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Supplementary Information for

E2F1 sumoylation as a protective cellular mechanism in oxidative stress response

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SI Materials and Methods

Cell culture, transfection, and treatments

Primary MEFs were isolated as previously described (1). HEK 293T, Lenti-X 293T, H1299, and primary MEFs were cultured in DMEM, while U2OS cells were cultured in McCoy's 5A modified media. All media was supplemented with 10% FBS, penicillin (50 IU/ml), and streptomycin (50 μg/ml). Stable cell lines containing doxycycline-inducible constructs were cultured in appropriate supplemented media with 10% tetracycline-free fetal bovine serum, penicillin (50 IU/ml), and streptomycin (50 μg/ml). All cells were maintained in a humidified incubator with 5% CO_2 at 37^{o}C. HEK 293T, Lenti-X 293T, and H1299 cells were transfected with the standard polyethylenimine method, while U2OS cells were transfected with PolyJet transfection reagent per manufacturer's guidelines (SignaGen). Treatments were done at the indicated doses and for indicated durations for t-BuOOH (Alfa Aesar), hydrogen peroxide (Fisher Scientific), and triptolide (Sigma).

Plasmid construction

CRISPR constructs were generated by cloning indicated sgRNA sequence into pLentiCRISPR v2 (addgene #52961) that had been linearized by BsmBI digestion. The following oligos were used and where relevant, cell clones resulting from these oligos are indicated: sgE2F1, 5'-CACCGGGAGATGATGACGATCTGCG-3' and 5'- AAACCGCAGATCGTCATCATCTCCC-3'; sgSENP3#1 that produced clone sgSENP3-A, 5'- CACCGCTATACAAGGGACCGGGTCC-3' and 5'- AAACGGACCCGGTCCCTTGTATAGC-3'; sgSENP3#2 that produced clones sgSENP3-B, -C and -D, 5'- CACCGCCAGGCGGGAGCGTCTTCGT-3' and 5'-AAACACGAAGACGCTCCCGCCTGGC-3'. K266R E2F1 was generated using QuikChange site-directed mutagenesis (Agilent) with the following primers: 5'-

AGCAGATGGTTATGGTGATCAGAGCCCCTCCTG-3' and 5'-CAGGAGGGGCTCTGATCACCATAACCATCTGCT-3'. Inducible E2F1 construct generation involved first mutating the sgE2F1 PAM site and sgRNA binding regions silently to create Cas9 resistant constructs that could be used to rescue E2F1 expression in sgE2F1 cells. Two sequential reactions were performed using quick change site directed mutagenesis on pCMV-Tag2B-E2F1 and pCMV-Tag2B-K266R E2F1. The first reaction used primers 5'-CTGCGGCTGCTCGACAGTTCGCAAATAGTCATCATC –3' and 5'-GATGATGACTATTTGCGAACTGTCGAGCAGCCGCAG-3' to silently mutate the sgE2F1 PAM site, then a subsequent reaction was performed with primers 5'-CTGCGGCTGCTCGACAGTTCACAAATAGTCATCATC-3' and 5'-GATGATGACTATTTGTGAACTGTCGAGCAGCCGCAG-3' to silently mutate sgE2F1 binding sequence adjacent to the PAM site. These sgE2F1 resistant E2F1 constructs, as well as pCMV-Tag2B empty vector were then amplified with 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGATTACAAGGATGACGACG-3' and 5-GGGGACCACTTTGTACAAGAAAGCTGGGTcGGGTACACTTACCTGGTACCTTAAT-3'. Products from this reaction were then cloned into pDONR221 and finally pInducer-20 using the gateway cloning system per manufacturer's instructions (Invitrogen). pDONR221 and pInducer-20 were provided as generous gifts from Dr. Nicholas Mitsiades.

Virus production and stable cell line generation

Lentivirus was produced in Lenti-X 293T cells by co-transfecting the lentiviral construct of interest with psPAX2 and pMD2.G. Media was changed 24 h after transfection. At 72 h and 96 h after transfection viral media was collected and filtered with a 0.45 μm PVDF filter. Stable inducible

cells cell lines were generated by transducing with appropriate virus concurrently with the addition of 8 µg/ml polybrene (Sigma). Seventy-two hours after transduction, 400 µg/ml G418 (VWR International) was added to select for stable cells. CRISPR-Cas9 knockout cells were generated by transfecting appropriate plenti-CRISPR v2 constructs into U2OS cells. Ninety-six hours after transfection cells were plated in serial dilutions and with 1 µg/ml puromycin (Gibco). Once colonies were visible, individual colonies were isolated, amplified and screened for knockout efficiency by western blotting.

