Supplemental Materials

Title: PDE4DIP contributes to colorectal cancer growth and chemoresistance through modulation of the NF1/RAS signaling axis

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Supplemental Figure Legends

Figure S1. Expression of PDE4DIP and its variants in CRC cells.

(A) Western blot analysis of the expression of PDE4DIP variants in nine CRC cell lines. Full-length PDE4DIP (PI) and isoform 5 (PI-5) were commonly expressed in CRC cells. (B) shRNA-mediated knockdown of PDE4DIP expression (PI and PI-5) was examined by Western blot analysis. DLD1 and SW480 cells were infected with lentivirus containing control shRNA (shNC) or shRNAs targeting PDE4DIP (shP1, shP2) for 72 h. (C) MTT assay showing that knockdown of PDE4DIP isoform 5 had no effect on CRC cell proliferation. DLD1 and SW480 cells were transfected with negative control siRNA (siNC) or either of two siRNAs targeting PDE4DIP isoform 5 (siPI5-1 and siPI5-2) for 48 h. Top, siRNA-mediated specific silencing of PDE4DIP isoform 5 (PI-5) was validated by Western blot analysis; bottom, data presented as the mean ± SD of three biological replicates. (D) MTT assay showing that overexpression of PDE4DIP isoform 5 had no effect on CRC cell proliferation. Top, ectopic overexpression of PDE4DIP isoform 5 (PI-5) was validated by Western blot analysis; bottom, data presented as the mean ± SD of three biological replicates.

Figure S2. Effects of PDE4DIP knockdown on PDE4D/PKA signaling and other signaling pathways in CRC cells.

(A) Western blot analysis of PDE4D/PKA signaling activity in PDE4DIP-silenced CRC cells. Cells were transfected with negative control siRNA (siNC) or either of two siRNAs targeting PDE4DIP (siP1, siP2) for 48 h. (B) GO analysis of the pathways enriched in PDE4DIP-silenced SW480 cells.

Figure S3. GSEA of the correlation between PDE4DIP expression and MAPK/AKT signaling in CRC.

(A) GSEA plots depicting enrichment in the MAPK or AKT/mTOR pathway based on PDE4DIP expression in CRC tumor samples from a TCGA dataset. The false discovery rate (FDR) was set at 0.25, and significance was defined as $P < 0.05$. (B-C) GSEA of enrichment in the MAPK and AKT/mTOR pathways based on PDE4DIP expression in CRC tumors from the GSE39582 cohort and GSE14333 cohort. The FDR was set at 0.25, and significance was defined as $P < 0.05$.

Figure S4. PDE4DIP promotes CRC growth through the RAS-ERK/AKT pathway.

(A) MTT assay showing that inhibition of ERK or AKT attenuated PDE4DIP-promoted CRC cell proliferation. DLD1 and SW480 cells transfected with empty vector (Vec) or the Myc-tagged PDE4DIP expression plasmid (PI) were treated with or without LY294002 (20 μM) or U0126 (20 μM) for 24 h. The data are representative of at least three independent experiments performed in triplicate. The data are presented as the means ± SDs, and the *P* values were determined by Student's *t* test. ** $P < 0.01$, *** $P < 0.001$. (B) Western blot showing that inhibition of RAS suppressed ERK/AKT phosphorylation in DLD1 and SW480 cells. Cells were treated with DMSO or FTS (salirasib, 75 μm) for 72 h. (C) Kaplan–Meier curves of overall survival for CRC patients stratified according to PDE4DIP expression and KRAS mutation status. CRC patients from the GSE40967 cohort were stratified into two groups based on KRAS mutation status: KRAS-mutated (left) or KRAS-wild-type (right). The significance of differences in survival was evaluated by the log-rank (Mantel–Cox) test.

Figure S5. NF1 suppresses oncogenic RAS/ERK signaling and KRAS-mutant CRC cell growth.

(A) Western blot showing that silencing NF1 suppressed RAS activity, ERK phosphorylation and AKT phosphorylation in KRAS-mutant DLD1 and SW480 cells. Cells were transfected with negative control siRNA (siNC) or siRNA targeting NF1 (siNF1). (B) Western blot showing that ectopic expression of NF1 suppressed RAS activity and ERK phosphorylation in DLD1 and SW480 cells. Cells were transfected with empty vector (Vec) or the NF1-GRD

overexpression plasmid for 48 h. (C) MTT assay showing that ectopic expression of NF1 suppressed DLD1 and SW480 cell proliferation. Cells were transfected with empty vector (Vec) or the indicated concentrations of the NF1-GRD plasmid. (D) Colony formation assay showing that presilencing of NF1 (siNF1) attenuated the inhibitory effect of PDE4DIP interference on cell proliferation. (C-D) The data are representative of at least three independent experiments performed in triplicate. The data are presented as the means ± SDs, and the *P* values were determined by Student's *t* test. ** $P < 0.01$, *** $P < 0.001$.

Figure S6. PKCε promotes the degradation of NF1 in CRC cells.

(A) Western blot analysis of the phosphorylation of MARCKS (pMARCKS) in control and PDE4DIP-overexpressing CRC cells. DLD1 and SW480 cells were transfected with empty vector (Vec) or the Myc-tagged PDE4DIP expression plasmid (PI) for 48 h. (B) Western blot showing that inhibition of PKC increased the NF1 level in CRC cells. DLD1 and SW480 cells were treated with RO-31-8220 (0, 5, or 10 μM) for 24 h. (C) Western blot showing that inhibition of PKCε increased the expression of NF1 in CRC cells. DLD1 and SW480 cells were treated with the PKCε inhibitor EV1-2 (1.0 μM) for the indicated times. (D) Western blot showing that inhibition of PKCε dose-dependently affected NF1 expression and ERK phosphorylation in DLD1 and SW480 cells. Cells were stimulated with the indicated concentrations of EV1-2 for 30 min. (E) Immunofluorescence staining showing the abundance of NF1 in control and EV1-2-treated CRC cells. Cells were stimulated with DMSO or 1.0 μM EV1-2 for 30 min. Scale bars, 20 μm. (F) Western blot analysis of NF1 ubiquitination in control (DMSO) and EV1-2-treated CRC cells (1.0 μM, 30 min). (G) MTT assay showing that knockdown of PKCε suppressed the proliferation of DLD1 and SW480 cells. Cells were transfected with negative control siRNA (siNC) or either of two siRNAs targeting PKCε (si-ε1, si-ε2). Top, siRNA-mediated knockdown of PKCε expression was validated by Western blot analysis; bottom, data presented as the mean ± SD of three biological replicates. The *P* values were determined by Student's *t* test. ** $P < 0.01$, *** $P < 0.001$.

