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Supplemental Information

Regulation of Cellular Senescence by Polycomb Chromatin Modifiers through Distinct DNA Damage- and Histone Methylation-Dependent Pathways

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SUPPLEMENTAL INFORMATION

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Lentivirus Vector Procedures

Supernatants collected from 293T cells were filtered through a 0.45 μM filter. Infections of HDF cells were done in the presence of 8 μg/ml polybrene (Stewart et al., 2003). pLKO.1 vectors were used for shRNA-mediated gene knockdowns (Moffat et al., 2006). The control used throughout this study was an shRNA against an irrelevant target (GFP, 5'-TACAACAGCCACAACGTCTAT). The following shRNA vectors were obtained from Open Biosystems: EZH2, TRCN0000018365 (#1 in Figure S1H), TRCN0000040075 (#2 in Figure S1H), TRCN0000040076 (#3 in Figure S1H); WNT2, TRCN0000033373 (#1 in Figure S6D), TRCN0000033372 (#2 in Figure S6D), MYC, TRCN0000010390 (#1 in Figure S6E), TRCN0000039642 (#2 in Figure S6E); and CDKN1A (p21), TRCN0000287021 (#1 in Figure 2F), TRCN0000287091 (#2 in Figure 2F). A pSUPER retrovirus vector was used for the knockdown of ATM (5'-GATACCAGATCCTTGGAGA, #1 in Figure 2H) (Ortega-Atienza et al., 2015) and lentivirus vector for knockdown of ATM was a gift from Didier Trono (Addgene plasmid #14542, shATM#2 in Figure 2H). TRCN0000040076 (EZH2 #3) was used throughout this study. TRCN0000033373 and TRCN0000033372 (WNT2) corresponds to shWnt2-5 and shWnt2-4, respectively, described and validated by (Ye et al., 2007). TRCN0000010390 (MYC) was described and validated by (Guney et al., 2006). For double knockdowns of p21 and EZH2, and ATM and EZH2, early passage LF1 cells were infected with p21 or ATM shRNA vectors, drug selected, grown up as pools, and superinfected with EZH2 shRNA vectors. Full-length *EZH2* cDNA was obtained from the ATCC; *MYC* cDNA was a kind gift of Dr. Michael Cole (Dartmouth Medical School); *EED* was obtained from Applied Biological Materials. *EZH2* and *MYC* were PCR cloned into pLX303 (plasmid 25897, Addgene) modified by removal of the Gateway Destination sequences and incorporation of multiple cloning sites and sequence verified. *EZH2* and *EED* were also PCR cloned into pLenti-puro, gift from le-Ming Shih (Addgene plasmid #39481). Empty pLX and pLenti-puro vectors were used as controls. For the expression of *HRAS*(G12V) we used the lentivirus vector pLenti CMV RasV12 Neo (plasmid 22259, Addgene, in Figure 1B) or retrovirus vector pQCXIN CMV ER:RasV12 (kind gift of Dr. Gregory David, New York University School of Medicine, in Figure 4A-4E). ER:RasV12 was activated by continuous treatment with 200 ng/ml of 4-hydroxytamoxifen. The empty vector (plasmid 17485, Addgene) was used as the control. In combined cDNA expression and knockdown experiments, cells were first infected with *EZH2* or *MYC* cDNAs, stable pools were selected as indicated above, and subsequently infected with shRNA vectors.

Immunoblotting

Cells were harvested in Laemmli sample buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT). Whole cell extracts were separated by SDS-PAGE and transferred onto immobilon-FL membranes (Millipore). Signals were detected using the LI-COR Odyssey infrared imaging system (LI-COR Biosciences). Immunoblots shown are representative images of three independent experiments.

Antibodies Used

The antibodies used were as follows: Millipore, active β-catenin (05-665, 1:1000), H3K27me3 (07-449, 1:1000), EED (09-774, 1:2000), MYC (06-340, 1:2000), phosphoserine (05-1000,

1:1000); Cell Signaling, GAPDH (5174, 1:1000), histone H3 (4499, 1:1000), EZH2 (5246, 1:1000), SUZ12 (3737, 1:1000), IκBα (4814, 1:1000); Santa Cruz, p16 (sc-756, 1:500), p21 (sc-397, 1:200), lamin A/C (sc-6215, 1:200); BD Bioscience, RAS (610001, 1:1000).

Chromatin Immunoprecipitation

After crosslinking, formaldehyde was quenched by adding glycine to 125 mM, and the dishes were washed three times in ice-cold phosphate-buffered saline (PBS) containing 1x protease inhibitor (Roche). Composition of the SDS lysis buffer was 1% SDS, 10 mM EDTA, 50 mM Tris pH 8.1, 1x protease inhibitor cocktail. Composition of the ChIP dilution buffer was 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl. After sonication the samples were centrifuged and the supernatants were diluted five-fold with ChIP dilution buffer. An aliquot (1%) of each chromatin preparation was saved as input. Samples were immunoprecipitated with 4 μg of antibody to MYC (Millipore 06-340) or H3K27me3 (Millipore 07-449). After pulldown the magnetic protein A beads were washed once with each of low salt, high salt and LiCl wash buffers, followed by two washes with TE buffer. Final yields of DNA were quantified with the Qubit 2.0 dsDNA HS assay kit (Invitrogen, Q32851). For the Chromatrap Premium ChIP qPCR protocol, the IP slurry containing 1 μg of the sheared chromatin and 2 μg of antibody to H3K27me3 (as above) or H3K27ac (Cell Signaling, 8173S) were diluted in wash buffer 1, transferred into the Chromatrap spin column, and incubated for 6 hrs. at 4° on a rocking platform. The Chromatrap spin columns were washed with wash buffers and the DNA was eluted with 50 μl of elution buffer.

Immunoprecipitations

Cells were lysed in cell lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF) for 30 min. on ice and then passed through a 26.5-gauge needle three times. Cell lysates were incubated with anti-EZH2 antibody (Cell Signaling, 5246, 1:300) or control IgG overnight at 4°C. Pre-washed magnetic beads (Thermo Fisher Scientific, 11203D) were added to each sample and incubated for 6 hrs. at 4 °C. The beads were washed five times with cell lysis buffer, boiled at 100°C for 5 min. in Laemmli sample buffer and processed for immunoblotting.

RNA-seq

Cells were infected with a lentivirus vector expressing shRNA #3 against *EZH2* (Figure 1E-H) and cells were harvested at 4 and 8 days after infection. Total RNA was harvested from cells using Trizol reagent (Invitrogen) and further purified using the Purelink RNA Mini kit (Invitrogen) with DNase I digestion. RNA library preparation with polyA selection and Illumina HiSeq 2x150bp sequencing was performed by GeneWiz Inc. Paired-end reads were quality trimmed using Trim galore v0.4.0 and subsequently aligned to the human reference genome, hg19, using HISAT2 v2.1.0. Reads mapping to annotated genes were quantified using featureCounts (Liao et al., 2014). Differential gene expression was determined using DESeq2 v1.12.4 (Love et al., 2014) and significance was defined as FDR-corrected p-values of <0.05. The log2 fold change for each gene was used to rank the list of genes for GSEAPreranked analysis (Subramanian et al., 2005). FPKM values were calculated using DESeq2 and Z-scores were generated from FPKMs. Upstream regulators were identified using Ingenuity Pathway Analysis (IPA, Qiagen) with default settings. The cutoffs used were p <0.05 and absolute

fold-change >1.75. The differential expression and alignment rates are shown in Table S4, and the DESeq2 output for the entire dataset in Table S5.

