

Supplementary Information for

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

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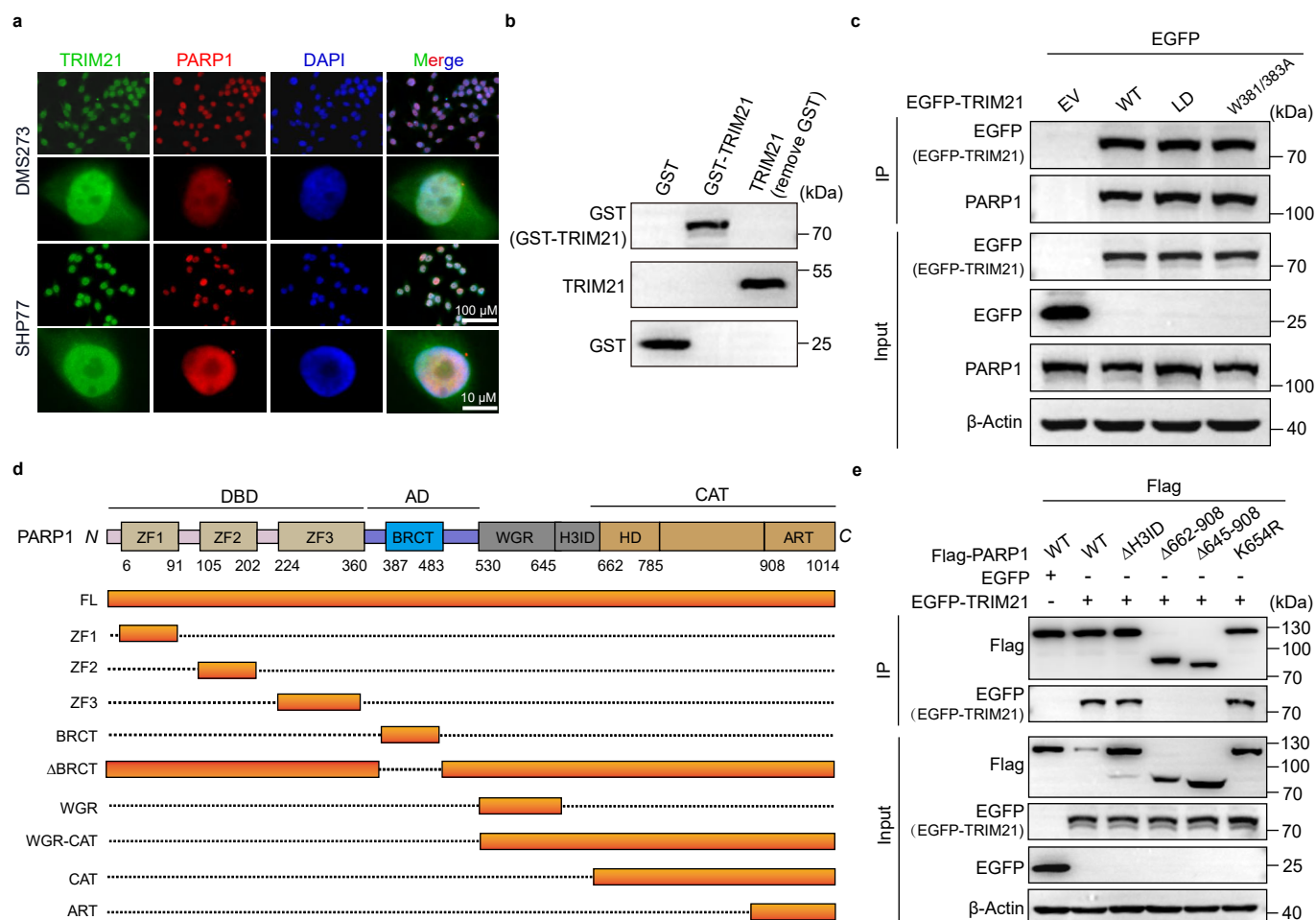
Running Title: PI3K/AKT-STAT5A-TRIM21-PARP1 axis Drives SCLC

#These authors contributed equally: Guozhen Cao, Gongfeng Li.

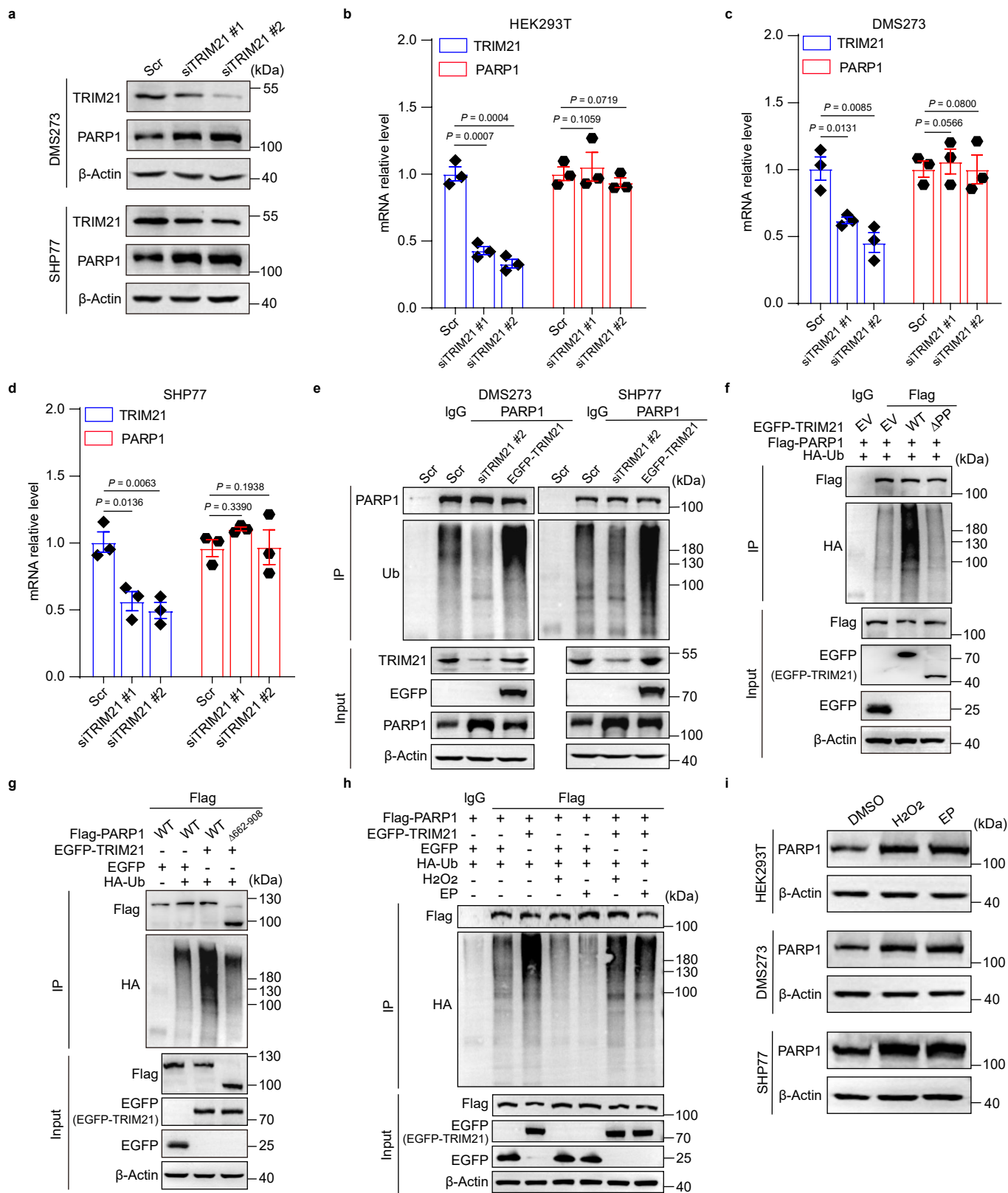
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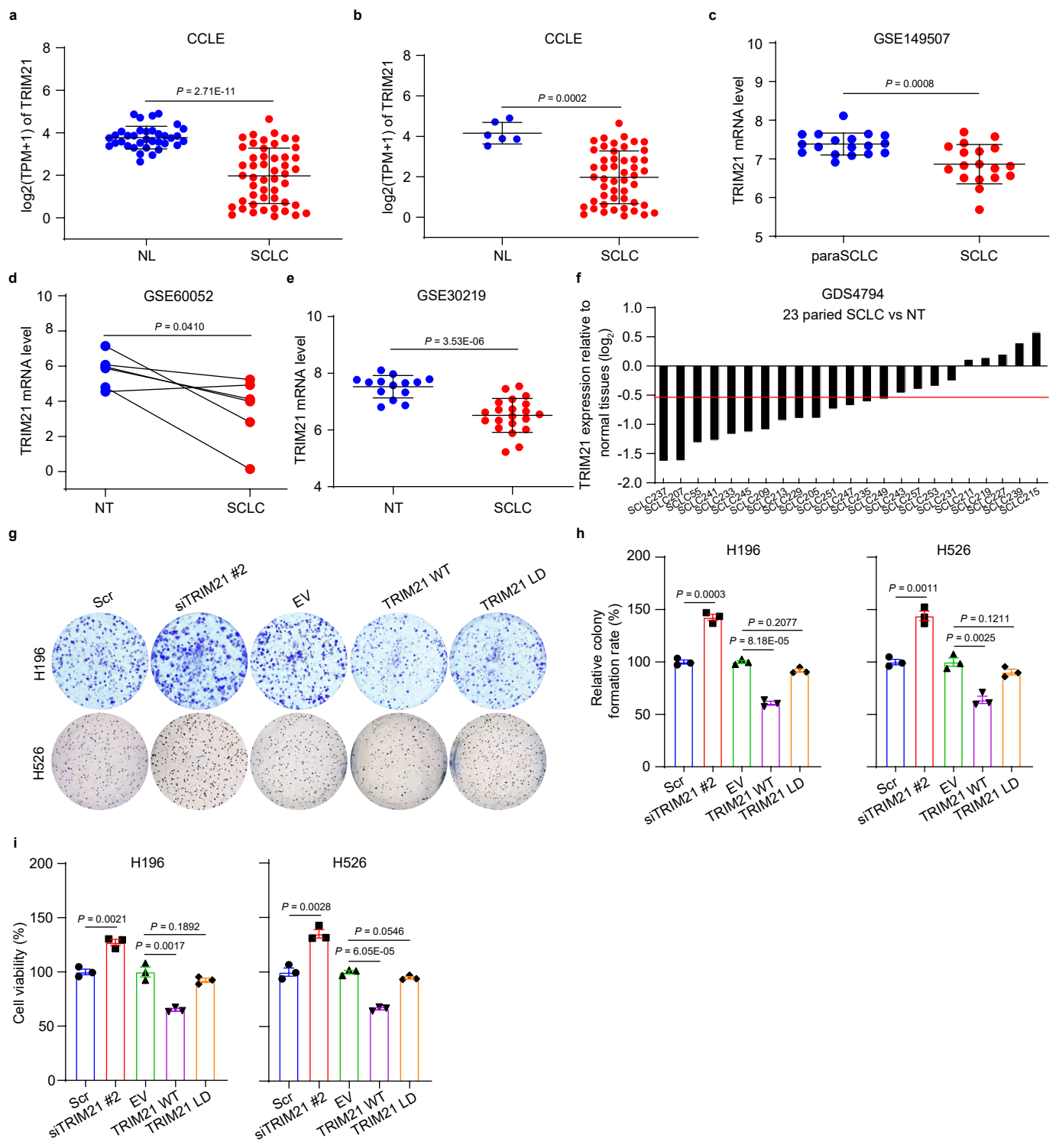


