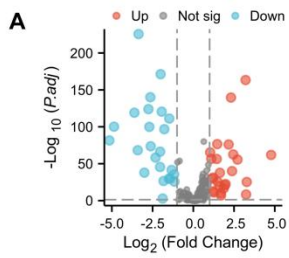


Figure S1: HSPA6 is a crucial suppressor protein associated with ferroptosis in TNBC.

A) The volcano map shows that ferroptosis-related genes are expressed differently in BRCA and non-cancer samples. A red dot indicates a gene with a high expression level, a blue dot indicates a gene with a low expression level, while a grey dot represented genes with no significance. **B)** The heat maps were generated to visualize key ferroptosis-related DEGs. **C)** A univariate Cox regression analysis was performed on 11 prognostic ferroptosis-related genes associated with overall survival. **D)** The optimal parameter (λ) was determined by using the minimum criteria set by the vertical lines in the LASSO Cox regression model. **E)** The LASSO coefficient of the LRGs signature. **F)** The gene set was optimized using regression coefficient calculations. **G)** Overall survival of BRCA patients based on ferroptosis-related score group by Kaplan–Meier analysis from the TCGA. **H)** Pie charts showing the Chi-squared test of clinicopathologic factors for ferroptosis-related score group in BRCA tumor samples from the TCGA. **I)** A comprehensive nomogram for predicting the survival probabilities of BRCA patients at 1-year, 3-year, and 5-year intervals based on ferroptosis score and clinicopathologic. **J)** Overall survival of BRCA patients based on ferroptosis-related score group by Kaplan–Meier analysis from the GSE58812 and GSE65216. **K)** Venn diagram exhibiting DEGs in ferroptosis score group in the TCGA, GSE58812, and GSE65216. **L)** Analysis of HSPA6 expression in samples with different grade and different prognosis from GSE31519. **M)** HSPA6 staining of unpaired BRCA from Human Protein Atlas (HPA) database. (Data are presented as mean \pm standard of error (SD) of three independent experiments. Statistical significance was determined using ANOVA with post-hoc Tukey multiple comparison, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns.: not significant.)



C

Gene	Log2(Fold Change)	Adjusted P-value
PTG2	1.088	0.003
High	5.0	0.002 (0.412 - 0.846)
Low	5.0	0.002 (0.412 - 0.846)
TF	1.088	0.004
High	5.0	0.015 (0.443 - 0.803)
Low	5.0	0.015 (0.443 - 0.803)
TRG	1.088	0.011
High	5.0	0.026 (0.471 - 0.906)
Low	5.0	0.026 (0.471 - 0.906)
ADH4	1.088	0.012
High	5.0	1.529 (0.188 - 1.107)
Low	5.0	1.529 (0.188 - 1.107)
MOG	1.088	0.027
High	5.0	1.451 (0.044 - 0.216)
Low	5.0	1.451 (0.044 - 0.216)
PRK2	1.088	0.030
High	5.0	0.730 (0.554 - 0.871)
Low	5.0	0.730 (0.554 - 0.871)
DDI	1.088	0.034
High	5.0	0.707 (0.505 - 0.875)
Low	5.0	0.707 (0.505 - 0.875)
LTF	1.088	0.04
High	5.0	0.719 (0.514 - 0.907)
Low	5.0	0.719 (0.514 - 0.907)
KP2A	1.088	0.043
High	5.0	1.464 (0.011 - 1.846)
Low	5.0	1.464 (0.011 - 1.846)
BCD1	1.088	0.043
High	5.0	0.719 (0.514 - 0.907)
Low	5.0	0.719 (0.514 - 0.907)

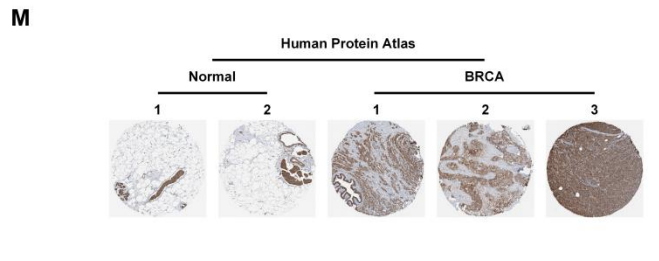
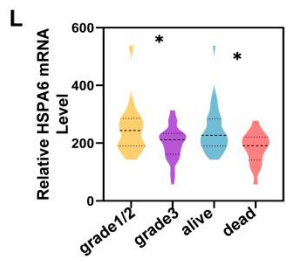
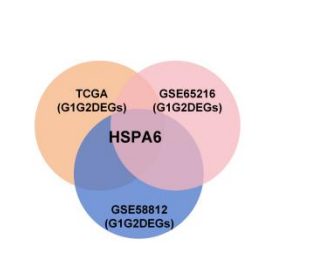
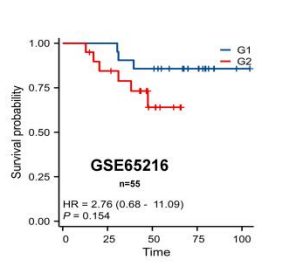
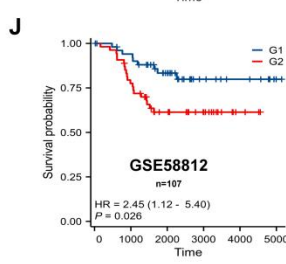
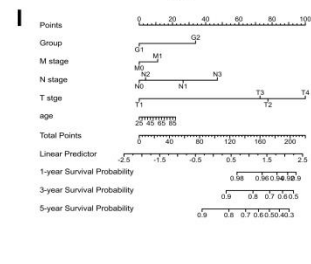
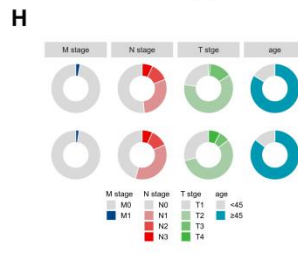
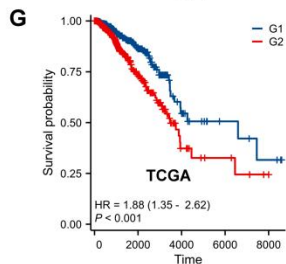
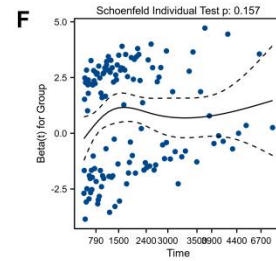
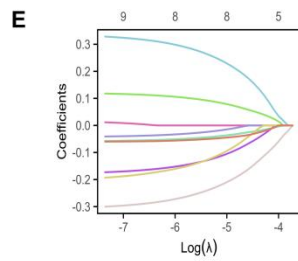
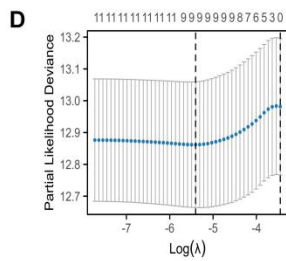
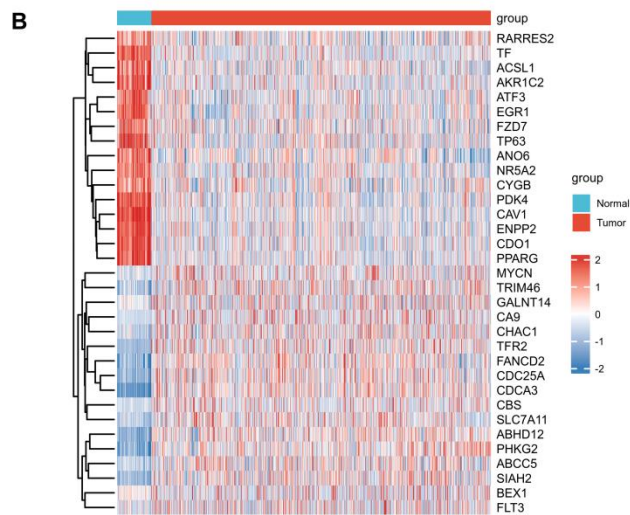


