p38 mediated ACSL4 phosphorylation drives stress-induced esophageal squamous cell carcinoma growth through Src myristoylation

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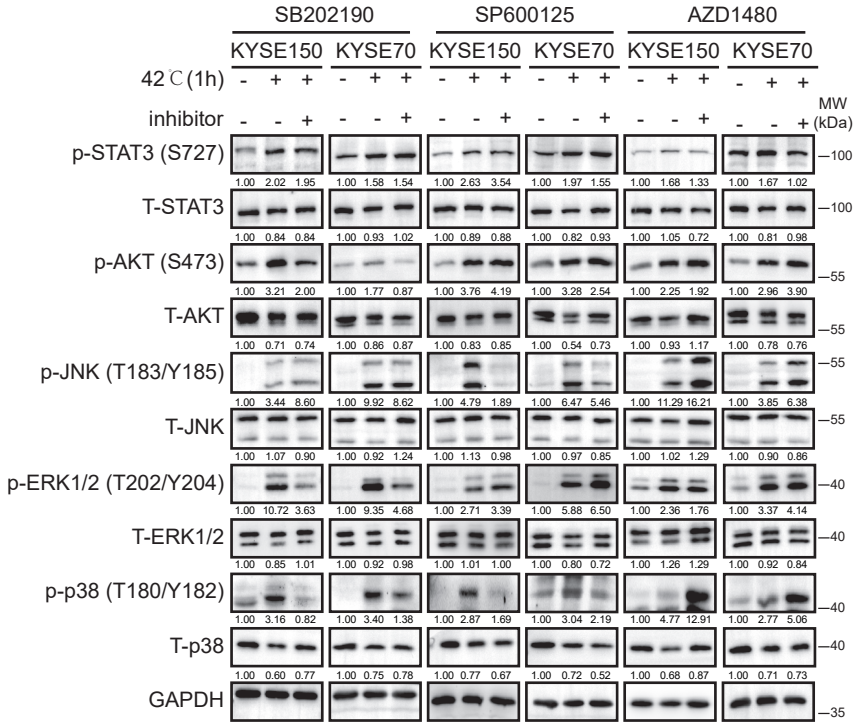
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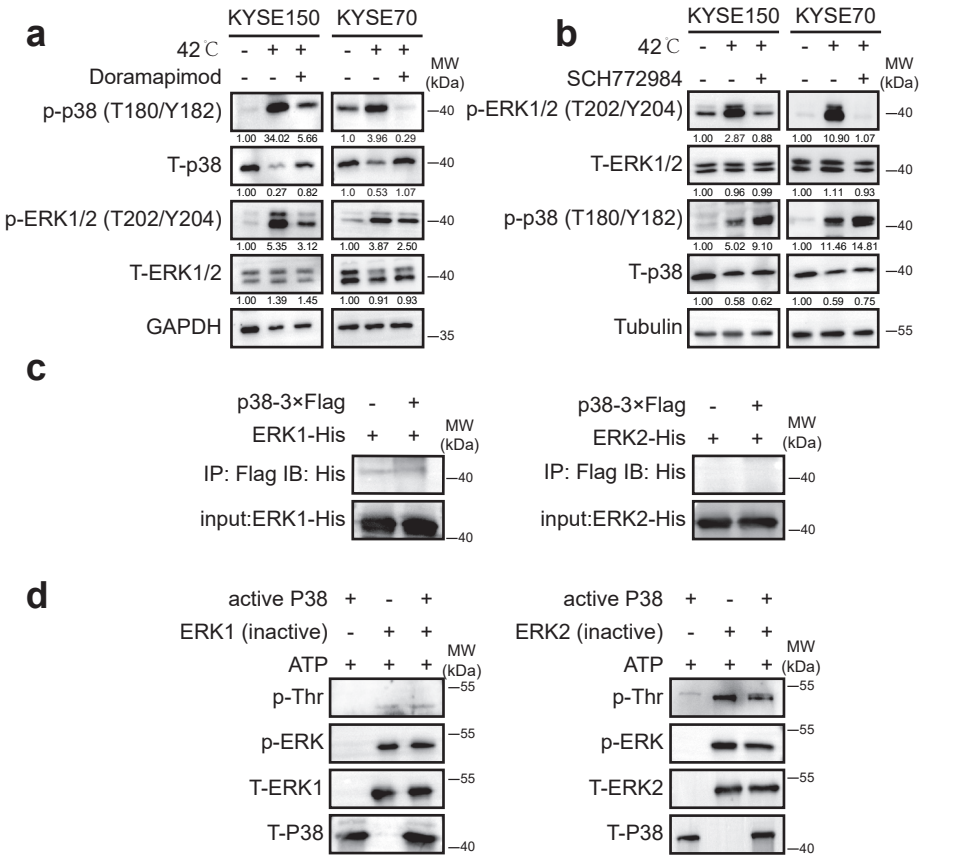
Supplementary Figure 1



Supplementary Fig. 1: p-ERK1/2 (T202/Y204) is down-regulated after treating with p38 inhibitor.

