**ΔNp63 activates the Fanconi anemia DNA repair pathway and**

limits the efficacy of cisplatin treatment in squamous cell carcinoma

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SUPPLEMENTARY FIGURES (S1-S7)

Supplementary Figure S1: High level p63 expression in squamous cell carcinomas
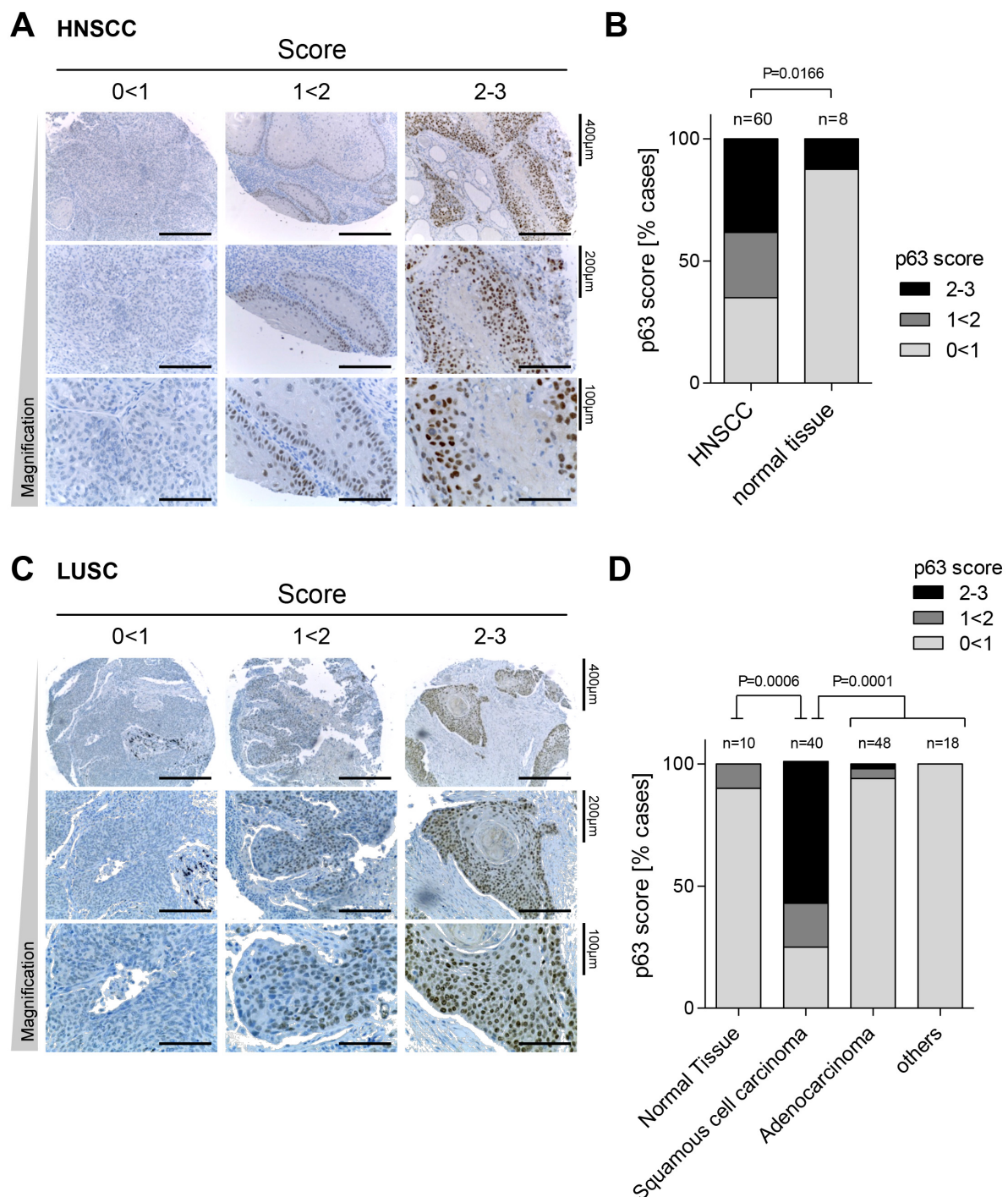
(A-D) Tissue microarrays of paraffin-embedded SCC samples **(A, B)** HNSCC; **(C, D)** LUSC were immunostained for p63. Protein levels were scored as low (0<1), intermediate (1<2) and high (2-3). **(A)** and **(C)**, Representative images. **(B)** and **(D)**, Distribution of p63 scores in tumor and normal tissues. n=number of samples analyzed; statistical significance tested by χ^2 test.

