

Supplementary Information

ACSL3 is an unfavorable prognostic marker in cholangiocarcinoma patients and confers ferroptosis resistance in cholangiocarcinoma cells

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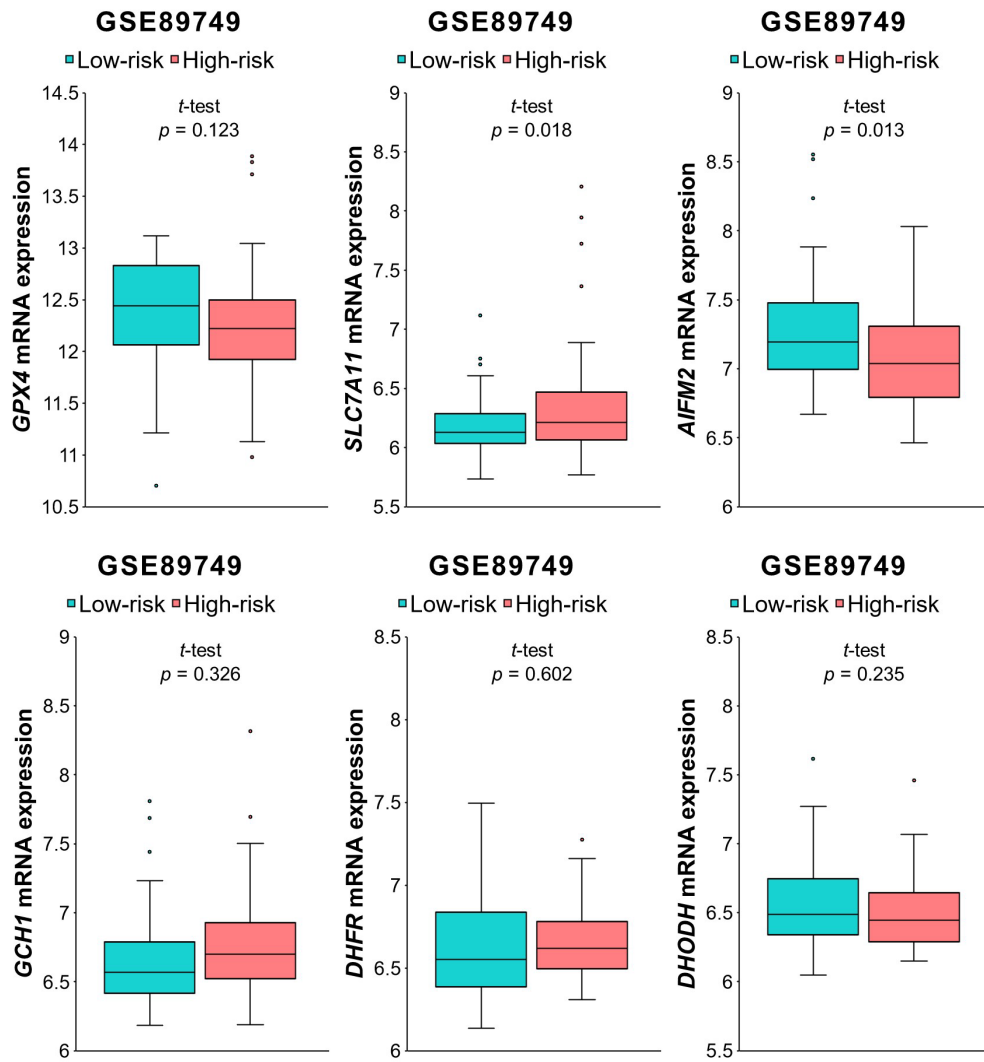
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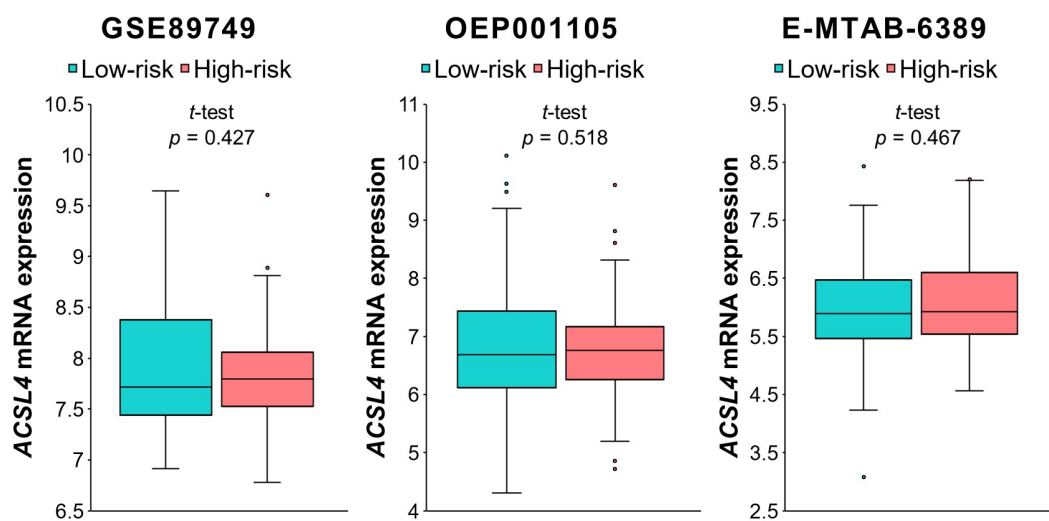
