Supplementary Information

Functional role of BTB and CNC Homology 1 gene in pancreatic cancer and its association with survival in patients treated with gemcitabine

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Supplementary Figure legends

Figure S1. Bioinformatics analysis of differentially expressed genes in CFPAC-1 cells with *BACH1* knockdown.

(A) GO biological processes and **(B)** GSEA of gene signatures regulated by *BACH1*. For GO analysis, $P<0.05$ was set as a criterion for significance (dotted line indicates $P=0.05$). For GSEA analysis, the significant level was FDR<0.25.

Figure S2. Validation of 7 genes regulated by *BACH1* which were identified by integrated analysis of ChIP-seq and microarray data in CFPAC-1 and BXPC-3 cells.

The levels of *FTL, FTH1, SQSTM1, TFE3, GCLC, NQO2* and *ITPR2* mRNA were examined by qRT-PCR. Results are mean ± SEM relative to *GAPDH*; *, $P<0.05$; **, $P<0.01$ and ***, $P<0.001$.

Figure S3. *BACH1* and *NRF2* regulate *HMOX1* expression in a competitive binding manner.

(A) Disturb the expression of *BACH1* has no effect on the *NRF2* expression in both mRNA and protein levels in CFPAC-1 and BXPC-3 cells. **(B)** Chromatin immunoprecipitation assays showing binding of *NRF2* to two enhancers (EN1 and EN2) in the upstream of *HMOX1* in CFPAC-1 and BXPC-3 cells. Overexpression of *BACH1* in these cells substantially reduced enrichment of *NRF2* in EN1 and EN2, while knockdown of *BACH1* substantially increased the enrichment in these two enhancers. Fold enrichment (mean ± SEM) represents DNA levels associated with *NRF2* or IgG relative to an input control from three independent experiments. IgG served as negative control. *, $P<0.05$; **, $P<0.01$ and ***, $P<0.001$ compared with Control or shControl.

Figure S4. *BACH1* inhibits PDAC cell proliferation via HO-1.

(A–B) Proliferation profile **(A)** and colony formation ability **(B)** of control CFPAC-1 and BXPC-3 cells and cells overexpressing *BACH1* transiently transfected with or without pcDNA3.1-HMOX1. Results are mean ± SEM from three experiments and each experiment had six replicates. *, $P<0.05$ and $^{\#}$, $P<0.001$ compared with corresponding control. **(C–D)** Proliferation profile **(C)** and colony formation ability **(D)** of control CFPAC-1 and BXPC-3 cells and cells with knockdown of *BACH1* transiently transfected with or without *HMOX1* siRNAs. Results are mean ± SEM from three experiments and each experiment had four replicates. *, $P<0.05$; $^{\$}$, $P<0.05$ and $^{\#}$, $P<0.05$ compared with the corresponding control.

Figure S5. Effects of *BACH1* overexpression or knockdown on the expression of some genes involved in the signaling pathways of proliferation and angiogenesis in CFPAC-1 and BXPC-3 cells.

Overexpression of *BACH1* suppressed mRNA expression of *HIF1A* **(A)** and *VEGF* **(B)** but promoted mRNA expression of *PTEN* **(C)**. The reverse results were observed in the same cells with knockdown of *BACH1* expression **(A–C)**. Results are mean ± SEM relative to *GAPDH*. *P*-values are for Student's *t*-test.

Figure S6. Northern blotting of miR-1257 isolated from CFPAC-1 and BXPC-3 cells.

The image shows the presence of miR-1257 and its expected molecular size.

Figure S7. DNA sequencing analysis of *BACH1* rs372883 genotype.

The results show different genotypes in BXPC-3 **(A)**, CFPAC-1 **(B)** and Capan-2 **(C)** cells.

Figure S8. The expression of genes involved in the drug resistant in PDAC cells depends on *BACH1* in an allele-specific manner.

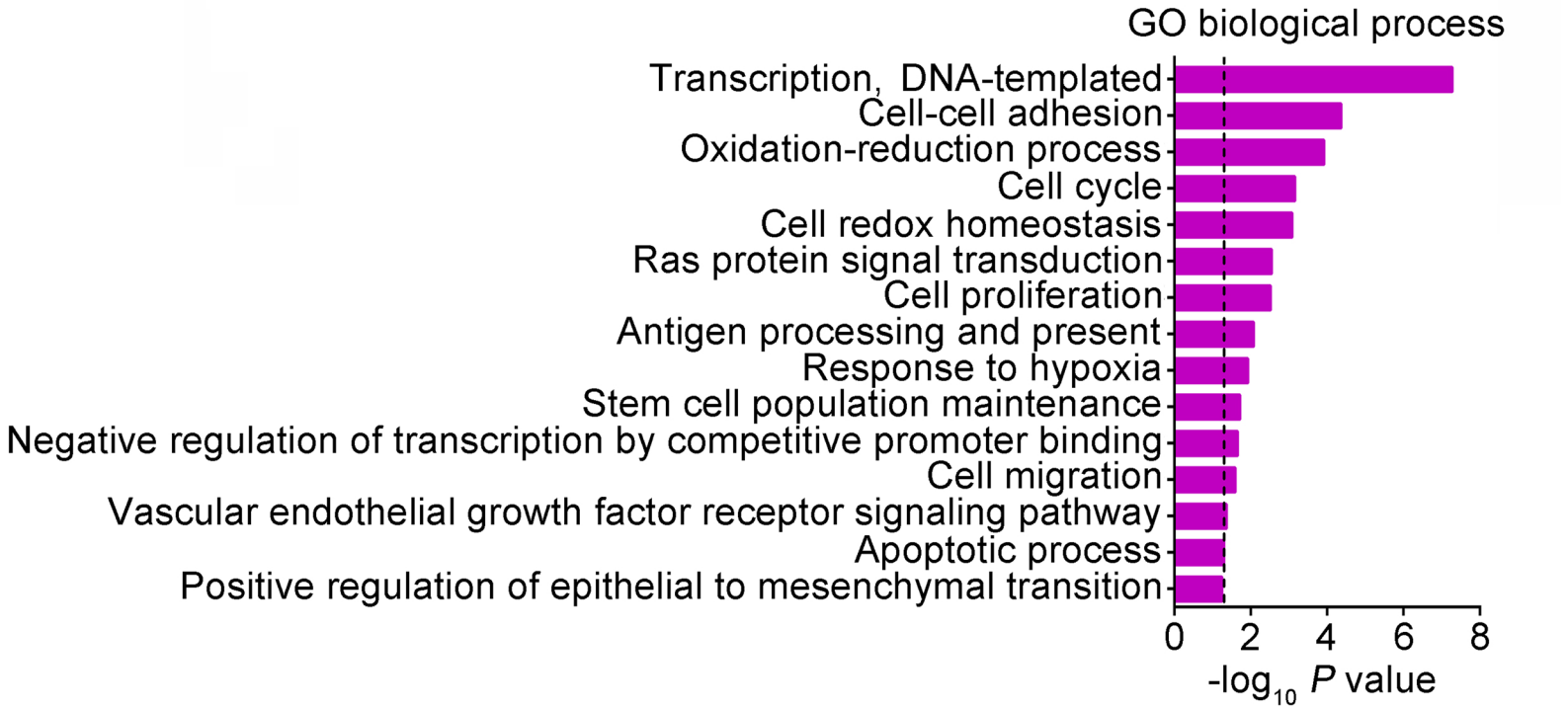
(A–C) Effect of miR-1257 on the expression levels of *ABCC2*, *MGST1* and *NQO1* in BXPC-3 **(A)**, CFPAC-1 **(B)** and Capan-2 **(C)** cells carrying the rs372883 CC, CT or TT genotype, respectively. Cells were transiently transfected with miR-1257 or its inhibitor, and gene expressions were examined by qRT-PCR. Results are mean ± SEM relative to *GAPDH*; *, $P<0.05$; **, $P<0.01$ and ***, $P<0.001$.