Figure S2: HSPA6 suppressed growth and metastasis of TNBC in vitro and in vivo.

A) Western blot was used to validate the knockdown of HSPA6 in TNBC cells. **B)** Representative images of plate colony formation assays after interfering of HSPA6 in MDA-MB-231, BT549 cells and the corresponding statistical graphs (n = 3). **C)** Representative images of transwell migration assays after the downexpression of HSPA6 in TNBC cells and the corresponding statistical graphs (n = 3). **D)** The expression levels of EMT-related biomarkers, including E-cadherin, N-cadherin, and Vimentin in MDA-MB-231 cells detected by Western blot. **E)** The viability of HSPA6-downexpressing and control cells determined by CCK-8 assays. **F)** Wound healing assay demonstrated that HSPA6 inhibits TNBC cell migration, (n=3). **G)** Bioluminescent imaging of metastasis for xenograft mice after tail vein injection of cells. **H)** The viability of TNBC cells with HSPA6 knockdown was determined by EdU assay. (Data are presented as mean \pm standard of error (SD) of three independent experiments. Statistical significance was determined using ANOVA with post-hoc Tukey multiple comparison, * P < 0.05, ** P < 0.01, *** P < 0.001, ns.: not significant.)

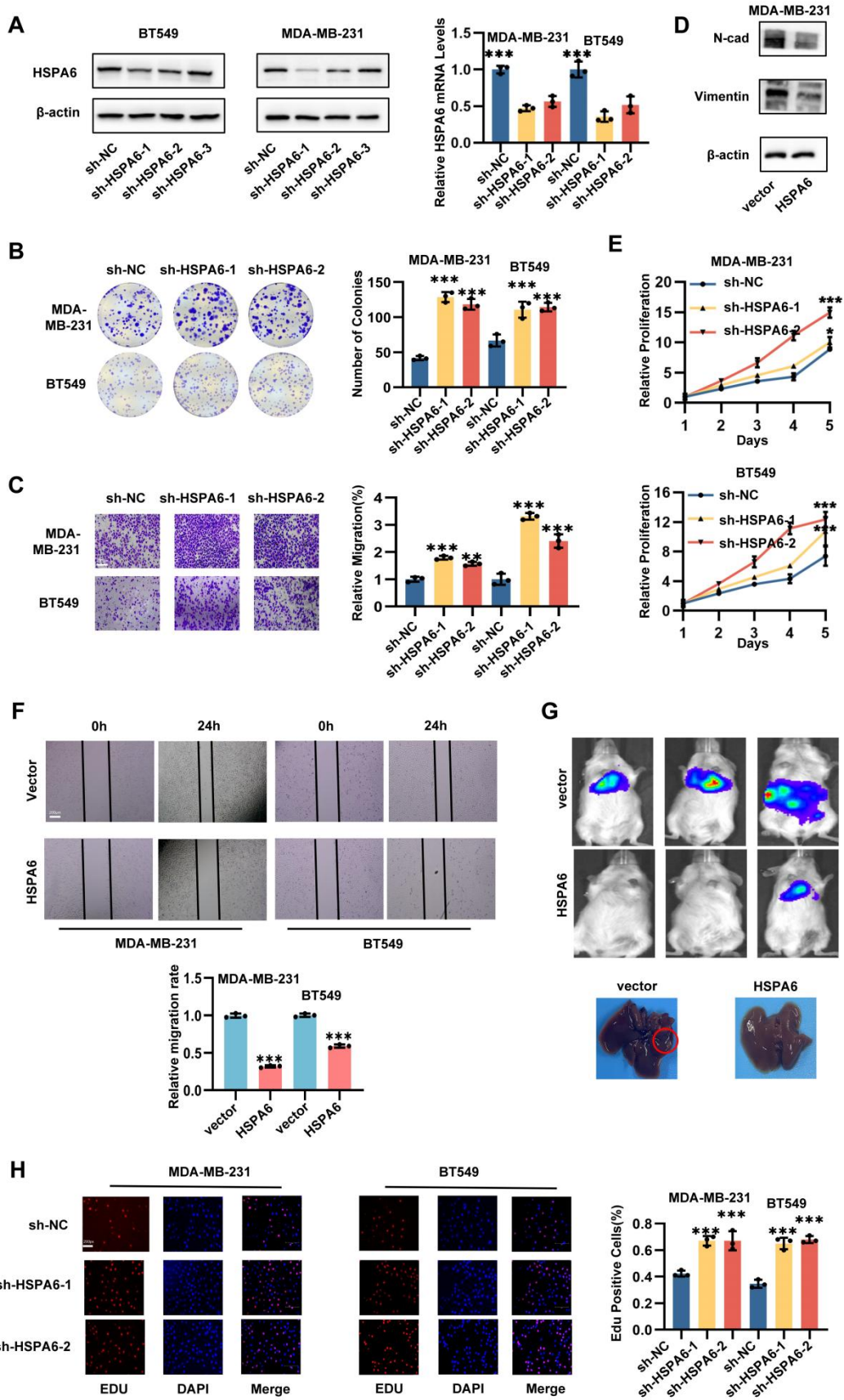


Figure S3: HSPA6 induces ferroptosis of TNBC in vitro and in vivo.

A) The viability of TNBC cells with different drug level was determined by CCK-8 assays (n = 5). **B-C)** The mRNA (B) and protein (C) expression levels of ferroptosis-related biomarker. **D)** Intracellular GSSG/GSH level of TNBC cells were measured by corresponding assay kits. **E)** Colony formation analysis of HSPA6-overexpressing TNBC cells and control cells treated with Fer-1 or Erastin. **F)** The expression of HSPA6, Ki67, ACSL4, FTH1, E-cadherin, and N-cadherin in xenografts of each group were assessed by immunochemistry. Scale bar, 100 μ m. **G)** The expression of ACSL4 and FTH1 in TNBC tissues with altered HSPA6 expression levels. Scale bars, 100 μ m. **H)** Immunofluorescence staining for HSPA6 (green), ACSL4 (red), FTH1 (orange) and DAPI (nucleus, blue) in TNBC tissues. Scale bar, 100 μ m. (Data are presented as mean \pm standard of error (SD) of three independent experiments. Statistical significance was determined using ANOVA with post-hoc Tukey multiple comparison, * P < 0.05, ** P < 0.01, *** P < 0.001, ns.: not significant.)

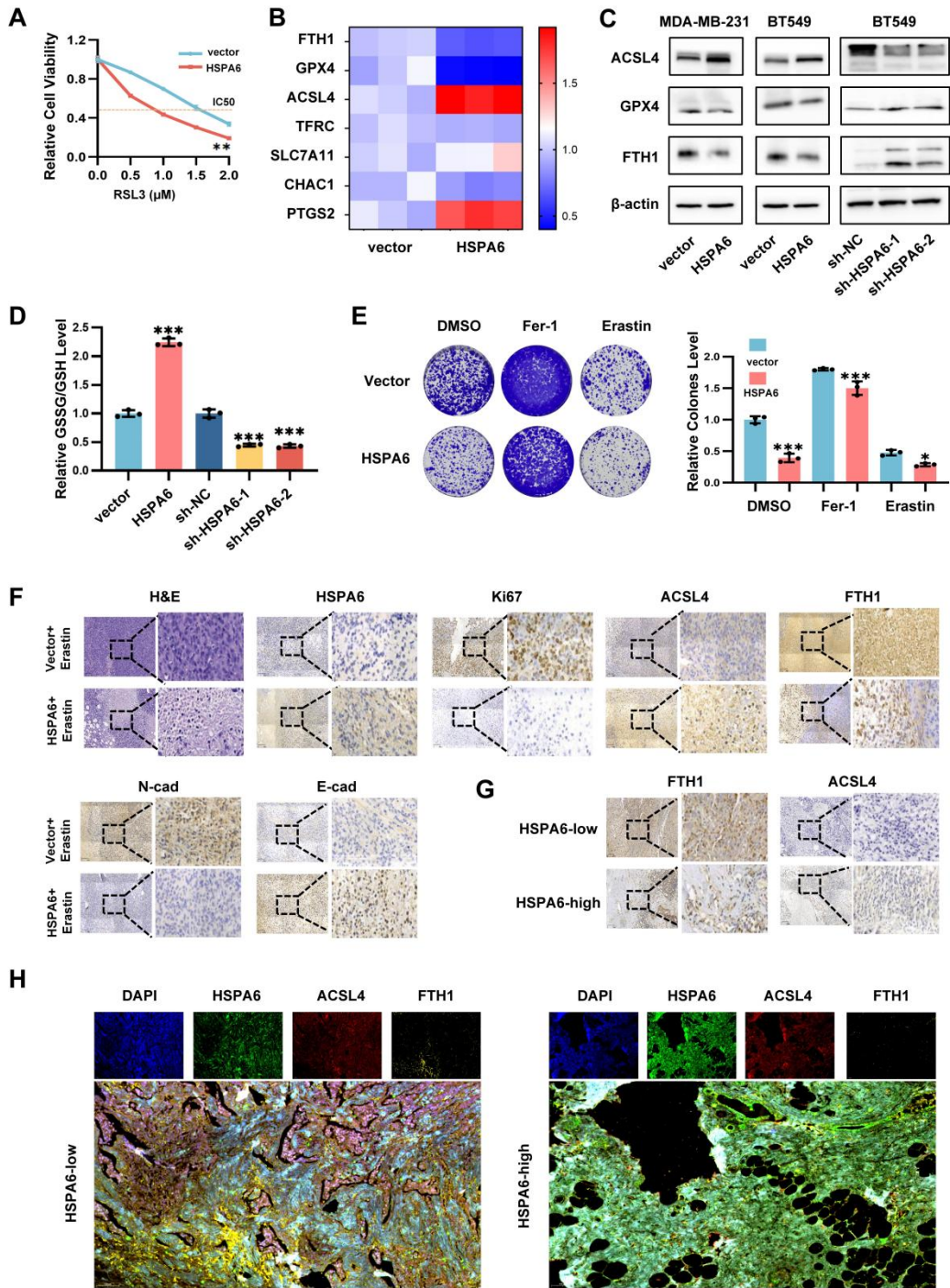


Figure S4: HSPA6 inhibits de novo lipogenesis and Lands cycles.

