SUPPLEMENTARY INFORMATION

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TGFβ1 Promotes Gemcitabine Resistance Regulating the LncRNA-LET/NF90/miR-145 Signaling Axis in Bladder Cancer

CONTENTS

SUPPLEMENTARY METHODS

Cell viability assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to assess cell survival. 1,500 cells per well were seeded to the 96-well plate in triplicate. GEM at various concentrations (2.5~20 μM) were added 24 h later. Three days post treatment, cells were washed with PBS and MTT (5 mg/ml) was added and incubated for 3 h at 37°C. Plates were shaken at room temperature for 15 min and absorbance at 490 nm was recorded with a microplate reader (BioTek Instruments, Winooski, VT). As for the collection of viable cells after GEM treatment, UBC cells were treated with either vehicle (control) or GEM for 3 days and allowed to recover in fresh media for another 3 days.

Flow cytometry. After UBC cells were trypsinized and prepared as single-cell population, CD44 (BD Biosciences, San Jose, CA, USA, 1:25) was incubated with cells in .PBS/0.5% BSA for 15 min at 4°C, followed by the analysis on a BD FACScan flow cytometer.

Plasmid construction and cell transfection. LncRNA-LET promoter and DNA with SMAD binding element deletion (ΔSBE) were generated from human genomic DNA by PCR and cloned into pGL3-basic vector (Promega) at BglII and HindIII sites. For lncRNA-LET overexpression, we cloned lncRNA-LET full length into vector pcDNA3.1 and lentiviral expression vector pCDH, respectively. For lncRNA-LET knockdown, shRNA targeting lncRNA-LET was inserted into the lentiviral expression vector pLKO.1 at AgeI and EcoRI sites. Recombinant lentiviruses were produced by transient transfection of 293FT packaging cells. DNA encoding NF90 was cloned into p3XFlag-CMV10 plasmid at BglII and KpnI sites. Pri-miR-145-WT and pri-miR-145-MUT were cloned into pcDNA3.1. HMGA2-3'UTR, HMGA2-3'UTR mutant, KLF4-3'UTR and KLF4-3'UTR mutant were constructed in psiCHECK2 at XhoI and NotI sites. For cell transfection, 20 μM siRNA or scramble control (Genepharma, Shanghai, China), 1 μg plasmids or empty vector, 50 μM mimic

miR-145 or mimic control (RIBOBIO, Guangzhou, China) were transfected into cells using Lipofectamine 3000. Analysis were performed 48 h post transfection. All sequences of siRNAs and mimic miRNAs were presented in Table S1.

Luciferase assay. Luciferase assays were performed using a luciferase assay kit (Promega). For microRNA, T24 cells in a 24-well plate were transfected with wild-type or mutant KLF4 or HMGA2 3'UTR plasmids with mimic miRNA negative control and mimic miR-145, respectively.