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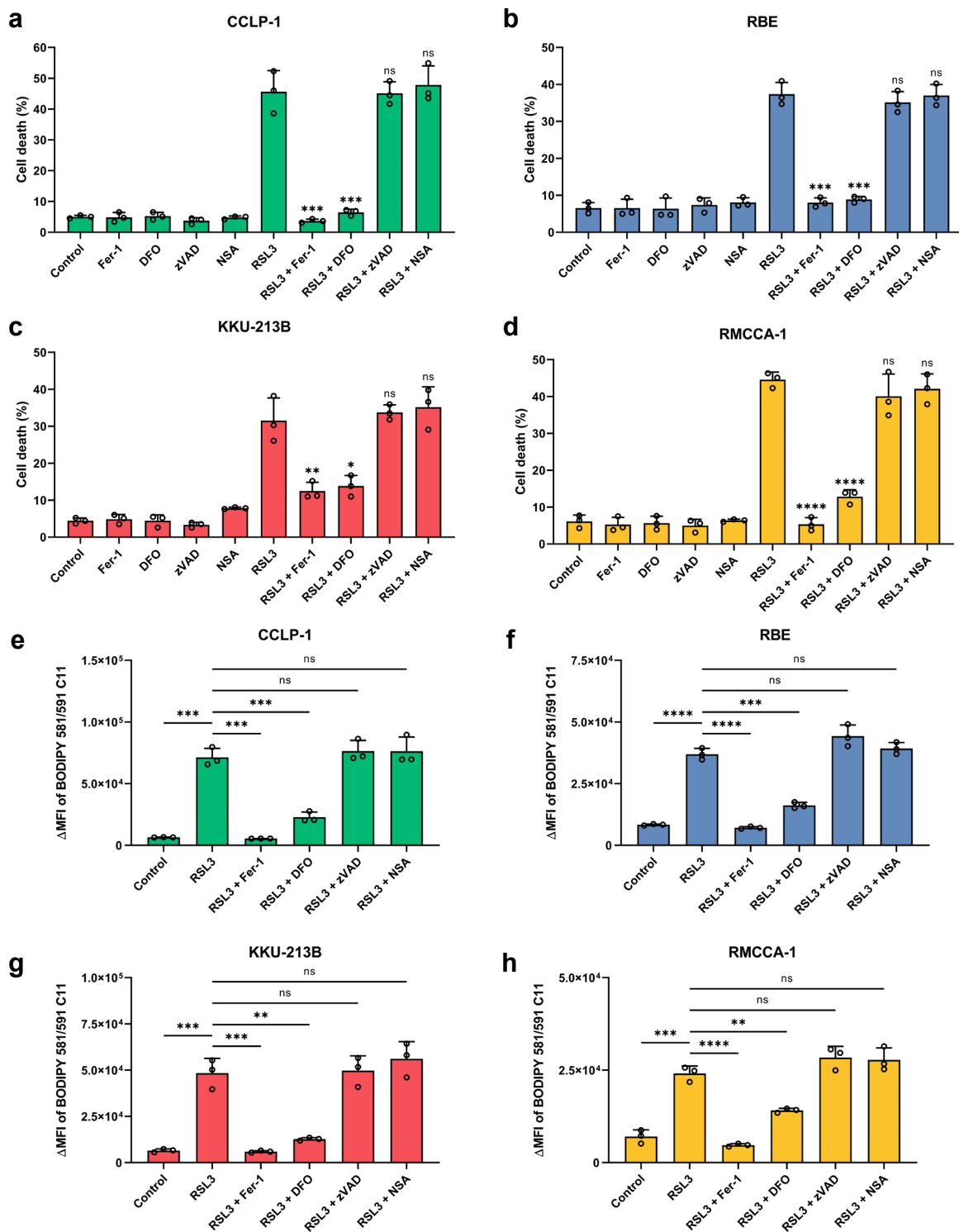
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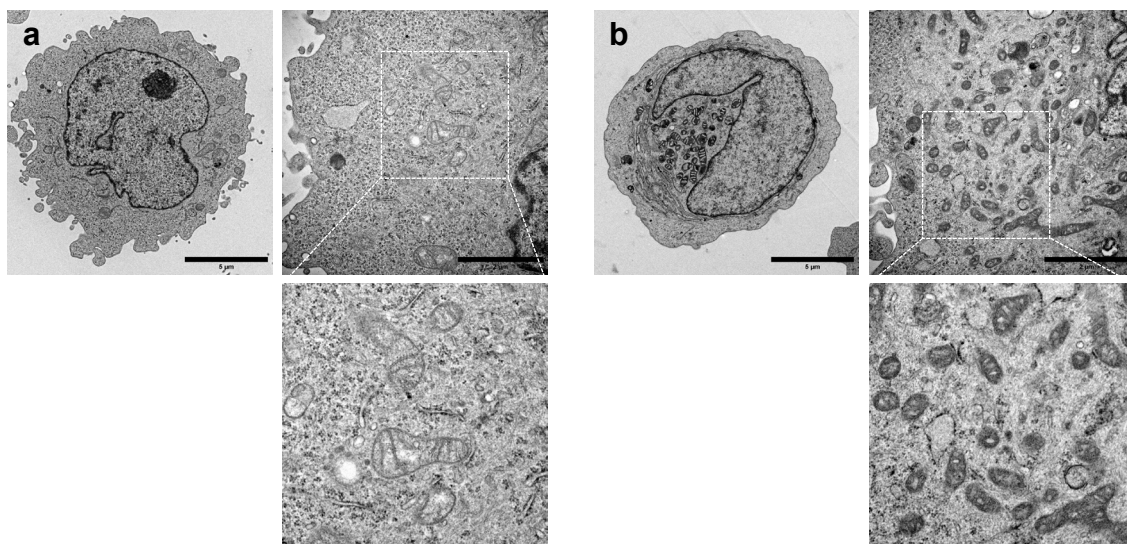
Supplementary Fig. 1 Gene expression in CCA patients. Box plots of ferroptosis inhibitory gene mRNA expression in CCA tumor tissues of high-risk CCA patients and low-risk CCA patients from the GSE89749 cohort (n = 111).