Supplementary Figure S2: Expression of p53 family members in HNSCC cell lines
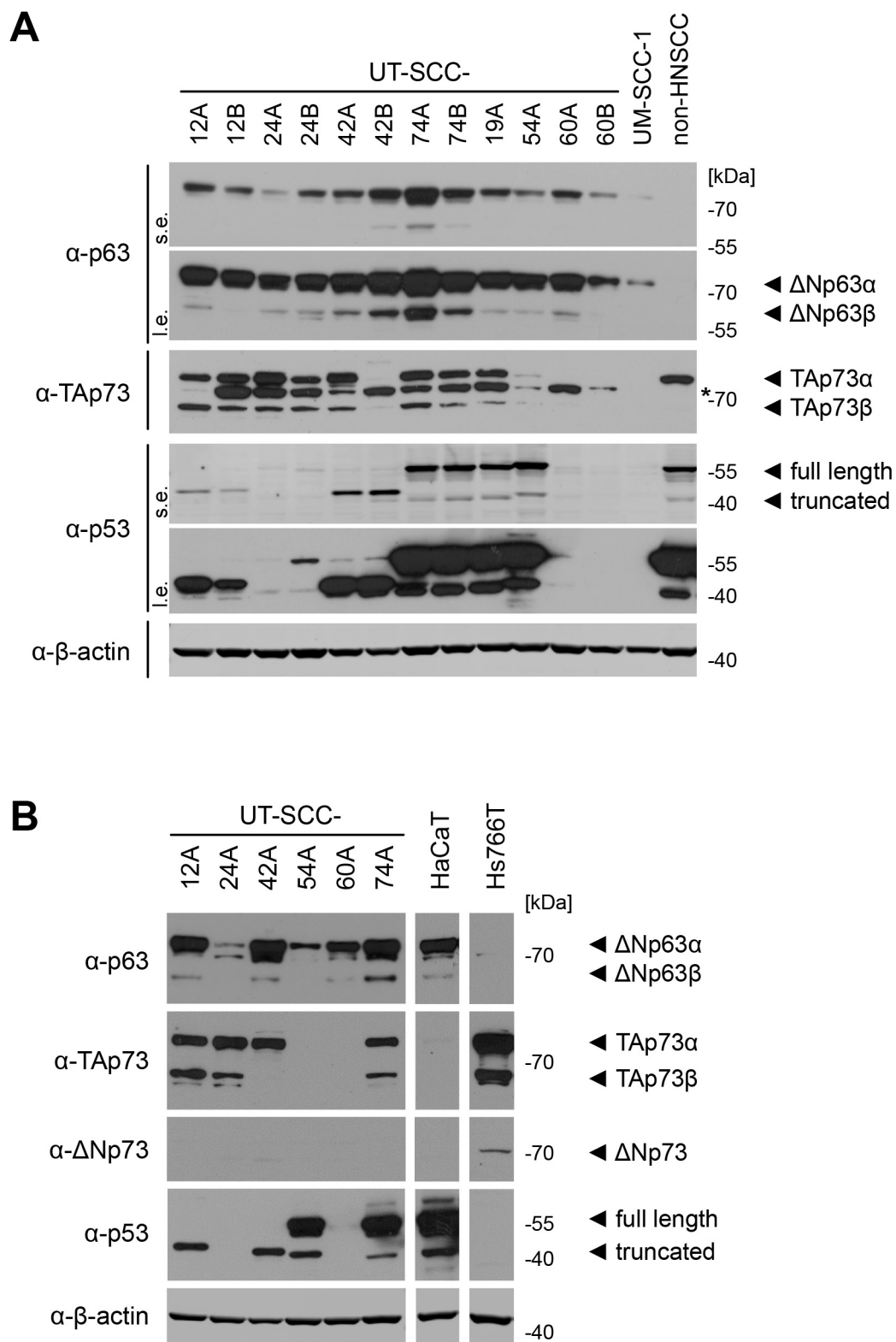
(A) Western Blot of a panel of established HNSCC cell lines evaluating p63, TAp73 and p53 expression. Arrows indicate isoforms previously validated by RNAi experiments (data not shown). non-HNSCC: A7 melanoma cell line. **(B)** Cell lines from A selected for further experiments were

analyzed in addition for expression of the ΔNp73 isoform. The human keratinocyte cell line HaCaT was used as a positive control for p63 expression, the pancreatic cancer cell line Hs766T for TAp73 and ΔNp73.

β-actin: loading control, s.e. short exposure, l.e. long exposure, * non-specific bands.

Supplementary Figure S3: Regulation of proliferation and cisplatin response by ΔNp63 is independent of TAp73.
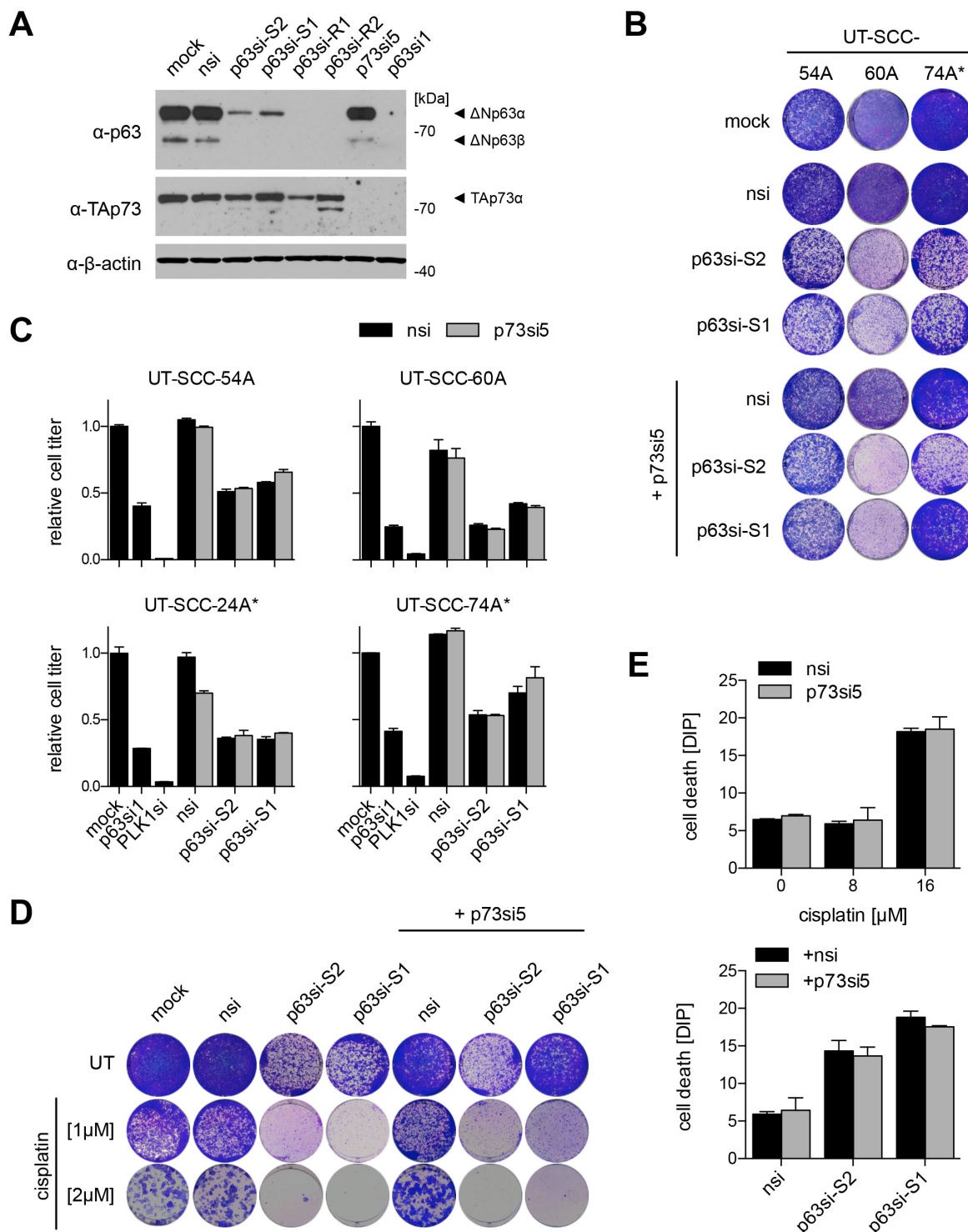
HNSCC cell lines were transfected with p63-targeting (p63si-S1, -S2, -R1, -R2) siRNAs with or without co-depletion of p73 (p63si1, p73si5). **(A)** Western Blot analyzing knockdown efficiency and specificity of siRNAs targeting p63 and p73. β-actin is shown as loading control. **(B)** Colony

