SUPPLEMENTAL INFORMATION

Estrogen Activates TAZ via GPER in Invasive Ductal Breast Carcinoma

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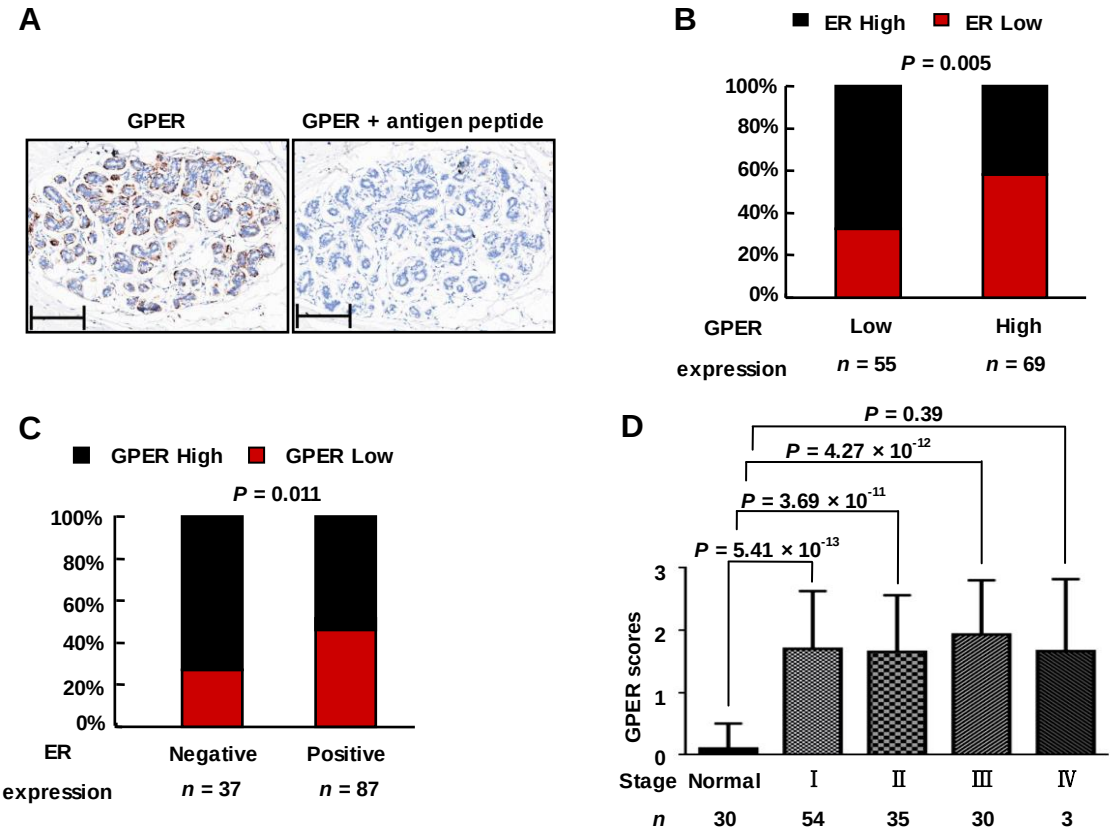
Inventory of Supplemental Information



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Statistical analysis of GPER expression levels in different stages of IDC				
Stage (*n*)	**Normal (30)**	**I (54)**	**II A & II B (35)**	**IIIA & IIIB & IIIC (30)**
I (54)	5.41×10^{-13}			
II A & II B (35)	3.69×10^{-11}	**1.00**		
IIIA & IIIB & IIIC (30)	4.27×10^{-12}	**0.79**	**0.72**	
IV (3)	**0.39**	**1.00**	**1.00**	**0.99**

Figure S1

GPER expression is elevated in invasive ductal carcinoma. (A) Characterization of the specificity of GPER antibody. For antigen competition experiments, two sections of the same breast tissue were stained with GPER antibody in the presence or absent of antigen peptides (for peptide information, see Supplement Methods). Two representative images are shown with scale bars as 50 μm. **(B)** Expression of GPER is inversely correlated with ER in IDC specimens. The 124 subjects were divided into two groups based on GPER expression scores in the tumors, representing low (scores 0, 1) and high (scores 2, 3). The percentage of ER-high expression (scores 2, 3) tumors in the two groups of subjects was analyzed using Pearson's $\chi 2$ test. **(C)** The expression of GPER is higher in ER-negative group than ER-positive group. The percentage of GPER-high expression (scores 2, 3) tumors in ER-negative (scores 0) and ER-positive (scores 1, 2, 3) groups of 124 subjects was analyzed using Pearson's $\chi 2$ test. **(D-E)** GPER expression correlates with initiation of IDC. 122 cases of IDC specimens were categorized into different stages based on WHO criterion. Statistical analysis of GPER expression between the adjacent normal breast tissues and the IDC specimens of different stages was performed by one-way ANOVA test with Games-Howell correction.