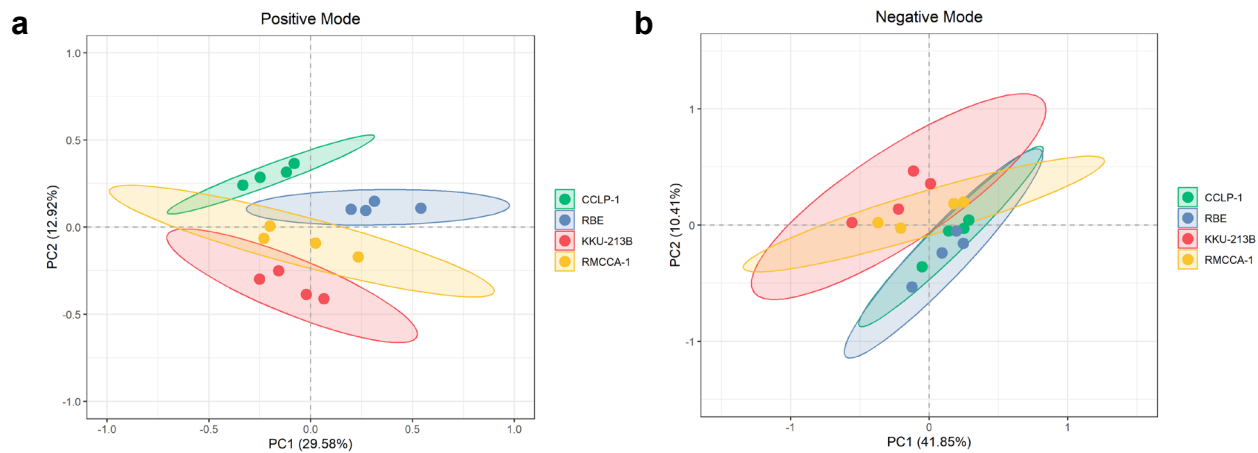
Supplementary Fig. 2 ACSL4 mRNA expression in CCA tumor tissues of high-risk CCA patients and low-risk CCA patients from the GSE89749 (n = 111), OEP001105 (n = 244), and E-MTAB-6389 (n = 75) cohorts.