Figure S7. PDE4DIP interacts with AKAP9 at the Golgi apparatus and promotes the

activation of PLCγ.

(A) Immunofluorescence analysis showing the colocalization of PDE4DIP (red) and GM130 (green) at the Golgi apparatus. DLD1 and SW480 cells were cotransfected with the GM130 and PDE4DIP expression plasmids for 48 h. Scale bars, 5 μm. (B) Coimmunoprecipitation of PDE4DIP with AKAP9 in DLD1 and SW480 cells. Cells were transfected with the Myc-tagged PDE4DIP expression plasmid, and cell lysates were immunoprecipitated with an anti-Myc antibody and subjected to Western blotting with anti-Myc and anti-AKAP9 antibodies. (C) Western blot showing the activation of PLCγ in PDE4DIP-overexpressing CRC cells. DLD1 and SW480 cells were transfected with empty vector (Vec) or the PDE4DIP expression plasmid (PI) for 48 h.

Figure S8. Inhibition of PLCγ activation sensitizes KRAS-mutant CRC cells to MEK inhibition.

(A) Dose–response curves of control and PDE4DIP-silenced DLD1 and SW480 cells treated with the MEKi trametinib. Cells infected with lentivirus containing control shRNA (shNC) or either of two shRNAs targeting PDE4DIP (shP1, shP2) were treated with increasing concentrations of trametinib for 72 h. The data are representative of at least three independent experiments performed in triplicate. The data are presented as the means ± SDs, and *P* values were determined by Student's *t* test. ** $P < 0.001$, *** $P < 0.001$ vs. control. (B) Colony formation assay of control and PDE4DIP-silenced DLD1 and SW480 cells treated with the indicated concentrations of trametinib for two weeks. The images are representative of at least three independent experiments. (C) Western blot analysis of the NF1 level and activation of PLCγ and ERK in AZD6244-resistant CRC cells treated with AZD6244 (1.0 μM) alone or in combination with a PLCγ inhibitor (U73122, 1.0 μM). Cells were collected for lysis at the indicated time points. UT, untreated. (D) Colony formation assay of DLD1 and SW480 cells treated with AZD6244 and/or a PLCγ inhibitor (U73122, 1.0 μM). The images are representative of at least three independent experiments.

Table S1. Correlation between alterations of PDE4DIP expression in CRC tumors and clinical and pathologic features of the individuals.

Clinical characteristic	Patient No.	PDE4DIP mRNA upregulation		χ^2	*P* value
		Yes (48)	No (12)		
Sex					
Male	32	27	5	0.82	0.365
Female	28	21	7		
Age (*y*)					
<60	24	22	2	2.296	0.13
≥60	36	26	10		
Differentiation grade					
Well	13	8	5	4.284	0.117
Moderate	33	27	6		
Poor	14	13	1		
Infiltration depth					
T1	7	1	6		
T2	28	24	4	21.716	<0.001
T3	12	11	1		
T4	13	12	1		
Lymph node metastasis					
N0	23	14	9	8.674	0.013
N1	19	17	2		
N2	18	17	1		
TNM stage					
I	19	12	7	8.8889	0.012
II	4	2	2		
III	37	34	3		
Tumor size (cm)					
<5	42	31	11	2.188	0.139
≥5	18	17	1		
Smoking					
Yes	22	18	4	0	1
No	38	30	8		
Drinking					
Yes	25	21	4	0.429	0.513
No	35	27	8		

Note: Using SPSS 20 statistical software, a chi-squared analysis was performed to analyze the correlations between PDE4DIP mRNA levels and clinical and pathologic parameters. The statistical significance of the individuals was determined with chi-squared test (χ^2). $P < 0.05$ was considered significant. Tumor size, differentiation grade, infiltration depth and lymph node metastasis were determined by the pathologists. Tumor stage was based on TNM.

Table S2. Sequences used for siRNA, shRNA interference, qRT-PCR and cloning.

Gene	Sequence (5'-3')	Application
PDE4DIP (siP1)	AGAGCGAGAUCAUGACUUATT	Gene silence
PDE4DIP (siP2)	AAGCAGAGAGACAGCUCUAUA	Gene silence
PDE4DIP (shP1)	AGAGCGAGAUCAUGACUUATT	Gene silence
PDE4DIP (shP2)	AAGCAGAGAGACAGCUCUAUA	Gene silence
PDE4DIP isoform 5 (siPI5-1#)	GGCUUCCGAUUCCAGUGAA	Gene silence
PDE4DIP isoform 5 (siPI5-2#)	GCCAGUAUGUAUCGGAAGATT	Gene silence
NF1 (siNF1)	CUUCGGAAUUCUGCCUCUG	Gene silence
AKAP9 (siA1/siAKAP9)	AACUUUGAAGUUAACUAUCAA	Gene silence
AKAP9 (siA2)	GCACAAUAAUUAUUGAAUUTT	Gene silence
PKCε (si-ε1)	UUGUAUAACCCAUGUUUAGCCTT	Gene silence
PKCε (si-ε2)	AGUGUAUACAGCUAAUUGCUGTT	Gene silence
Nontarget control siRNA (siNC)	UUCUCCGAACGUGUCACGUTT	Gene silence
Nontarget control shRNA (shNC)	UUCUCCGAACGUGUCACGUTT	Gene silence
PDE4DIP	Forward: 5'-GAGAACTCCAGGACAAGAAACAGCAT-3' Reverse: 5'-GGATTCCTCCTGCAGAAGCTGG-3'	qRT-PCR
NF1	Forward: 5'-CAGAATTCCCCCCTCAACTTCGAAGT-3' Reverse: 5'-TGCGTGCTGCATCAAAGTTGCTTTTCAC-3'	qRT-PCR

