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Supplemental Information

The RNA-Binding Protein CELF2 Inhibits Ovarian Cancer Progression by Stabilizing FAM198B

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Supplementary Figures

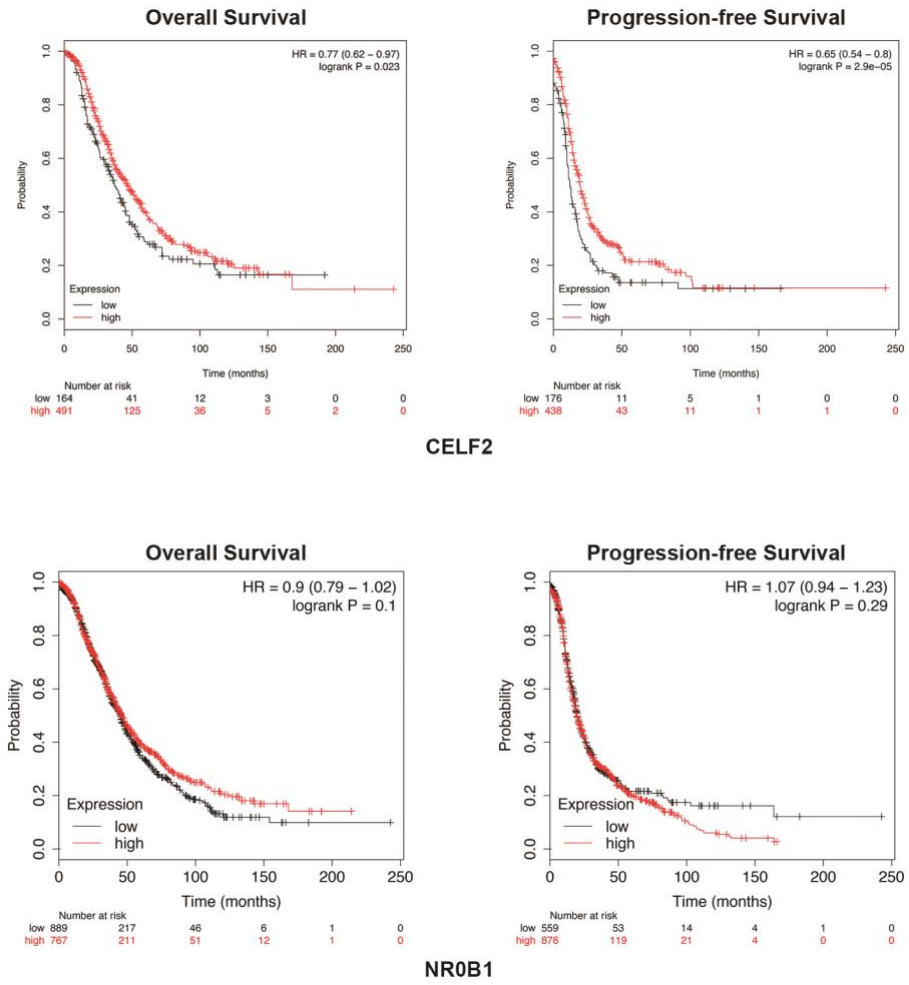

Figure S1. Kaplan-Meier analysis of the OS and PFS of patients with ovarian cancer and the expression of CELF2 (upper panel) and NR0B1 (lower panel) using an online tool.

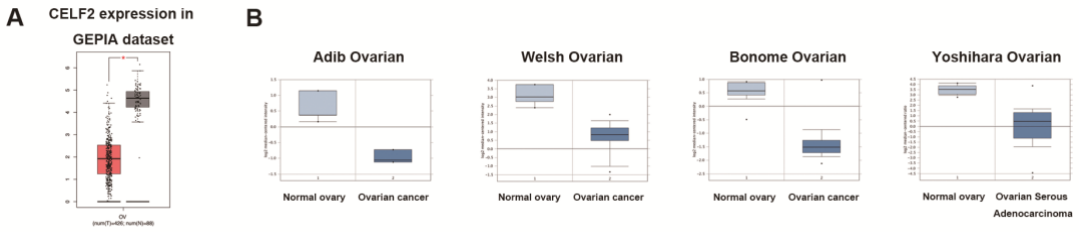

Figure S2. CELF2 expression in samples contained in databases. (A) CELF2 expression in normal ovarian tissues and ovarian cancer tissues in the GEPIA database. (B) CELF2 expression in normal ovarian tissues and ovarian cancer tissues in four other publicly available datasets in the Oncomine database.