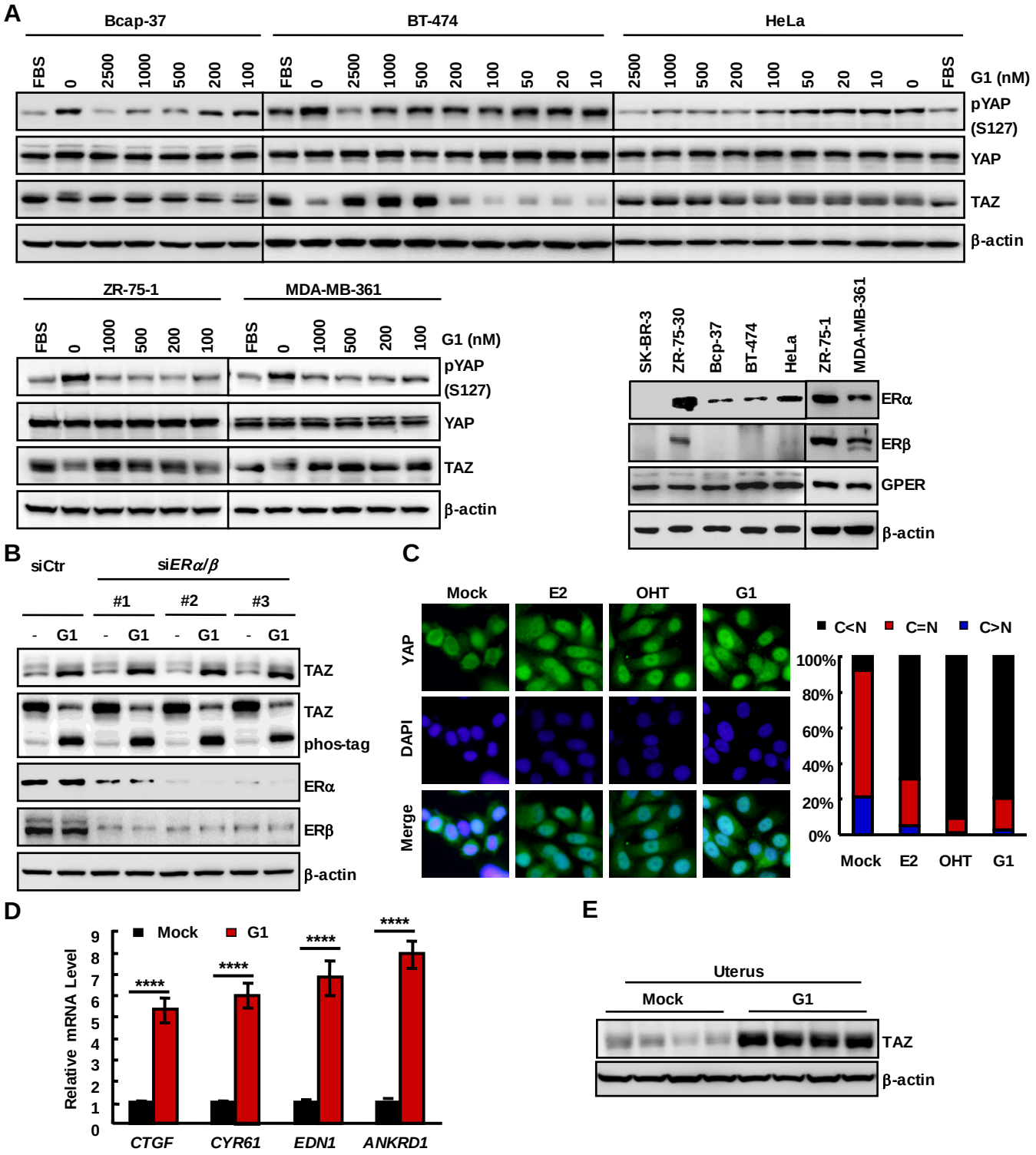

Figure S2

GPER Stimulation of GPER activates YAP/TAZ. (A) G1 induces YAP dephosphorylation and TAZ accumulation in a dose dependent manner in multiple cells. Serum-starved cells were treated with G1 at various concentrations for 2 hrs and subjected to immunoblotting with the indicated antibodies. The expression of ERs and GPER in all the cells used were detected. **(B)** ER is not involved in G1-inducd TAZ activation. ERα/β were silenced in ZR-75-30 cells by specific siRNAs. 40 hrs after transfection, cells were serum-starved for 8 hrs and stimulated with G1 for 2 hrs. Immunoblotting was performed to detect the knockdown efficiency, TAZ protein level and TAZ phosphorylation. **(C)** GPER agonists stimulate YAP nuclear localization. SK-BR-3 cells were serum-starved for 8 hrs and then stimulated with 100nM β-estradiol, 200 nM OHT or 100 nM G1 for 2 hrs. Immunofluorescence staining was performed to determine YAP (green) subcellular localization; Quantifications are shown in the right panel. **(D)** GPER activation induces the expression of YAP/TAZ target genes. Serum starved ZR-75-30 cells were treated with mock or G1 as indicated. The mRNA levels of *CTGF*, *CYR61*, *EDN1* and *ANKRD1* were determined by qPCR, data are represented as mean ± SD, $n = 3$, by Student's t test, **** denotes $P < 0.0001$. **(E)** G1 stimulates TAZ protein accumulation in uterus tissue. Mice were injected with G1 as described in Supplemental Methods and subjected to immunoblotting as indicated. Data are representative of at least 3 independent experiments.

