Supplementary Material

Zinc-finger protein p52-ZER6 accelerates colorectal cancer cell proliferation and tumour progression through promoting p53 ubiquitination

Can Huang, Shourong Wu, Wenfang Li, Arin Herkilini, Makoto Miyagishi, Hezhao Zhao, Vivi Kasim*

*Email: vivikasim@cqu.edu.cn (V.K.)

Supplementary Figure S1

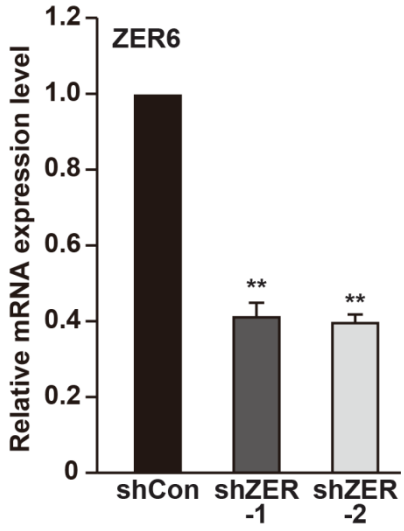

Fig. S1. The efficacy of shRNA expression vectors targeting ZER6. ZER6 mRNA expression level in HCT116 cells transfected with shRNA expression vectors targeting different sites of ZER6, as determined using quantitative RT-PCR (qPCR). Cells transfected with control vector (shCon) were used as control. β-Actin was used for qPCR normalization. Data were shown as mean ± SEM of three independent experiments. $^{**}P < 0.01$ (ANOVA).

Supplementary Figure S2

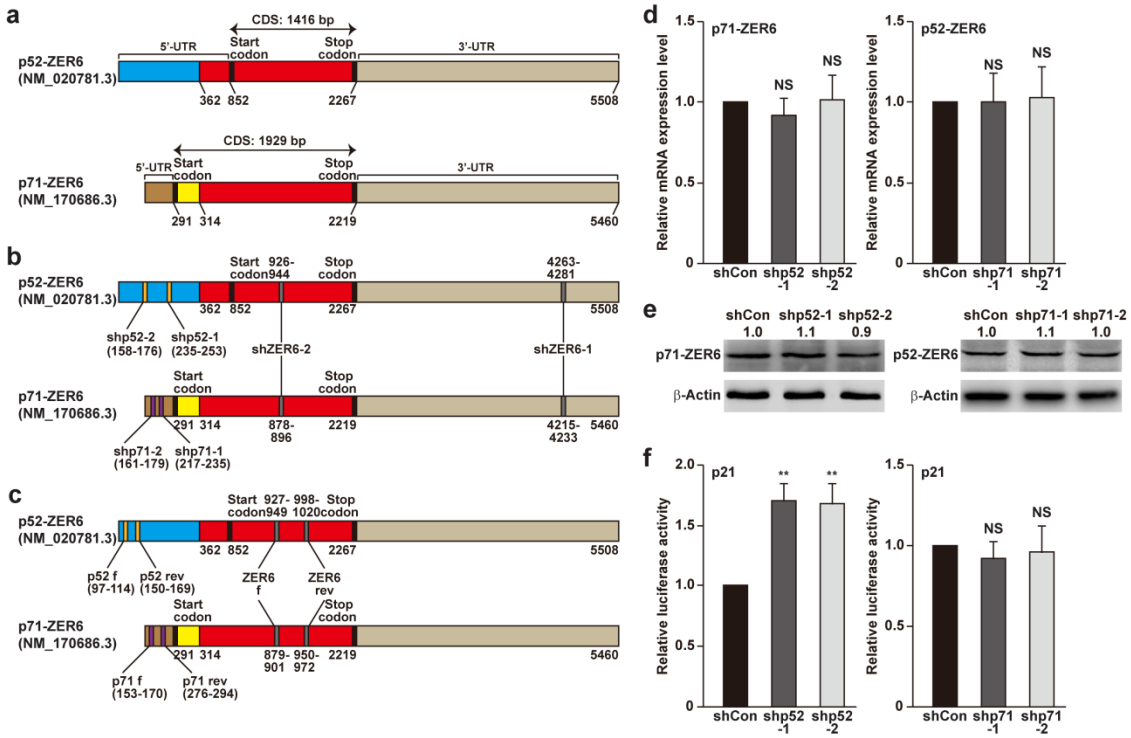

Fig. S2. p52-ZER6 regulates p21 transcriptional activity. a Schematic diagram of the nucleotide sequences of ZER6 isoforms mRNA. Homologous sequences were shown in same colours. **b** Schematic diagram of the shRNA target sites targeting the whole ZER6, and shRNA target sites specifically targeting p52-ZER6 and p71-ZER6. **c** Schematic diagram of the location of qPCR primer pairs for detection of the whole ZER6, and for specific detections of p52-ZER6 and p71-ZER6. **d** p71-ZER6 mRNA expression level in *p52-ZER6*-silenced HCT116 cells (left); and p52-ZER6 mRNA expression level in *p71-ZER6*-silenced HCT116 cells (right), as determined using qPCR. **e** p71-ZER6 protein expression level in *p52-ZER6*-silenced HCT116 cells (left); and p52-ZER6 protein expression level in *p71-ZER6*-silenced HCT116 cells (right), as determined using western blotting. **f** Activity of p21 reporter in *p52-ZER6*- (left) and *p71-ZER6*-silenced HCT116 cells (right), as determined using dual luciferase assay. Cells transfected with control vector (shCon) were used as control. β-Actin was used for qPCR normalization. Data were shown as mean ± SEM of three independent experiments. ***P* < 0.01; NS: not significant (ANOVA).