Figure S9. *BACH1* regulates expression of EMT and stemness associated genes.

(A) Western blot analysis of *E-cadherin*, *ZO-1*, *ZEB1*, *Vimentin* and *Slug* in CFPAC-1 and BXPC-3 cells with overexpression or knockdown of *BACH1*. **(B)** Western blot analysis of *OCT4*, *ABCG2*, *ALDH1* and *TRA-1-60* in CFPAC-1 and BXPC-3 cells with overexpression or knockdown of *BACH1*. **(C–D)** Correlation between *BACH1* and *ABCG2*, *CXCR4* mRNA levels in pancreatic cancer tissues. Data are from Oncomine database generated by Pei et al. [27] **(C)** and Badea et al. [28] **(D)**.

Figure S1

A


GO biological process
Transcription, DNA-templated
Cell-cell adhesion
Oxidation-reduction process
Cell cycle
Cell redox homeostasis
Ras protein signal transduction
Cell proliferation
Antigen processing and present
Response to hypoxia
Stem cell population maintenance
Negative regulation of transcription by competitive promoter binding
Cell migration
Vascular endothelial growth factor receptor signaling pathway
Apoptotic process
Positive regulation of epithelial to mesenchymal transition
0
2
4
6
8
-log10 P value

B

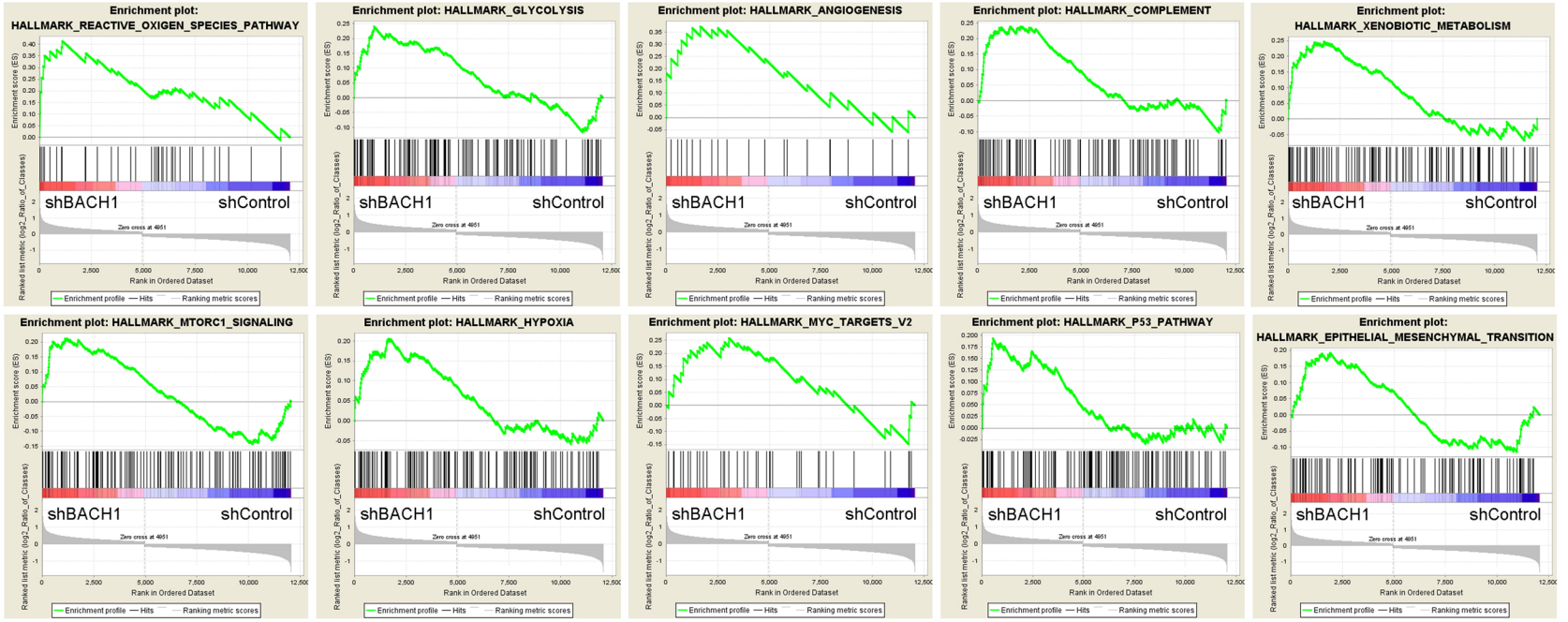

Enrichment plot: HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY
Enrichment plot: HALLMARK_GLYCOLYSIS
Enrichment plot: HALLMARK_ANGIOGENESIS
Enrichment plot: HALLMARK_COMPLEMENT
Enrichment plot: HALLMARK_XENOBIOTIC_METABOLISM
Enrichment plot: HALLMARK_MTORC1_SIGNALING
Enrichment plot: HALLMARK_HYPOXIA
Enrichment plot: HALLMARK_MYC_TARGETS_V2
Enrichment plot: HALLMARK_P53_PATHWAY
Enrichment plot: HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
shBACH1
shControl
Enrichment score (ES)
Ranked list metric (log2_Ratio_of_Classes)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

