Supporting Information

TRIM21-mediated ubiquitination of PARP1 suppresses small cell lung cancer progression through the PI3K/AKT/STAT5A axis

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Figure S1. PARP1 binds with TRIM21

A) Immunofluorescence assay demonstrating the cellular localization of TRIM21 and PARP1 in DMS273 and SHP77 cells. **B**) Western blot analysis of TRIM21 after removal of the GST tag from GST-TRIM21 recombinant protein. **C**) Immunoprecipitation (IP)-western analysis of the interaction between PARP1 and TRIM21. HEK293T cells were transfected with either EGFP-empty, EGFP-TRIM21 WT, EGFP-TRIM21 LD (C16A, C31A, and H33W), or EGFP-TRIM21 W381/383A mutant, the interaction between TRIM21 and PARP1 was detected through immunoprecipitation followed by western blot analysis. **D**) Schematic diagram illustrating the domains of PARP1 protein alongside deletion constructs. **E**) IP-western analysis demonstrating the domain of PARP1 required for its interaction with TRIM21. Flag-PARP1 WT, Flag-PARP1 ΔHIR, Flag-PARP1 ΔHD, Flag-PARP1 Δ645-809, or Flag-PARP1 K654R was co-transfected with EGFP-TRIM21 or EGFP-empty in HEK293T cells, and then the interaction between TRIM21 and PARP1 was evaluated by IP and immunoblotting.

Figure S2. TRIM21 facilitates the ubiquitination and degradation of PARP1

A) Western blot analysis of PARP1 following *TRIM21* knockdown using siRNAs targeting *TRIM21*. **B, C, D**) RT-qPCR analysis of *PARP1* expression in response to *TRIM21* knockdown by siRNAs in HEK293T (**B**), DMS273 (**C**), and SHP77 (**D**) cells, normalized to β-Actin. **E**) *In vivo* ubiquitination of PARP1. Immunoprecipitation (IP) followed by western blot was employed to detect total ubiquitinated

PARP1 in both *TRIM21* knockdown and overexpressed DMS273 and SHP77 cells. **F**, **G**) IP-western analysis showing the requirement of the TRIM21-PARP1 interaction for TRIM21-mediated ubiquitination and degradation of PARP1. HEK293T cells were co-transfected with Flag-PARP1, HA-Ub, and either EGFP-TRIM21 or EGFP-TRIM21 ΔPP (**F**), or co-transfected with HA-Ub, EGFP-TRIM21, and either Flag-PARP1 or Flag-PARP1 ΔHD (**G**). PARP1 ubiquitination levels were assessed via IP-western analysis. **H**, **I**) Assessment of PARP1 ubiquitination and PARP1 protein under DNA damage-inducing conditions. HEK293T cells were co-transfected with Flag-PARP1, HA-Ub, and either EGFP-TRIM21 or EGFP-empty vector. After treatment with etoposide (EP, 40 μM, 24 h) or H_2O_2 (2 μM, 5 min), PARP1 ubiquitination levels of PARP1 were assessed via IP-WB (**H**), while PARP1 accumulation was analyzed by WB (**I**). Data are mean ± s.e.m., and statistical analysis was performed using two-tailed unpaired Student's *t*-tests in (**B**), (**C**), and (**D**). Statistical significance is indicated as **P* < 0.05; ***P* < 0.01; *****P* < 0.0001; 'ns' indicates no significant difference.

Figure S3. TRIM21 is downregulated in SCLC

A, B) Scatter plot analysis of the CCLE dataset showing significantly reduced expression of *TRIM21* in SCLC cells (*n* = 49) compared to normal tissue cell lines (NL) (*n* = 35, *P* < 0.0001) (**A**), and normal lung cell lines (NL) (*n* = 6, *P* = 0.0002) (**B**). **C**) Analysis of *TRIM21* mRNA expression in SCLC and adjacent normal tissues (paraSCLC) (*n* = 18, *P* < 0.0001). **D**) Scatter plot analysis of the expression of *TRIM21* in SCLC tissues compared to paired normal lung tissues (NT) (*n* = 6, *P* = 0.0410). **E**) Analysis of *TRIM21* mRNA expression levels in 21 SCLC tissues and 14 normal tissues (NT) (*P* < 0.0001). **F**) Relative *TRIM21* expression in human SCLC tissues compared to the normal lung tissues (NT) from the GDS4794 data set (n=23, the red line means more than a 1.5-fold increase). Data are mean ± s.d., and statistical analysis was performed using two-tailed unpaired Student's *t*-tests in (**A**), (**B**), (**C**), and (**E**). Data across (**D**) was statistically analyzed utilizing two-tailed paired Student's *t*-tests.

Figure S4. TRIM21 suppresses the survival of SCLC cells by decreasing PARP1 accumulation

A) Western blot analysis investigating the compensatory effects of *PARP1* on altered *TRIM21* expression. PARP1 and TRIM21 protein levels were assessed following the treatment described in Figure **4A**. **B**) Quantitative analysis of colony formation shown in Figure **4A**. **C**) Western blot analysis of TRIM21, full-length PARP1, and its mutants in *TRIM21*-overexpressing cells. The protein levels of TRIM21, PARP1, and its mutants were analyzed following the treatment described in Figure **4C**. **D**) Quantitative analysis of colony formation is shown in Figure **4C**. Data are mean ± s.e.m., and statistical analysis was performed using two-tailed unpaired Student's *t*-tests in (**B**) and (**D**). Statistical significance is indicated as **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001; 'ns' indicates no significant difference.