A) TNBC cells were treated with FASNi (0, 1, 2 μ m) for 48 h for Western blot analyses. **B)** Viability assay of human TNBC cells with FASNi treated detected by CCK8. **C)** The FASN expression were detected following sh-HSPA6 and FASN i transfection. **D)** Oil red O staining for lipid droplets in TNBC cells. **E-F)** Intracellular TG levels (E) and CHOL (F) of TNBC cells were measured by corresponding assay kits. **G)** Intracellular lipid peroxidation and ROS content of TNBC cells were quantified by flow cytometry. **H)** Mitochondrial membrane potential was measured using the JC-1 probe. The distribution of JC-1 aggregates (PE channel) and monomers (FITC channel) was determined by flow cytometry. **I)** Representative images of plate colony formation assays after the knockdown of HSPA6 or treated with FASNi in TNBC cells and the corresponding statistical graphs (n = 3). **J, K, L)** Intracellular GSH levels (J), NAD⁺/NADPH levels (K) and MDA levels (L) of TNBC cells were measured by corresponding assay kits. **M)** Incorporation of arachidonic acid (AA) alkyne in the indicated cell lines treated as indicated. (Data are presented as mean \pm standard of error (SD) of three independent experiments. Statistical significance was determined using ANOVA with post-hoc Tukey multiple comparison, * P < 0.05, ** P < 0.01, *** P < 0.001, ns.: not significant.)

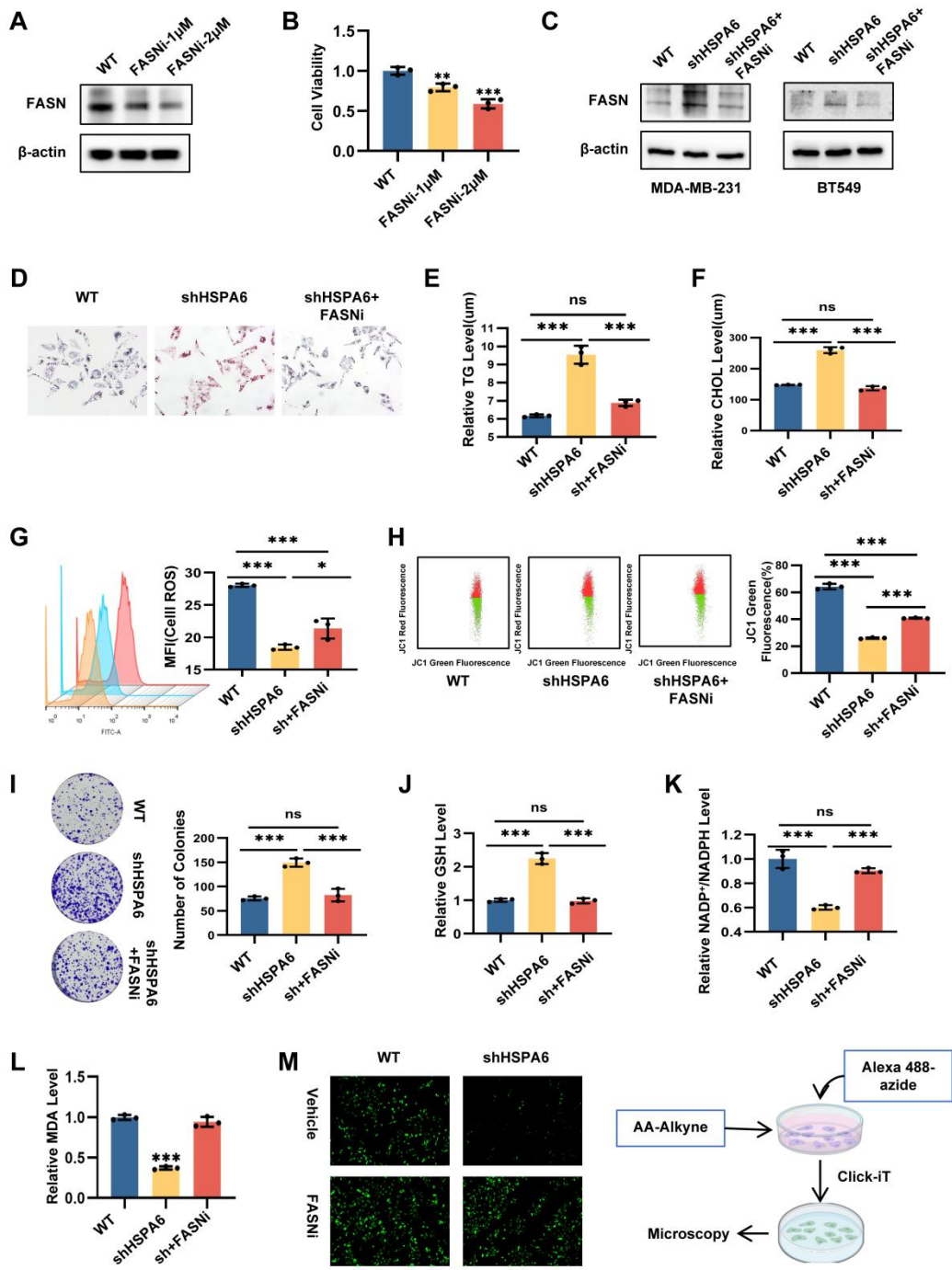


Figure S5: HSPA6 inhibits de novo lipogenesis and Lands cycles.

A) Representative LPCAT1, cPLA2, and p-cPLA2 IHC staining of different HSPA6 level tissues from TMUCIH. Scale bars, 100 μ m. **B)** Representative images of LPCAT1, cPLA2, and p-cPLA2 IHC staining xenografts. Scale bar, 100 μ m. **C)** Representative images of immunofluorescence staining for HSPA6 (green), LPCAT1 (red), and DAPI (nucleus, blue) in tissue sections from TNBC patients. Scale bar: 100 μ m. (Data are presented as mean \pm standard of error (SD) of three independent experiments. Statistical significance was determined using ANOVA with post-hoc Tukey multiple comparison, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns.: not significant.)