Cells were treated with p38 inhibitor (20 μM SB202190), JNK inhibitor (20 μM SP600125) or JAK2 inhibitor (50 μM AZD1480) for 2 h following by heat stress for 1 h and processed for Western blot. Source data are provided as a Source Data file.

Supplementary Figure 2



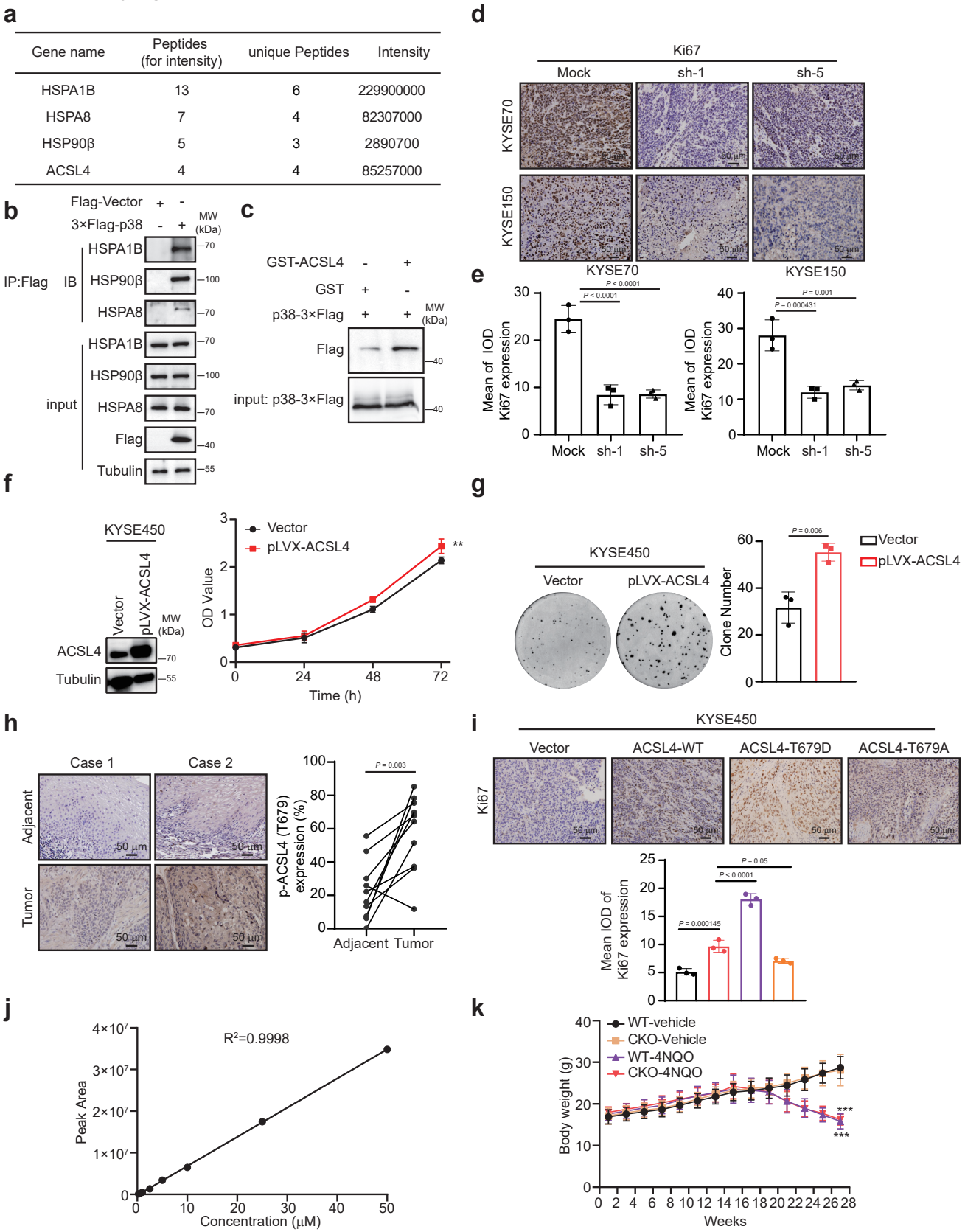
Supplementary Fig. 2: p38 shows no affinity with ERK1/2 and directly phosphorylation ERK1/2.