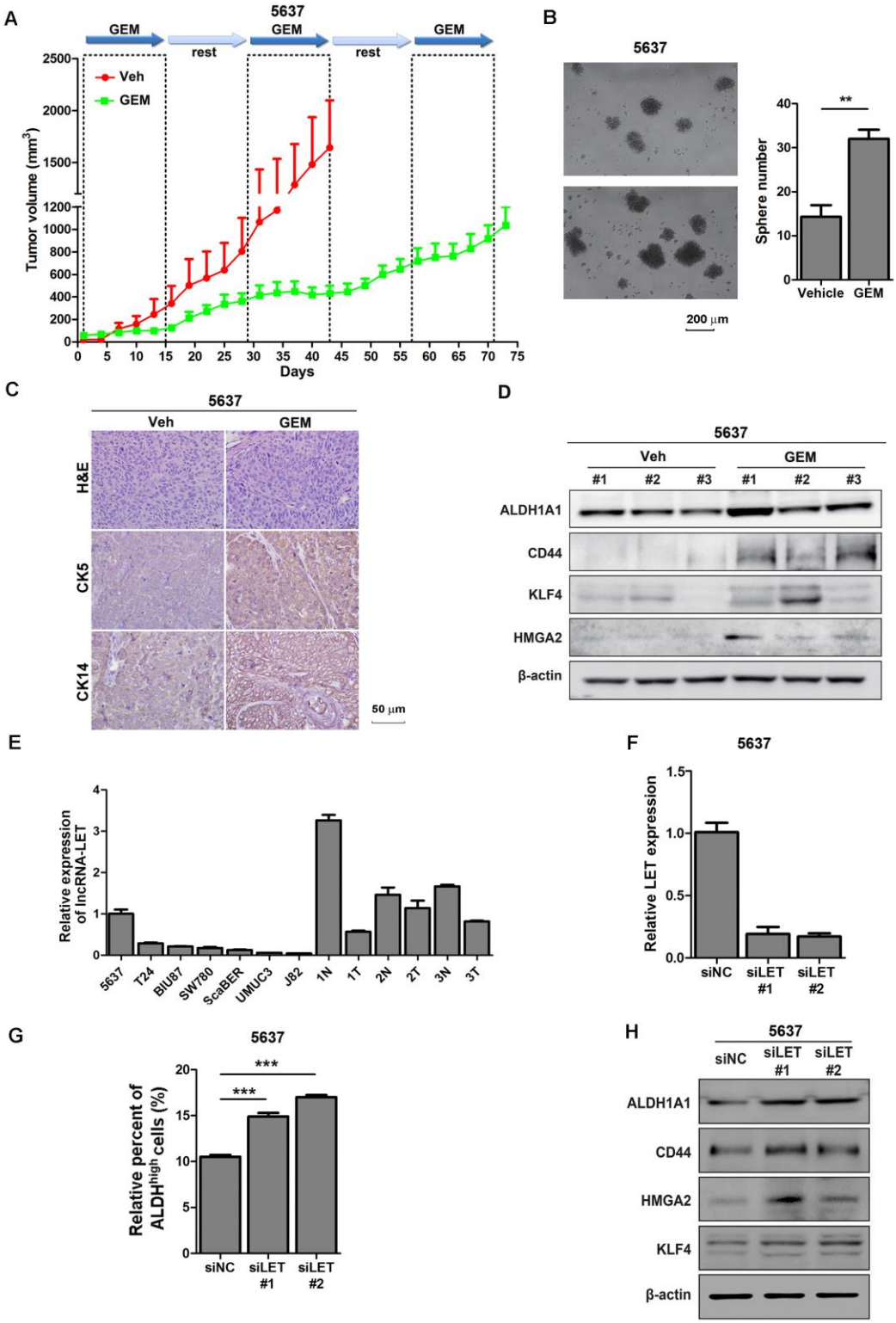

Figure S1. Increased CSC markers in 5637 xenografts treated with GEM *in vivo* and the downregulation of lncRNA-LET is required for 5637 UBC stemness. (A) *In vivo* GEM chemotherapy simulates clinical regimen with multiple treatment cycles (dashed boxes) and gap periods. Tumor sizes of 5637 xenografts were measured for GEM treatment and vehicle control group (n = 6 per group). **(B)** Sphere formation assay of primary cells derived from 5637 xenografts of control group (Veh) and GEM (n=3 per group). **(C)** Representative H&E and IHC data showing the expression levels of CSC

markers (CK5 and CK14) in 5637 xenografts of control and GEM groups (n = 6 per group). **(D)** Western blotting of CSC markers in 5637 xenografts of control and GEM groups. **(E)** The levels of lncRNA-LET in a panel of UBC cell lines and 3 pairs of adjacent normal bladder (N) and UBC (T) samples. **(F)** Knockdown efficiency of lncRNA-LET in 5637 cells. **(G, H)** $ALDH^{high}$ population **(G)** and CSC markers **(H)** were determined by flow cytometer and Western blotting in 5637 cells with and without lncRNA-LET depletion (n=3 per group). Data are shown as mean ± SD and represent three independent experiments with similar results. ****** $P < 0.01$; ******* $P < 0.001$ (Student's unpaired two-tailed *t*-test).