Figure S2

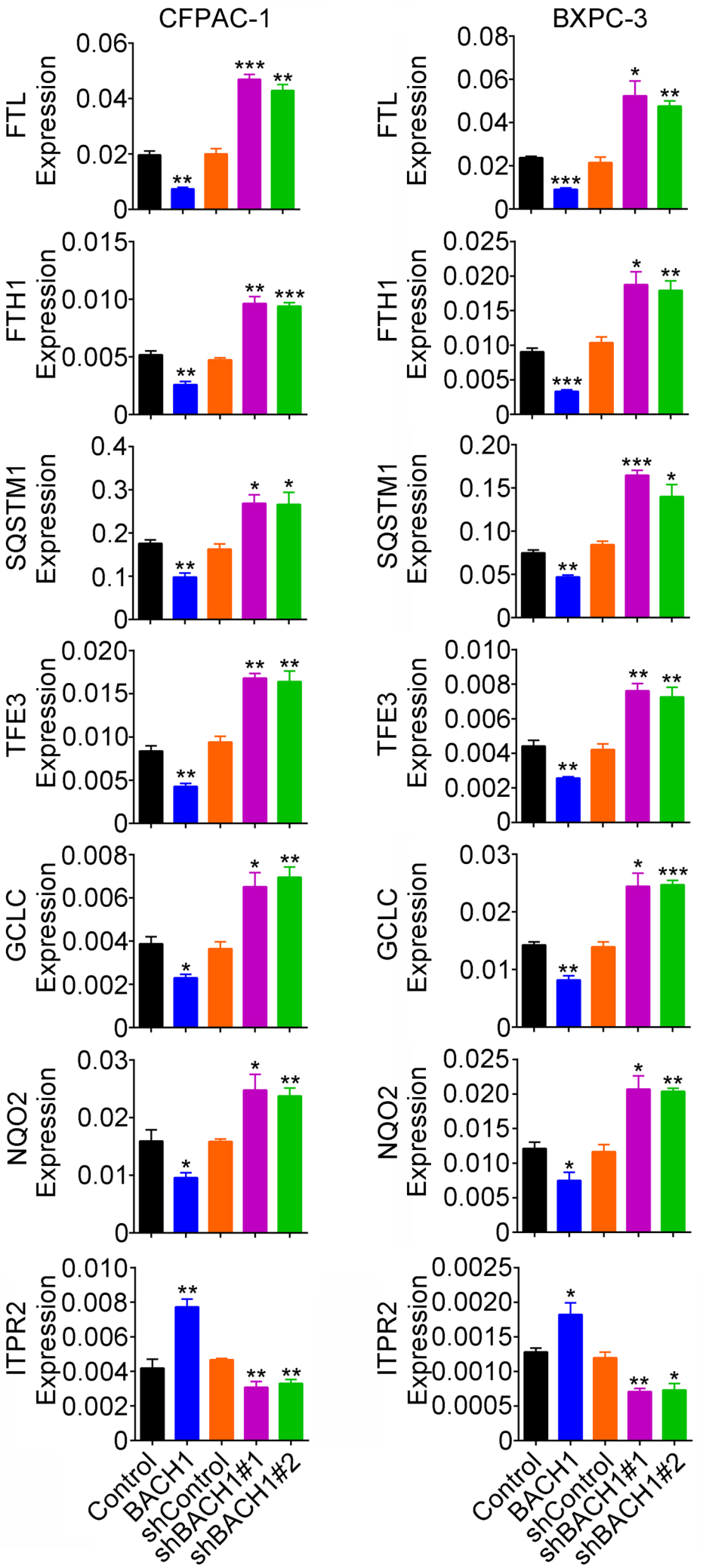

CFPAC-1
BXPC-3
FTL Expression
FTH1 Expression
SQSTM1 Expression
TFE3 Expression
GCLC Expression
NQO2 Expression
ITPR2 Expression
Control
BACH1
shControl
shBACH1#1
shBACH1#2

Figure S3

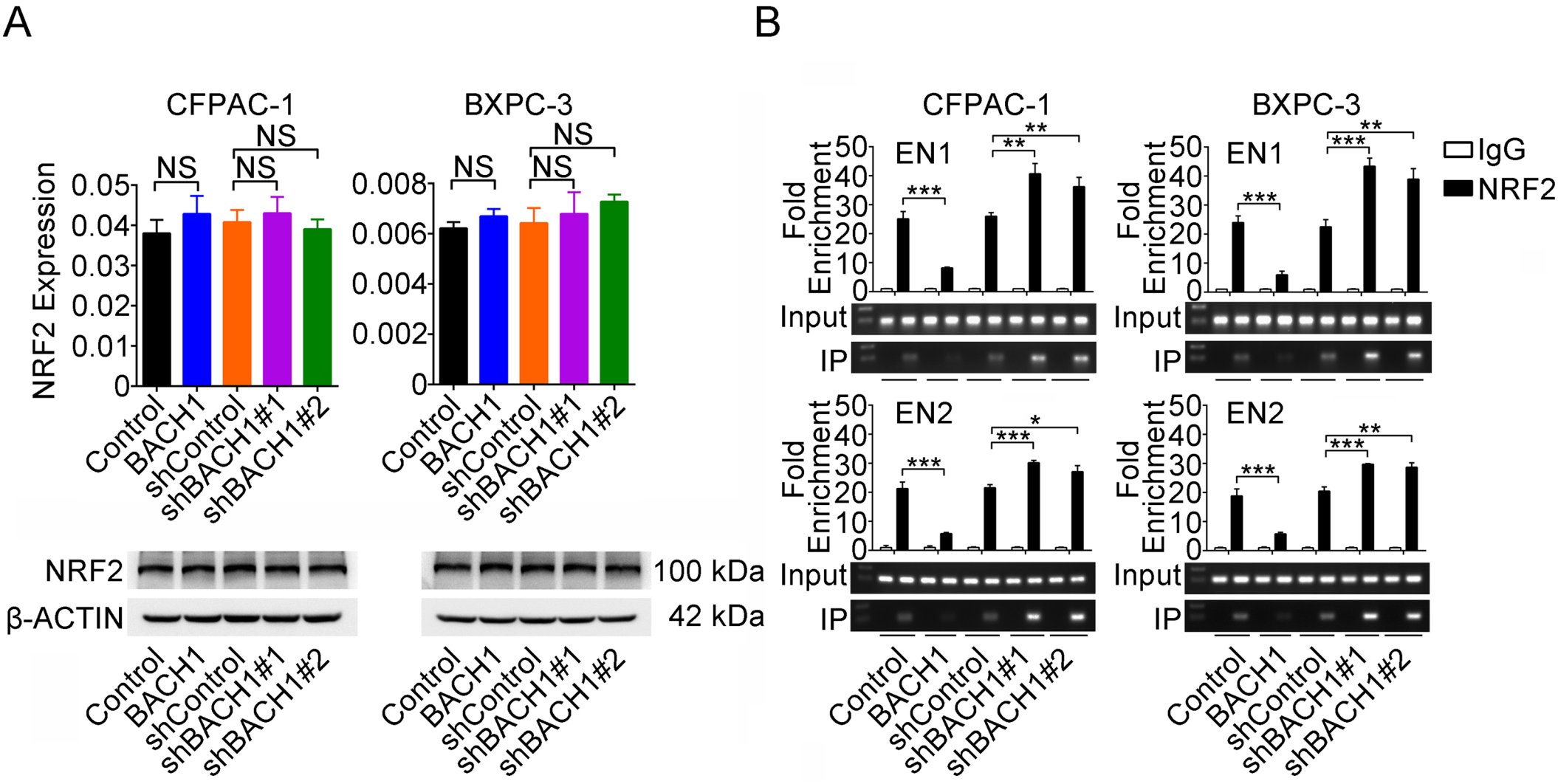

A
CFPAC-1
BXPC-3
NRF2 Expression
NS
0.05
0.04
0.03
0.02
0.01
0
0.008
0.006
0.004
0.002
Control
BACH1
shControl
shBACH1#1
shBACH1#2
NRF2
β-ACTIN
100 kDa
42 kDa
B
EN1
EN2
Fold Enrichment
50
40
30
20
10
0
***
**
*
Input
IP
IgG
NRF2