(a) Western blot analysis was performed on KYSE 70 and KYSE150 cells induced with 20 μM Doramapimod for 4 h followed by heat stress for 1 h. (b) Western blot analysis was performed on KYSE 70 and KYSE150 cells induced with SCH772984 for 2 h followed by heat stress for 1 h. (c) p38 has no affinity with ERK1/2. Beads coated with p38-Flag fusion were incubated with bacterially expressed ERK1-his (left) or ERK2-his (right) fusion protein. The Western blot analysis was carried out using the indicated antibody. (d) Active p38 was incubated with recombinant ERK1 (left) or ERK2 (right) protein in kinase reaction buffer for 30 min at 30℃ and stopped by 6 × SDS loading buffer. Phosphorylation signals were detected by Western blot. Source data are provided as a Source Data file.

Supplementary Figure 3

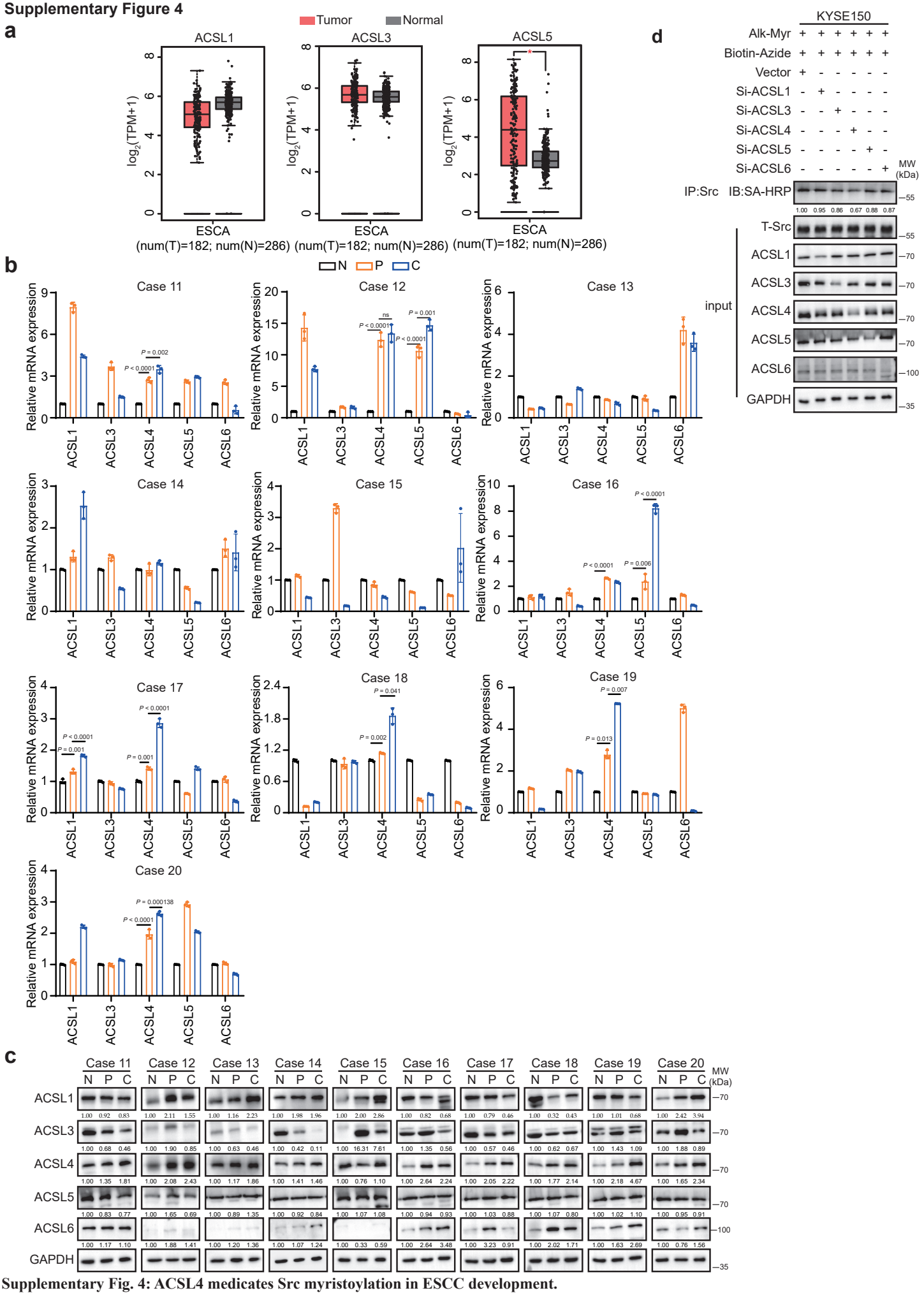
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Gene name	Peptides (for intensity)	unique Peptides	Intensity
HSPA1B	13	6	229900000
HSPA8	7	4	82307000
HSP90β	5	3	2890700
ACSL4	4	4	85257000