formation of HNSCC cell lines following p63/p73 depletion. **(C)** Relative cell titer of p63-depleted HNSCC cell lines. Comparison of p73si5- and nsi- co-transfected samples is shown. Bars show relative cell titer as mean +SD (n=3) normalized to mock-treated cells. PLK1si was used as a positive control. **(D)** Colony formation following cisplatin treatment of p63/p73 depletion. Co-depletion of p73 does not rescue from p63si-induced sensitization towards cisplatin. **(E)** Cell death measurement by DAPI intensity per pixel [DIP] analysis (see Supplementary Fig. 3). UT-SCC-74A were transfected with siRNAs for 48 h, treated with cisplatin at indicated concentration for 24 h and analyzed by DIP. Each bar shows mean +SD (n=2). Top: p73 depletion has no effect on cisplatin response. Bottom: p73 co-depletion does not rescue p63si-induced sensitization towards cisplatin.

* TAp73 high-expressing cell line.

Supplementary Figure S4: DIP method for quantification of cell death on a single cell level

UT-SCC-74A were treated with increasing doses of cisplatin and analyzed for cell death induction by measuring the DAPI intensity per pixel (DIP) on a single-cell level in a 96-well plate format. **(A)** Representative immunofluorescence images of DAPI-methanol stained cells. Small bright spots indicate dead cells. **(B)** Quantification of the DAPI intensity per pixel [DIP] as a measure for cell death. Each region of interest (ROI), i.e. cell, was quantified for its DAPI intensity and size in pixel. An increase in the ratio DAPI intensity / pixel (DIP) indicates cell death. Left: DIP cell-to-cell variability. Distribution of DIP values for each ROI are shown as box plots, whiskers indicate 10th and 90th percentile. n: number of ROIs (cells) analyzed. Right: Comparison of mean DIP values from three independent wells showed high reproducibility. **(C)** Correlation of DIP analysis with propidium iodide-staining for quantification of dead cells.

UT: untreated control, AU: arbitrary units.

Supplementary Figure S5: Cell death response to cisplatin of G1-arrested cells
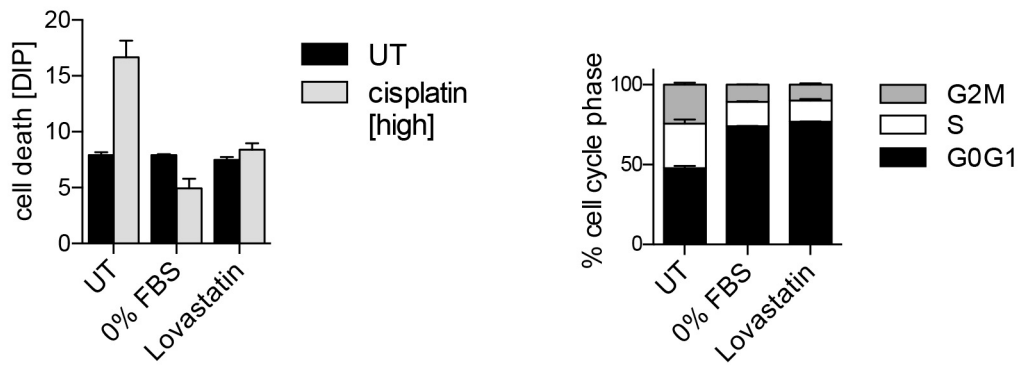
UT-SCC-74A were arrested in G0/G1-phase by Lovastatin treatment [40 μM] or serum-deprivation (0% FBS) for 48 h followed by cisplatin treatment for 24 h. *Left*: Cell death analysis by DIP (mean +SD, n=4). *Right*: Cell cycle analysis of propidium iodide-stained cells by flow cytometry prior to cisplatin treatment (mean +SD, n=3).

