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Supplemental Information

Timeless-Stimulated miR-5188-FOXO1/β-Catenin-c-Jun Feedback Loop Promotes Stemness via Ubiquitination of β-Catenin in Breast Cancer

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Supplemental Figures

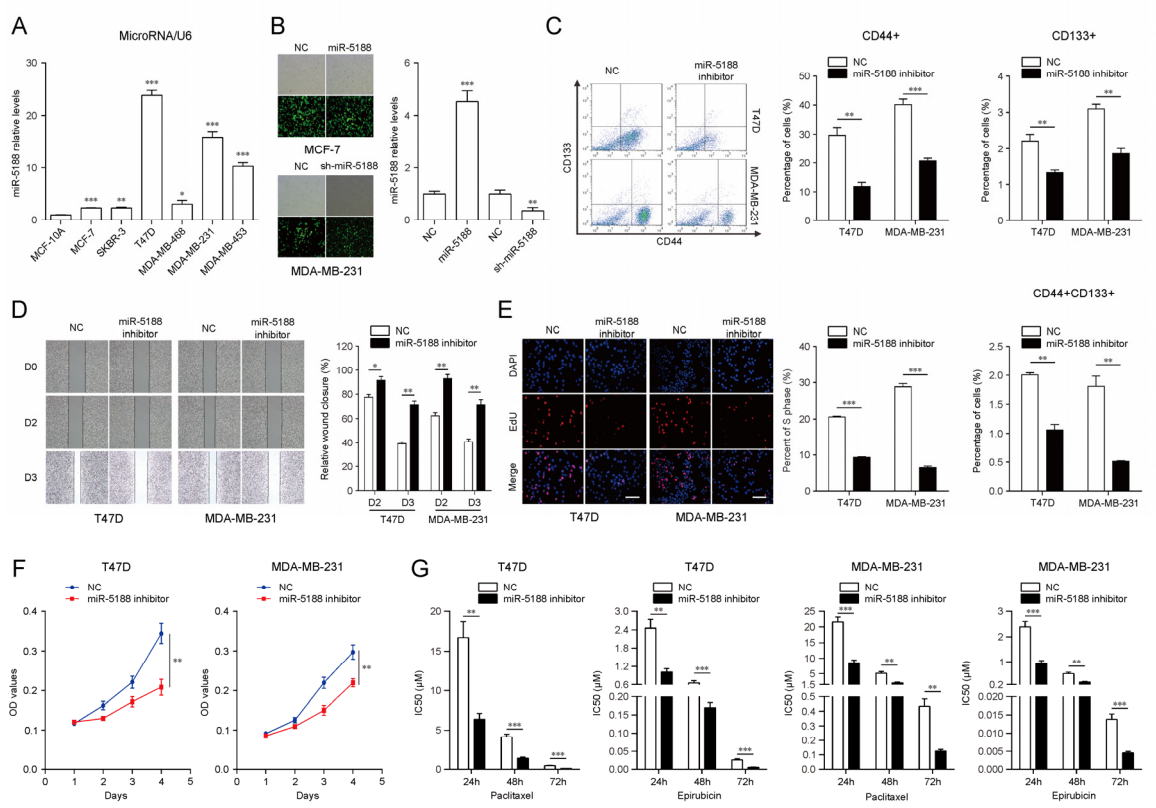

Figure S1. miR-5188 knockdown suppresses breast cancer stemness, metastasis, proliferation, and chemoresistance. (A) qRT-PCR analysis of miR-5188 expression in MCF-7, SKBR-3, T47D, MDA-MB-468, MDA-MB-231, MDA-MB-453, and MCF-10A cells. (B) miR-5188 expression in breast cancer cells treated with lentivirus particles carrying has-miR-5188 precursor (miR-5188), miR-5188 shRNA (sh-miR-5188) or their control (NC). (C) Flow cytometry, (D) wound healing assays, (E) EdU incorporation assays (Scale bar: 100 μm), (F) MTT assays, and (G) drug sensitivity tests of miR-5188-silenced T47D and MDA-MB-231 cells, and their control cells. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

