Supplementary Figure legends.

Supplementary Figure S1. Morphological effects of melphalan on HPV16 positive tonsillar cancer cells. (A) Pictures of C33A2 cells treated with DMSO or 100uM of melphalan for the indicated time periods. **(B)** Pictures of HN26 cells treated with DMSO or 100uM of melphalan for the indicated time periods.

Supplementary Figure S2. Morphological effects of melphalan on HPV16 negative tonsillar cancer cells. Pictures of HN7 cells treated with DMSO or 100uM of melphalan for 9hrs.

Supplementary Figure S3. Melphalan induces apoptosis in HPV16 positive tonsillar cancer cells. (A)(B) Western blots with monospecific antibodies to caspase 3 or caspase 8 on extracts from HN26 or C33A2 cells treated with DMSO or 100uM melphalan for the indicated time points. The locations in the gels of the full-length caspase proteins as well as the apoptosis-induced cleavage products are indicated. **(C)** Apoptotic DNA fragmentation was detected by separation on agarose gels of total DNA extracted from C33A2 cells or HN26 cells treated with 100uM melphalan for the indicated time periods.

Supplementary Figure S4. Melphalan induces a DNA damage response in HPV16 positive tonsillar cancer cells. (A) Western blots on extracts from HN26 cells incubated with DMSO (D) or 100uM melphalan (M) for 22hrs with monospecific antibodies to two phosphorylated forms or BRCA1 (p-BRCA1 (Ser988)) and (p-BRCA1 (Ser1423)), BRCA1, PARP1, general splicing factor SF3b and actin. **(B)** Western blot with monospecific antibody to phosphorylated BRCA1 (p-BRCA1 (Ser988)) or BRCA1 on extracts from HN26 cells incubated in 100uM melphalan for the indicated time periods. **(C)** Western blot on extracts from HN26 cells incubated with DMSO (D), 100uM melphalan (M) or 100uM etoposide (E) for 22hrs with monospecific antibody to phosphorylated Chk1 (p-Chk1) or actin. Fold induction is shown below blots. **(D)** Western blot with monospecific antibody to BARD1 or actin on extracts from HN26 cells or C33A2 cells incubated in 100uM melphalan for the indicated time periods. **(E, F)** RT-PCR with BARD1 mRNA specific primers on total RNA extracted from two independent preps from HN26 cells, C33A2 cells and HeLa cells treated with DMSO alone or 100uM of melphalan for 3hrs. The full-length BARD1 mRNA is indicated with an arrow. Multiple smaller bands likely representing previously described alternatively spliced BARD1 mRNAs were also detected. MW, molecular weight marker.

Supplementary Figure S5. Staining of melphalan treated HN26 cells with antibodies to DDR factors. (A)(B) Immunofluorescence with antibodies to phosphorylated Chk1 or histone γH2AX on DMSO- or melphalan-treated HN26 cells. 100uM melphalan was used. pChk1 was detected with Alexa Fluor 488 donkey anti-goat IgG, and γH2AX was detected with Alexa Fluor 488 goat anti-rabbit IgG(H+L). **(C)** Enlargement of pictures from (A). **(D)** Enlargement of pictures from (B). **(E)** Number of γH2AX-positive foci/nucleus in HN26 cells treated with DMSO or 100uM melphalan at the indicated time points. Average number of foci are indicated in the graph.

Supplementary Figure S6. HPV16 genome and mRNAs. (A) Schematic representation of the HPV16 genome that is present in the HN26 cell line. Rectangles represent open reading frames, promoters p97 and p670 are indicated as arrows, and the early and late polyadenylation signals pAE and pAL as vertical lines. **(B)** A subset of the alternatively spliced early mRNAs produced by the HPV16 genome is indicated. Arrows represent RT-PCR primers.

Supplementary Figure S7. HeLa and SiHa cells. (A) RT-PCR on total RNA extracted from HeLa cells (left) or SiHa cells (right) treated with DMSO alone or 100uM of melphalan for the

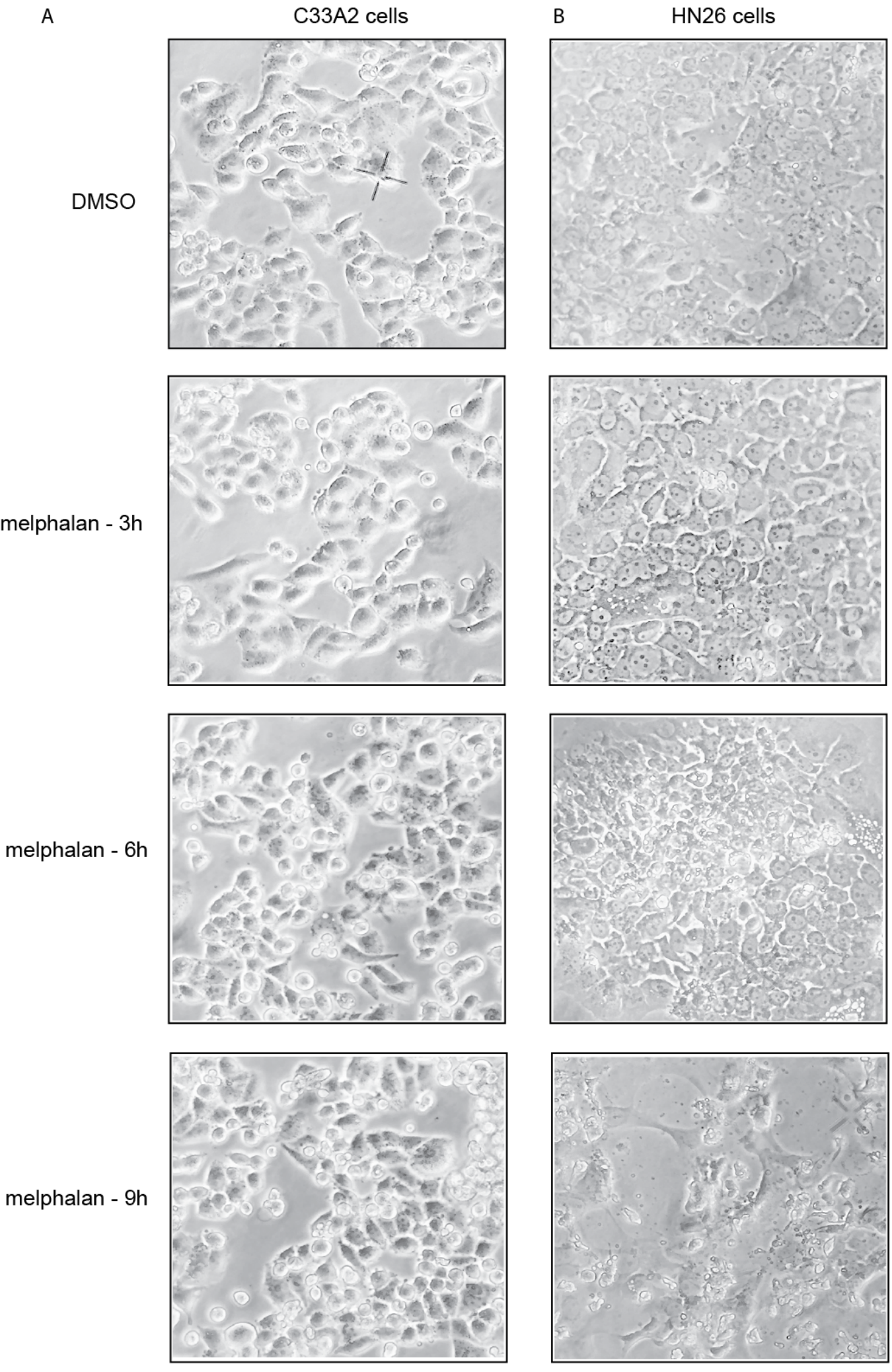
indicated time periods. HPV18 E6, E6*I/E7 and E6*II/E7 mRNAs in HeLa cells were monitored with HPV18-specific RT-PCR primers and HPV16 E6, E6*I/E7 and E6*II/E7 mRNAs in SiHa cells were monitored with HPV16-specific RT-PCR primers. For primer sequences see Supplementary Table 1. **(B)** Western blots with monospecific antibody to γH2AX on extracts from HeLa cells (left) or SiHa cells (right) treated with DMSO or 100uM melphalan for the indicated time points. **(C)** Western blots with monospecific antibody to caspase 3 on extracts from HeLa cells (left) or SiHa cells (right) treated with DMSO or 100uM melphalan for the indicated time points. **(D)** Western blots with monospecific antibody to PARP1 on extracts from HeLa cells treated with DMSO or 100uM melphalan for 9hrs.