Supplementary Figure S6: Identification of ΔNp63-regulated pathways in SCC by transcriptome-wide profiling

UT-SCC-74A were transfected with a set of p63-targeting siRNAs (p63si-S1/2, -R1/2) and controls (mock, nsi-1, nsi-2) and mRNA expression levels were analyzed by a cDNA microarray. **(A)** RNA samples for microarray analysis from two biological replicates were analyzed for ΔNp63 levels by qPCR. Relative mRNA levels are expressed as x-fold of mock-treated sample (n=3). **(B)** Enrichment plot of results from a gene set enrichment analysis (GSEA) (1) of p63-depleted (p63si = p63si-S1/2, -R1/2) versus control (nsi = nsi-1, -2) samples. Gene sets from the C5 collection (gene ontology terms)

of the Molecular Signatures Database MSigDB (2) were analyzed for enrichment and visualized by Cytoscape using the Enrichment Map Plugin (3). Significantly enriched gene sets (nodes) are depicted (P-value >0.005, FDR Q-value >0.1, overlap cutoff 0.5), edges (green lines) represent common genes.

(C) Enrichment plot as described in B) but using C2.CP gene set collection of canonical pathways.

Supplementary Figure S7: Expression of FANCD2 and RAD18 during the cell cycle
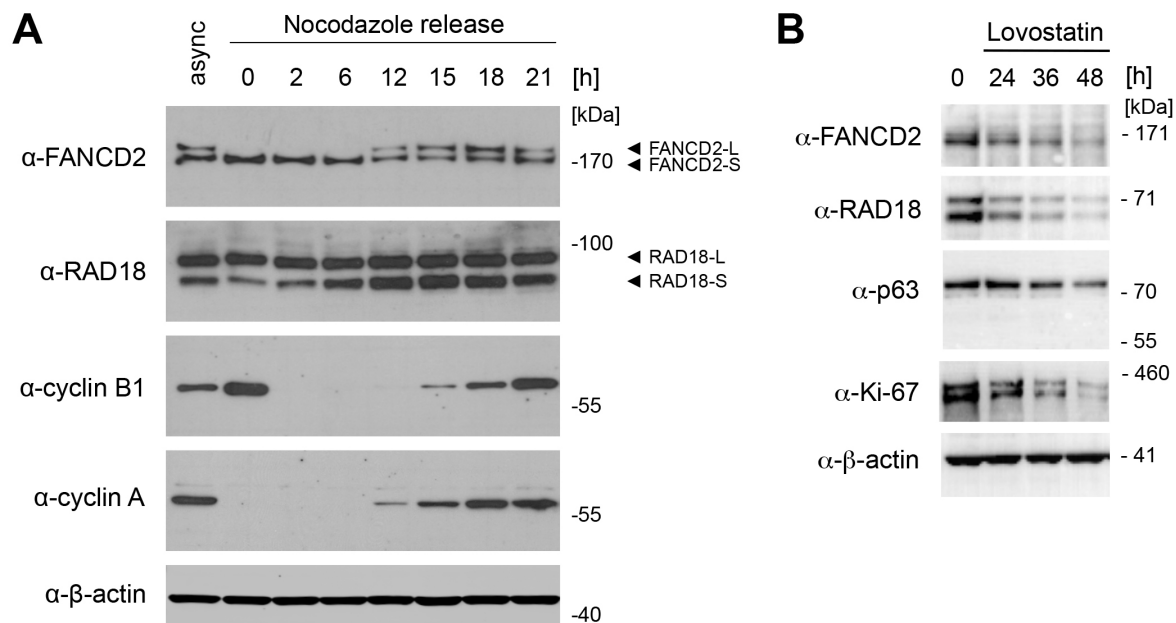
(A) UT-SCC-74A were treated with nocodazole [100 ng/ml] for 18 h to induce a G2/M arrest. Mitotic cells were detached by shake-off and re-plated in fresh growth medium without nocodazole. To analyze protein expression during the cell cycle, cells were harvested at indicated time points after release from nocodazole and analyzed by Western Blot. Only the mono-ubiquitinated form of FANCD2 (FANCD2-L) is regulated in a cell cycle-dependent manner without affecting basal levels of unmodified FANCD2 and RAD18. Cyclin B1 and cyclin A are shown as indicators for G2/M and S-phase, respectively.

(B) UT-SCC-74A cell were treated with lovastatin [20 µM] resulting in progressive exit from the cell cycle (G0 arrest). To analyze protein expression when cells were undergoing arrest, cells were harvested at indicated time points and analyzed by Western Blot for expression of FANCD2 and RAD18. Ki-67 was analyzed as a marker for non-G0 cells. Expression of p63 was measured in parallel, revealing concomitant downregulation of FANCD2, RAD18 and p63 upon cell cycle exit.

β-actin: loading control. -L: long isoform, -S: short isoform.