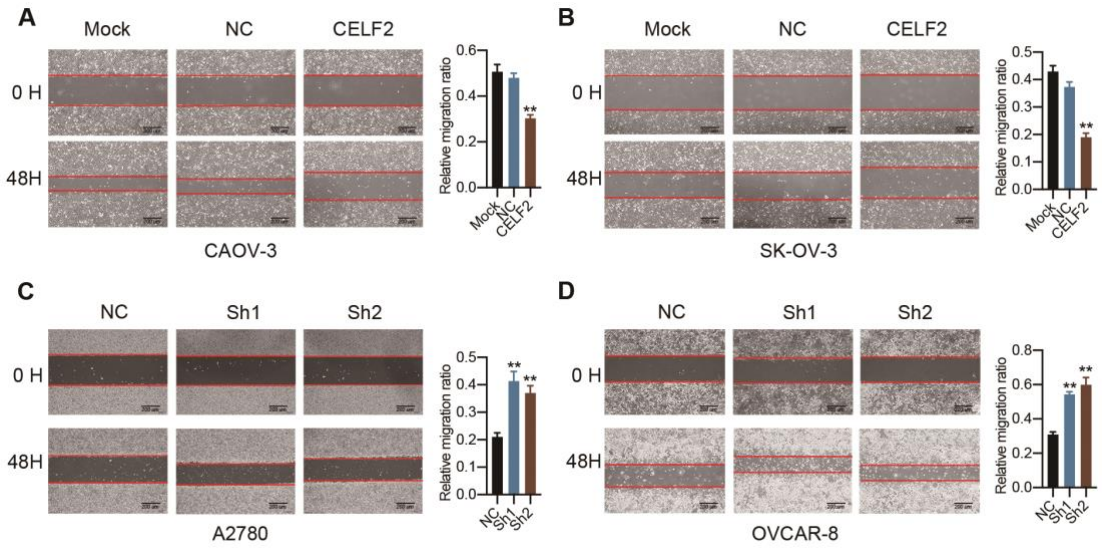

Figure S3. Representative images (scale bar, 200 μm) of wound healing assays in CAOV-3 (A), SK-OV-3 (B), A2780 (C), and OVCAR-8 (D) cells (**$p < 0.01$).