Supplementary Figure S8. Quality control of extracts used for DNA-protein gel shifts. Western blot of actin on extracts from untreated HN26 cells or HN26 cells treated with DMSO or 100uM melphalan.

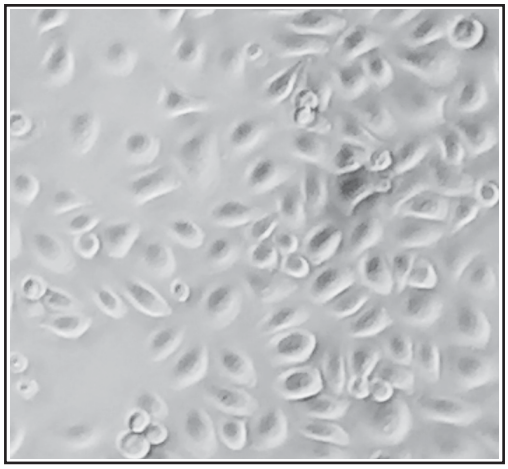
Supplementary Figure S9. Actinomycin D causes rapid degradation of HPV E6 and E7 oncogene mRNAs in HeLa cells but not in SiHa cells. (A) RT-PCR on total RNA extracted from HeLa cells treated with DMSO alone or 1.5uM of actinomycin D for the indicated time periods. HPV18 E6 and E6*I/E7 mRNAs were monitored as well as spliced cellular gapdh mRNAs. **(B)** RT-PCR on total RNA extracted from SiHa cells treated with DMSO alone or 1.5uM of actinomycin D for the indicated time periods. HPV16 E6 and E6*I/E7 mRNAs were monitored as well as spliced cellular gapdh mRNAs.

Supplementary Figure S10. Determination of maximum tolerated dose (MTD) of cisplatin and melphalan in non-tumor bearing nude mice. The indicated doses of **(A)** melphalan, and **(B)** cisplatin were injected on day 0 (N=5). Controls were injected with physiological NaCl. Body weight was measured for 16 days and related to the weight at day 0. Error bars indicate standard error of the mean.

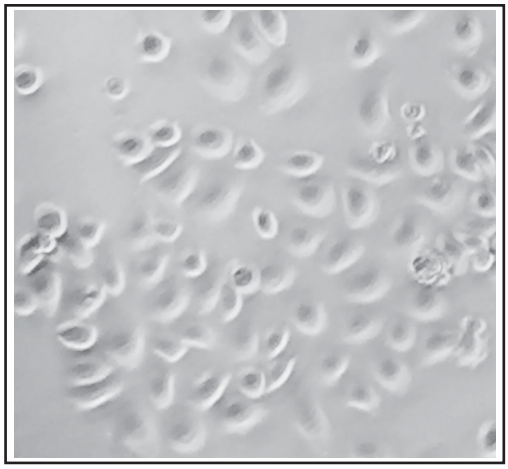
Supplementary Figure S11. A xenograft of HN26, was sectioned and stained with hematoxylin and eosin. Photograph was taken with a 20× objective.