Figure S5. TRIM21 deficiency enhances DNA damage responses by induction of PARP1 accumulation

A) Immunostaining staining showing the formation of γH2AX foci in SHP77 cells following the protocol described in Figure **5C**. Scale bars, 10 μm. **B**) Quantification of the γH2AX foci formation in (**A**). *n* = 80 cells. **C**) Comet assays depicting the effect of *PARP1* or *TRIM21* knockdown on DNA damage in SHP77 cells. Comet assays were performed in SHP77 cells, following a protocol similar to that described in Figure **5E**. Scale bars, 100 μm. **D**) Quantification of the tail moment of comet assay in (**C**). *n* = 100 cells. **E**) Quantitative analysis of colony formation shown in Figure **5G**. **F**, **H**, **J**) Colony formation assays measuring the clonogenic capacity of the DMS273 or SHP77 cells treated by CPT or EP with indicated concentrations. **G**, **I**, **K**) Quantitative analysis of colony formation shown in (**F**), (**H**), and (**J**). Data show

mean ± s.d. in (**B**) and (**D**). Data are mean ± s.e.m. in (**E**), (**G**), (**I**), and (**K**). Statistical analysis was performed using two-tailed unpaired *t*-tests. Statistical significance is indicated as **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

Figure S6. PI3K/AKT facilitates PARP1 stabilization by blocking TRIM21 expression

A) Correlation analysis of *TRIM21* and *STAT5A* mRNA levels with the activity of various signaling pathways in 81 SCLC tissues from the European Genome-phenome Archive (EGAS00001000925). **B**) A similar correlation analysis to (**A**) in 107 SCLC tissues from the GSA database (HRA003419). **C**) Analysis of the correlation between PI3K/AKT pathway activity and *TRIM21* mRNA levels in 107 SCLC tissues from the GSA database (HRA003419). **D**) Dose-response analysis of protein levels of AKT, p-AKT, TRIM21, and PARP1 in DMS273 and SHP77 cells treated with a gradient of PKI-587 or SC-79 concentrations for 24 h, and these protein levels were subsequently assessed by western blot.

Figure S7. STAT5A acts as a transcription factor inhibiting TRIM21 via PI3K/AKT pathway

A) RT-qPCR analysis of *TRIM21*, *STAT5A*, *SIX5*, and *TCF21* expression after knocking down *STAT5A*, *SIX5*, or *TCF21* in HEK293T cells. **B**) Identification of STAT5A peaks in the promoter of the TRIM21 gene from the ENCODE (Encyclopedia of DNA Elements) database. **C**) The consensus DNA-binding motif of STAT5A derived from the JASPAR database. **D**) Analysis of the correlation between PI3K/AKT pathway activity scores and *STAT5A* mRNA levels in 81 SCLC tissues obtained from the European Genome-phenome Archive (EGAS00001000925). **E**) Analysis of the correlation between *TRIM21* and *STAT5A* mRNA levels in 107 SCLC tissues using data from the OMIX002489 database. **F**) Correlation analysis of TRIM21 and STAT5A protein levels in 112 SCLC tumor tissues acquired from the OMIX database (OMIX002489). **G**) Dose-response analysis of mRNA levels of *STAT5A*, *TRIM21*, and *PARP1* in SCLC cells by RT-qPCR. DMS273 and SHP77 cells were treated with varying concentrations of PKI-587 or SC-79 for 24 h, and the mRNA levels of *PARP1*, *TRIM21*, and *STAT5A* were subsequently measured by RT-qPCR. The data are expressed as mean ± s.e.m. and statistical analysis was performed using two-tailed unpaired Student's *t*-tests in (**A**) and (**G**). Statistical significance is indicated as **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001; 'ns' indicates no significant difference.

Figure S8. Inhibition of PI3K/AKT synergize with PARPi

A, B) Growth inhibition curves of BMN673 (**A**) and PKI-587 (**B**) in DMS273 and SHP77 cells. The cells were treated with different concentrations of BMN673 or PKI-587 for 72 h. The cell viability was then assessed by the CellTiter-Glo assay. The Half-lethal dose (IC_{50}) values were determined from the sigmoidal dose-response curves. **C, E**) Clonogenic assays illustrating the effect of BMN673 (**C**) and PKI-587 (**E**) on DMS273 and SHP77 cells. (**D**) Quantification of colony formation as shown in (**C**). (**F**) Quantification of clone formation as shown in (**E**). **G**) Drug-response curves shown in Figure **8C**. **H**) Body weight curves of xenograft mice during treatment with PKI-587, BMN673, or a combination of PKI-587 and BMN673. **I**) Representative images of HE staining and immunohistochemistry for Ki67, p-AKT, p-STAT5A, TRIM21, PARP1, and γH2AX within tumor sections. Scale bars represent 40 μm. Data are mean ± s.e.m. in (**A**), (**B**), (**D**), (**F**), (**G**), and (**H**).