Supplementary Fig. 1 PARP1 binds with TRIM21. **a** Immunofluorescence assay demonstrating the cellular localization of TRIM21 and PARP1 in DMS273 and SHP77 cells. **b** Western blot analysis of TRIM21 after removal of the GST tag from GST-TRIM21 recombinant protein. **c** Immunoprecipitation (IP)-Western 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 IP followed by Western blot analysis. **d** Schematic diagram illustrating the domains of the 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* Δ H3ID, Flag-*PARP1* Δ 662-908, Flag-*PARP1* Δ 645-908, 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. Source data are provided as a Source Data file.

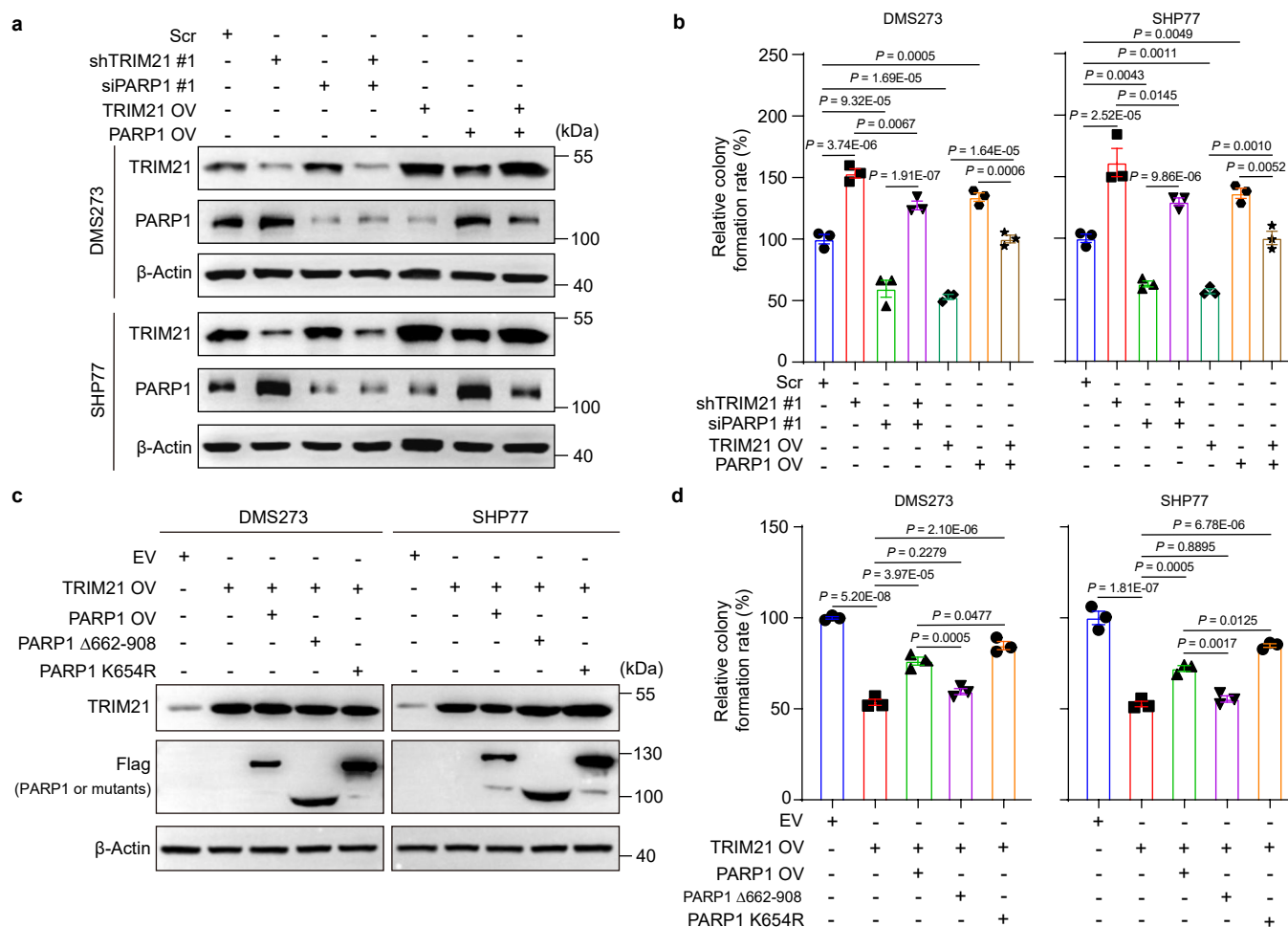


Supplementary Fig. 2 TRIM21 facilitates the ubiquitination and degradation of PARP1.