Figure S4

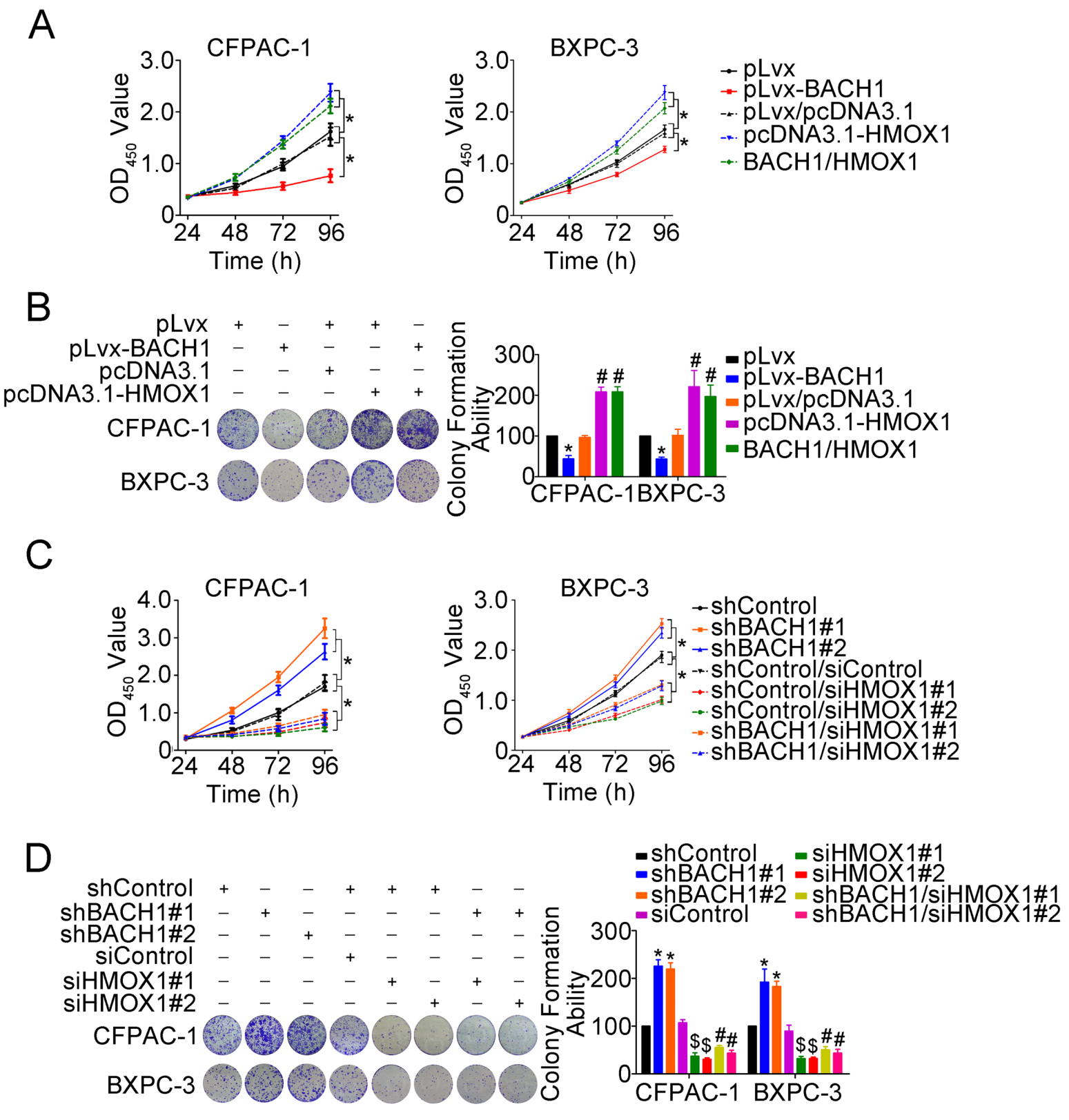

A
CFPAC-1
BXPC-3
OD450 Value
Time (h)
pLvx
pLvx-BACH1
pLvx/pcDNA3.1
pcDNA3.1-HMOX1
BACH1/HMOX1
B
pLvx
pLvx-BACH1
pcDNA3.1
pcDNA3.1-HMOX1
CFPAC-1
BXPC-3
Colony Formation Ability
C
shControl
shBACH1#1
shBACH1#2
shControl/siControl
shControl/siHMOX1#1
shControl/siHMOX1#2
shBACH1/siHMOX1#1
shBACH1/siHMOX1#2
D
shControl
shBACH1#1
shBACH1#2
siControl
siHMOX1#1
siHMOX1#2
shBACH1/siHMOX1#1
shBACH1/siHMOX1#2

