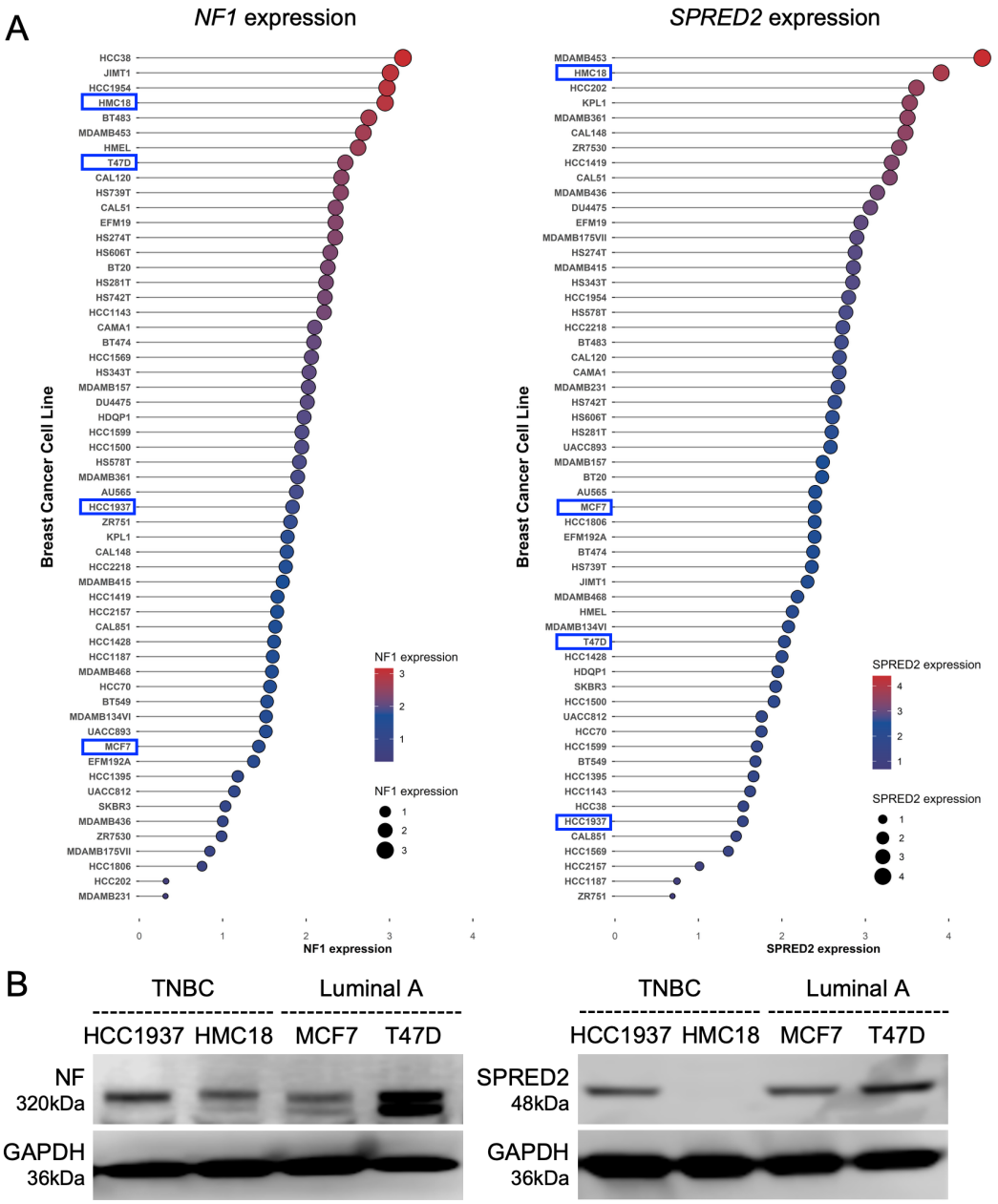

Figure 1. Expression of NF and SPRED2 in human breast cancer cell lines. (A) Normalized RNA-seq expression values of *NF1* (left) and *SPRED2* (right) were obtained from CCLE breast cancer cell lines. Raw read counts were processed in R (v4.5.1) using the *edgeR* package with trimmed mean of M-values normalization and transformed to $\log_2$ counts per million. Cell lines are ranked in descending order of expression. Dot size and color indicate normalized expression levels. The cell lines selected for subsequent experiments are highlighted by blue boxes. (B) Cell lysates were prepared from the indicated cell lines, and NF and SPRED2 protein expression was assessed by Western blotting. Representative images from three independent experiments are shown.