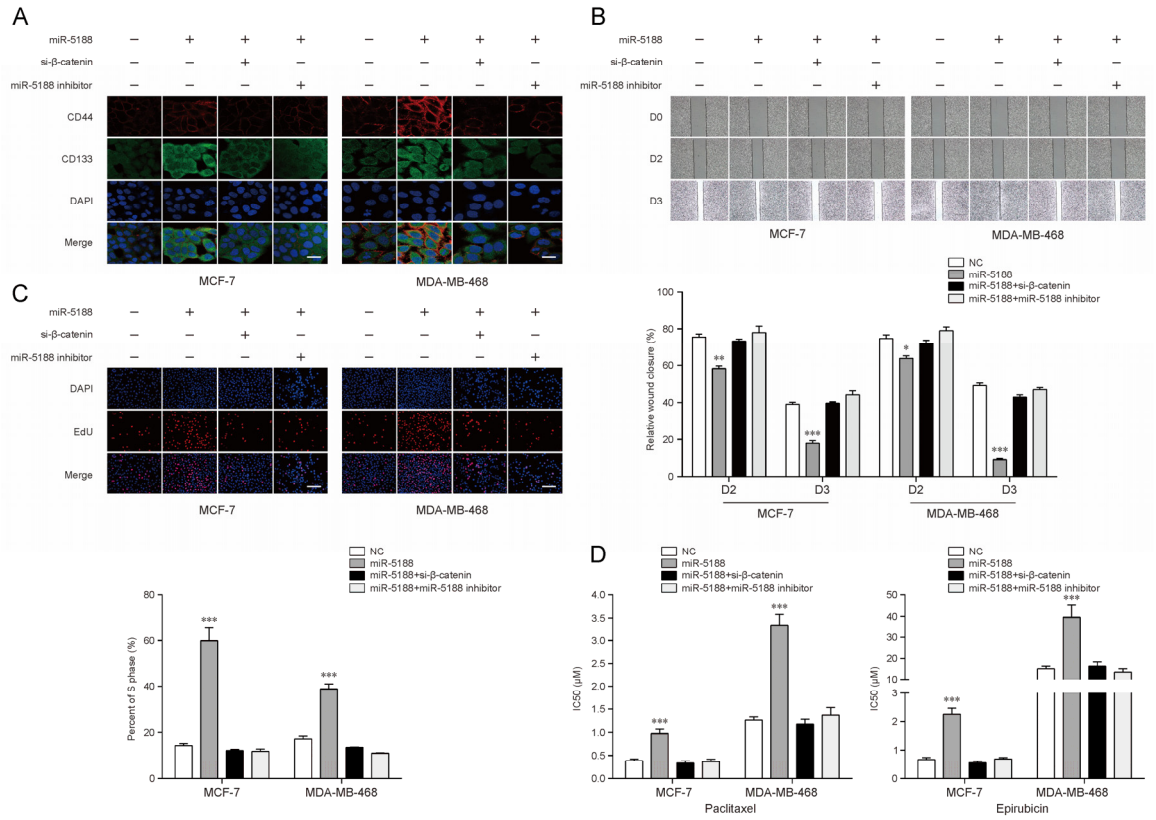

Figure S2. miR-5188 accelerates β-catenin-mediated breast cancer stemness, metastasis, proliferation, and chemoresistance. (A) Immunofluorescence analysis (Scale bar: 10 μm), (B) wound healing assays, (C) EdU incorporation assays (Scale bar: 100 μm), and (D) drug sensitivity tests of miR-5188-overexpressed breast cancer cells, miR-5188-overexpressed breast cancer cells with β-catenin knockdown, miR-5188-overexpressed breast cancer cells with miR-5188 knockdown, and their control cells. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