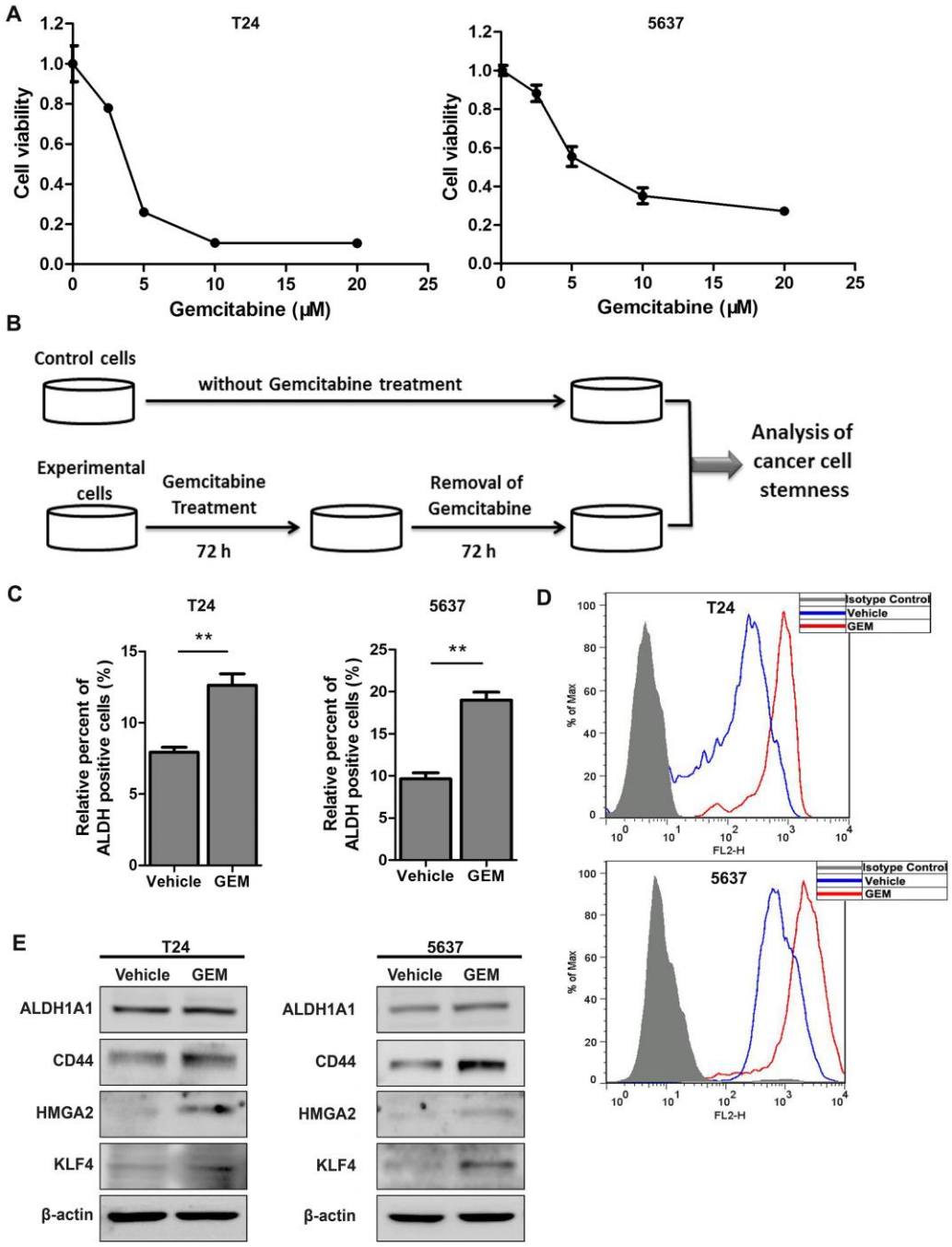

Figure S2. Increase of cancer stemness markers in cells treated with chemotherapeutic agents *in vitro*. (A) Survival of T24 and 5637 cells treated with GEM at the indicated concentrations (n=5 per group). **(B)** Schematic illustration of GEM treatment to enrich CSCs *in vitro*. **(C,D)** The changes of $ALDH^{high}$ (**C**) and $CD44^{+}$ (**D**) population of T24 and 5637 cells after 3.8 and 6.4 μM GEM treatment, respectively (n=3 per group). **(E)** The protein levels of CSC markers in T24 and 5637 cells treated with vehicle or GEM. Data are shown as mean ± SD and represent at least two independent experiments with similar results. ****** $P < 0.01$ (Student's unpaired two-tailed *t*-test).