Figure S1

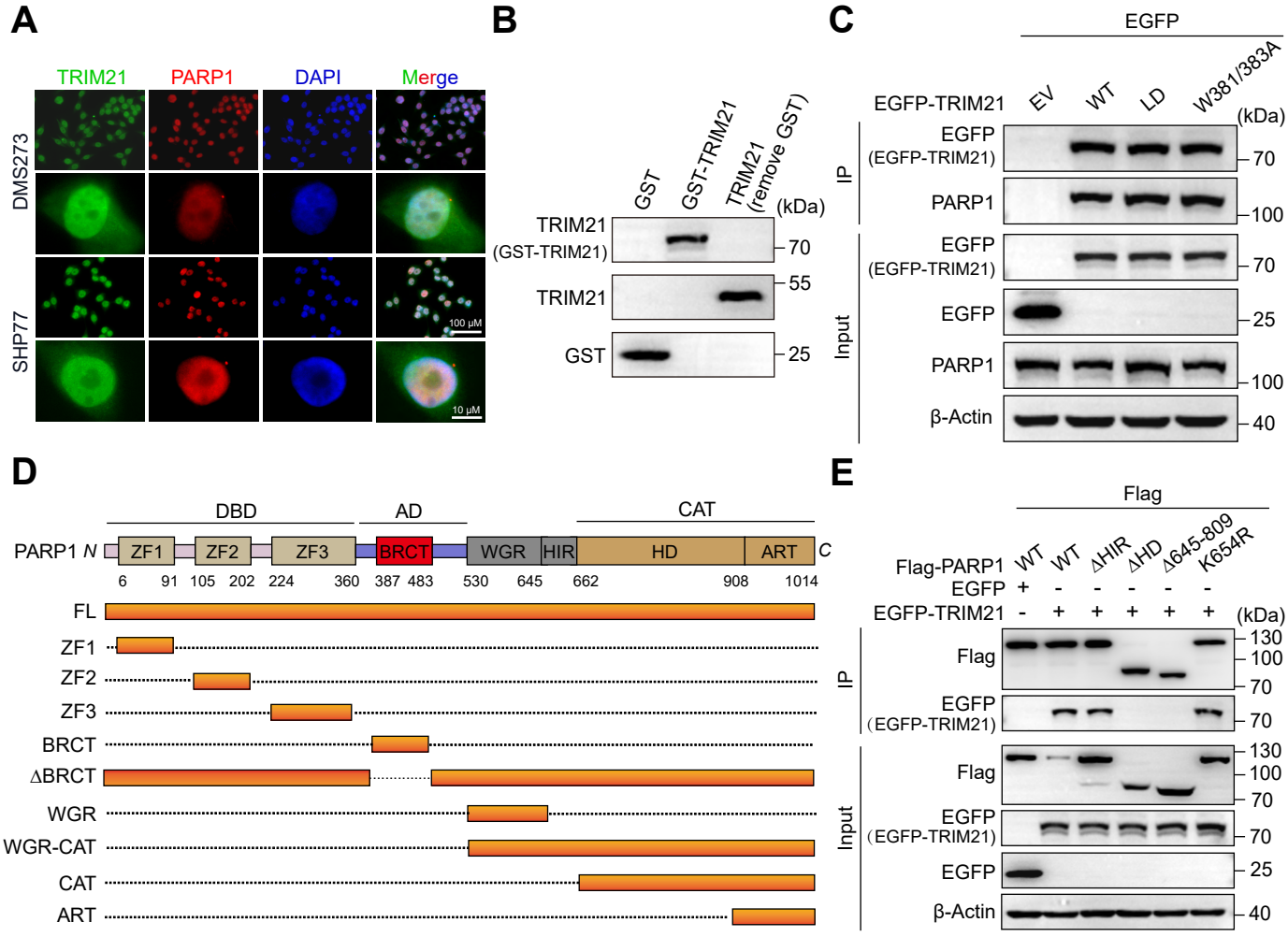

A
TRIM21
PARP1
DAPI
Merge
DMS273
SHP77
100 μM
10 μM
B
GST
GST-TRIM21
TRIM21 (remove GST)
(kDa)
TRIM21 (GST-TRIM21)
70
TRIM21
55
GST
25
IP
Input
C
EGFP
EGFP-TRIM21
EV
WT
LD
W381/383A
(kDa)
EGFP (EGFP-TRIM21)
70
PARP1
100
EGFP (EGFP-TRIM21)
70
EGFP
25
PARP1
100
β-Actin
40
D
DBD
AD
CAT
PARP1 N
ZF1
ZF2
ZF3
BRCT
WGR
HIR
HD
ART
C
6
91
105
202
224
360
387
483
530
645
662
908
1014
FL
ZF1
ZF2
ZF3
BRCT
ΔBRCT
WGR
WGR-CAT
CAT
ART
E
Flag
Flag-PARP1
WT
WT
ΔHIR
ΔHD
Δ645-809
K654R
EGFP
+
-
-
-
-
-
EGFP-TRIM21
-
+
+
+
+
+
(kDa)
Flag
130
100
70
EGFP (EGFP-TRIM21)
70
Flag
130
100
70
EGFP (EGFP-TRIM21)
70
EGFP
25
β-Actin
40
IP
Input