Immunofluorescence and ImmunoFISH

Nonspecific binding solution contained 4% bovine serum albumin (BSA; Thermo Fisher Scientific), 2% donkey serum, and 0.1% Triton X-100 in PBS. The antibodies used were as follows: Santa Cruz, p16 (sc-756, 1:100), p21 (sc-397; 1:100); Millipore, γH2AX (05-636, 1:100); Becton Dickinson, 53BP1 (NB100-304, 1:100); Cell Signaling, phospho-ATM (Ser1981, 4526, 1:100), EZH2 (5246, 1:100). The rabbit polyclonal antibody against human mH2A was a kind gift from Dr. Peter Adams (University of Glasgow) and was used as indicated before (Kreiling et al., 2011). For the TIF assay (Herbig et al., 2004), cells were first fixed, permeabilized and immuno-stained for 53BP1 as described above. The samples were then dehydrated in a 70%, 90%, and 100% ethanol (3 min. each) and air dried. Nuclear DNA was denatured for 5 min. at 80°C in hybridization buffer containing 0.5 µg/ml Cy5-conjugated peptide nucleic acid (PNA) telomere C probe (PNA bio, #F1003), 70% formamide, 12 mM Tris HCl (pH 8), 5 mM KCl, 1 mM $MgCl_2$, 0.001% Triton X-100, and 2.5 mg/ml acetylated BSA. After denaturation, incubation was continued for 14 hrs. at room temperature in a humidified chamber. Cells were washed two times for 15 min. with 70% formamide in 2x SSC (0.3 M NaCl, 30 mM Na-citrate), followed by a 10 min. wash with 2x SSC, and a 10 min. wash with PBS. For EdU incorporation, cells were treated for 24 hours with EdU and processed as instructed by the manufacturer (Life Technologies, #C10337).

Cytokine Array

Cultures were washed three times with PBS and incubated in serum-free medium for 24 hrs. The conditioned media (CM) were collected and centrifuged at 1000 x g for 10 min. at 4°C. The CM were diluted in proportion to the cell number and analyzed using the Quantibody Human Cytokine Array 1 (QAH-CYT-1, RayBiotech) as per manufacturer's instructions. The signals were detected with a GenePix 4200B microarray scanner.

Detection of SA-β-Gal Activity

Cells grown on coverslips were fixed with 0.2% glutaraldehyde and 2% formaldehyde for 5 min., washed twice with PBS, and incubated overnight at 37°C in a staining solution (Debacq-Chainiaux et al., 2009) containing 5-bromo-4-chloro-3-indolyl-β-galactopyranoside.

Microscopy

Imaging was done on either a Zeiss Axiovert 200M fluorescence microscope equipped with a Roper CoolSnap HQ monochrome camera controlled by MetaMorph software 6.1 (Molecular Devices), or a Zeiss LSM 710 Confocal Laser Scanning Microscope. Image analysis was performed using ImageJ open source software from the NIH (http://rsbweb.nih.gov/ij/).

Site-directed mutagenesis

EZH2 mutant (S652A and S734A), EZH2 deletion (EZH2 Δ SET) and EED mutant (F97A, W364A, and Y365A) were generated by using the QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies). All constructs were confirmed by sequencing.

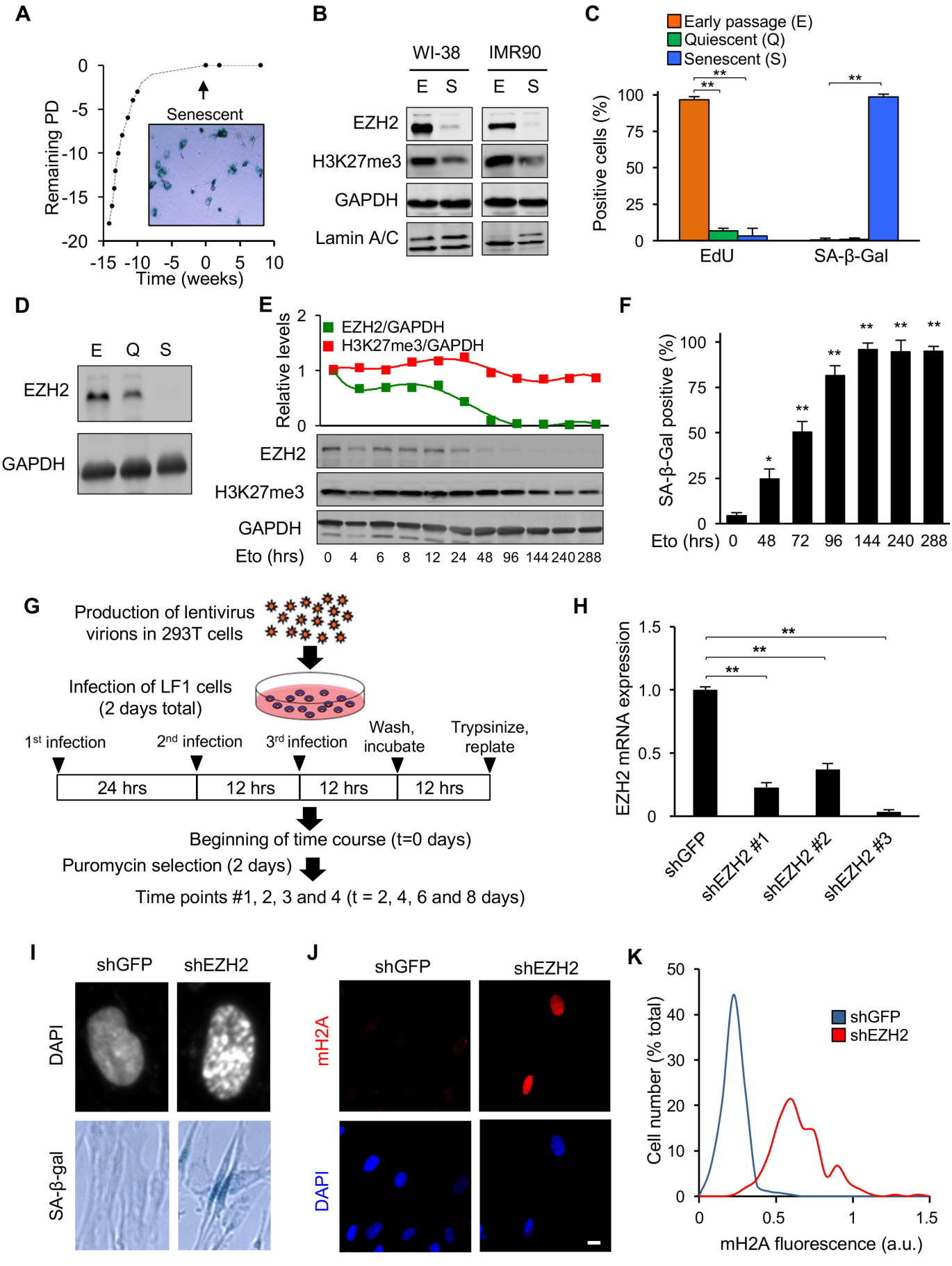
A
Remaining PD
Senescent
Time (weeks)
B
WI-38
IMR90
E S E S
EZH2
H3K27me3
GAPDH
Lamin A/C
C
Early passage (E)
Quiescent (Q)
Senescent (S)
Positive cells (%)
EdU
SA-β-Gal
D
E Q S
EZH2
GAPDH
E
EZH2/GAPDH
H3K27me3/GAPDH
Relative levels
EZH2
H3K27me3
GAPDH
Eto (hrs) 0 4 6 8 12 24 48 96 144 240 288
F
SA-β-Gal positive (%)
Eto (hrs) 0 48 72 96 144 240 288
G
Production of lentivirus virions in 293T cells
Infection of LF1 cells (2 days total)
1st infection
2nd infection
3rd infection
Wash, incubate
Trypsinize, replate
24 hrs
12 hrs
12 hrs
12 hrs
Beginning of time course (t=0 days)
Puromycin selection (2 days)
Time points #1, 2, 3 and 4 (t = 2, 4, 6 and 8 days)
H
EZH2 mRNA expression
shGFP
shEZH2 #1
shEZH2 #2
shEZH2 #3
I
shGFP
shEZH2
DAPI
SA-β-gal
J
shGFP
shEZH2
mH2A
DAPI
K
Cell number (% total)
shGFP
shEZH2
mH2A fluorescence (a.u.)

Figure S1, Related to Figure 1

Expression and Knockdown of EZH2 and Phenotypes of Senescent Cells

(A) Cells were serially propagated under standard culture conditions. Onset of senescence was designated as the zero time point, at which >95% of the cells in the culture were SA-β-Gal positive. The points in the graph correspond to the lanes shown in Figure 1D.