MTT assay

Cells were seeded at 4,000-6,000 cell/well in a 96-well plate. 16-24 h after seeding, media was replaced with media pre-treated with peroxide at the indicated concentrations. At time of assaying viability, MTT reagent (0.5 mg/ml, Sigma) was added to the treatment media and incubated with cells for 4 h in the culture incubator. Duration of peroxide treatment for MTT assays corresponds to time from peroxide addition to time of MTT reagent addition. After incubation with MTT reagent, all treatment media and MTT reagent was removed, and 100 µl of DMSO was added to the wells. Plates were placed on a rocker for 15 min to solubilize MTT crystals, and optical density was measured with a plate reader at 490 and 630 nm.

Colony formation assay

500-1,000 cells were seeded per well into 6-well plates, and were treated 16 h after seeding. Media was changed after 24 h of peroxide treatment, and every 3 days for the remainder of the assay. When the majority of colonies were $\geq$ 50 cells, cells were fixed with 4% formaldehyde in PBS, and subsequently stained with 5% crystal violet in PBS. Colonies were counted using ImageJ software.

Caspase activity assay

Twenty-four hours after seeding, cells were treated with t-BuOOH at a concentration of 100 μM. After 8 h of treatment, caspase-3/7 activity was measured using the Promega Caspase- Glo™3/7 assay per manufacturer's guidelines.

Cell synchronization

Sixteen hours after seeding, CDK1 inhibitor RO-3306 (Tocris) was added to culture media at a concentration of 5 μM. After a 24 h incubation, media was removed and cells were washed twice with PBS, followed by the addition of fresh media with 20% FBS. After 12 h, peroxide was added for various times. At time of harvest, cells were fixed and subject to cell cycle analysis.

Calcein-AM viability assay

Cell viability was determined by treating cells with indicated doses of hydrogen peroxide for 24 h followed by harvesting via trypsinization. Cells were washed twice with PBS, then resuspended in PBS containing 2 μM calcein-AM and 2 μM propidium iodide and incubated on ice for 15 min. Subsequently, samples were analyzed via flow cytometry with the criteria of dead cells being defined as calcein-AM negative and propidium iodide positive.

Flow cytometry

Flow cytometry was performed in the BCM cytometry and cell sorting facility on a BD FACSCanto II. All experiments done using flow cytometry had $\geq$ 10,000 cells assayed, and within a single experiment equivalent cell numbers were interrogated. Data analysis including cell cycle modeling was performed using FlowJo software.

Antibodies for co-immunoprecipitation, nickel pulldowns, and immunoblotting

Antibodies against E2F1 (C-20, KH95), GAPDH (6C5), HA (Y11), p53 (FL-39), and Rb (C-15) were purchased from Santa Cruz Biotechnology. Antibodies against β-Actin (13E5), BNIP3 (D7U1T), Histone H3 (D1H2), LC3B (D11), $p27^{Kip1}$ (D69C12), p62 (D5E2), pS15 p53 (16G8), and SENP3 (D20A10) were purchased from Cell Signaling Technologies. Anti-PARP antibody was purchased from BD Transduction Laboratories. Anti-FLAG antibodies were purchased from Sigma, while the RGS·His antibody was purchased from Qiagen, and the Anti-6X His antibody was purchased from Clontech.

Chromatin binding assay

Detergent-based biochemical fractionation was performed as described by Andegekok et al. (2), and modified by Liu et al. (3). After fractionation, equal cell equivalents were pooled between fractions 1+2 and fractions 3+4, followed by SDS-PAGE analysis.

Chromatin-immunoprecipitation

Treated cells were fixed, harvested, and had nuclei isolated as previously described (4). Nuclei were suspended in shearing buffer (0.1 mM EDTA, pH 8.0, 0.1 mM EGTA, 10 mM Tris-HCl, pH 6.8, 100 mM NaCl, 0.1% sodium deoxycholate, 0.5% N-lauroylsarcosine, protease inhibitors) and sonicated until an average fragment size of 800 bp was reached. Chromatin concentrations were calculated based on processed aliquots, and equal chromatin amounts were used in each subsequent immunoprecipitation. For ChIP studies, protein G magnetic dynabeads (Invitrogen) were used with the corresponding antibodies. For studies at physiological levels, antibodies used for ChIP include CBX4 from Bethyl, E2F1 (C-20) from Santa Cruz Biotechnology, H3K27me3

from Active Motif, SUMO2/3 from Abcam, and IgG from Thermo. For rescue studies in sgE2F1 cells, anti-FLAG antibody purchased from Sigma was used. All chromatin immunoprecipitations, washes, and downstream processing were performed as described previously (4). Samples were analyzed via qPCR, with ChIP primer sequences as indicated in SI Appendix, Table S2. Primer sequence for Gene Desert was designed by Active Motif.

RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

RNA extraction was done using the standard trizol method (Invitrogen) and qPCR was performed on an MX3005P thermal cycler with SYBR green and Rox reference dye as previously described (5). All primers for primary transcripts were validated by confirming sensitivity to RNA polymerase II inhibitor triptolide. Primer pairs used for RT-qPCR studies are described in SI Appendix, Table S2.

Luciferase reporter assay

Luciferase reporter assays were performed using the Promega Dual-Luciferase Reporter Assay System per manufacturer's guidelines. Cells were co-transfected with the indicated E2F1 target gene firefly luciferase reporter construct and either pRL(renilla luciferase)-TK or pCMV-β-galactosidase. After treatments, cells were lysed in passive lysis buffer supplemented with protease inhibitors. Samples were then assayed for firefly luciferase signal, followed by renilla luciferase signal, or were processed for β-galactosidase activity assay. Data was analyzed by normalizing individual sample firefly luciferase activity to its respective renilla luciferase activity or β-galactosidase activity.

Proximity ligation assay

Proximity ligation assays (PLA) were performed using the Duolink In Situ Red Kit for Mouse/Rabbit purchased from Sigma. Cells were seeded on 8-well chamber slides from Thermo Fisher that had been coated with collagen. 36 h after seeding, samples were fixed and processed per manufacturer's guidelines. Antibodies used for immunostaining were E2F1 (KH95) and SUMO2/3 (18H8), purchased from Santa Cruz Biotechnology and Cell Signaling Technologies respectively. No primary antibody addition was used as a negative control. Prior to mounting slides, nuclei were stained with Hoechst 33258. Images were captured with a Zeiss fluorescent microscope, and the same exposure time per channel was used between fields.

Statistical analysis

Two-tailed *t* test was performed to evaluate the differences between experimental groups. We analyzed the survival data using the Kaplan-Meier method. The difference in survival times by SENP3 level was examined by the log-rank test. Proteomics datasets were downloaded from PRIDE (Proteomics Identifications Database, https://www.ebi.ac.uk/pride/). The lists of correlative genes were analyzed in GSEA server, https://www.gsea-msigdb.org/gsea/index.jsp). *P* values less than 0.05 were considered statistically significant.

Figure S1

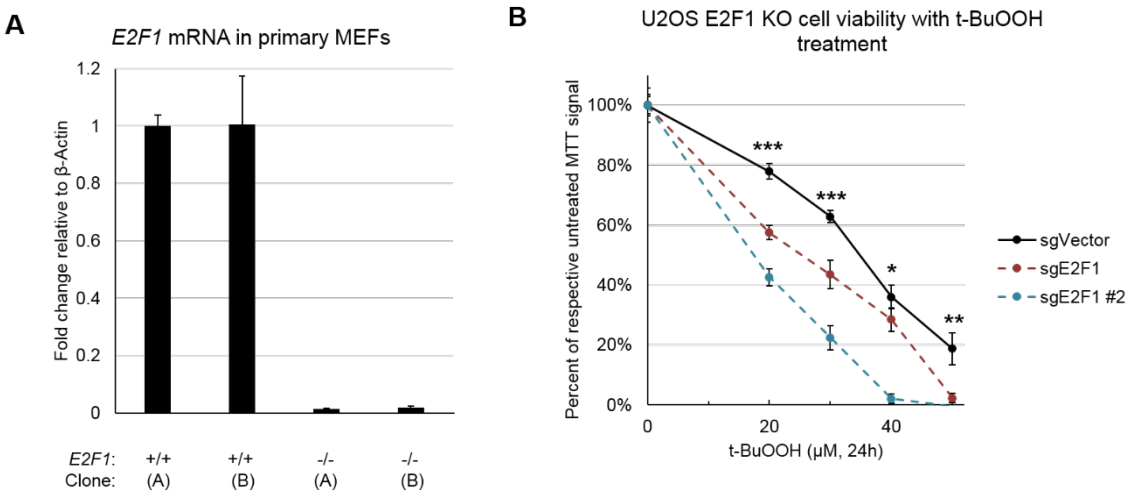

Figure S1

A. RT-qPCR analysis of *e2f1* mRNA expression in $e2f1^{+/+}$ and $e2f1^{-/-}$ primary MEFs.

B. MTT viability assay in wild-type or E2F1 knockout U2OS cell lines after 24 h of t-BuOOH treatment. Error bars represent mean$\pm$SD ($n \geq 4$). *$p<0.05$, **$p<0.005$, ***$p<5x10^{-6}$.