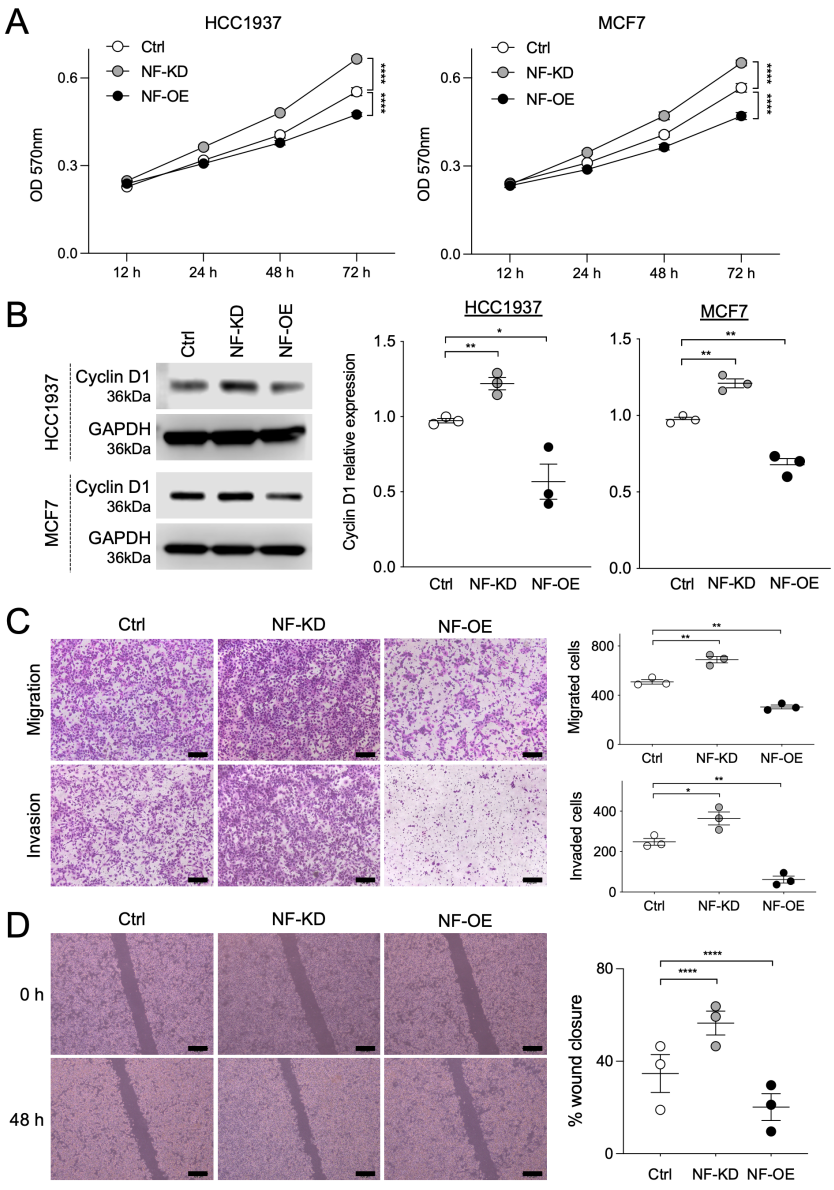

Figure 2. NF negatively regulates BC cell proliferation, migration, and invasiveness. NF was knocked down (NF-KD) or overexpressed (NF-OE) in HCC1937 and MCF7 cells. (A) Cell proliferation was evaluated by MTT assay in each cell line. (B) Cell lysates were prepared from control (Ctrl), NF-KD and NF-OE cells, and cyclin D1 expression was analyzed by Western blotting. Representative images are shown on the left, and the band intensities were quantified and semi-quantitated from three independent experiments on the right. Cyclin D1 expression levels were normalized to GAPDH. (C) Cell migration and invasion assays were performed using HCC1937 cells. Representative images are shown on the left (scale bars: 100 μm). For quantification, cells in three randomly selected low-power fields (20× magnification) per membrane were counted (three independent experiments). (D) Scratch assays were performed using MCF7 cells. Representative images captured with an inverted microscope are shown on the left (scale bars: 100 μm). The wound gap distance was measured at the indicated time points using Image J software (three independent experiments). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ (two-tailed unpaired t-test).