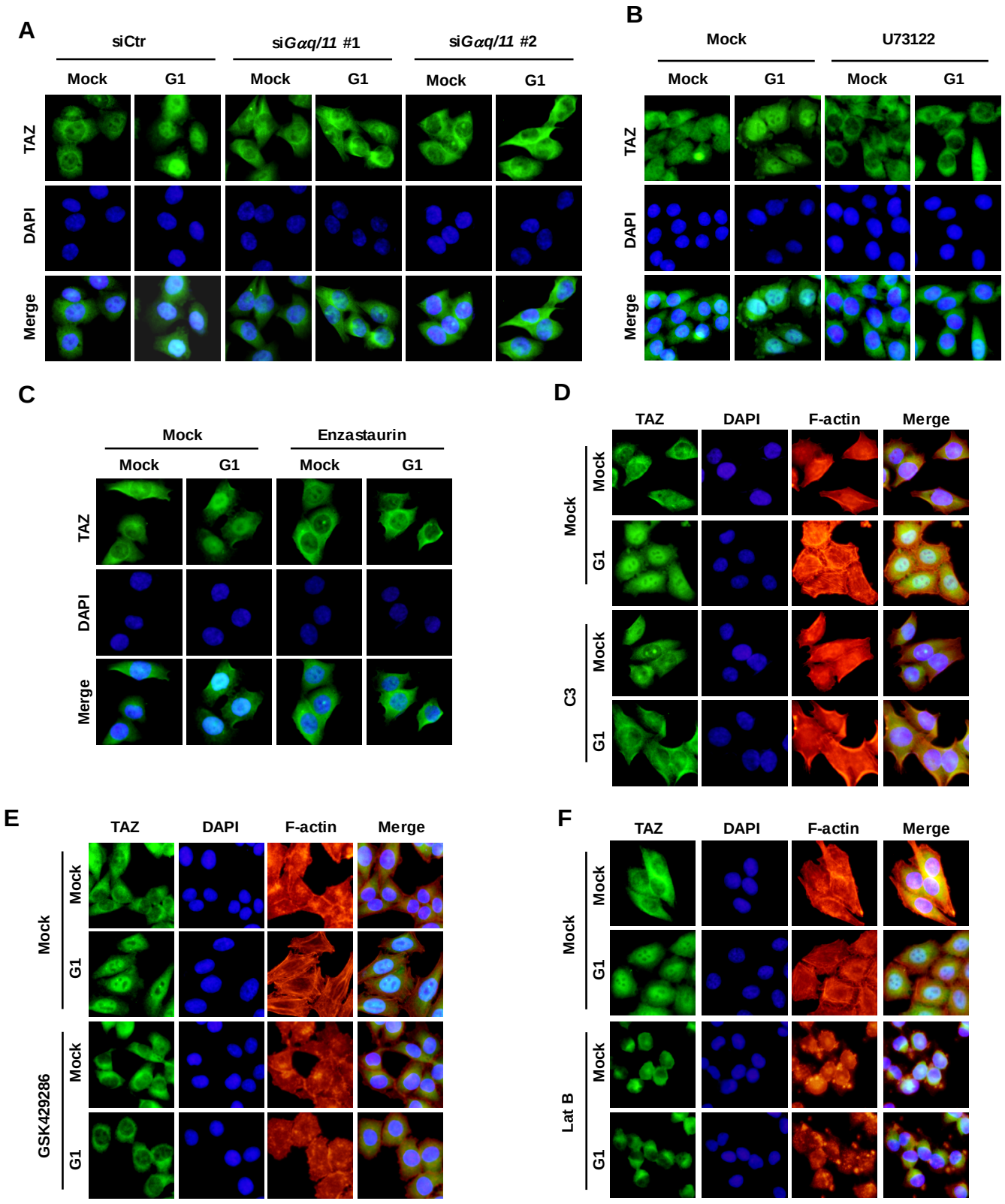

Figure S3

GPER acts through Gαq/11, PLCβ-PKC and Rho-ROCK to stimulate TAZ. (A) Gαq/11 is required for G1 to activate TAZ. ZR-75-30 cells were transfected with control or *Gαq/11* siRNA. Immunofluorescence staining was carried out to detect the subcellular localization of TAZ (green); DAPI (blue) was used for cell nuclei. **(B-E)** PLCβ, PKC, Rho and ROCK are required for G1-induced TAZ nuclear localization. Serum-starved ZR-75-30 cells were pretreated with PLCβ inhibitor (U73122), PKC inhibitors (Enzastaurin), Rho inhibitor (C3) or ROCK inhibitor (GSK429286) for 4 hrs and then stimulated with 100 nM G1 for 2 hrs. Subcellular localization of endogenous TAZ was detected by immunofluorescence staining. **(F)** Disruption of actin cytoskeleton blocks G1-induced TAZ activation. Serum-starved ZR-75-30 cells were pretreated with Lat B for 15 min and then stimulated with 100 nM G1 for 2hrs. Immunofluorescence staining is performed to detect subcellular localization of TAZ. Data are representative of at least 3 independent experiments.