Figure S2

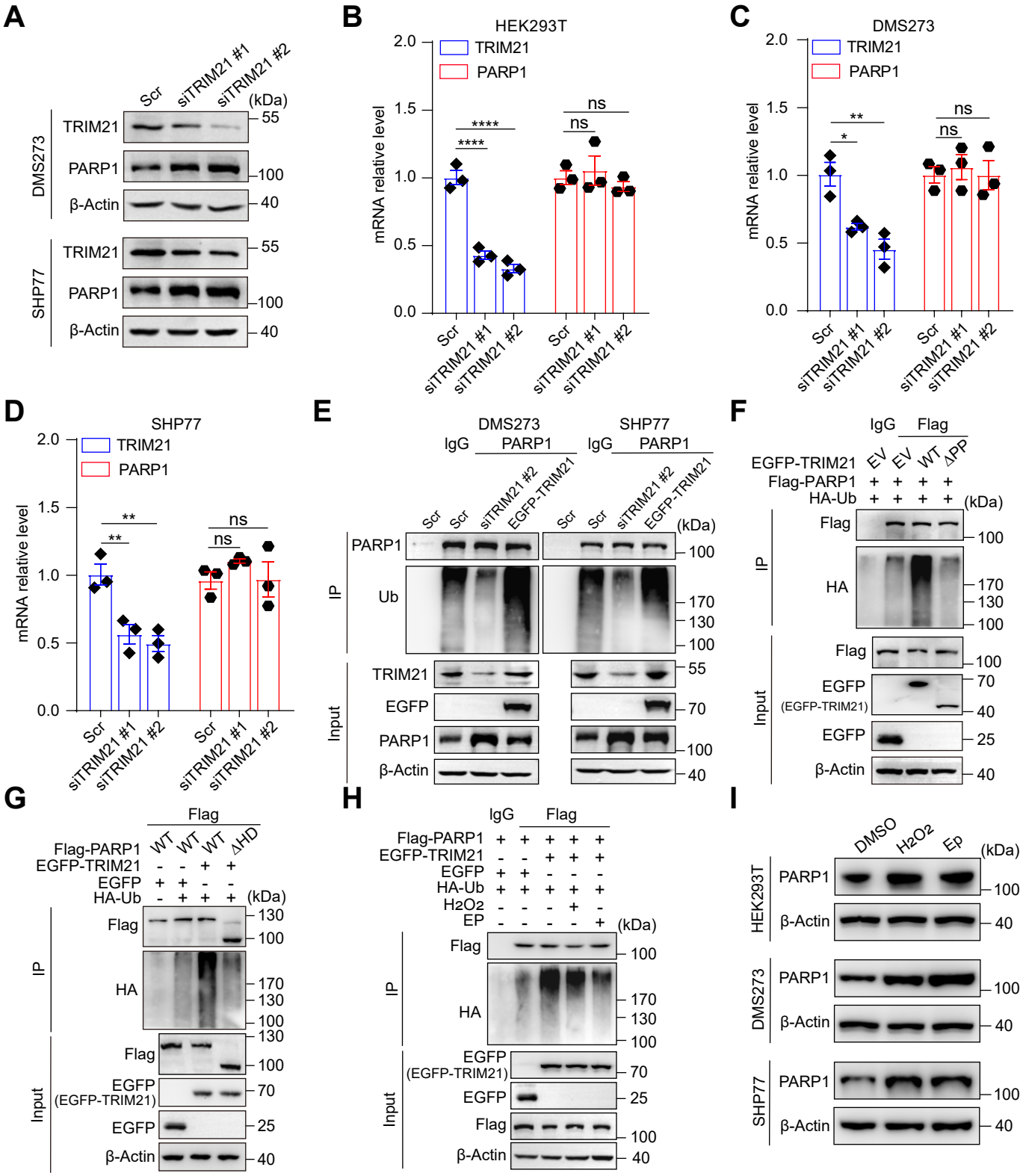

A
Scr
siTRIM21 #1
siTRIM21 #2
(kDa)
DMS273
TRIM21
PARP1
β-Actin
55
100
40
SHP77
TRIM21
PARP1
β-Actin
55
100
40
B
HEK293T
TRIM21
PARP1
mRNA relative level
2.0
1.5
1.0
0.5
0.0
****
****
ns
ns
Scr
siTRIM21 #1
siTRIM21 #2
C
DMS273
TRIM21
PARP1
mRNA relative level
**
*
ns
ns
D
SHP77
TRIM21
PARP1
mRNA relative level
**
**
ns
ns
E
DMS273
SHP77
IgG
PARP1
Scr
siTRIM21 #2
EGFP-TRIM21
(kDa)
IP
PARP1
Ub
170
130
100
Input
TRIM21
55
EGFP
70
PARP1
100
β-Actin
40
F
IgG
Flag
EGFP-TRIM21
EV
EV
WT
ΔPP
Flag-PARP1
HA-Ub
(kDa)
IP
Flag
HA
100
170
130
100
Input
Flag
EGFP
(EGFP-TRIM21)
70
40
EGFP
25
β-Actin
40
G
Flag
Flag-PARP1
WT
WT
WT
ΔHD
EGFP-TRIM21
EGFP
HA-Ub
(kDa)
IP
Flag
130
100
HA
170
130
100
Input
Flag
130
100
EGFP
(EGFP-TRIM21)
70
EGFP
25
β-Actin
40
H
IgG
Flag
Flag-PARP1
EGFP-TRIM21
EGFP
HA-Ub
H2O2
EP
(kDa)
IP
Flag
100
HA
170
130
100
Input
EGFP
(EGFP-TRIM21)
70
EGFP
25
Flag
100
β-Actin
40
I
DMSO
H2O2
Ep
(kDa)
HEK293T
PARP1
β-Actin
100
40
DMS273
PARP1
β-Actin
100
40
SHP77
PARP1
β-Actin
100
40

Figure S3

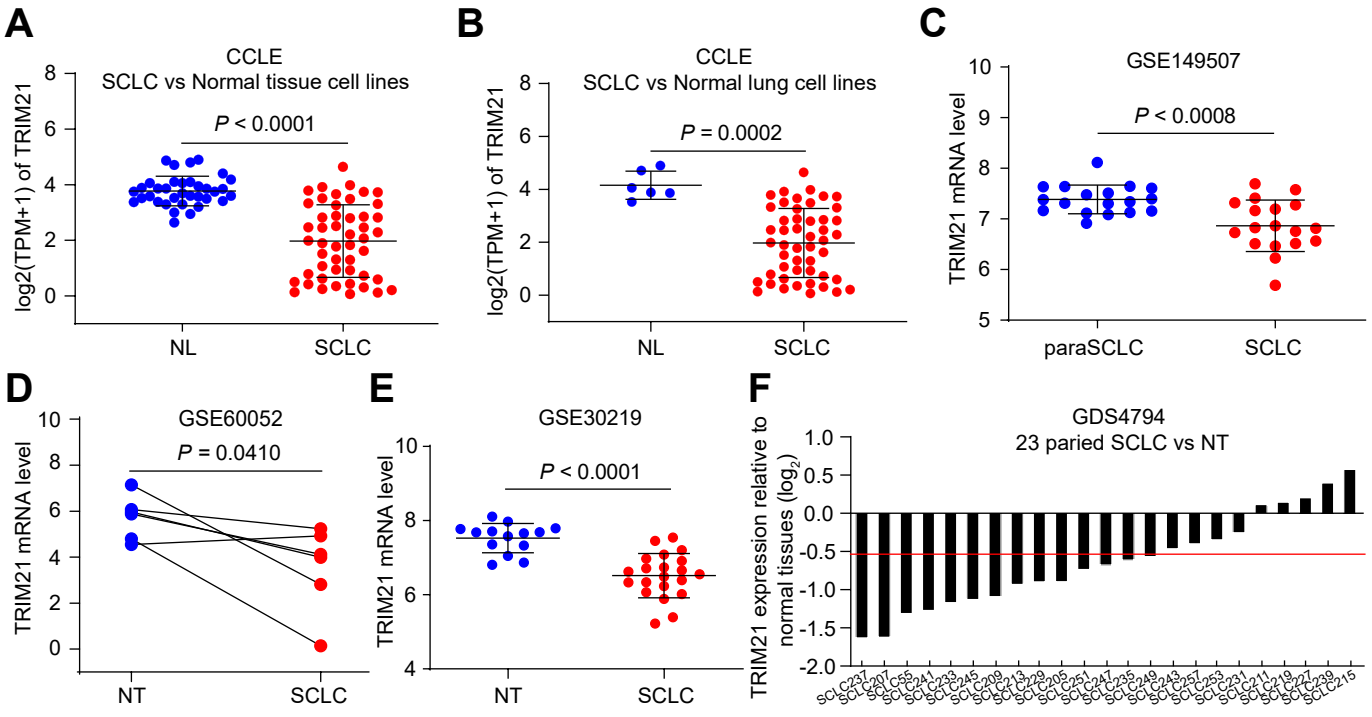

A
CCLE
SCLC vs Normal tissue cell lines
P < 0.0001
log2(TPM+1) of TRIM21
NL
SCLC
B
CCLE
SCLC vs Normal lung cell lines
P = 0.0002
log2(TPM+1) of TRIM21
NL
SCLC
C
GSE149507
P < 0.0008
TRIM21 mRNA level
paraSCLC
SCLC
D
GSE60052
P = 0.0410
TRIM21 mRNA level
NT
SCLC
E
GSE30219
P < 0.0001
TRIM21 mRNA level
NT
SCLC
F
GDS4794
23 paried SCLC vs NT
TRIM21 expression relative to normal tissues (log2)