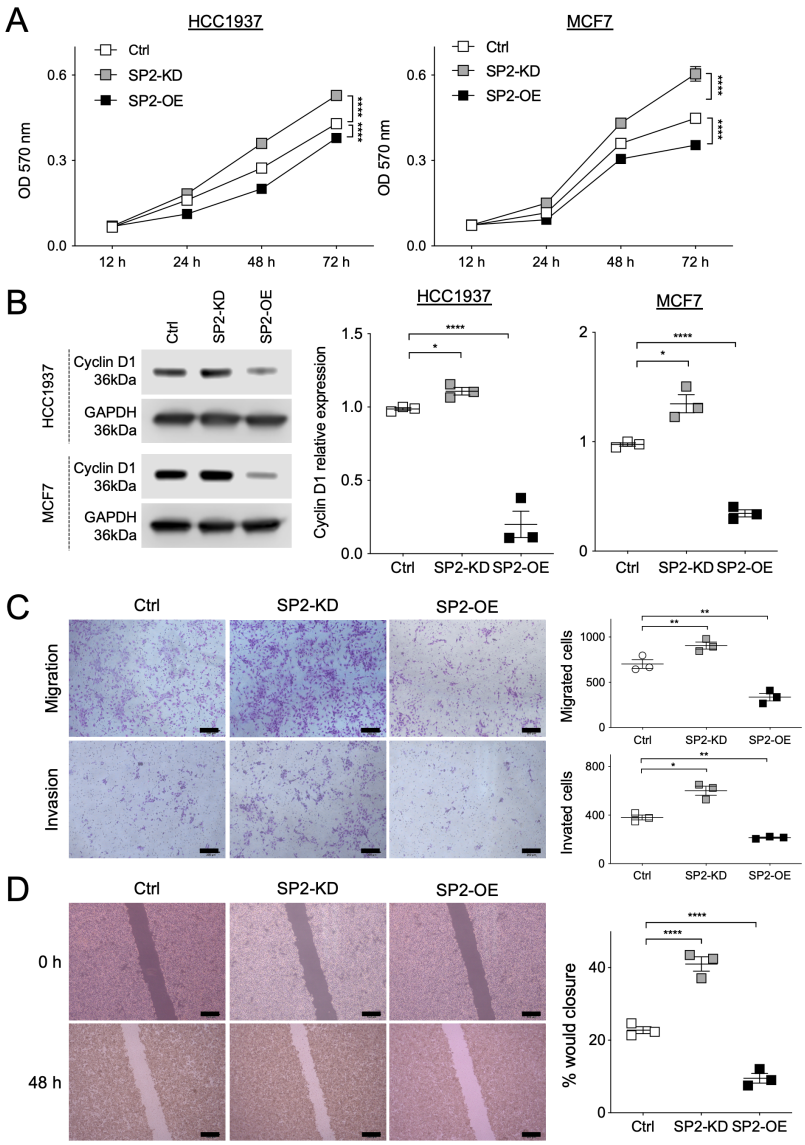

Figure 3. SPRED2 negatively regulates cancer cell proliferation, migration, and invasiveness. SPRED2 was knocked down (SP2-KD) or overexpressed (SP2-OE) in HCC1937 and MCF7 cells. (A) Cell proliferation was evaluated by MTT assay in each cell line. (B) Cell lysates were prepared from control (Ctrl), SP2-KD and SP2-OE cells, and cyclin D1 expression was analyzed by Western blotting. Representative images are shown on the left, and band intensities were quantified and semi-quantitated from three independent experiments on the right. Cyclin D1 expression levels were normalized to GAPDH. (C) Cell migration and invasion assays were performed using HCC1937 cells. Representative images are shown on the left (scale bars: 100 μm). For quantification, cells in three randomly selected low-power fields (20× magnification) per membrane were counted (three independent experiments). (D) Scratch assays were performed using MCF7 cells. Representative images captured with an inverted microscope are shown on the left (scale bars: 100 μm). The wound gap distance was measured at the indicated time points using Image J software (three independent experiments). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ (two-tailed unpaired t-test).