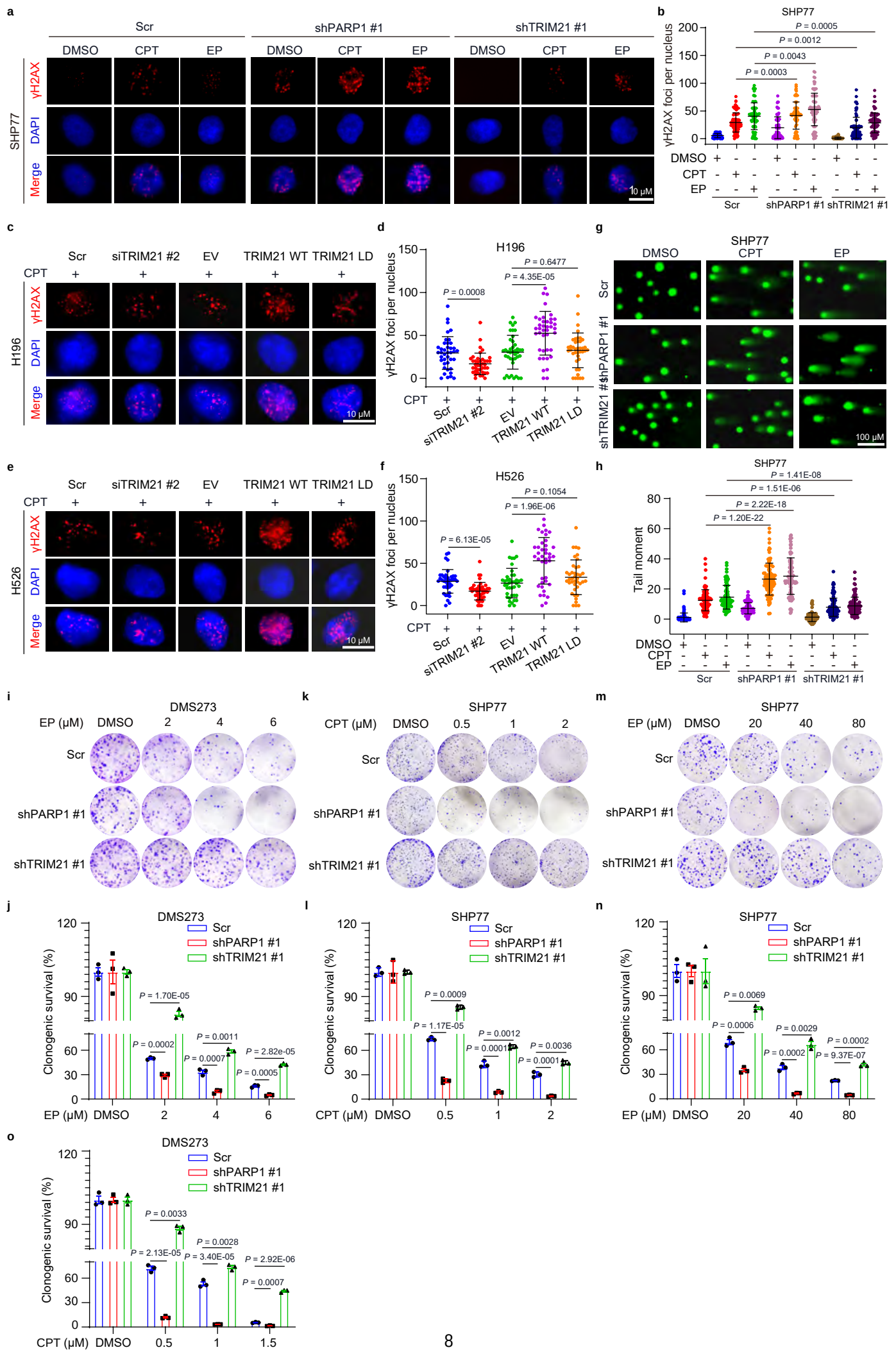
a Western blot analysis of PARP1 following *TRIM21* knockdown using siRNAs targeting *TRIM21*. **b–d** RT-qPCR analysis of *PARP1* expression in response to *TRIM21* knockdown by siRNAs in HEK293T (**b**), DMS273 (**c**), and SHP77 (**d**) cells, normalized to β -Actin. **e** *In vivo* ubiquitination 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* Δ PP (**f**), or co-transfected with HA-Ub, EGFP-*TRIM21*, and either Flag-*PARP1* or Flag-*PARP1* Δ 662-908 (**g**). PARP1 ubiquitination levels were assessed via IP-Western analysis. **h** Assessment of PARP1 ubiquitination levels 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 μ M, 24 hours) or H₂O₂ (2 μ M, 5 min), the ubiquitination and protein levels of PARP1 were evaluated by IP followed by Western blotting. **i** Analysis of PARP1 protein accumulation under DNA damage-inducing conditions. HEK293T, DMS273, and SHP77 cells were treated with EP (40 μ M, 24 hours) or H₂O₂ (2 μ M, 5 min), and PARP1 accumulation was analyzed by Western blotting. Data from three independent experiments are shown as mean $\pm$ SEM in (**b–d**). Statistical analysis was performed using two-tailed unpaired Student's *t*-tests. Source data are provided as a Source Data file.



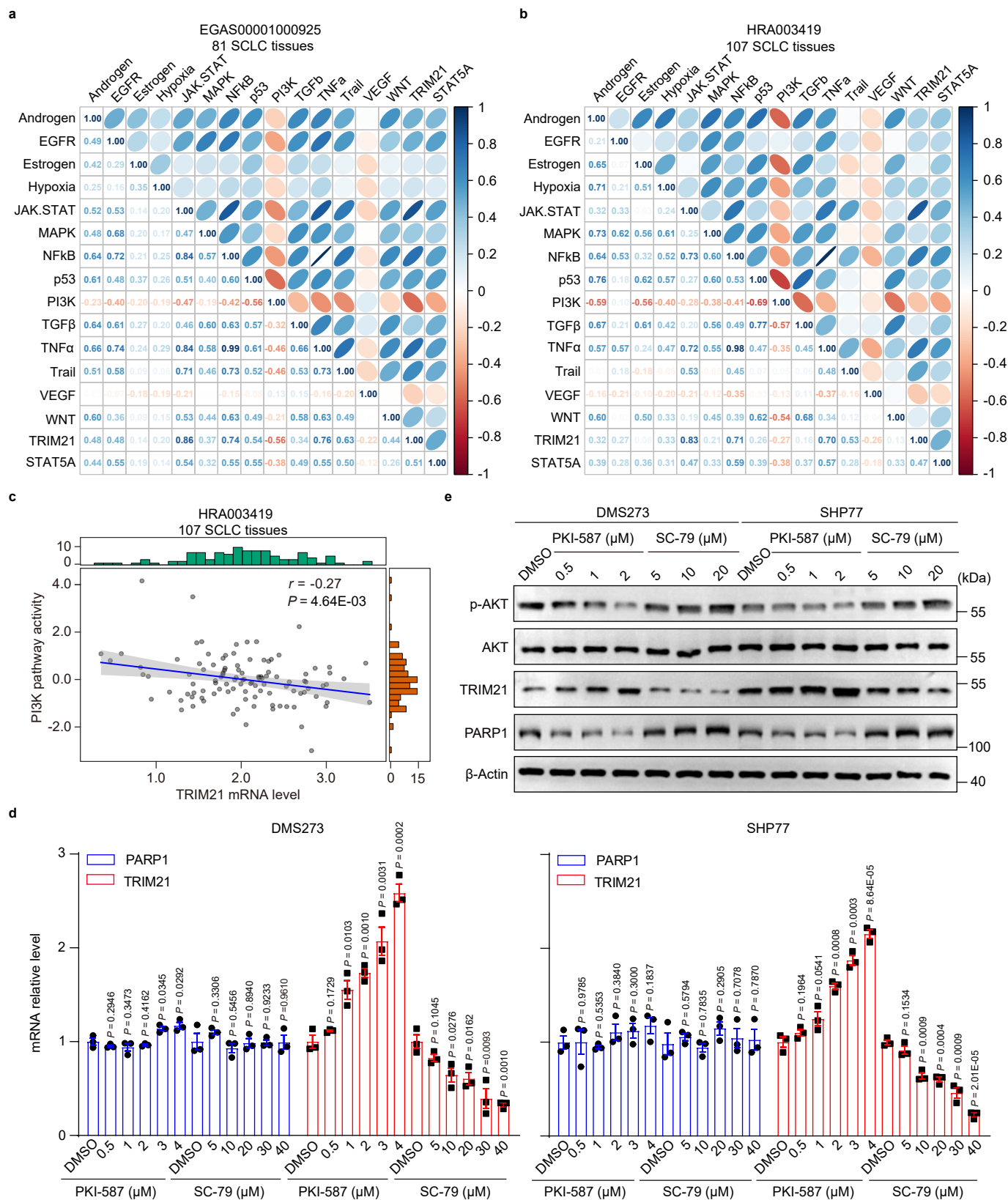
Supplementary Fig. 3 TRIM21 is downregulated in SCLC. **a, b** Scatter plot analysis of the CCLE dataset showing significantly reduced expression of *TRIM21* in SCLC cells ($n = 49$) compared to normal tissue cell lines (NL) ($n = 35$) (**a**), and normal lung cell lines (NL) ($n = 6$) (**b**). **c** Analysis of *TRIM21* mRNA expression in SCLC and adjacent normal tissues (paraSCLC) from the GSE149507 dataset ($n = 18$). **d** Scatter plot comparing *TRIM21* expression in SCLC tissues versus paired normal lung tissues (NT) from the GSE60052 dataset ($n = 6$). **e** Analysis of *TRIM21* mRNA expression levels in 21 SCLC tissues and 14 normal tissues (NT) from the GSE30219 dataset. **f** Relative *TRIM21* expression in human SCLC tissues compared to the normal lung tissues (NT) from the GDS4794 dataset ($n = 23$, the red line means more than a 1.5-fold increase). **g** Colony formation assays in H526 and H196 cells following either *TRIM21* knockdown (si*TRIM21*) or overexpression of wild-type (WT) or ligase-deficient mutant (LD, C16A, C31A, and H33W) *TRIM21*. For the suspension cell line H526 in soft agar colony formation assays. **h** Quantification of clonogenic survival from (**g**) using ImageJ software. **i** Cell viability measured by CellTiter-Glo assay in H526/H196 cells with si*TRIM21*, *TRIM21* WT, or *TRIM21* LD expression. Data are shown as mean $\pm$ SD in (**a–c**, **e**). Data from three independent experiments are shown as mean $\pm$ SEM in (**h**, **i**). Statistical analysis was performed using two-tailed unpaired Student's *t*-tests in (**a–c**, **e**, **h**, **i**); data in (**d**) were analyzed by two-tailed paired Student's *t*-tests. Source data are provided as a Source Data file.