Figure S4

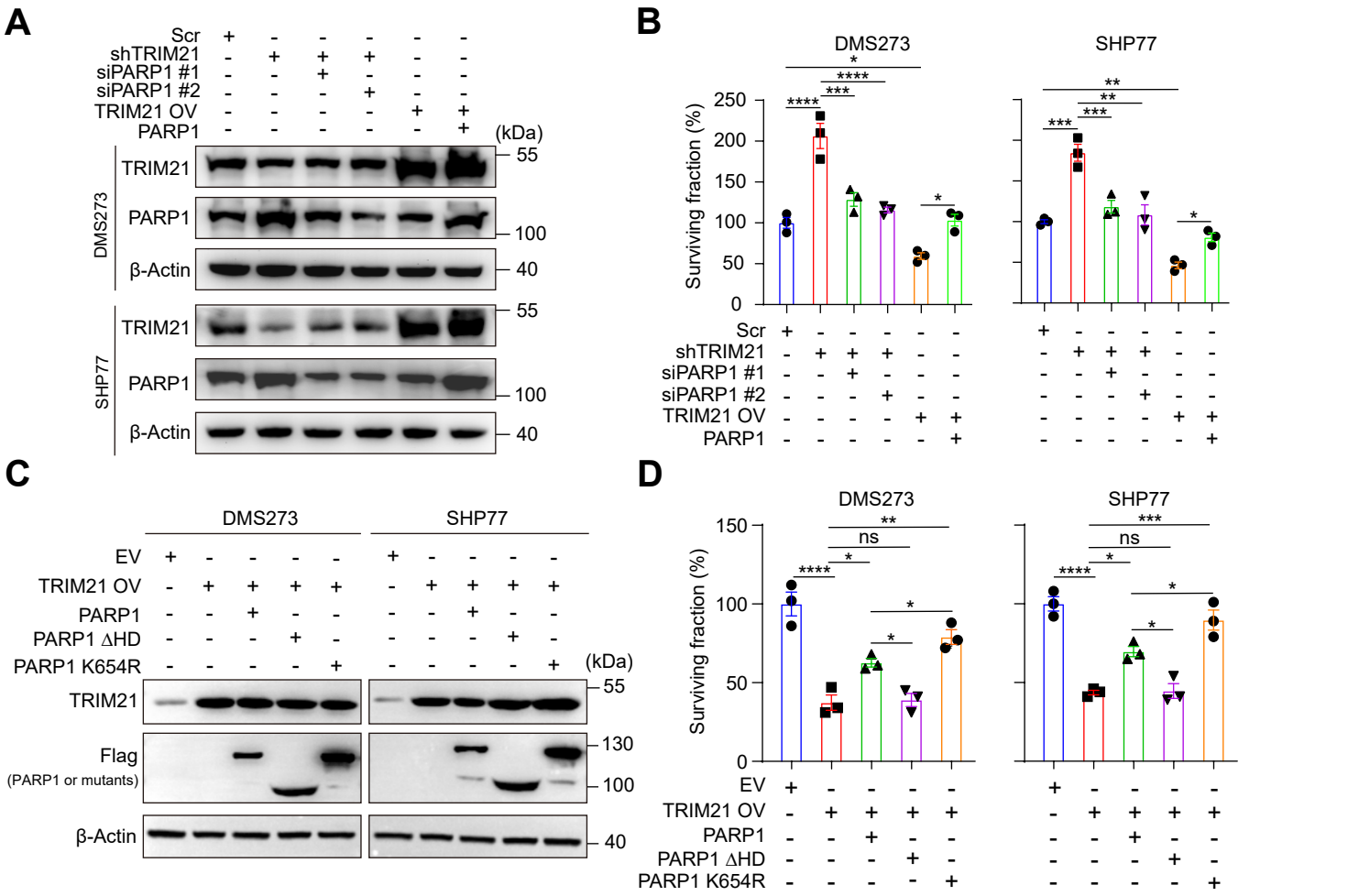

A
Scr
shTRIM21
siPARP1 #1
siPARP1 #2
TRIM21 OV
PARP1
(kDa)
DMS273
TRIM21
PARP1
β-Actin
55
100
40
SHP77
TRIM21
PARP1
β-Actin
55
100
40
B
DMS273
SHP77
Surviving fraction (%)
250
200
150
100
50
0
Scr
shTRIM21
siPARP1 #1
siPARP1 #2
TRIM21 OV
PARP1
C
DMS273
SHP77
EV
TRIM21 OV
PARP1
PARP1 ΔHD
PARP1 K654R
(kDa)
TRIM21
Flag
(PARP1 or mutants)
β-Actin
55
130
100
40
D
DMS273
SHP77
Surviving fraction (%)
150
100
50
0
ns
EV
TRIM21 OV
PARP1
PARP1 ΔHD
PARP1 K654R