Figure S5

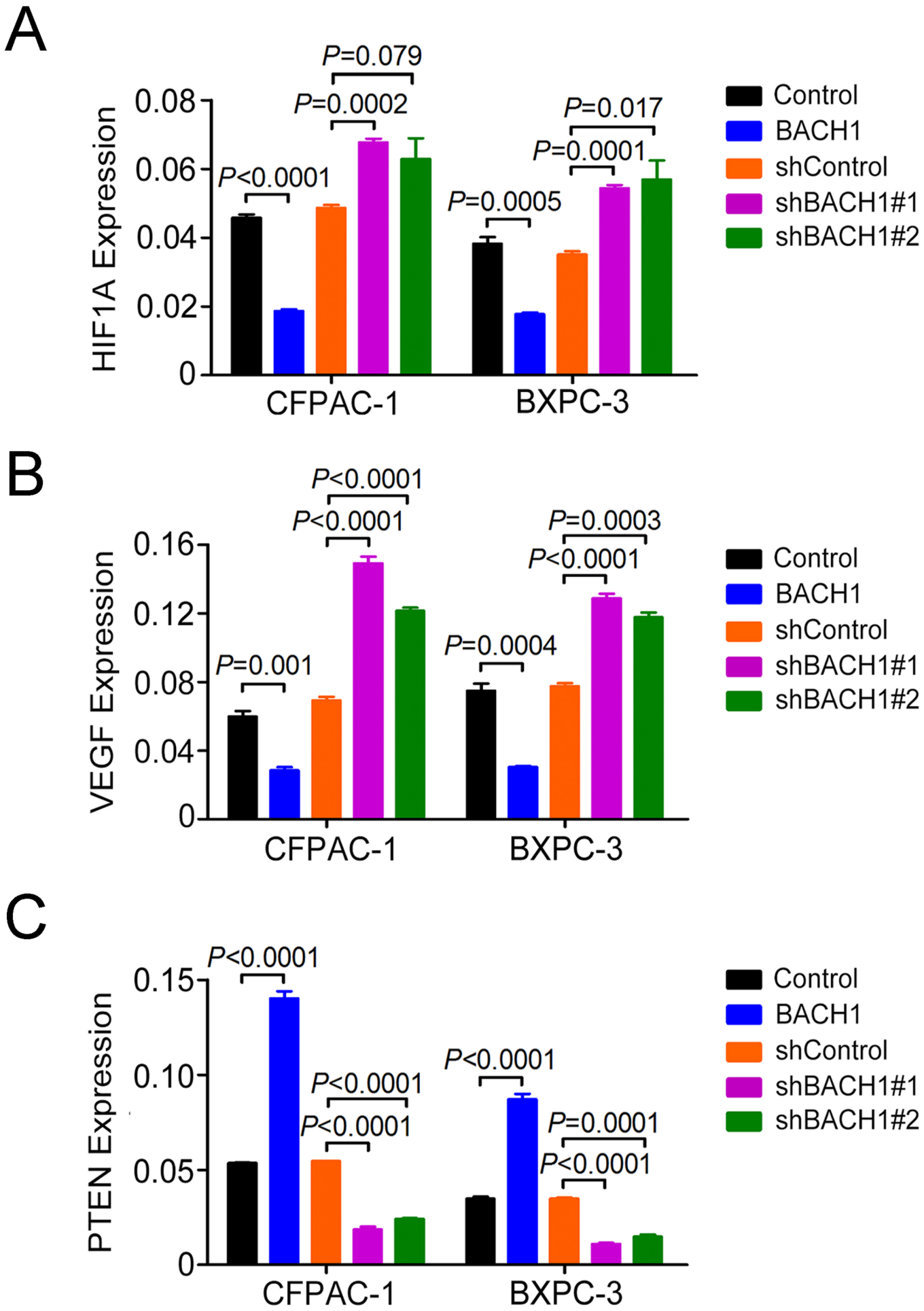

A
HIF1A Expression
0.08
0.06
0.04
0.02
0
P=0.079
P=0.0002
P<0.0001
P=0.017
P=0.0001
P=0.0005
CFPAC-1
BXPC-3
Control
BACH1
shControl
shBACH1#1
shBACH1#2
B
VEGF Expression
0.16
0.12
0.08
0.04
0
P<0.0001
P<0.0001
P=0.0003
P<0.0001
P=0.001
P=0.0004
CFPAC-1
BXPC-3
Control
BACH1
shControl
shBACH1#1
shBACH1#2
C
PTEN Expression
0.15
0.10
0.05
0
P<0.0001
P<0.0001
P<0.0001
P<0.0001
P=0.0001
P<0.0001
CFPAC-1
BXPC-3
Control
BACH1
shControl
shBACH1#1
shBACH1#2

Figure S6

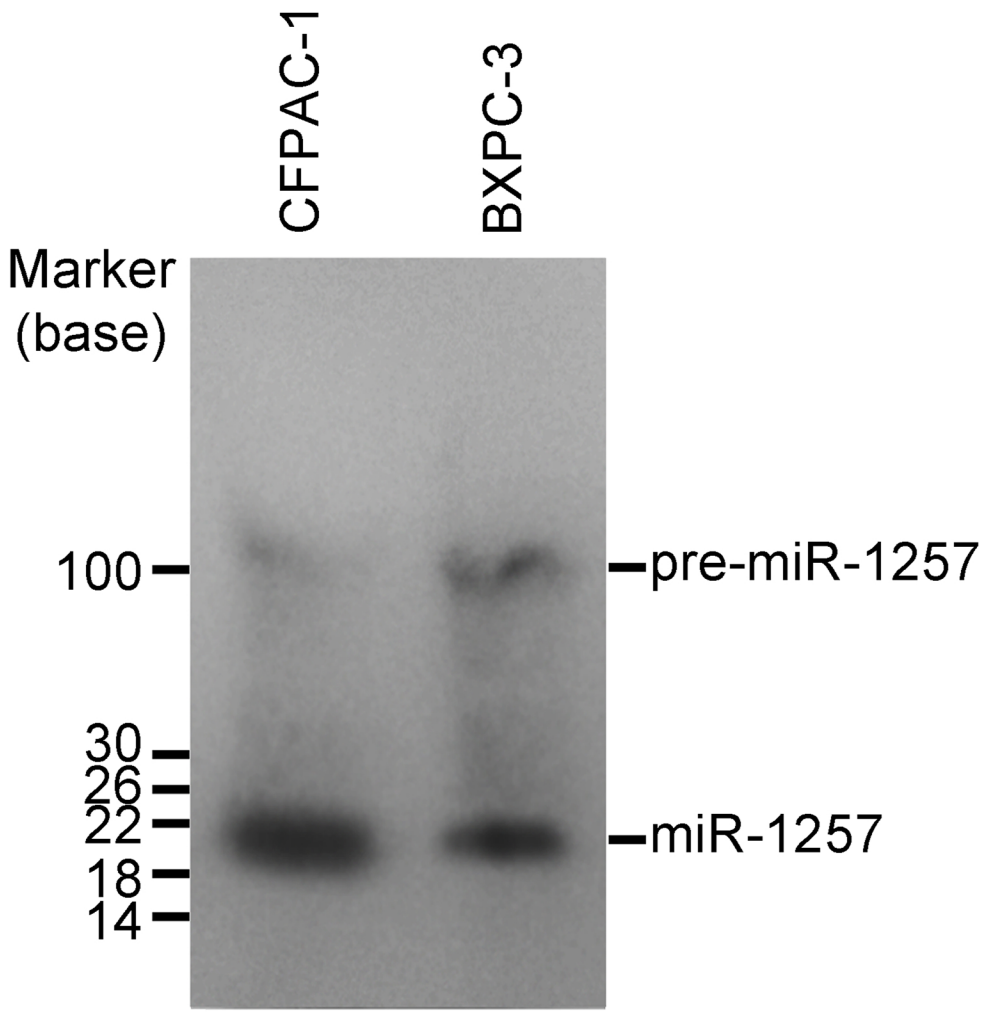
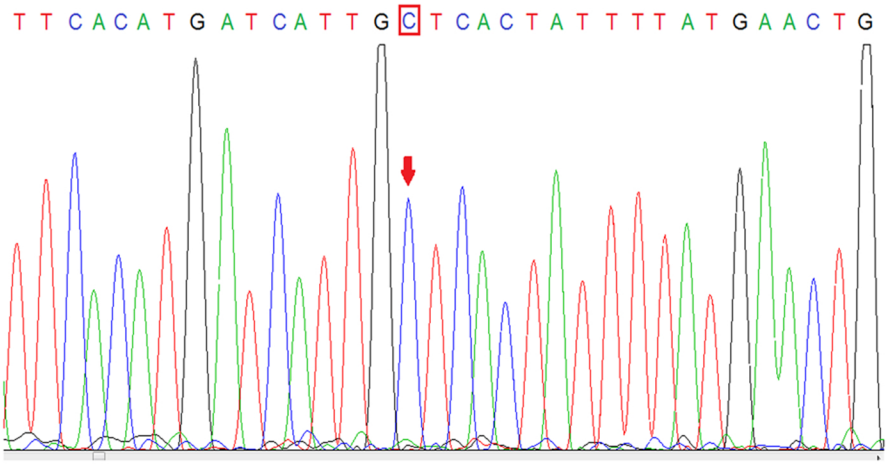
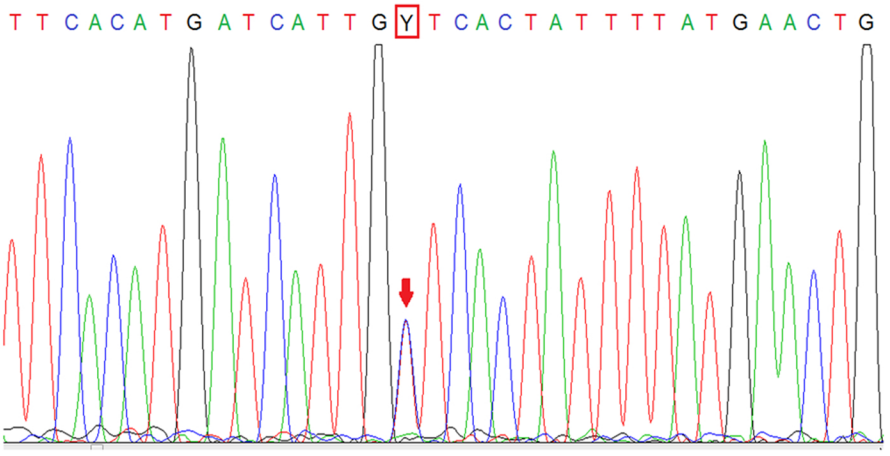