Supplementary Figure S3

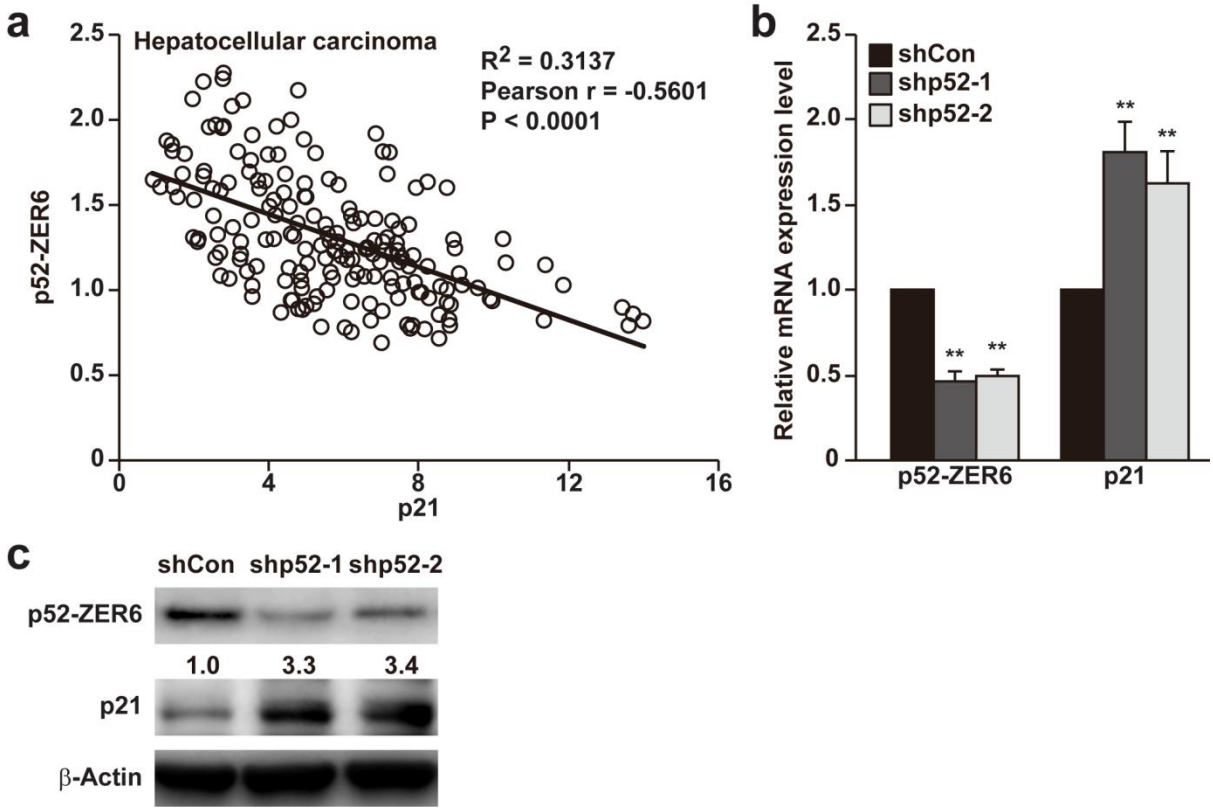

Fig. S3. p52-ZER6 expression is negatively correlated with p21. a Correlation analysis of ZER6 and p21 in hepatocellular carcinoma specimens (GEO data set: GSE25097, n = 184). **b** p52-ZER6 and p21 mRNA expression levels in *p52-ZER6*-silenced HCT116 stable cell lines HCT116/shp52-1 and HCT116/shp52-2, as determined using qPCR. **c** p52-ZER6 and p21 protein expression levels in *p52-ZER6*-silenced HCT116 stable cell lines HCT116/shp52-1 and HCT116/shp52-2, as analysed using western blotting. The stable cell lines were obtained using two shRNA expression vectors targeting different sites of p52-ZER6 (shp52-1 and shp52-2), and were established from single clones. Stable cell line obtained using control vector (shCon) was used as control. β-Actin was used for qPCR normalization and as western blotting loading control. Quantitative data were shown as mean ± SEM of three independent experiments. ***P* < 0.01 (ANOVA).