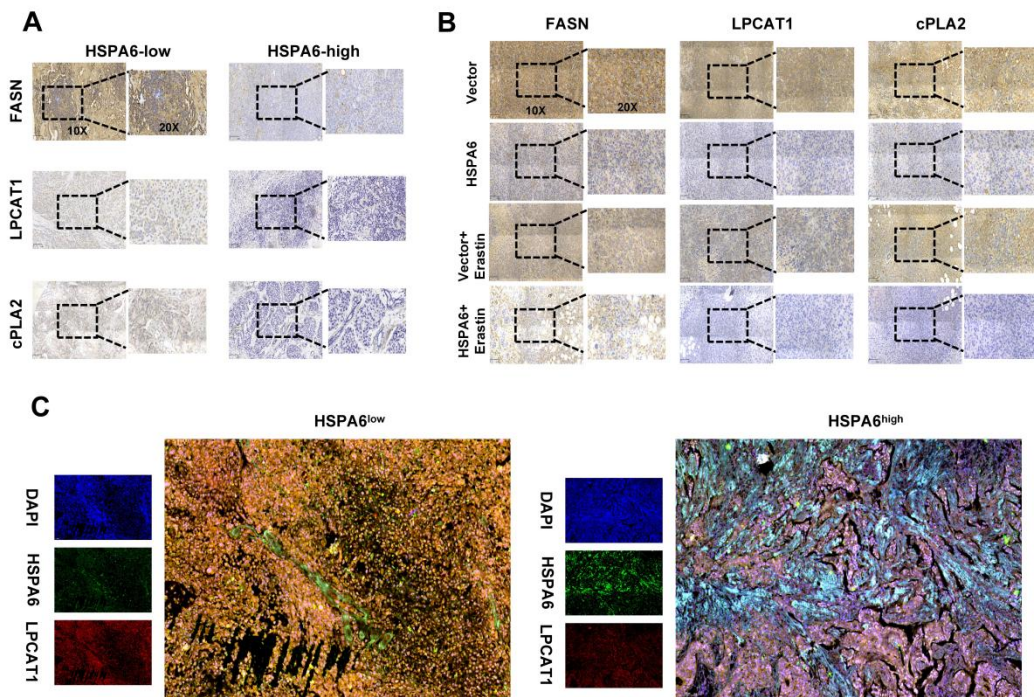


Figure S6: HSPA6 inhibits translocation of p65 protein into nucleus in an importin-dependent manner.

A) Co-IP assays using an anti-HSPA6 antibody detect the interaction between endogenous HSPA6 and importin- α , importin- β with HSPA6 in TNBC cells. **B)** The wild-type p65 (p65^{WT}) and a NLS-mutated p65 (p65 ^{Δ NLS}) were transfected into TNBC cells and the localization of p65 was determined by Western blot. **C)** The expression level of p65 in TNBC cells was determined by Western blot. **D)** The vectors with wild-type p65 (p65^{WT}) and a mutation (p65 ^{Δ NLS}) that disrupted p65 translocation into the nucleus were transfected into TNBC cells. **E)** The subcellular localization of p65 was determined by immunofluorescence staining. **F)** Colony formation analysis of p65^{WT} or p65 ^{Δ NLS}-overexpressing TNBC cells, as well as the control cells. **G)** The overexpression of HSPA6 does not alter the phosphorylation status of p65-Ser564. **H)** The mRNA level of FASN was performed by using RT-qPCR in different groups. **I)** The relationship between p65 and FASN from TCGA BRCA data. **J)** Public ChIP-seq datasets indicated p65 occupancy at the FASN promoter. **K)** EMSA assay showed the binding ability of p65 with biotin-labeled oligonucleotides containing GGTGGTTTCC motif from FASN. **L)** In 293T cells, p65 increased the promoter luciferase activity of FASN. The luciferase activity of the reporter gene was normalized to that of Renilla luciferase. **M)** ChIP-qPCR were used to verify the binding of p65 to the FASN promoter (up). ChIP-qPCR analysis of p65 binding at the FASN promoter upon HSPA6/p65^{WT}/p65 ^{Δ NLS} overexpression (down). **N)** The expression levels of ferroptosis-related biomarkers, including GPX4, FTH1, and ACSL4 in TNBC cells detected by western blot. **O)** RT-qPCR and western blot were used to detect the rescue effect of HSPA6 upregulation on FASN knockdown induced by T p65^{WT}/p65 ^{Δ NLS} overexpression. **P)** RT-qPCR and western blot were used to detect the rescue effect of HSPA6 upregulation on FASN knockdown induced by T p65^{WT}/p65 ^{Δ NLS}/p65^{S468A} overexpression. **Q)** The expression levels of ferroptosis-related biomarkers, including GPX4, FTH1, and ACSL4 in TNBC cells detected by western blot. **R)** The expression of H&E, p65, and FASN of each group were assessed by immunohistochemistry. Scale bar, 100 μ m. (Data are presented as mean \pm standard of error (SD) of three independent experiments. Statistical significance was determined using ANOVA with post-hoc Tukey multiple comparison, * P < 0.05, ** P < 0.01, *** P < 0.001, ns.: not significant.)