Figure S5

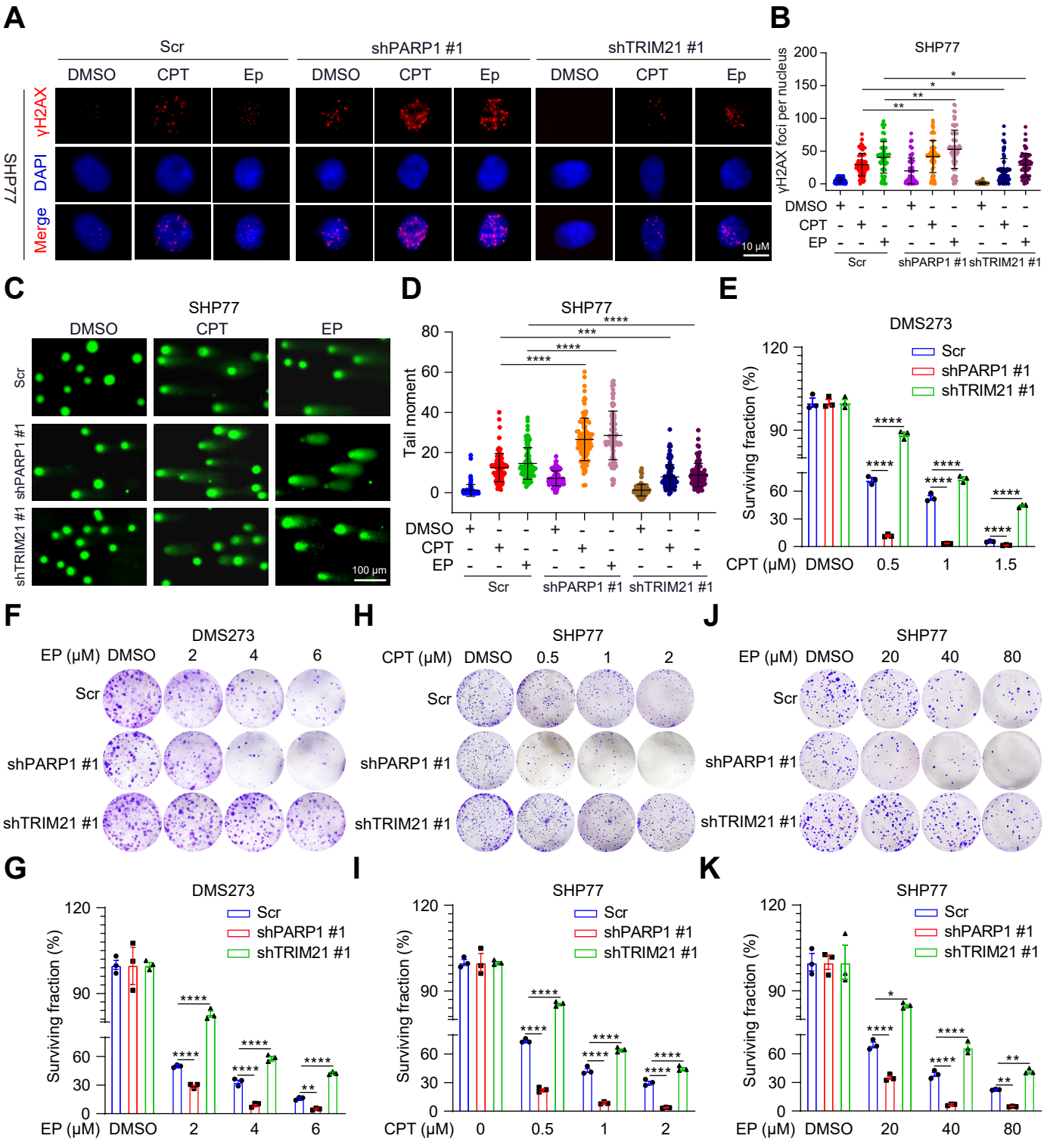

A
Scr
shPARP1 #1
shTRIM21 #1
DMSO
CPT
Ep
γH2AX
DAPI
Merge
SHP77
10 μM
B
SHP77
γH2AX foci per nucleus
DMSO
CPT
EP
Scr
shPARP1 #1
shTRIM21 #1
C
SHP77
DMSO
CPT
EP
100 μm
D
SHP77
Tail moment
E
DMS273
Surviving fraction (%)
CPT (μM)
F
DMS273
EP (μM)
G
DMS273
H
SHP77
CPT (μM)
I
SHP77
J
SHP77
EP (μM)
K
SHP77