Figure S2

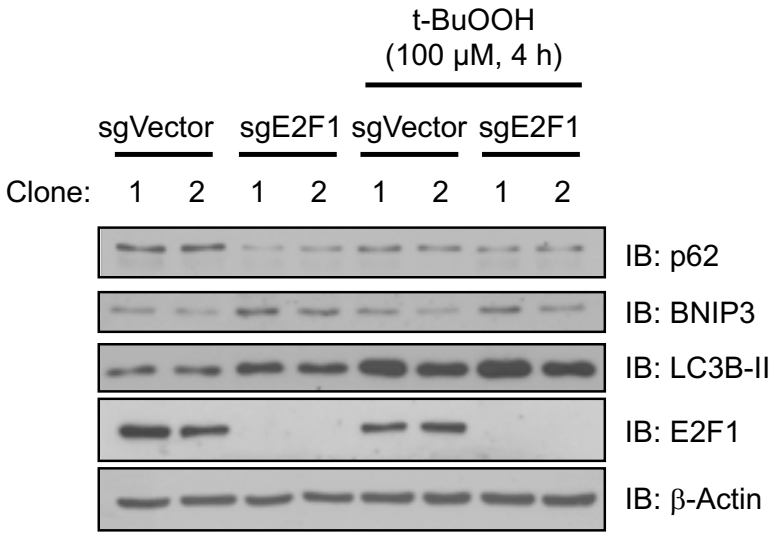

Figure S2

Western blot analysis of autophagy and mitophagy markers in two sgVector and sgE2F1 U2OS clones after four hours of t-BuOOH treatment.

Figure S3

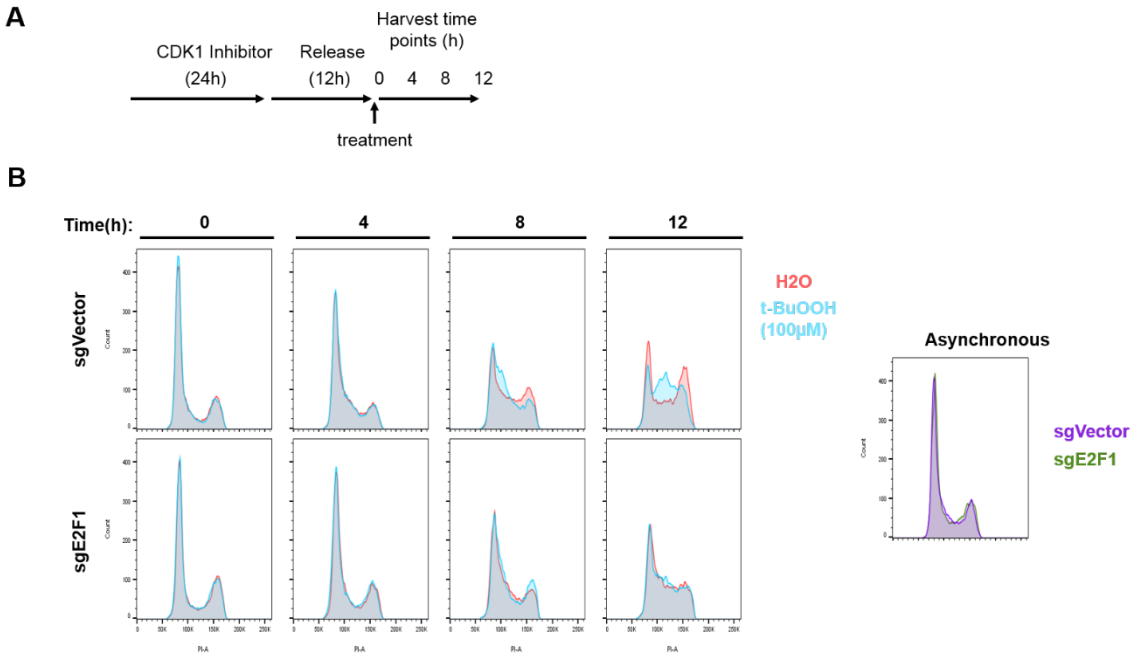

Figure S3

A. Experimental design schematic with timepoints indicating cell cycle synchronization, release, t-BuOOH addition, and harvest points.

B. Vector control or E2F1 knockout U2OS cells were subjected to cell cycle synchronization and release followed by t-BuOOH treatment when enriched for G1-phase cells. t-BuOOH was added at the "0 h" timepoint, and cells were harvested every 4 h up until 12 h to monitor cell cycle progression status.