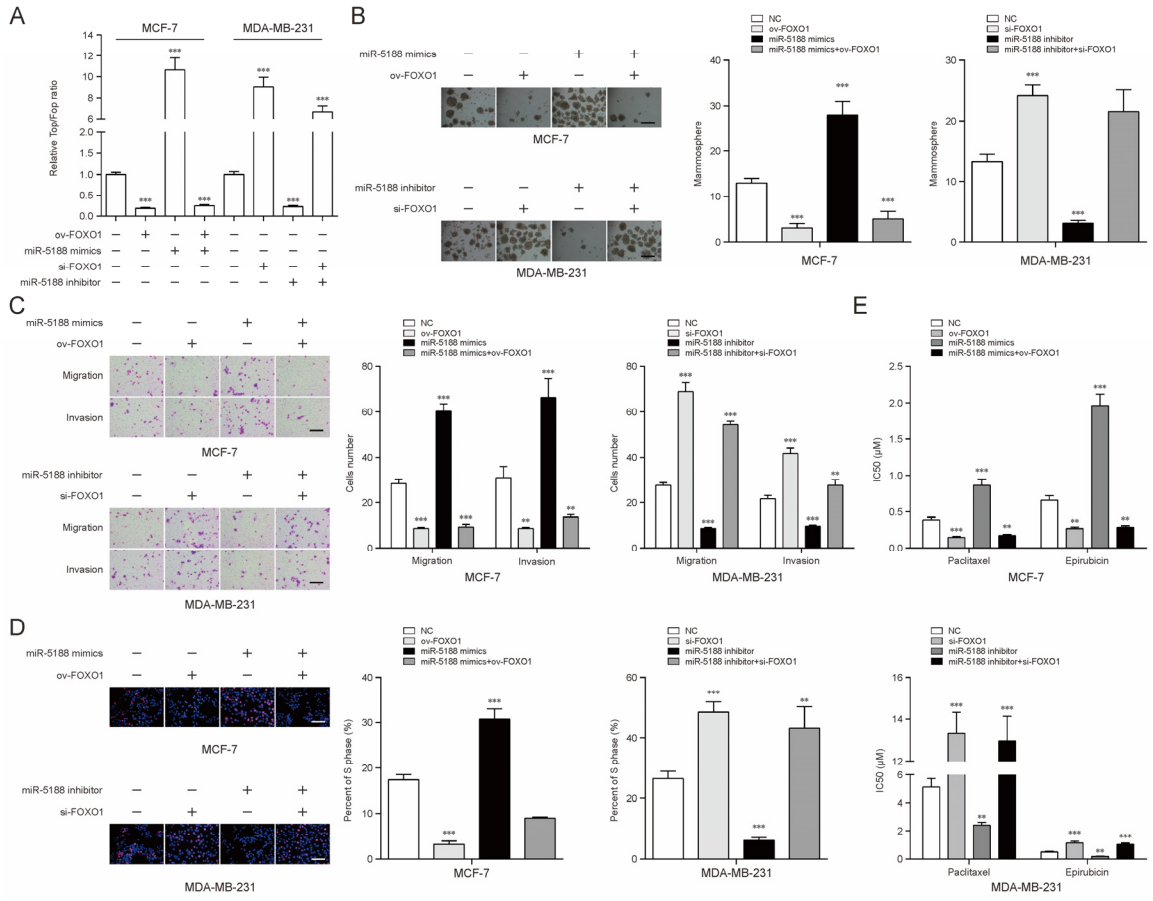

Figure S3. miR-5188 regulates FOXO1-mediated breast cancer stemness, metastasis, proliferation, chemoresistance and Wnt/β-catenin/c-Jun signaling. (A) TOP/FOP luciferase reporter assays of Wnt/β-catenin signaling activity in FOXO1-overexpressed MCF-7 cells, miR-5188-overexpressed MCF-7 cells, miR-5188-overexpressed MCF-7 cells with FOXO1 overexpression, FOXO1-silenced MDA-MB-231 cells, miR-5188-silenced MDA-MB-231 cells, miR-5188-silenced MDA-MB-231 cells with FOXO1 knockdown, and their control cells. (B) Mammosphere formation analysis (Scale bar: 40 μm), (C) transwell assays (Scale bar: 40 μm), (D) EdU incorporation assays (Scale bar: 100 μm), and (E) drug sensitivity tests of FOXO1-overexpressed MCF-7 cells, miR-5188-overexpressed MCF-7 cells, miR-5188-overexpressed MCF-7 cells with FOXO1 overexpression, FOXO1-silenced MDA-MB-231 cells, miR-5188-silenced MDA-MB-231

cells, miR-5188-silenced MDA-MB-231 cells with FOXO1 knockdown, and their control cells. $^{**}p < 0.01$; $^{***}p < 0.001$.