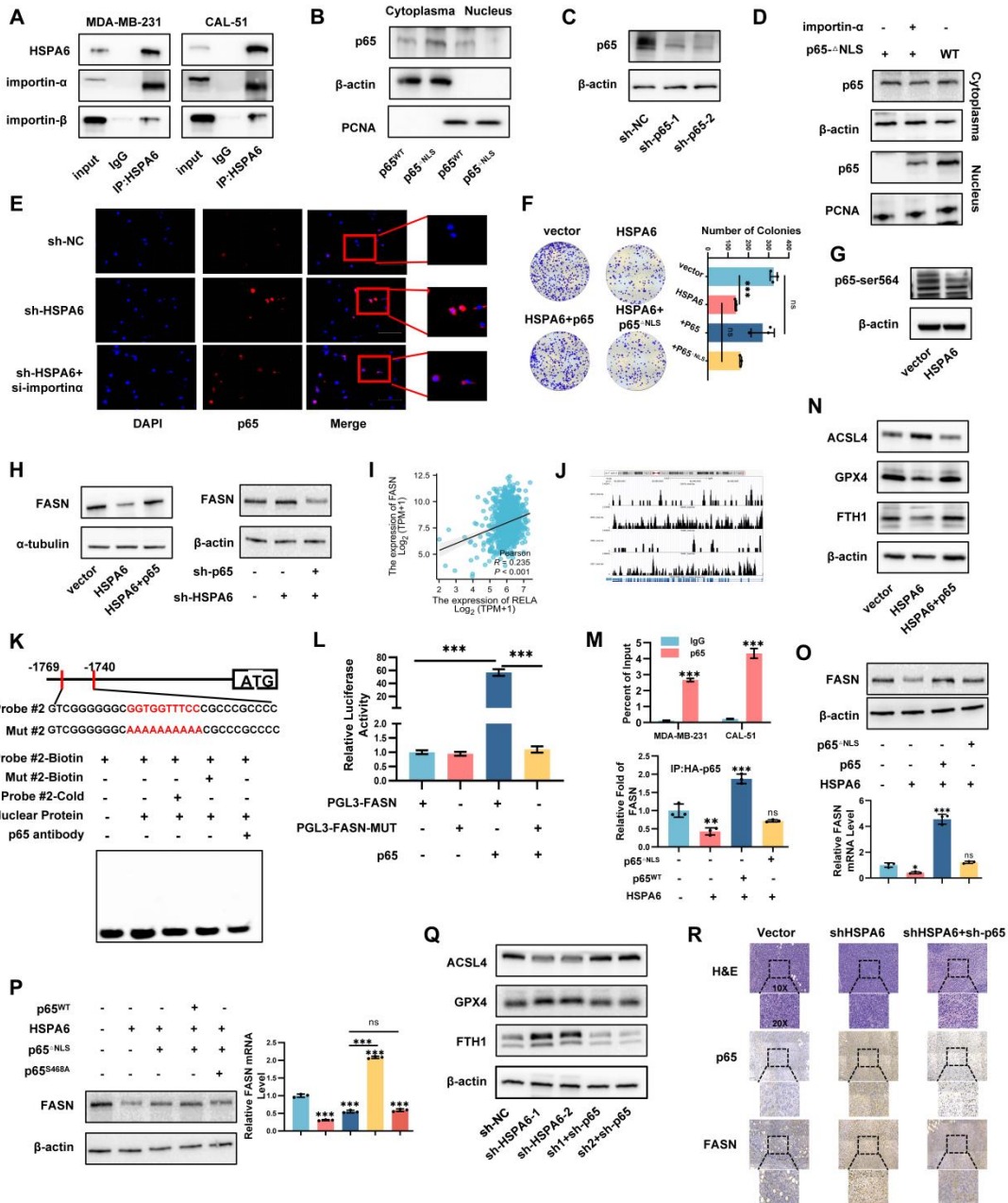


Figure S7: HSPA6 functions through the SBD domain combined with the p65.

A) Correlation between HSPA6 and ANKIB1 expression in public BRCA databases. **B)** Western blot was used to validate the knockdown of ANKIB1 in TNBC cells. **C)** The expression of ANKIB1 in breast cancer and adjacent-healthy tissues from TCGA database. **D)** Western blot analysis of palmitoylation levels in cells transfected with ANKIB1 and its site-specific mutants (C184S, C281S, C338S). **E)** The expression of HSPA6 in si-ANKIB1 TNBC and control cells treated with CHX (50 µg/ml) for the indicated times determined by Western blot. **F)** Diagrammatic representation of HSPA6 and its truncated forms. 293T cells were transfected with the indicated constructs subjected to immunoprecipitation with anti-Flag, which was detected by WB for indicated targets. **G)** Co-IP assays using an anti-Flag antibody detect HSPA6 and its domains ubiquitination mediated by ANKIB1, which was detected by WB for indicated targets. Co-IP assay revealed SBD domains were required for the ubiquitination activity of HSPA6. **H)** Co-IP assay using an anti-Flag antibody was applied to assess the ubiquitination level of HSPA6 affected by the Ub-HA or Ub-K48R plasmids, which was detected by WB for indicated targets. (Data are presented as mean ± standard of error (SD) of three independent experiments. Statistical significance was determined using ANOVA with post-hoc Tukey multiple comparison, * P < 0.05, ** P < 0.01, *** P < 0.001, ns.: not significant.)