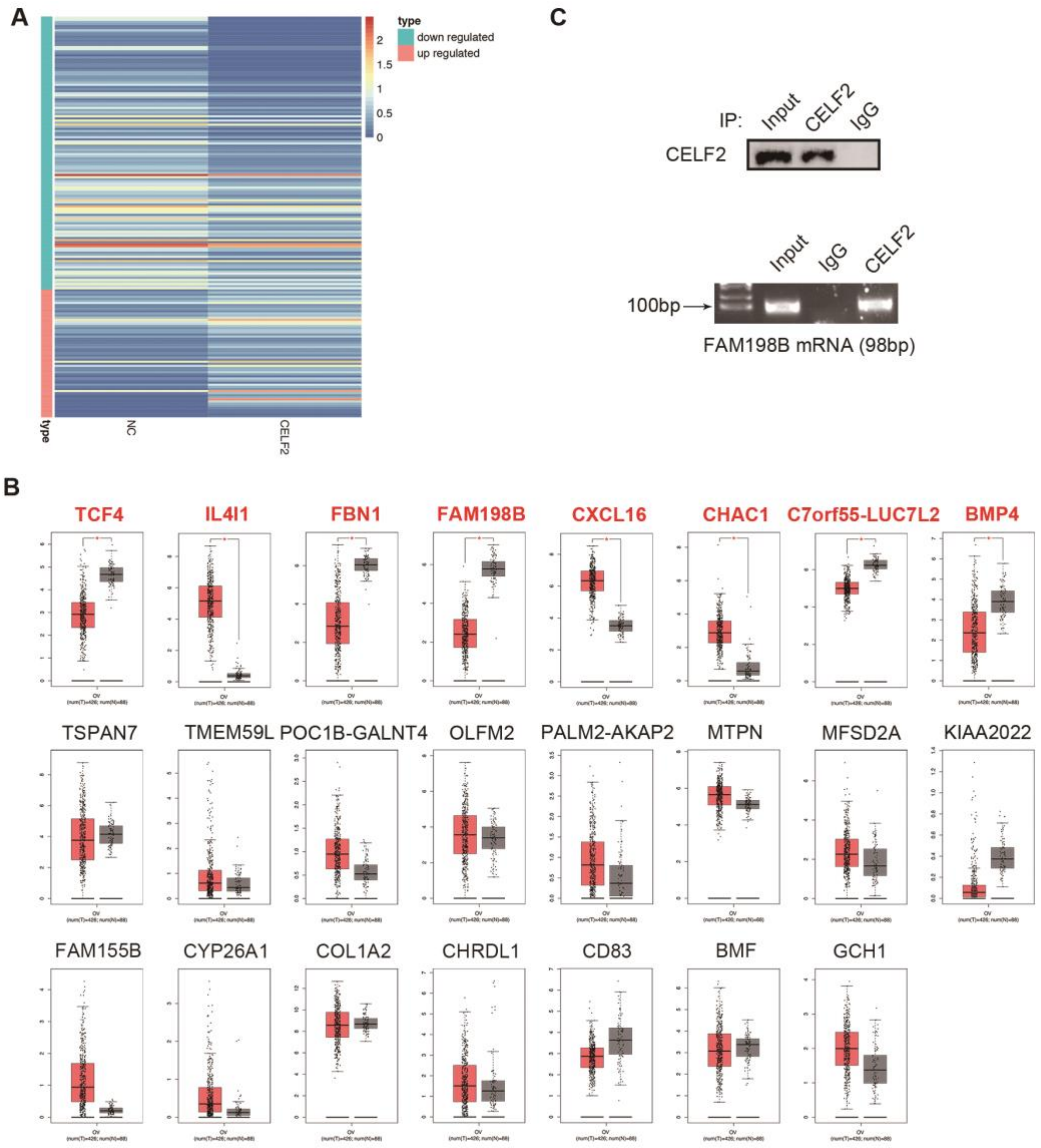

Figure S4. Validation of downstream target genes of CELF2. (A) The differentially expressed genes in CELF2-overexpressing (CELF2) cells compared with the vector-transfected (NC) cells are shown in the heat map. (B) The mRNA expression of 23 candidate CELF2 target transcripts in normal ovarian tissues and ovarian cancer tissues in the GEPIA database. (C) Western blot and RIP-qPCR analyses following CELF2 immunoprecipitation in A2780 cells.

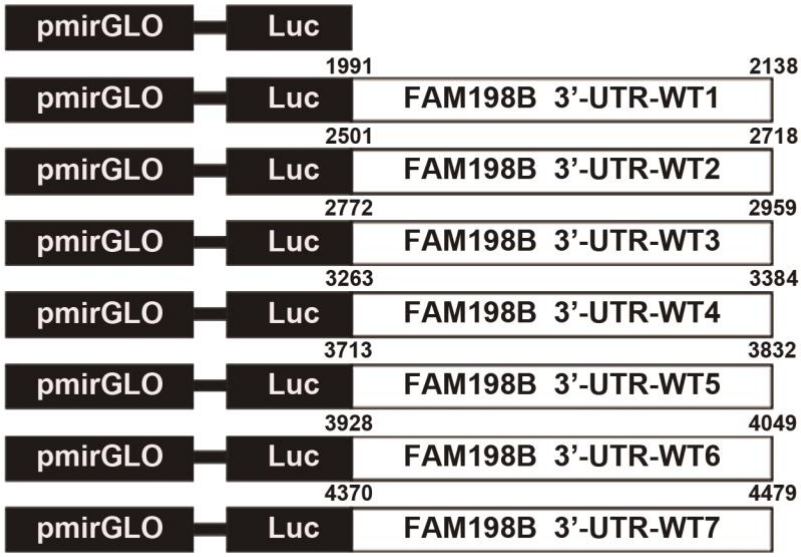

Figure S5. Schematics outlining the construction of various regions in the 3'-UTR reporter for FAM198B. Both were compared relative to a control vector with no additional 3' UTR sequence.