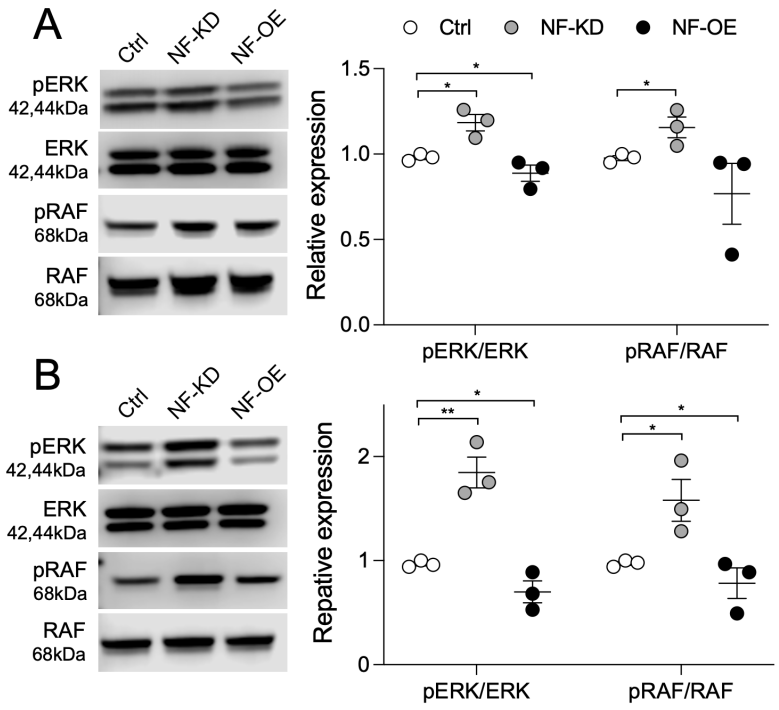

Figure 4. NF negatively regulates ERK and RAF activation. NF was knocked down (NF-KD) or overexpressed (NF-OE) in HCC1937 and MCF7 cells. ERK and RAF phosphorylation in HCC1937 cells (A) and MCF7 cells (B) was analyzed by Western blotting. Representative images are shown on the left, and band intensities were quantified and semi-quantitated from three independent experiments on the right. $^{*}p < 0.05$, $^{**}p < 0.01$ (two-tailed unpaired t-test).

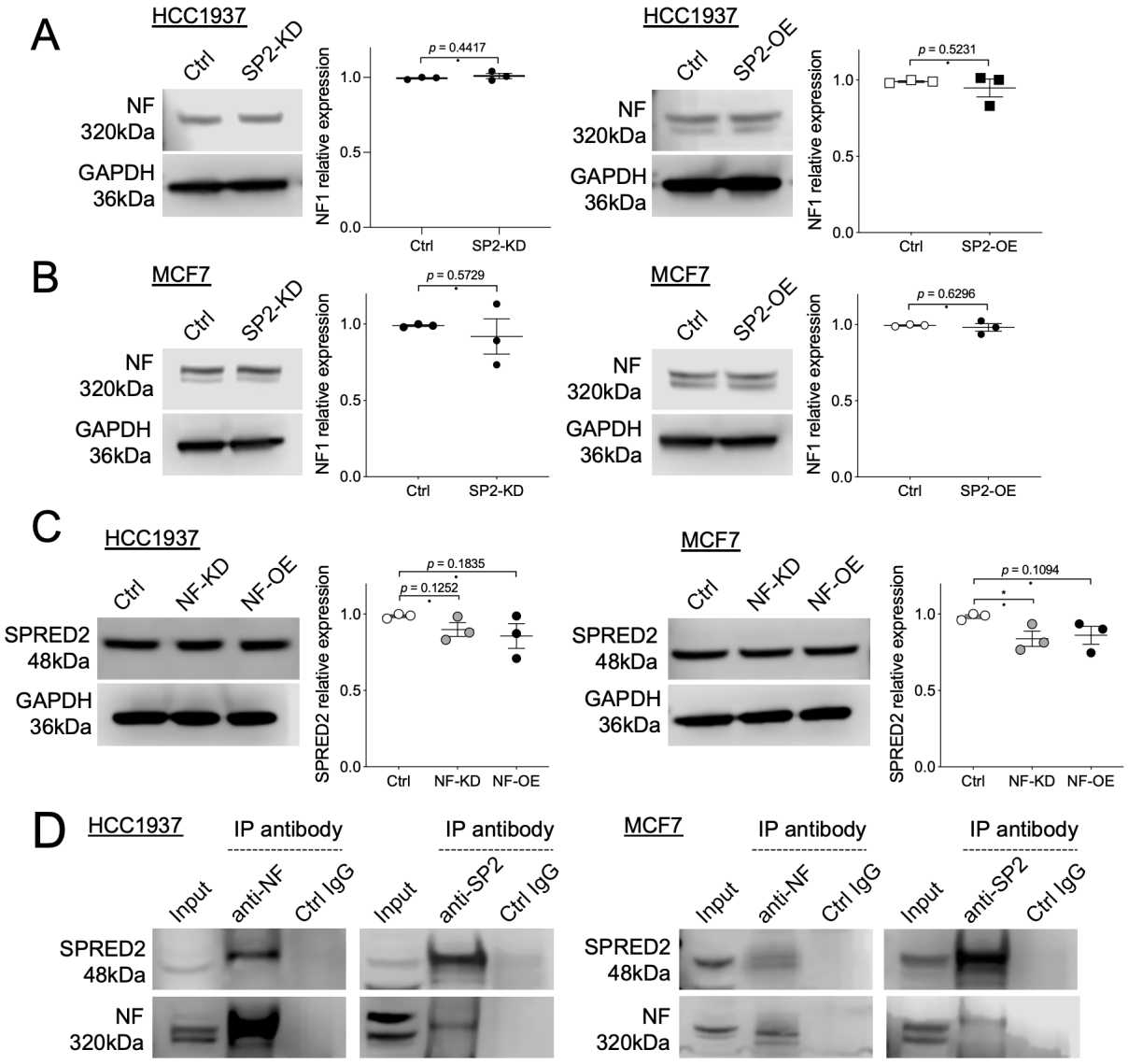

Figure 5. Interaction between NF and SPRED2 in BC cells. (A-C) NF was knocked down (NF-KD) or overexpressed (NF-OE) in HCC1937 and MCF7 cells. Cell lysates were prepared, and the protein expression of NF and SPRED2 was analyzed by Western blotting. Representative images are shown on the left, and band intensities were quantified and semi-quantitated from three independent experiments on the right. (D) Cell lysates (1 mg) from HCC1937 and MCF7 cells were incubated with anti-NF or anti-SPRED2 antibody. The immunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western blotting using anti-SPRED2 or anti-NF antibody, respectively. Representative images are shown. $^{*}p < 0.05$ (two-tailed unpaired t-test.)