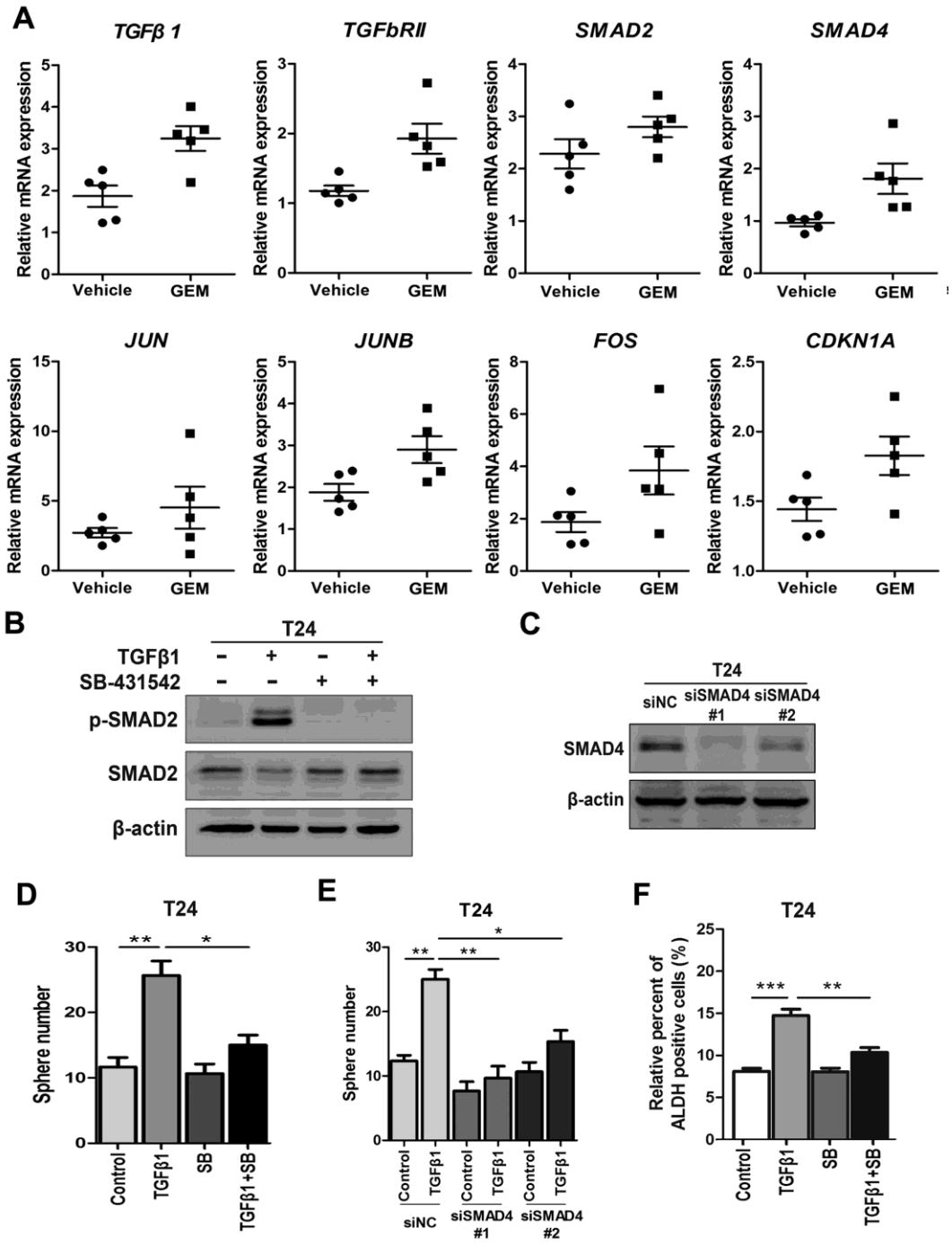

Figure S3. Activation of canonical TGFβ1 signaling promotes UBC stemness. (A) Expression of the key components in TGFβ1/SMAD pathway in T24 xenografts treated with vehicle or GEM (n=5 per group). **(B)** Western blotting showing the levels of p-SMAD2 and SMAD2 in T24 cells treated with or without TGFβ1 and/or TGFβRI inhibitor, SB-431542. **(C)** Knockdown efficiency of Smad4 in T24 cells by Western blotting. Sphere numbers **(D)** and ALDH^{high} population **(F)** were determined in T24 cells treated with or without TGFβ1 and/or TGFβRI inhibitor, SB-431542 (n=3 per group). **(E)** Sphere numbers in T24 cells transfected with control (siNC) or 2 different RNAi of SMAD4 (siSMAD4#1, siSMAD4#2), followed by vehicle or TGFβ1 treatment (n=3 per group). Data are shown as mean ± SD and represent at least two independent experiments with similar results. ***** $P < 0.05$, ****** $P < 0.01$, ******* $P < 0.001$ (Student's unpaired two-tailed *t*-test).

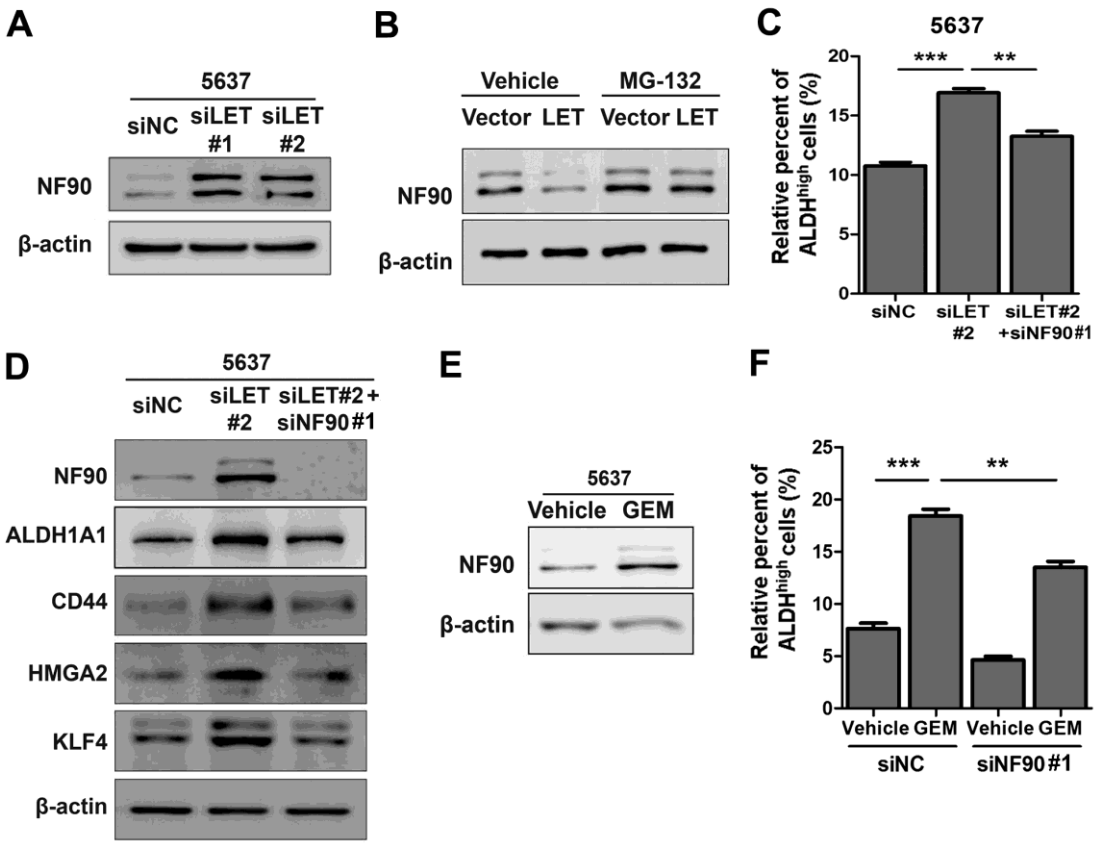

Figure S4. The stabilized NF90 by the reduced lncRNA-LET is required for cancer cell stemness. (A) Western blotting showing NF90 protein level in 5637 cells depleted of lncRNA-LET. **(B)** Protein level of NF90 in J82 cells over-expressed lncRNA-LET, followed by 20 μM MG-132 treatment determined by Western blotting. ALDH^{high} population **(C)** and expression of CSC markers **(D)** were determined by flow cytometer and Western blotting in control (siNC), lncRNA-LET knockdown (siLET#2), and simultaneous knockdown lncRNA-LET and NF90 (siLET#2 + siNF90) of 5637 cells (n=3 per group). **(E)** Protein level of NF90 was determined by Western blotting in vehicle -treated and GEM resistant 5637 cells. **(F)** The ALDH^{high} population was determined by flow cytometer in control (siNC) and NF90 knockdown (siNF90) 5637 cells treated with or without GEM. Data are shown as mean ± SD and represent at least two independent experiments with similar results. ****** $P < 0.01$, ******* $P < 0.001$ (Student's unpaired two-tailed *t*-test).