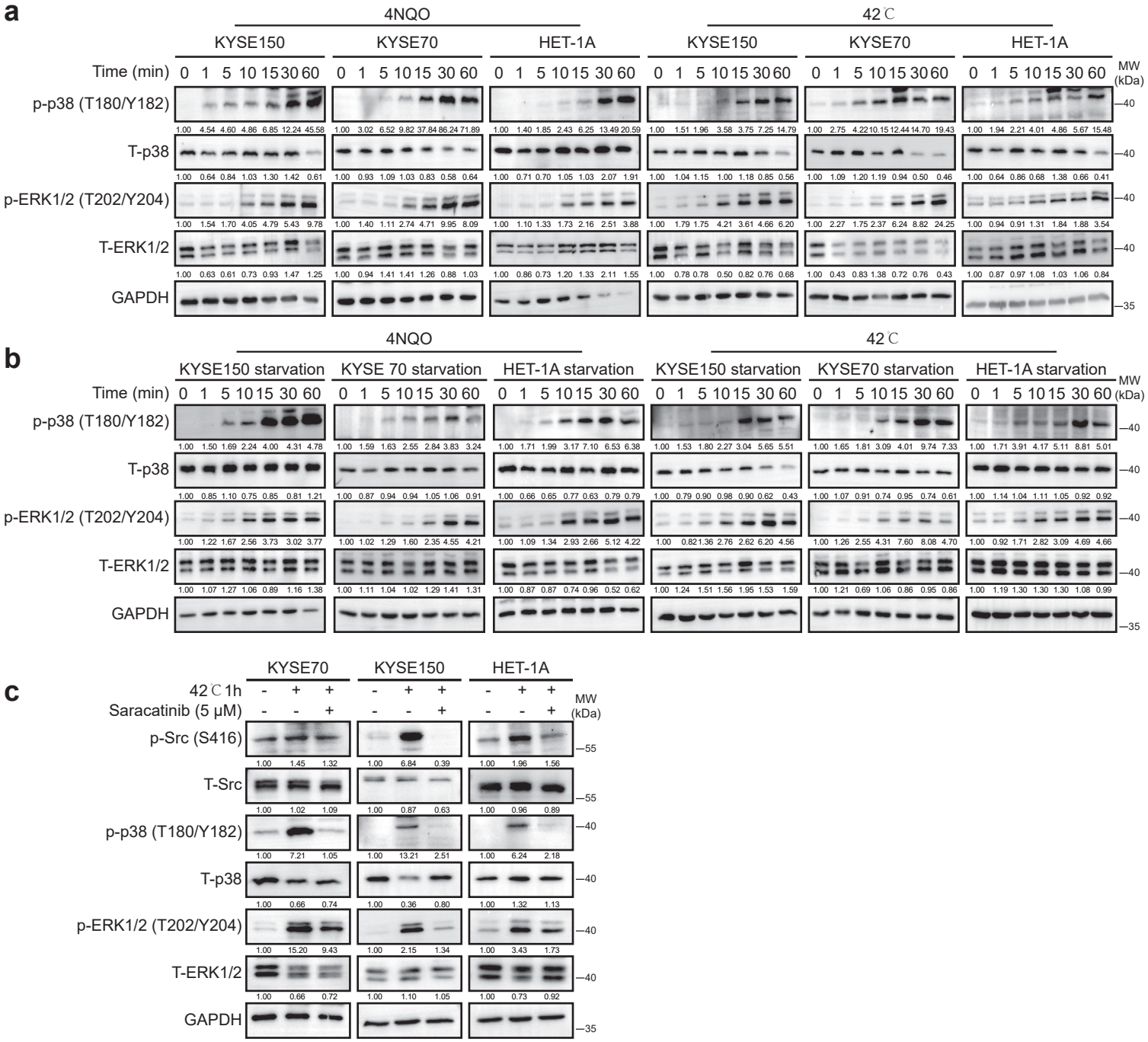
Supplementary Fig. 3: ACSL4 promotes cell proliferation in ESCC.
(a) The potential interaction proteins with p38 were shown in the table according to the intensity and the number of identified peptides (>2). (b) Beads coated with p38-3 × Flag fusion protein were incubated with KYSE 150 cell lysate. Endogenous HSPA1A, HSPA8, and HSP90β were detected by Western blot. (c) Interaction between p38 and ACSL4. GST-ACSL4 fusion proteins were prepared from escherichia coli and p38-flag protein was extracted in HEK293F cells. Beads coated with GST-ACSL4 or GST protein were incubated with flag-p38. Western blot analysis was carried out using p38 antibody. (d, e) Tumor samples were stained with Ki67 in ACSL4 knock down CDX models. (d) Representative pictures of CDX models. (e) The results of Ki67 staining in different groups (n = 3 samples detected from 3 independent experiments, mean ± SD). *P* values were determined by One-way ANOVA. (f) KYSE450 cells with ACSL4 overexpression were established and (left) Western blot was performed to determine the protein levels of ACSL4. Cell proliferation was measured by MTT assay on cells (right), and (g) plate colony formation assay was measured. (f) n = 5 biological replicate, mean ± SD. Statistical significance was conducted by two-sided unpaired Student's t-test, ** $P = 0.004$. (g) n = 3 biological replicates, mean ± SD. *P* value was determined by two-sided unpaired Student's t-test. (h) p-ACSL4 at T679 IHC staining in ESCC tissues and paired adjacent tissues. Statistical significance was conducted by two-sided paired Student's t-test. (i) Tumor samples were stained with Ki67 in ACSL4 overexpression CDX models (n=3 biological replicates, mean ± SD). Statistical significance was conducted by One-way ANOVA. (j) C14:0 CoA was quantified by LC-ESI-MS/MS, and C15:0 CoA was used as internal standard. (k) The body weights of mice were recorded in different groups. Statistical significance was conducted by One-way ANOVA. WT-vehicle vs WT-4NQO, *** $P = 0.00019$; WT-vehicle vs CKO-4NQO, *** $P = 0.000338$; WT-4NQO vs CKO-4NQO, ns $P = 0.929$. Source data are provided as a Source Data file.