Supplementary Figure S4

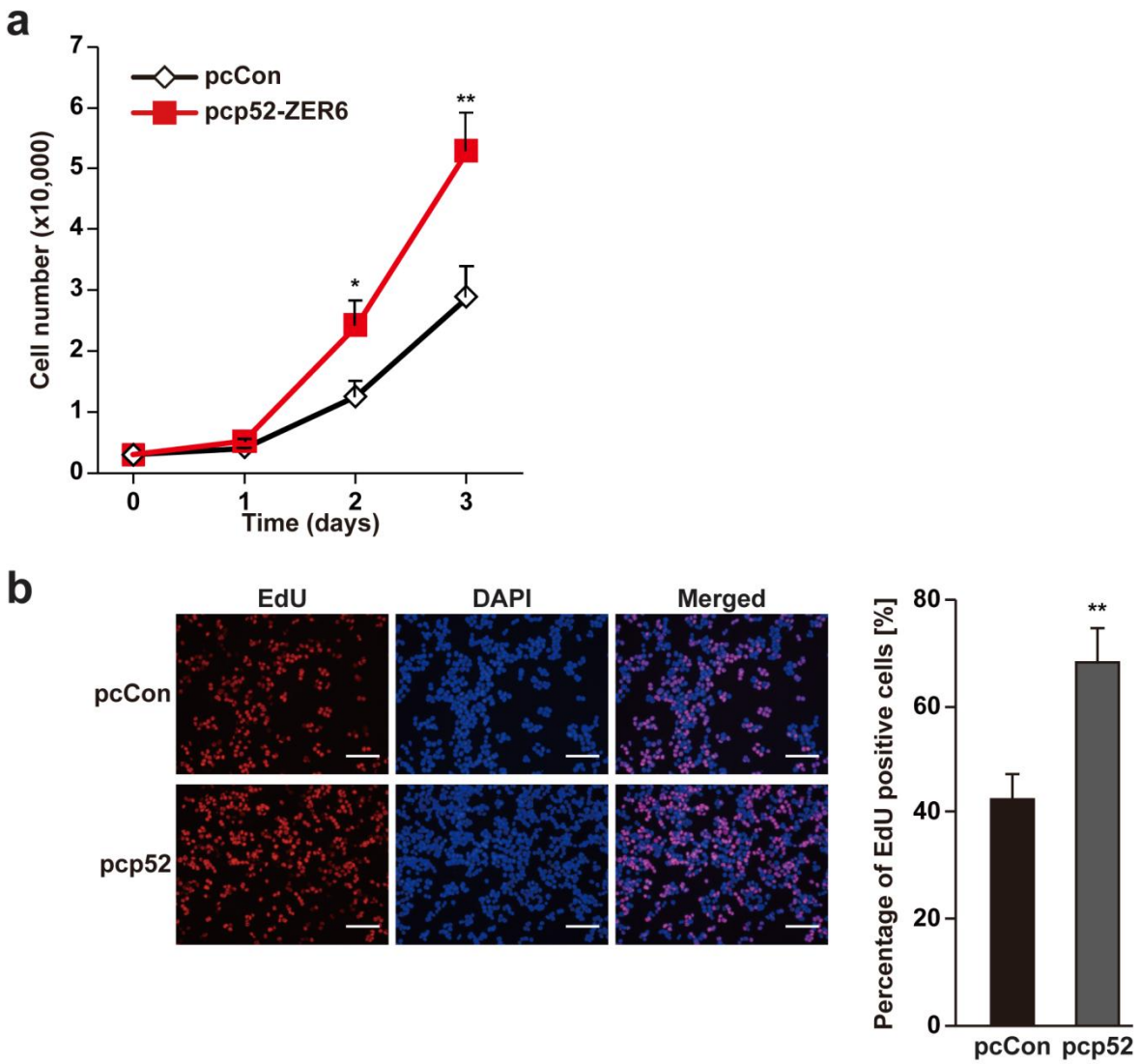

Fig. S4. *p52-ZER6* overexpression enhances tumor cells proliferation. a Total cell number of HCT116 cells overexpressing *p52-ZER6* at the indicated time points. **b** Number of proliferative HCT116 cells overexpressing *p52-ZER6*, as determined by the EdU incorporation assay. Representative images (left) and the percentage of EdU-positive cells to DAPI positive cells (right) were shown. Quantitative data were shown as mean ± SEM of three independent experiments. **P* < 0.05; ***P* < 0.01 (ANOVA); pcp52: p52-ZER6 overexpression vector.