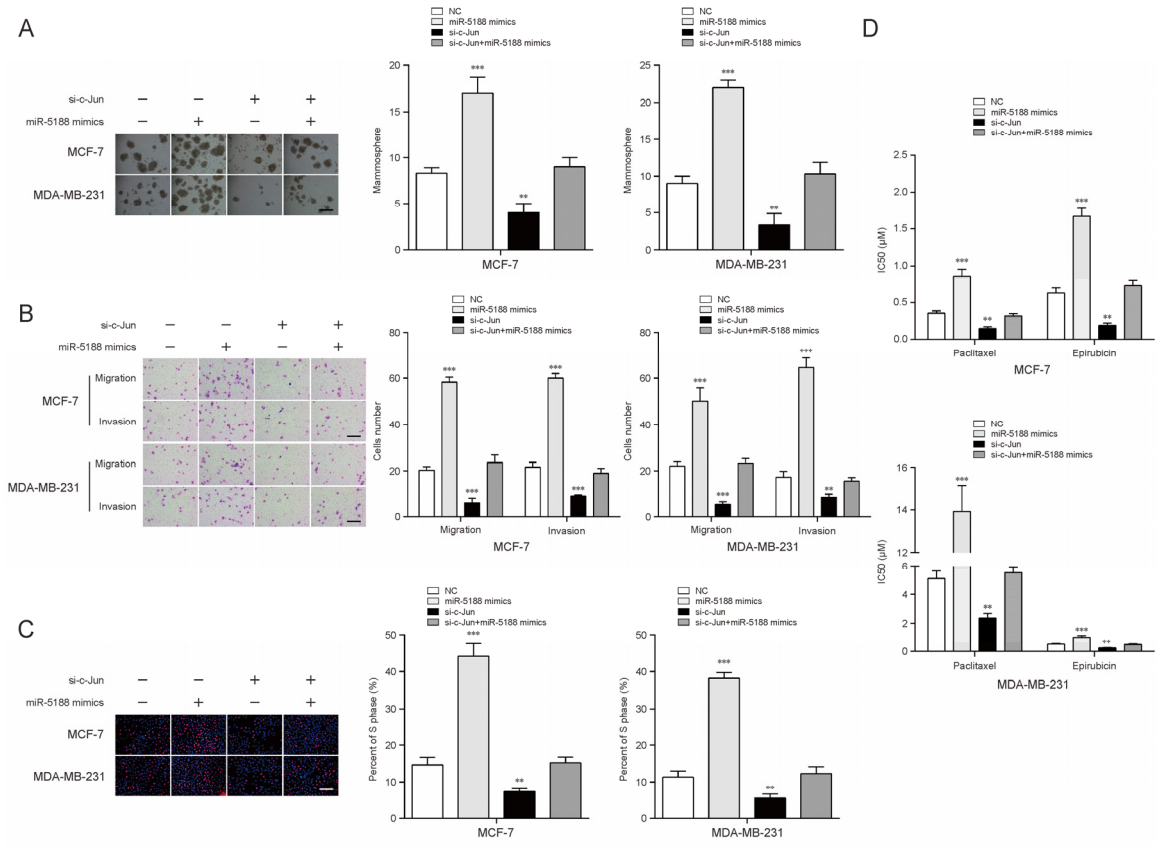

Figure S4. c-Jun knockdown impaired miR-5188-mediated breast cancer stemness, metastasis, proliferation, and chemoresistance. (A) Mammosphere formation assays (Scale bar: 40 μm), (B) transwell assays (Scale bar: 40 μm), (C) EdU incorporation assays (Scale bar: 100 μm) and (D) drug sensitivity tests of c-Jun-silenced MCF-7 and MDA-MB-231 cells, miR-5188-overexpressed MCF-7 and MDA-MB-231 cells, c-Jun-silenced MCF-7 and MDA-MB-231 cells with miR-5188 overexpression, and their control cells. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