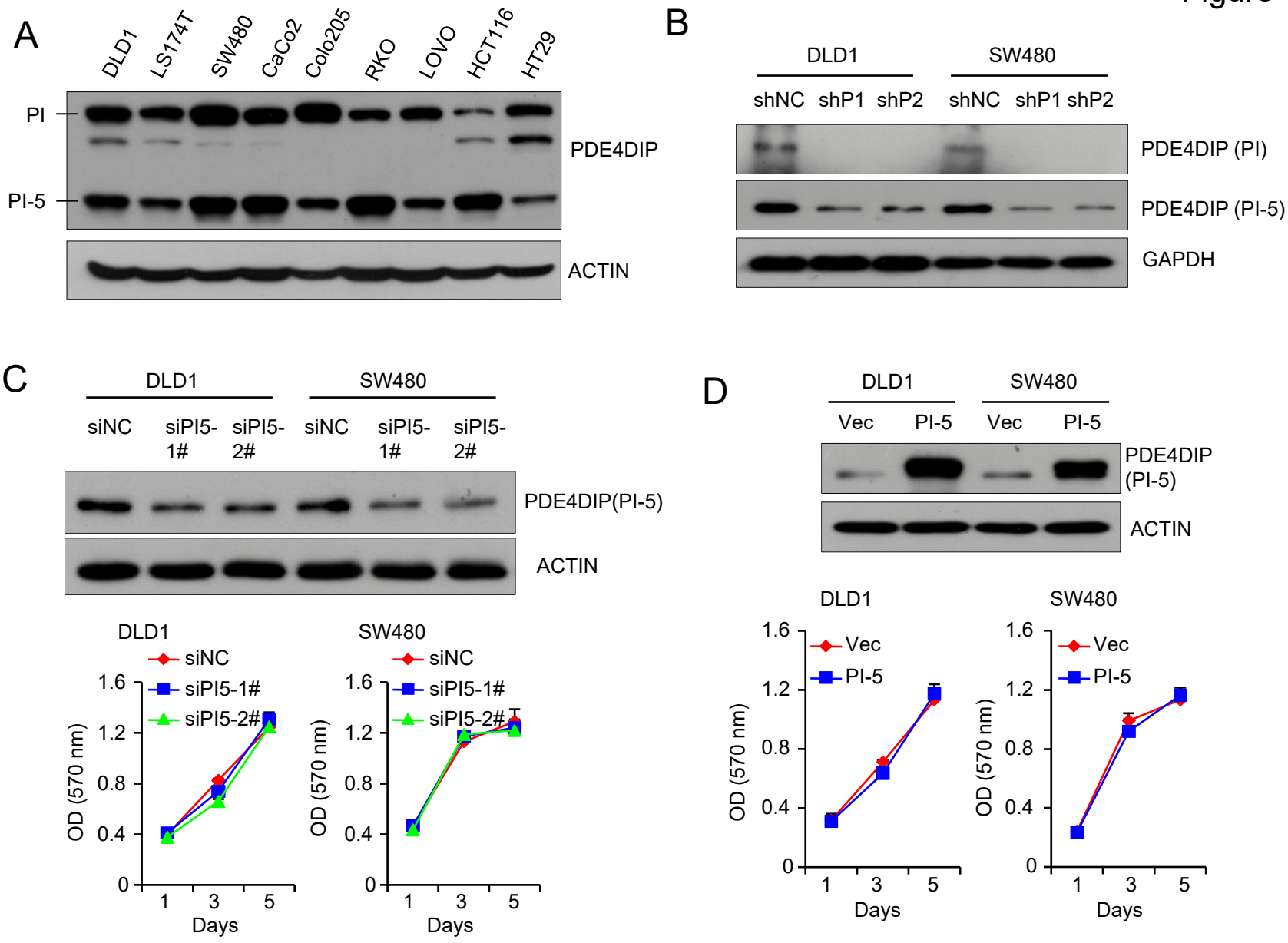
NF1-GRD	Forward: 5’-CCAAGCTTGCCACCATGGGTTACCACAAGGATCTCCAG-3’ Reverse: 5’-CGTCTAGAGTGCTCTGGAGGACCCAGGTA-3’	Cloning
PDE4DIP isoform 5 (PI5)	Forward: 5’-CGGATCCGCCACCATGAAGGAGATTTGCAGGATC-3’ Reverse: 5’-CCTCGAGTAGTTGCTGGTGACTATGGTGT-3’	Cloning

Table S3. Primary antibodies used.

Antibody	LOT	KD	Host	Company	Application
PDE4DIP	A89686	150-300	Rabbit	Sigma	WB 1:2000 IHC 1:200 IF 1:200
Phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204)	#9101	42,44	Rabbit	CST	WB 1:2000
p44/42 MAPK (Erk1/2) Antibody	#9102	42,44	Rabbit	CST	WB 1:2000
β-actin	#4970	45	Rabbit	CST	WB 1:2000
Phospho-Akt (Ser473) Antibody	#9271	60	Rabbit	CST	WB 1:2000
Akt (pan)(C67E7) Rabbit mAb	#4691	60	Rabbit	CST	WB 1:2000
Phospho-p38 MAPK (Thr180/Tyr182) Antibody	#9211	38	Rabbit	CST	WB 1:2000
P38 alpha/beta MAPK(A-12)	sc-7972	38	Mouse	Santa	WB 1:2000
Phospho-SAPK/JNK (Thr183/Tyr185)(G9) Mouse mAb	#9255	46,54	Mouse	CST	WB 1:2000
SAPK/JNK Antibody	#9252	46,54	Rabbit	CST	WB 1:2000
Phospho-MEK1/2 (Ser217/221) (E4M5C) Rabbit mAb	#86128	45	Rabbit	CST	WB 1:2000
MEK1/2 (L38C12) Mouse mAb	#4694	45	Mouse	CST	WB 1:2000
GAPDH (14C10) Rabbit mAb	#2118	37	Rabbit	CST	WB 1:6000
Tubulin Antibody	#2144	52	Rabbit	CST	WB 1:6000
PTEN (138G6) Rabbit	#9559	54	Rabbit	CST	WB 1:2000

p-EGFR (Tyr1068) (D7A5)	#3777	175	Rabbit	CST	WB 1:2000
EGFR	#2232	175	Rabbit	CST	WB 1:2000
Neurofibromin (D)	sc-67	250	Rabbit	Santa	WB 1:1000 IF 1:200
Myc-tag Rabbit mAb	#2278		Rabbit	CST	WB 1:2000 IF 1:200
Myc-tag Mouse mAb	#2276		Mouse	CST	WB 1:2000 IF 1:200
HA-probe (F-7)	sc-7392		Mouse	Santa	WB 1:1000
PCNA (D3H8P) XP® Rabbit mAb	#13110	36	Rabbit	CST	WB 1:2000
Phospho-MARCKS (Ser159/163) (D13D2) Rabbit mAb	#11992	80	Rabbit	CST	WB 1:1000
MARCKS (D88D11)	#5607	75	Rabbit	CST	WB 1:2000
p-PKC-ε (Ser729)	sc-12355	90	Mouse	Santa	WB 1:1000
PKC ε (E-5)	sc-1681	90	Mouse	Santa	WB 1:1000 IF 1:200
Phospho-PKC (pan) (zeta Thr410) (190D10) Rabbit mAb	#2060	76,85	Rabbit	CST	WB 1:1000
AKAP9	611518	450	Mouse	BD	WB 1:800 IP 1:100
Phospho-PLCγ (Tyr783) Antibody	#2821	155	Rabbit	CST	WB 1:1000
PLC γ (E-12)	sc-7290	155	Mouse	Santa	WB 1:1000 IF 1:200
PDE4D(H-69)	sc-25814	68	Rabbit	Santa	WB 1:2000
Phospho-PKA C (Thr197) Antibody	#4781	42	Rabbit	CST	WB 1:2000
PKA C-α Antibody	#4782	43	Rabbit	CST	WB 1:2000
Phospho-CREB (Ser133) (87G3) Rabbit mAb	#9198	43	Rabbit	CST	WB 1:2000
CREB (48H2) Rabbit mAb	#9197	43	Rabbit	CST	WB 1:2000
GM130 (D6B1) XP® Rabbit mAb	#12480		Rabbit	CST	IF 1:200
Normal Rabbit IgG	#2729		Rabbit	CST	IP 1:100
Mouse mAb IgG1 Isotype Control	#5415		Mouse	CST	IP 1:100