Supplementary Figure 4



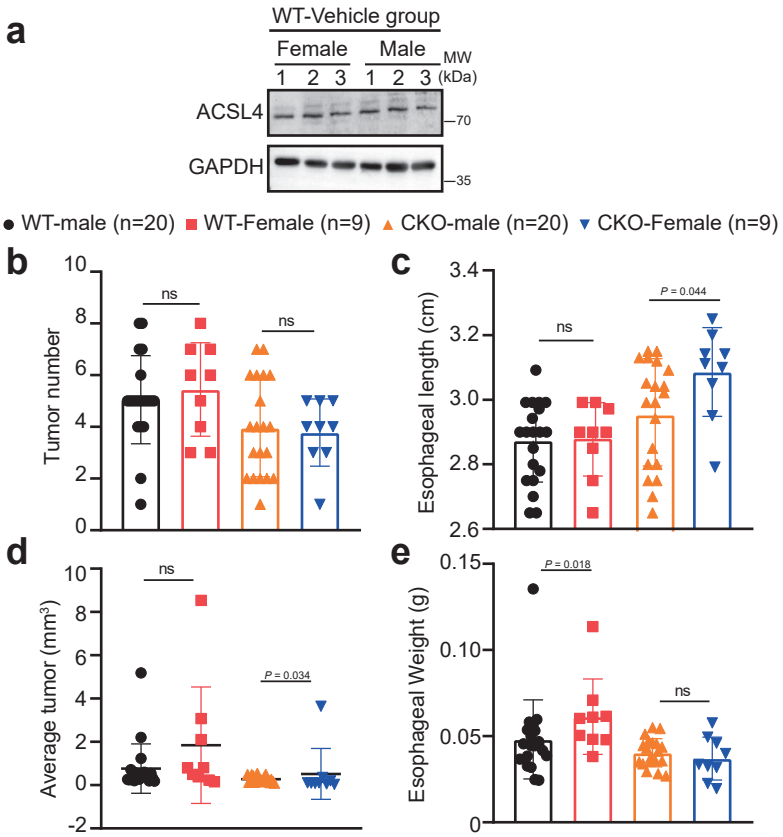
Supplementary Fig. 4: ACSL4 medicates Src myristoylation in ESCC development.
(a) Plots showing the ACSL1, ACSL3 and ACSL5 transcript expression levels in two paired adjacent-tumor ESCC datasets from GPEIA database. Characteristics of the boxplot in a (median of log_2TPM+1): ACSL1 in normal tissue (5.69), tumor tissue (5.08); ACSL3 in normal tissue (5.58), tumor tissue (5.68); ACSL5 in normal tissue (2.73), tumor tissue (4.39) The database lacks information on ACSL6 expression. The database lacks information on ACSL6 expression. (b) The mRNA expression levels of ACSL family members were determined in another 10 cases of patients with ESCC (n=3 samples detected from 3 independent experiments, mean ± SD). Statistical analysis and exact *P* values were shown in Source data. (c) The protein levels of ACSL family members were determined in another 10 cases of patients with ESCC. (d) Src myristoylation was detected in cells with ACSL1 or ACSL3, ACSL4, ACSL5, ACSL6 knock down respectively. Source data are provided as a Source Data file.