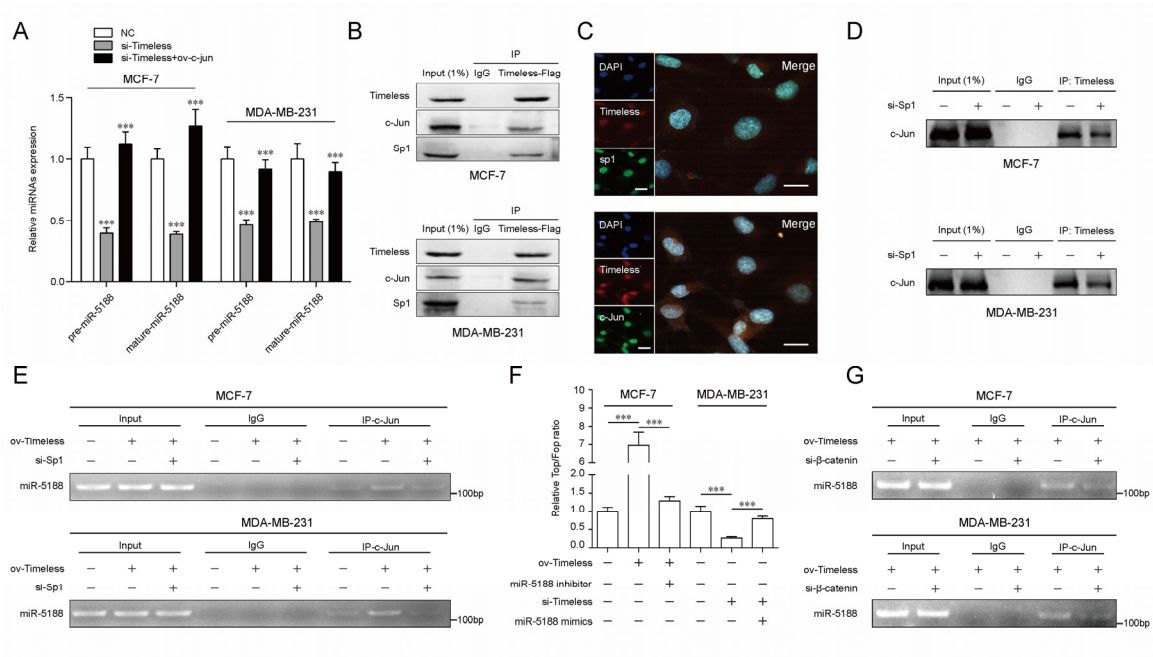

Figure S5. Timeless facilitates miR-5188-mediated breast cancer stemness, metastasis, proliferation, chemoresistance, and Wnt/β-catenin/c-Jun signaling. (A) qRT-PCR analysis of pre-miR-5188 and mature miR-5188 expression in Timeless-depleted MCF-7 and MDA-MB-231 cells, Timeless-depleted MCF-7 and MDA-MB-231 cells with c-Jun overexpression, and their control cells. (B) Exogenous and endogenous co-immunoprecipitation analysis of the interaction between Timeless and Sp1, and Timeless and c-Jun. (C) Immunofluorescence co-staining of Timeless and Sp1, and Timeless and c-Jun were performed to detect their colocalization. The fluorescence intensities along the dark arrow crossing the cytoplasm were calculated to show the colocalization of Timeless and Sp1, and Timeless and c-Jun. (D) Co-immunoprecipitation analysis of the effect of Sp1 on the interaction between Timeless and c-Jun. (E) Chromatin immunoprecipitation analysis of c-Jun binding to miR-5188 promoter in Timeless-overexpressed MCF-7 and MDA-MB-231 cells, Timeless-overexpressed MCF-7 and MDA-MB-231 cells with Sp1 knockdown, and their control cells. (F) TOP/FOP luciferase reporter assays of Wnt/β-catenin signaling activity in Timeless-overexpressed MCF-7, Timeless-overexpressed MCF-7 with miR-5188 knockdown, Timeless-silenced MDA-MB-231 cells,

Timeless-silenced MDA-MB-231 cells with miR-5188 overexpression, and their control cells. (G) Chromatin immunoprecipitation analysis of c-Jun binding to miR-5188 promoter in Timeless-overexpressed MCF-7 cells, Timeless-overexpressed MCF-7 cells with β-catenin knockdown, Timeless-silenced MDA-MB-231 cells, Timeless-silenced MDA-MB-231 cells with β-catenin overexpression, and their control cells. $^{***}p < 0.001$.