A
C33A2 cells
B
HN26 cells
DMSO
melphalan - 3h
melphalan - 6h
melphalan - 9h

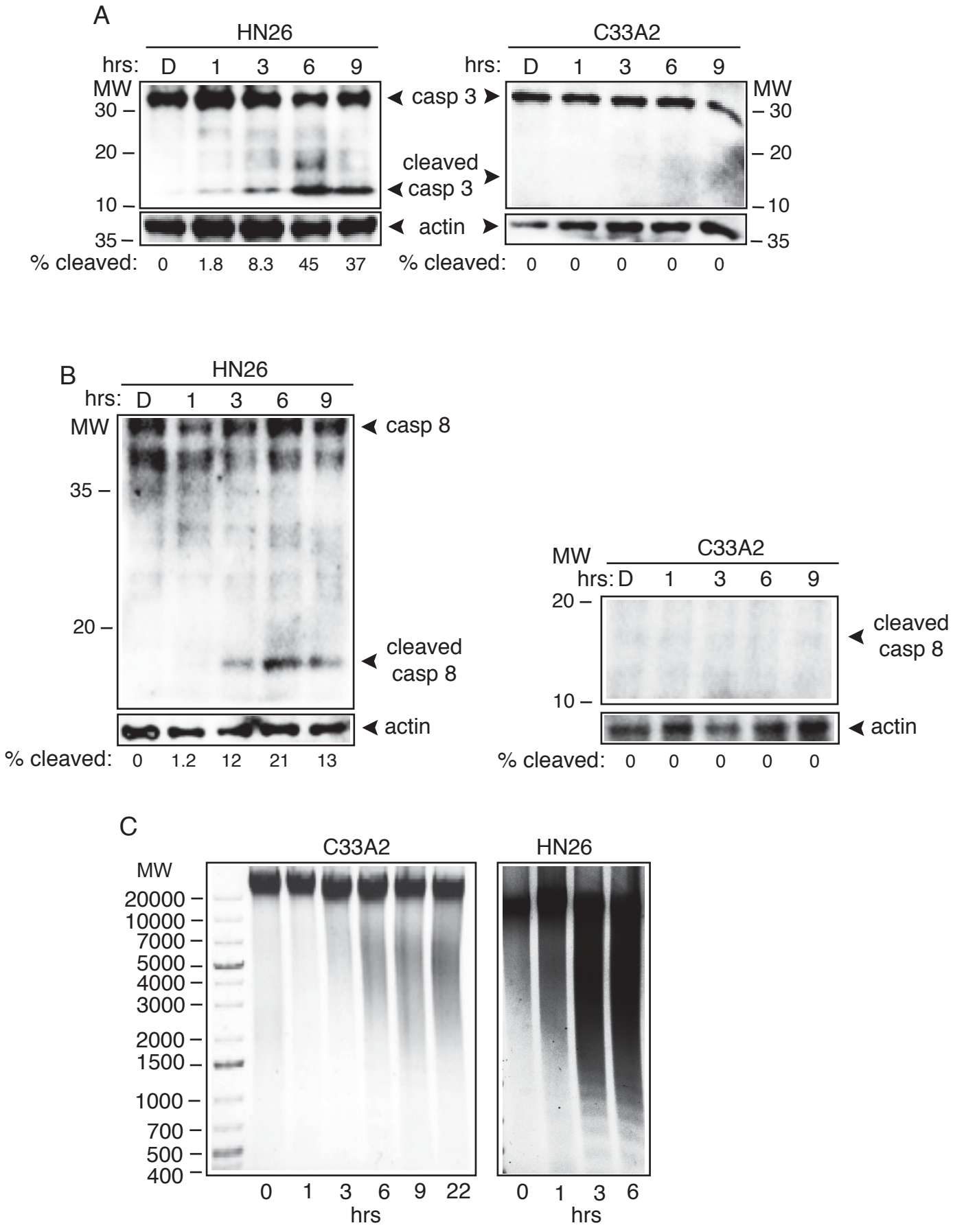
HN7 cells



DMSO



melphalan - 9h


A
HN26
C33A2
hrs: D 1 3 6 9
MW
30 –
20 –
10 –
35 –
casp 3
cleaved
casp 3
actin
% cleaved: 0 1.8 8.3 45 37
% cleaved: 0 0 0 0 0
B
HN26
hrs: D 1 3 6 9
MW
35 –
20 –
casp 8
cleaved
casp 8
actin
% cleaved: 0 1.2 12 21 13
C33A2
MW
hrs: D 1 3 6 9
20 –
10 –
cleaved
casp 8
actin
% cleaved: 0 0 0 0 0
C
C33A2
HN26
MW
20000 –
10000 –
7000 –
5000 –
4000 –
3000 –
2000 –
1500 –
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700 –
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400 –
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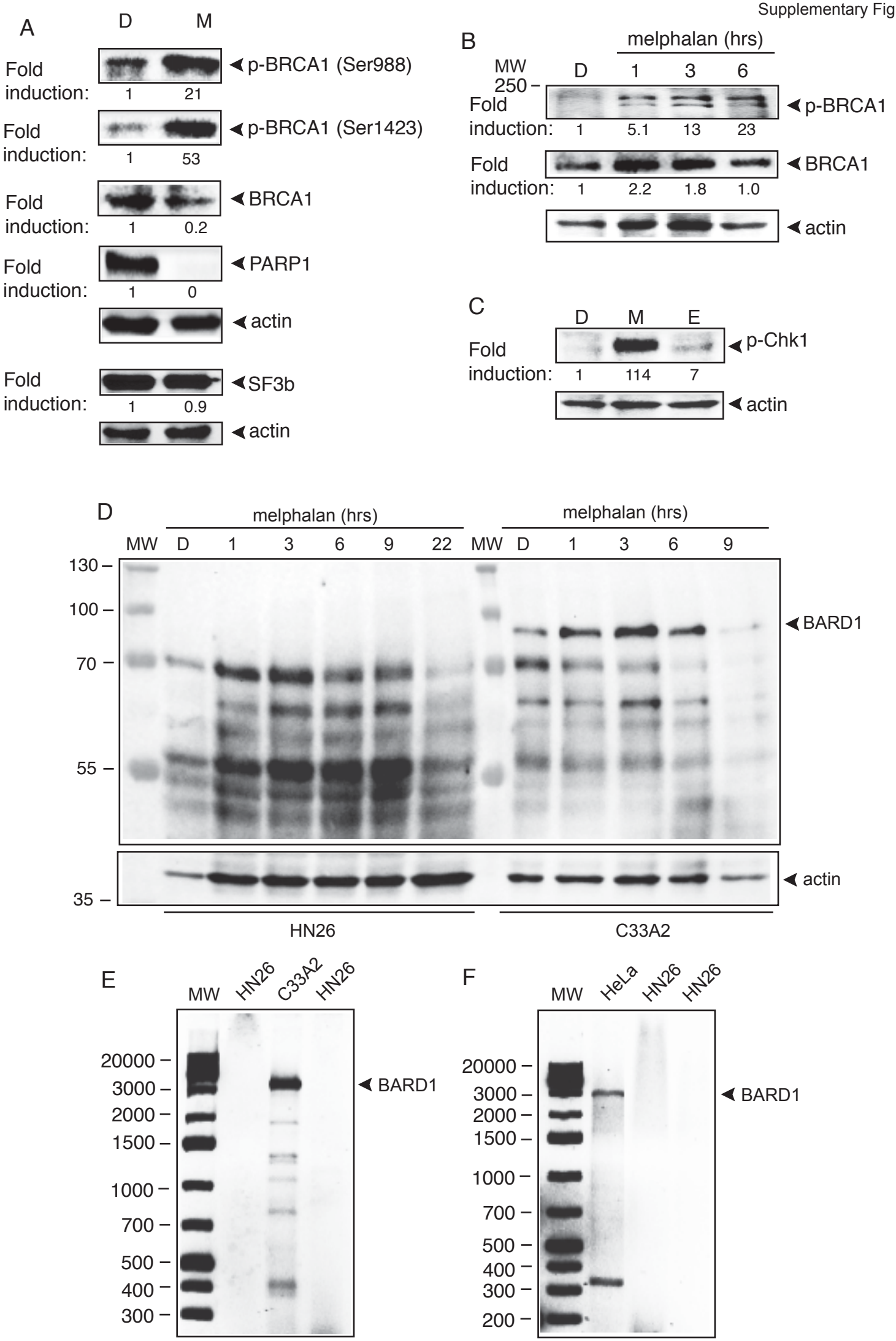
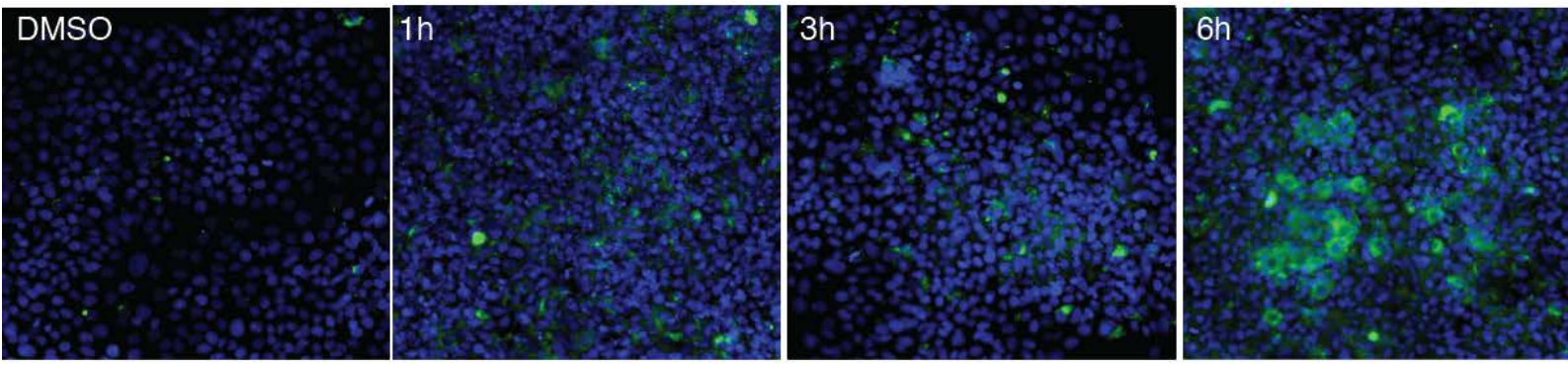
hrs
0 1 3 6
hrs


A
D
M
Fold induction:
1
21
p-BRCA1 (Ser988)
Fold induction:
1
53
p-BRCA1 (Ser1423)
Fold induction:
1
0.2
BRCA1
Fold induction:
1
0
PARP1
actin
Fold induction:
1
0.9
SF3b
actin
B
melphalan (hrs)
MW
D
1
3
6
250 –
Fold induction:
1
5.1
13
23
p-BRCA1
Fold induction:
1
2.2
1.8
1.0
BRCA1
actin
C
D
M
E
Fold induction:
1
114
7
p-Chk1
actin
D
melphalan (hrs)
melphalan (hrs)
MW
D
1
3
6
9
22
MW
D
1
3
6
9
130 –
100 –
70 –
55 –
35 –
BARD1
actin
HN26
C33A2
E
MW
HN26
C33A2
HN26
20000 –
3000 –
2000 –
1500 –
1000 –
700 –
500 –
400 –
300 –
BARD1
F
MW
HeLa
HN26
HN26
20000 –
3000 –
2000 –
1500 –
1000 –
700 –
500 –
400 –
300 –
200 –
BARD1