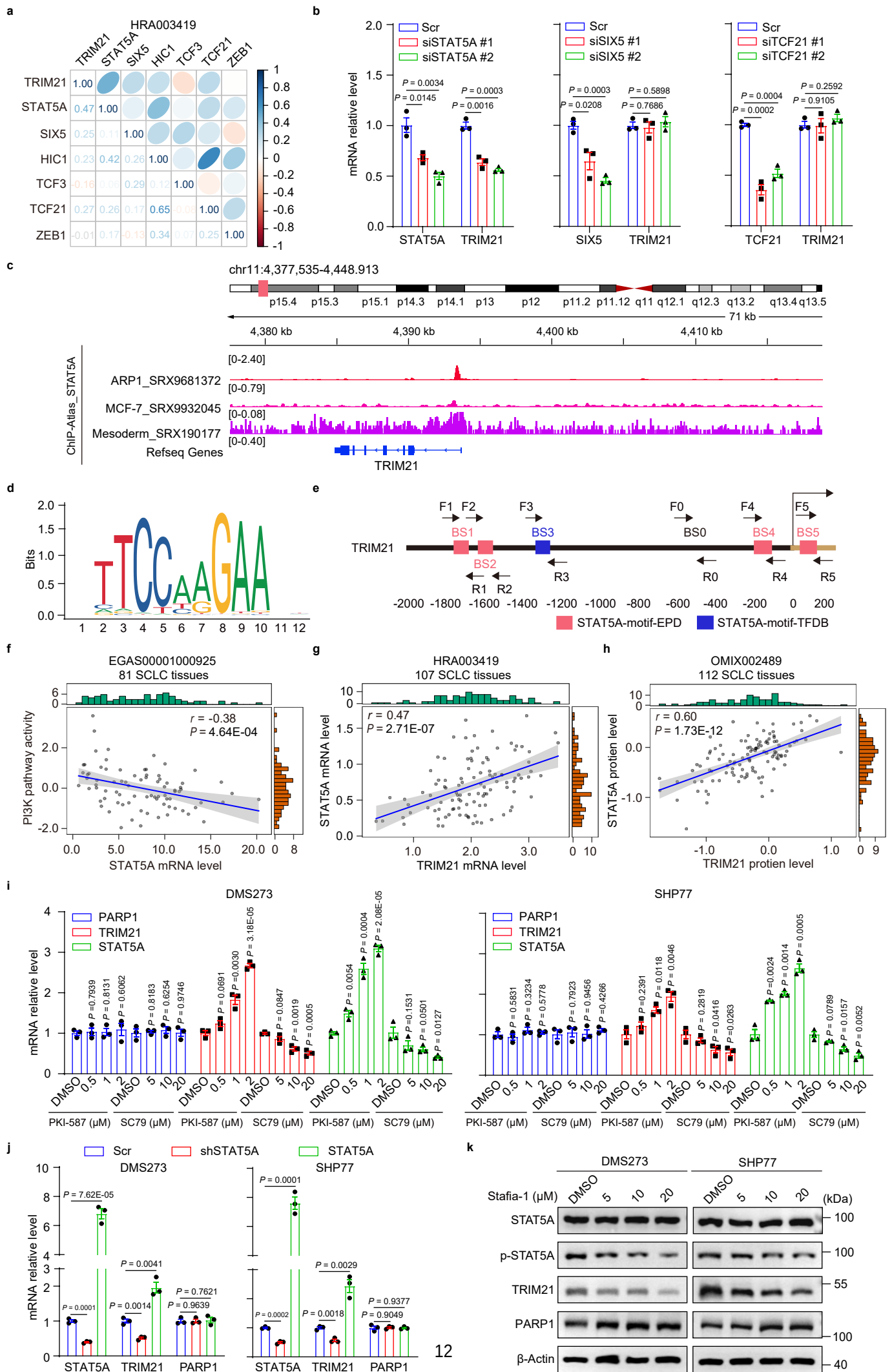
Supplementary Fig. 4 TRIM21 suppresses the survival of SCLC cells by decreasing PARP1 accumulation. **a** Western blot analysis investigating the compensatory effects of *PARP1* on altered *TRIM21* expression. *PARP1* and *TRIM21* protein levels were assessed following the treatment described in Fig. 4a. **b** Quantitative analysis of colony formation shown in Fig. 4b. **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 Fig. 4c. **d** Quantitative analysis of colony formation is shown in Fig. 4d. Data from three independent experiments are shown as mean $\pm$ SEM in (b, d). Statistical analysis was performed using two-tailed one-way ANOVA. Source data are provided as a Source Data file.



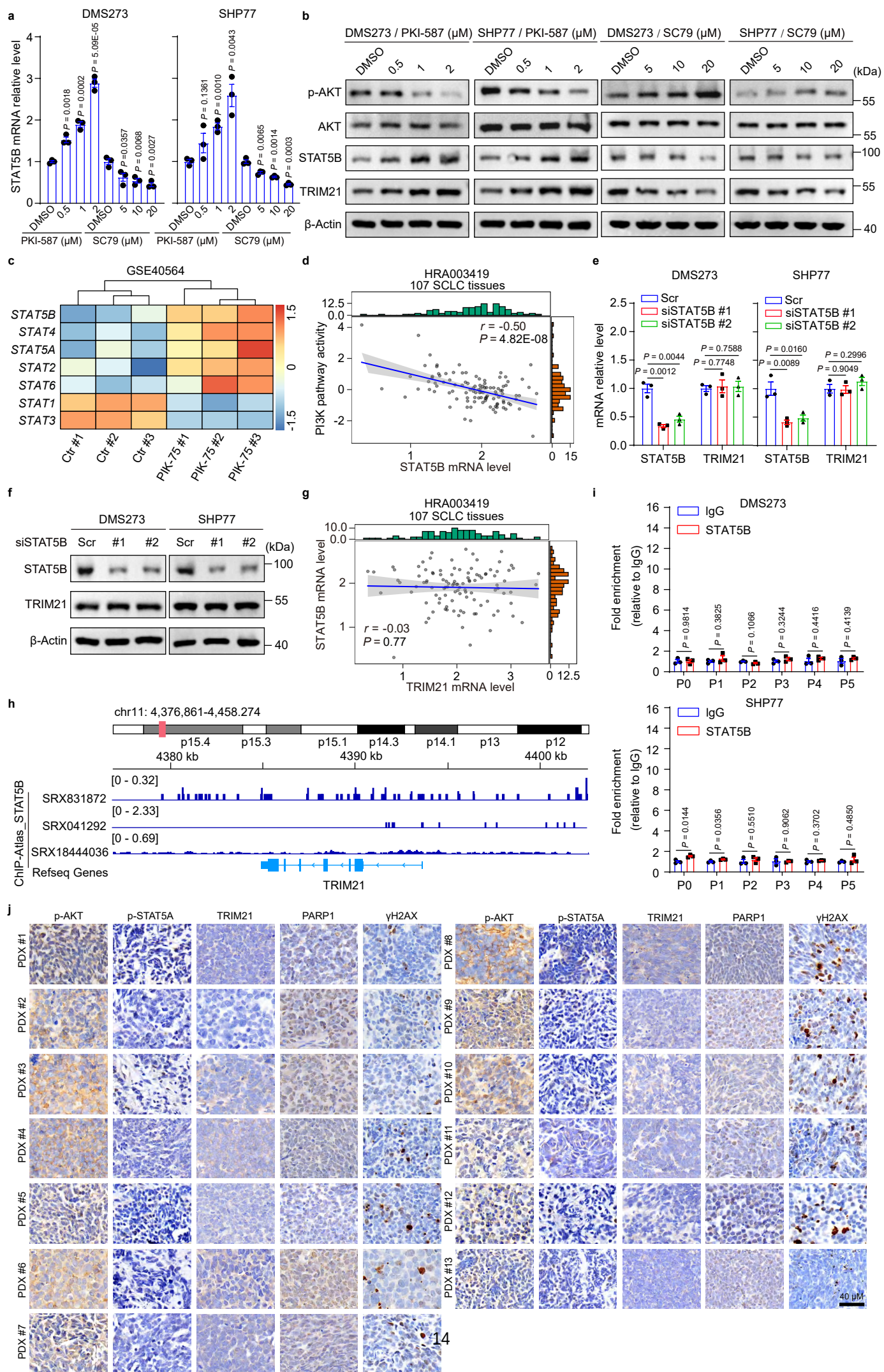
Supplementary Fig. 5 TRIM21 deficiency enhances DNA damage responses by induction of PARP1 accumulation. **a** Immunostaining showing the formation of γ H2AX foci in SHP77 cells following the protocol described in Fig. 5c. Scale bars, 10 μ m. **b** Quantification of the γ H2AX foci formation in (a). **c, e** Immunofluorescence analysis of γ H2AX foci in H196 (c) and H526 (e) cells treated with 2 μ M Camptothecin (CPT, 1 hour) followed by 4 hours recovery under si*TRIM21*, overexpression of wild-type (WT) *TRIM21*, or ligase-deficient mutant (LD, C16A, C31A, and H33W) *TRIM21* conditions. Scale bars, 10 μ m. **d, f** γ H2AX foci quantification from (c, e). **g** 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 Fig. 5e. Scale bars, 100 μ m. **h** Quantification of the tail moment of the comet assay in (g). **i, k, m** Colony formation assays measuring the clonogenic capacity of the DMS273 or SHP77 cells treated by CPT or EP with indicated concentrations. **j, l, n** Quantitative analysis of colony formation shown in (i, k, m). **o** Quantitative analysis of colony formation shown in Fig. 5g. Data in (b) ($n = 80$ cells), (d, f) ($n = 40$ cells), and (h) ($n = 100$ cells) are presented as mean $\pm$ SD. In (j, l, n, o), data from three independent experiments are shown as mean $\pm$ SEM. Statistical analysis was performed using two-tailed unpaired Student's *t*-tests. Source data are provided as a Source Data file.