Figure S4

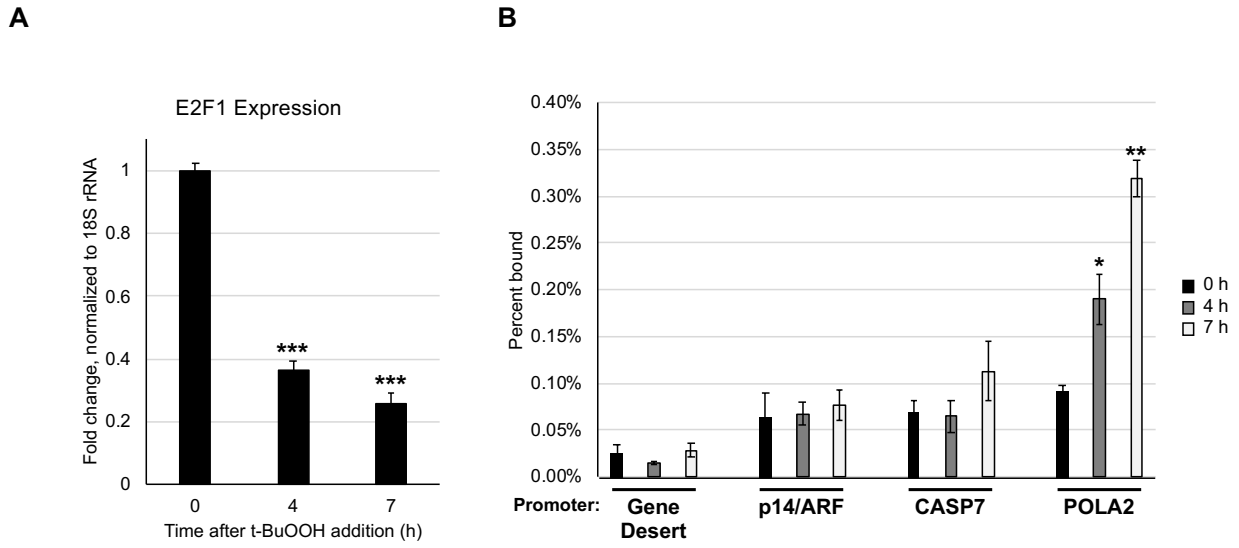

Figure S4

A. RT-qPCR analysis of E2F1 mature transcript in U2OS cells after treatment with t-BuOOH (500 μM) for the indicated time. Data is reported as a fold change relative to 0 h, normalized to respective 18S rRNA expression levels. Error bars represent mean±SD ($n \geq 3$). ***$p<5x10^{-6}$.

B. Percentage of the indicated promoters bound by E2F1 as determined by chromatin-immunoprecipitation of E2F1 under varying lengths of t-BuOOH treatment (500 μM) in U2OS cells. Signal for gene desert enrichment is used as a negative control. Error bars represent mean±SD ($n \geq 3$). *$p<0.05$, **$p<0.005$.

Figure S5

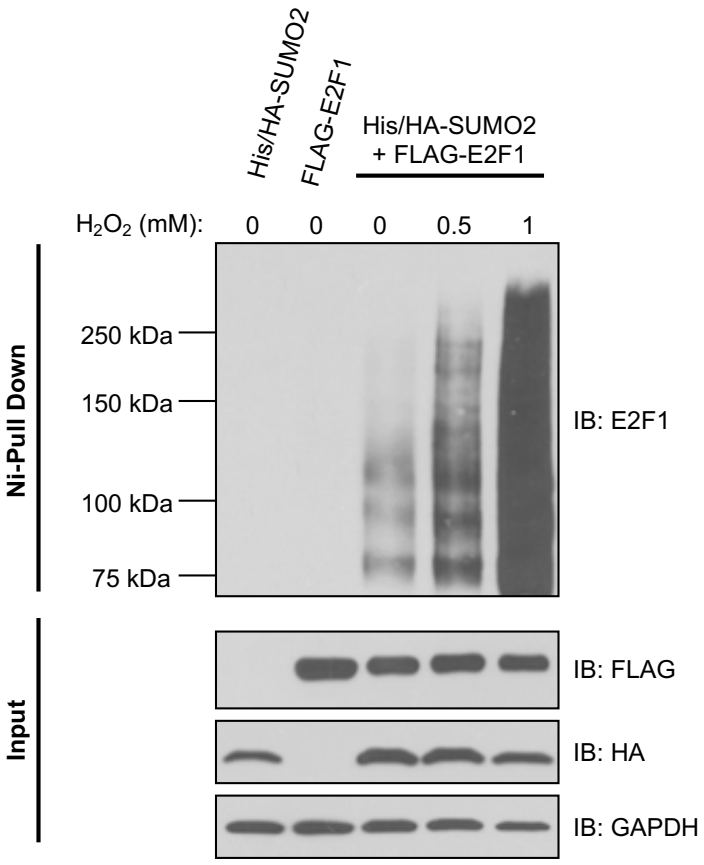

Figure S5

Nickel pulldown of HEK 293T cells transfected with FLAG-E2F1, His/HA-SUMO2, or FLAG-E2F1 and His/HA-SUMO2. Cells were treated with indicated dose of hydrogen peroxide for 8 h prior to harvest.