Figure S6

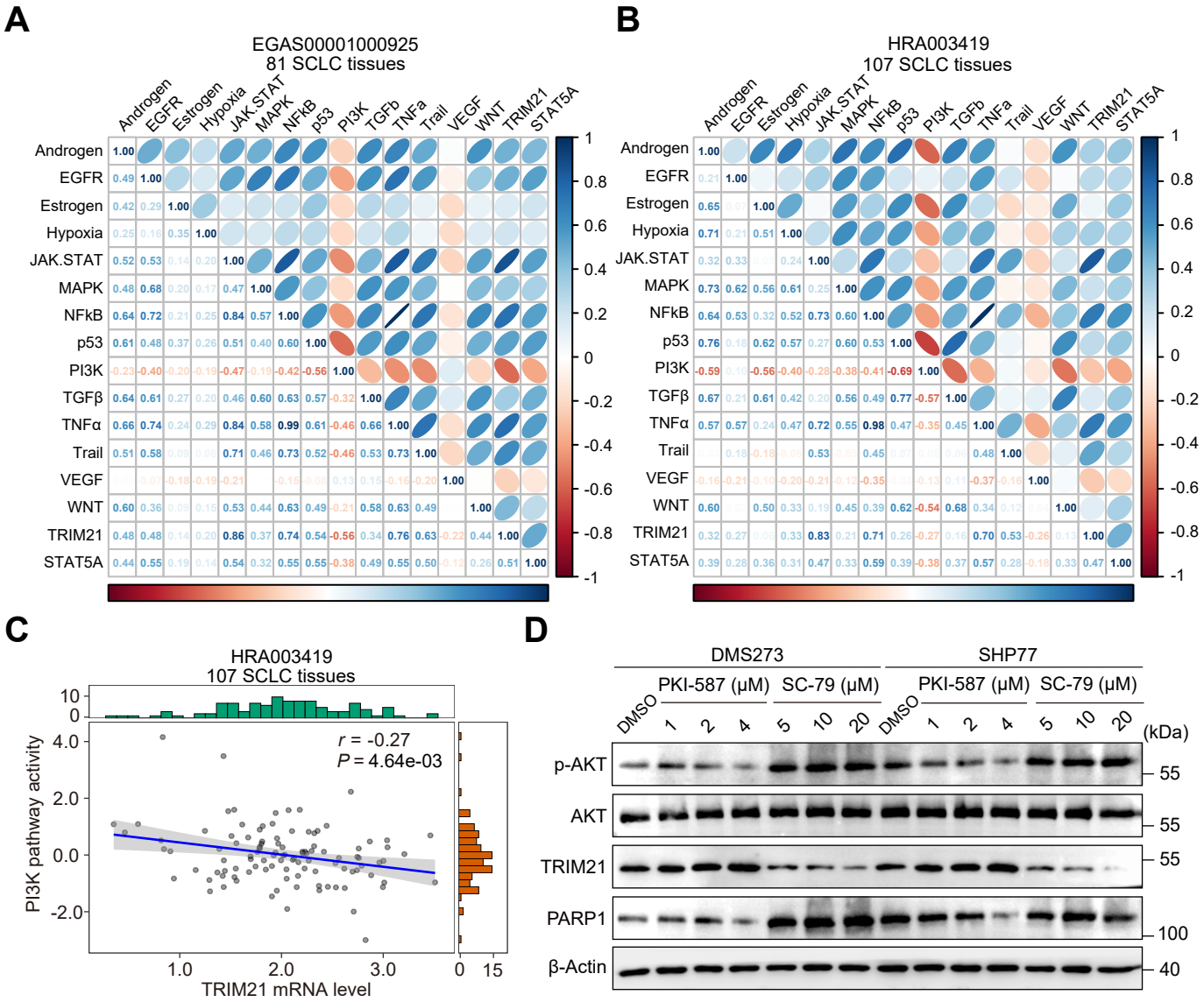

A
EGAS00001000925
81 SCLC tissues
B
HRA003419
107 SCLC tissues
Androgen
EGFR
Estrogen
Hypoxia
JAK.STAT
MAPK
NFkB
p53
PI3K
TGFb
TNFa
Trail
VEGF
WNT
TRIM21
STAT5A
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
C
HRA003419
107 SCLC tissues
r = -0.27
P = 4.64e-03
PI3K pathway activity
TRIM21 mRNA level
D
DMS273
SHP77
PKI-587 (μM)
SC-79 (μM)
DMSO
1
2
4
5
10
20
(kDa)
p-AKT
AKT
TRIM21
PARP1
β-Actin
55
55
55
100
40