A p-Chk1



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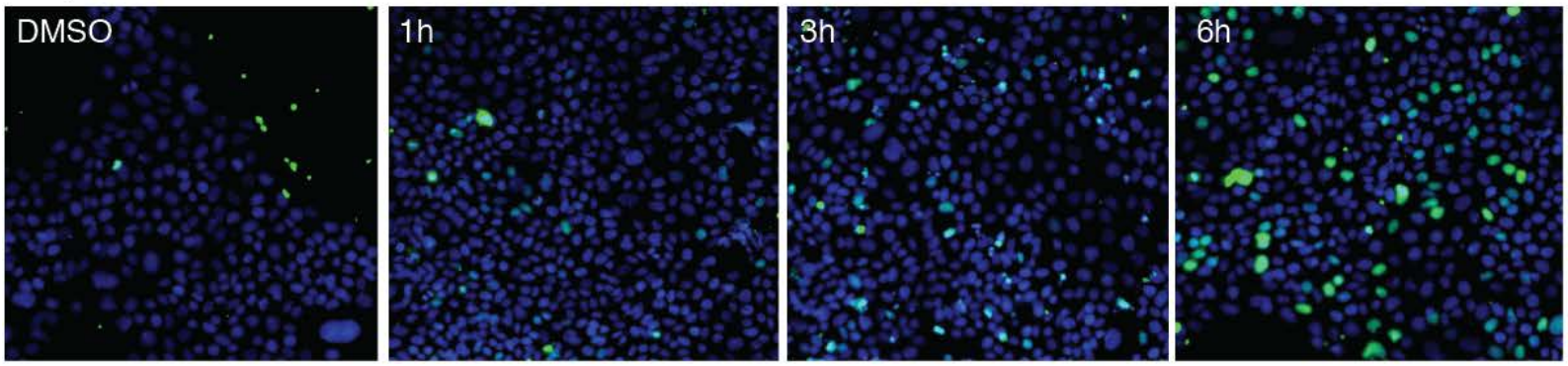
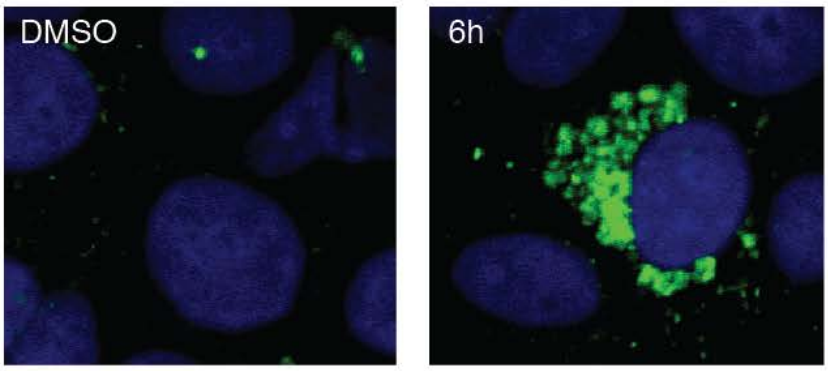
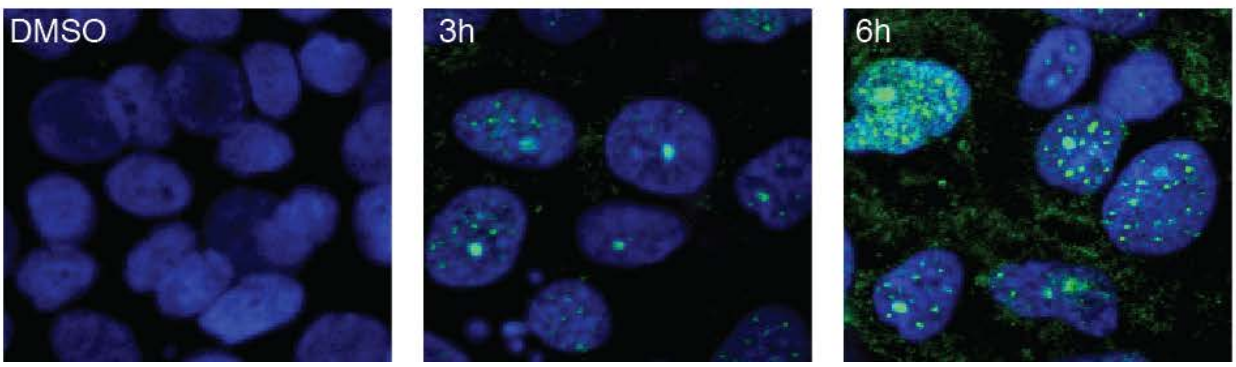