Figure S6

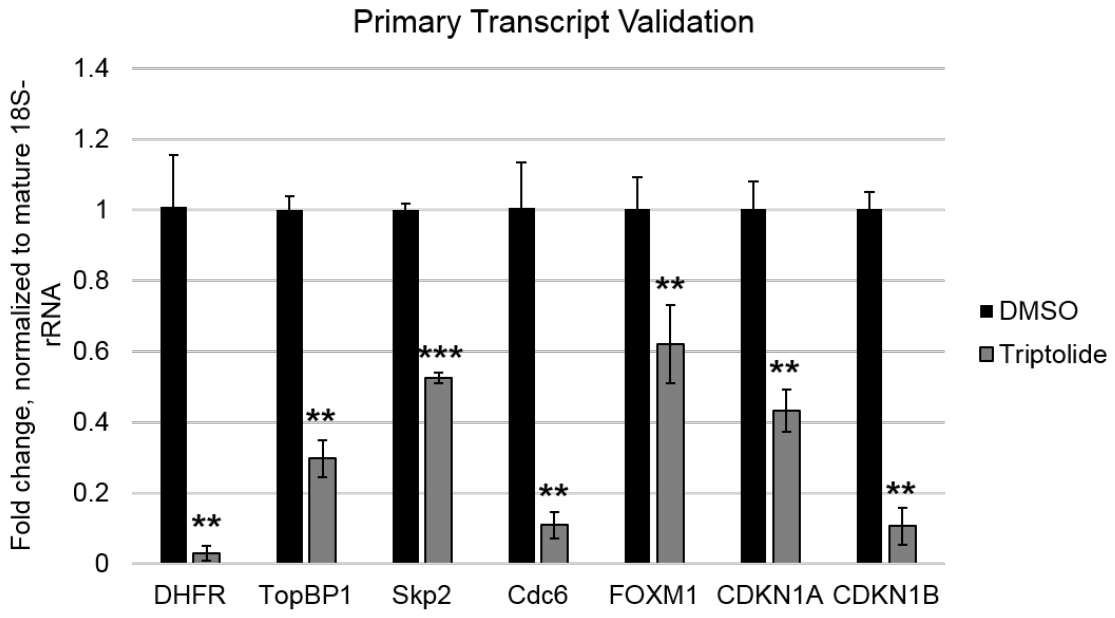

Figure S6

Validation of primary transcript primers via RT-qPCR of sgE2F1-pInducer20-Empty cells after 1 h of 1 μM Triptolide treatment or DMSO control. Error bars represent mean±SD (n=3). **p<0.005, ***p<$5x10^{-6}$.

Figure S7

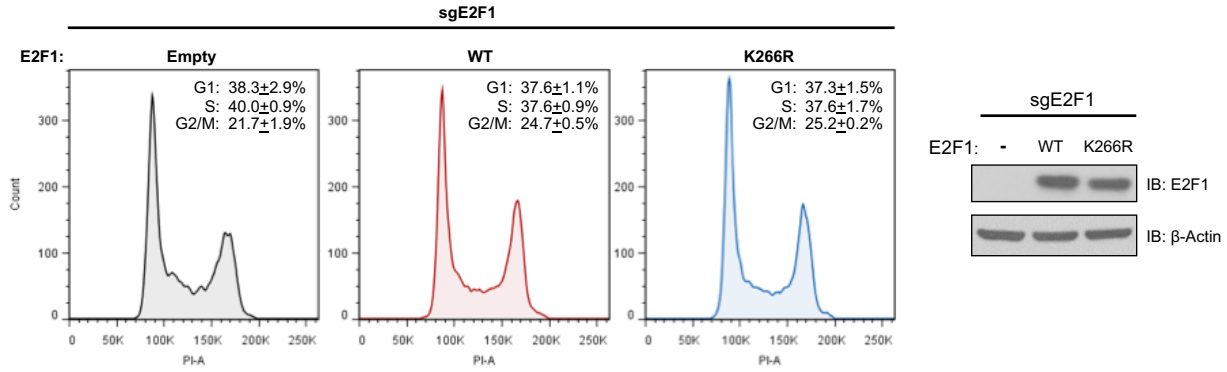

Figure S7

Cell cycle analysis of sgE2F1 cells rescued with an empty vector, WT, or K266R E2F1. Representative profiles are shown. Error bars represent mean±SD (n=3).

