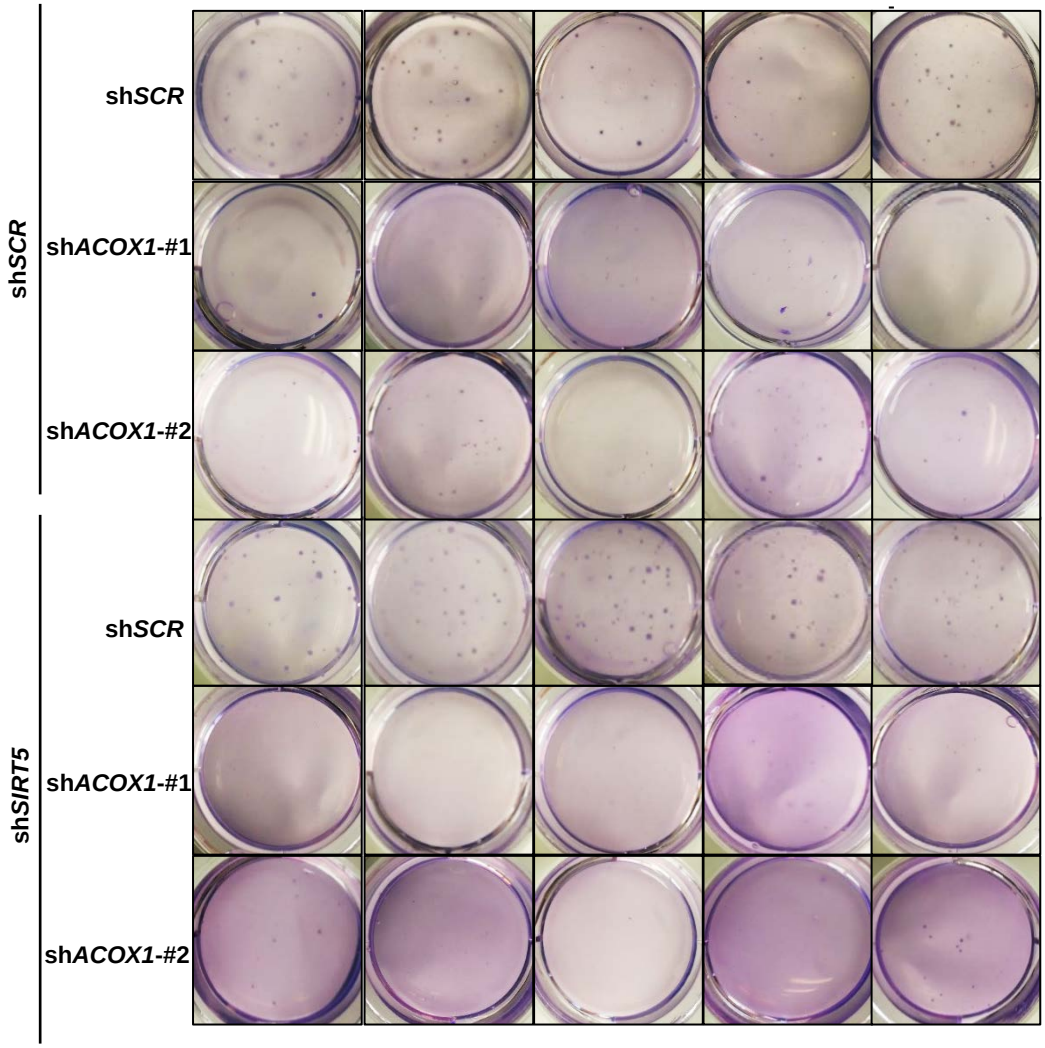
Appendix Figure S10. Succinylation of multiple lysine residues affects ACOX1 activity



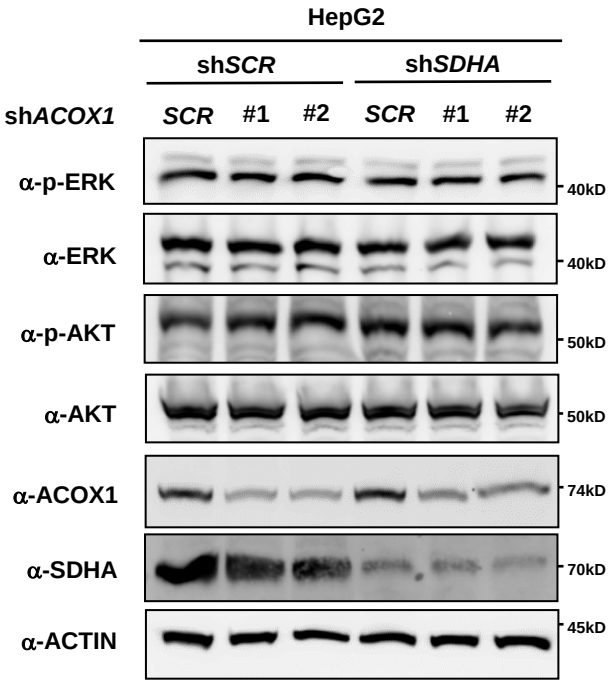
Appendix Figure S11. The ERK/AKT phosphorylation pathway is not affected by *SIRT5* knockdown in HepG2 liver cells