(B) Levels of EZH2 protein and the H3K27me3 mark were examined by immunoblotting in HDF strains WI-38 and IMR90 at early passage (E) and 5 weeks after the onset of senescence (S).

(C) LF1 early passage, replicatively senescent, and serum-starved quiescent cells were assayed for SA-β-Gal activity and EdU incorportation (**$p<0.01$, n=3).

(D) EZH2 protein levels were examined by immunoblotting in samples from panel (C).

(E) Cells were treated with etoposide (Eto, 40 μM) at the zero time point and levels of EZH2 protein and H3K27me3 marks were examined by immunoblotting. EZH2 and H3K27me3 levels were normalized to GAPDH (green and red plots, respectively).

(F) SA-β-Gal positive cells were scored in the etoposide treatment experiment shown in panel (E) (*$p<0.05$, **$p<0.01$, n=3).

(G) Schematic overview and timeline of lentivirus shRNA infection experiments. Cells were infected with lentiviral particles for a total of 2 days. After a wash and 12 hrs further incubation the cultures were replated, and this time point was designated as t=0 for all the time courses presented in this study. Cells were treated with puromycin for 2 days, at which time all sensitive cells had been effectively removed. Cells were harvested at successive time points as indicated in individual experiments.

(H) LF1 cells were infected with lentivirus vectors expressing three different shRNAs against the EZH2 transcript. The effectiveness of knockdowns was examined by RT-qPCR for the EZH2 mRNA 2 days after infection (**$p<0.01$, n=3). shRNA against GFP was used as the control. The shRNAs used are listed in the Extended Experimental Procedures.

(I) EZH2 was knocked down as described in Figure 1E. Representative images of cells displaying SAHF and SA-β-Gal activity are shown. SAHF formation was visualized by DAPI staining. Images were acquired 2 days after infection with EZH2 shRNA.

(J) EZH2 was knocked down as in panel I and mH2A expression was examined using IF microscopy.

(K) mH2A nuclear signals were quantified in the IF images using ImageJ software and plotted as histograms of cell number (% of total) against fluorescent intensity in arbitrary units (a.u.). At least 260 nuclei were observed for each condition.

Error bars represent SD.

A

shEZH2: 4 days after infection

GO Process (Upregulated)	NES	FDR q-val
SYNAPSE_ASSEMBLY	2.223	<0.001
ORGANIC_HYDROXY_COMPOUND_TRANSPORT	2.188	<0.001
TEMPERATURE_HOMEOSTASIS	2.145	<0.001
SYNAPSE_ORGANIZATION	2.141	<0.001
DOPAMINE_METABOLIC_PROCESS	2.107	<0.001

GO Process (Downregulated)	NES	FDR q-val
DNA_STRAND_ELONGATION_INVOLVED_IN_DNA_REPLICATION	-2.564	<0.001
DNA_REPLICATION_INITIATION	-2.541	<0.001
DNA_DEPENDENT_DNA_REPLICATION	-2.517	<0.001
DNA_STRAND_ELONGATION	-2.467	<0.001
DANG_REGULATED_BY_MYC_UP	-2.463	<0.001

B

shEZH2: 8 days after infection

GO Process (Upregulated)	NES	FDR q-val
SASP	2.551	<0.001
CHEMOKINE_MEDIATED_SIGNALING_PATHWAY	2.297	<0.001
LEUKOCYTE_CHEMOTAXIS	2.271	<0.001
CELL_CHEMOTAXIS	2.269	<0.001
MYELOID_LEUKOCYTE_MIGRATION	2.183	<0.001

GO Process (Downregulated)	NES	FDR q-val
SISTER_CHROMATID_SEGREGATION	-3.066	<0.001
CHROMOSOME_SEGREGATION	-3.033	<0.001
NUCLEAR_CHROMOSOME_SEGREGATION	-2.992	<0.001
SISTER_CHROMATID_COHESION	-2.904	<0.001
MITOTIC_SISTER_CHROMATID_SEGREGATION	-2.833	<0.001

C

Top upstream regulators assessed by IPA

Upstream Regulator	Predicted Activation State	Activation z-score	p-value of overlap
ERBB2	Inhibited	-4.95	4.73E-39
TP53	Activated	4.242	9.06E-38
NUPR1	Activated	5.803	7.48E-24
CCND1	Inhibited	-3.408	1.43E-23
MYC	Inhibited	-5.465	3.94E-23
CDKN1A (p21)	Activated	2.544	5.42E-22
TBX2	Inhibited	-6.265	5.90E-22
FOXO3	Activated	3.333	7.78E-22

Genes fold change 1.75 and p<0.05

Figure S2, Related to Figure 1

RNA-seq Analysis of Global Gene Expression Changes Elicited by Knockdown of EZH2

(A-B) EZH2 was knocked down as described in Figure 1E and RNA-seq was performed on RNA harvested on day 4 and 8 after infection. The data were analyzed using GSEA and the top 5 upregulated and downregulated GO process pathways, ranked by normalized enrichment score (NES), are shown for 4 days (A) and 8 days (B) after infection.

(C) Upstream Regulator analysis was performed using IPA and the 8 most activated or inhibited regulators are shown at 8 days after EZH2 knockdown.

See also Tables S4, S5.

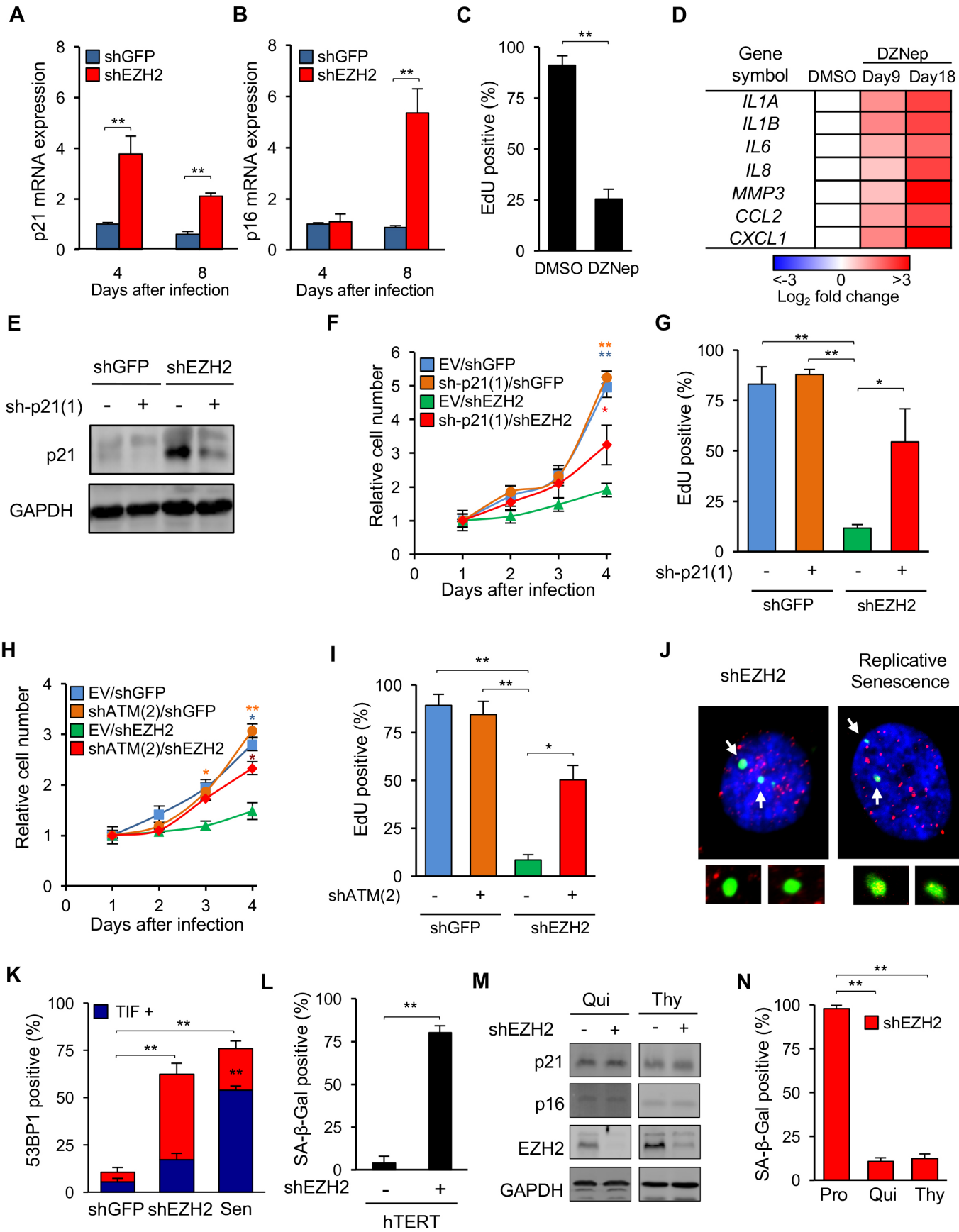
A
p21 mRNA expression
shGFP
shEZH2
Days after infection
B
p16 mRNA expression
C
EdU positive (%)
DMSO DZNep
D
Gene symbol
DZNep
DMSO Day9 Day18
IL1A
IL1B
IL6
IL8
MMP3
CCL2
CXCL1
Log2 fold change
E
shGFP shEZH2
sh-p21(1)
p21
GAPDH
F
Relative cell number
EV/shGFP
sh-p21(1)/shGFP
EV/shEZH2
sh-p21(1)/shEZH2
Days after infection
G
EdU positive (%)
sh-p21(1)
shGFP shEZH2
H
Relative cell number
EV/shGFP
shATM(2)/shGFP
EV/shEZH2
shATM(2)/shEZH2
Days after infection
I
EdU positive (%)
shATM(2)
shGFP shEZH2
J
shEZH2
Replicative Senescence
K
53BP1 positive (%)
TIF +
shGFP shEZH2 Sen
L
SA-β-Gal positive (%)
shEZH2
hTERT
M
Qui Thy
shEZH2
p21
p16
EZH2
GAPDH
N
SA-β-Gal positive (%)
shEZH2
Pro Qui Thy