Figure S1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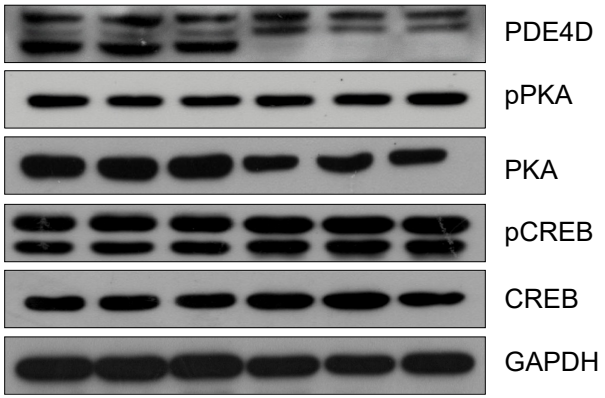
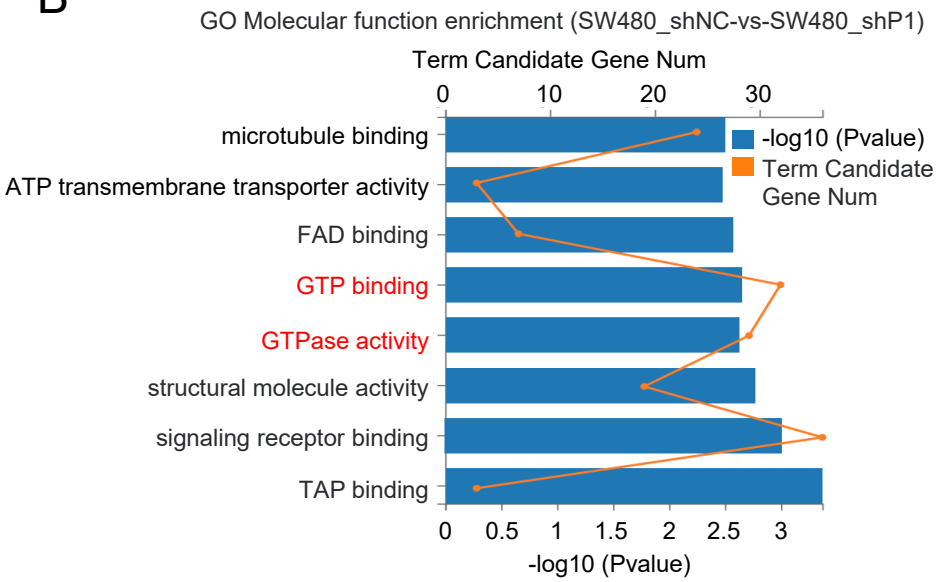
SW480
CaCo2
Colo205
RKO
LOVO
HCT116
HT29
PI
PI-5
PDE4DIP
ACTIN
B
DLD1
SW480
shNC
shP1
shP2
shNC
shP1
shP2
PDE4DIP (PI)
PDE4DIP (PI-5)
GAPDH
C
DLD1
SW480
siNC
siPI5-1#
siPI5-2#
siNC
siPI5-1#
siPI5-2#
PDE4DIP(PI-5)
ACTIN
DLD1
SW480
siNC
siPI5-1#
siPI5-2#
OD (570 nm)
Days
D
DLD1
SW480
Vec
PI-5
Vec
PI-5
PDE4DIP (PI-5)
ACTIN
DLD1
SW480
Vec
PI-5
OD (570 nm)
Days

Figure S2

A


DLD1
SW480
siNC
siP1
siP2
siNC
siP1
siP2
PDE4D
pPKA
PKA
pCREB
CREB
GAPDH

B


GO Molecular function enrichment (SW480_shNC-vs-SW480_shP1)
Term Candidate Gene Num
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10
20
30
microtubule binding
ATP transmembrane transporter activity
FAD binding
GTP binding
GTPase activity
structural molecule activity
signaling receptor binding
TAP binding
-log10 (Pvalue)
Term Candidate Gene Num
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0.5
1
1.5
2
2.5
3
-log10 (Pvalue)