Figure S8

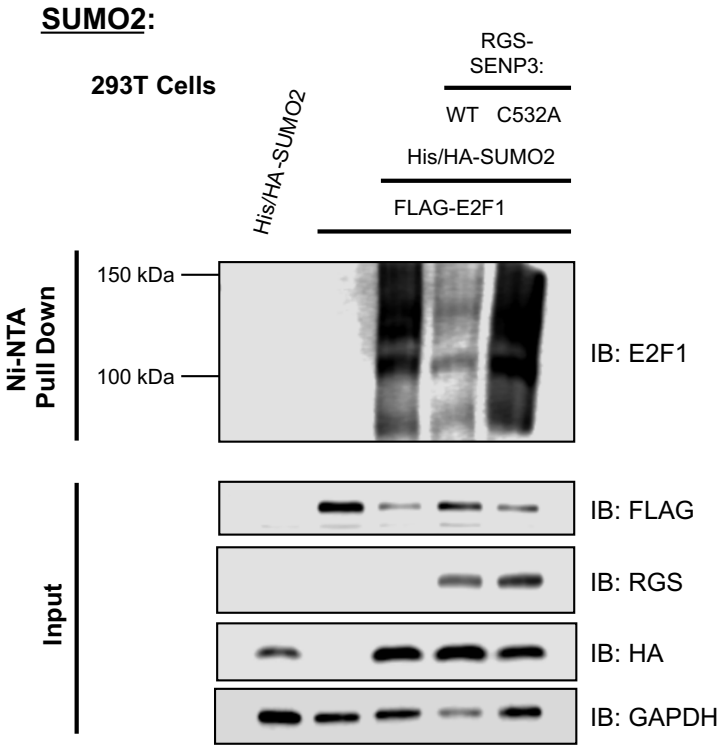

Figure S8

Nickel pulldown of HEK 293T cells transfected with FLAG-E2F1, His/HA-SUMO2, and empty vector, WT, or C532 RGS-SENP3.

Table S1. Patients and tumor characteristics

Sample ID	Path-ology	Size (cm)	Grade	Nodes	Stage	race	ER	PR	Her2	Age	DFS	DFS Status	OS	OS Status	TopBP1
SENP3 LOW															
03T	D+L	2	2	0/2	1	W	Pos	Pos	Neg	60	59	0	59	0	-
04T	D	6	2	4/12	3	B	Pos	Pos	Pos	56	60	0	60	0	+
s07T	L	5.5	2	1/6	3	W	Pos	Pos	Neg	43	86	0	86	0	-
08T	L	5.5	1	4/8	3	W	Pos	Neg	Neg	67	60	0	60	0	-
09T	L		3	6/6	4	W	Pos	Neg		28	36	1	192	1	-
17T	D+L	3.5	2	0/3	2	W	Pos	Neg	Neg	61	54	0	54	0	+
20T	D	1.8	3	0/2	1	W	Neg	Neg	Neg	48	55	0	55	0	-
22T	D+L	6.5	3	18/19	3	B	Pos	Neg	Pos	48	61	0	61	0	+
41T	D		2	0/3	2	W	Pos	Pos	Neg						
45T	D+L	3.2	1	1/8	2	W	Pos	Pos	Neg	86	46	0	46	0	-
46T	D	6.6	2	10/21	3	W	Pos	Neg	Neg	53	24	1	31	0	-
49T	D		3	10/21	3	W				53					+
70T	D	14	3	0/17	3	A	Neg	Pos	Pos	57	36	0	36	0	+
71T	D	2.2	3	0/8	2	B	Pos	Neg	Neg	49	36	0	36	0	-
75T	D	3.3	2	1/11	3	W	Pos	Pos	Neg	56	42	0	42	0	-
78T	L	6.5	2	14/19	3	W	Pos	Pos	Neg	81	20	1	34	0	+
79T	L	5	3	4/9	3	W	Pos	Pos	Neg	57	11	1	32	1	-
80T	D	8.5	3	3/18	3	B	Neg	Neg	Pos	50	33	0	33	0	+
SENP3 HIGH															
01T	Other	3.9	3	1/23	2	B				64					+
02T	L	8	2	12/13	3	W	Pos	Neg	Neg	60	9	1	33	1	+
06T	L	10.5	3	13/21	3	W	Pos	Neg	Neg	61	65	0	65	0	-
10T	D	10	3	2/10	2	A	Neg	Neg	Neg	57	13	1	20	1	+
14T1	D	3.5	3	10/14	3	H	Neg	Pos	Neg	28	17	1	34	1	+
16T															+
18T	D	1	3	0/2	1	B	Neg	Neg	Neg	48	12	0	12	0	+
19T	Other			0/14		W				55					+
24T	L	4	3	0/6	2	W	Pos	Neg	Neg	35	25	0	25	0	+
26T	D	1.8	2	0/3	1	W	Pos	Pos	Pos	43	48	0	48	0	+
28T	D	15	3	5/6	3	H	Pos	Pos	Neg	49	11	1	17	1	+
39T	D		3	1/5	3					51					+

Abbreviation: D, ductal; L: lobular; B, black; W, white; A, Asian; H, Hispanic; Pos, positive; Neg, negative; Nodes, number of nodes involved/number of nodes examined; ER, estrogen receptor; PR, progesterone receptor; Her2, Her2/Neu status positivity is defined by FISH high level amplification and/or immunohistochemistry 3+; DFS, disease-free survival (number of months); DFS Status, status = 0, disease-free; status = 1, recurred; OS, overall survival; OS Status, status = 0, alive; status = 1, dead; TopBP1, (-) not overexpressed, (+) overexpressed. Pathology of 01T: medullary carcinoma; 19T: recurrent Phyllodes tumor.