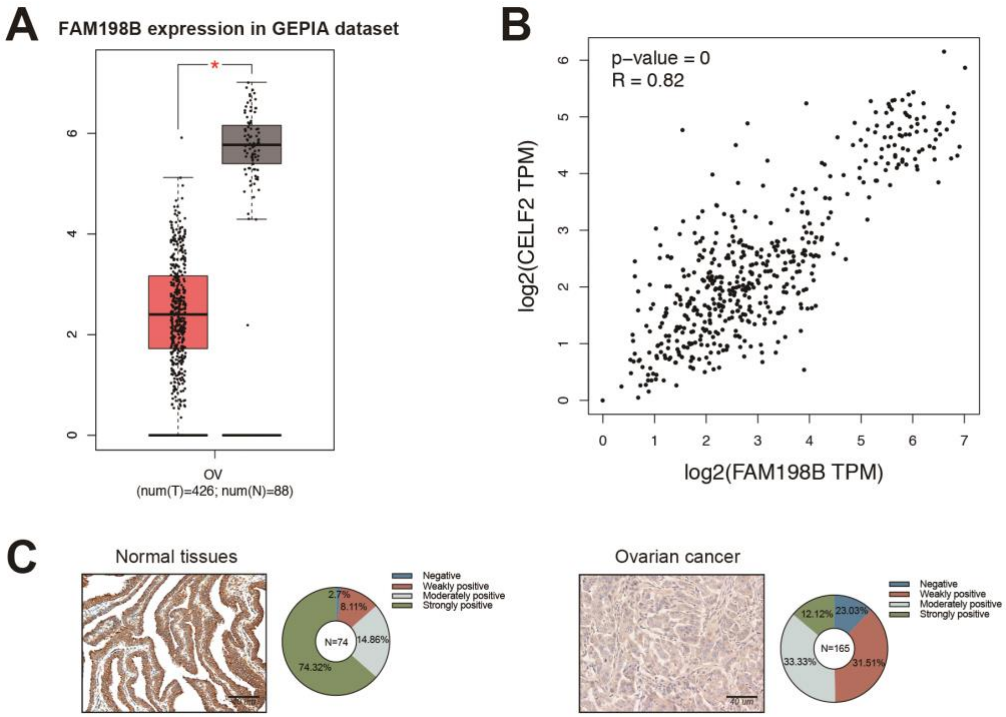

Figure S6. Expression of FAM198B in ovarian cancer. (A) The expression of the FAM198B mRNA in the GEPIA database. (B) Correlation between the expression of the CELF2 and FAM198B mRNAs in ovarian tumors in the GEPIA database. (C) Representative images and proportions of negative, weakly positive, moderately positive and strongly positive immunohistochemical staining in normal ovarian tissues (left panel) and ovarian cancer tissues (right panel) (scale bar, 40 μm).

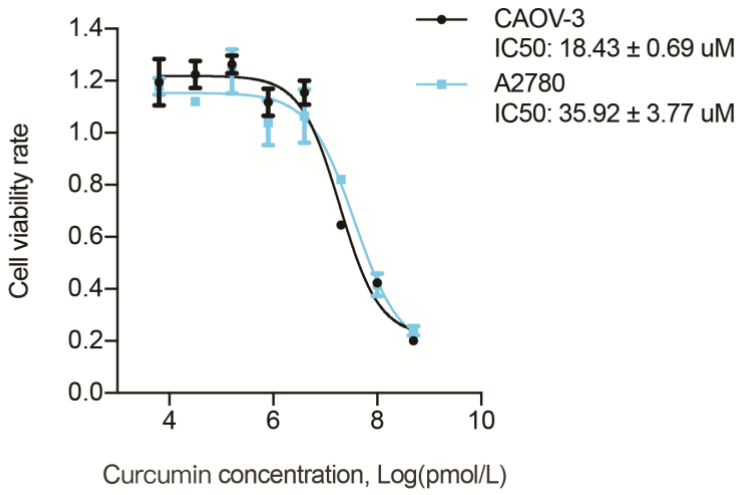

Figure S7. The 50% inhibitory concentration (IC50) value of curcumin was determined in CAOV-3 and A2780 cells.