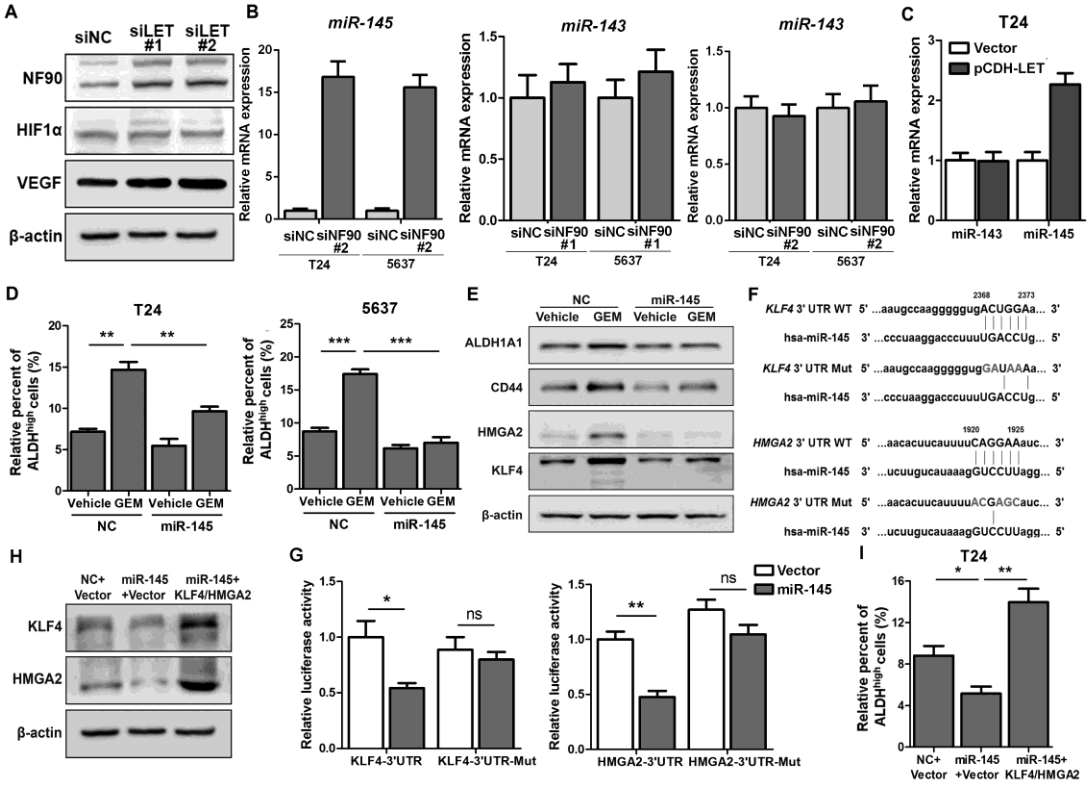

Figure S5. NF90/miR-145 inhibits UBC cell stemness through HMGA2 and KLF4. (A) Protein levels of NF90, HIF1α and VEGF were determined in T24 cells transfected with control (siNC) or lncRNA-LET knockdown (siLET-#1 and siLET-#2) by Western blotting. **(B)** The qRT-PCR showing the levels of miR-145 and miR-143 in control (siNC) and NF90 knockdown (siNF90 #1 and #2) T24 and 5637 cells. **(C)** The qRT-PCR showing the levels of miR-143 and miR-145 in control (Vector) and lncRNA-LET stable overexpression (pCDH-LET) T24 cells. **(D)** ALDH^{high} population was determined by flow cytometry in control (NC), mimic miR-145, and vehicle or GEM treated T24 and 5637 cells. (**E**) Protein levels of stemness markers were analyzed in control (NC), mimic miR-145, and vehicle or GEM treated T24 cells by Western blotting. **(F)** HMGA2 and KLF4 were predicted as miR-145 targets. Sequences of wild type (KLF4-3'UTR-WT, HMGA2-3'UTR-WT) and mutated 3'UTR *Renilla* luciferase reporters (KLF4-3'UTR-Mut, HMGA2-3'UTR-Mut) were listed. **(G)** Relative luciferase activity was measured in control (NC) and mimic miR-145 transfected T24 cells, which were simultaneously transfected with KLF4-3'UTR-WT or KLF4-3'UTR-Mut, HMGA2-3'UTR-WT or HMGA2-3'UTR-Mut. **(H,I)** expression of CSC markers **(H)** and ALDH^{high} population **(I)** were determined by Western blotting and flow cytometer in control (NC) and miR-145 overexpressed (miR-145) T24 cells, transfected with vector or KLF4/HMGA2 expression plasmids (n=3 per group). Data are shown as mean ± SD and represent at least two independent experiments with similar results. ***** P < 0.05, ****** P < 0.01, ******* P < 0.001 (Student's unpaired two-tailed *t*-test).