Supplementary Figure S5

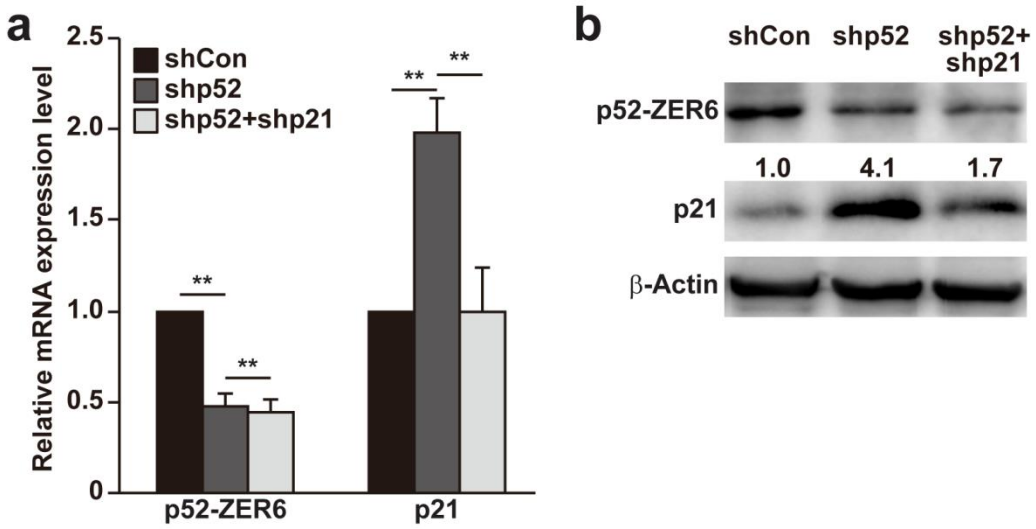

Fig. S5. Establishment of the *p52-ZER6* and *p21* double-knockdown system. a p52-ZER6 and p21 mRNA expression levels in HCT116 cells transfected with both shRNAs targeting *p52-ZER6* and *p21*, as determined using qPCR. **b** p52-ZER6 and p21 protein expression levels in HCT116 cells transfected with both shRNAs targeting *p52-ZER6* and *p21*, as analysed using western blotting. Cells transfected with control vector (shCon) were used as controls. β-Actin was used for qPCR normalization and as western blotting loading control. Quantitative data were shown as mean ± SEM of three independent experiments. ***P* < 0.01 (ANOVA).