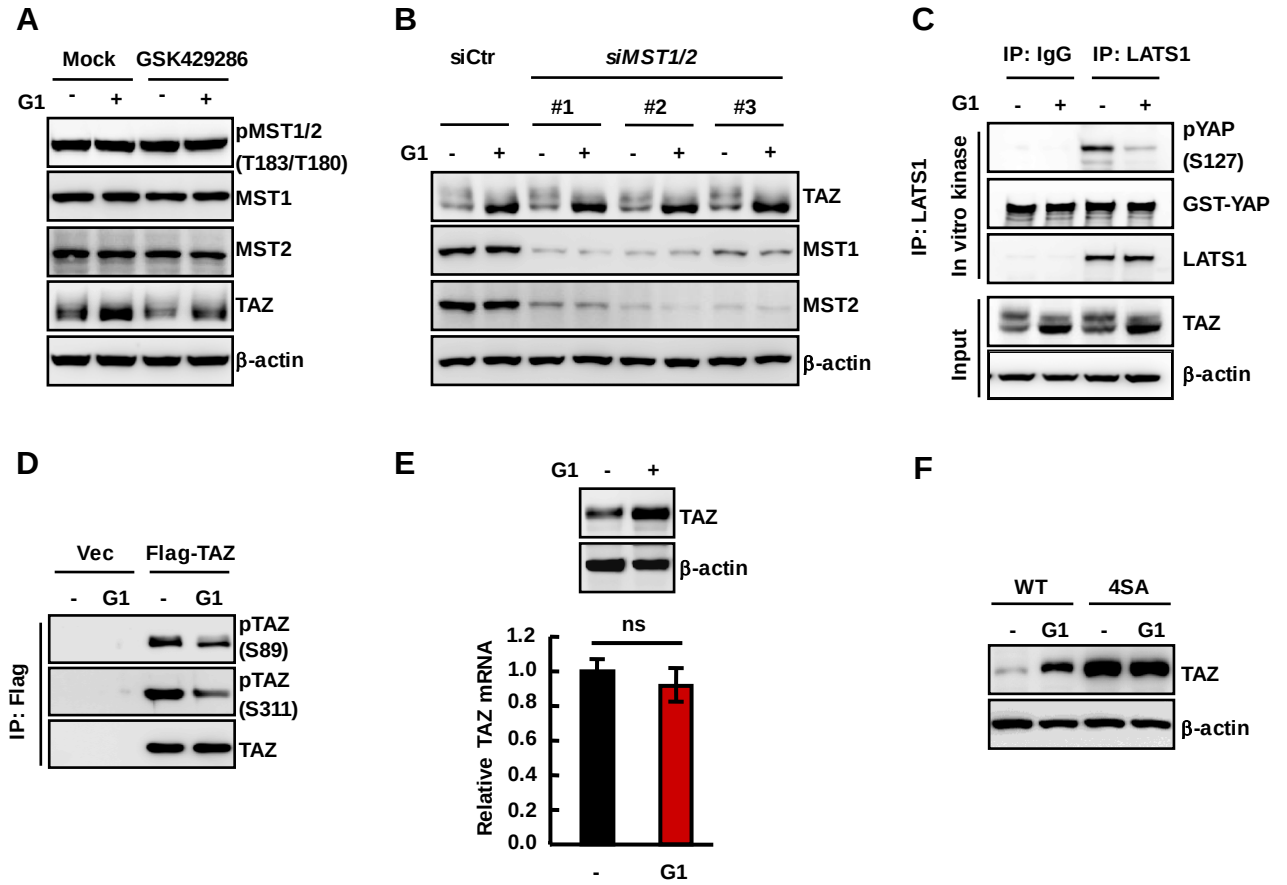

Figure S4

GPER activates TAZ via LATS inhibition. (A-B) MST is not required for G1-induced TAZ accumulation. Serum-starved ZR-75-30 cells were pretreated with 200 nM GSK429286 for 4 hrs, followed by treatment with 100 nM G1 for 2hrs (A); *MST1/2* were knocked-down by three independent siRNAs in ZR-75-30 cells. These cells were stimulated with 100 nM G1 as indicated (B). Immunoblotting was performed. **(C)** LATS1 kinase activity is inhibited by G1. ZR-75-30 cells were treated with 100 nM G1 as indicated. The immunoprecipitated LATS1 was subjected to in vitro kinase assay. YAP phosphorylation was detected by phospho-YAP(S127) antibody. **(D)** Phosphorylation of TAZ at Ser89 and Ser311 is inhibited by G1 treatment. ZR-75-30 cells transfected with Flag-TAZ or Vector were serum-starved for 8 hrs and stimulated with 100 nM G1 for 2 hrs. TAZ was immunoprecipitated and the phosphorylation of TAZ at Ser89 and Ser311 was assessed. **(E)** G1 has no effect on TAZ transcription level. ZR-75-30 cells were serum starved for 8 hrs and then treated with 100 nM G1 for 2 hrs. The cells were divided into two parts and used for immunoblotting (top panel) and qPCR (bottom panel), respectively. Data are represented as mean ± SD, $n = 3$, by Student's t test, ns denotes no significance. **(F)** TAZ^{4SA} mutant is resistant to G1 stimulation. ZR-75-30 cells stably expressed Flag-TAZ^{WT} or Flag-TAZ^{4SA} were serum-starved for 8 hrs and stimulated with 100 nM G1 for 2 hrs. Data are representative of at least 3 independent experiments.