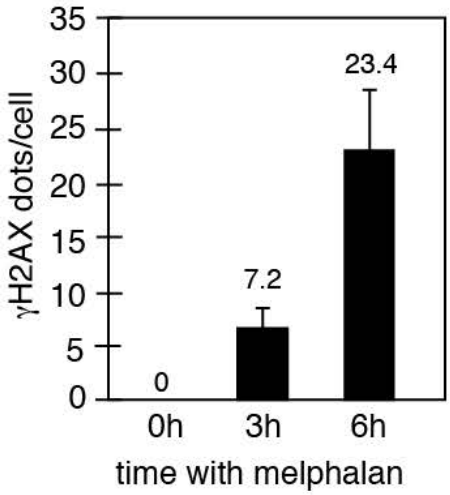
C p-Chk1

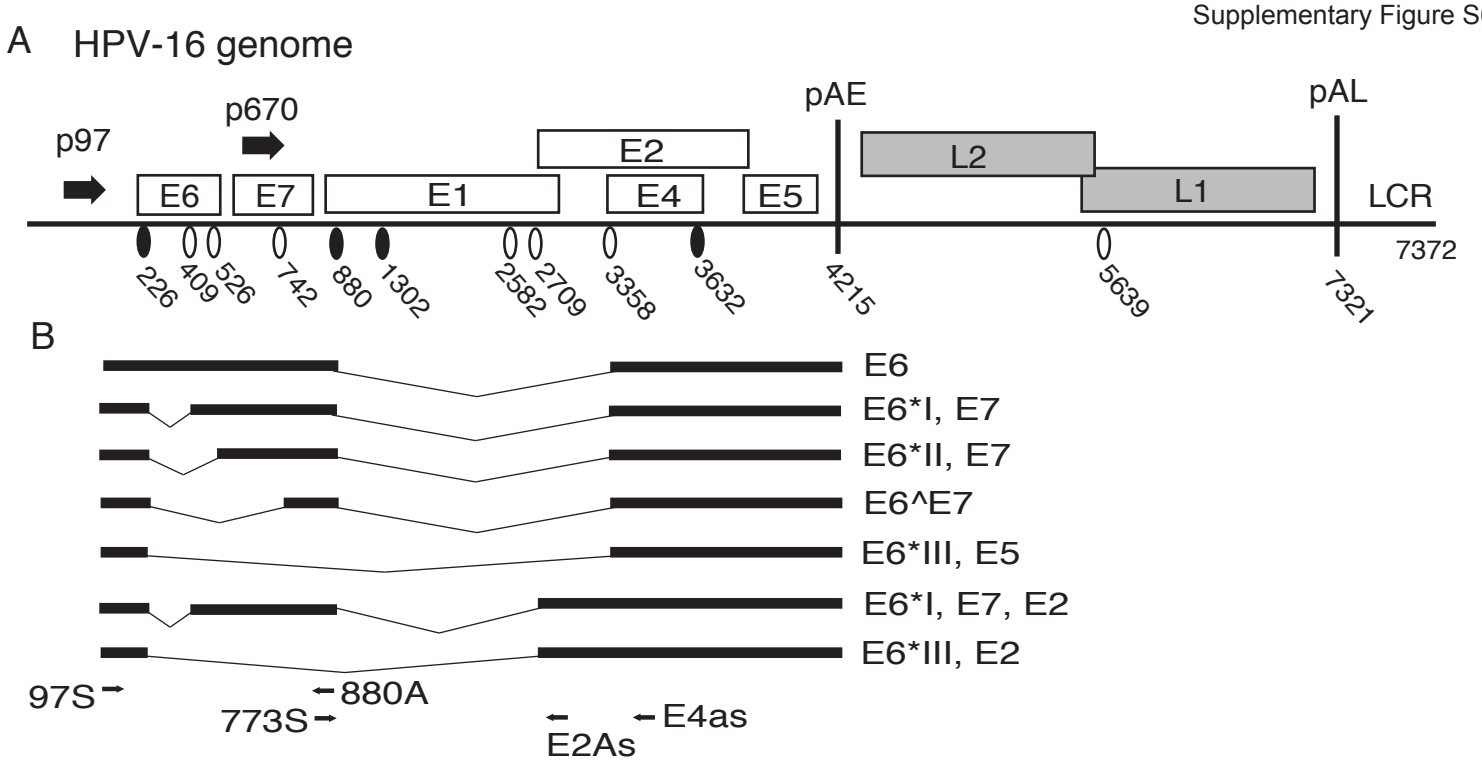


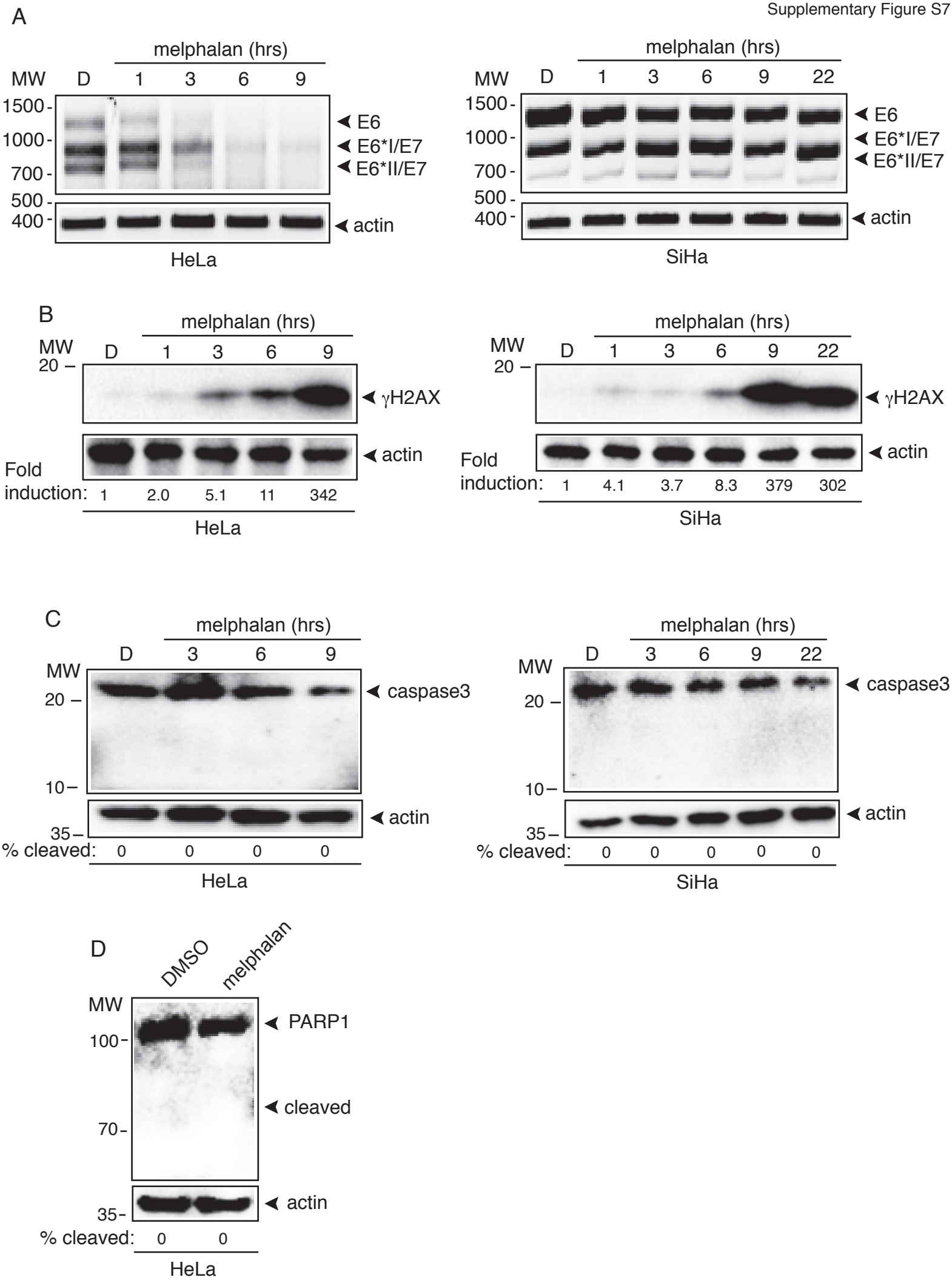
D γH2AX

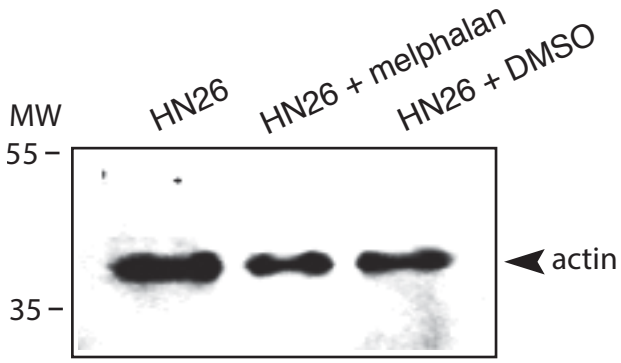


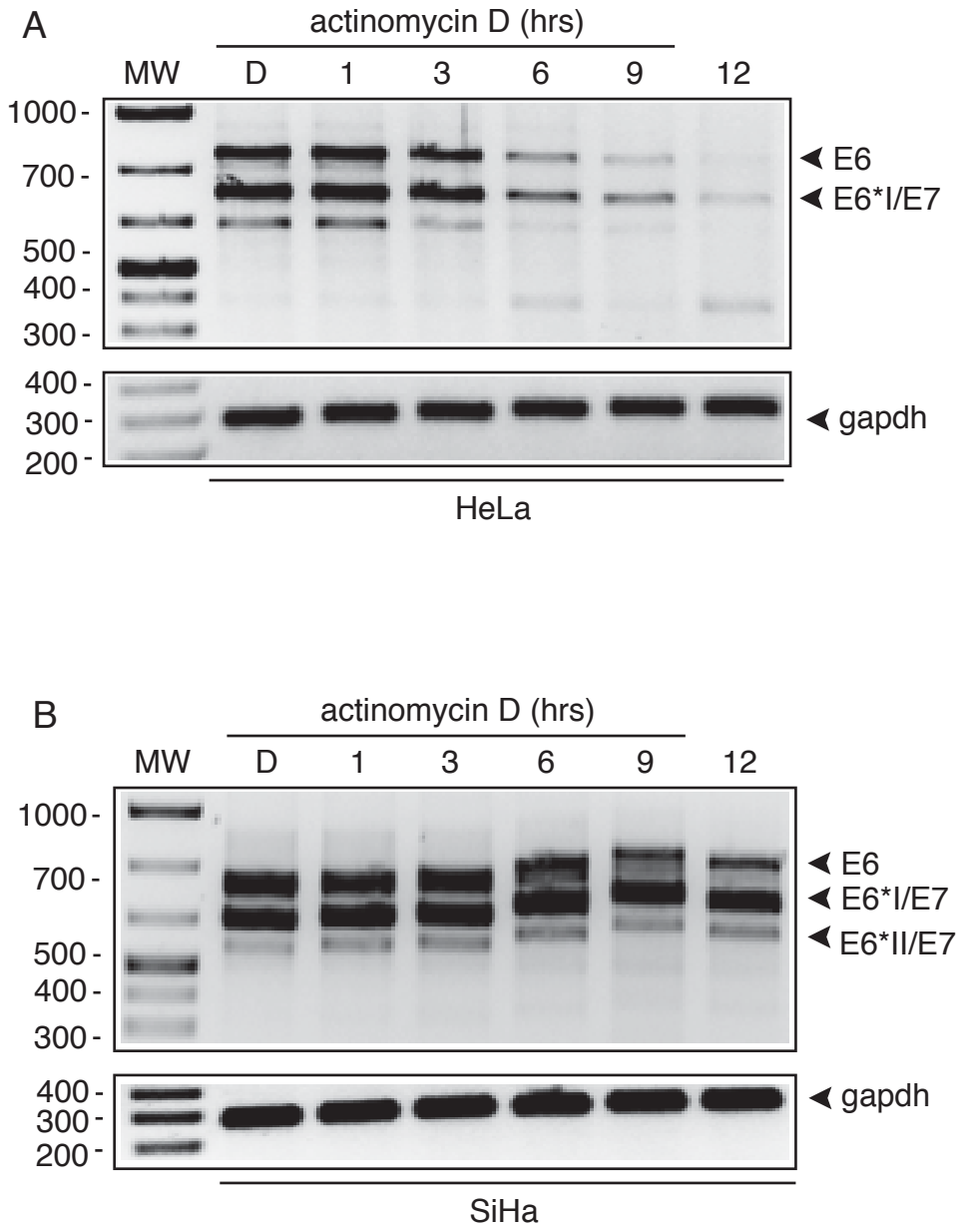
E



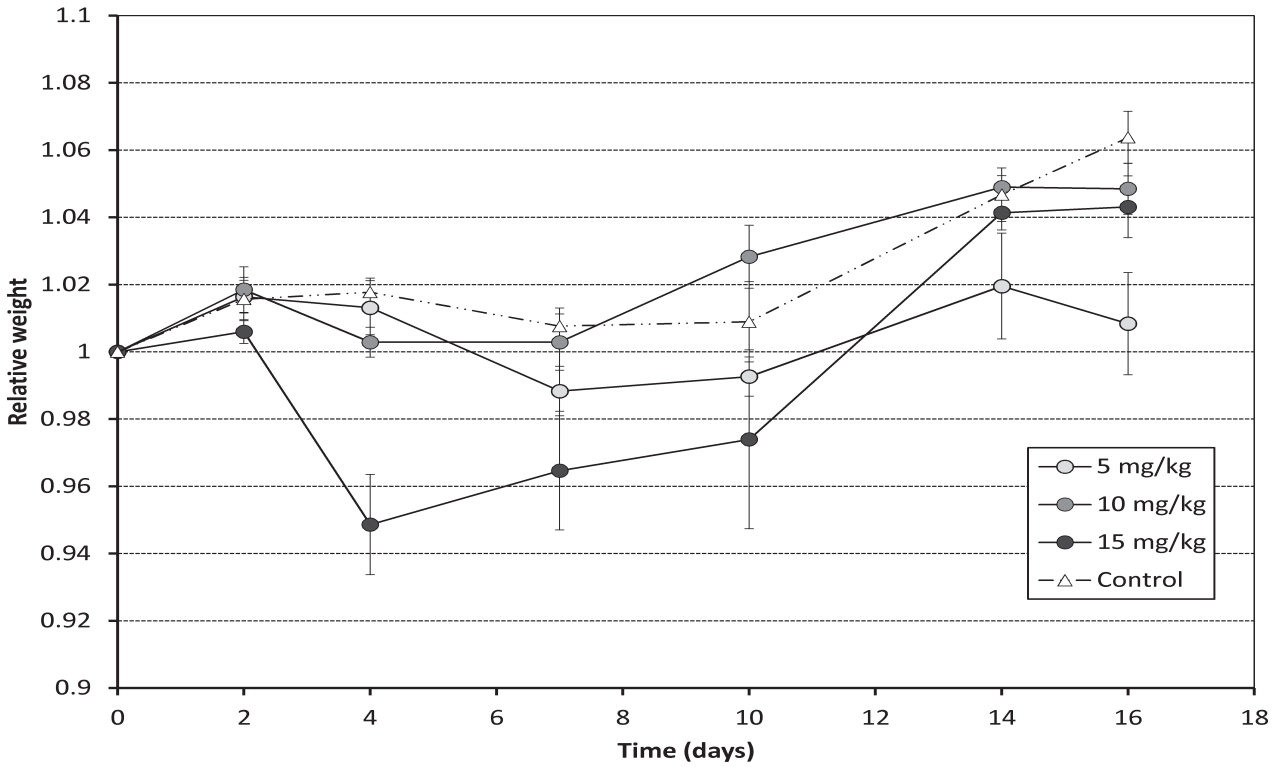

A
HPV-16 genome
p97
p670
E6
E7
E1
E2
E4
E5
pAE
pAL
L2
L1
LCR
7372
226
409
526
742
880
1302
2582
2709
3358
3632
4215
5639
7321
B
E6
E6*I, E7
E6*II, E7
E6^E7
E6*III, E5
E6*I, E7, E2
E6*III, E2
97S
880A
773S
E2As
E4as


A
melphalan (hrs)
MW D 1 3 6 9
1500
1000
700
500
400
E6
E6*I/E7
E6*II/E7
actin
HeLa
melphalan (hrs)
MW D 1 3 6 9 22
1500
1000
700
500
400
E6
E6*I/E7
E6*II/E7
actin
SiHa
B
melphalan (hrs)
MW D 1 3 6 9
20
γH2AX
actin
Fold induction: 1 2.0 5.1 11 342
HeLa
melphalan (hrs)
MW D 1 3 6 9 22
20
γH2AX
actin
Fold induction: 1 4.1 3.7 8.3 379 302
SiHa
C
melphalan (hrs)
D 3 6 9
MW
20
10
35
caspase3
actin
% cleaved: 0 0 0 0
HeLa
melphalan (hrs)
D 3 6 9 22
MW
20
10
35
caspase3
actin
% cleaved: 0 0 0 0 0
SiHa
D
DMSO
melphalan
MW
100
70
35
PARP1
cleaved
actin
% cleaved: 0 0
HeLa