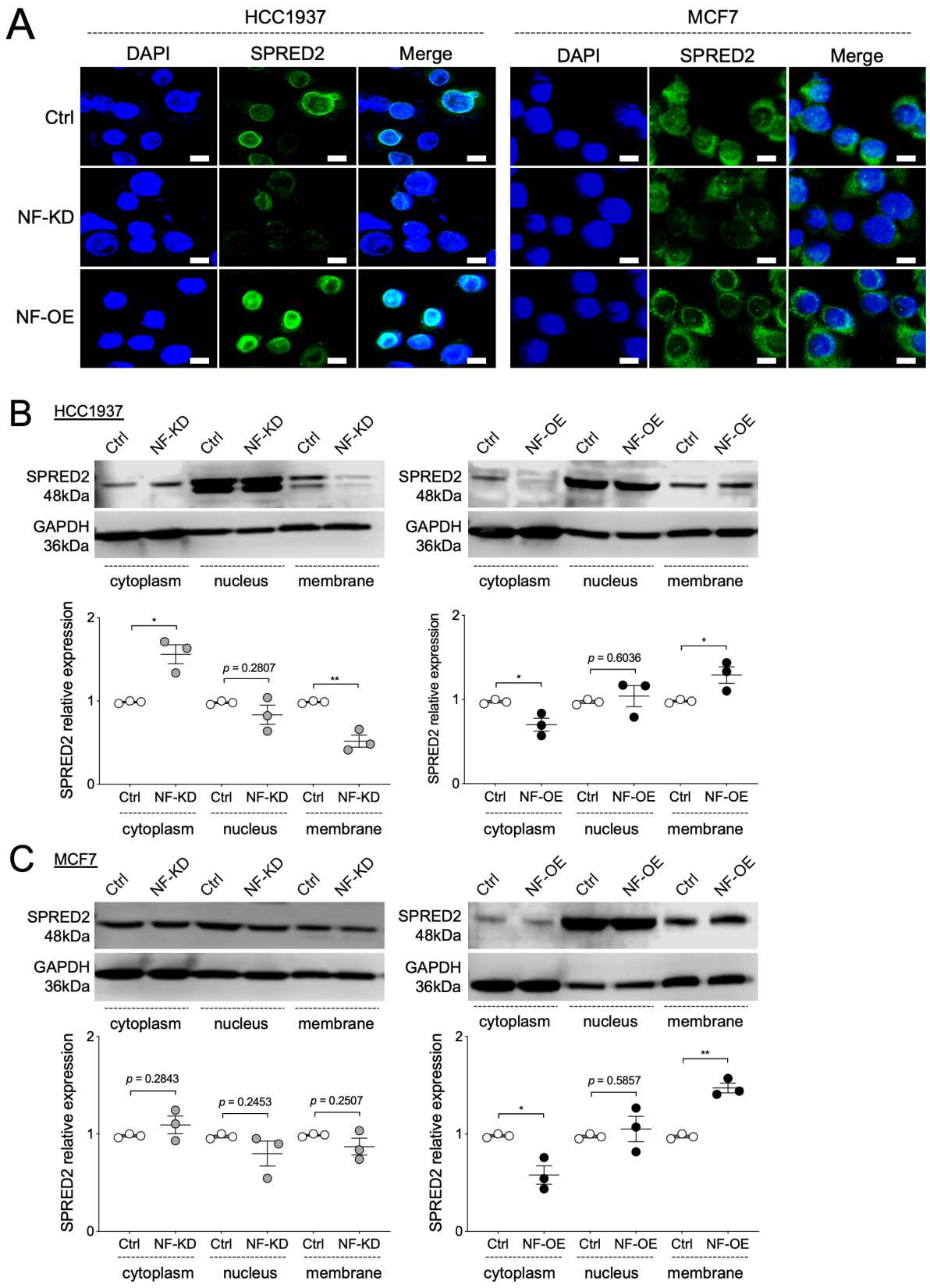
A
HCC1937
MCF7
DAPI
SPRED2
Merge
Ctrl
NF-KD
NF-OE
B
HCC1937
SPRED2
48kDa
GAPDH
36kDa
cytoplasm
nucleus
membrane
SPRED2 relative expression
p = 0.2807
p = 0.6036
C
MCF7
p = 0.2843
p = 0.2453
p = 0.2507
p = 0.5857