Figure S3

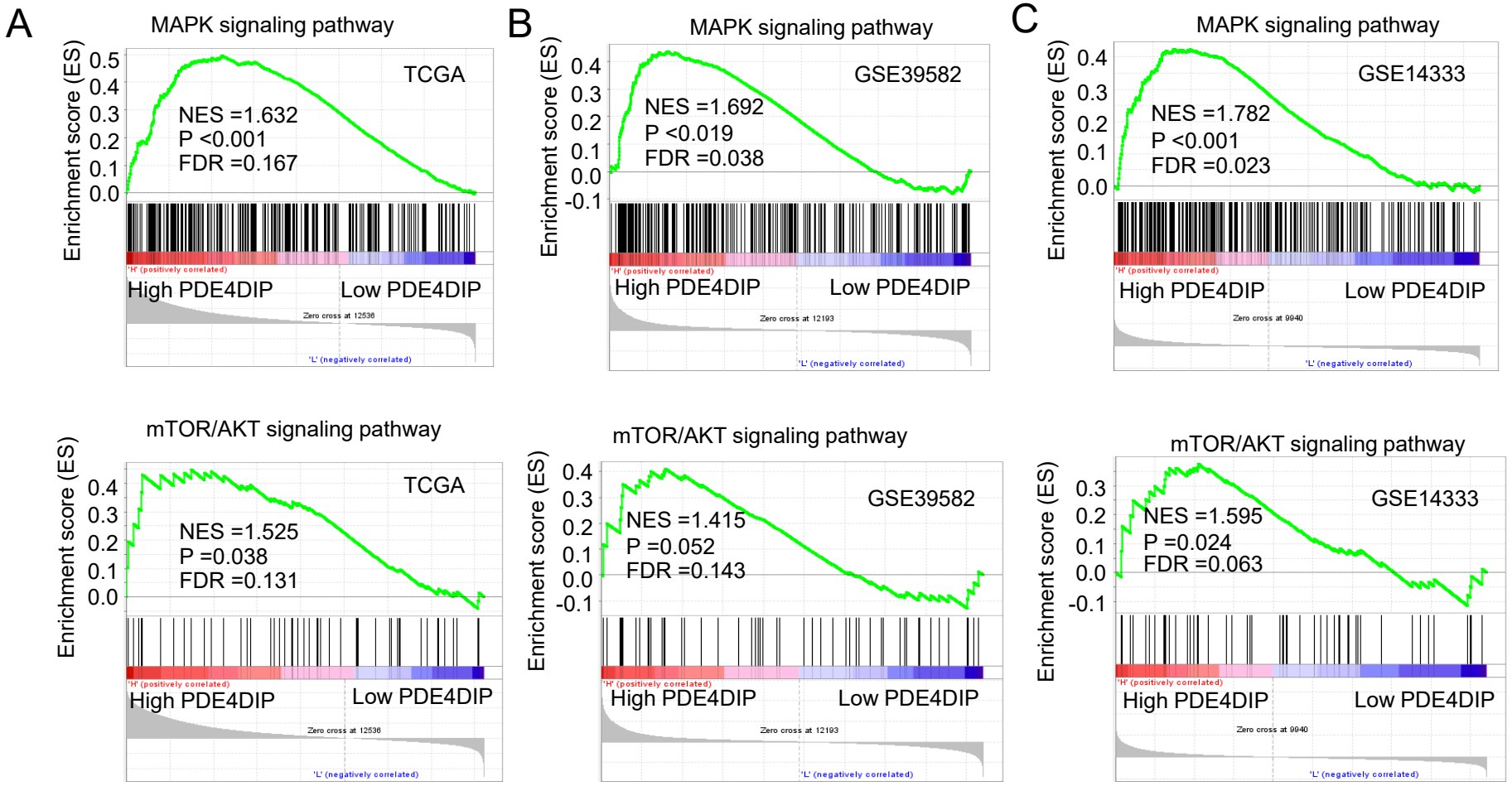

A
MAPK signaling pathway
TCGA
NES =1.632
P <0.001
FDR =0.167
Enrichment score (ES)
'H' (positively correlated)
High PDE4DIP
Low PDE4DIP
Zero cross at 12536
'L' (negatively correlated)
mTOR/AKT signaling pathway
TCGA
NES =1.525
P =0.038
FDR =0.131
B
MAPK signaling pathway
GSE39582
NES =1.692
P <0.019
FDR =0.038
mTOR/AKT signaling pathway
GSE39582
NES =1.415
P =0.052
FDR =0.143
C
MAPK signaling pathway
GSE14333
NES =1.782
P <0.001
FDR =0.023
mTOR/AKT signaling pathway
GSE14333
NES =1.595
P =0.024
FDR =0.063

Figure S4

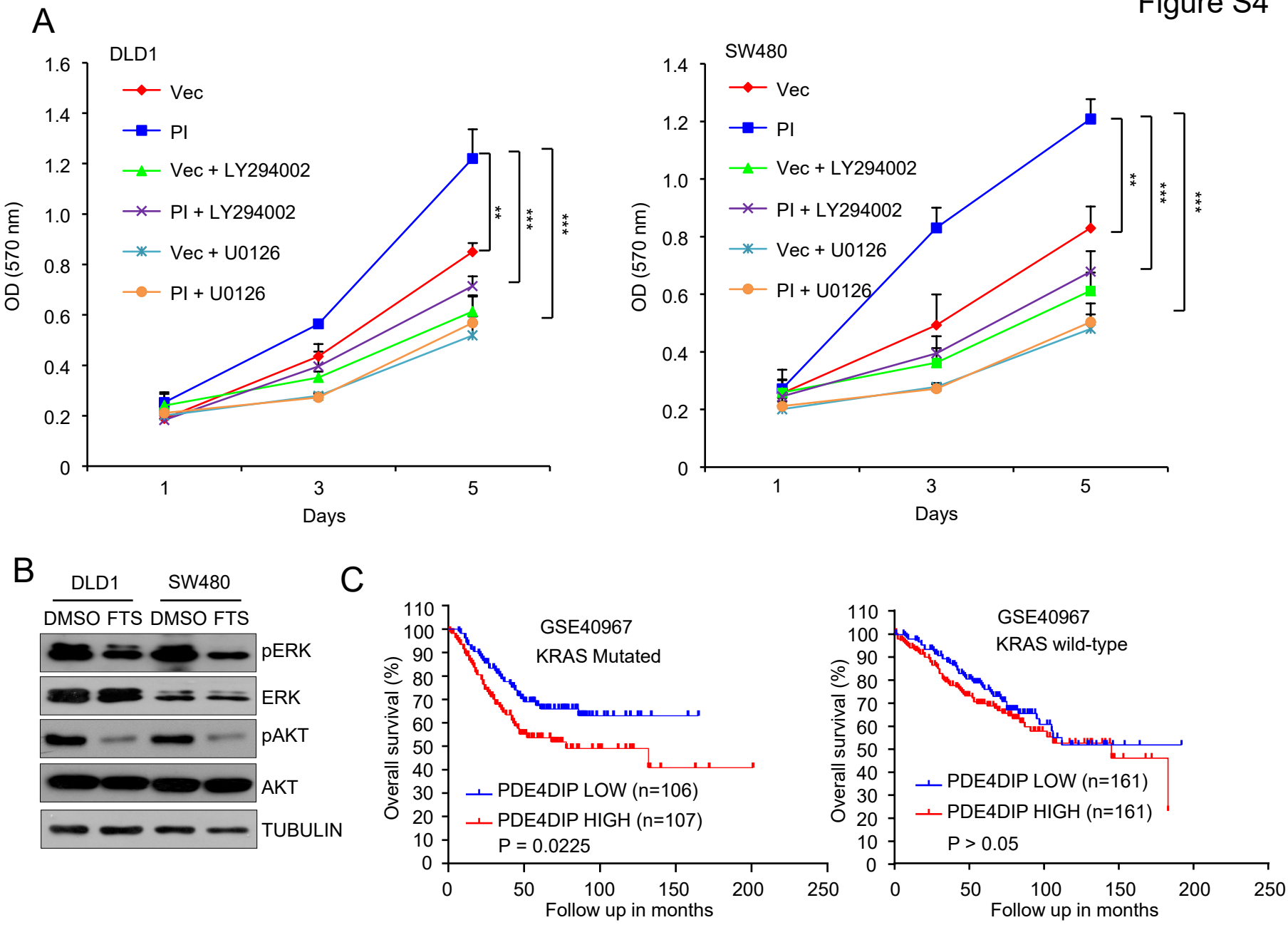

A
DLD1
Vec
PI
Vec + LY294002
PI + LY294002
Vec + U0126
PI + U0126
OD (570 nm)
Days
SW480
B
DLD1
SW480
DMSO FTS DMSO FTS
pERK
ERK
pAKT
AKT
TUBULIN
C
GSE40967
KRAS Mutated
PDE4DIP LOW (n=106)
PDE4DIP HIGH (n=107)
P = 0.0225
Overall survival (%)
Follow up in months
GSE40967
KRAS wild-type
PDE4DIP LOW (n=161)
PDE4DIP HIGH (n=161)
P > 0.05