Figure S3, Related to Figure 2

Cellular Phenotypes Elicited by Knockdown of EZH2

(A) EZH2 was knocked down as in Figure 1E and expression of p21 mRNA was measured by RT-qPCR at 4 and 8 days after infection (**p<0.01, n=3).

(B) Expression of p16 was determined in the experiment shown in (A).

(C) Cells grown continuously in the presence of DZNep (5 μM) for 9 days were assayed for EdU incorporation (**p<0.01, n=3).

(D) Expression of the indicated genes was determined by RT-qPCR in cells treated with DZNep for 9 and 18 days. The data are represented as heat maps relative to vehicle (DMSO) control (n=3).

(E) A double knockdown of EZH2 and p21 was performed as described in Figure 2F, and the levels of p21 protein were determined by immunoblotting. The control for EZH2 knockdown was shGFP, and for p21, the empty vector.

(F-G) A double knockdown of EZH2 and p21 was performed as above and proliferation was assessed by cell number (F) or EdU staining (G) (*p<0.05, **p<0.01, n=3).

(H-I) A double knockdown of EZH2 and ATM was performed and proliferation was assessed by cell numbers (H) or EdU staining (I) (*p<0.05, **p<0.01, n=3).

(J) Replicatively senescent cells or cells made senescent by knockdown of EZH2 were processed for the simultaneous visualization of 53BP1 DNA damage foci (by IF) and telomeres (by FISH). Colocalization of 53BP1 and telomere signals are scored as Telomere dysfunction Induced Foci (TIFs). DNA was counterstained with DAPI (blue). Arrows point to representative enlarged images of 53BP1 DNA damage foci.

(K) The experiment in (J) above was quantified by counting DNA damage foci and TIFs in >150 nuclei per condition. A cell with at least 1 clearly visible 53BP1 focus was scored as a 53BP1-positive cell, and a cell in which ≥50% of 53BP1 foci colocalized with a telomere was scored as a TIF-positive cell. (**p<0.01, n=3). The increase in TIFs in cells infected with shEZH2 was not significant compared to the control (cells infected with shGFP).

(L) EZH2 was knocked down as in (A) in LF1 cells immortalized with human telomerase (hTERT) and SA-β-Gal-positive cells were scored 4 days after infection (**p<0.01, n=3).

(M-N) EZH2 was knocked down as in Figure 2I, and p21, p16 and EZH2 protein levels were determined by immunoblotting (M) or assayed for SA-β-Gal activity (N) 4 days after infection (**p<0.01, n=3).

Error bars represent SD.

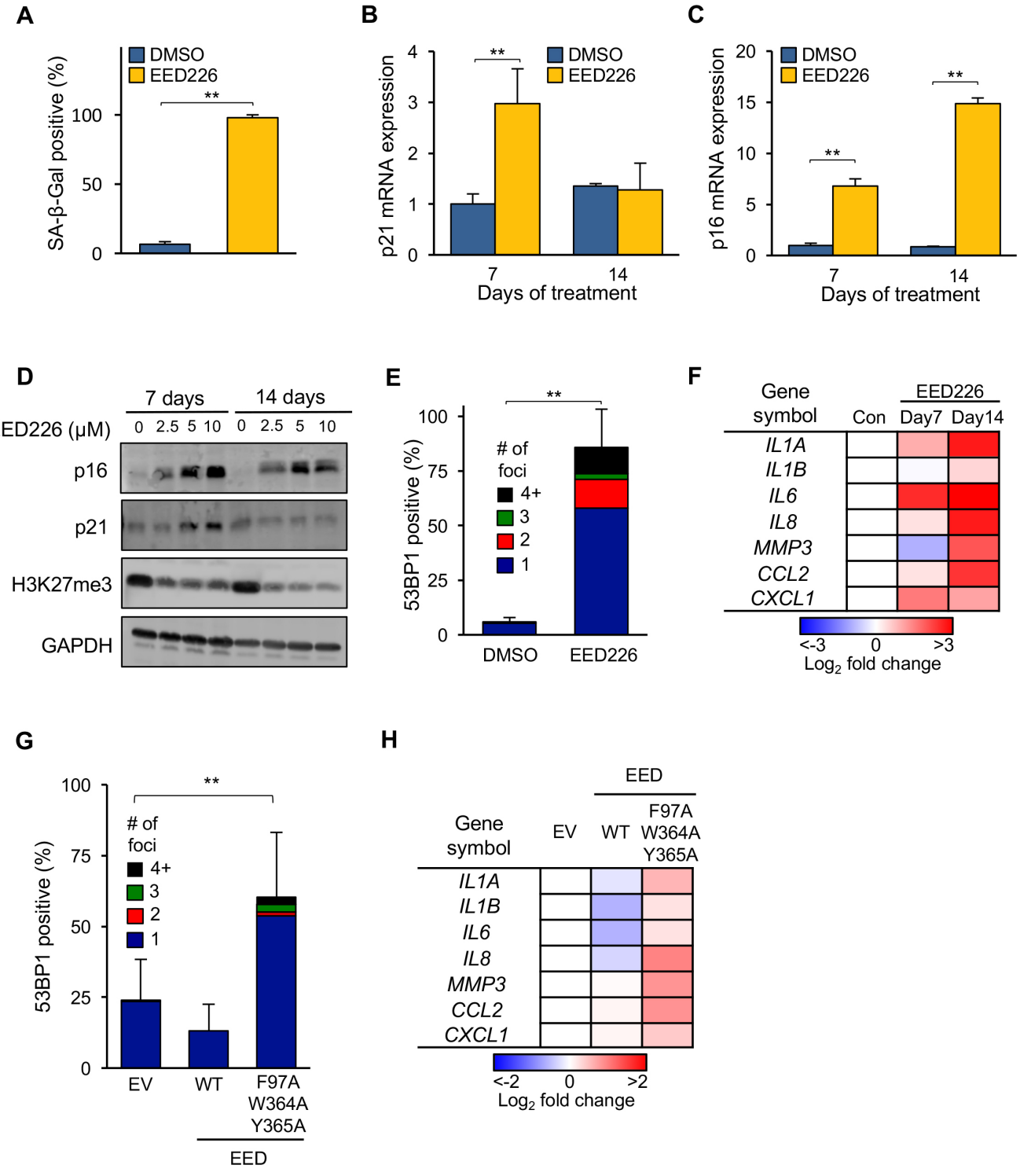
A
SA-β-Gal positive (%)
DMSO
EED226
**
B
p21 mRNA expression
DMSO
EED226
**
7
14
Days of treatment
C
p16 mRNA expression
DMSO
EED226
**
**
7
14
Days of treatment
D
7 days
14 days
EED226 (μM)
0 2.5 5 10 0 2.5 5 10
p16
p21
H3K27me3
GAPDH
E
53BP1 positive (%)
**
# of foci
4+
3
2
1
DMSO
EED226
F
Gene symbol
Con
EED226
Day7
Day14
IL1A
IL1B
IL6
IL8
MMP3
CCL2
CXCL1
<-3
0
>3
Log2 fold change
G
53BP1 positive (%)
**
# of foci
4+
3
2
1
EV
WT
F97A W364A Y365A
EED
H
EED
Gene symbol
EV
WT
F97A W364A Y365A
IL1A
IL1B
IL6
IL8
MMP3
CCL2
CXCL1
<-2
0
>2
Log2 fold change