Supplementary Figure S6

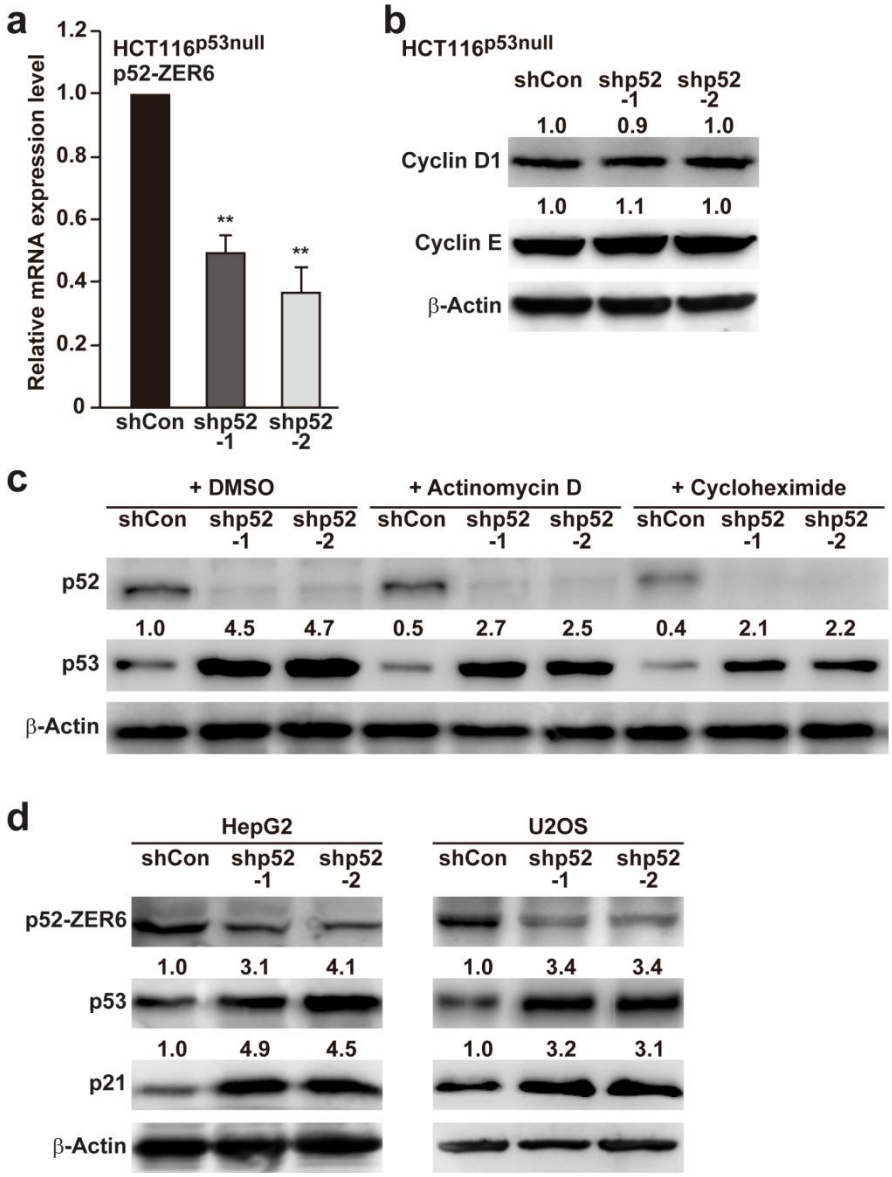

Fig. S6. p52-ZER6 suppresses p21 expression level through p53 post-translational regulation. a p52-ZER6 mRNA expression level in HCT116^{p53null} cells transfected with shRNA vectors targeting *p52-ZER6*, as determined using qPCR. **b** The protein expression levels of cyclin D1 and cyclin E in HCT116^{p53null} cells transfected with shRNA vectors targeting *p52-ZER6*, as determined using western blotting. **c** p53 protein expression level in *p52-ZER6* silenced HCT116 cells treated with transcriptional inhibitor actinomycin D, or *de novo* protein synthesis inhibitor cycloheximide (30 μg/ml), as analysed using western blotting. Cells treated with DMSO were used as control. **d** p53 and p21 protein expression levels in *p52-ZER6*-silenced human HepG2 hepatocellular carcinoma cell line (left) and human U2OS sarcoma cell line (right), as analysed using western blotting. β-Actin was used for qPCR normalization and as western blotting loading control. Cells transfected with shCon were used as control. Quantitative data were shown as mean ± SEM of three independent experiments. $^{**}P < 0.01$ (ANOVA).

Supplementary Figure S7

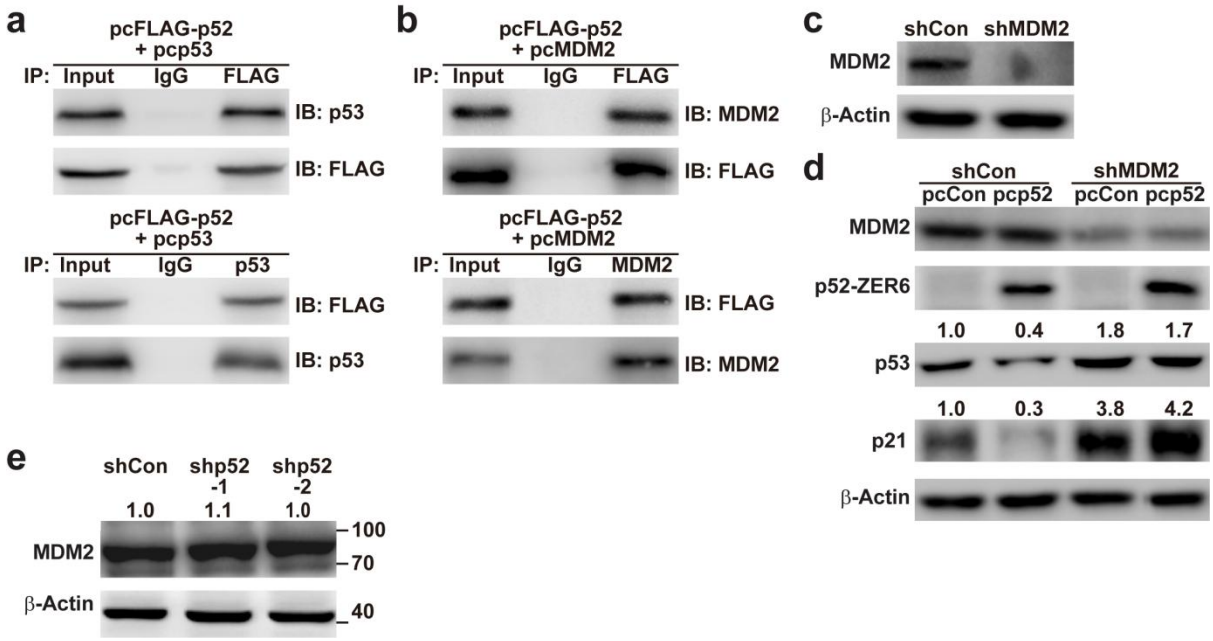

Fig. S7. MDM2 is necessary for p52-ZER6 regulation on p53 protein accumulation. a–b Physical interactions between p52-ZER6 and p53 (a) or MDM2 (b) in HCT116 cells overexpressing *p52-ZER6* and *p53* or *MDM2*, as determined by anti-p53 or anti-MDM2 immunoblotting of cell lysate immunoprecipitated with anti-FLAG antibody, and *vice versa*. **c** Protein expression level of MDM2 in *MDM2*-silenced HCT116 cells, as determined using western blotting. **d** p53 and p21 protein expression levels in *MDM2*-silenced HCT116 cells overexpressing *p52-ZER6*, as analysed using western blotting. **e** MDM2 protein expression level in *p52-ZER6*-silenced HCT116 cells, as determined using western blotting. β-Actin was used as western blotting loading control.