Figure S5

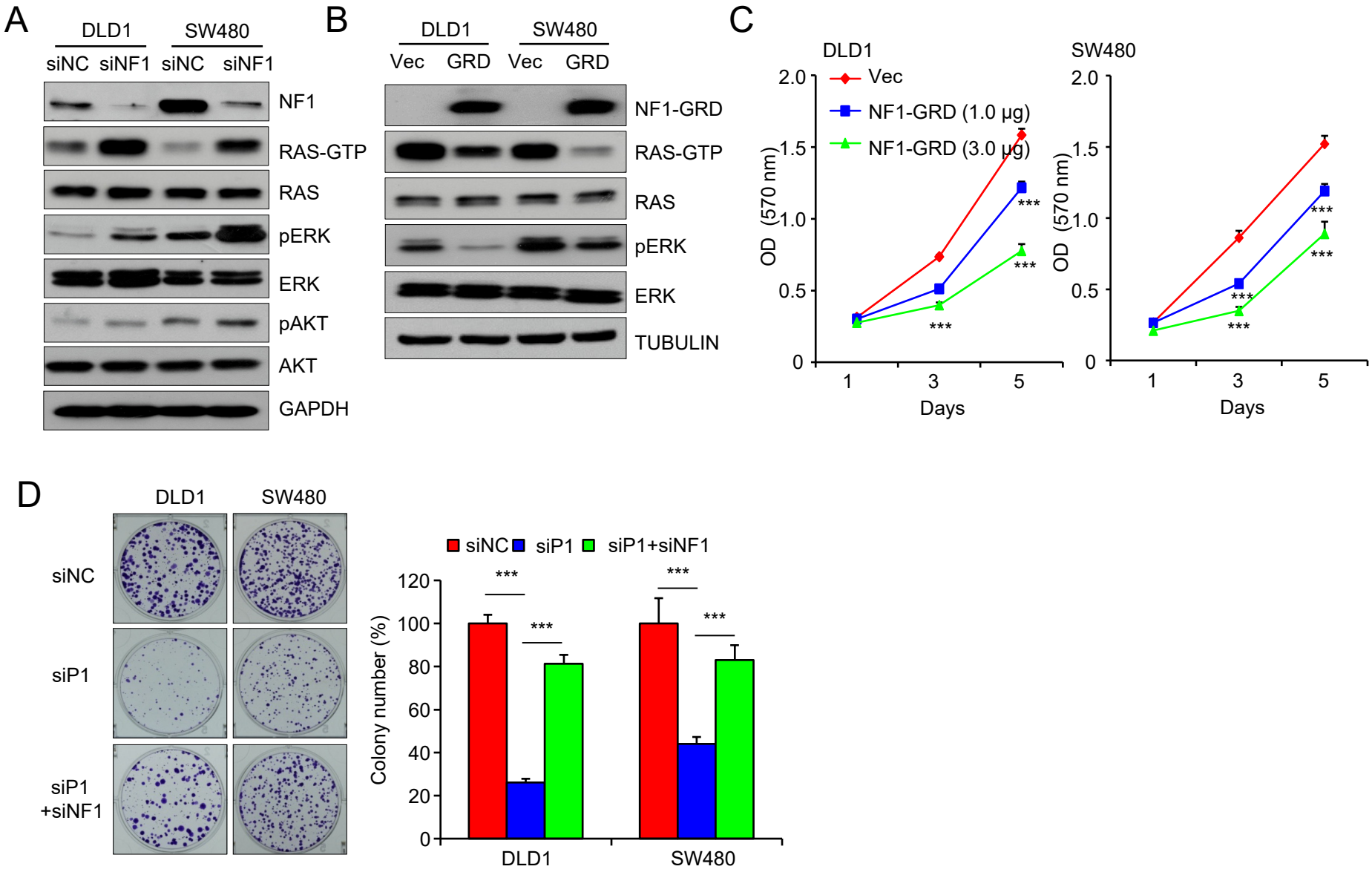

A
DLD1
SW480
siNC
siNF1
siNC
siNF1
NF1
RAS-GTP
RAS
pERK
ERK
pAKT
AKT
GAPDH
B
DLD1
SW480
Vec
GRD
Vec
GRD
NF1-GRD
RAS-GTP
RAS
pERK
ERK
TUBULIN
C
DLD1
SW480
Vec
NF1-GRD (1.0 μg)
NF1-GRD (3.0 μg)
OD (570 nm)
Days
D
DLD1
SW480
siNC
siP1
siP1
+siNF1
siNC
siP1
siP1+siNF1
Colony number (%)
DLD1
SW480