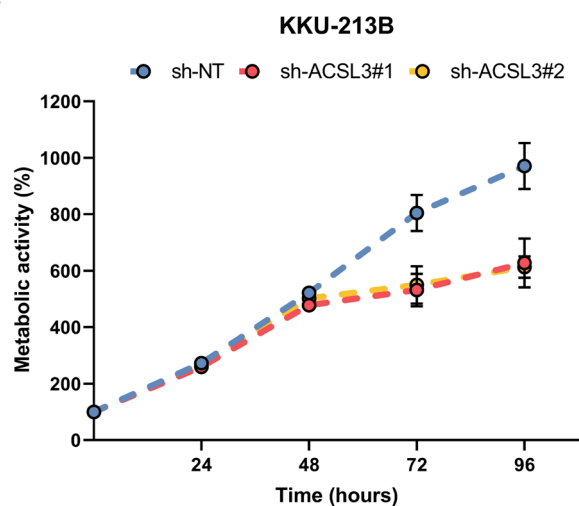
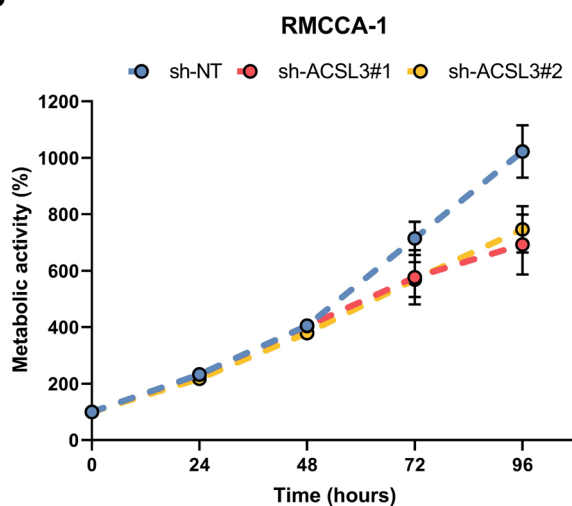
Supplementary Fig. 3 Confirmation of ferroptosis induction in CCA cell lines. (a-d) CCA cell lines were pretreated with 5 μ M Fer-1, 10 μ M DFO, 20 μ M zVAD-fmk, or 1 μ M NSA, then exposed for 48 h to RSL3 (0.05 μ M for CCLP-1, 0.2 μ M for RBE, 4 μ M for KKKU-213B, and 2 μ M for RMCCA-1) and cell death was determined by flow cytometry using Annexin V/PI staining. (e-h) Lipid peroxidation in CCA cell lines pretreated with 5 μ M Fer-1, 10 μ M DFO, 20 μ M zVAD-fmk, or 1 μ M NSA, and exposed to RSL3 (0.05 μ M for CCLP-1, 0.2 μ M for RBE, 4 μ M for KKKU-213B, and 2 μ M for RMCCA-1) for 6 h was by flow cytometry using BODIPYTM 581/591 C11 staining. All experiments were repeated independently three times. The data are shown as mean values $\pm$ S.D. and *p*-values were calculated using two-tailed unpaired Student's *t*-test. ns, not significant; **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.