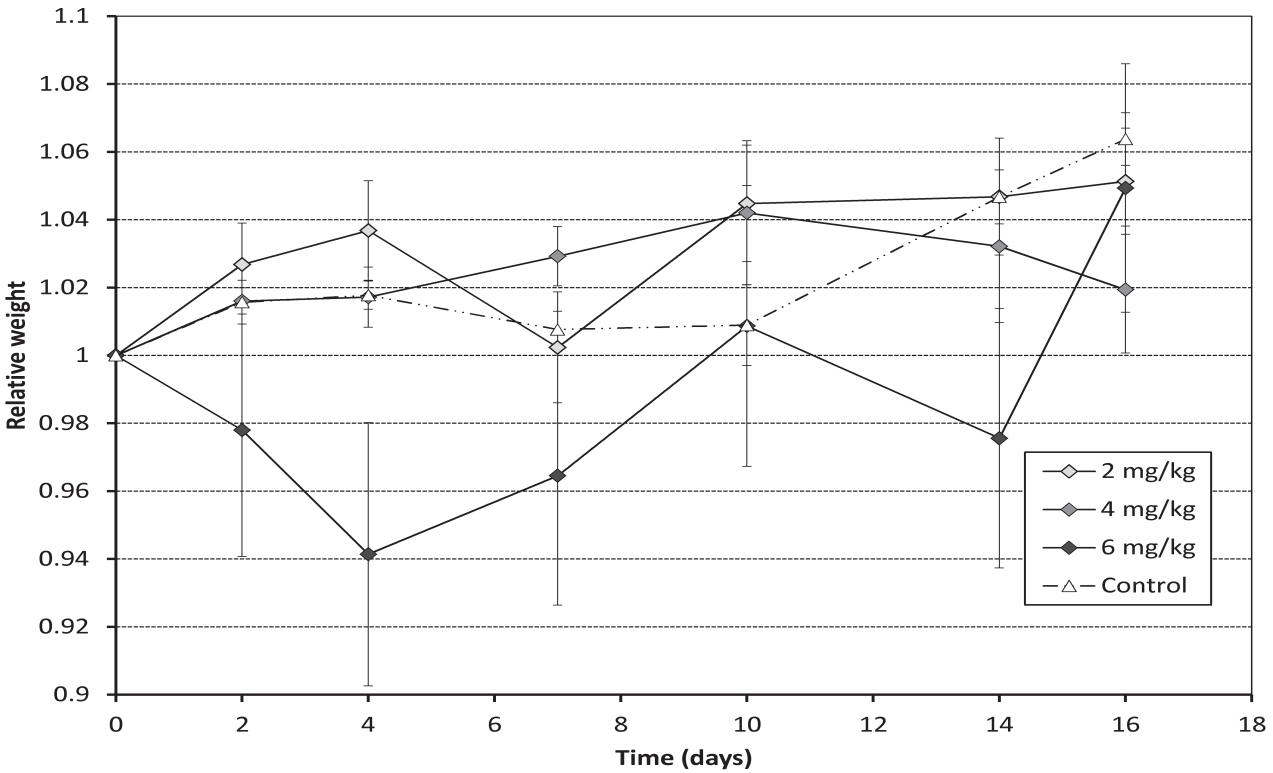

MW
HN26
HN26 + melphalan
HN26 + DMSO
55 –
35 –
actin


A
actinomycin D (hrs)
MW
D
1
3
6
9
12
1000
700
500
400
300
E6
E6*I/E7
400
300
200
gapdh
HeLa
B
actinomycin D (hrs)
MW
D
1
3
6
9
12
1000
700
500
400
300
E6
E6*I/E7
E6*II/E7
400
300
200
gapdh
SiHa

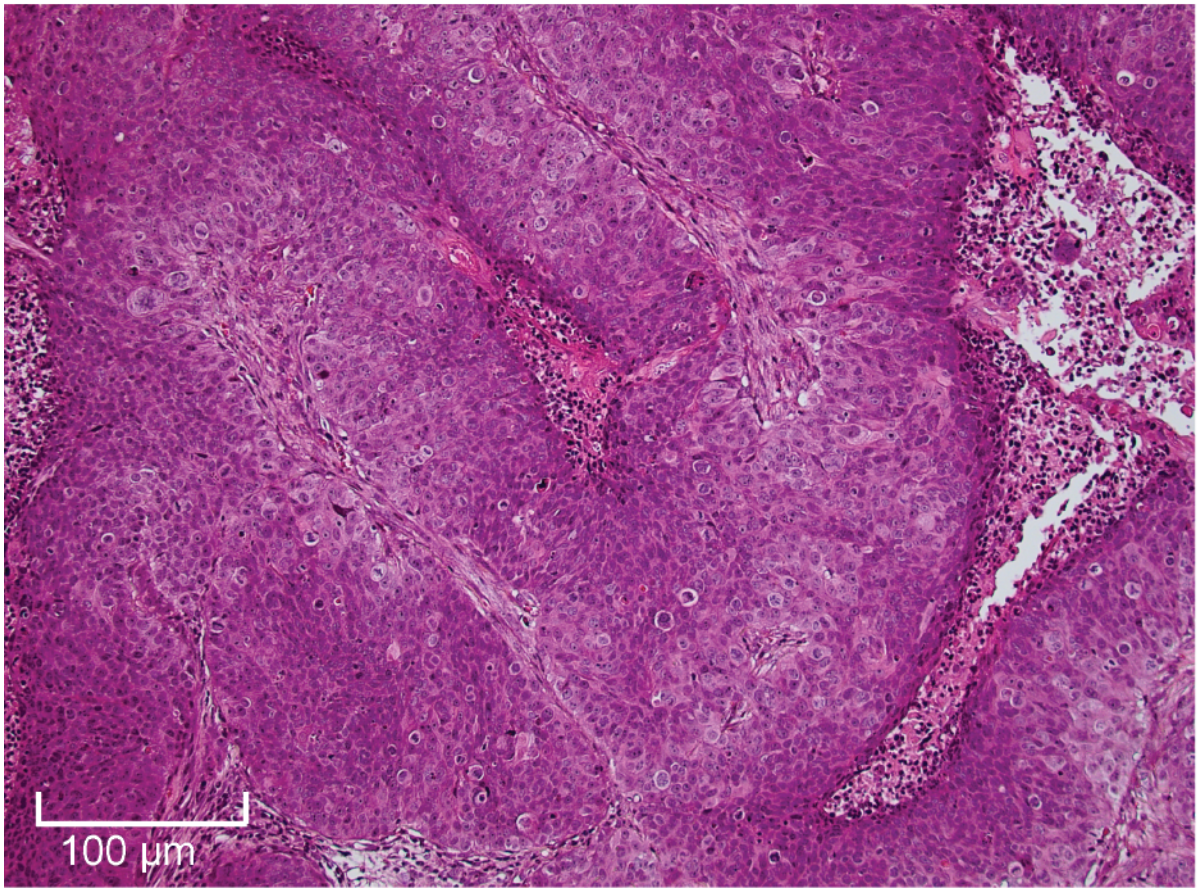
A


1.1
1.08
1.06
1.04
1.02
1
0.98
0.96
0.94
0.92
0.9
Relative weight
0
2
4
6
8
10
12
14
16
18
Time (days)
5 mg/kg
10 mg/kg
15 mg/kg
Control

B


1.1
1.08
1.06
1.04
1.02
1
0.98
0.96
0.94
0.92
0.9
Relative weight
0
2
4
6
8
10
12
14
16
18
Time (days)
2 mg/kg
4 mg/kg
6 mg/kg
Control

Supplementary Figure S11



Supplementary Table 1. List of PCR primers used in the current work.

RT-PCR primers	Amplified region	Sequence 5'-3'
773s	E4, L1, E2	GCACACACGTAGACATTCGTACTTTG
E4as	E4	TGCTGCCTAATAGTTTCAGGAGAGG
E2as	E2	CCTGACCACCCGCATGAACTTCC
F-GAPDH	GAPDH	ACCCAGAAGACTGTGGATGG
R-GAPDH	GAPDH	TTCTAGACGGCAGGTCAGGT
casp3S	Caspase3	GTGAGGCGGTTGTAGAAGAGT
casp3A	Caspase3	TCACGGCCTGGGATTTCAAG
Bcl2S	Bcl-2	GTGGAGGAGCTCTTCAGG
Bcl2A	Bcl-2	AGGCACCCAGGGTGATGCAA
BARD1S	BARD1	GAGGAGCCTTTCATCCGAAG
BARD1A	BARD1	CAGCTGTCAAGAGGAAGCAAC
p97F	E6/E7	AAACTAAGGGCGTAACCGAAA
880As	E6/E7	GATCAGCCATGGTAGATTATGGTTTC
E1F	E1	AGTAGAGCTGCAAAAAGGAGATTA
E1R	E1	CTGACTACATGGTGTTTCAGTCTC
E2F	E2	CTGGAAATCCTTTTTCTCAAGG
E2R	E2	CATTTTCATAATGTGTTAGTATTTTGTC
L2F	L2	GACCCTGCTTTTGTAACCACTC
L2R	L2	ATGCTGGCCTATGTAAAGCAAC
L1SF	L1S	CCTTTAGTATCAGGTCCTGATATACCC
L1SR	L1S	GCAACATATTCATCCGTGCTTACAACC
18154S	HPV18 E6/E7	TGTGCACGGAACTGAACACT
18570AS	HPV18 E6/E7	TTCTCTGCGTCGTTGGAGTC
7860S	P97 probe	ACCGTTTTGGGTTACACATTTACAAGC
160A	P97 probe	CAGCTCTGTGCATAACTGTGGTAAC

Supplementary Table 2. List of antibodies used in the current work.