Figure S6

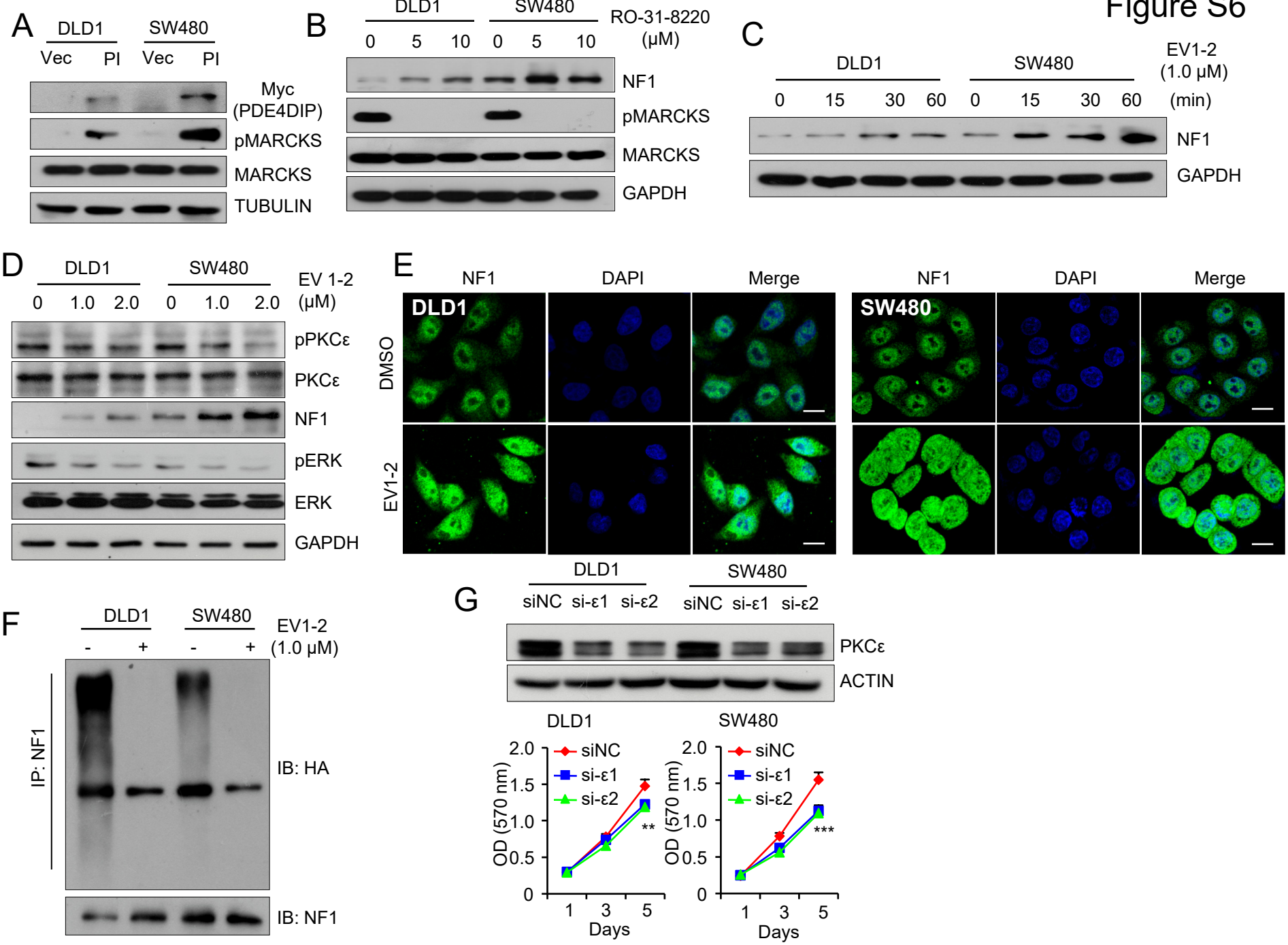

A
DLD1
SW480
Vec
PI
Vec
PI
Myc (PDE4DIP)
pMARCKS
MARCKS
TUBULIN
B
DLD1
SW480
RO-31-8220 (μM)
0
5
10
0
5
10
NF1
pMARCKS
MARCKS
GAPDH
C
DLD1
SW480
EV1-2 (1.0 μM)
0
15
30
60
0
15
30
60
(min)
NF1
GAPDH
D
DLD1
SW480
EV 1-2 (μM)
0
1.0
2.0
0
1.0
2.0
pPKCε
PKCε
NF1
pERK
ERK
GAPDH
E
NF1
DAPI
Merge
NF1
DAPI
Merge
DLD1
SW480
DMSO
EV1-2
F
DLD1
SW480
EV1-2 (1.0 μM)
-
+
-
+
IP: NF1
IB: HA
IB: NF1
G
DLD1
SW480
siNC
si-ε1
si-ε2
siNC
si-ε1
si-ε2
PKCε
ACTIN
DLD1
SW480
siNC
si-ε1
si-ε2
OD (570 nm)
2.0
1.5
1.0
0.5
0
**
***
1
3
5
Days