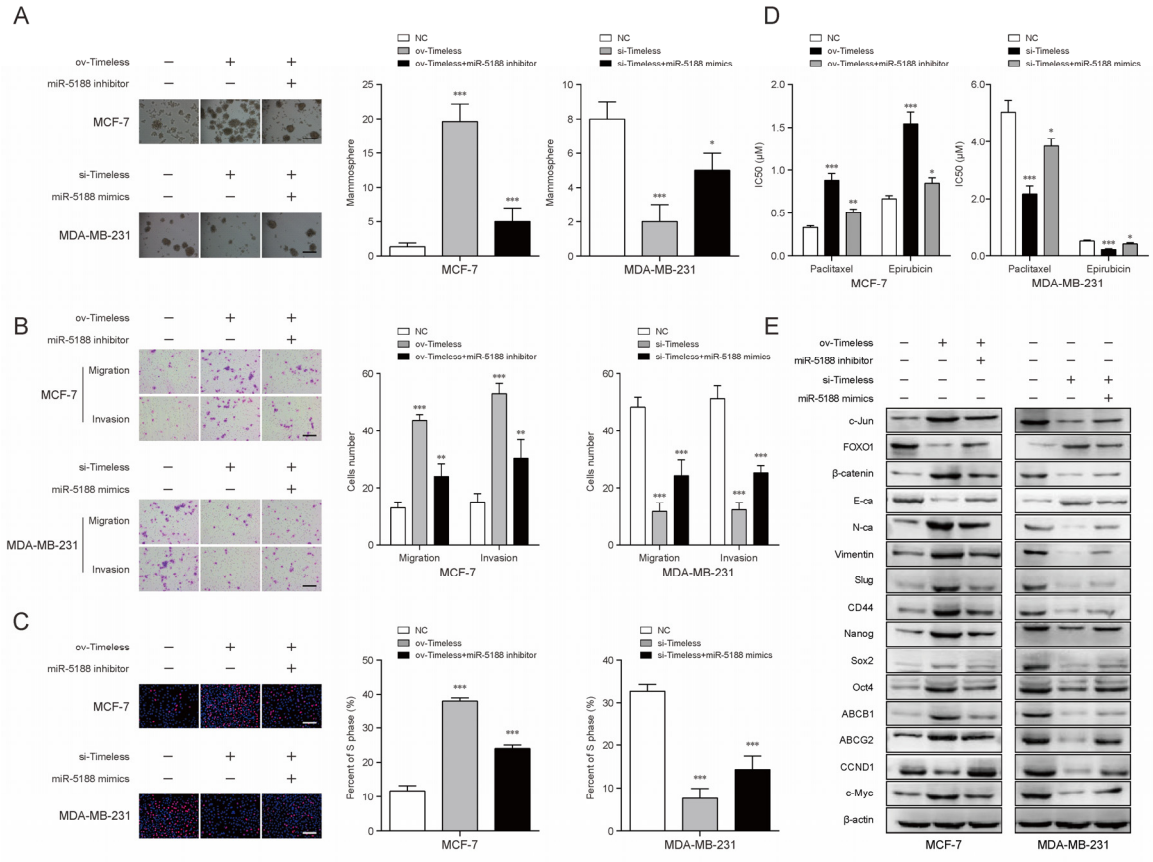

Figure S6. Timeless accelerates miR-5188-mediated breast cancer stemness, metastasis, proliferation, and chemoresistance. (A) Mammosphere formation assays (Scale bar: 40 μm), (B) transwell assays (Scale bar: 40 μm), (C) EdU incorporation assays (Scale bar: 100 μm) and (D) drug sensitivity tests of Timeless-overexpressed MCF-7 cells, Timeless-overexpressed MCF-7 cells with miR-5188 knockdown, Timeless-silenced MDA-MB-231 cells, Timeless-silenced MDA-MB-231 cells with miR-5188 overexpression, and their control cells. (E) Western blot analysis of stemness, metastasis, proliferation, chemoresistance, and Wnt/β-catenin signaling-associated proteins expression in Timeless-overexpressed MCF-7 cells, Timeless-overexpressed MCF-7 cells with miR-5188 knockdown, Timeless-silenced MDA-MB-231 cells, Timeless-silenced MDA-MB-231 cells with miR-5188 overexpression, and their control cells. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