Table S1: Correlation of CELF2 expression and clinicopathological parameters in ovarian cancer tissues.

Characteristics	No.	CELF2-Low	CELF2-High	χ2	*P* Value
Patient age (years)				0.02	0.887
≤ 55	78	43	35		
> 55	87	47	40		
Tumor diameter (cm)				0.657	0.418
≤ 5	56	33	23		
> 5	109	57	52		
LVSI				0.107	0.744
No	69	36	33		
Yes	71	39	32		
Poor histologic differentiation				0.341	0.559
No	82	44	38		
Yes	79	46	33		
FIGO stage				6.238	0.013*
I-II	16	4	12		
III–IV	149	86	63		
Lymph node metastasis				2.716	0.099
No	14	6	8		
Yes	61	43	18		
Intestinal metastasis				0.829	0.363
No	79	46	33		
Yes	86	44	42		
Diaphragmatic metastasis				3.847	0.035*
No	82	51	31		
Yes	83	39	44		

Abbreviations: LVSI, lymph-vascular space invasion; FIGO, Federation of Gynecology and Obstetrics; *$p < 0.05$.

Table S2. Univariate and multivariate Cox regression of overall survival for patients with ovarian cancer.

Characteristics	Univariate analysis			Multivariate analysis		
	HR	95%CI	P value	HR	95%CI	P value
Patient age (≤55, >55)	1.169	0.801-1.704	0.418			
Tumor diameter (≤ 5cm, >5cm)	0.813	0.549-1.205	0.303			
LVSI (No, Yes)	1.147	0.762-1.726	0.512			
Poor histologic differentiation (No, Yes)	0.978	0.669-1.430	0.91			
FIGO stage (I-II, III–IV)	0.98	0.538-1.786	0.947			
Lymph node metastasis (No, Yes)	1.837	0.825-4.089	0.137			
Intestinal metastasis (No, Yes)	1.761	1.195-2.594	0.004*	1.608	1.069-2.419	0.023*
Diaphragmatic metastasis (No, Yes)	1.769	1.202-2.604	0.004*	1.674	1.111-2.523	0.014*
CELF2 expression (Low, High)	0.603	0.410-0.886	0.01*	0.527	0.356-0.780	0.001*

Abbreviations: HR, hazard ratio; LVSI, lymph-vascular space invasion; FIGO, Federation of Gynecology and Obstetrics; CI, confidence interval. *$p < 0.05$.

Table S3. Univariate and multivariate Cox regression of progression-free survival for patients with ovarian cancer.

Characteristics	Univariate analysis			Multivariate analysis		
	HR	95%CI	P value	HR	95%CI	P value
Patient age (≤55, >55)	1.201	0.838-1.721	0.318			
Tumor diameter (≤ 5cm, >5cm)	0.736	0.506-1.072	0.11			
LVSI (No, Yes)	1.053	0.715-1.552	0.792			
Poor histologic differentiation (No, Yes)	0.963	0.669-1.384	0.837			
FIGO stage (I-II, III–IV)	1.245	0.668-2.319	0.49			
Lymph node metastasis (No, Yes)	1.75	0.855-3.581	0.126			
Intestinal metastasis (No, Yes)	1.219	1.051-1.745	0.038*	1.007	0.608-1.494	0.973
Diaphragmatic metastasis (No, Yes)	1.516	1.051-2.187	0.026*	1.752	1.159-2.647	0.008*
CELF2 expression (Low, High)	0.552	0.380-0.803	0.002*	0.488	0.332-0.718	<0.001*

Abbreviations: HR, hazard ratio; LVSI, lymph-vascular space invasion; FIGO, Federation of Gynecology and Obstetrics; CI, confidence interval. *$p < 0.05$.

Table S4. shRNA and siRNA sequences used in the study.