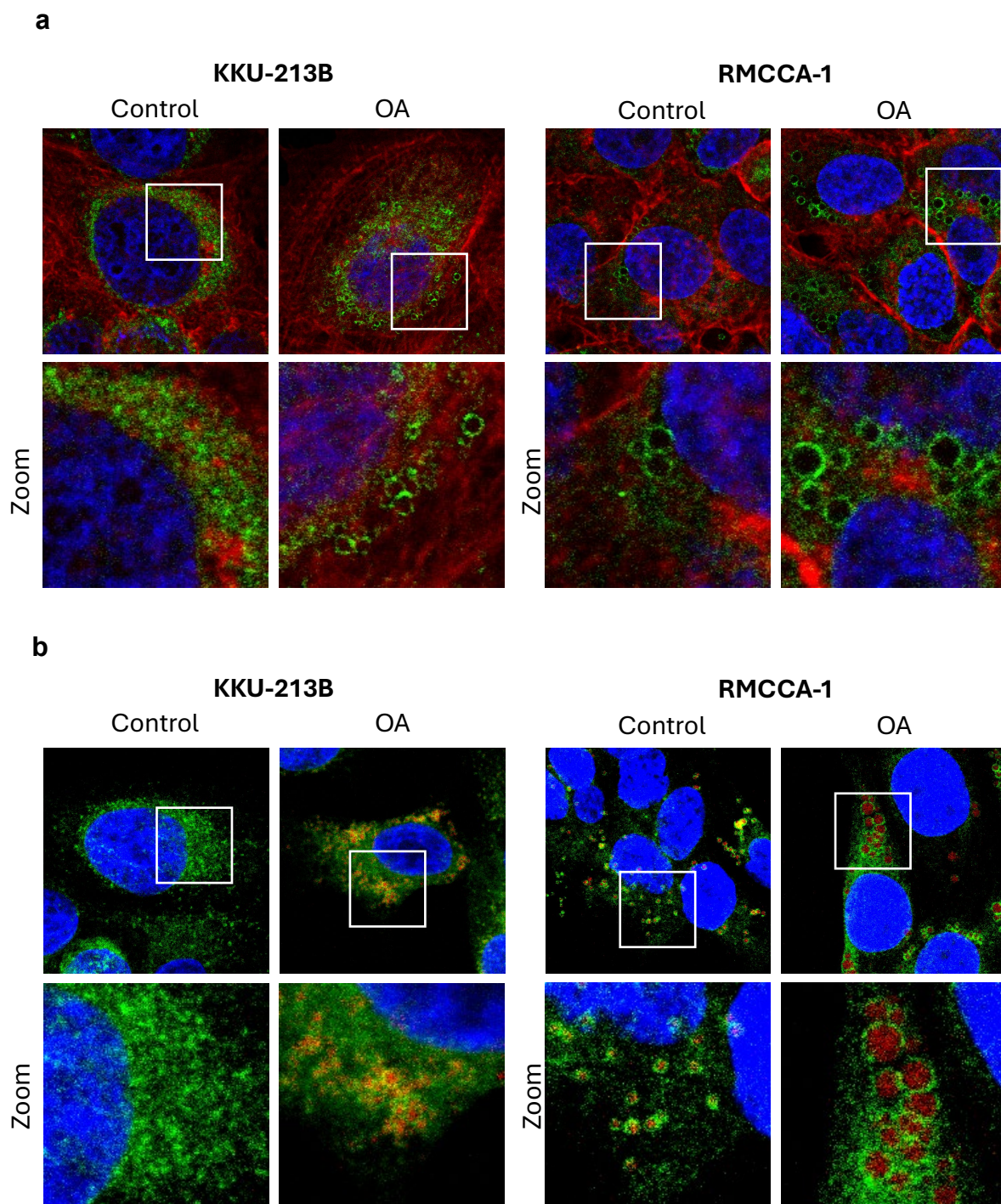
Supplementary Fig. 4 TEM micrographs of CCLP-1 cells treated with vehicle alone (**a**) or 25 nM RSL3 (**b**) for 8 h. Note increased cristae density and dilated ER in (**b**). Scale bars: 5 μ m (overview) and 2 μ m (close-up).