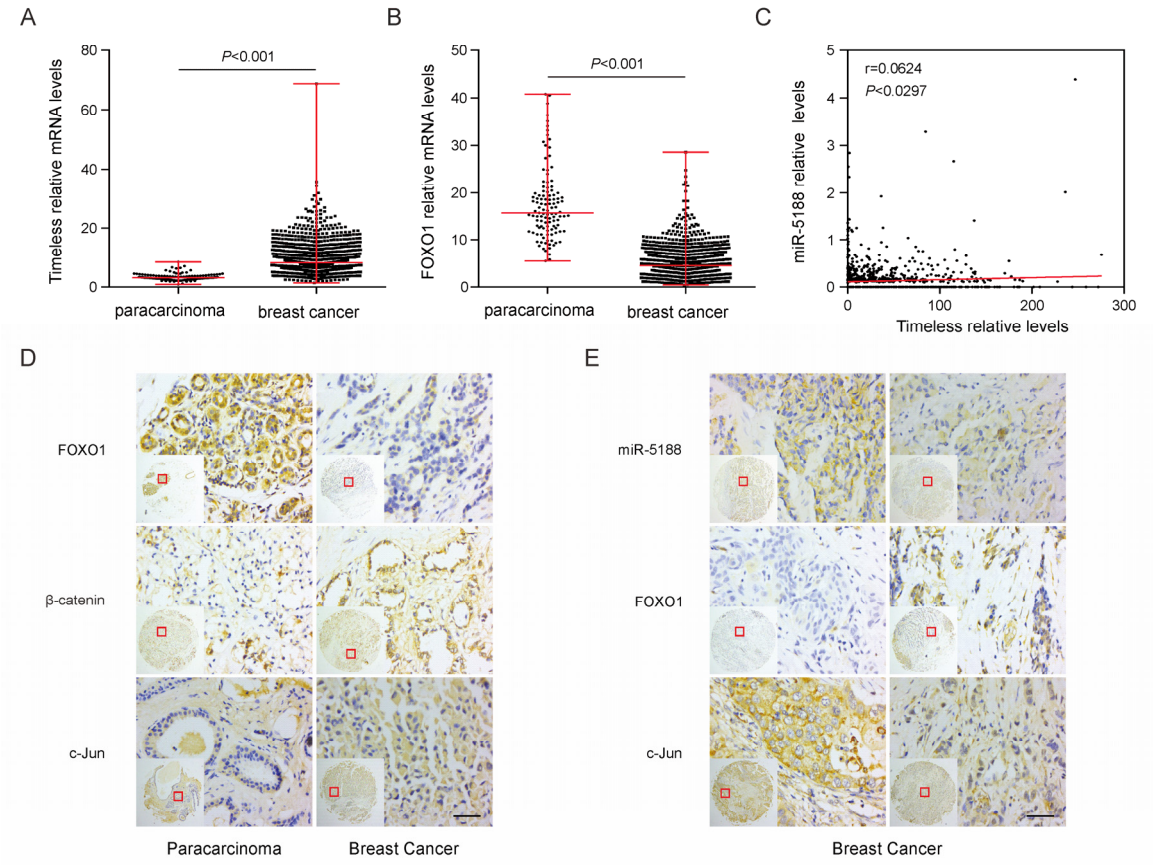

Figure S7. The bioinformatics analysis, immunohistochemical and *in situ* hybridization analysis of miR-5188, FOXO1, c-Jun, and β-catenin expression were performed in breast cancer. (A-B) Comparison of Timeless and FOXO1 expression between breast cancer and para-carcinoma tissues. (C) The relationships between Timeless and miR-5188 expression (Spearman's rank correlation test). (D) Comparison of FOXO1, c-Jun, and β-catenin expression between breast cancer tissues and para-carcinoma tissues. (E) Correlations among FOXO1, c-Jun and miR-5188 expression in breast cancer tissues (Spearman's rank correlation test). The lines indicate median values, and the whiskers indicate minimum and maximum values (A-B), Wilcoxon rank sum test. Scale bar: 40 μm.

Supplemental Tables

Table S1. Relationships between miR-5188, FOXO1, β-catenin, and c-Jun expression, and clinicopathological features of breast cancer patients

1. miR-5188 expression in breast cancer and para-carcinoma				
Group	**Cases（n）**	**miR-5188 expression**		***P* value**
		Low	High	
Breast cancer	140	53 (37.9%)	87 (62.1%)	0.002
Para-carcinoma	77	46 (59.7%)	31 (40.3%)	

2. Correlation between miR-5188 and FOXO1 expression in breast cancer					
miR5188 expression	**FOXO1 expression**		**Total**	***Kappa***	***P* value**
	Low	**High**			
Low	26 (49.1%)	27 (50.9%)	53	-0.244	0.001
High	67 (77.0%)	20 (23.0%)	87		
Total	93	47	140		

3. Correlation between c-Jun and miR-5188 expression in breast cancer					
c-Jun expression	**miR-5188 expression**		**Total**	***Kappa***	***P* value**
	Low	**High**			
Low	28 (48.3%)	30 (51.7%)	58	0.180	0.033
High	25 (30.5%)	57 (69.5%)	82		
Total	53	87	140		

4. Correlation between FOXO1 and nuclear β-catenin expression in breast cancer					
FOXO1 expression	**Nuclear β-catenin expression**		**Total**	***Kappa***	***P* value**
	Negative	**Positive**			
Low	76 (81.7%)	17 (18.3%)	93	-0.139	0.057
High	44 (93.6%)	3 (6.4%)	47		
Total	120	20	140		

5. Correlation between miR-5188 and β-catenin expression in breast cancer					
miR-5188 expression	**β-catenin expression**		**Total**	***Kappa***	***P* value**
	Low	**High**			
Low	35 (66.0%)	18 (34.0%)	53	0.152	0.055
High	43 (49.4%)	44 (50.6%)	87		
Total	78	62	140		

Table S2. The sequences used in this study.

c-Jun	1	Sense	5’ GGCACAGCUUAAACAGAAA dTdT 3’
		Antisense	3’ dTdT CCGUGUCGAAUUUGUCUUU 5’
	2	Sense	5’ CGCAGCAGUUGCAAACAUU dTdT 3’
		Antisense	3’ dTdT GCGUCGUCAACGUUUGUAA 5’
FOXO1	1	Sense	5’CUGCAUCCAUGGACAACAA dTdT 3’
		Antisense	3’ dTdTGACGUAGGUACCUGUUGUU 5’
	2	Sense	5’ CCAGAUGCCUAUACAAACA dTdT 3’
		Antisense	3’ dTdT GGUCUACGGAUAUGUUUGU 5’
β-catenin	1	Sense	5’ GAUGGUGUCUGCUAUUGUA dTdT 3’
		Antisense	3’ dTdT CUACCACAGACGAUAACAU 5’
	2	Sense	5’ GGACAAGGAAGCUGCAGAA dTdT 3’
		Antisense	3’ dTdT CCUGUUCCUUCGACGUCUU 5’
miR-5188 mimics		Sense	5‘AAUCGGACCCAUUUAAACCGGAG 3’
		Antisense	3’UUAGCCUGGGUAAAUUUGGCCUC 5’
Negative control		Sense	5’ UUUGUACUACACAAAAGUACUG 3’
		Antisense	3’ AAACAUGAUGUGUUUUCAUGAC 5’
miR-5188 inhibitor			5’ CUCCGGUUUAAAUGGGUCCGAUU 3’
Inhibitor negative control			5’ CAGUACUUUUGUGUAGUACAAA 3’
miR-5188 precursor			5’GGGAGGCAUGGAAAUUUCUCUGGUUUCAAUGGGUA CGAUUAUUGUAAGCAGGAUCCAUUCAAUAAUCGGACC CAUUUAAACCGGAGAUUUUAAAAGACAGGAAUAGAA UCCCA 3’