Supplementary Figure S8: Enhancer-associated histone modifications of the *FANCD2* −10kb region
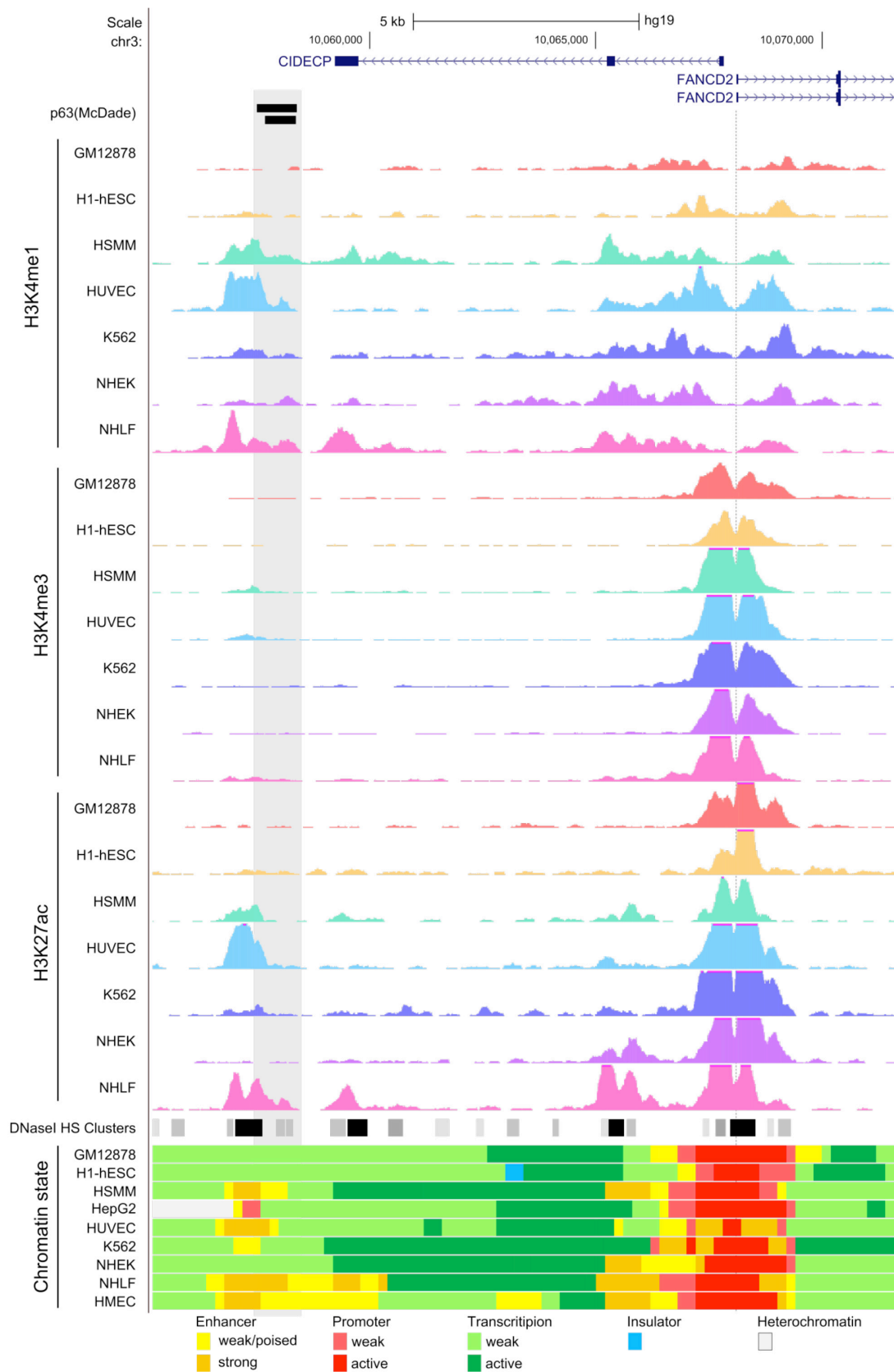
The *FANCD2* -10kb upstream region was analyzed for its chromatin state using publicly available data sets from the Encyclopedia of DNA elements (ENCODE) (4, 5). UCSC genome browser view shows read alignments from ChIPseq of the histone modifications H3K4me1, H3K4me3 and H3K27ac in indicated cell lines (6). DNase I hypersensitive (HS) clusters of 125 cell types and chromatin states of selected cell lines are illustrated below. Peaks of p63-bound sites identified by McDade et al. (7) are indicated as black bars above the plots. *FANCD2* transcription start site (dashed line) and p63-bound -10kb enhancer region (grey rectangle) are highlighted.

NHEK: primary normal human epidermal keratinocyctes.

Supplementary Figure S9: Ectopic expression of FANCD2 is not sufficient to rescue ΔNp63-depleted cells from cisplatin-induced cell death
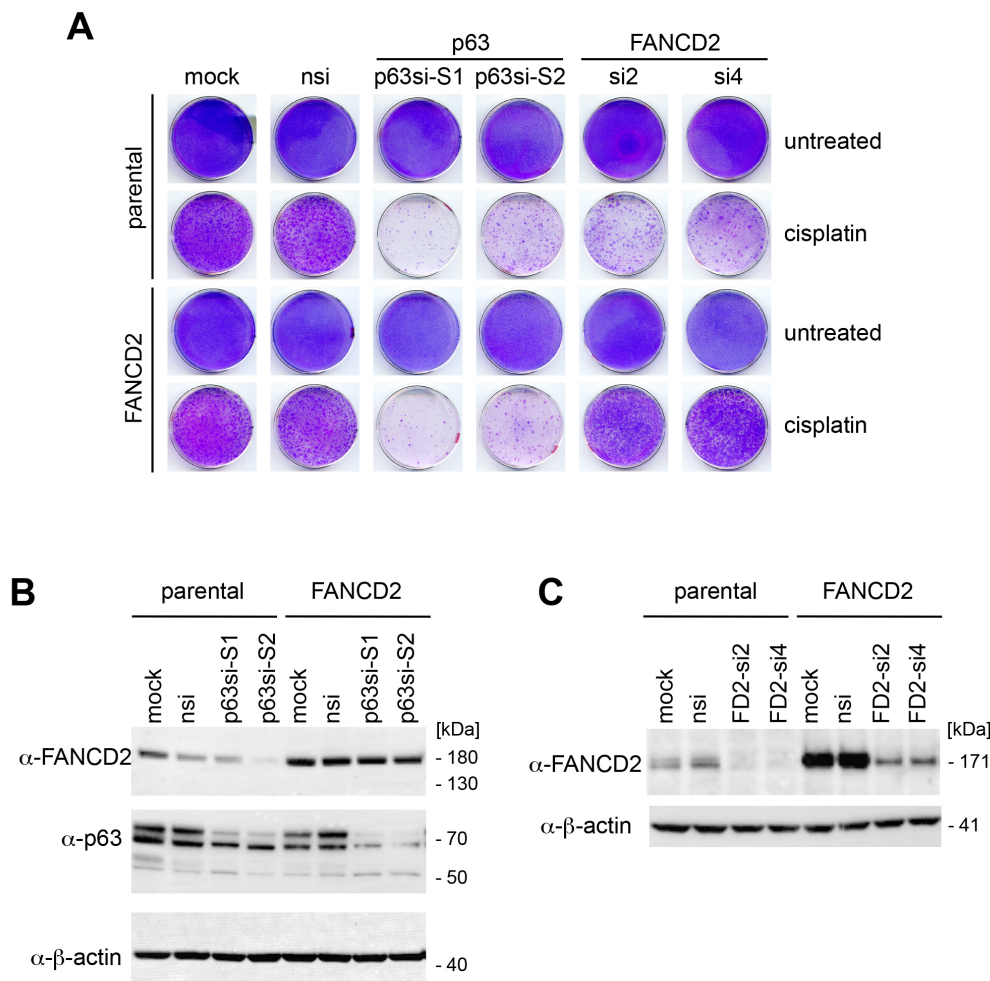
UT-SCC-74A cells were stably transduced with FANCD2 expressing lentiviruses. **(A)** Parental and FANCD2-transduced cells were transfected with the two p63-targeting siRNAs p63si-S1 and p63si-S2, respectively. Two independent FANCD2-targeting siRNAs (FD2-si2 and FD2-si4) served as a positive control. Non-targeting siRNA (nsi) and mock-transfected cells were included as negative controls. Colony formation of cells with or without 24-h cisplatin treatment [2 μM] was measured after 10 days. FANCD2-depleted cells were treated with 1 μM cisplatin because of higher cisplatin sensitivity. Efficient rescue of FANCD2-depleted cells from cisplatin-induced cell death by ectopically expressed FANCD2 validates the functionality of transfected FANCD2. **(B)** Overexpression and knockdown of FANCD2 and p63 were validated by Western blot. β-actin: loading control.