Supplementary Fig. 5 Principal component analysis. **(a)** PCA of lipidomics data obtained for the CCA cell lines in positive mode. **(b)** PCA of lipidomics for the CCA cell lines in negative mode.

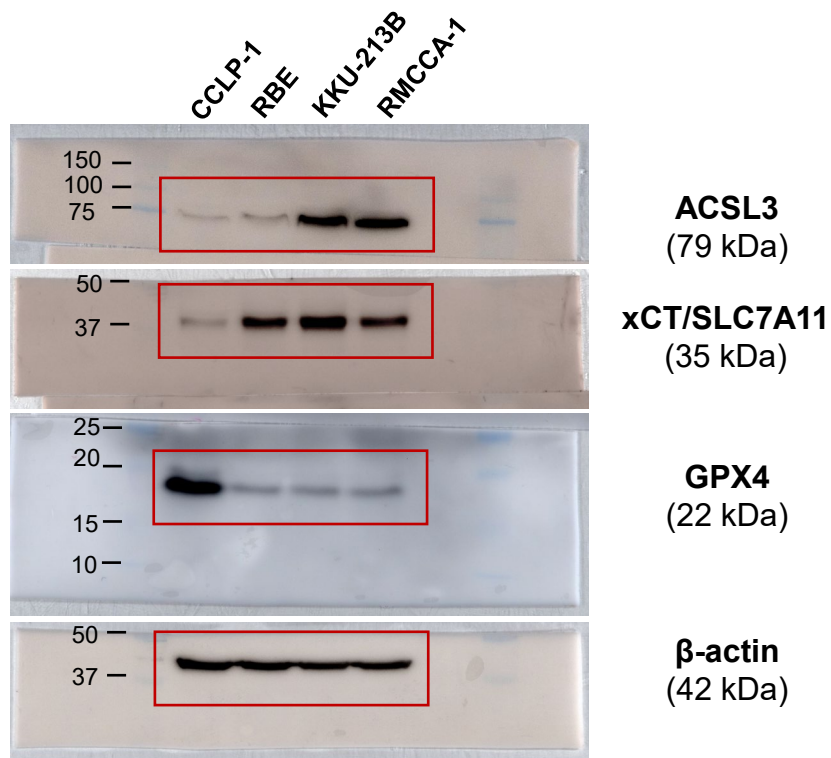
a**b**

Supplementary Fig. 6 ACSL3 silencing compromises cell viability in CCA cells after 48 h of cell culture while no differences are seen up to 48 h. ACSL3-expressing and ACSL3-silenced KKU-213B cells (**a**) and RMCCA-1 cells (**b**) were cultured for 24, 48, 72, and 96 h and cell viability was determined using the MTT assay. All experiments were repeated independently three times. The data are shown as mean values $\pm$ S.D.