Table S3: **The primers used in this study.**

Primers name		Sequence (5'-3')
c-Jun	Forward	TCAGACAGTGCCCGAGATG
	Reverse	CTGCTGCGTTAGCATGAGTT
FOXO1	Forward	TGAACCGCCTGACCCAA
	Reverse	CAATGAACATGCCATCCAAG
β-catenin	Forward	GGCCCAGAATGCAGTTCGCCTT
	Reverse	AATGGCACCCTGCTCACGCA
β-actin	Forward	CTCGCTGTCCACCTTCCA
	Reverse	ACCTTCACCGTTCCAGTTTT
miR-5188		AATCGGACCCATTTAAACCGGAG
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT
Pre-miR-5188	Forward	TCTGGTTTCAATGGGTACG
	Reverse	TCTCCGGTTTAAATGGGTC
promoter of miR-5188-A	Forward	TGCGACGGAGAAAAGCC
	Reverse	GGGACCCTGACGTGAAGTT
promoter of miR-5188-B	Forward	GAGTCACCCAAGTCCCGTCCTA
	Reverse	AGCGAGCGTCCTGATCCTTC
promoter of miR-5188-C	Forward	TGCGAGATGGACGGGTCTT
	Reverse	AGGCTCAGGGAGGTTGAAGG

Table S4: A list of antibodies used for WB, RIP, ChIP, EMSA, Co-IP, ICC and IHC.

Antibodies name	Cat. No	Company	Species	Dilution
Flag	F7425	Sigma	Rabbit	1:1000 (WB); 1:20 (Co-IP)
Ki67	ab16667	Abcam	Rabbit	1:100 (IHC)
Ago2	03-110	Millipore	Mouse	1:20 (RIP)
c-Jun	9165	CST	Rabbit	1:1000 (WB); 1:50 (ChIP); 1:15 (EMSA); 1:20 (Co-IP); 1:300 (IHC)
β-catenin	8480	CST	Rabbit	1:1000 (WB); 1:100 (ICC); 1:100 (IHC)
β-catenin	2677	CST	Mouse	1:200 (ICC)
CD44	3570	CST	Mouse	1:1000 (WB)
ABCG2	42078	CST	Rabbit	1:1000 (WB)
FOXO1	2880	CST	Rabbit	1:1000 (WB); 1:20 (Co-IP); 1:100 (ICC); 1:100 (IHC)
Slug	9585	CST	Rabbit	1:1000 (WB)
Ubiquitin	3933	CST	Rabbit	1:1000 (WB)
PCNA	13110	CST	Rabbit	1:1000 (IHC)
c-Myc	10828-1-AP	Proteintech	Rabbit	1:1000 (WB)
CCND1	60186-1-Ig	Proteintech	Mouse	1:1000 (WB)
SOX2	20118-1-AP	Proteintech	Rabbit	1:1000 (WB); 1:100 (IHC)
OCT4	11263-1-AP	Proteintech	Rabbit	1:1000 (WB); 1:100 (IHC)
Nanog	14295-1-AP	Proteintech	Rabbit	1:1000 (WB); 1:100 (IHC)
ABCB1	22336-1-AP	Proteintech	Rabbit	1:1000 (WB)
E-cadherin	60335-1-Ig	Proteintech	Mouse	1:1000 (WB); 1:100 (IHC)
N-cadherin	66219-1-Ig	Proteintech	Mouse	1:1000 (WB); 1:100 (IHC)
Vimentin	10366-1-AP	Proteintech	Rabbit	1:1000 (WB); 1:1000 (IHC)
CD133	18470-1-AP	Proteintech	Rabbit	1:1000 (WB)
β-actin	6008-1-Ig	Proteintech	Mouse	1:5000 (WB)
Histone	17168-1-AP	Proteintech	Rabbit	1:1000 (WB)

Table S5. The sequences used in Electrophoretic mobility shift assay.

miR-5188	probes	wild type	5’ ACGGGTGACGTCACGACAGCCCTAGAGTCACCCAAAT TTTTGGGTCACATTT 3’
	competitors	wild type	5’ ACGGGTGACGTCACGACAGCCCTAGAGTCACCCAAAT TTTTGGGTCACATTT 3’
		site 1 mutant	5’ ACGGGCTGTTCTTCGACAGCCCTAGAGTCACCCAAAT TTTTGGGTCACATTT 3’
		site 2 mutant	5’ ACGGGTGACGTCACGACAGCCCTCATTCTTCCCAAATT TTTGGGTCACATTT 3’
		site 3 mutant	5’ACGGGTGACGTCACGACAGCCCTAGAGTCACCCAAA TTTTCAATGTTCATTT 3’
		all sites mutant	5’ ACGGGCTGTTCTTCGACAGCCCTCATTCTTCCCAAATT TTCAATGTTCATTT 3’