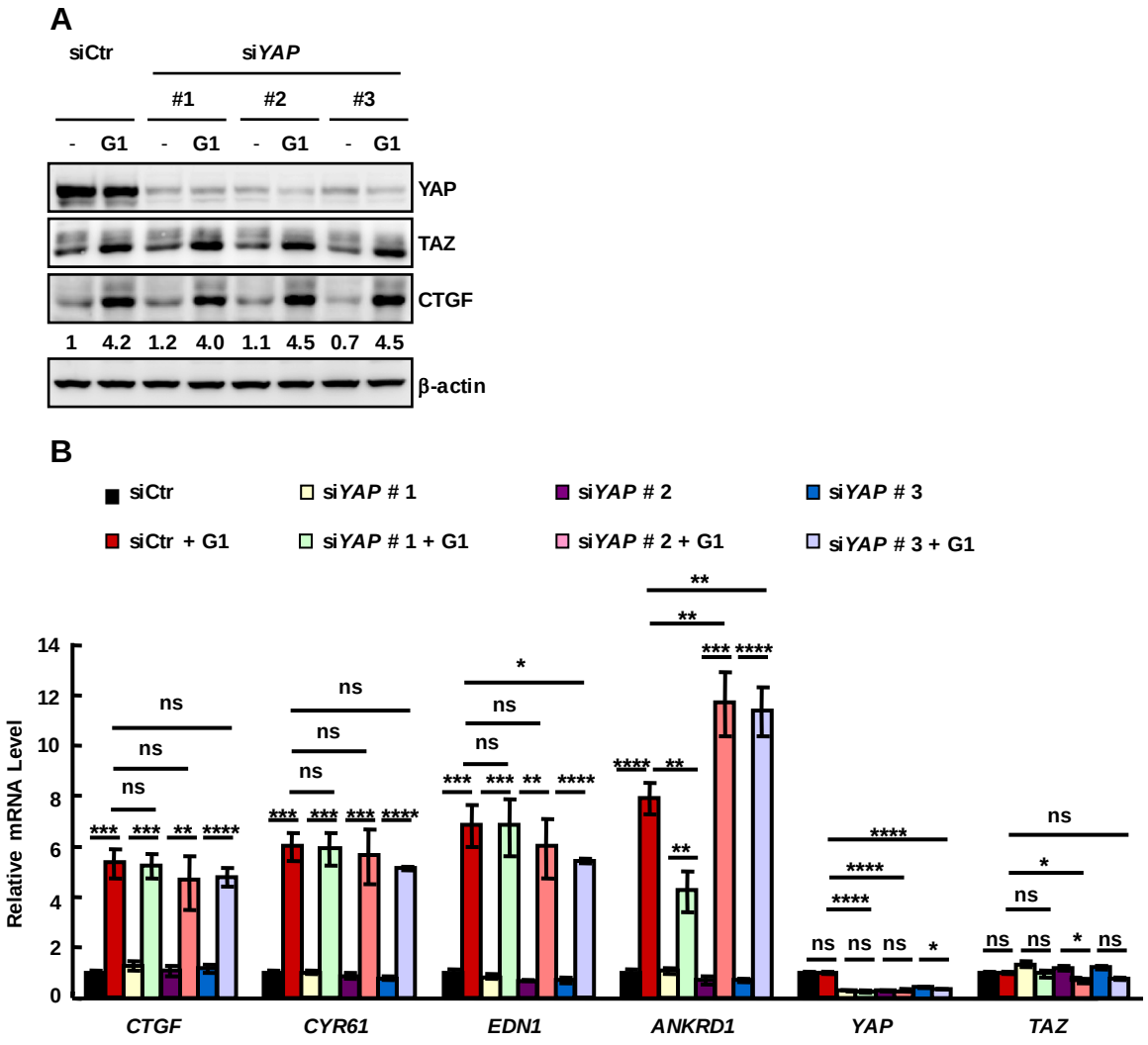

Figure S5

YAP/TAZ mediate the physiological functions of GPER activation in target gene induction and cell migration. (A-B) YAP is not involved in G1-induced gene transcription. ZR-75-30 cells were transiently transfected with control or *YAP* siRNAs. Serum starved cells were treated with 100 nM G1 for 2 hrs as indicated. Protein levels of CTGF and the knockdown efficiency of YAP were determined by immunoblotting. The mRNA levels of the indicated target genes were measured by qPCR. Data are represented as mean ± SD, $n = 3$. Data was analyzed by one-way ANOVA test with LSD correction. * denotes $P < 0.05$; ** denotes $P < 0.01$; *** denotes $P < 0.001$; **** denotes $P < 0.0001$; ns stands for no significance. The blots shown are representative of at least 3 independent experiments.