Supplementary Figure S8

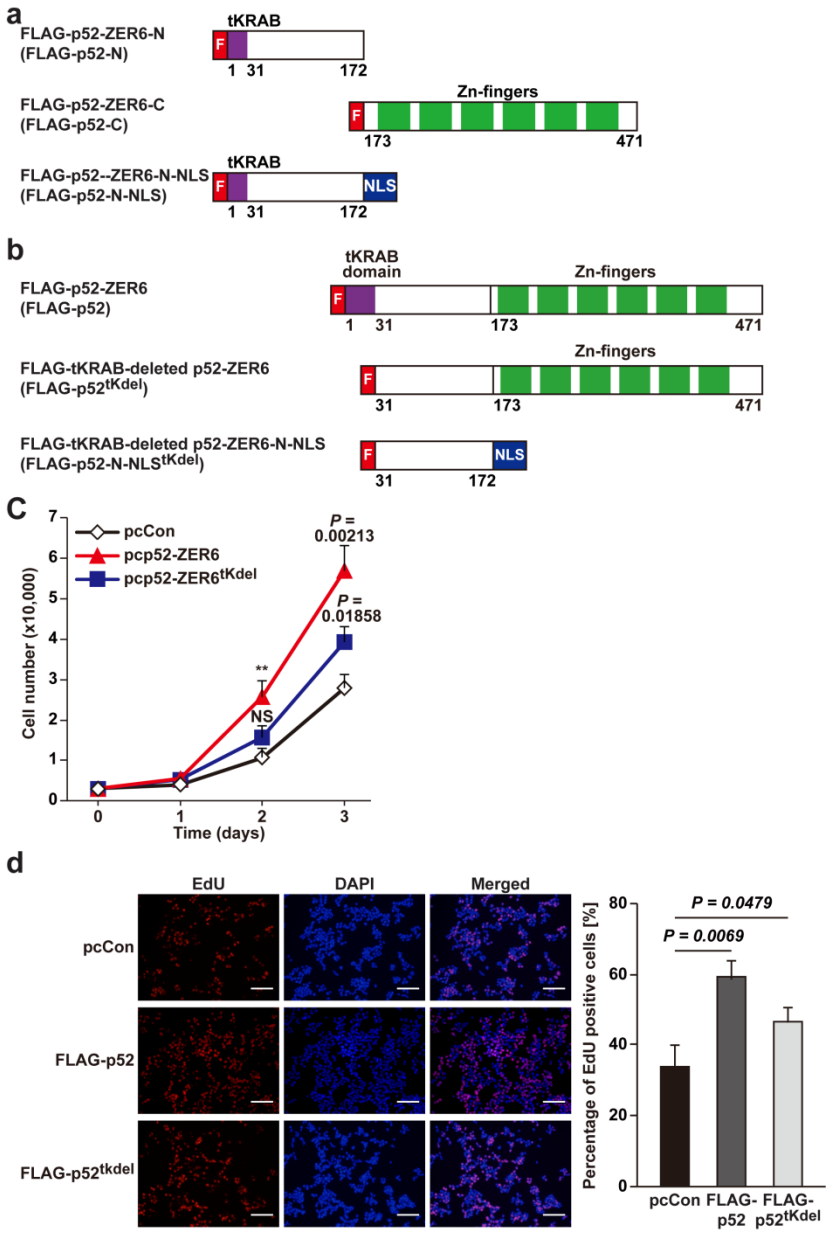

Fig. S8. The role of tKRAB domain in p52-ZER6-mediated tumour cells proliferation. a Schematic diagram of the FLAG-conjugated N terminal and C terminal fragments of p52-ZER6 (FLAG-p52-N and FLAG-p52-C, respectively), and NLS-fused N terminal fragment of p52-ZER6 (FLAG-p52-N-NLS). **b** Schematic diagram of FLAG-conjugated p52-ZER6 fragment lacking tKRAB domain (FLAG-p52^{tKdel}) and NLS-fused N terminal fragment of p52-ZER6 lacking tKRAB domain (FLAG-p52-N-NLS^{tKdel}). **c** Total cell number of HCT116 cells overexpressing *FLAG-p52^{tKdel}* at the indicated time points. **d** Number of proliferative HCT116 cells overexpressing *p52^{tKdel}*, as determined by the EdU incorporation assay. Representative images (left) and the percentage of EdU-positive cells to DAPI positive cells (right) were shown. Quantitative data were shown as mean ± SEM of three independent experiments. $^{**}P < 0.01$; NS: not significant; *P* values were analysed using ANOVA.