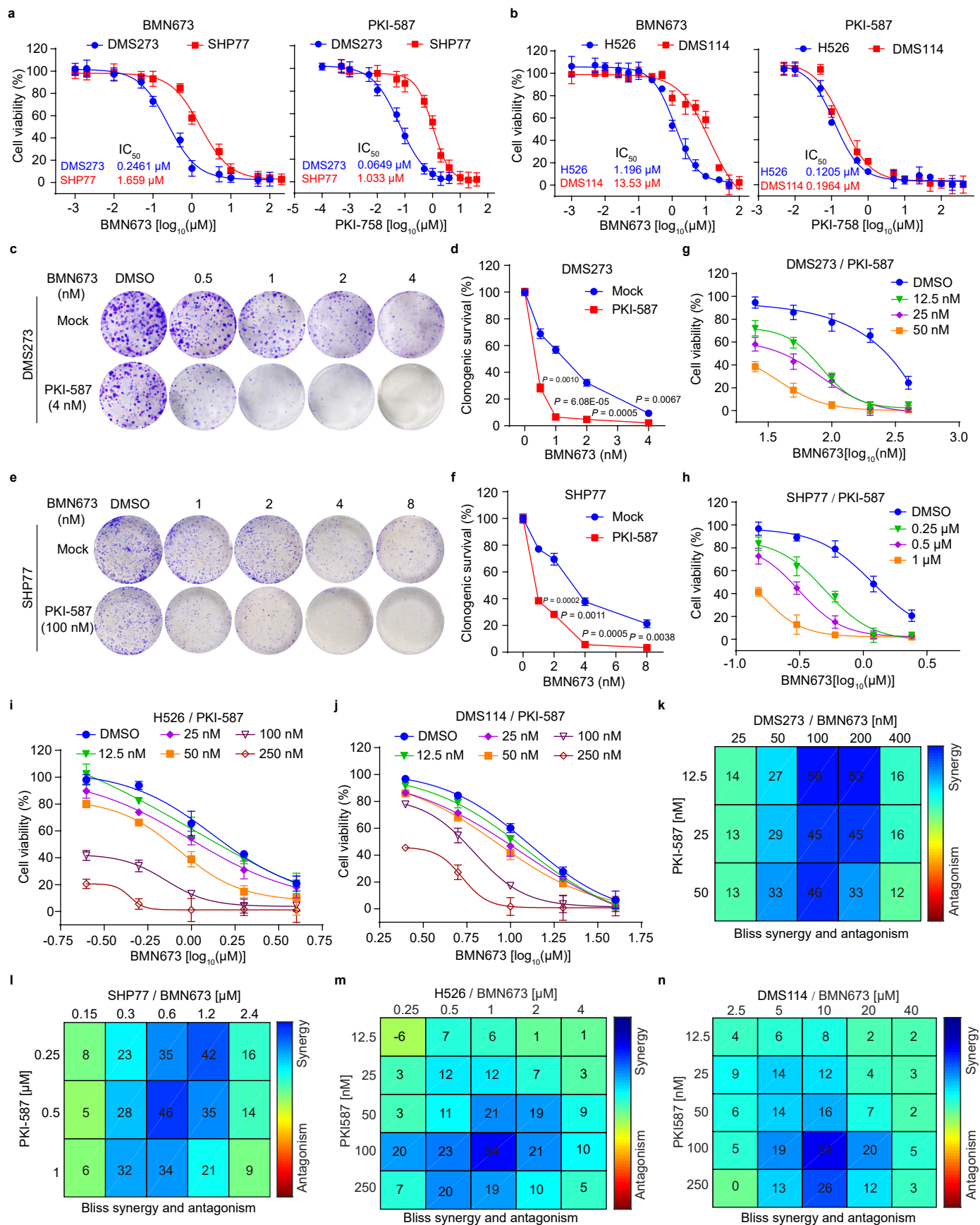
Supplementary Fig. 6 PI3K/AKT facilitates PARP1 stabilization by blocking TRIM21 expression. **a** Correlation analysis of *TRIM21* and *STAT5A* mRNA levels with the activity of various signaling pathways in 81 SCLC tissues from the European Genome-phenome Archive database (EGAS00001000925). **b** A similar correlation analysis to **(a)** in 107 SCLC tissues from the Genome Sequence Archive (GSA) database (HRA003419). **c** Analysis of the correlation between PI3K/AKT pathway activity and *TRIM21* mRNA levels in SCLC tissues from the GSA database (HRA003419, $n = 107$; 95% confidence interval, -0.44 to -0.09). Two-tailed P value is reported. **d** RT-qPCR analysis demonstrating the effects of PKI-587 or SC-79 on the expression of *TRIM21* and *PARP1* in SCLC cells. DMS273 and SHP77 cells were treated with a gradient of different concentrations of PKI-587 or SC-79 for 24 hours, followed by measurement of *TRIM21* and *PARP1* mRNA levels. **e** 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 hours, and these protein levels were subsequently assessed by Western blot. In **(d)**, data from three independent experiments are shown as mean $\pm$ SEM. Statistical analysis was performed using two-tailed unpaired Student's t -tests. Source data are provided as a Source Data file.



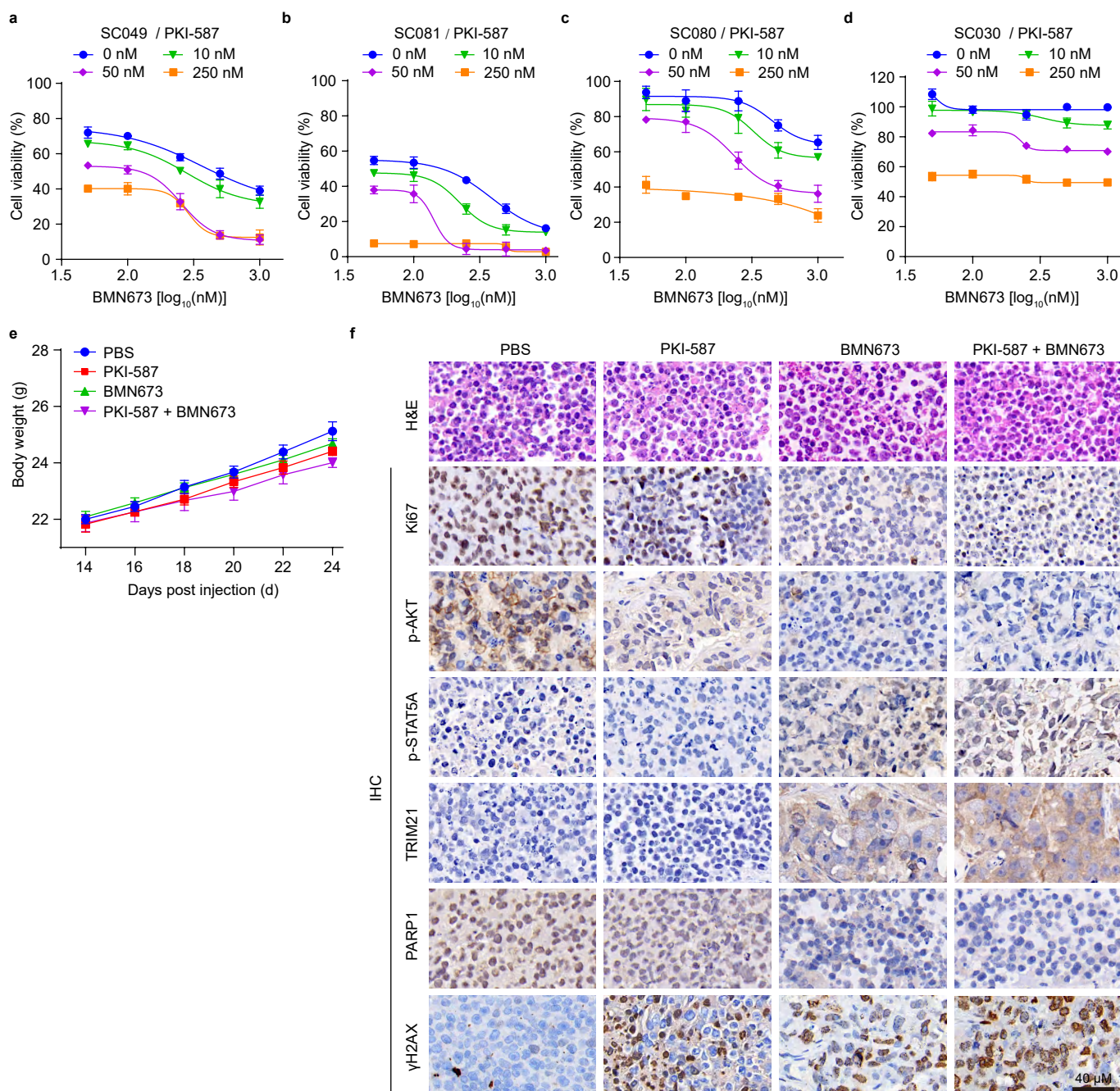
Supplementary Fig. 7 STAT5A acts as a transcription factor inhibiting *TRIM21* via the PI3K/AKT pathway. **a** Correlations between the *TRIM21* mRNA levels and the expression of potential transcription factors (TFs) ZEB1, HIC1, SIX5, STAT5A, TCF3, and TCF21 in 107 SCLC tissues from the HRA003419 dataset. **b** RT-qPCR analysis of *TRIM21*, *STAT5A*, *SIX5*, and *TCF21* expression after knocking down *STAT5A*, *SIX5*, or *TCF21* in HEK293T cells. **c** Identification of STAT5A peaks in the promoter of the *TRIM21* gene from the ChIP-Atlas database. **d** The consensus DNA-binding motif of STAT5A derived from the JASPAR database. **e** Schematic diagram of primers design strategy for STAT5A binding sites (BSs) in the *TRIM21* promoter. Five putative STAT5A BSs were predicted using the Eukaryotic Promoter Database (EPD) and AnimalTFDB 4.0 (TFDB). BS0 serves as a randomly selected negative control. **f** Correlation between PI3K/AKT pathway activity and *STAT5A* mRNA levels in SCLC tissues from the European Genome-phenome Archive database (EGAS00001000925, $n = 81$; 95% confidence interval (CI), -0.55 to -0.18). **g** Correlation between *TRIM21* and *STAT5A* mRNA levels in SCLC tumor tissues from the GSA database (HRA003419, $n = 107$; 95% CI, 0.31 to 0.61). **h** Correlation between *TRIM21* and *STAT5A* protein levels in SCLC tumor tissues from the OMIX database (OMIX002489, $n = 112$; 95% CI, 0.47 to 0.71). Two-tailed P values are reported in (**a**, **f–h**). **i** Dose-response of *STAT5A*, *TRIM21*, and *PARP1* mRNA levels in SCLC cells. DMS273 and SHP77 cells were treated with the indicated concentrations of PKI-587 or SC-79 for 24 hours, followed by RT-qPCR analysis of the respective mRNA. **j** *STAT5A*, *TRIM21*, and *PARP1* mRNA expression after *STAT5A* knockdown and rescue. mRNA levels were assessed by RT-qPCR in DMS273 and SHP77 cells stably expressing sh*STAT5A*, followed by *STAT5A* overexpression. **k** Western blot analysis of *STAT5A*, p-*STAT5A*, *TRIM21*, and *PARP1* in DMS273 and SHP77 cells treated with increasing concentrations of Stafia-1 for 24 hours. Data in (**b**, **i**, **j**) are from three independent experiments and are presented as mean $\pm$ SEM. Statistical significance was determined using two-tailed unpaired Student's t -tests. Source data are provided as a Source Data file.