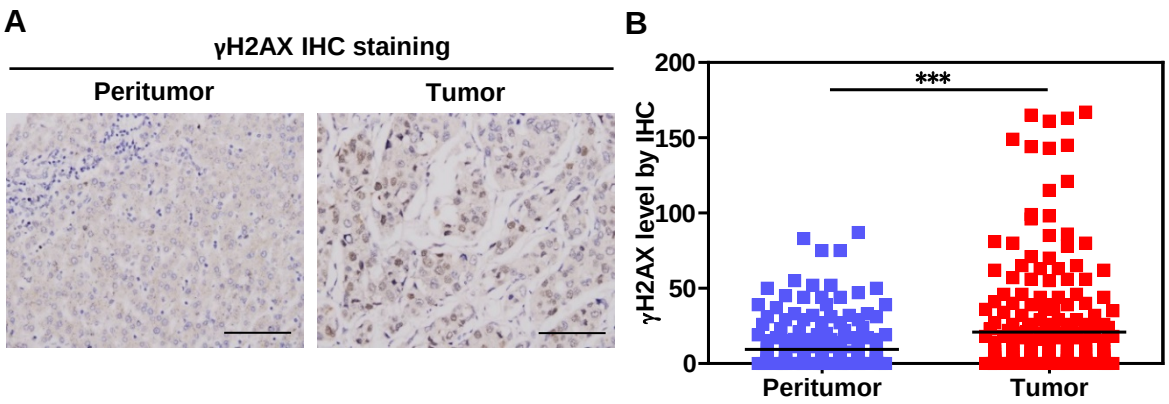
Appendix Figure S12. SIRT5-regulated succinylation activates ACOX1 to promote anchorage-independent cell growth in HepG2 liver cells



Appendix Figure S13. The ERK/AKT phosphorylation pathway is not affected by single or double knockdown of *SDHA*/*ACOX1* in HepG2 liver cells



Appendix Figure S14. γH2AX is broadly increased in HCC tumors



Appendix Table S1.
Potential peroxisomal substrates of SIRT5-regulated lysine succinylation

Protein	Description
ABCD3	ATP-binding cassette sub-family D member 3
ACOX1	Peroxisomal acyl-coenzyme A oxidase 1
ACOX2	Peroxisomal acyl-coenzyme A oxidase 2
ACSL1	Long-chain-fatty-acid--CoA ligase 1
AMACR	Alpha-methylacyl-CoA racemase
CATA	Catalase
DECR2	Peroxisomal 2,4-dienoyl-CoA reductase
DHB4	Peroxisomal multifunctional enzyme type 2
DHRS4	Dehydrogenase/reductase SDR family member 4
ECHP	Peroxisomal bifunctional enzyme
GSTK1	Glutathione S-transferase kappa 1
HAOX1	Hydroxyacid oxidase 1
HYES	Bifunctional epoxide hydrolase 2
IDHC	Isocitrate dehydrogenase [NADP] cytoplasmic
NUDT7	Peroxisomal coenzyme A diphosphatase NUDT7
PAHX	Phytanoyl-CoA dioxygenase, peroxisomal
PECR	Peroxisomal trans-2-enoyl-CoA reductase
PRDX5	Peroxiredoxin-5, mitochondrial
SOX	Peroxisomal sarcosine oxidase
THIKA	3-ketoacyl-CoA thiolase A, peroxisomal
THIKB	3-ketoacyl-CoA thiolase B, peroxisomal
URIC	Uricase

Appendix Table S2.
SIRT5 expression is associated with a worse outcome in HCC patients

Clinicopathological index		SIRT5		
		Low	High	P†
Sex	Female	43	9	.303
	Male	201	63	
Age (year)	≤50	99	26	.496
	>50	145	46	
HBsAg	Negative	36	7	.274
	Positive	208	65	
HCV	Negative	242	72	.441
	Positive	2	0	
AFP	≤20	85	31	.204
	>20	159	41	
γ-GT(U/L)	≤54	129	34	.400
	>54	115	38	
Liver cirrhosis	No	49	12	.519
	Yes	195	60	
Tumor number	Single	202	58	.663
	Multiple	42	14	
Tumor size (cm)	≤5	140	44	.572
	>5	104	28	
Tumor encapsulation	complete	119	46	.024
	none	125	26	
Microvascular invasion	absence	164	44	.337
	present	80	28	
Tumor differentiation	I+II	179	59	.138
	III+IV	65	13	
TNM stage	I	139	37	.402
	II+III	105	35	
BCLC stage	0+A	132	36	.540
	B+C	112	36	

Abbreviations: AFP, α-fetoprotein; γ-GT, γ-glutamyltransferase;
TNM, tumor-nodes-metastasis; BCLC, Barcelona Clinic Liver Cancer; HR, hazard ratio;
CI, confidential interval; Boldface type indicates significant values.
†Cox proportional hazards regression.