Gene Name		Target Sequence (5'-3')
CELF2	shRNA1	CCTCTCTCGGGACTCTGCAAGGACT
	shRNA2	GAGCCACTGTTGGACTGAATAATAT
FAM198B	siRNA1	GGUUAAGAUUGGAGAGCGAdTdT
	siRNA2	GCUUAUCGAUGUAAUAGAAdTdT

Table S5. Primer sequences used in the study.

Gene Name	Direction	Primer Sequence (5'-3')
CELF2	Forward	ACCTGGGTGCGTTCAGCGGCATTCA
	Reverse	CCATTCGTCAAGCCTGTGGCGCCAA
TCF4	Forward	ACGGACAAAGAGCTGAGTGA
	Reverse	CCCTGCTAGTCATGTGGTCA
IL4I1	Forward	ATCACTCAGGGGAGGAACGAT
	Reverse	CGACGGAAACTCAGAAAAACC
FBN1	Forward	AGTCGGGCCAAGAGAAGAGGCG
	Reverse	TCCATCCAGGGCAACAGTAAGCAT
FAM198B	Forward	GATGGCACTCTTTGATTTTTTGTTAC
	Reverse	GGCCTCAATCCATTCTGTACACA
CXCL16	Forward	GGCCCACCAGAAGCATTTAC
	Reverse	CTGAAGATGCCCCCTCTGAG
CHAC1	Forward	GGTTCTGCTCCCCTTGCA
	Reverse	CGTGTGGTGACGCTCCTTG
C7orf55-LUC7L2	Forward	AGAAGGACAGGACAATGGCG
	Reverse	TTCACTGGTGACCCGATGTG
BMP4	Forward	GGGATTCCCGTCCAAGCTAT
	Reverse	ACGGAATGGCTCCATAGGTC
β-actin	Forward	AGTCATTCCAAATATGAGATGCGTT
	Reverse	TGCTATCACCTCCCCTGTGT

Supplemental materials and methods

Stable and transient transfections

For lentivirus production, lentiviral vectors were cotransfected into HEK293T cells with the packaging vectors psPAX2 and pMD2.G using Lipofectamine 3000 (Invitrogen, CA, USA). Virus particles were harvested 48 h after transfection. Lentiviruses were transduced into cells with polybrene (2 μg/ml) to increase the infection efficiency. Then, the positive cells were selected with 3 μg/ml puromycin for 2-3 weeks to establish stable cell lines. For the FAM198B siRNA transfection, cells were seeded in 6-well plates overnight and then transfected with FAM198B-siRNAs (si1 and si2) or the NC siRNA using the Lipofectamine 2000 transfection agent (Invitrogen, CA, USA) according to the manufacturer's instructions. The transfection efficiencies were verified by RT-qPCR and Western blotting.

Immunohistochemistry

IHC staining with antibodies against CELF2 (1:100, Abcam, MA, USA), FAM198B (1:50, Sigma-Aldrich, MO, USA) and Ki67 (1:10 000, Proteintech, IL, USA) was performed to detect protein expression levels using standard procedures. Images of randomly selected fields in each section were captured at 40× and 200× magnification. A well-established IRS was used to calculate the protein expression level. First, the staining intensity (SI) was scored using a 4-point scale ranging from 0 to 3 points, with 0 indicating no staining. The scores for weak, moderate, and strong staining were 1, 2

and 3, respectively. Second, the percentage of positive cells was scored into five categories: no staining, 1–10%, 11–50%, 51–80%, and 81–100% positive cells. The scores were 0, 1, 2, 3 and 4 points, respectively. An IRS was calculated by multiplying the percentage by the SI score, resulting in a scale ranging from 0 to 12 points. The IRS was divided into four groups: 0 (IRS of 0–1 point), 1 (IRS of 2–3 points), 2 (IRS of 4–7 points) and 3 (IRS of 8–12 points).

Preparation of curcumin and cisplatin stock solutions

Curcumin (Selleck, TX, USA) was suspended in dimethyl sulfoxide (DMSO, Sigma-Aldrich, MO, USA) to prepare a stock solution of 40 mM. The curcumin stock solution was then diluted in complete DMEM to prepare stock and final working concentrations. Cisplatin (Selleck, TX, USA) was suspended in dimethylformamide (DMF, Sigma-Aldrich, MO, USA) to prepare a stock solution with a concentration of 20 mM and then diluted in complete DMEM. The solution was stored at -80°C until further use.