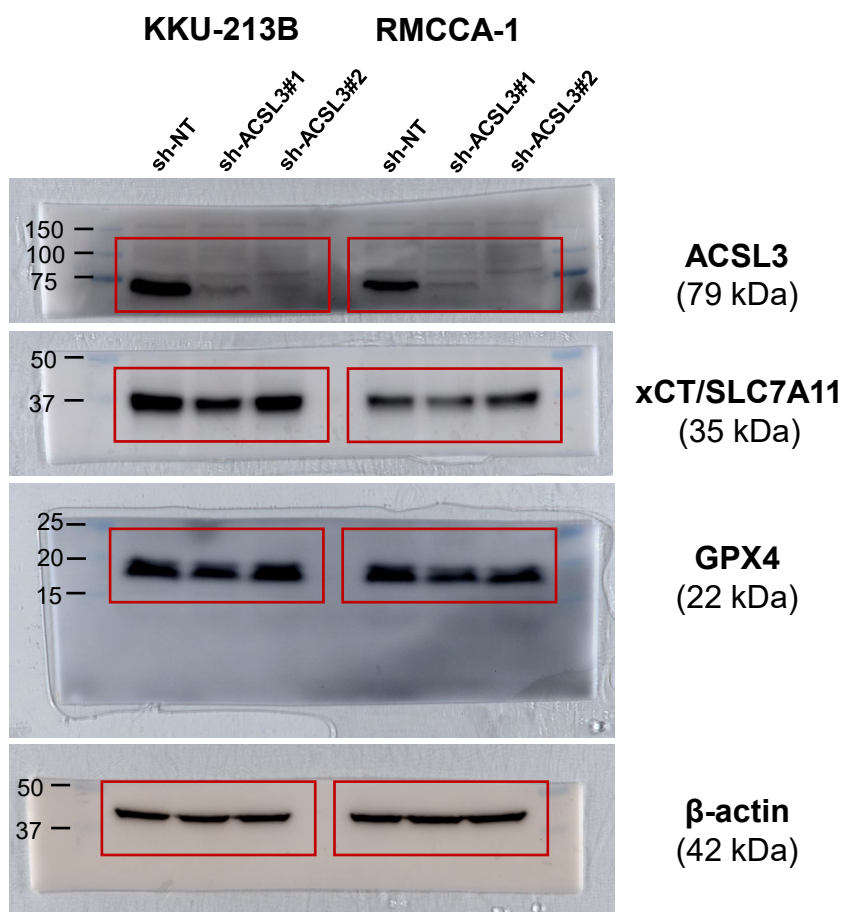


Supplementary Fig. 7 ACSL3 is localized to lipid droplets. The images shown here are a duplication of the images in Figure 7a, b with zoom-in images added showing the localization of ACSL3. **(a)** ACSL3 localization in KKU-213B and RMCCA-1 cells after exposure to vehicle control or OA (100 μ M) for 24 h. Cells were stained with antibodies specific for ACSL3 (green) and counterstained with Hoechst 33342 (blue) (cell nuclei) and phalloidin-iFluor 647 (red). **(b)** Co-localization studies of ACSL3 and lipid droplets in KKU-213B and RMCCA-1 cells after exposure to vehicle control or OA (100 μ M) for 24 h. Cells were stained with antibodies against ACSL3 (green) and cell nuclei were counterstained with Hoechst 33342 (blue), while lipid droplets were stained using Nile Red (red).

a



b



Supplementary Fig. 8 Original data for Western blot. The images shown here are uncropped and unprocessed Western blots corresponding to Figure 2b (a) and Figure 4a, b (b).