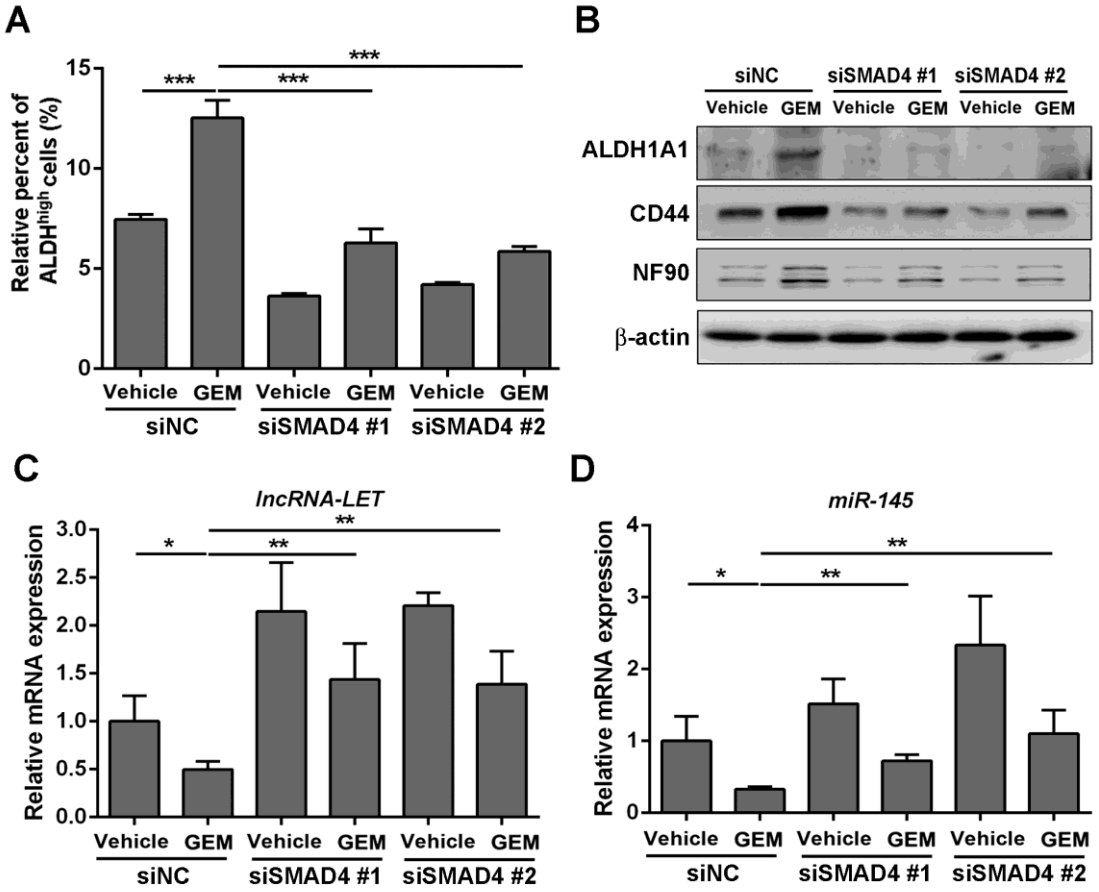

Figure S6. Inhibition of TGFβ1 signaling pathway suppressed GEM-induced UBC stemness. (**A**) The ALDH^{high} population was determined by flow cytometer in control (siNC) and SMAD4 knockdown (siSMAD4 #1 and #2) T24 cells treated with or without GEM**.** (**B**) Western blotting showing the levels of CSC markers and NF90 in control (siNC) and SMAD4 knockdown (siSMAD4 #1 and #2) T24 cells, followed by the treatment with or without GEM**.** (**C-D**) mRNA levels of lncRNA-LET (**C**) and miR-145 (**D**) were measured by qRT-PCR in control (siNC) and SMAD4 knockdown (siSMAD4 #1 and #2) T24 cells treated with or without GEM**.** Data are shown as mean ± SD and represent at least two independent experiments with similar results. ***** $P < 0.05$, ****** $P < 0.01$, ******* $P < 0.001$ (Student's unpaired two-tailed *t*-test).