IC50 assay

IC50 values of curcumin or cisplatin in cell lines were assessed using a CCK-8 assay. Cells were plated in 96-well plates, incubated overnight, and treated with serial dilutions (6.4 nM-500 μM) of each compound for 48 h. Ten microliters of CCK-8 reagent were added to the wells and incubated for an additional 2 h. The absorbance was measured at 450 nm. IC50 values were calculated from the dose-response curves and defined as the concentration of curcumin or cisplatin that caused 50% inhibition of

ovarian cancer cell growth.

RNA-seq

RNA-seq was performed by Huada Genomics Institute (Wuhan, China) using the BGISEQ platform. Reads were filtered (SOAPnuke, version 1.5.2) and mapped (HISAT2, version 2.0.4) to the human transcriptome. Then, Bowtie2 (v2.2.5) was applied to align the clean reads to the reference coding gene set, and the gene expression level was calculated using RSEM (v1.2.12). A fold change > 2 was set as the threshold for significantly different expression.

Dual-luciferase reporter assay

Sequences of multiple regions of AREs within the FAM198B 3′-UTR and mutated sequences of CELF2 target binding sites were synthesized and amplified by PCR. The PCR fragments were separately subcloned into the dual-luciferase reporter pmirGLO vector (Promega, WI, USA). The mutant construct containing the predicted CELF2 binding sites (WT3 and WT4) was replaced by complementary sequences of AREs in the original sequences. The constructs were named Mutant 3 and Mutant 4, respectively. A dual-luciferase reporter assay was performed according to the manufacturer's protocol (Promega, WI, USA). The experimental group and the control group of CAOV-3 and A2780 cells were seeded in a 24-well plate at a concentration of 6×10^4 cells and 1×10^5 cells, respectively, and cultured overnight. Then, the above plasmids were cotransfected into these cells. After 48 h, the cells were harvested, and the firefly

luciferase activity and Renilla activity were determined using a dual-luciferase reporter assay system (Promega, WI, USA). For each experiment, the level of firefly luciferase activity was normalized to the level of Renilla luciferase activity.

Cell proliferation and colony formation assay

Cell proliferation was detected using Cell Counting Kit-8 (CCK-8, Dojindo, Japan) according to the manufacturer's protocol. Cells were plated in 96-well plates, and 10 μl of CCK-8 were added to each well at the same time of each day and incubated at 37°C for 2 h. The absorbance was measured at 450 nm using a microplate reader. Each treatment was assayed in triplicate wells. For colony formation assays, aliquots of 1×10^3 viable cells were seeded in triplicate into 6-cm dishes after the appropriate treatment. After 2 weeks of cultivation, colonies were fixed with ethanol and stained with 1% crystal violet (Solarbio, Beijing, China), and colonies (foci containing > 50 cells) were counted and photographed.

Transwell assay and wound healing assay

Transwell chambers (Corning, NY, USA) were placed in 24-well plates and used for in vitro cell migration assays. Cells were seeded in the upper chamber and cultured with 250 μl of serum-free DMEM. The lower chamber contained 500 μl of medium supplemented with 20% FBS. After the plates were cultured for 24-48 h, migrated cells (on the lower side of the membranes) were washed, fixed, stained and imaged. The number of migrating cells was counted in randomly selected fields using a microscope

at 200× magnification. For the wound healing assay, cells were seeded into 6-well plates and cultured until they reached 100% confluence. Artificial wounds were generated using 1-ml pipette tips to generate a straight scratch. Serum-free medium was subsequently added, and culture plates were incubated at 37°C for 48 h. Wound healing was observed within the scrape line at 0 and 48 h, and representative images of the scrape lines in the same field were captured at 40× magnification.

Analysis of mRNA stability

Cells were seeded in 6-well plates to assess the stability of the FAM198B mRNA. After 24 h, cells were treated with 5 μg/ml Act D (Sigma-Aldrich, MO, USA) for the indicated periods. RNA extraction, cDNA generation, and RT-qPCR were performed as described above. The mRNA levels were calibrated to the 0 h time point.