CFPAC-1
BXPC-3
Marker
(base)
100
30
26
22
18
14
pre-miR-1257
miR-1257

Figure S7

BXPC-3 rs372883 CC genotype



CFPAC-1 rs372883 CT genotype



Capan-2 rs372883 TT genotype

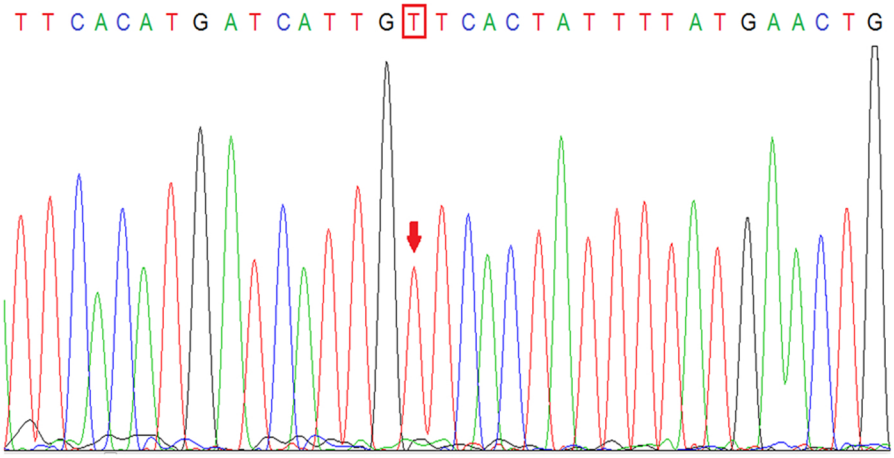


Figure S8

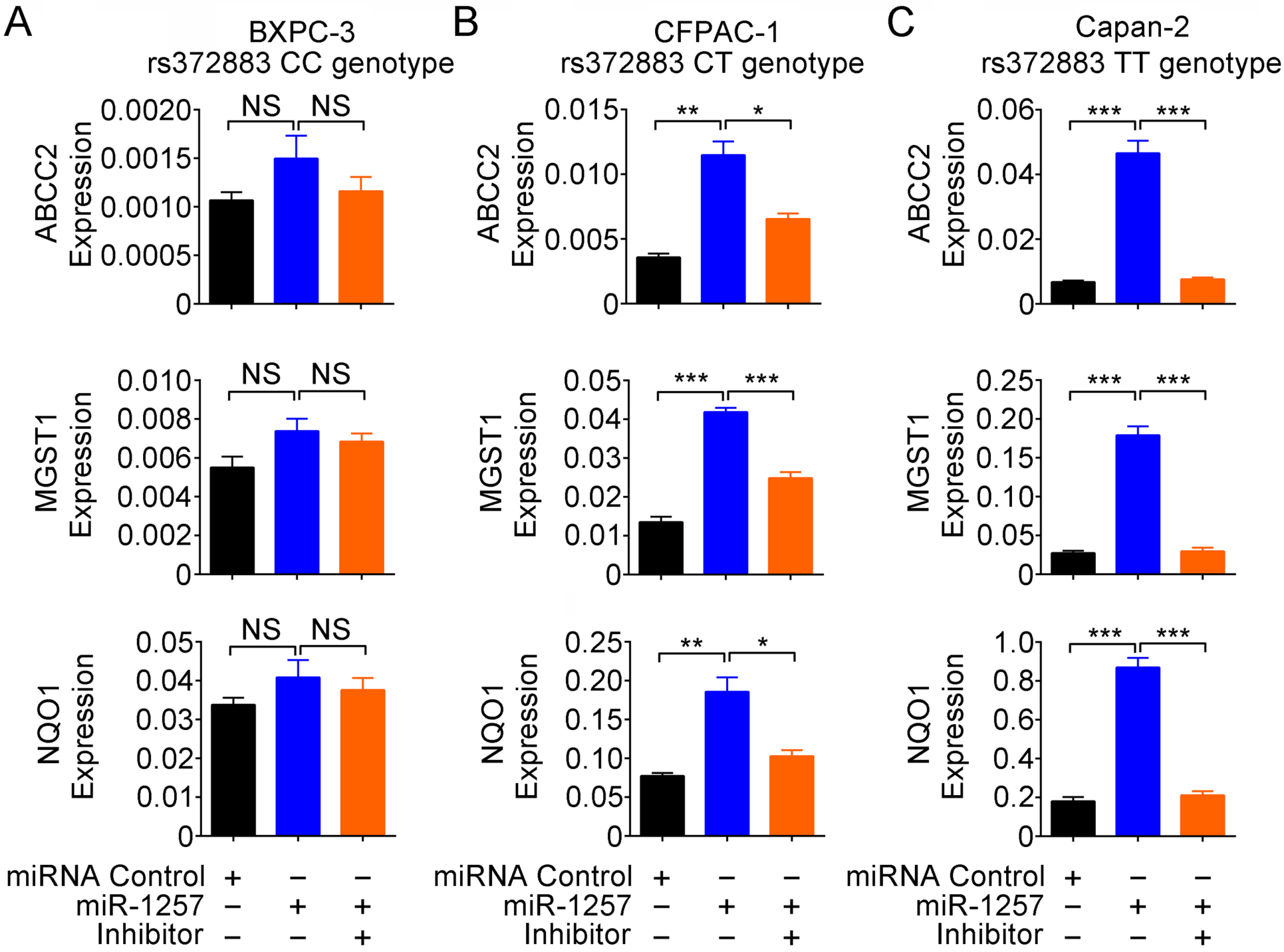
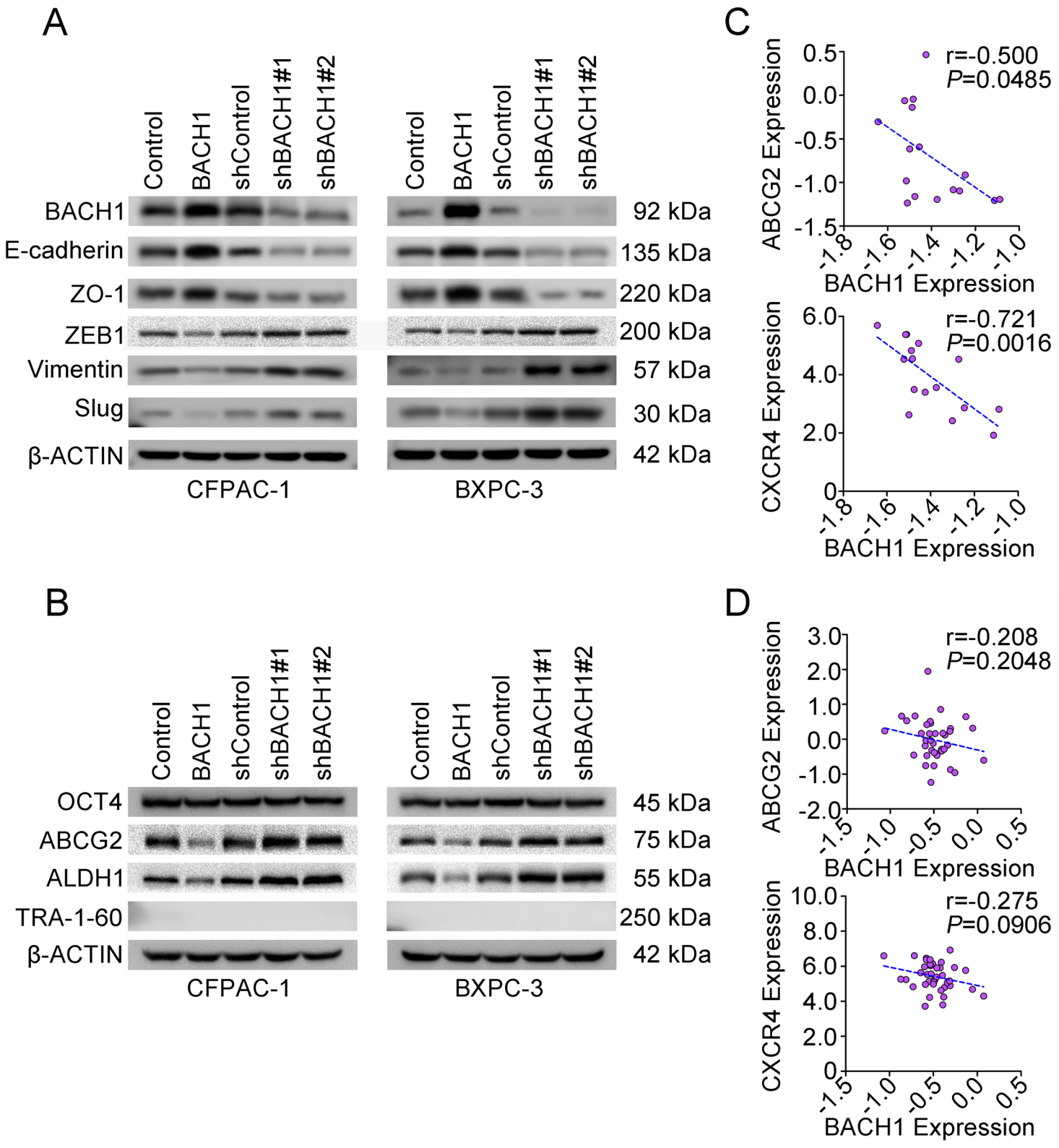

A
BXPC-3
rs372883 CC genotype
B
CFPAC-1
rs372883 CT genotype
C
Capan-2
rs372883 TT genotype
ABCC2 Expression
MGST1 Expression
NQO1 Expression
NS
**
*
***
miRNA Control
miR-1257
Inhibitor

Figure S9


A
Control
BACH1
shControl
shBACH1#1
shBACH1#2
BACH1
E-cadherin
ZO-1
ZEB1
Vimentin
Slug
β-ACTIN
92 kDa
135 kDa
220 kDa
200 kDa
57 kDa
30 kDa
42 kDa
CFPAC-1
BXPC-3
B
OCT4
ABCG2
ALDH1
TRA-1-60
β-ACTIN
45 kDa
75 kDa
55 kDa
250 kDa
42 kDa
C
ABCG2 Expression
r=-0.500
P=0.0485
BACH1 Expression
CXCR4 Expression
r=-0.721
P=0.0016
D
r=-0.208
P=0.2048
r=-0.275
P=0.0906

Supplementary Table S1 Demographic and clinical characteristics of 102 individuals with PDAC in this study