Figure 6. NF promotes the membrane translocation of SPRED2. (A- C) NF was knocked down (NF-KD) or overexpressed (NF-OE) in HCC1937 and MCF7 cells by transfection with control siRNA (Ctrl) or NF-specific siRNA, and with control plasmid (Ctrl) or NF-overexpression plasmid, respectively. (A) SPRED2 localization in HCC1937 (left) and MCF7 (right) cells was examined by immunofluorescence using a confocal microscope. Representative images are shown (scale bars: 10 μm). (B, C) Cytoplasmic, nuclear, and membrane protein fractions were isolated from the HCC1937 (B) and MCF7 (C) cells. The expression of each protein was analyzed by Western blotting. Representative images are shown in the upper panels, and band intensities were quantified and semi-quantitated from three independent experiments in the lower panels. $^{*}p < 0.05$, $^{**}p < 0.01$ (two-tailed unpaired t-test).

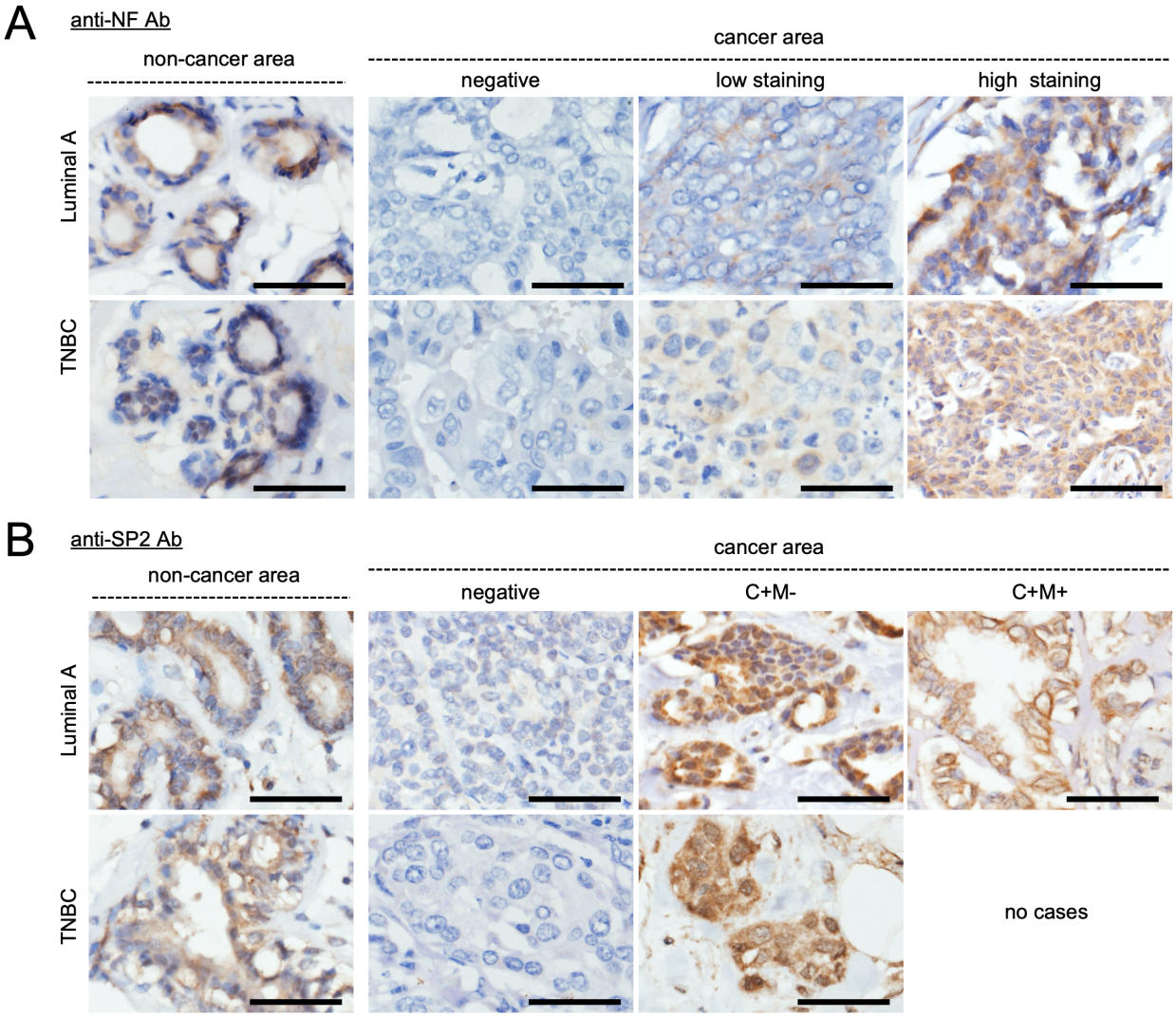