Figure S7

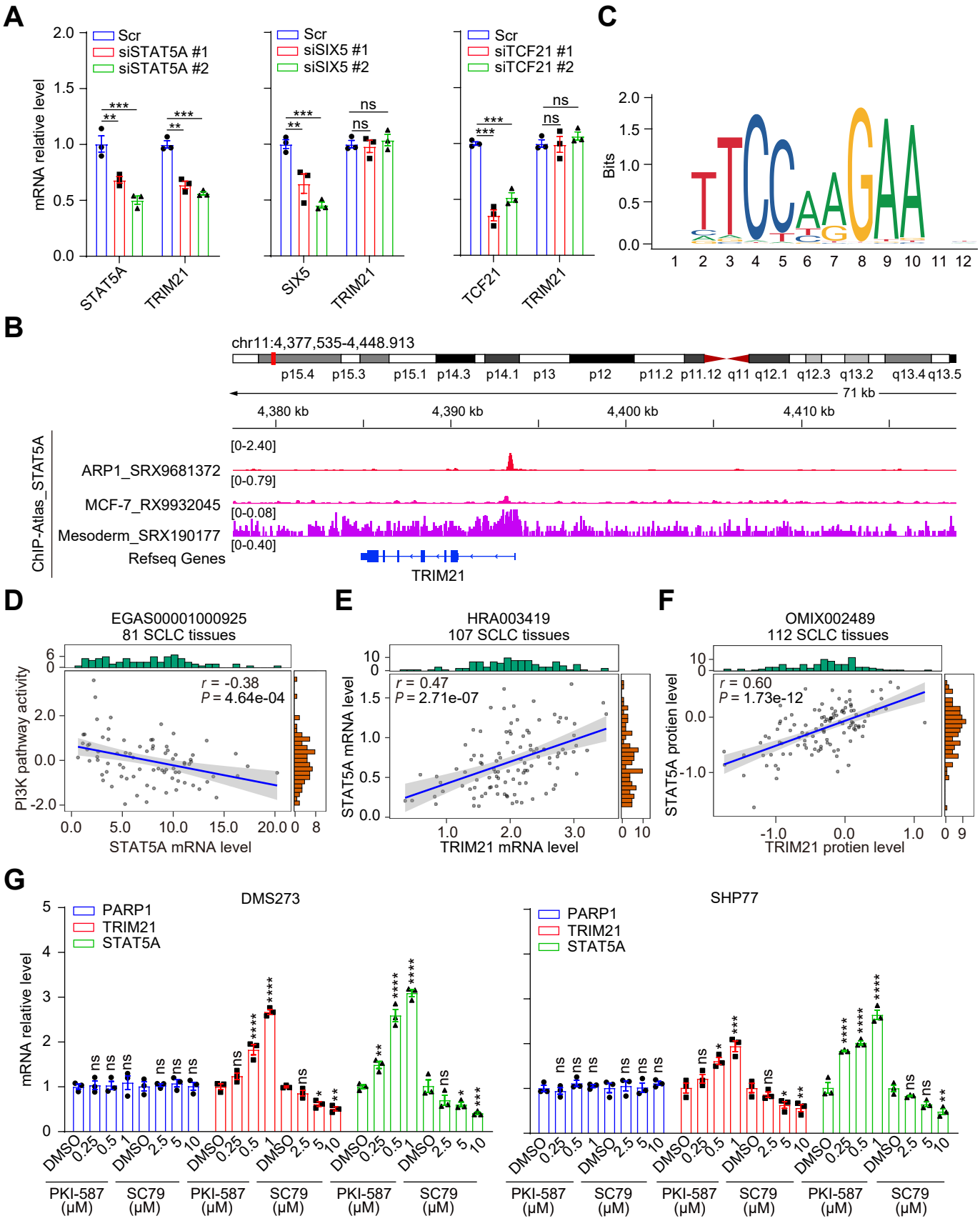
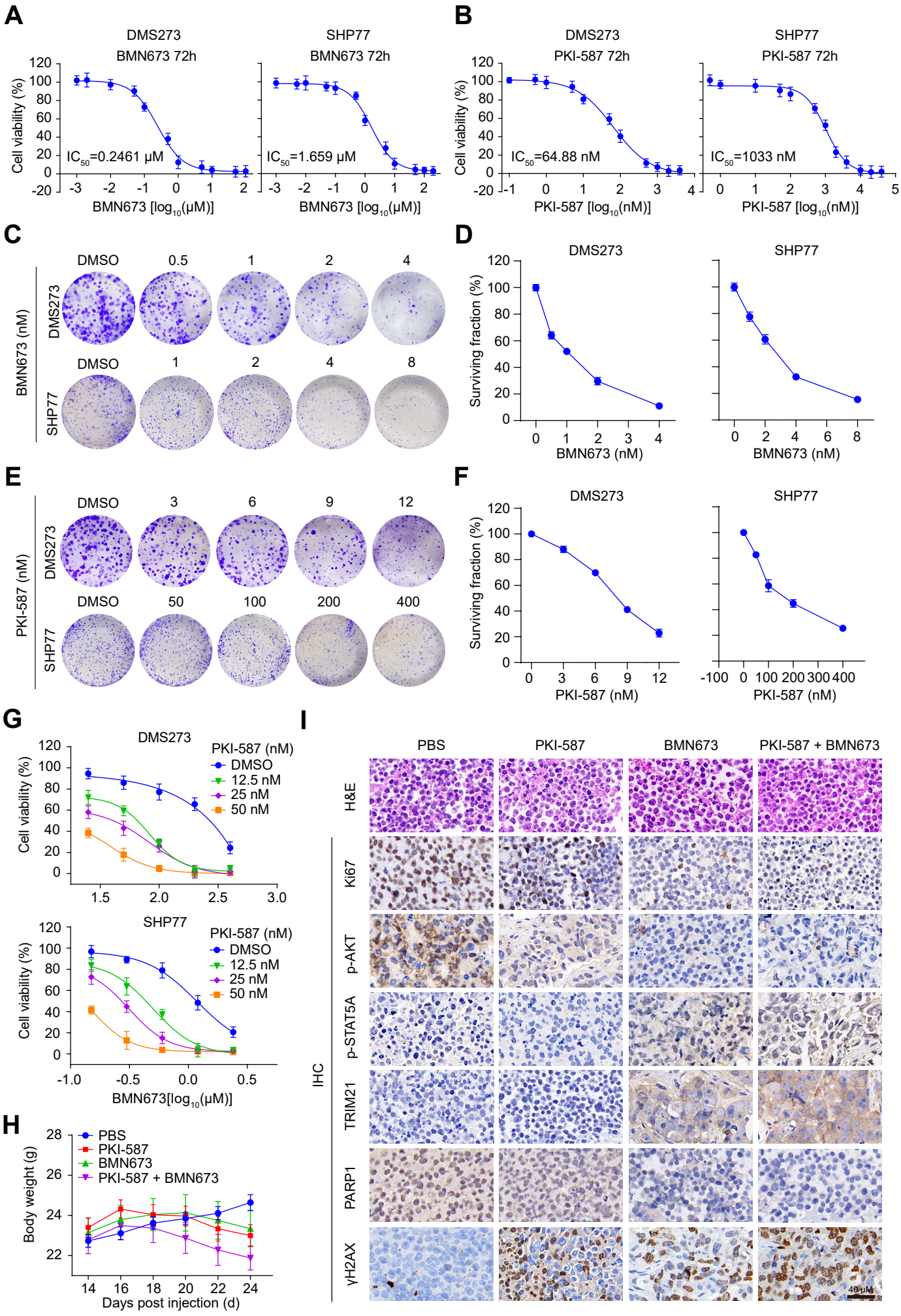

A
Scr
siSTAT5A #1
siSTAT5A #2
mRNA relative level
STAT5A
TRIM21
Scr
siSIX5 #1
siSIX5 #2
SIX5
TRIM21
Scr
siTCF21 #1
siTCF21 #2
TCF21
TRIM21
C
Bits
B
chr11:4,377,535-4,448.913
71 kb
4,380 kb
4,390 kb
4,400 kb
4,410 kb
ChIP-Atlas_STAT5A
ARP1_SRX9681372
MCF-7_RX9932045
Mesoderm_SRX190177
Refseq Genes
TRIM21
D
EGAS00001000925
81 SCLC tissues
r = -0.38
P = 4.64e-04
PI3K pathway activity
STAT5A mRNA level
E
HRA003419
107 SCLC tissues
r = 0.47
P = 2.71e-07
STAT5A mRNA level
TRIM21 mRNA level
F
OMIX002489
112 SCLC tissues
r = 0.60
P = 1.73e-12
STAT5A protien level
TRIM21 protien level
G
DMS273
SHP77
PARP1
TRIM21
STAT5A
mRNA relative level
PKI-587 (μM)
SC79 (μM)

Figure S8


A
DMS273
BMN673 72h
SHP77
BMN673 72h
Cell viability (%)
IC50=0.2461 μM
IC50=1.659 μM
BMN673 [log10(μM)]
B
DMS273
PKI-587 72h
SHP77
PKI-587 72h
IC50=64.88 nM
IC50=1033 nM
PKI-587 [log10(nM)]
C
BMN673 (nM)
DMS273
SHP77
DMSO
0.5
1
2
4
8
D
DMS273
SHP77
Surviving fraction (%)
BMN673 (nM)
E
PKI-587 (nM)
DMSO
3
6
9
12
50
100
200
400
F
PKI-587 (nM)
G
PKI-587 (nM)
DMSO
12.5 nM
25 nM
50 nM
BMN673[log10(μM)]
H
PBS
PKI-587
BMN673
PKI-587 + BMN673
Body weight (g)
Days post injection (d)
I
PBS
PKI-587
BMN673
PKI-587 + BMN673
IHC
H&E
Ki67
p-AKT
p-STAT5A
TRIM21
PARP1
γH2AX
40 μM