Characteristics	No. (%)	MST*	$P^{\S}$	Responders	Non-responders	$P^{\#}$
No. (%)	102 (100%)			16 (15.7)	86 (84.3)	
Status						
Dead	85 (83.3)	9.3		-	-	
Censored	17 (16.7)			-	-	
Sex			0.440			0.558
Male	64 (62.7)	9.9		9 (56.3)	55 (64.0)	
Female	38 (37.3)	8.7		7 (43.7)	31 (36.0)	
Age			0.657			0.732
<57	47 (46.1)	10.0		8 (50.0)	39 (45.3)	
≥57	55 (53.9)	8.7		8 (50.0)	47 (54.7)	
Stage			0.006			0.921
Local disease	12 (11.8)	14.4		2 (12.5)	10 (11.6)	
Local advanced disease	25 (24.5)	10.0		3 (18.8)	22 (25.6)	
Metastatic disease	65 (63.7)	7.8		11 (68.7)	54 (62.8)	
Treatment			0.005			0.512
Surgery and gemcitabine	20 (19.6)	15.0		4 (25.0)	16 (18.6)	
Gemcitabine alone	82 (80.4)	8.2		12 (75.0)	70 (81.4)	
Response			0.175			
Responder (CR+PR)*	16 (15.7)	12.3		-	-	
Non-responder (SD+PD)*	86 (84.3)	8.2		-	-	

*MST, median survival time (month); CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.
§Log-rank test; $^{\#}\chi^2$ test or Fisher exact test.