Figure S4, Related to Figure 2

Inhibition of H3K27me3 Mark Recognition by EED Phenocopies EZH2 Knockdown

(A) Cells were continuously treated with the drug EED226 (10 μM) or vehicle (DMSO) for 7 days and assayed for SA-β-Gal activity (**p<0.01, n=3).

(B-C) Cells were treated with EED226 (10 μM) or DMSO and expression of p21 (B) and p16 (C) mRNA was measured by RT-qPCR at 7 and 14 days (**p<0.01, n=3).

(D) Cells were treated with varying concentrations of EED226 and p16, p21 and H3K27me3 levels were determined by immunoblotting.

(E) Cells were treated as in (A) and 53BP1 foci were visualized by IF after 7 days of drug treatment (**p<0.01, n=3).

(F) Expression of the indicated genes was determined by RT-qPCR in cells treated with EED226 (10 μM) or DMSO control at 7 and 14 days. The data are represented as heat maps relative to the DMSO control (n=3).

(G) Cells were infected with lentivirus vectors to ectopically express WT *EED* cDNA or *EED*(F97A/W364A/Y365A) mutated cDNA and p16, p21 and H3K27me3 levels were scored for 53BP1 foci as in (E) (**p<0.01, n=3).

(H) Cells ectopically expressing WT *EED* or *EED*(F97A/W364A/Y365A) cDNAs (experiment in (G) above) were assessed for the expression of the indicated genes as in (F).

Error bars represent SD.

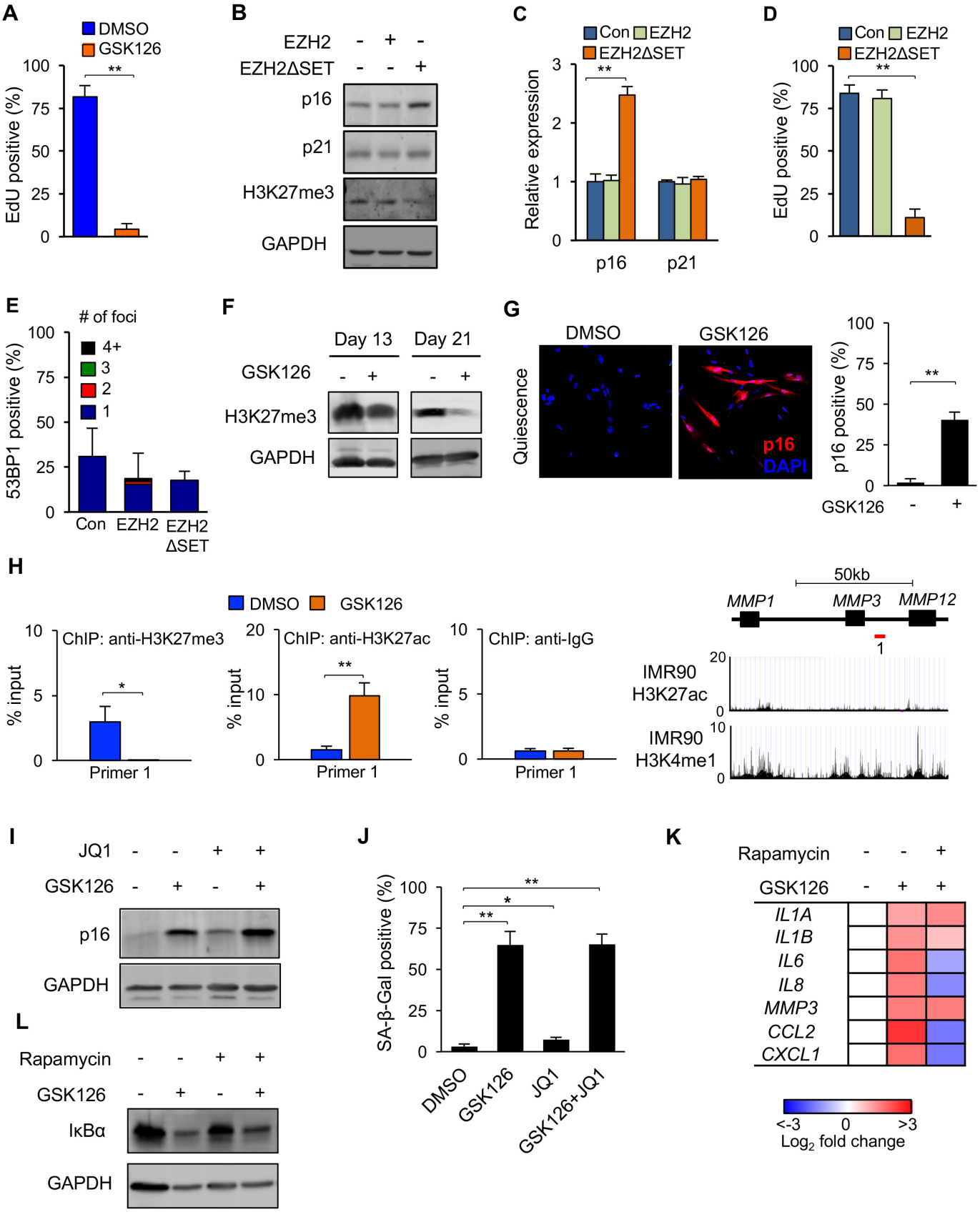
A
DMSO
GSK126
EdU positive (%)
100
75
50
25
0
**
B
EZH2 - + -
EZH2ΔSET - - +
p16
p21
H3K27me3
GAPDH
C
Con EZH2
EZH2ΔSET
Relative expression
3
2
1
0
**
p16
p21
D
Con EZH2
EZH2ΔSET
EdU positive (%)
100
75
50
25
0
**
E
# of foci
4+
3
2
1
53BP1 positive (%)
100
75
50
25
0
Con
EZH2
EZH2 ΔSET
F
Day 13
Day 21
GSK126 - + - +
H3K27me3
GAPDH
G
DMSO
GSK126
Quiescence
p16
DAPI
p16 positive (%)
100
75
50
25
0
**
GSK126 - +
H
DMSO
GSK126
ChIP: anti-H3K27me3
% input
10
5
0
*
Primer 1
ChIP: anti-H3K27ac
% input
20
10
0
**
Primer 1
ChIP: anti-IgG
% input
10
5
0
Primer 1
50kb
MMP1
MMP3
MMP12
1
IMR90
H3K27ac
20
0
IMR90
H3K4me1
10
0
I
JQ1 - - + +
GSK126 - + - +
p16
GAPDH
J
SA-β-Gal positive (%)
100
75
50
25
0
**
*
**
DMSO
GSK126
JQ1
GSK126+JQ1
K
Rapamycin - - +
GSK126 - + +
IL1A
IL1B
IL6
IL8
MMP3
CCL2
CXCL1
<-3
0
>3
Log2 fold change
L
Rapamycin - - + +
GSK126 - + - +
IκBα
GAPDH

Figure S5, Related to Figure 3

Inhibition of EZH2 Activity Induces Senescence by Depleting H3K27me3 Marks and Activating p16 and SASP Genes

(A) Cells were continuously treated with the drug GSK126 (5 μM) or vehicle (DMSO) for 20 days and then assayed for EdU incorporation (** $p<0.01$, n=3).