Supplementary Figure S9

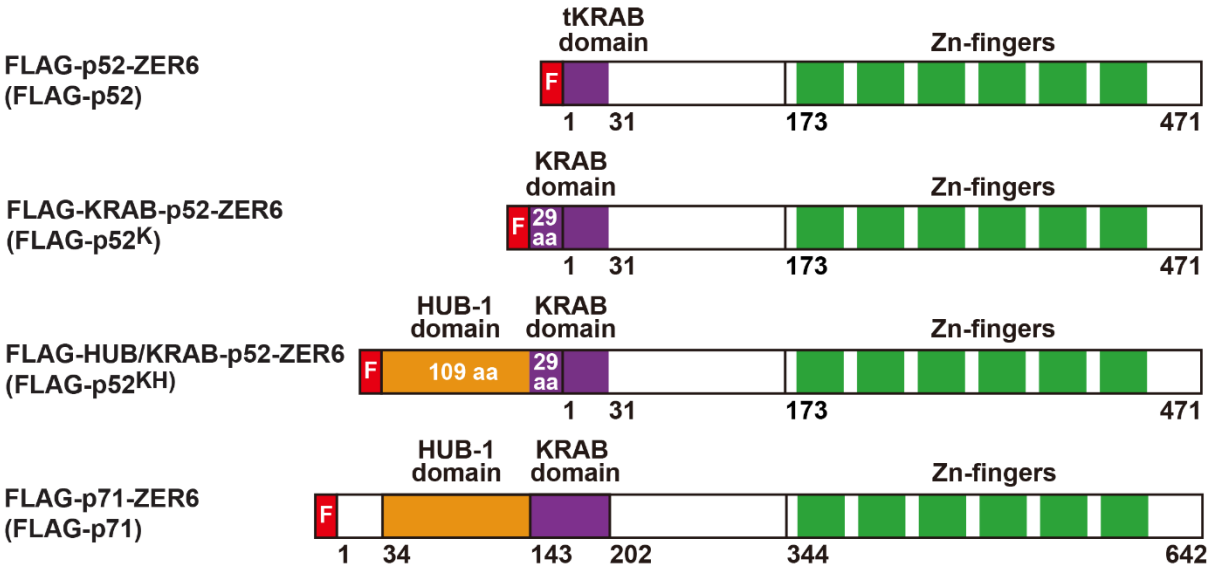

Fig. S9. Schematic diagram of various p52-ZER6 and p71-ZER6 fragments. Schematic diagram of various fragments with intermediate length and sequences between p52-ZER6 and p71-ZER6 conjugated with FLAG: FLAG-p52-ZER6, FLAG-KRAB-p52-ZER6 (FLAG-p52^{K}), FLAG-HUB-1/KRAB-p52-ZER6 (FLAG-p52^{KH}) and FLAG-p71-ZER6. F: FLAG.