Supplementary Figure 5



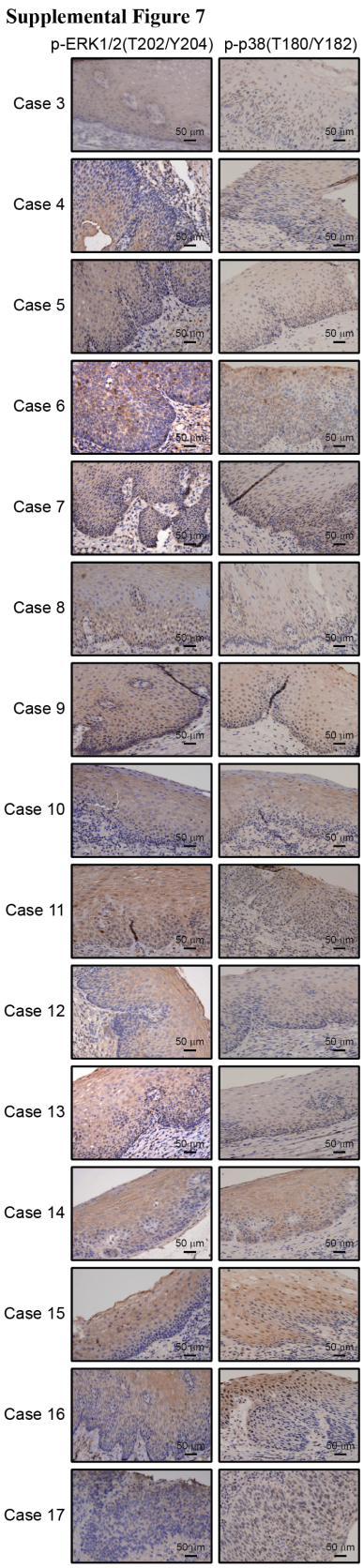
Supplementary Fig. 5: The persistence of heat stress or 4NQO exposure induces activation of p38 and ERK1/2.
(a) The KYSE150, KYSE70 and HET-1A cells were subjected to 4NQO or heat stress to examine the change of p-p38 (T180/Y182) and p-ERK1/2 (T202/Y204). (b) The KYSE150, KYSE70 and HET-1A cells were subjected to 4NQO or heat stress to examine the change of p-p38 (T180/Y182) and p-ERK1/2 (T202/Y204) with starvation conditions. (c) A crosstalk between p38 and Src. Cells were treated with Src inhibitor Saracatinib (20 μM) for 2 h following by heat stress for 1 h and processed for Western blot analysis. Source data are provided as a Source Data file.