(B) Cells were infected with lentivirus vectors expressing *EZH2* or *EZH2ΔSET* cDNAs, or empty vector as control (as in Figure 3G), and p16, p21 and H3K27me3 levels were determined by immunoblotting 9 days after infection.

(C-D) In the experiment shown in (B) above, expression of p16 and p21 mRNAs was determined by RT-qPCR (C) and proliferation was assessed by EdU incorporation (D) (** $p<0.01$, n=3).

(E) In the experiment shown in (B) above, 53BP1 foci were visualized by IF (n=3).

(F) Cells were made quiescent by incubation in medium supplemented with 0.25% FBS and then treated with GSK126 (5 μM) for the indicated times. Levels of the H3K27me3 mark were determined by immunoblotting.

(G) Cells were treated with GSK as in (F) above for 21 days and immunostained with antibodies to p16. Nuclei were counterstained with DAPI. The frequency of p16-expressing cells is shown in the right panel (% of total cells, random fields, >200 cells per condition, **$p<0.01$).

(H) Cells were treated with GSK126 (5 μM) or DMSO for 10 days and H3K27me3 and H3K27ac enrichment at the enhancer between the *MMP3* and *MMP12* loci was determined by ChIP-qPCR. Normal rabbit IgG was used as the IP control (** $p<0.01$, n=3). Location of the primer pair is indicated in the schematic on right. The IMR90 H3K27ac and H3K4me1 tracks were obtained from ENCODE (GEO ID: GSM469966 and GSM521895).

(I) Cells were treated with GSK126 (5 μM), JQ1 (100 nM) or both, and levels of the p16 protein were determined by immunoblotting after 10 days.

(J) Cells were treated as in (I) and the frequency of SA-β-Gal positive cells was determined (* $p<0.05$, ** $p<0.01$, n=3).

(K) Cells were treated with GSK126 for 10 days, followed by 4 days of combined treatment with GSK126 and rapamycin (12.5 nM). Expression of the indicated SASP genes was determined by RT-qPCR (n=3). The data are expressed as heat maps relative to control (vehicle-treated) cells.

(L) Cells were treated as in (K) and IκBα protein levels were determined by immunoblotting. Error bars represent SD.

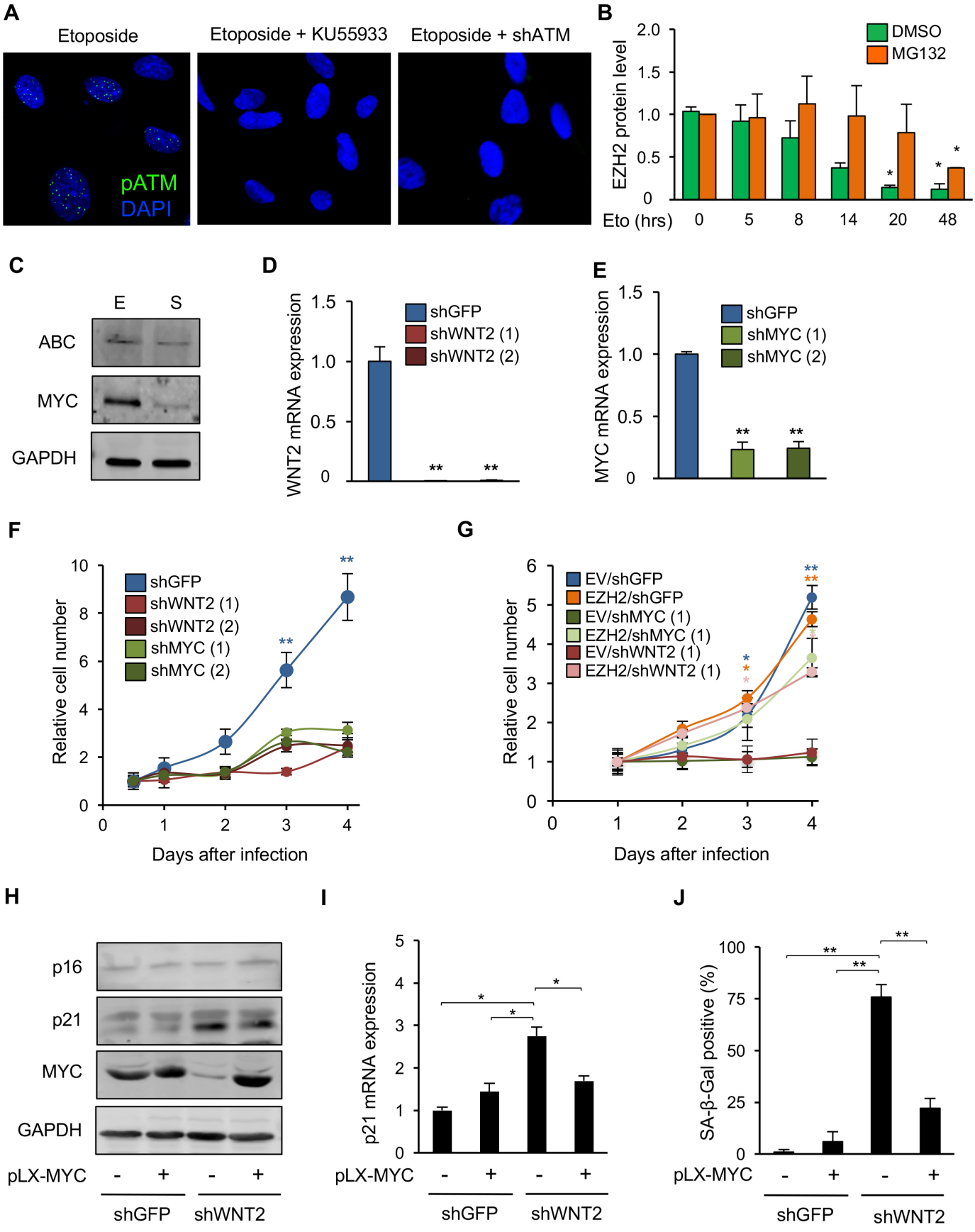
A
Etoposide
Etoposide + KU55933
Etoposide + shATM
pATM
DAPI
B
DMSO
MG132
EZH2 protein level
Eto (hrs)
0
5
8
14
20
48
C
E
S
ABC
MYC
GAPDH
D
shGFP
shWNT2 (1)
shWNT2 (2)
WNT2 mRNA expression
E
shGFP
shMYC (1)
shMYC (2)
MYC mRNA expression
F
shGFP
shWNT2 (1)
shWNT2 (2)
shMYC (1)
shMYC (2)
Relative cell number
Days after infection
G
EV/shGFP
EZH2/shGFP
EV/shMYC (1)
EZH2/shMYC (1)
EV/shWNT2 (1)
EZH2/shWNT2 (1)
Relative cell number
Days after infection
H
p16
p21
MYC
GAPDH
pLX-MYC
shGFP
shWNT2
I
p21 mRNA expression
pLX-MYC
shGFP
shWNT2
J
SA-β-Gal positive (%)
pLX-MYC
shGFP
shWNT2

Figure S6, Related to Figure 5 and Figure 6

Regulation of EZH2 Expression by ATM and WNT-MYC Signaling

(A) Cells were treated with etoposide (40 μM, left panel), etoposide plus the ATM inhibitor KU-55933 (10 μM, middle panel), or with etoposide and infected with shRNA against ATM (right panel), and immunostained with antibodies to phospho-ATM (S1981). Nuclei were counterstained with DAPI.