Supplementary Fig. 8 STAT5B is not a transcriptional regulator of TRIM21. **a** RT-qPCR analysis of *STAT5B* expression in DMS273 and SHP77 cells treated with a concentration gradient of PKI-587 or SC-79 for 24 hours. **b** Western blot analysis of p-AKT, AKT, STAT5B, and TRIM21 in DMS273 and SHP77 cells following treatment with PKI-587 or SC-79 for 24 hours. **c** Heatmap of STAT family members expression (*STAT1*, *STAT2*, *STAT3*, *STAT4*, *STAT5A/B*, and *STAT6*) from RNA-seq data (GSE40564 dataset) in SCLC cells treated with the PI3K/AKT inhibitor PIK-75. **d** Correlation analysis between PI3K/AKT pathway activity scores and *STAT5B* mRNA levels in SCLC tissues (HRA003419, $n = 107$; 95% confidence interval (CI), -0.63 to -0.34). **e, f** RT-qPCR and Western blot analyses of *STAT5B* and *TRIM21* expression following *STAT5B* knockdown (si*STAT5B*). The mRNA (**e**) and protein (**f**) levels of *STAT5B* and *TRIM21* were measured. **g** Correlation analysis between PI3K/AKT pathway activity scores and *STAT5B* mRNA levels in SCLC tissues (HRA003419, $n = 107$; 95% CI, -0.22 to 0.16). **h** Identification of *STAT5B* peaks in the promoter of the *TRIM21* gene from the ChIP-Atlas database. **i** *STAT5B* did not bind to the *TRIM21* promoter assessed by ChIP-qPCR in DMS273 and SHP77 cells. **j** IHC profiling of SCLC patient-derived xenograft (PDX) models. 13 PDX models (PDX #1–#13) were analyzed by IHC for key biomarkers: p-AKT, p-STAT5A, *TRIM21*, PARP1, and γ H2AX. Scale bars, 40 μ m. Data in (**a**, **e**, **i**) are from three independent experiments and are presented as mean $\pm$ SEM. Statistical significance was determined using two-tailed unpaired Student's *t*-tests. Two-tailed *P* values are reported in (**d**, **g**). Source data are provided as a Source Data file.



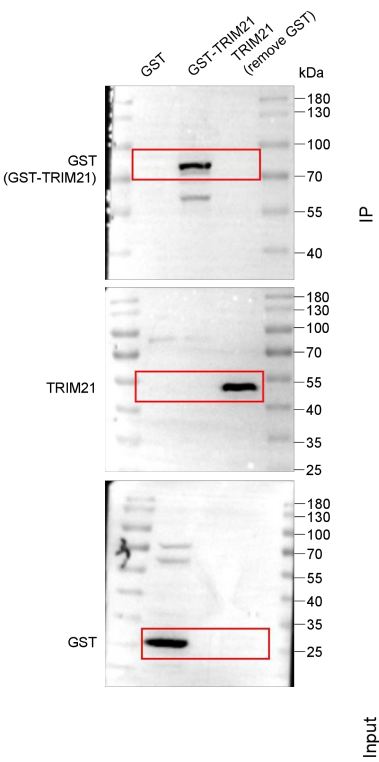
Supplementary Fig. 9 Inhibition of PI3K/AKT synergizes with PARPi in SCLC cells. **a, b** Growth inhibition curves of BMN673 and PKI-587 in DMS273, SHP77, H526, and DMS114 cell lines. The cells were treated with different concentrations of BMN673 or PKI-587 for 72 hours. The cell viability was then assessed by the CellTiter-Glo assay. The Half-lethal dose (IC_{50}) values were determined from the sigmoidal dose-response curves. **c, e** Colony formation assays demonstrating the effect of PKI-587 on the cytotoxicity of PARPi in SCLC cells. DMS273 (**c**) and SHP77 (**e**) cells were exposed to BMN673 in the presence of PKI-587 for a period of 7–10 days. **d, f** Drug-response curves for the colony formation assays presented in (**c**) and (**e**). **g–j** Dose-response profiles of BMN673 combined with PKI-587 in DMS273 (**g**), SHP77 (**h**), H526 (**i**), and DMS114 (**j**) cells. Cells were treated with increasing concentrations of BMN673 and PKI-587 for 72 hours, followed by cell viability measurement using CellTiter-Glo luminescent assay. **k–n** Bliss synergy analysis of the combination of BMN673 and PKI-587 in DMS273 (**k**), SHP77 (**l**), H526 (**m**), and DMS114 (**n**) cells. Synergy indices were calculated using Combenefit software (CRUK Cambridge Institute) based on the dose-response data from (**g–j**). Data in (**a, b, d, f, g–j**), derived from three independent experiments, are presented as mean $\pm$ SEM. Source data are provided as a Source Data file.



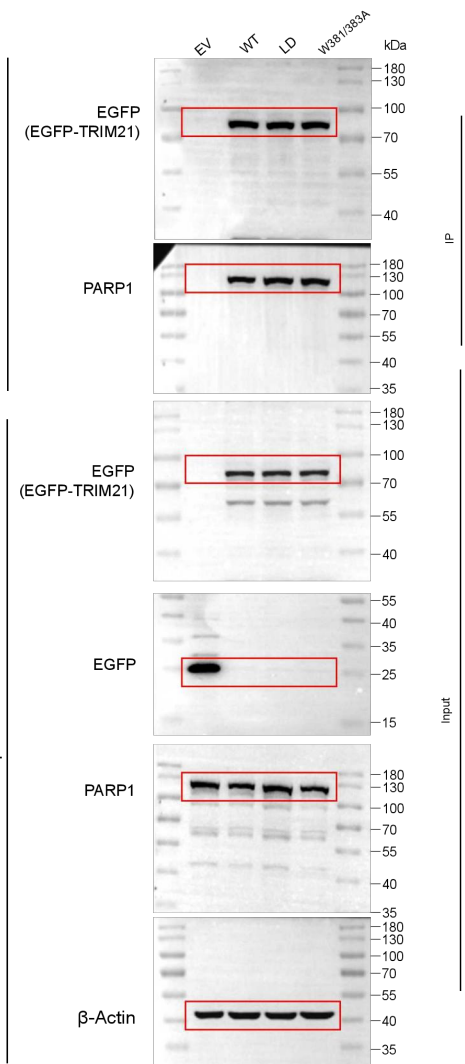
Supplementary Fig. 10 Co-inhibition of PI3K/AKT and PARP results in synergy *in vivo*. **a–d** Combinatorial dose-response profiles illustrating the effects of BMN673 and PKI-587 co-treatment across all four subtypes (A: SC049, **a**; A + N: SC081, **b**; P: SC080, **c**; Y: SC030, **d**) following 72 hours of exposure, measured by CellTiter-Glo viability assays. **e** Body weight curves of xenograft mice during treatment with PKI-587, BMN673, or a combination of PKI-587 and BMN673. **f** Representative images of HE staining and immunohistochemistry for Ki67, p-AKT, p-STAT5A, TRIM21, PARP1, and γ H2AX within tumor sections. Scale bars represent 40 μ m. Data in (**a–d**) are from three independent experiments, and data in (**e**) are from 7 mice per group. All data are presented as mean $\pm$ SEM. Source data are provided as a Source Data file.