Figure 7. Expression of NF and SPRED2 in BC tissues. A total of 94 invasive BC tissue specimens were stained with anti-NF or anti-SPRED2 antibodies. Representative images of NF (A) and SPRED2 (B) staining are shown (original magnification, 400×). (A) NF expression was classified based on staining intensity as negative, low, or high. (B) SPRED2 staining was categorized as negative, cytoplasm positive (C+), membrane negative (M-), or membrane positive (M+). Scale bars: 50 μm.

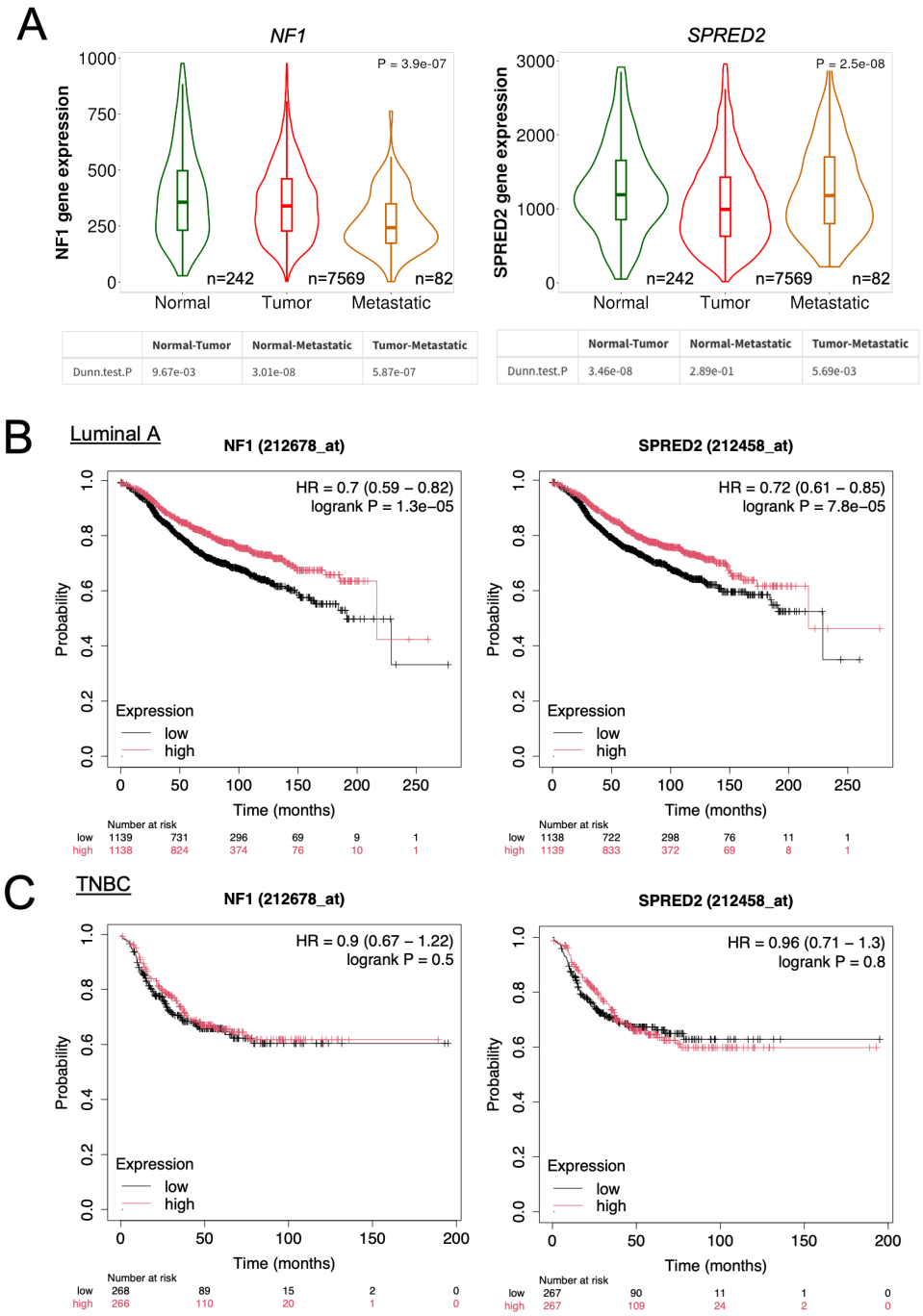

Figure 8. Database analysis of *NF1* and *SPRED2* mRNA expression and their prognostic value in BC. (A) *NF1* and *SPRED2* mRNA expression levels in normal breast tissues, primary breast cancer tissues, and metastatic tissues were obtained from TNMplot. Statistical significance was assessed using Dunn's multiple comparison test. (B, C) The prognostic significance of *NF1* and *SPRED2* mRNA expression in invasive luminal A BC (B) and TNBC (C) was evaluated using Kaplan–Meier Plotter. A log-rank p-value < 0.05 was considered statistically significant.