Supplementary Figure S10: Ectopic expression of FANCD2 is not sufficient to sustain proliferation of ΔNp63-depleted cells
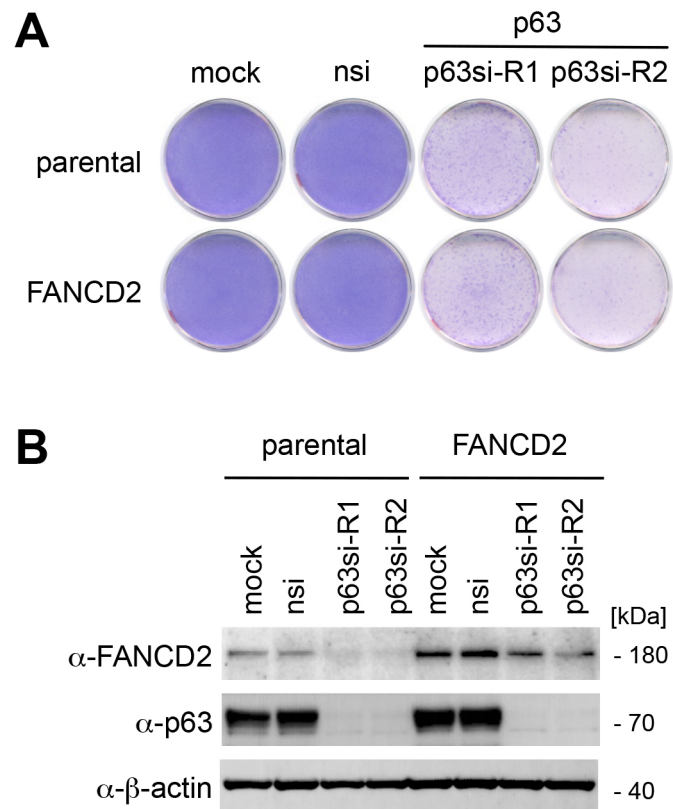

UT-SCC-74A cells were stably transduced with FANCD2 expressing lentiviruses. **(A)** Parental and FANCD2-transduced cells were transfected with the two siRNAs p63si-R1 and p63si-R2, respectively. Non-targeting siRNA (nsi) and mock-transfected cells were included as negative controls. Colony formation of cells was measured after 10 days. **(B)** Overexpression of FANCD2 and knockdown of p63 were validated by Western blot. β-actin: loading control.

Supplementary Figure S11: Depletion of multiple FA pathway components sensitizes SCC cells to cisplatin
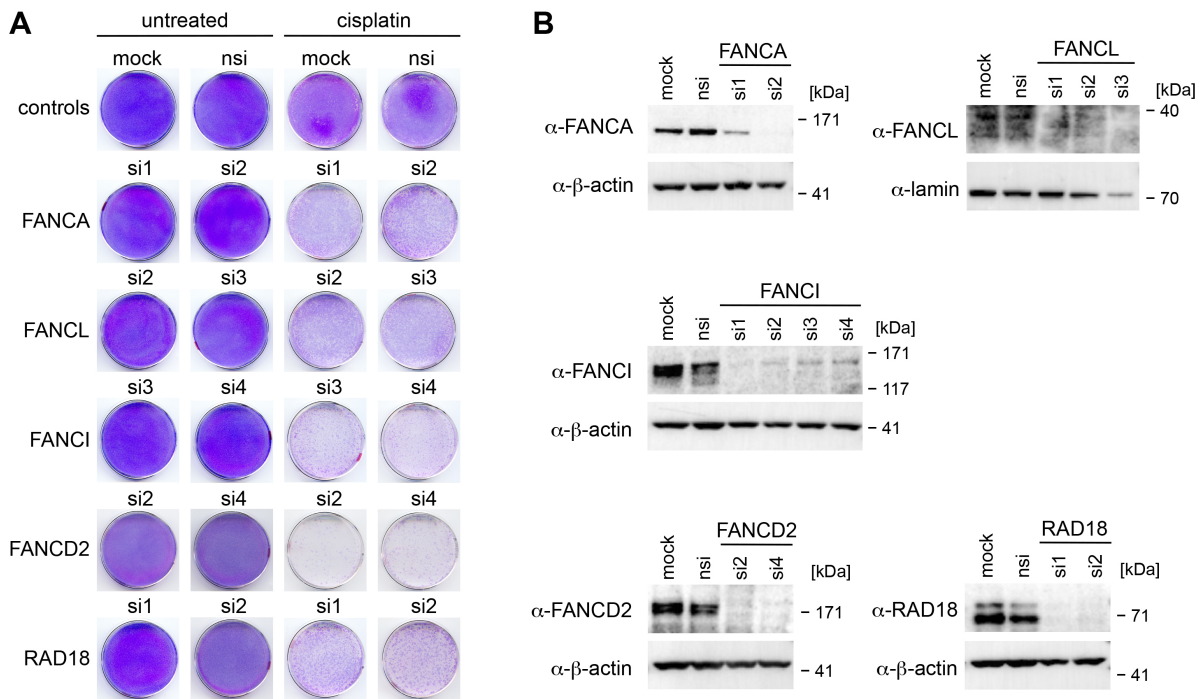
UT-SCC-74A cells were transfected with siRNAs targeting FANCA, FANCL, FANCI, FANCD2 and RAD18. Non-targeting siRNA (nsi) and mock-transfected cells were included as negative controls. **(A)** Colony formation of cells with or without 24-h cisplatin treatment [2 µM] was measured after 10 days. **(B)** Knock-down efficiencies were validated by Western blot. β-actin, lamin: loading controls.