(B) Cells were treated with etoposide (40 μM) with or without the proteasome inhibitor MG132 (10 μM) and levels of EZH2 protein were determined by immunoblotting. GAPDH was used as the loading control (* $p<0.05$, calculated relative to t=0 time point, n=2).

(C) Levels of active β-catenin (ABC) and MYC protein were examined by immunoblotting in early passage cells and 2 weeks after the onset of replicative senescence.

(D-E) Cells were infected with lentivirus vectors expressing two different shRNAs against the WNT2 or MYC transcripts. The effectiveness of knockdowns was examined by RT-qPCR for the WNT2 (D) or MYC (E) mRNAs 2 days after infection (** $p<0.01$, n=3). shRNA against GFP was used as the control. The shRNAs used are listed in the Extended Experimental Procedures.

(F) WNT2 or MYC were knocked down as in (D) and (E) above and proliferation was assessed by counting cell numbers (** $p<0.01$, n=3).

(G) Ectopic expression of *EZH2* cDNA was combined with a shRNA knockdown of WNT2 or MYC and proliferation was assessed by counting cell numbers (** $p<0.01$, n=3).

(H) Ectopic expression of *MYC* cDNA was combined with a shRNA knockdown of WNT2. Controls were empty pLX lentivirus vector for *MYC* (–) and shGFP for WNT2. Levels of p16, p21, and MYC proteins were determined by immunoblotting. Note that while endogenous MYC expression is effectively downregulated by shWNT2 (lane 3), ectopic expression is maintained by the pLX-MYC vector (lane 4).

(I) p21 mRNA expression was determined by RT-qPCR in the experiment shown in (H) (* $p<0.05$, n=3).

(J) The presence of SA-β-Gal positive cells was scored in the experiment shown in (H) (** $p<0.01$, n=3).

Error bars represent SD.

Table S1, Related to Experimental Procedures

List of primer sequences for RT-qPCR

Gene	Orientation	Sequences (5' to 3')
EZH2	Sense	GCAACACCCAACACTTATAAGC
	Antisense	CTCCCTCCAAATGCTGGTA
WNT2	Sense	TGGCAGGAAGGCTGTAAAGC
	Antisense	ATGGCCAGCCAGCATGTC
MYC	Sense	GGATTCTCTGCTCTCCTCGAC
	Antisense	TTGTTCCTCCTCAGAGTCGC
IL1A	Sense	GAGAGCATGGTGGTAGTAGCAA
	Antisense	AGGCTTGATGATTTCTTCCTCTGA
IL1B	Sense	CGCCAGTGAAATGATGGCTTAT
	Antisense	CTGGAAGGAGCACTTCATCTGT
MMP3	Sense	CCCACCTTACATACAGGATTGTGA
	Antisense	CCCAGACTTTCAGAGCTTTCTCA
IL6	Sense	CACTGGCAGAAAACAACCTGAA
	Antisense	ACCAGGCAAGTCTCCTCATTGA
CXCL8 (IL8)	Sense	GTTTTTGAAGAGGGCTGAGAATTC
	Antisense	CCCTACAACAGACCCACACAATAC
CCL2	Sense	AAGACCATTGTGGCCAAGGA
	Antisense	TTCGGAGTTTGGGTTTGCT
CXCL1	Sense	CAATCCTGCATCCCCCATAG
	Antisense	CAGCCACCAGTGAGCTTCCT
CDKN2A (p16-INK4A)	Sense	CGGAAGGTCCCTCAGACATC
	Antisense	CCCTGTAGGACCTTCGGTGA
CDKN1A (p21-CIP1)	Sense	GAGACTCTCAGGGTCGAAAACG
	Antisense	TTCCTGTGGGCGGATTAGG
GAPDH	Sense	GGAGTCAACGGATTTGGTCGT
	Antisense	GTTGAGGTCAATGAAGGGGTCA

Table S2, Related to Experimental Procedures

List of primer sequences for ChIP-qPCR

Gene/location	Orientation	Sequences (5' to 3')	Notes
ARF Exon 1β	Sense	GTGGGTCCCAGTCTGCAGTTA	Fig. 2A, #1
	Antisense	CCTTTGGCACCAGAGGTGAG	
15 kb downstream of *ARF* promoter	Sense	GCACTTGCCCTTCCAGGTATA	Fig. 2A, #2
	Antisense	TGATAGTTCAAGGCCCTATGCC	
200 bp upstream *p16-INK4A* TSS	Sense	ACCCCGATTCAATTTGGCAG	Fig. 2A, #3
	Antisense	AAAAAGAAATCCGCCCCCG	
p16-INK4A, exon 1α	Sense	AGAGGGTCTGCAGCGG	Fig. 2A, #4
	Antisense	TCGAAGCGCTACCTGATTCC	
200 bp downstream of *p16-INK4A* exon 1α	Sense	GCCAAGGAAGAGGAATGAGGAG	Fig. 2A, #5
	Antisense	CCTTCAGATCTTCTCAGCATTCG	
678 bp upstream of *IL1A*	Sense	GCTGACTCAAACGCCAATGAAA	Fig. 3J, #1
	Antisense	CGTTTTGACGACGCACTTGTAG	
11490 bp upstream of *IL1A*	Sense	CAGGACCCATGCTAGACACATT	Fig. 3J, #2
	Antisense	CGAAGTAAAGCAGAAAGAGCGT	
20187 bp upstream of *IL1A*	Sense	GTGCCTGTTGACAGTAAGGCAT	Fig. 3J, #3
	Antisense	TGGCATCCAAAACAGGTGTCAA	
28917 bp upstream of *IL1A*	Sense	TACTAGGGCCCAGGAGAGTTAC	Fig. 3J, #4
	Antisense	CCTCACGCCATAGCTATTCACA	
5567 bp downstream of *IL1B*	Sense	CCACATGCTGCATTTCATGGTT	Fig. 3J, #5
	Antisense	GGAAGTCCAGAAGCACTCCTTT	
3756 bp upstream of *IL1B*	Sense	CCAGCTAAGAGGAGCCCTAATG	Fig. 3J, #6
	Antisense	ATGAGTCACTTCCACCCTCCTA	
32953 bp upstream of *IL1B*	Sense	AGTGAGCAAGAGTTCTGGATTGT	Fig. 3J, #7
	Antisense	ACCAGTCACTGCTTTGTTTCTG	
45347 bp upstream of *IL1B*	Sense	AATCATAAGTCAGGAGGGCCAC	Fig. 3J, #8
	Antisense	TGTCTGGCCATTTAAGTCACGA	
6898 bp upstream of *MMP3*	Sense	TATCTGGTGGCAGTGATTGAGA	Fig. S5H, #1
	Antisense	TATCTGCAGTCCTTCGGGTTG	
EZH2, 1034 bp upstream of TSS	Sense	ACATTGCTGCCATTTCAGAC	Fig. 6C, #1
	Antisense	AGTGAACAGTGCTCATCTTGA	
EZH2, 156 bp upstream of TSS	Sense	TTCGCTGTAAGGGACGC	Fig. 6C, #2
	Antisense	TGTGTTCAGCGAAAGAACAA	
EZH2 TSS	Sense	CCAATCGCCATCGCTTTTAT	Fig. 6C, #3
	Antisense	GGGCCCTGTGATTGGAC	