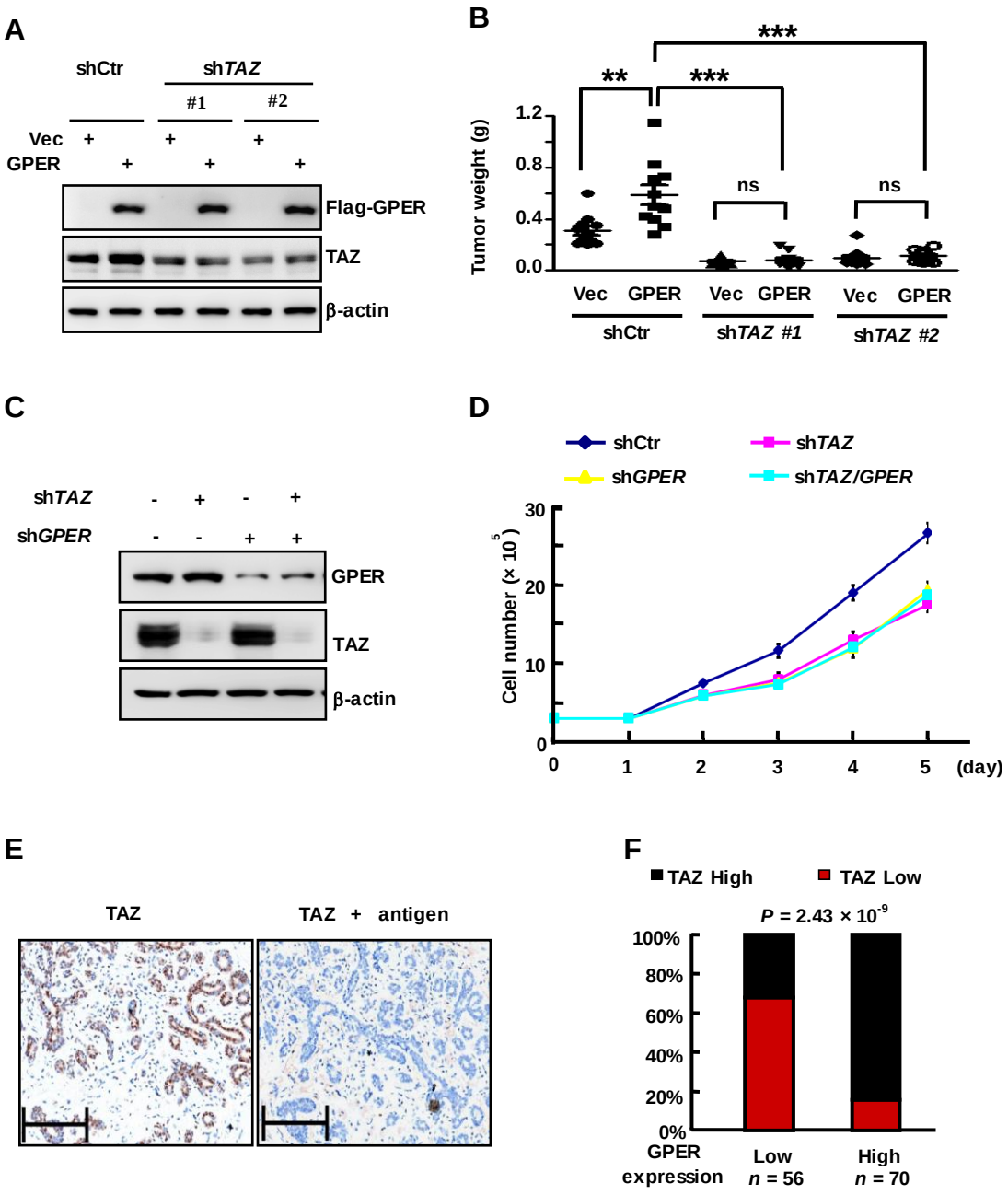

Figure S6

TAZ mediates the tumorigenic effect of GPER and its expression correlates with GPER in invasive ductal carcinomas. (A) *TAZ* knockdown in ZR-75-30 cells. GPER-expressing ZR-75-30 cells were infected with scramble or sh*TAZ* retroviruses. Expression of GPER and TAZ were determined by immunoblotting. **(B)** TAZ is required for GPER to promote tumor growth in a xenograft mouse model. Xenograft was performed as described in Figure 6B. Tumor weight was measured for the indicated groups. The *P* value was calculated by one-way ANOVA test with LSD correction and shown as dot plot (** denotes $P < 0.01$; *** denotes $P < 0.001$; ns stands for no significance). $n = 11$ in each group. **(C-D)** No synergistic effect of GPER and TAZ on ZR-75-30 cell. *TAZ* or/and *GPER* were knocked down in ZR-75-30 cells and cell growth was determined (D). Error bars represent cell numbers ± SD for triplicate experiments. The knockdown efficiency was shown (C). **(E)** Characterization of the TAZ antibody for its application in IHC. Immunohistochemical staining was performed in normal breast tissue with TAZ-specific antibody. To validate the specificity, antibody to TAZ was incubated with recombinant His-TAZ protein for 1 hr prior to immunohistochemical staining. Scale bars, 50 μm. **(F)** The percentage of TAZ high expression tumors in $GPER^{high}$ (scores 2, 3) or $GPER^{low}$ (scores 0, 1) groups of subjects were analyzed using Pearson's $\chi 2$ test. Data are representative of at least 3 independent experiments.

Table S1. Association between GPER intensity and various clinicopathologic variables in invasive ductal carcinoma

Factor	Patient	GPER intensity		*P**
		Low	High	
	n	*n (%)*		
All	126			
Node status				
Negative	72	37(51%)	35(49%)	0.057
Positive	50	17(34%)	33(66%)	
Missing	4			
Tumor size				
≤20mm	88	43(49%)	45(51%)	0.219
＞20mm	33	12(36%)	21(64%)	
Missing	5			
Histological grade				
1	40	17(43%)	23(57%)	0.920
2	78	35(45%)	43(55%)	
3	8	4(50%)	4(50%)	
Missing				

Factor	Patient	GPER intensity		*P**
		Low	High	
	n	*n (%)*		
TNM stage				
Ⅰ	54	27(50%)	27(50%)	0.428
Ⅱ	35	16(46%)	19(54%)	
Ⅲ	30	10(33%)	20(67%)	
Ⅳ	3	2(67%)	1(33%)	
Missing	4			
ER				
Low	58	18(31%)	40(69%)	0.005
High	66	37(56%)	29(44%)	
Missing	2			
PR				
Low	83	33(40%)	50(60%)	0.143
High	41	22(54%)	19(46%)	
Missing	2			
HER2				
Low	65	29(45%)	36(55%)	0.866
High	58	25(43%)	33(57%)	
Missing	3			

*****Chi-square test

Table S2. Summary of quantification for TAZ protein levels

Fig. \ lane	1	2	3	4	5	6	7	8	9	10
2B	1	1.08 ± 0.13	1.56 ± 0.13	2.51 ± 0.18	3.44 ± 0.21					
2C	1	3.36 ± 0.22	0.94 ± 0.06	1.53 ± 0.07	1.01 ± 0.04	1.46 ± 0.05	0.96 ± 0.12	1.39 ± 0.13		
2D	1	3.40 ± 0.07	0.81 ±0.14	1.01 ± 0.09						
2G	1	3.78 ± 0.01	0.07 ± 0.01	0.07 ±0.01	0.08 ±0.01					
3A	1	3.15 ± 0.08	1.02 ± 0.13	1.13 ± 0.15	0.99 ± 0.09	1.08 ± 0.21				
3B	1	3.17 ± 0.21	0.80 ± 0.06	0.86 ± 0.08	1	3.29 ± 0.17	0.82 ± 0.03	1.09 ± 0.19		
3C	1	3.43 ± 0.08	0.92 ± 0.05	1.00 ± 0.07	1	3.44 ± 0.48	0.81 ± 0.32	0.91 ± 0.16		
3D	1	3.57 ± 0.15	0.83 ± 0.12	0.92 ± 0.09	1	3.35 ± 0.14	0.93 ± 0.09	0.94 ± 0.19	0.98 ± 0.10	0.99 ± 0.08
3E	1	3.45 ± 0.33	0.91 ± 0.02	1.40 ± 0.15	1	3.31 ± 0.05	0.78 ± 0.09	0.91 ± 0.06		
3F	1	3.33 ± 0.29	0.51 ± 0.14	0.64 ± 0.16	1	3.24 ± 0.01	0.89 ± 0.02	1.09 ± 0.07		
4B	1	3.80 ± 0.48	3.21 ± 0.68	3.97 ± 0.47	2.85 ± 0.45	3.89 ± 0.32	2.92 ± 0.46	3.72 ± 0.63		
5A	1	3.42 ± 0.18	0.47 ± 0.04	0.60 ± 0.06	0.48 ± 0.07	0.62 ± 0.07	0.47 ± 0.06	0.61 ± 0.09		
5C	1	3.47 ± 0.15	0.96 ± 0.01	3.20 ± 0.15	0.96 ± 0.08	3.42 ± 0.12				

SUPPLEMENTAL METHODS

Chemicals

The following chemicals were used in this study: E2 (β-Estradiol), OHT (4-Hydroxytamoxifen), U-73122, Chelerythrin chloride, Latrunculin B, Cytochalasin D and Tetramethylrhodamine B isothiocyanate-conjugated Phalloidin were purchased from Sigma-Aldrich. G1 and G15 were from Cayman Chemical. ET-18-OCH3 was purchased from Millipore. Enzastaurin, GSK429286 and Y27632 were obtained from Selleckchem. C3 was from Cytoskeleton Inc. Phos-tag conjugated acrylamide was purchased from Wako chemicals. DAPI was purchased from Invitrogen. Liquid DAB + Substrate Chromogen System used in IHC was obtained from Dako. The antigen peptides with the sequence as MDVTSQARGVGLEMYPGTAQPAAPNTTSPELNL SHPLLGTALANGTGELSEHQQYVIGLFLS were purchased from Shanghai GL Biochem Ltd..