SUPPLEMENTARY METHODS

siRNA

siRNAs were provided by Dharmacon and Qiagen.

Name / Gene	siRNA #	target sequence	modification[1]	supplier
nsi [2]	nsi-1	UGGUUUACAUGUCGACUAA	OTP	Dharmacon
	nsi-2	UGGUUUACAUGUUGUGUGA	OTP	Dharmacon
	nsi-3	UGGUUUACAUGUUUUCUGA	OTP	Dharmacon
	nsi-4	UGGUUUACAUGUUUUCCUA	OTP	Dharmacon
p63si-S1		GGGUGAGCGUGUUAUUGAUGCU	OTP	Dharmacon
p63si-S2		AGAAAGAGAGAGAGGGACU	OTP	Dharmacon
p63si-R1		GGACAGCAGCAUUGAUCAA	siG	Dharmacon
p63si-R2		CGACAGUCUUGUACAAUUU	siG	Dharmacon
p63si1		AACUGAAGAAACUCUACUGCC	HPP	Qiagen
p73si5		GCAAGCAGCCCAUCAAGGA	OTP	Dharmacon
FANCA	si1	GCAGGUCACGGUUGAUGUA	OTP	Dharmacon
	si2	GUUAGAGUUUGCUCAGUAU	OTP	Dharmacon
FANCD2	si1	UGGAUAAGUUGUCGUCUAU	OTP	Dharmacon
	si2	CAACAUACCUCGACUCAUU	OTP	Dharmacon
	si3	GGAUUUACCUGUGAUAAUA	OTP	Dharmacon
	si4	GGAGAUUGAUGGUCUACUA	OTP	Dharmacon
FANCI	si1	ACAGAGUGGUGACGAGCUA	OTP	Dharmacon
	si2	GCAGAAAGAAAUAGCGUCU	OTP	Dharmacon
	si3	GAUACUUGUCCUUCGGAAA	OTP	Dharmacon
	si4	ACGAAGACCUAGAUGAUAU	OTP	Dharmacon
FANCL	si1	GCGGAUACCUGCUUCAGUA	OTP	Dharmacon
	si2	AGUGUUGCCUGAAGAUUUA	OTP	Dharmacon
	si3	GCAAUAGAAUCACUAAAGG	OTP	Dharmacon
PLK1 [2]	si9	GCACAUACCGCCUGAGUCU	OTP	Dharmacon
	si10	CCACCAAGGUUUUCGAUUG	OTP	Dharmacon
	si11	GCUCUUCAAUGACUCAACA	OTP	Dharmacon
	si12	UCUCAAGGCCUCCUAAUAG	OTP	Dharmacon
RAD18	si1	CAUAUUAGAUGAACUGGUA	siG	Dharmacon
	si2	GAUAAUAUGACCUCAGUAA	siG	Dharmacon

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[1] Modifications: siG siGenome, OTP ON-TARGET*plus,* HPP
[2] Used as siRNA pool of four different siRNAs.

Antibodies

Name	application	Clone / order no.	source
β-actin	WB	AC-15 / ab6276	Abcam
ΔNp73	WB	EP051710	Eurogentec
γH2AX (pS139)	IF, WB	- / ab11174	Abcam
cyclin A	WB	C-19 / sc-596	Santa Cruz Biotechnology
cyclin B1	WB	GNS1 / sc-245	Santa Cruz Biotechnology
p53	WB	DO1 / -	B. Vojtesek
p63	ChIP, IF, WB	4A4 / sc-8431	Santa Cruz Biotechnology
p63	IHC	4A4 / 559951	BD Pharmingen
FANCA	WB	- / A301-908A	Bethyl Lab.
FANCD2	WB	FI17 / sc-20022	Santa Cruz Biotechnology
FANCD2	IHC	- / ab111269	Abcam
FANCI	WB	- / A301-254A	Bethyl Lab.
FANCL	WB	H-197 / sc-66887	Santa Cruz Biotechnology
H3K4me1	ChIP	- / ab8895	Abcam
H3K4me3	ChIP	- / ab8580	Abcam
H3K27ac	ChIP	- / ab4729	Abcam
HA-tag	EMSA	16B12 / MMS-101R	Covance
Ki-67	WB	- /ab15580	Abcam
mouse IgG (normal)	ChIP	- / 12-371	Merck Millipore
mouse IgG-HRP	WB	- / NA9310	GE Healthcare
mouse IgG-Alexa Fluor 488	IF	- /A11029	Molecular Probes
mouse IgG-Alexa Fluor 647	IF	- / A21236	Molecular Probes
Pt-[GG]	IF	R-C18 / -	Thomale J. (2, 8)
rabbit IgG (normal)	ChIP	- / sc.2027	Santa Cruz Biotechnology
rabbit IgG-HRP	WB	- / NA9340	GE Healthcare
rabbit IgG-Alexa Fluor 488	IF	- / A11008	Molecular Probes
rabbit IgG-Alexa Fluor 647	IF	- / A-21057	Molecular Probes
rat IgG-Cy3	IF	- / 312-165-003	Dianova
mouse IgG-Biotin	IHC	- / EO46401	Dako
rabbit IgG-Biotin	IHC	- / EO43201	Dako

RAD18	WB	- / ab79763	Abcam
RAD18	IHC	3H7 / H00056852-M01	Abnova
RPA32, pS33	IF	- / A300-246A	Bethyl Lab.
TAp73	WB	- / A300-126A	Bethyl Lab.
USP1	WB	- / A301-700A	Bethyl Lab.