Supplementary Table S2 Primers and probes used for genotyping, vector construction, ChIP-qPCR, Northern blot or qRT-PCR analysis

rs372883 genotyping	
rs372883 genotyping-F	CCTTTTCCTTCATGCAGAATTTTG
rs372883 genotyping-R	CAAACATTGAGAAGGCCAGTTCAT
rs372883 C allele probe	FAM-TGATCATTGCTCACTATT-MGB
rs372883 T allele probe	HEX-TGATCATTGTTCACTATTT-MGB
Reporter gene construction	Sequence (5'→ 3')
BACH1-3'UTR-F	CCGCTCGAGACTTGCATTCACTTCCTTCA
BACH1-3'UTR-R	ATAAGAATGCGGCCGCTTCAATTAGAAGCAATTTTAGAG
rs372883T>C-F	GCATATTCACATGATCATTGCTCACTATTTTATGAACTGGCC
rs372883T>C-R	GGCCAGTTCATAAAATAGTGAGCAATGATCATGTGAATATGC
Lentiviral vector construction	
pLvx-BACH1-F	CCGCTCGAGGCCACCATGTCTCTGAGTGAG
pLvx-BACH1-R	ATAAGAATGCGGCCGCTTACTCATCAGTAGTAC
shBACH1-1	GATCCCCAGGTCAAAGGACTTTCACTTCCTGTCAGATGAAAGTCCTTTGACCTGGTTTTTG
shBACH1-2	GATCCGCAGATGACTGATAAATGTCTTCCTGTCAGAACATTTATCAGTCATCTGCTTTTTG
HMOX1 vector construction	
pcDNA3.1-HMOX1-F	CGGGATCCGCCACCATGGAGCGTCCGCAACC
pcDNA3.1-HMOX1-R	CCGCTCGAGCGGTCACATGGCATAAAGCCCTAC
HMOX1 siRNA	
siControl	UUCUCCGAACGUGUCACGU
siHMOX1-1	GGGUGAUAGAAGAGGCCAA
siHMOX1-2	GGGUCCUUACACUCAGCUU
HMOX1 ChIP-qPCR	
EN1-F	CACGGTCCCGAGGTCTATT
EN1-R	TAGACCGTGACTCAGCGAAA
EN2-F	GAAGGCGGATTTTGCTAGATTT
EN2-R	CTCCTGCCTACCATTAAAGCTG

miR-1257 Northern blot	
miR-1257 complementary probe	GGTCAGAACCCATCATTCACT-DIG
qRT-PCR	Sequence (5'→ 3')
BACH1-F	GAAAGATGTGCTGTGCGATG
BACH1-R	CACACTTCATCCACATTCTCTTTAC
HMOX1-F	CAGCGGGCCAGCAACAAAGT
HMOX1-R	ACCCATCGGAGAAGCGGAGC
HIF1A-F	TACCCTAACTAGCCGAGGAAGAA
HIF1A-R	ACACTGAGGTTGGTTACTGTTGG
VEGF-F	AGGGCAGAATCATCACGAAGT
VEGF-R	AGGGTCTCGATTGGATGGCA
PTEN-F	CTGAAAGACATTATGACACCGC
PTEN-R	TATCATTACACCAGTTCGTCCCT
NRF2-F	TTCCCGGTCACATCGAGAG
NRF2-R	TCCTGTTGCATACCGTCTAAATC
FTL-F	CAGCCGTCAACAGCCTGGTCAAT
FTL-R	CTCACGCCTTCCAGAGCCACATC
FTH1-F	CGAGGTGGCCGAATCTTCC
FTH1-R	GTTTGTGCAGTTCCAGTAGTGA
SQSTM1-F	GACTACGACTTGTGTAGCGTC
SQSTM1-R	AGTGTCCGTGTTTCACCTTCC
TFE3-F	CCGTGTTCGTGCTGTTGGA
TFE3-R	GCTCGTAGAAGCTGTCAGGAT
GCLC-F	GGAAGTGGATGTGGACACCAGA
GCLC-R	GCTTGTAGTCAGGATGGTTTGCG
NQO2-F	GTACTCATTGTCTATGCACACCA
NQO2-R	TGCCTGCTCAGTTCATCTACA
ITPR2-F	GGTTGGAGACTATCAGCTCGCT
ITPR2-R	GCATCATTGGGCTGAACTGGTG
ABCC2-F	CCCTGCTGTTCGATATACCAATC
ABCC2-R	TCGAGAGAATCCAGAATAGGGAC
MGST1-F	ATTGGCCTCCTGTATTCCTTGA

MGST1-R	GTGCTCCGACAAATAGTCTGAAG
NQO1-F	GAAGAGCACTGATCGTACTGGC
NQO1-R	GGATACTGAAAGTTCGCAGGG
GAPDH-F	TTGGCCAGGGGTGCTAAG
GAPDH-R	AGCCAAAAGGGTCATCATCTC
miR-1257-F	AGTGAATGATGGGTTCTGACCAAA
U6-F	CTCGCTTCGGCAGCACA
Universal reverse primer	GCTGTCAACGATACGCTACCTA

Supplementary Table S3 Gene set enrichment analysis (GSEA) of differentially expressed genes in PDAC cells with *BACH1* knockdown

Gene set name	ES	NES	Nom *P*-value	FDR
HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY	0.41	2.05	0.001	0.006
HALLMARK_GLYCOLYSIS	0.24	1.58	0.005	0.087
HALLMARK_ANGIOGENESIS	0.37	1.51	0.068	0.089
HALLMARK_COMPLEMENT	0.24	1.51	0.001	0.111
HALLMARK_XENOBIOTIC_METABOLISM	0.25	1.59	0.004	0.125
HALLMARK_MTORC1_SIGNALING	0.21	1.40	0.021	0.159
HALLMARK_HYPOXIA	0.21	1.37	0.029	0.163
HALLMARK_MYC_TARGETS_V2	0.26	1.30	0.102	0.218
HALLMARK_P53_PATHWAY	0.19	1.28	0.070	0.218
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	0.19	1.26	0.103	0.222

ES: Enrichment score; NES: Normalized enrichment score; Nom *P*-value: nominal *P*-value; FDR: false discovery rate