Supplementary Table S1. List of oligonucleotide sequences

	Direction	Sequences (5'-3')
mRNA primers		
LncRNA-LET	Forward	CCTGCTACATATTAGGACTC
	Reverse	TGAGGAAGGTGGTATTGG
KLF4	Forward	CCCACATGAAGCGACTTCCC
	Reverse	CAGGTCCAGGAGATCGTTGAA
HMGA2	Forward	ACCCAGGGGAAGACCCAAA
	Reverse	CCTCTTGGCCGTTTTTCTCCA
CD44	Forward	CTGCCGCTTTGCAGGTGTA
	Reverse	CATTGTGGGCAAGGTGCTATT
ALDH1A1	Forward	CTGTGTTCCAGGAGCCGAAT
	Reverse	TGCCTTGTCAACATCCTCCTTA
NANOG	Forward	CTGCAGAGAAGAGTGTCGCA
	Reverse	ACCAGGTCTTCACCTGTTTGT
OCT4	Forward	CAAAGCAGAAACCCTCGTGC
	Reverse	CTCGGACCACATCCTTCTCG
β-actin	Forward	CATGTACGTTGCTATCCAGGC
	Reverse	CTCCTTAATGTCACGCACGA
miRNA primers		
miR-145-5p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGGGATTC
	qPCR	ACACTCCAGCTGGGGTCCAGTTTTCCCAGG
miR-143-3p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGAGCTACA
	qPCR	ACACTCCAGCTGGGTGAGATGAAGCACTGT
RNU6	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAACGCTTC
	qPCR	ACACTCCAGCTGGGACGCAAATTCGTGAAG
PCR primers for subcloning		
pGL3-LET-promoter	Forward	GAAGATCTGTAAGAGCATCCCTACAAAATAG
	Reverse	CCCAAGCTTTGAGGGAGCACCAGATGTC
pGL3-LET-promoter-ΔSBE	Overlap Forward	TGGTGTAGCCCCTGTACTGTTCTCAGAAAA
	Overlap Reverse	TTTTCTGAGAACAGTACAGGGGCTACACCA
pcDNA3.1^{+}-LET	Forward	GGGGTACCCTCACAGACAAAGGAGAGTCTG
	Reverse	TGCTCTAGATGGGTGTTTTCATGTAGGAAATG
p3XFlag-CMV10-NF90	Forward	CGCGAATTCATCGATAGATCTAATGCGTCCAATGCGAATTTT
	Reverse	CCTCTAGAGTCGACTGGTACCCTAGGAAGACCCAAAATCAT
pcDNA3.1-pri-miR-145-wt	Forward	GGGGTACCCCCTGGAAAGCCACTAGTAC

	Reverse	TGCTCTAGACTGGCTGCATTCCAAATCG
pcDNA3.1-pri-miR-145-Mut	Overlap Forward	GGATTCCTGGAAATACTGCCCTTGAGGTCATGG
	Overlap Reverse	CCATGACCTCAAGGGCAGTATTTCCAGGAATCC
psiCheck2-KLF4-3'UTR-wt	Forward	CTCGAGCACACTGTCTTCCCGATGAGG
	Reverse	GCGGCCGCATGCAAAATACAAACTCCACAAAA
psiCheck2-KLF4-3'UTR-Mut	Overlap Forward	AAGGGCGTGGATAAACGTTGTGGATATCAGGGTAT
	Overlap Reverse	CGTTTATCCACGCCCTTGGCATTTTGTAAGT
psiCheck2-HMGA2-3'UTR-wt	Forward	AATTCTAGGCGATCGCTCGAGTGATAAGCAAGAGTGGGCGG
	Reverse	ATTTTATTGCGGCCAGCGGCCGCACCTCCTGGCCCAGTTGATA
psiCheck2-HMGA2-3'UTR-Mut	Overlap Forward	CACTTCATTTTACGAGCATCTAC
	Overlap Reverse	GGAAGTAGATGCTCGTAAAATG
pCDH-LET	Forward	CTAGCTAGCTGGGTGTTTTCATGTAGGAAATG
	Reverse	ATTTGCGGCCGCCTCACAGACAAAGGAGAGTCTG
shRNA primers		
shLET	Forward	CCGGGAGCTGAAATCTTAGGTTATTCTCGAGAATAACCTAAGATTTCAGCTCTTTTTG
	Reverse	AATTCAAAAAGAGCTGAAATCTTAGGTTATTCTCGAGAATAACCTAAGATTTCAGCTC
siRNA sequences		
siLET#1	Sense	GGAGUAAAGGGAAAGAGTT
	Anti-sense	CUCUUUCCCUUUACUCCTT
siLET#2	Sense	GUGCAUGUGGUAGGUUAGATT
	Anti-sense	UCUAACCUACCACAUGCACTT
siSMAD4#1	Sense	UAAAGAAGCUGAAGGAGAATT
	Anti-sense	UUCUCCUUCAGCUUCUUUATT
siSMAD4#2	Sense	GCACAAGGUUGGUUGCUAATT
	Anti-sense	UUAGCAACCAACCUUGUGCTT
siNF90 #1	Sense	GCUCAAAGCUGUGUCCGACUGGATT
	Anti-sense	UCCAGUCGGACACAGCUUUGAGCTT
siNF90 #2	Sense	AAGCCACUGAUGCUAUUGGGCTT
	Anti-sense	GCCCAAUAGCAUCAGUGGCUUTT

Supplementary Table S2. Fold change of lncRNAs in T24 and 5637 xenografts treated with gemcitabine.