Supplementary Figure 6



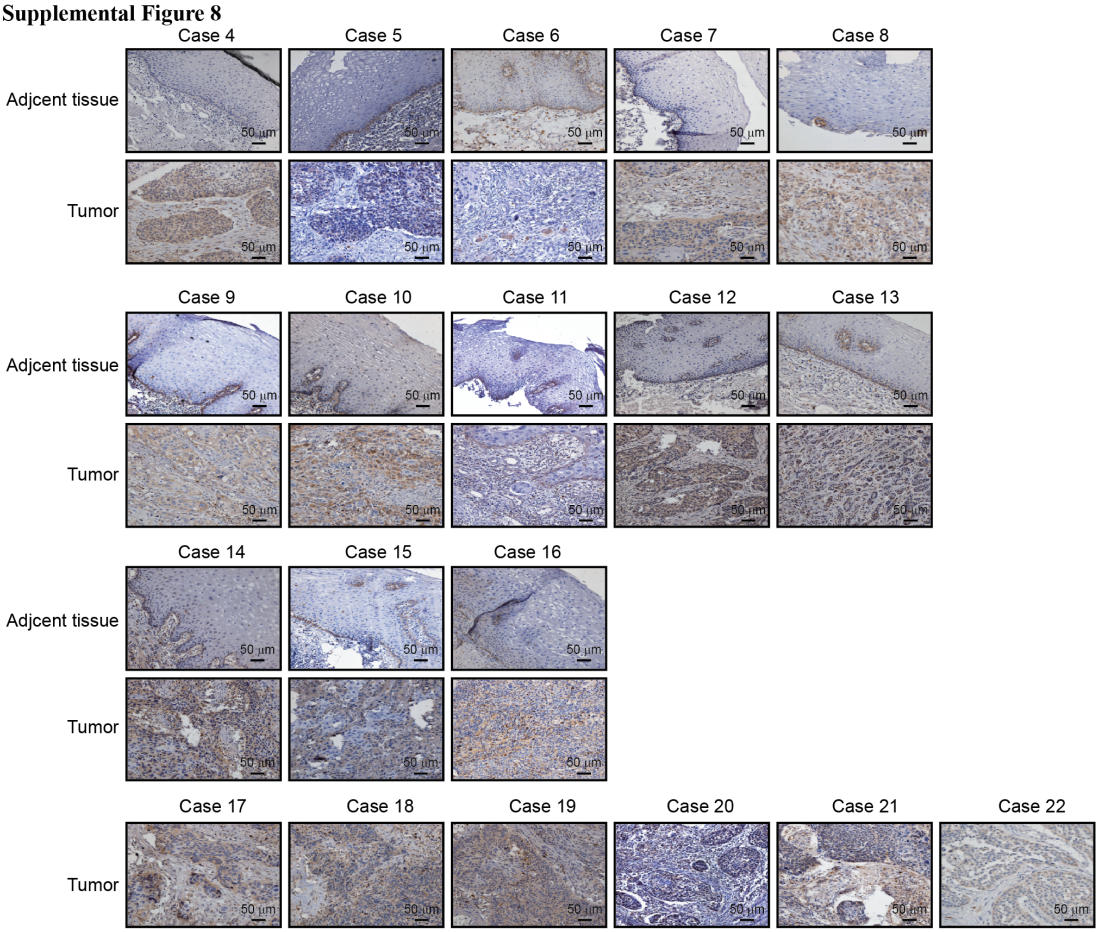
Supplementary Fig. 6: Differences of ACSL4 protein level and ESCC formation between genders.

(a) Protein levels of ACSL4 in female and male of mice. Comparison 4NQO-induced ESCC in situ between female and male in tumor number (b) The length of the esophageal (c), average tumor volumes (d), esophageal weight (e). The mean esophageal weight is 0.048 g in WT-male group, and 0.061 g in WT-female group. Data were presented as means ± SD (ns denotes not significant). Statistical analysis and exact *P* values were provided as a Source data file. Source data are provided as a Source Data file.



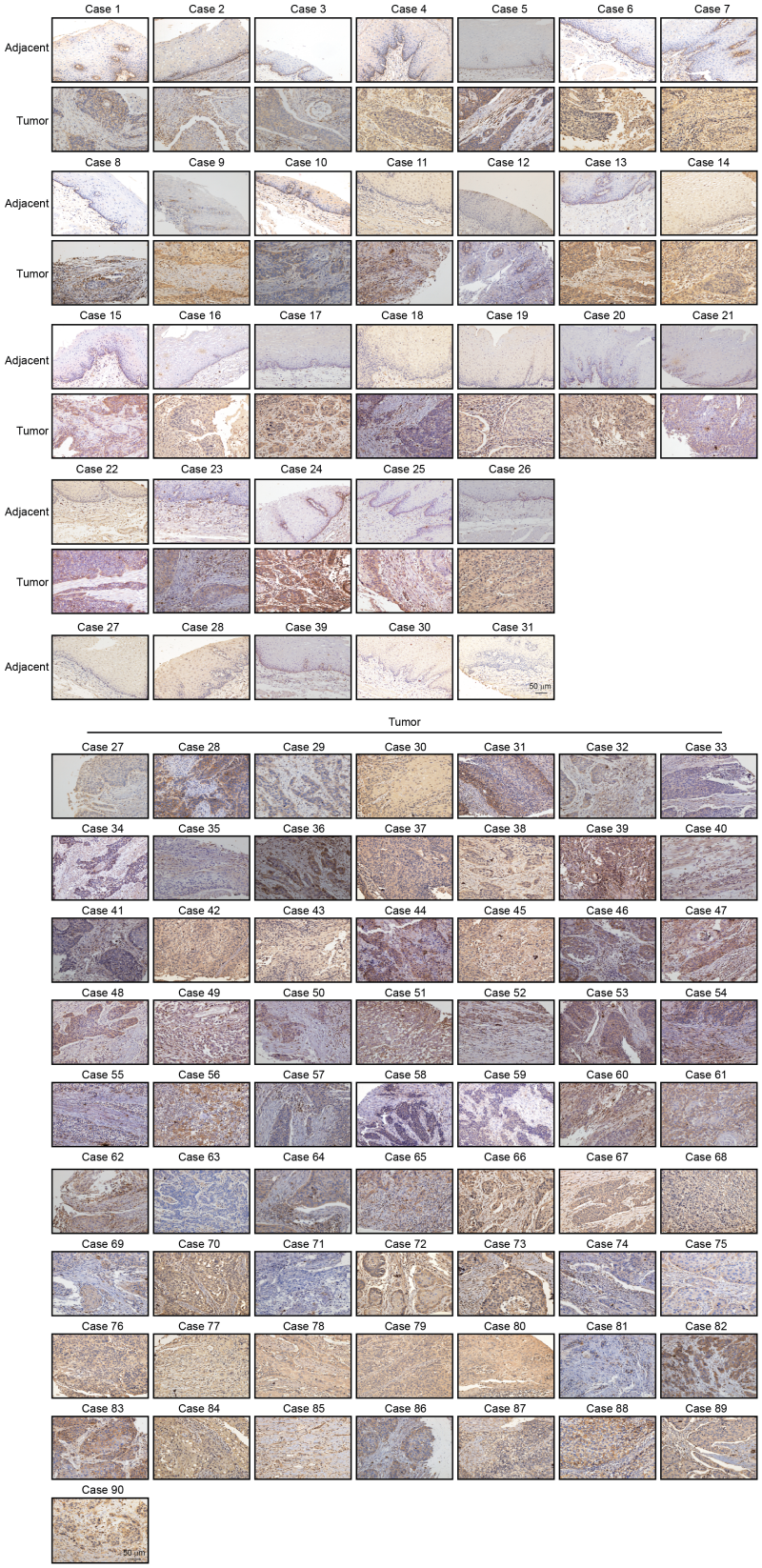
Supplemental Fig. 7: Representative p-p38 (T180/Y182) and p-ERK1/2 (T202/Y204) IHC staining on human precancerous lesions tissue. Expect for 2 cases in text, the other 15 cases were shown.