Oligonucleotides

Gene / Region	Application	sense sequence	anti-sense sequence
28S	qPCR	CCTGAGCGCAAGTACTCTGTGT	GCTGATCCACATCTGCTGGAA
β-actin	qPCR	TTGAAAATCCGGGGGAGAG	ACATTGTTCCAACATGCCAG
ΔNp63	qPCR	GGAGCCAGAAGAAAGGACAGCAGC	CCAGGTTCGTGTACTGTGGCTCA
FANCA	qPCR	AAAAATGCCGCAGGTCACG	CCTGTACTCCAGCAGCCAAA
FANCB	qPCR	TTCGGGAATTACGGCAGCAT	GACATTCCTTCTCCTCTGCACT
FANCC	qPCR	ACACTCAAAGGCGAATGGCT	GGGGTCAACATCTGTCAGGG
FANCD2	qPCR	GGAACTACTCAGCCAGAGCGTCCA	CCCACTTGGCCACACCCGAC
RAD18	qPCR	TCTGTATGCATGGGACAGGA	TCAGGTTCCAATTCCTCTGG
USP1	qPCR	GGTTGAACAGCTCCAGGCTA	AGGGTTGAGTTCCCTCAGTG
FANCD2 -10kb	ChIP	GCTGTCTGGCAAGTTAGGATGG	CAAGCTGTAAGGCATTTCCCCG
FANCD2 TSS	ChIP	GGAAAGTCGAAAACTACGGGC	TCGAGAGACTACGACCATTGC
control site (CDKN1A +11443)	ChIP	TCTGTCTCGGCAGCTGACAT	AACACAAAGATCAAGGTGAGTGA
FANCD2 −10kb_WT	EMSA	GGGATTACAGGCATGAGCCAGCACGCCCAGCCC	GGGCTGGGCGTGCTGGCTCATGCCTGTAATCCC
FANCD2 −10kb_MUT	EMSA	GGGATTATAGTTATTAGTCATTACTCCCAGCCC	GGGCTGGGAGTAATGACTAATAACTATAATCCC
FANCD2 −10kb_WT-scrambled	EMSA	GGGGTTAAACGGCGCCATACCACCGACGAGCCC	GGGCTCGTCGGTGGTATGGCGCCGTTTAACCCC
FANCD2 −10kb_MUT-scrambled	EMSA	GGGGATTATTAATCGTCTACATAGATTCTCCCC	GGGGAGAATCTATGTAGACGATTAATAATCCCC
FANCD2 −10kb_WT	Luciferase reporter assay	GATCATTACAGGCATGAGCCAGCACGCCC	GATCGGGCGTGCTGGCTCATGCCTGTAAT
FANCD2 −10kb_MUT	Luciferase reporter assay	GATCATTATAGTTATTAGTCATTACTCCC	GATCGGGAGTAATGACTAATAACTATAAT

SUPPLEMENTARY REFERENCES

1. Subramanian,A., Tamayo,P., Mootha,V.K., Mukherjee,S., Ebert,B.L., Gillette,M.A., Paulovich,A., Pomeroy,S.L., Golub,T.R., Lander,E.S., *et al.* (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA*, **102**, 15545–15550.

2. Liberzon,A., Subramanian,A., Pinchback,R., Thorvaldsdottir,H., Tamayo,P. and Mesirov,J.P. (2011) Molecular signatures database (MSigDB) 3.0. *Bioinformatics*, **27**, 1739–1740.

3. Merico,D., Isserlin,R., Stueker,O., Emili,A. and Bader,G.D. (2010) Enrichment map: a network-based method for gene-set enrichment visualization and interpretation. *PLoS ONE*, **5**, e13984.

4. ENCODE Project Consortium (2011) A user's guide to the encyclopedia of DNA elements (ENCODE). *PLoS Biol*, **9**, e1001046.

5. Ernst,J., Kheradpour,P., Mikkelsen,T.S., Shoresh,N., Ward,L.D., Epstein,C.B., Zhang,X., Wang,L., Issner,R., Coyne,M., *et al.* (2012) Mapping and analysis of chromatin state dynamics in nine human cell types. *Nature*, **473**, 43–49.

6. Rosenbloom,K.R., Armstrong,J., Barber,G.P., Casper,J., Clawson,H., Diekhans,M., Dreszer,T.R., Fujita,P.A., Guruvadoo,L., Haeussler,M., *et al.* (2015) The UCSC Genome Browser database: 2015 update. *Nucleic Acids Res*, **43**, D670–81.

7. McDade,S.S., Patel,D., Moran,M., Campbell,J., Fenwick,K., Kozarewa,I., Orr,N.J., Lord,C.J., Ashworth,A.A. and McCance,D.J. (2014) Genome-wide characterization reveals complex interplay between TP53 and TP63 in response to genotoxic stress. *Nucleic Acids Res*, **42**, 6270–6285.

8. Liedert,B. (2006) Adduct-specific monoclonal antibodies for the measurement of cisplatin-induced DNA lesions in individual cell nuclei. *Nucleic Acids Res*, **34**, e47–e47.