T24	
lncRNA	**Folds**
HOTAIRM1	968.085
lincRNA-P21	25.8637
PCGEM1	15.3191
H19	15.1928
HIF1A-AS1	4.19863
HULC	3.49282
ZEB2NAT	3.38106
BANCR	3.03472
GADD7	3.02559
AK126698	2.60184
ncRNA	2.29128
HOTAIR	2.23088
HOTTIP	2.17063
NEAT1	2.06226
PTENP1-AS	1.98990
GACTA1	1.95995
PCAT1	1.83319
PLncRNA-1	1.81818
lncRNA-VLDLR	1.76112
LSINCT5	1.75851
SRA1	1.71965
PTENP1	1.62827
lncRNA-ATB	1.58384
CRNDE	1.57309
RERT	1.45241
Xist	1.44360
DICER	1.43210
FAS-AS1	1.39815
ASAP1-IT1	1.39147
ANRIL	1.33230
Kcnq1OT1	1.31342
AFAP1-AS1	1.25962
SPRY4-IT1	1.24536
lncRNA-MVIH	1.22728
MALAT1	1.19549
PCAT6	1.18903

5637	
lncRNA	**Folds**
lncRNA-VLDLR	24.6862
ASAP1-IT1	5.24484
Loc285194	4.29563
CTBP1-AS	3.13102
HOTAIRM1	2.98165
GAS3	2.89049
ZEB2NAT	2.63146
PCAT114	1.84122
GAS5	1.66768
HOTAIR	1.55595
PTENP1-AS	1.52120
lncRNA-DQ786227	1.47010
HIF1A-AS1	1.45590
SchLAP1	1.28188
SCAL1	1.26987
CCAT1	1.26285
HOTTIP	1.23853
NEAT1	1.16509
SPRY4-IT1	1.08999
FAS-AS1	0.99941
MIR155HG	0.97079
AK126698	0.96750
MIR7-3HG	0.96260
RP11-462C24.1	0.95516
SCA8	0.93470
ERIC	0.91406
PANDAR	0.91255
MALAT1	0.90032
lncRNA-APTR	0.87858
PTENP1	0.84269
UCA1	0.83083
NRON	0.82808
DICER	0.82507
SOX2OT	0.80058
ncRNA	0.79977
PCAT6	0.79069

NRON	1.14729
PCAT114	1.05698
MIR155HG	1.01104
lncRNA-APTR	1.00267
PANDAR	0.99371
CTBP1-AS	0.97694
PRNCR1	0.95200
RP11-462C24.1	0.94493
MIR31HG	0.92699
SOX2OT	0.87793
lncRNA-Dreh	0.85341
JPX	0.83278
SCAL1	0.83007
lncRNA-DQ786227	0.81171
CCAT1	0.78678
GAS3	0.76875
MIR7-3HG	0.75764
linc-UBC1	0.71866
SCA8	0.71453
TUG1	0.69632
MEG3	0.67537
PVT1	0.65532
lncRNA-JADE	0.56974
RMRP	0.53230
ERIC	0.53031
GAS5	0.45898
lncRNA-LET	0.44831
UCA1	0.30248
Loc285194	0.26241
DANCR	0.00097
SchLAP1	#DIV/0!
IGF2-AS	#DIV/0!
CCAT2	#DIV/0!
GAS6-AS1	#DIV/0!
lincRNA-RoR	#DIV/0!
ADAMTS9-AS2	#DIV/0!

ADAMTS9-AS2	0.77532
lncRNA-MVIH	0.75666
PVT1	0.74824
HULC	0.74507
MEG3	0.72881
Xist	0.72109
BANCR	0.67967
AFAP1-AS1	0.66690
H19	0.65967
DANCR	0.64432
lncRNA-ATB	0.61326
TUG1	0.59243
CRNDE	0.56775
linc-UBC1	0.55794
PCAT1	0.54220
JPX	0.53319
GADD7	0.52179
PRNCR1	0.51929
PCGEM1	0.50860
RERT	0.49072
CCAT2	0.49004
Kcnq1OT1	0.46226
PLncRNA-1	0.42211
lncRNA-LET	0.42126
ANRIL	0.41980
MIR31HG	0.33195
RMRP	0.31347
SRA1	0.26843
GAS6-AS1	0.26729
lncRNA-JADE	0.26435
lincRNA-RoR	0.14575
GACTA1	0.09590
lincRNA-P21	0.07118
lncRNA-Dreh	#DIV/0!
IGF2-AS	#DIV/0!
LSINCT5	#DIV/0!

Supplementary Table S3: Correlation between lncRNA-LET expression and clinicopathological characteristics of 60 UBCs

Characteristic	Patients (n = 60)		lncRNA-LET expression		
	Number	%	Low (n = 30)	High (n = 30)	*P* value
Age					0.267
<60	19	31.7	7	12	
≥60	41	68.3	23	18	
Gender					0.146
Male	51	85.0	23	28	
Female	9	15.0	7	2	
Tumor number					0.399
Single	18	30.0	11	7	
Multiple	42	70.0	19	23	
Tumor stage					**0.039**
<II	16	26.7	4	12	
≥II	44	73.3	26	18	
Tumor grade					0.424
Low	7	11.7	2	5	
High	53	88.3	28	25	
Microvasclular invasion					**0.003**
Absent	38	63.3	13	25	
Present	22	36.7	17	5	
Lymph-node metastasis					**0.012**
Absent	50	83.3	21	29	
Present	10	16.7	9	1	
Recurrence					**0.005**
Absent	46	76.7	18	28	
Present	14	23.3	12	2	

Note: Bold values indicate $P < 0.05$. Fisher's exact test was used to analyze dichotomous variables. The median expression level was used as the cutoff point. Low expression of lncRNA-LET in 30 patients was classified as values below the 50th percentile. High lncRNA-LET expression in 30 patients was classified as values at or above the 50th percentile.