Uncropped scans of Supplementary Figure 1

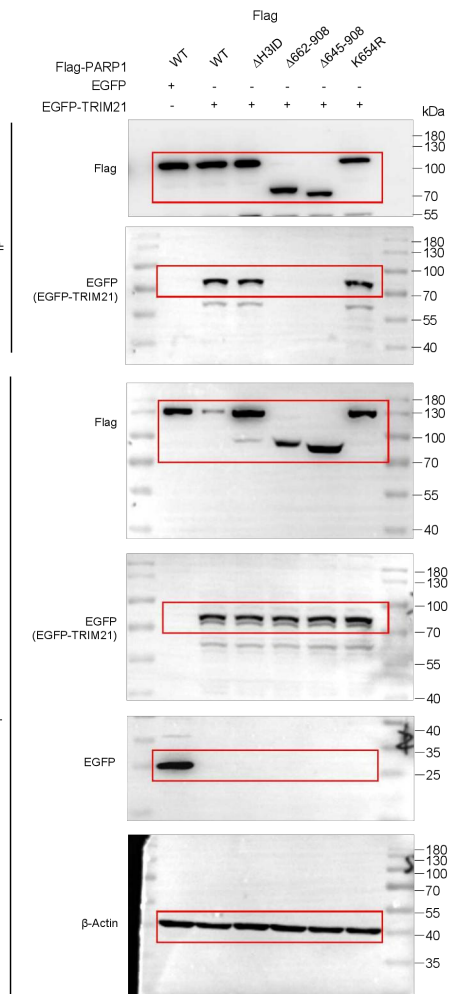
Supplementary Fig. 1b



Supplementary Fig. 1c

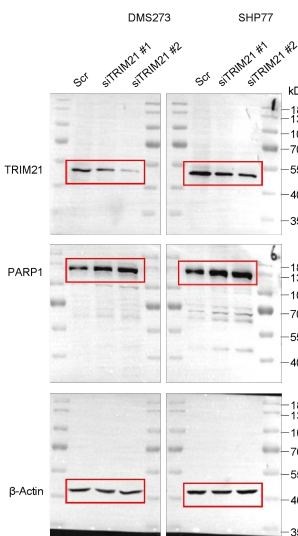


Supplementary Fig. 1e

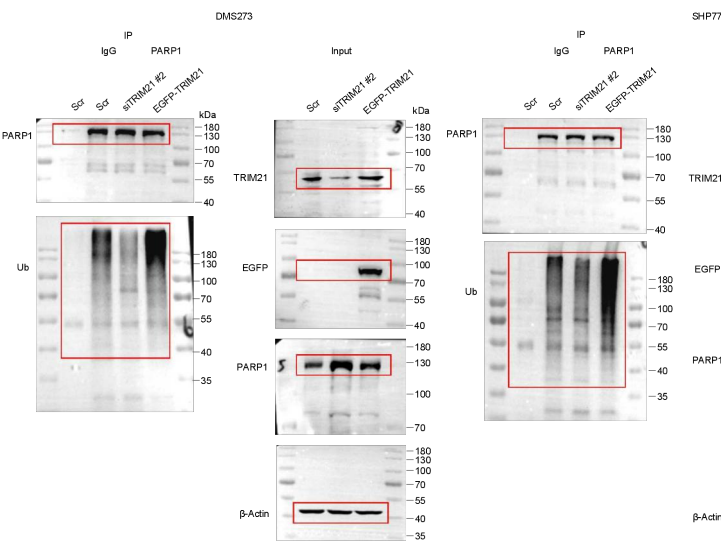


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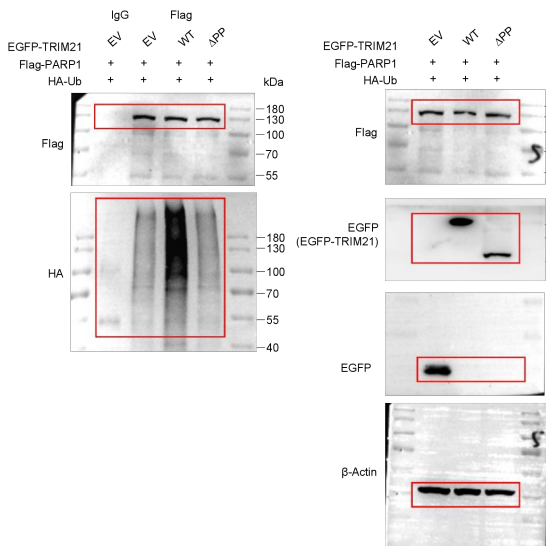
Supplementary Fig. 2a



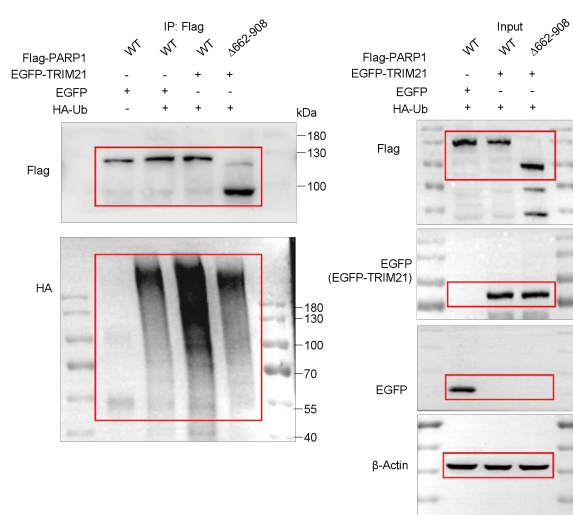
Supplementary Fig. 2e



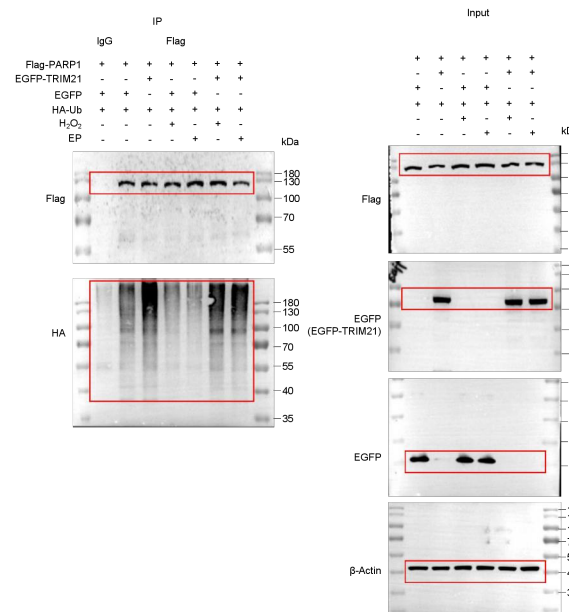
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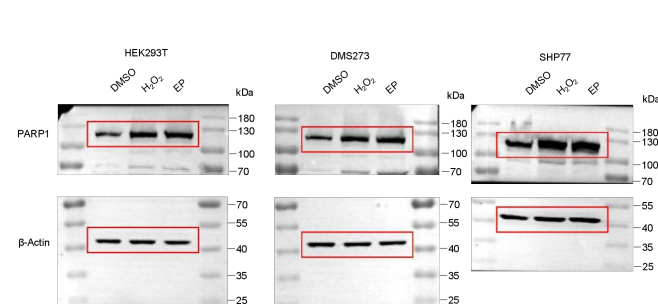
Supplementary Fig. 2g