Table 1. NF staining in breast cancer patients

	total cases	negative	low	high	*p*
TNBC	49	21 (42.9%)	20 (40.8%)	8 (16.3%)	0.0213
Luminal A	45	10 (22.2%)	21 (46.7%)	14 (31.1%)	

p: chi-squared test

Table 2. SPRED2 staining in breast cancer patients

	total cases	C-M-	C+M-	C+M+	*p*
TNBC	49	11 (22.4%)	38 (77.6%)	0 (0%)	<0.0001
Luminal A	45	6 (13.3%)	21 (46.7%)	18 (40.0%)	

C: cytoplasm, M: membrane
p: chi-squared test

Table 3. SPRED2 staining in breast cancer patients

	NF-negative		NF-positive		
	C+M-	C+M+	C+M-	C+M+	*p*
TNBC	21	0	28	0	0.0213
Luminal A	10	0	17	18	

C: cytoplasm, M: membrane
p: chi-squared test

Table 4. Primary antibodies used in this study

Antigen	Company (Cat. number)
*Neurofibromin (*NF)	Proteintech (27249 -1-AP) [1,2]
	Santa Cruz Biotechnology (sc-376886) [1,2]
SPRED2	Proteintech (24091-1-AP) [1,2]
	Santa Cruz Biotechnology (sc-517018) [1,2]
p44/42 MAPK (ERK1/2)	Cell Signaling Technology (4695) [1]
Phospho-p44/42 MAPK (pERK1/2)	Cell Signaling Technology (4370) [1]
c-RAF (D4B3J)	Cell Signaling Technology (53745) [1]
Phospho-c-RAF (Ser338) (56A6)	Cell Signaling Technology (9427) [1]
CyclinD1	Cell Signaling Technology (92G2) [1]
GAPDH	Cell Signaling Technology (5174) [1]
Estrogen Receptor α (D6R2W)	Cell Signaling Technology (13258) [1]
Progesterone Receptor A/B (D8Q2J)	Cell Signaling Technology (8757) [1]
HER2/ErbB2 (29D8)	Cell Signaling Technology (2165) [1]
Mouse IgG Isotype control	Cell Signaling Technology (14269S) [1]
Normal Rabbit IgG	Cell Signaling Technology (5415S) [1]
HRP-goat anti-rabbit IgG	Cell Signaling Technology (7074) [1,2]
HRP-anti-mouse IgG	Cell Signaling Technology (7076) [1,2]
Goat anti-Rabbit IgG, Alexa Fluor 488 conjugated	Thermo Fisher Scientific (A-11008) [1]

[1]Antibodies used for Western blotting
[2]Antibodies used for *Co-immunoprecipitation assay*
Cell signaling Technology: Danvers, MA, USA
Proteintech: Rosemont, IL, USA
Thermo Fisher Scientific: Waltham, MA, USA
Santa Cruz Biotechnology: Dallas, Texas, USA

Table 5. The clinicopathological characteristics of the patients with invasive breast cancer

Characteristics		n	(%)
Age	≤49	29	30.85
	≥50	65	69.15
Tumor size	pT1 (≤5mm)	6	6.38
	pT1b (6-10mm)	14	14.89
	pT1c (10-20mm)	46	48.94
	pT2 (20-50mm)	24	25.53
	pT3 (>50mm)	4	4.25
Lymph node metastasis	pN0	69	73.40
	pN1	15	15.96
	pN2	3	3.19
	pN3	5	5.32
	not accessible	2	2.13
Histologic grade	Grade 1 and 2	52	55.32
	Grade 3	42	44.68
Estrogen receptor	Negative	49	52.13
	Positive	45	47.87
Progesterone receptor	Negative	49	52.13
	Positive	45	47.87
Her2 overexpression	Negative	94	100
	Positive	0	0
Ki67 index	≤20%	51	54.26
	>20%	43	45.74
Intrinsic subtypes	Luminal A	45	47.87
	Triple negative	49	52.13
Histologic type	No special type (NST)	94	100
	Invasive lobular carcinoma	0	0
	Mucinous carcinoma	0	0
	Invasive micropapillary	0	0
	Other special types	0	0
Outcome	Disease free survival[1]	70	74.47
	Overall survival[2]	74	78.72
	Death	19	15.79

[1]Median follow up for disease free survival was 2607 days.

[2]Median follow up for overall survival was 3681 days.