Table S3, Related to Figure 3

Assay of Conditioned Media for Cytokine Production

CONDITION[1]	GENE SYMBOL	CONCENTRATION (pg/ml)
CTR #1	IL1A	117.875
CTR #1	IL1B	140.9496
CTR #1	IL2	15.0576
CTR #1	IL4	39.3584
CTR #1	IL5	24.906
CTR #1	IL6	22.4068
CTR #1	IL8	3.4572
CTR #1	IL10	96.278
CTR #1	IL12	8.7362
CTR #1	IL13	6.1605
CTR #1	CSF2	27.6012
CTR #1	CXCL1	77.3528
CTR #1	IFNG	153.7699
CTR #1	CCL2	90.0836
CTR #1	CCL3	314.4603
CTR #1	CCL4	12.4705
CTR #1	MMP9	131.8152
CTR #1	CCL5	27.0336
CTR #1	TNF	16.115
CTR #1	VEGF	918.7487
CTR #2	IL1A	115
CTR #2	IL1B	136.6784
CTR #2	IL2	14.1165
CTR #2	IL4	39.3584
CTR #2	IL5	24.906
CTR #2	IL6	20.6832
CTR #2	IL8	3.0444
CTR #2	IL10	89.999
CTR #2	IL12	8.0465
CTR #2	IL13	5.8867
CTR #2	CSF2	27.6012
CTR #2	CXCL1	66.3024
CTR #2	IFNG	147.2265
CTR #2	CCL2	74.1396
CTR #2	CCL3	304.9312
CTR #2	CCL4	12.1142
CTR #2	MMP9	127.8208
CTR #2	CCL5	27.0336
CTR #2	TNF	16.4373
CTR #2	VEGF	680.2715
GSK126+JQ1 #1	IL1A	117.875
GSK126+JQ1 #1	IL1B	136.6784
GSK126+JQ1 #1	IL2	15.9987
GSK126+JQ1 #1	IL4	40.516
GSK126+JQ1 #1	IL5	25.499
GSK126+JQ1 #1	IL6	96.5216
GSK126+JQ1 #1	IL8	7.0692
GSK126+JQ1 #1	IL10	96.278
GSK126+JQ1 #1	IL12	8.9661
GSK126+JQ1 #1	IL13	6.4343
GSK126+JQ1 #1	CSF2	30.1104
GSK126+JQ1 #1	CXCL1	103.5975
GSK126+JQ1 #1	IFNG	153.7699
GSK126+JQ1 #1	CCL2	217.6356
GSK126+JQ1 #1	CCL3	323.9894

GSK126+JQ1 #1	CCL4	12.8268
GSK126+JQ1 #1	MMP9	135.8096
GSK126+JQ1 #1	CCL5	27.0336
GSK126+JQ1 #1	TNF	17.7265
GSK126+JQ1 #1	VEGF	524.3441
GSK126+JQ1 #2	IL1A	115
GSK126+JQ1 #2	IL1B	145.2208
GSK126+JQ1 #2	IL2	15.3713
GSK126+JQ1 #2	IL4	50.9344
GSK126+JQ1 #2	IL5	25.499
GSK126+JQ1 #2	IL6	109.8795
GSK126+JQ1 #2	IL8	5.16
GSK126+JQ1 #2	IL10	104.65
GSK126+JQ1 #2	IL12	8.7362
GSK126+JQ1 #2	IL13	7.2557
GSK126+JQ1 #2	CSF2	29.4831
GSK126+JQ1 #2	CXCL1	95.3097
GSK126+JQ1 #2	IFNG	150.4982
GSK126+JQ1 #2	CCL2	235.174
GSK126+JQ1 #2	CCL3	314.4603
GSK126+JQ1 #2	CCL4	12.8268
GSK126+JQ1 #2	MMP9	131.8152
GSK126+JQ1 #2	CCL5	27.8784
GSK126+JQ1 #2	TNF	17.4042
GSK126+JQ1 #2	VEGF	454.0239
GSK126 #1	IL1A	132.25
GSK126 #1	IL1B	140.9496
GSK126 #1	IL2	17.8809
GSK126 #1	IL4	42.8312
GSK126 #1	IL5	30.243
GSK126 #1	IL6	10455.3576
GSK126 #1	IL8	278.2788
GSK126 #1	IL10	119.301
GSK126 #1	IL12	9.6558
GSK126 #1	IL13	8.8985
GSK126 #1	CSF2	43.911
GSK126 #1	CXCL1	1164.4359
GSK126 #1	IFNG	173.4001
GSK126 #1	CCL2	2134.1044
GSK126 #1	CCL3	343.0476
GSK126 #1	CCL4	14.6083
GSK126 #1	MMP9	135.8096
GSK126 #1	CCL5	29.568
GSK126 #1	TNF	19.0157
GSK126 #1	VEGF	3321.8651
GSK126 #2	IL1A	115
GSK126 #2	IL1B	166.5768
GSK126 #2	IL2	16.9398
GSK126 #2	IL4	40.516
GSK126 #2	IL5	23.72
GSK126 #2	IL6	5046.2699
GSK126 #2	IL8	224.1504
GSK126 #2	IL10	100.464
GSK126 #2	IL12	8.7362
GSK126 #2	IL13	8.4878
GSK126 #2	CSF2	37.0107
GSK126 #2	CXCL1	868.8377
GSK126 #2	IFNG	170.1284
GSK126 #2	CCL2	1387.128
GSK126 #2	CCL3	323.9894
GSK126 #2	CCL4	13.1831

GSK126 #2	MMP9	135.8096
GSK126 #2	CCL5	28.7232
GSK126 #2	TNF	18.3711
GSK126 #2	VEGF	1872.6575
shEZH2 #1	IL1A	140.875
shEZH2 #1	IL1B	149.492
shEZH2 #1	IL2	20.3905
shEZH2 #1	IL4	45.1464
shEZH2 #1	IL5	31.429
shEZH2 #1	IL6	24071.7976
shEZH2 #1	IL8	758.8296
shEZH2 #1	IL10	198.835
shEZH2 #1	IL12	10.5754
shEZH2 #1	IL13	12.4579
shEZH2 #1	CSF2	148.6701
shEZH2 #1	CXCL1	16943.0258
shEZH2 #1	IFNG	199.5737
shEZH2 #1	CCL2	5057.4368
shEZH2 #1	CCL3	371.6349
shEZH2 #1	CCL4	17.1024
shEZH2 #1	MMP9	159.776
shEZH2 #1	CCL5	32.9472
shEZH2 #1	TNF	22.561
shEZH2 #1	VEGF	4073.9855
shEZH2 #2	IL1A	126.5
shEZH2 #2	IL1B	158.0344
shEZH2 #2	IL2	19.4494
shEZH2 #2	IL4	48.6192
shEZH2 #2	IL5	27.871
shEZH2 #2	IL6	12237.9909
shEZH2 #2	IL8	617.7552
shEZH2 #2	IL10	182.091
shEZH2 #2	IL12	10.5754
shEZH2 #2	IL13	10.8151
shEZH2 #2	CSF2	124.2054
shEZH2 #2	CXCL1	13926.2666
shEZH2 #2	IFNG	179.9435
shEZH2 #2	CCL2	3494.1276
shEZH2 #2	CCL3	343.0476
shEZH2 #2	CCL4	15.3209
shEZH2 #2	MMP9	143.7984
shEZH2 #2	CCL5	30.4128
shEZH2 #2	TNF	19.9826
shEZH2 #2	VEGF	2432.1617

[1] CTR, Control.

Table S4, Related to Figures 1 and S2.

Alignment Rates of RNA-seq Data

Sample	Number of reads	Reads after trimming	% of reads remaining	HISAT2 alignment %	% aligned concordantly exactly 1 time	# aligned concordantly exactly 1 time	FeatureCount gene count
shGFP_1	39,544,533	39,493,427	99.87	96.17	87.73	34,647,571	32,177,852
shGFP_2	33,331,456	33,293,441	99.89	96.1	88.73	29,541,575	27,440,073
shGFP_3	31,621,901	31,581,385	99.87	96.01	88.33	27,894,550	25,876,556
shEZH2_4hr_1	30,174,636	30,145,492	99.90	95.72	88.56	26,696,692	24,665,826
shEZH2_4hr_2	27,559,330	27,532,883	99.90	95.33	87.76	24,163,287	22,260,670
shEZH2_4hr_3	32,452,763	32,420,262	99.90	96.2	88.15	28,578,966	26,016,533
shEZH2_8hr_1	34,542,173	34,496,893	99.87	94.39	86.3	29,771,809	27,425,389
shEZH2_8hr_2	34,805,705	34,770,894	99.90	94.27	86.55	30,094,531	27,588,381
shEZH2_8hr_3	33,358,345	33,326,622	99.90	94.86	88.64	29,540,951	27,126,907

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