Antibodies

Antibodies for YAP (#4912), Phospho-YAP(S127) (#4911), MST1 (#3682), MST2 (#3952), Phospho-MST1(T183)/MST2(T180) (#3681), Phospho-LATS1(S909) (#9157) were purchased from Cell Signaling Technology. The LATS1 (A300-477A), LATS2 (A300-479A) and YAP (A302-308A) antibodies used in immunoprecipitation were from Bethyl Laboratories. GPER (HPA027052) and TAZ (HPA007415) antibodies were obtained from Sigma-Aldrich. Anti-TEF-1 (610922) was purchased from BD Transduction Laboratories. Antibodies for Gαq/11 (SC392),

14-3-3θ(SC732), CTGF (SC14939), Phospho-TAZ(S89) (SC17610) and HA (SC7392) were from Santa Cruz Biotechnology. Anti-β-actin (A00702) and anti-Flag were purchased from GeneScript. Phospho-TAZ(S311) was generated in rabbits by Shanghai Genomics. Anti-rabbit detection reagent (HRP) used in IHC was obtained from Shanghai Changdao Biotech Co., Ltd.. All antibodies were used according to the manufacturer recommended dilutions in immunoblotting and immunofluorescence staining analysis.

Plasmids

GPER plasmid was purchased from Missouri S&T cDNA Resource Center and sub-cloned into pQCXIH vector. The oligos targeting *GPER* or *TAZ* were annealed following the protocol from Addgene and cloned into pMKO.1 vector at AgeI and EcoRI sites. The sequences of all shRNAs used in this study were shown below:

sh*GPER* #1:**CCGGCTCCCTCATTGAGGTGTTCAACTCGAGTTGAACACCTCAATGAGGGAGTTTTTTG**

sh*GPER* #2:**CCGGCGCTCCCTGCAAGCAGTCTTTCTCGAGAAAGACTGCTTGCAGGGAGCGTTTTTTG**

sh*GPER* #3:**CCGGCTTCAGCGAATCTCACTCCTTCTCGAGAAGGAGTGAGATTCGCTGAAGTTTTTTG**

sh*TAZ* #1: **CCGGCCAGGAACAAACGTTGACTTACTCGAG TAAGTCAACGTTTGTTCCTGGTTTTTG**

sh*TAZ* #2: **CCGGGCGTTCTTGTGACAGATTATACTCGAGTATAATCTGTCACAAGAACGCTTTTTG**

Cell culture and transfection

SK-BR-3, ZR-75-30, ZR-75-1 and HeLa cells were maintained in DMEM (Invitrogen), BT-474, Bcap-37 and MDA-MB-361 were cultured in RPMI-1640. 10% FBS (Gibco) and 50 μg/mL penicillin/ streptomycin (P/S) were supplemented into the culture medium. All cell lines were cultured in 37°C incubator with 5% CO_2. For

serum starvation, cells were incubated in DMEM supplemented with 0.1% FBS. Cells were transfected with plasmid DNA using PolyJet in vitro DNA transfection reagent (SignaGen Laboratories). siRNAs were delivered into cells following the manufacturer's instructions for Lipofectamine RNAi MAX reagent (Invitrogen). To generate stable *TAZ* knockdown in ZR-75-30 cells, retroviruses carrying pMKO-shCtr or pMKO-sh*TAZ* that produced in HEK293T cells using VSVG and GAG as packaging plasmids were used to infect ZR-75-30 cells. Stable cell pools were selected with 1 μg/mL puromycin (Amresco) for 5 days. These cells were subsequently infected with retroviruses encoding pQCXIH-GPER and screened with 50 μg/mL hygromycinB (Amresco) for 5 days. The cells that are resistant to both puromycine and hygromycine B were maintained and used in further experiments, such as cell growth assay and xenograft experiment.

G1 administration

Adult female C57 mice, about 10 weeks old, were used in the study. Mice were injected intraperitoneally (IP) with 80 mg/kg G1 solved in 0.9% NaCl solution or with vehicle only in the case of control group. 2 hrs after the injection, mice were sacrificed and IHC was performed. All animal-related procedures were performed under the Division of Laboratory Animal Medicine regulations of Fudan University.

Immunoblotting

Cells were lysed in NP-40 buffer or SDS sample buffer and denatured by heating on 99^{o}C for 10 min. Proteins were separated in 8-10% Bis-Tris polyacrylamide gel and immunoblotting was performed according to the standard protocol. Images were

captured by Image Quant LAS4000 mini (GE healthcare) when the nitrocellulose membranes were developed with Clarity Western ECL substrates (Bio-rad Laboratories). All the antibodies used in immunoblotting were listed in Supplemental Methods. The phos-tag reagents were purchased from Wako Chemicals. The preparation and usage of the gels containing phos-tag and $MnCl_2$ followed the manufacturer's instructions. On the phos-tag gels, the dephosphorylated form of TAZ proteins migrated much faster than the phosphorylated TAZ. Total TAZ protein levels were quantified by immunoblotting on a regular gel.

Immunoprecipitation

Cells were lysed using mild lysis buffer (50 mM Tris at pH 7.5, 150 mM NaCl, 0.5% NP-40 and supplemented with protease inhibitors and phosphatase inhibitors). Cell lysates were centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatants were incubated with antibodies of TAZ (Sigma-Aldrich) for 2 hrs at 4°C, and for another 2 hrs after addition of protein A conjugated beads. Immunoprecipitates were washed three times with lysis buffer, and proteins were boiled with SDS sample buffer.