Figure S7

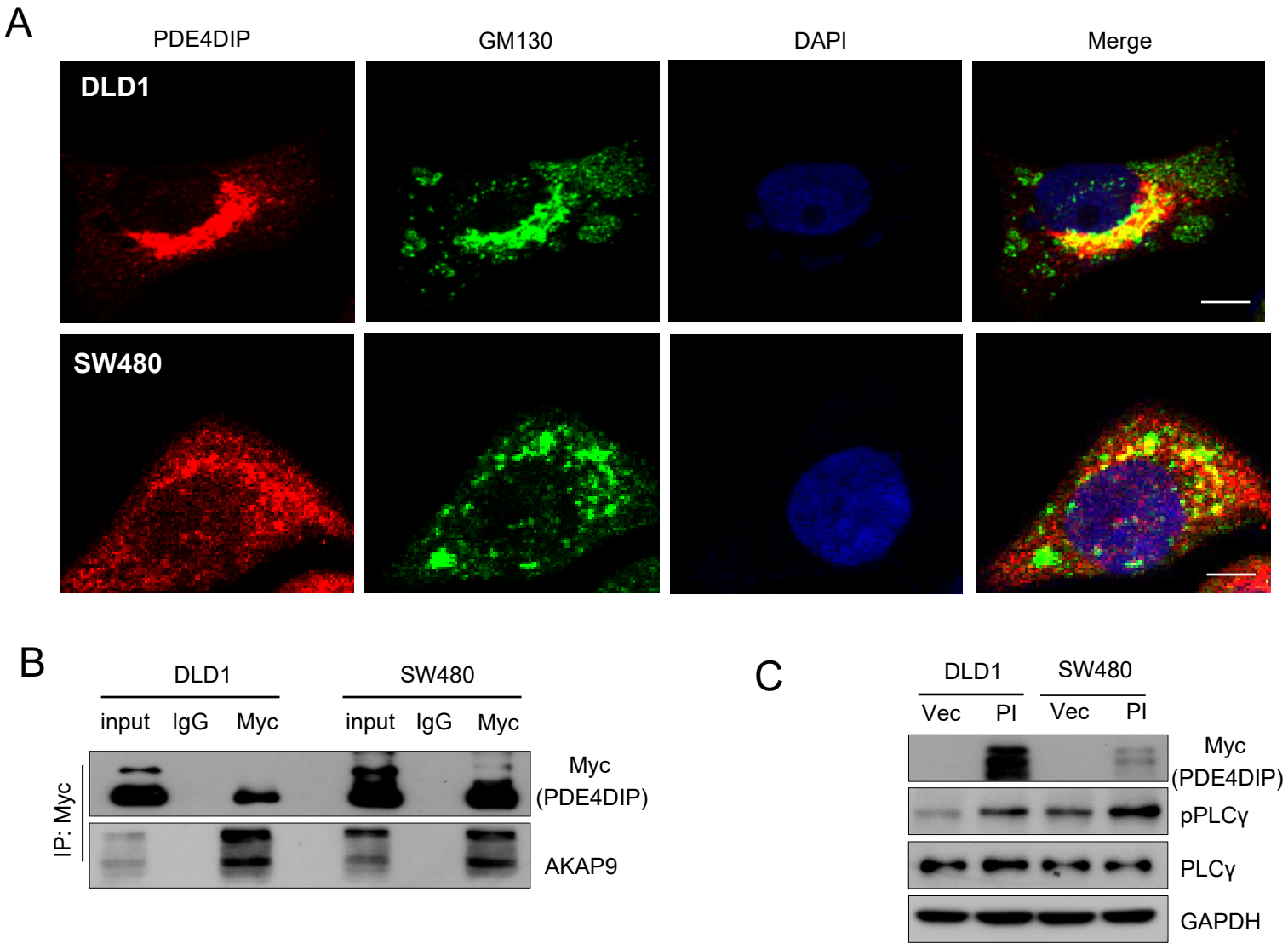
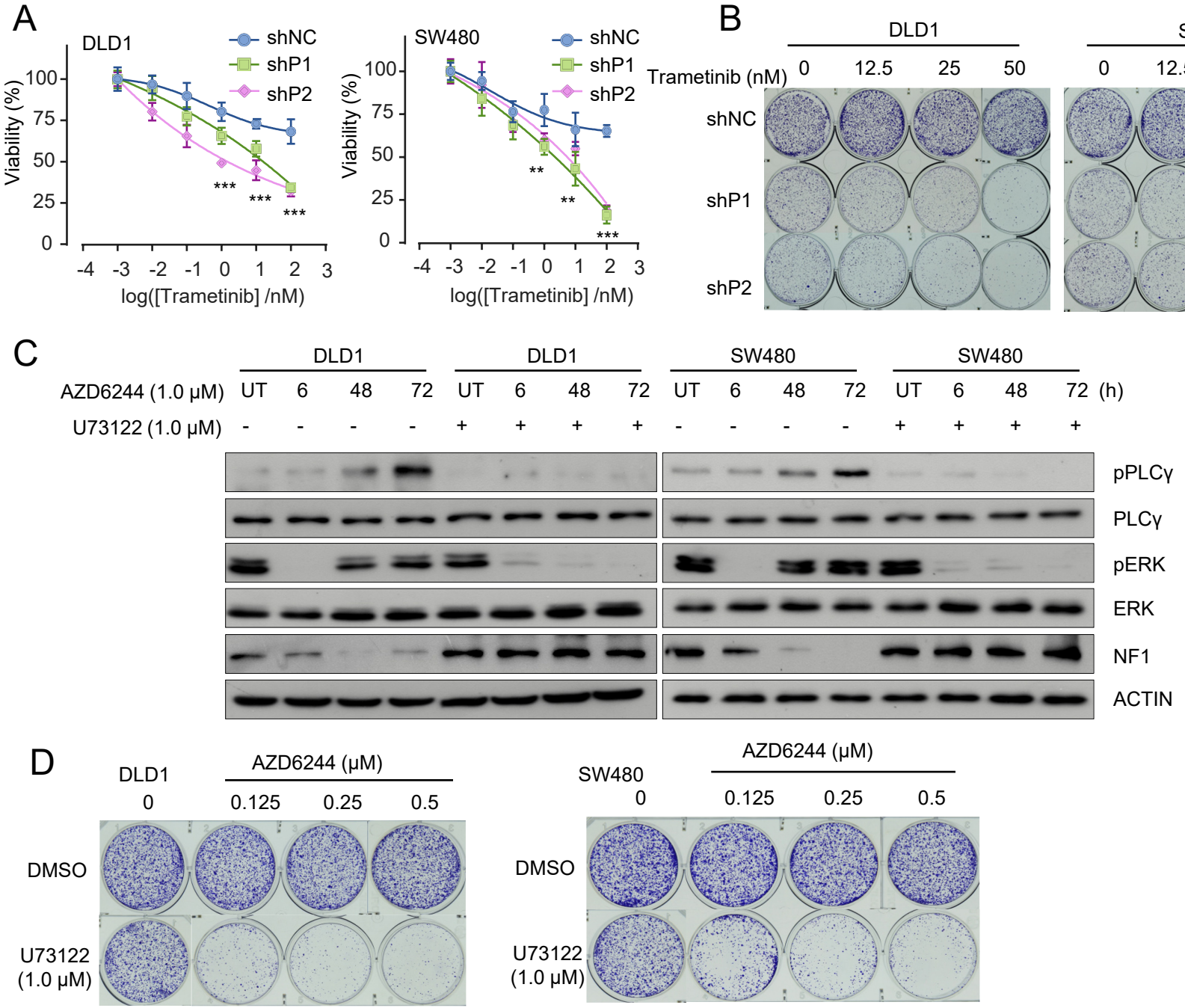

A
PDE4DIP
GM130
DAPI
Merge
DLD1
SW480
B
DLD1
SW480
input
IgG
Myc
input
IgG
Myc
IP: Myc
Myc
(PDE4DIP)
AKAP9
C
DLD1
SW480
Vec
PI
Vec
PI
Myc
(PDE4DIP)
pPLCγ
PLCγ
GAPDH

Figure S8


A
DLD1
shNC
shP1
shP2
Viability (%)
100
75
50
25
0
-4 -3 -2 -1 0 1 2 3
log([Trametinib] /nM)
***
***
***
SW480
shNC
shP1
shP2
Viability (%)
100
75
50
25
0
-4 -3 -2 -1 0 1 2 3
log([Trametinib] /nM)
**
**
***
B
DLD1
SW480
Trametinib (nM)
0 12.5 25 50
0 12.5 25 50
shNC
shP1
shP2
C
DLD1
DLD1
SW480
SW480
AZD6244 (1.0 μM)
UT 6 48 72 UT 6 48 72 UT 6 48 72 UT 6 48 72 (h)
U73122 (1.0 μM)
- - - - + + + + - - - - + + + +
pPLCγ
PLCγ
pERK
ERK
NF1
ACTIN
D
DLD1
AZD6244 (μM)
0
0.125
0.25
0.5
DMSO
U73122
(1.0 μM)
SW480
AZD6244 (μM)
0
0.125
0.25
0.5
DMSO
U73122
(1.0 μM)