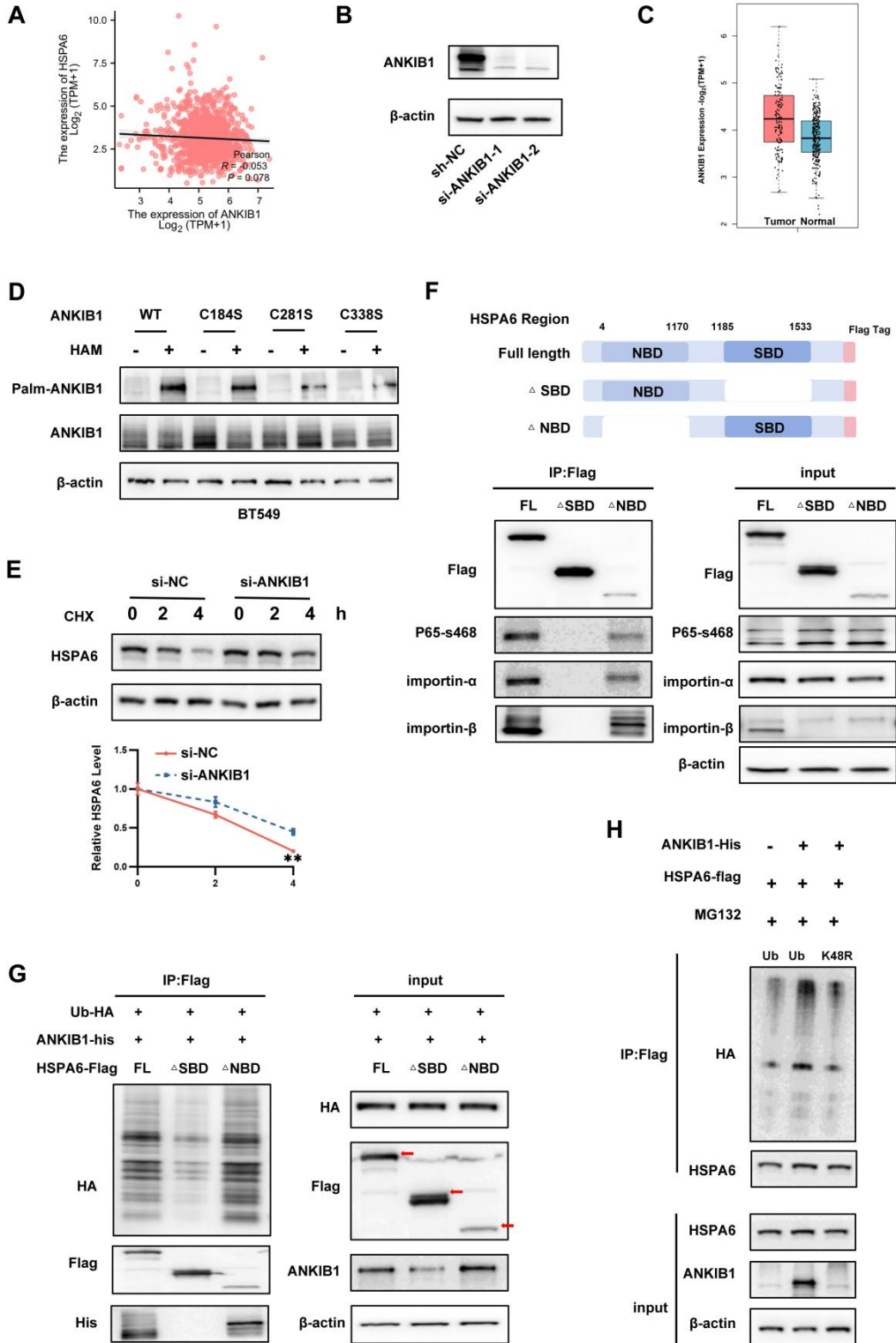


Figure S8: HSPA6 facilitates ferroptosis by inhibiting the p65/FASN/ANKIB1 pathway in vitro

A) Intracellular ROS levels in overexpressing ANKIB1^{WT}, ANKIB1^{C281S}, or ANKIB1^{C338S} TNBC cells were detected by flow cytometry using DCFH-DA probe. **B)** Mitochondrial membrane potential was measured using the JC-1 probe. The distribution of JC-1 aggregates (PE channel) and monomers (FITC channel) in each group TNBC cells were determined by flow cytometry. **C)** Intracellular ROS levels in TNBC cells were detected by flow cytometry using DCFH-DA probe. **D)** Mitochondrial membrane potential was measured using the JC-1 probe. The distribution of JC-1 aggregates (PE channel) and monomers (FITC channel) in each group TNBC cells were determined by flow cytometry. **E)** Transwell assays to assess the effect of FASNi, ANKIB1, and HSPA6 on TNBC cell invasion. **F)** Clonogenic assays to examine the effects of each group on TNBC cell proliferation. **G)** The Intracellular MDA level were measured by corresponding assay kits. **H)** The Intracellular GSH level were measured by corresponding assay kits. **I)** The Intracellular Iron content were measured by corresponding assay kits. **J)** Transmission electron microscopy was used to detect the morphological changes in cellular mitochondria, including smaller size, reduced cristae, and even membrane rupture. Scale bars, 100 μ m. **K)** Representative xenograft images from nude mice subcutaneously implanted with different group cells. **L)** Representative images of H&E staining staining of xenografts. Scale bar, 100 μ m. (Data are presented as mean \pm standard of error (SD) of three independent experiments. Statistical significance was determined using ANOVA with post-hoc Tukey multiple comparison, * P < 0.05, ** P < 0.01, *** P < 0.001, ns.: not significant.)