Kinase assay

For the LATS1 kinase assay, ZR-75-30 cells were treated as indicated and LATS1 was immunoprecipitated as described in Supplemental Methods above. The immunoprecipitates were washed three times with lysis buffer, followed by a single wash with wash buffer (40 mM HEPES, 200 mM NaCl) and once with kinase assay buffer (30 mM HEPES, 50 mM potassium acetate, 5 mM $MgCl_2$). The purified His-TAZ or GST-YAP was used as the substrate for the immunoprecipitated LATS1

in the reaction mixture which contained 500 μM cold ATP. The in vitro reaction was carried out at 30°C for 30 min and terminated with SDS sample buffer. The phosphorylation levels of His-TAZ at Ser89 residue or GST-YAP at Ser127 in the reaction mixture were determined by immunoblotting using Phospho-TAZ(S89) or Phospho-YAP(S127) antibody.

Immunofluorescence staining

ZR-75-30 and SK-BR-3 cells were seeded in 6-well cell-culture plates. Cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min followed the treatment as indicated. Permeabilization was carried out with 0.1% Triton X-100 for 5 min. Cells then were blocked with 5% BSA for 30 min and subsequently incubated with primary antibodies overnight at 4°C. Cells were rinsed with PBS, and Alexa Fluor labled secondary antibodies were added for 2 hrs at room temperature. DAPI and Tetramethylrhodamine B isothiocyanate-conjugated Phalloidin were used to stain the nucleus and the actin filaments, respectively. Photos were taken by an Olympus microscope.

RNA interference

RNAi-mediated down-regulation of human *ESRα, ESRβ, Gαq, Gα11, MST1*(*STK4*), *MST2*(*STK3*), *LATS1*, *LATS2*, *YAP*, *TAZ*(*WWTR1*) and *TEAD1/3/4* were performed by transfecting siRNA oligonucleotides into ZR-75-30 cells in accordance with the manufacturer's instructions of lipofectamin RNAi MAX reagent (Invitrogen). A non-targeting control siRNA duplex (5'-CCAUUCCGAUCCUGAUCCG-3') was included as a negative control. The knockdown efficiency was assessed 48 hrs after

transfection by immunoblotting or qPCR. The sequences of all small interfering RNAs (siRNAs) used in this study were shown below:

si*ESRα* #1: UCAUCGCAUUCCUUGCAAA

si*ESRα* #2: GCAGGATTGTTGTGGCTACTA

si*ESRα* #3: CTCTACTTCATCGCATTCCTT

si*ESRβ* #1: CCCAAAUGUGUUGUGGCCAACACCU

si*ESRβ* #2: ATGTGTTGTGGCCAACACCT

si*ESRβ* #3: TGTAGACAGCCACCATGAATA

si*GNAQ* #1: GACACCGAGAATATCCGCTTT

si*GNAQ* #2: CTATGATAGACGACGAGAATA

si*GNA11* #1: GCTCAAGATCCTCTACAAGTA

si*GNA11* #2: GCTCAACCTCAAGGAGTACAA

si*MST1* #1:AGTTGAGTGATAGCTGGGAAA

si*MST1* #2:CCGGCCAGATTGTTGCTATTA

si*MST1* #3:GCCCTCATGTAGTCAAATATT

si*MST2* #1: GTCATTTCCTAAGCTACATAT

si*MST2* #2: GAATGCCAAACCTGTATCAAT

si*MST2* #3*:* CGGATGAAGATGAGCTGGATT

si*LATS1* #1: CACGGCAAGATAGCATGGATT

si*LATS1* #2: CAAGTCAGAAATCCACCCAAA

si*LATS1* #3: CGGCAAGAUAGCAUGGAUUUCAGTA

si*LATS2* #1: GCCATGAAGACCCTAAGGAAA

si*LATS2* #2: GGAAAUCAGAUAUUCCUUGUUGCCT

si*LATS2* #3: CTACTCGCCATACGCCTTTAA

si*YAP1* #1: CCCAGTTAAATGTTCACCAAT

si*YAP1* #2: GCCACCAAGCTAGATAAAGAA

si*YAP1* #3: CAGGTGATACTATCAACCAAA

si*TAZ* #1: CAGCCAAATCTCGTGATGAAT

si*TAZ* #2: CCAGGAACAAACGTTGACTTA

si*TAZ* #3: GCGTTCTTGTGACAGATTATA

si*TEAD1/3/4* #1: ATGATCAACTTCATCCACAAG

si*TEAD1/3/4* #2: GATCAACTTCATCCACAAGCT

RNA isolation, reverse transcription, and real-time PCR

Cells were washed with cold PBS and the total cellular RNA was isolated using Trizol reagent following the manufacturer's instructions (Invitrogen). 2-5 μg RNA was reverse transcribed to complementary DNA (cDNA) with oligo-dT primers. cDNA was then diluted and proceeded for real-time PCR with gene-specific primers in the presence of SYBR Premix Ex Taq (TaKaRa). Assays were performed in triplicate, and the results were normalized for *ACTB*. Relative abundance of mRNA was determined in 7500 real-time PCR system (Applied biosystems). The primer pairs used in this study were:

ACTB-F: GCCGACAGGATGCAGAAGGAGATCA

ACTB-R: AAGCATTTGCGGTGGACGATGGA

CTGF-F: CCAATGACAACGCCTCCTG

CTGF-R: TGGTGCAGCCAGAAAGCTC

CYR61-F: AGCCTCGCATCCTATACAACC

CYR61-R: TTCTTTCACAAGGCGGCACTC

EDN1-F: TGTGTCTACTTCTGCCACCT

EDN1-R: CCCTGAGTTCTTTTCCTGCTT

ANKRD1-F: CACTTCTAGCCCACCCTGTGA

ANKRD1-R: CCACAGGTTCCGTAATGATTT

TAZ-F: GGCTGGGAGATGACCTTCAC

TAZ-R: AGGCACTGGTGTGGAACTGAC

YAP-F: CCACAGGCAATGCGGAATATC

YAP-R: GGTGCCACTGTTAAGGAAAGG