Table S2. Primer sequences used for RT-qPCR and ChIP-qPCR

Application	Gene	Species	Direction	Sequence (5' to 3')
Mature Transcript	E2F1	Human	Forward	CCGCCATCCAGGAAAAGG
		Human	Reverse	GCCCTCAAGGACGTTGGT
	18S rRNA	Human	Forward	GGCCCTGTAATTGGAATGAGTC
		Human	Reverse	CCAAGATCCAACTACGAGCTT
	β-Actin	Human	Forward	CACCAACTGGGACGACAT
		Human	Reverse	ACAGCCTGGATAGCAACG
	E2F1	Mouse	Forward	GAGGGCATCCAGCTCATTG
		Mouse	Reverse	GGTCCCCAAAGTCACAGTC
	β-Actin	Mouse	Forward	TGTTACCAACTGGGACGACA
		Mouse	Reverse	GGGGTGTTGAAGGTCTCAAA
Primary Transcript	Cdc6	Human	Forward	AAGCTGTCTCGGGCATTGAA
		Human	Reverse	AAAAGAAAGGTCACGAGCTGC
	CDKN1A	Human	Forward	GTGGACCTGTCACTGTCTTG
		Human	Reverse	CCCTTGGACCATGGATTCTG
	CDKN1B	Human	Forward	TTTGGTGGACCCAAAGACTG
		Human	Reverse	GCACTGAACACCTAAGACCA
	DHFR	Human	Forward	TTTCCAGAGAATGACCACAACCT
		Human	Reverse	TGCTCGTGCGTTGACATACA
	FOXM1	Human	Forward	GACTGACTACACACCTTGCC
		Human	Reverse	AGTGGGCCCAACAAATTCAT
	Skp2	Human	Forward	GCTGAACCTCTCCTGGTGTT
		Human	Reverse	GGGACTAACACATGCACTGGA
	TopBP1	Human	Forward	AACGCCACTAAAAGGGTCAC
		Human	Reverse	AAAGGCTGGATTTGAGATGGA
Promoter	Caspase-7	Human	Forward	TTTGGGCACTTGGAGCGCG
		Human	Reverse	AAGAGCCCAAAGCGACCCGT
	CDKN1A	Human	Forward	GTGGCTCTGATTGGCTTTCTG
		Human	Reverse	CTGAAAACAGGCAGCCCAAG
	CDKN1B	Human	Forward	TTCTGGGTTAAGGCTGAGCG
		Human	Reverse	CAACAAACCTGCTCTGGCTG
	E2F1	Human	Forward	AGGAACCGCCGCCGTTGTTCCCGT
		Human	Reverse	CTGCCTGCAAAGTCCCGGCCACTT
	Gene Desert	Human	Forward	TGAGCATTCCAGTGATTTATTG
		Human	Reverse	AAGCAGGTAAAGGTCCATATTTC
	p14/ARF	Human	Forward	CCAGGCGTCCGGCCCCTGGGCCGT
		Human	Reverse	CACGCGGGAAGGGCTGCCGGAGGC
	POLA2	Human	Forward	TTAGATAGTCGAAGGCACGC
		Human	Reverse	AGAACGTCCTGCTTCCCAAAA

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1. Liu K, Lin FT, Ruppert JM, & Lin WC (2003) Regulation of E2F1 by BRCT-domain containing protein TopBP1. *Mol Cell Biol* 23(9):3287-3304.
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3. Liu K, Lin FT, Graves JD, Lee YJ, & Lin WC (2017) Mutant p53 perturbs DNA replication checkpoint control through TopBP1 and Treslin. *Proc Natl Acad Sci U S A* 114(19):E3766-E3775.
4. Paik JC, Wang B, Liu K, Lue JK, & Lin WC (2010) Regulation of E2F1-induced apoptosis by the nucleolar protein RRP1B. *J Biol Chem* 285(9):6348-6363.
5. Liu K, Graves JD, Scott JD, Li R, & Lin WC (2013) Akt switches TopBP1 function from checkpoint activation to transcriptional regulation through phosphoserine binding-mediated oligomerization. *Mol Cell Biol* 33(23):4685-4700.