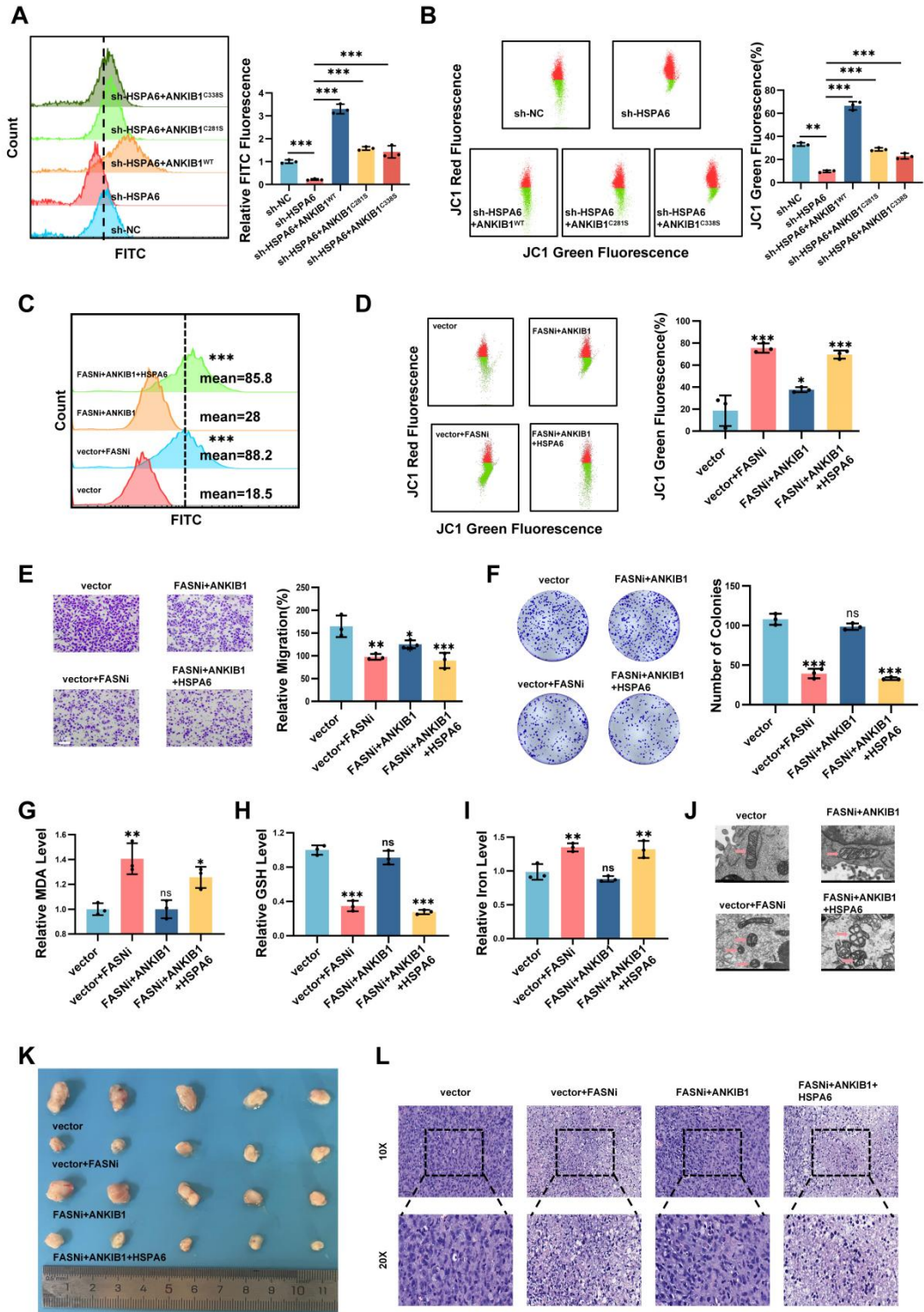


Table S1

gene	Forward Primer	Reverse Primer
β -actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
HSPA6	CAAGGTGCGCGTATGCTAC	GCTCATTGATGATCCGCAACAC
FASN	AAGGACCTGTCTAGGTTTGATGC	TGGCTTCATAGGTGACTTCCA
LPCAT1	ACATCCCGATCTGGGGAAC	GGCCACTTTCCGTTGGACT
LPCAT3	GGCTGGATACTATTACACTGCC	GATCTTCCCTCCGTCAAAGTAG
	CHIP-Forward Primer	CHIP-Reverse Primer
FASN	ACAAAGGTGAGGAGATGGAGCT	TCGGAGAACTTGCAGGAGT

Table S2

gene_name	log2FoldChange	pvalue	padj
CA9	4.776067588	1.28361E-62	4.14421E-61
BEX1	3.28019549	4.96434E-26	4.26861E-25
FGF21	3.239928996	4.4526E-09	1.50197E-08
CDCA3	3.207793321	4.3405E-164	1.7094E-161
CDKN2A	2.713512705	2.43348E-56	6.50124E-55
DPEP1	2.452082681	2.80016E-63	9.26303E-62
GDF15	2.376512331	1.05383E-40	1.6493E-39
EZH2	2.312766241	2.4256E-140	5.4053E-138
CDC25A	2.169858977	8.92292E-77	4.53936E-75
CBS	1.980660485	3.41675E-23	2.57533E-22
IDO1	1.935642105	2.07262E-21	1.42043E-20
IFNG	1.865027839	4.21943E-17	2.32662E-16
CYP4F8	1.700467625	9.66235E-08	2.93396E-07
BRDT	1.685986908	1.03562E-10	3.92594E-10
HSPB1	1.63028177	2.19357E-38	3.16622E-37
FADS2	1.605755211	1.11339E-20	7.36307E-20
CHAC1	1.494221688	3.39927E-26	2.9417E-25
FANCD2	1.468998461	2.77028E-77	1.43003E-75
IDH2	1.437997078	3.80224E-57	1.03951E-55
FLT3	1.266019996	9.66451E-12	3.9305E-11
CISD3	1.225791335	7.96853E-32	8.90713E-31
GALNT14	1.206231695	2.05004E-13	9.23429E-13
ABCC5	1.145109934	1.48303E-30	1.56324E-29
ABHD12	1.102640653	2.36282E-57	6.51437E-56
CFL1	1.032325053	4.41792E-66	1.60181E-64
FZD7	-1.159058393	2.12987E-26	1.85689E-25
ACSL4	-1.178431713	3.48831E-36	4.64471E-35
CYGB	-1.330778014	2.96901E-42	4.92684E-41
CPEB1	-1.428442108	1.50914E-30	1.59003E-29
ANO6	-1.475561415	7.0466E-112	8.724E-110
DPP4	-1.493056357	1.78108E-30	1.87266E-29
AIFM2	-1.779799962	1.76358E-97	1.61135E-95
EGFR	-1.825781462	2.5849E-27	2.35659E-26
IFNA8	-1.882618297	0.001437795	0.002817978
EPAS1	-1.938304757	3.1795E-121	5.0434E-119
ACO1	-2.00635269	5.3673E-172	2.6366E-169
CREB5	-2.035931897	4.90857E-67	1.83806E-65
ATF3	-2.040186596	2.94336E-46	5.67361E-45
AKR1C3	-2.319299122	4.85403E-59	1.40438E-57
EGR1	-2.615190328	2.28686E-74	1.08713E-72
DDR2	-2.637250588	8.5437E-141	1.9322E-138
ENPP2	-2.698110746	1.26665E-100	1.22338E-98

ACSL1	-2.75286351	1.2327E-124	2.125E-122
AKR1C2	-3.00457869	1.33786E-38	1.9477E-37
CAV1	-3.360559709	2.5222E-226	7.6817E-223
AKR1C1	-3.41072927	5.76658E-69	2.29878E-67
CDO1	-3.616772231	7.8951E-120	1.2144E-117
FABP4	-4.867568213	6.8333E-101	6.64194E-99
ADIPOQ	-5.152572434	2.90123E-82	1.75317E-80