Supplementary Figure S10

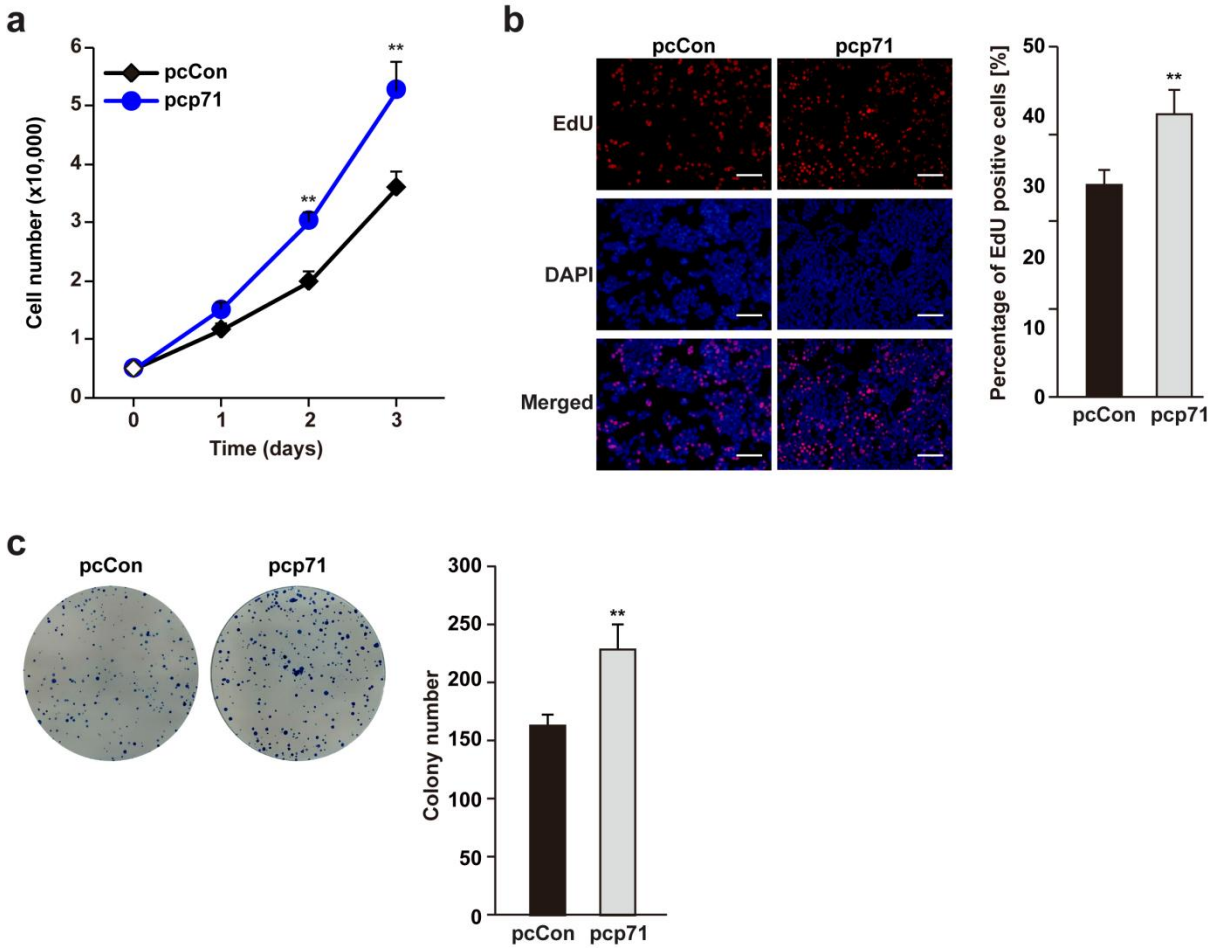

Fig. S10. p71-ZER6 enhances cell proliferation and colony formation potentials. a Total cell number of HCT116 cells overexpressing *p71-ZER6* at the indicated time points. **b** Number of proliferative HCT116 cells overexpressing *p71-ZER6*, as determined by the EdU incorporation assay. Representative images (left) and the percentage of EdU-positive cells to DAPI positive cells (right) were shown. **c** Colony formation potential of HCT116 cells overexpressing *p71-ZER6*. Representative images (left) and quantification results (right) were shown. Cells transfected with pcCon were used as controls. $^{**}P < 0.01$ (ANOVA).

Supplementary Table S1.

Primer pairs used for gene quantification by quantitative PCR.

Gene	RefSeq No.	Forward (5'-3')	Reverse (5'-3')
p21	NM_000389.4	TCACTGTCTTGTACCCTTGTGC	GGCGTTTGGAGTGGTAGAAA
ZER6	Common for NM_170686.2 & NM_020781.3	CCAGAAGGGGAACATAATACAGA	CTGATGTTGAAATACCAGGCTCT
p52-ZER6	Specific for NM_020781.3	TGCGGTCGAAGACGAGAT	TCCTGCTCTCTCCATGTTCA
p71-ZER6	Specific for NM_170686.2	GTCCGTGCGGGGAAGGCA	CCATGGCCCTGCGCTGTCT
β-Actin	NM_001101.3	CGAGCGCGGCTACAGCTT	TCCTTAATGTCACGCACGATTT

Supplementary Table S2.

Antibodies used for western blotting, immunohistochemistry, immunofluorescence and immunoprecipitation.

Antibody	Maker	Product number	Experiment	Dilution
anti-β-Actin	Proteintech	60008-1- Ig	Western Blotting	1/50000
anti-ZNF398 (anti-ZER6)	GeneTex	GTX107221	Western Blotting	1/1000
anti-p21	Proteintech	10355-1-AP	Western Blotting Immunohistochemistry	1/1000 1/50
anti-Cyclin D1	Santa Cruz Biotechnology	sc-8396	Western Blotting	1/500
anti-Cyclin E	Santa Cruz Biotechnology	sc-481	Western Blotting	1/300
anti-p53	Proteintech	10442-1-AP	Western Blotting Immunoprecipitation Immunohistochemistry Immunofluorescence	1/1000 5 μg/ml cell lysate 1/50 1/100
anti-MDM2	Santa Cruz Biotechnology	sc-965	Western Blotting Immunoprecipitation	1/100 5 μg/ml cell lysate
anti-FLAG	Sigma-Aldrich	F1804	Western Blotting	1/1000
anti-FLAG	Proteintech	66008-2-Ig	Immunoprecipitation Immunofluorescence	5 μg/ml cell lysate 1/100
anti-Ubiquitin	Proteintech	10201-2-AP	Ubiquitination assay	1/1000
Goat Anti-Rabbit IgG	ZSGB-BIO	ZB2301	Western Blotting	1/10000
Goat Anti-Mouse IgG	ZSGB-BIO	ZB2305	Western Blotting	1/10000
Alexa Fluor 488 Donkey Anti-rabbit IgG	Invitrogen	A21206	Immunofluorescence	1/500
Alexa Fluor 568 Goat Anti-mouse IgG	Invitrogen	A11004	Immunofluorescence	1/100
Goat Anti-Mouse/Rabbit Immunoglobulins/HRP	DAKO	K5007	Immunohistochemistry	not diluted
Mouse Anti-Rabbit IgG Light Chain	Abbkine	A25022	Western Blotting	1/10000
Goat Anti-Mouse IgG Light Chain	Abbkine	A25012	Western Blotting	1/10000