Supplementary Fig. 2h

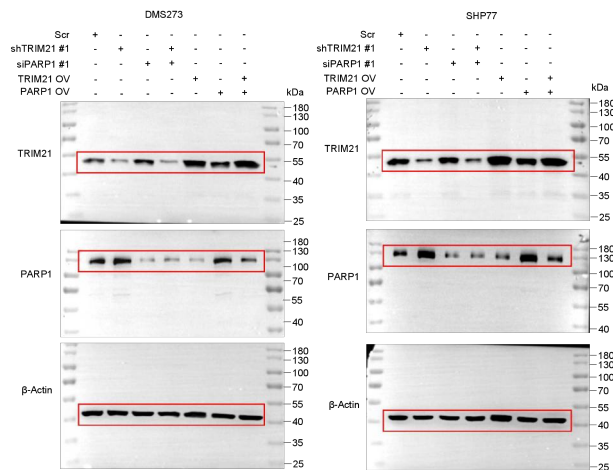


Supplementary Fig. 2i

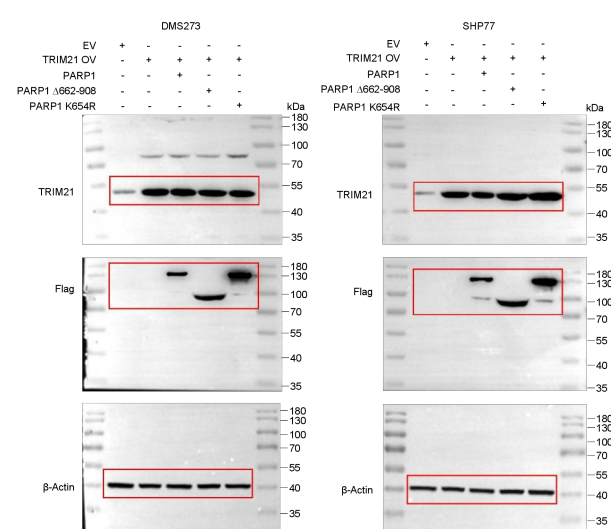


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Supplementary Fig. 4a

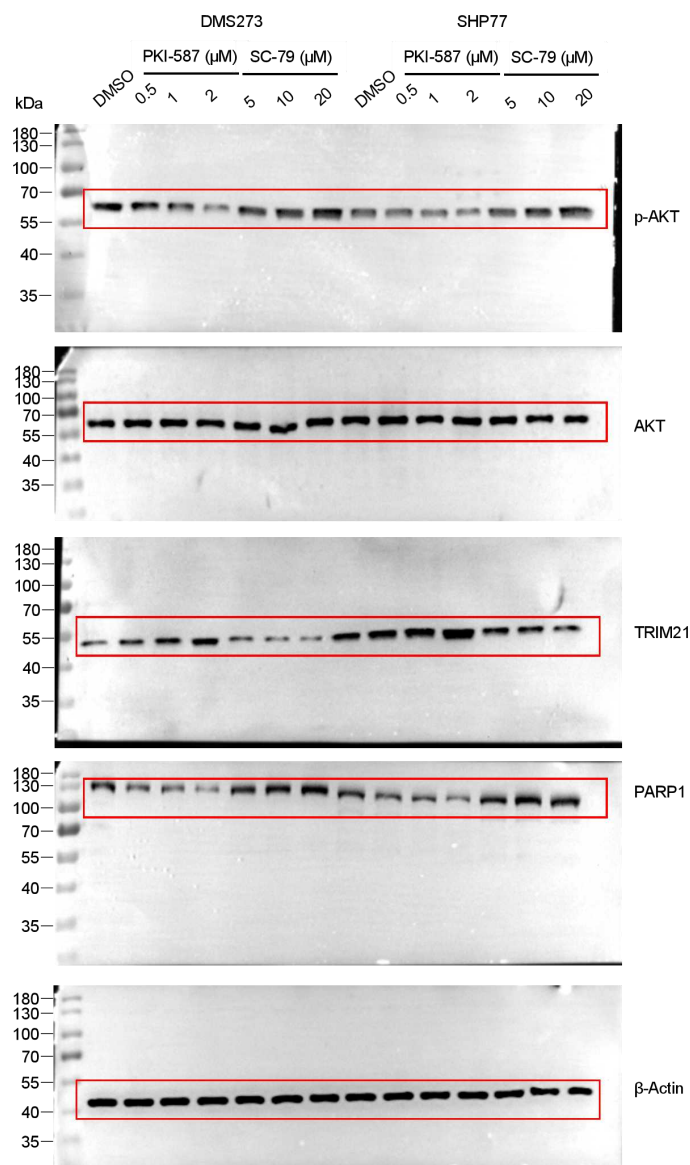


Supplementary Fig. 4c



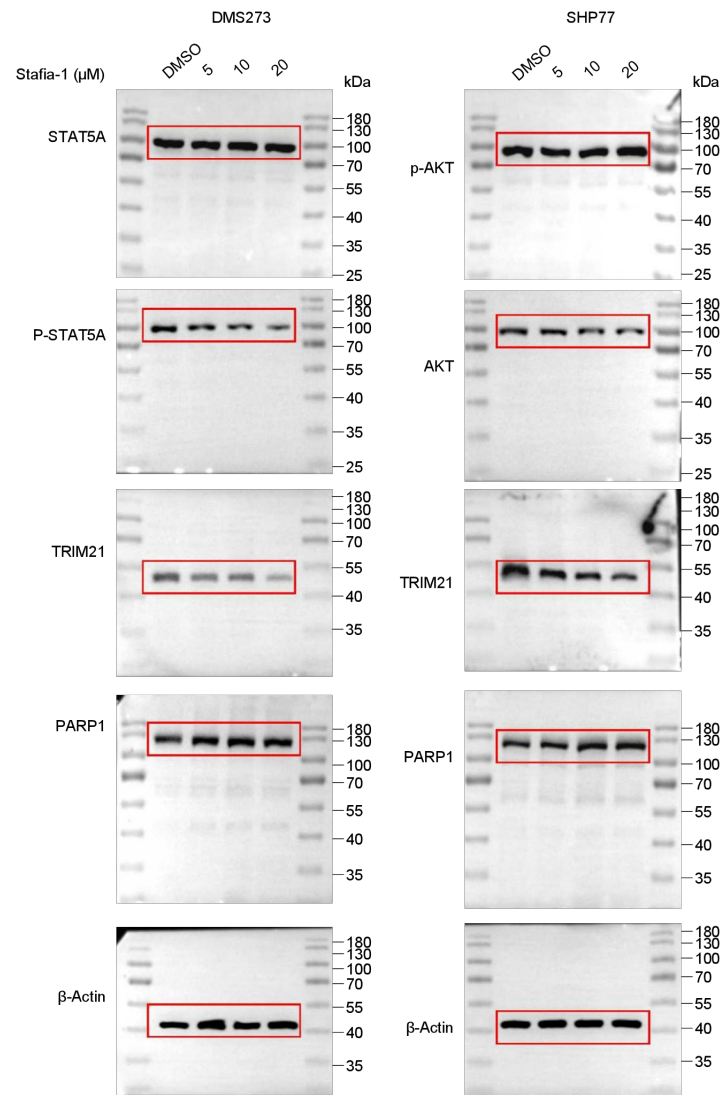
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Supplementary Fig. 6e



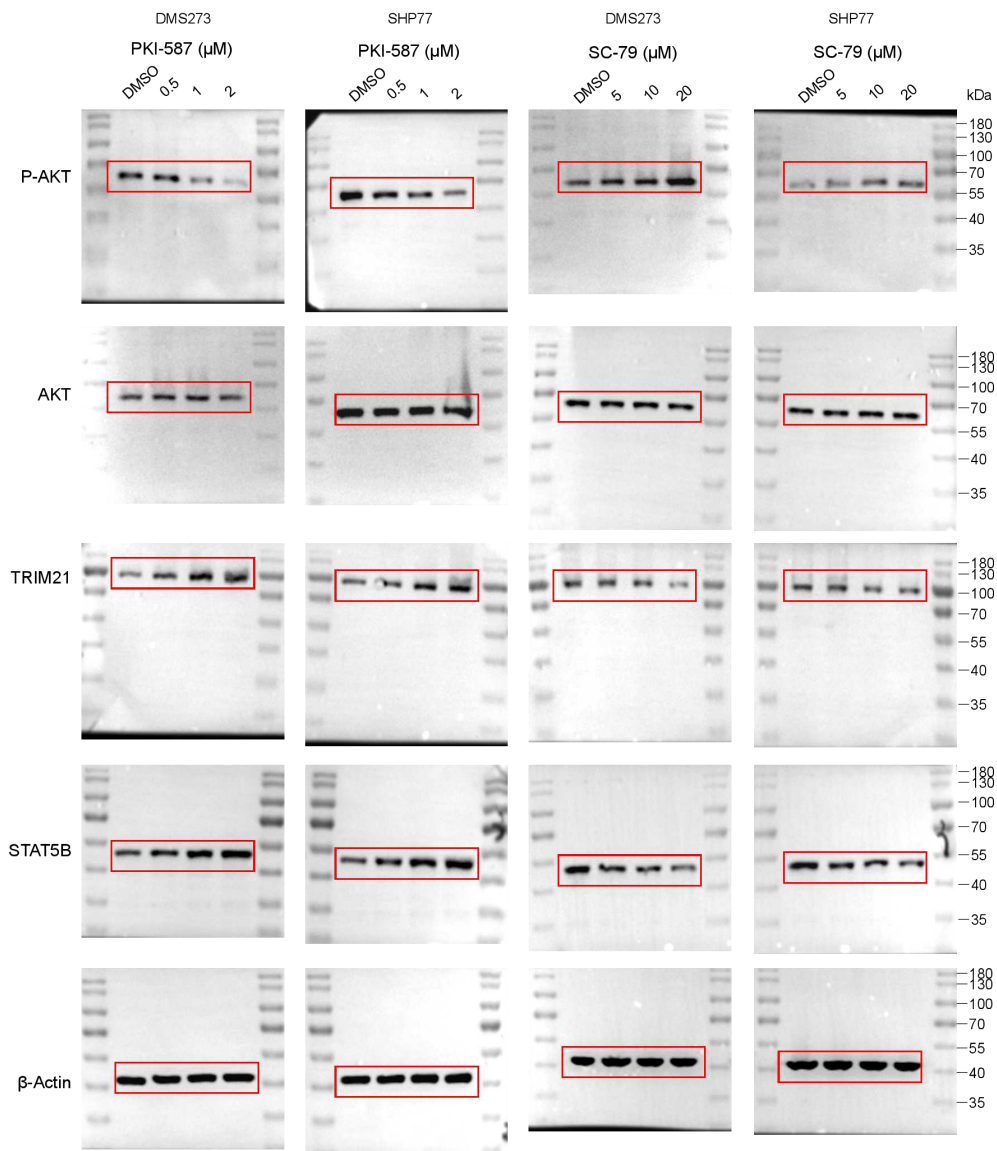
Uncropped scans of Supplementary Figure 7

Supplementary Fig. 7k



Uncropped scans of Supplementary Figure 8

Supplementary Fig. 8b



Supplementary Fig. 8f