Supplemental Figure 8



Supplemental Fig. 8: IHC staining of human ESCC tissues and adjacent tissues elucidated by ACSL4 antibody. Expect for 3 cases in text, the other 19 cases of tumor and 13 cases of adjacent tissues were shown.

Supplemental Figure 9



Supplemental Fig. 9: Immunohistochemical staining of ESCC tissues and adjacent tissues in human tissue microarray with ACSL4 antibody. Total 90 cases of tumor, and 31 cases of adjacent tissues.

Supplementary Table 1: The primer sequences of siRNA.

ACSL1	Forward 5'-GCCAAAUGUAUUUCAGGGC-3'
	Reverse 5'-GCCCUGAAAUACAUUUGGC-3'
ACSL3	Forward 5'-AGGUUAUUCUUGGACAGUA-3'
	Reverse 5'-UACUGUCCAAGAAUAACCU-3'
ACSL4	Forward 5'-GCAGAGAUAUCUUGCUUUA-3'
	Reverse 5'-UAAAGCAAGAUAUCUCUGC-3'
ACSL5	Forward 5'-CCAAAUACUUUCGGACCCA-3'
	Reverse 5'-UGGGUCCGAAAGUAUUUGG-3'
ACSL6	Forward 5'-GCAGGUUAAAGCCAUUCAC-3'
	Reverse 5'-GUGAAUGGCUUUAACCUGC-3'

Supplementary Table 2: The primer sequences of shRNA and sgRNA.

ACSL4 sh#1	5’-GCAGTAGTTCATGGGCTAAAT-3’
ACSL4 sh#5	5’-GCAGAGTGAATAACTTTGGAA-3’
ACSL4 sg#3	5’-TTAGGATTTCCCTGGTCCCA-3’

Supplementary Table 3: The primer sequences of qRT-PCR.

ACSL1	Forward 5'-CTTATGGGCTTCGGAGCTTTT-3'
	Reverse 5'-CAAGTAGTGCGGATCTTCGTG-3'
ACSL3	Forward 5'-ATGGAAAACCAACCTCATAGCAA-3'
	Reverse 5'-GCCATCCCAGTTATACCAGCAA-3'
ACSL4	Forward 5'-CATCCCTGGAGCAGATACTCT-3'
	Reverse 5'-TCACTTAGGATTTCCCTGGTCC-3'
ACSL5	Forward 5'-CTCAACCCGTCTTACCTCTTCT-3'
	Reverse 5'-GCAGCAACTTGTTAGGTCATTG-3'
ACSL6	Forward 5'-CGCTACATCATCAATACAGCGG-3'
	Reverse 5'-GCATGGACTTAATGACCACCC-3'
GAPDH	Forward 5'-CCATGTTCGTCATGGGTGTG-3'
	Reverse 5'-GGTGCTAAGCAGTTGGTGGTG-3'