Target protein	Antibody	Vendor
RNA polymerase II	ab817	Abcam
caspase 3	ab90437	Abcam
caspase 8	ab86296	Abcam
PAR	ab14459	Abcam
p53	sc-6423	Santa Cruz
BRCA1	sc-642	Santa Cruz
phosphorylated BRCA1 (Ser1423)	sc-101647	Santa Cruz
phosphorylated BRCA1 (Ser988)	sc-12888	Santa Cruz
Sp1	ab13370	Abcam
Chk1	sc-8408	Santa Cruz
phosphorylated Chk1	sc-17922	Santa Cruz
γH2AX	ab2893	Abcam
ATM	ab32420	Abcam
p-ATM	sc-47739	Santa Cruz
TFIID/TBP	sc-421	Santa Cruz
Cytochrome c	ab13575	Abcam
actin	sc-1616	Santa Cruz

Supplementary Table T3. Effects of melphalan on the various cell line

Properties:	detachment of cells	DDR activation	apoptosis induction	p53 induction	E6/E7 degradation
C33A2	-/+	+	-	- [a]	NA
HN7	-	+	-	+	NA
HN26	+++	+++[b]	+++	+++	+++
HeLa	-	+++	-	-	+++
SiHa	-/+	+++	-	ND	-
3310	-	+++	-	-	+++

NA, not applicable; ND, not done.

[a]C33A2 cells produce constitutively high levels of negative mutant p53 protein, [b]DDR activation may be incomplete due to BARD1 deficiency,

Supplementary Methods

Cell lines

HPV16-positive tonsillar cancer cell line HN26 was propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/mL penicillin, and 100 units/mL streptomycin sulfate. C33A2 and C33A cells were cultured in Dulbecco's modified Eagle medium (GE Healthcare Life Science HyClone Laboratories) with 10% heat-inactivated foetal bovine calf serum (GE Healthcare Life Sciences HyClone Laboratories) and penicillin/streptomycin (Gibco Thermo Fisher Science). The 3310 cell line has been described previously and was generated by transfection of normal human primary keratinocytes with full-length HPV16 genome plasmid pHPV16AN. The 3310 cells were cultured in EpiLife Medium supplemented with Human Keratinocyte Growth Supplement (Gibco Thermo Fisher Science). Propagation of neonatal human epidermal keratinocytes has been described previously.

Mitochondrial cytochrome c release assay to monitor apoptosis

Two million HN26 cells treated with either DMSO or 100uM melphalan were pelleted and the cytoplasmic fraction free of mitochondria was prepared using the mitochondria isolation kit for cultured cells (Thermo Scientific 89874). The cytosolic fraction was analysed by Western blotting with antibody to cytochrome c. Antibodies are listed in Table

DNA preparation and DNA fragmentation analysis

Total DNA was prepared by lysis of cells in 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA, 0.5 % SDS, 250 ug/ml proteinase K followed by incubation for 4 hours at 37°C. An equal volume of phenol/chisam solution was added to the samples that were mixed for two minutes, and centrifuged at 13000 rpm for two minutes at room temperature. Following centrifugation, the aqueous phase was transferred to new tubes, containing $1/10^{th}$ the volume of the sample with 3M sodium acetate pH 5.2. The DNA was ethanol precipitated and air dried followed by RNaseA treatment for 1h at 37°. Five ug of each DNA sample were analysed on 1% agarose gels.

MTT assay

MMT stock solution (5mg/ml) (Sigma-Aldrich) is added to each C33A2 or HN26 cell culture being assayed to equal one tenth the original culture volume and incubated for 3 to 4hrs. At the end of the incubation period the medium is removed and the converted dye is solubilized with acidic isopropanol (0.1N HCl in absolute isopropanol). Absorbance of converted dye is measured at a wavelength of 570 with background subtraction at 630-690 nm.

Chromatin immunoprecipitation (ChIP) supplementary information

The cross-linking reaction was stopped by the addition of glycine to a final concentration of 0.125M. Cells were lysed in Buffer A and the nuclei were recovered by centrifugation. Nuclei were dissolved in Buffer B and MNase was added to digest the DNA at a final concentration of 0.125 U/ul. Incubation time and amount of enzyme had been previously titrated to achieve mono- and dinucleosomes. Nuclei were resuspended in ChIP buffer, incubated on ice for 10 min and sonicated 15s on and 45s off, 10 cycles, using a Diagenode Bioruptor with high setting. Nuclei were observed in a microscope during sonication to ensure that sufficient lysis had occurred. Samples were thereafter subjected to centrifugation and the nuclear extract was recovered. A small aliquot was RNase and proteinase K treated prior to spin column purification to determine the size and concentration of the DNA. For immunoprecipitation, nuclear extract corresponding to 10–15ug of DNA was diluted in ChIP buffer and antibodies indicated in the figures were added for an overnight end-over- end incubation at 4°C. Immune complexes were precipitated through a two-hour end-over-end incubation at 4°C with Protein G Magnetic Beads. Beads were washed three times with ChIP buffer, one time with ChIP buffer supplemented with 500 mM NaCl and finally twice with ChIP buffer supplemented with 250 mM LiCl. Each wash was incubated end- over-end for 5 min at 4°C. Samples were eluted from the beads with elution buffer (1% SDS, 0.1 M $NaHCO_3$) and treated with proteinase K prior to purification of the DNA on spin columns followed by DNA qPCR.

C33A2 cells were cross-linked in 1% formaldehyde for 10min at room temperature. 2% of the extract used for immunoprecipitation was purified and used as input control. Sequences of HPV16 primers E1F and E1R (E1), E2F and E2R (E2), L2F and L2R (L2) or L1F and L1R (L1) used for the ChIP-PCR are listed in **Supplementary Table T1**. Real-time PCR was carried out using SYBR green PCR mix (BIO-RAD, SsAdvanced™ Universal SYBR Green Supermix) according to the manufacturer's instructions. The relative proportions of coimmunoprecipitated gene fragments were determined on the basis of the threshold cycle (Ct) for each PCR product. The Ct values obtained from control IgG were subtracted from Ct values obtained from immunoprecipitations with specific antibodies. The resulting values were further used to calculate the amount of each immunoprecipitation relative to the input according to following formula: Relative amount = $2^{-Ct(\text{specific antibody-IgG})}/2^{-Ct(\text{input-IgG})}$, for each PCR amplicon. For Melphalan treatments, the fold change compared to DMSO-treated samples was calculated according to Fold change = $(2^{-Ct(\text{specific antibody-IgG})}/2^{-Ct(\text{input-IgG})})_{drug}/(2^{-Ct(\text{specific antibody-IgG})}/2^{-Ct(\text{input-IgG})})_{DMSO}$. For every analysed gene fragment, each sample was quantified in duplicate from at least three independent ChIP analyses.

Immunofluorescence

HN26 cells were grown to 70–80% confluency on 8-well chamber glass slides ($5x10^4$ cells/well) (Thermo Fisher Scientific). Cells treated with DMSO or melphalan for 1-, 3- or 6-hours were fixed with 3.5% formaldehyde for 10min at 4 °C and permeabilized in 0.25% Triton X-100 and 5% FBS for 15 min and blocked with 5% FBS for 1h at room temperature. Slides were incubated with primary antibodies in 5% FBS overnight at 4°C, washed with PBS for 3 x 10min at room temperature followed by incubation with FITC labelled secondary antibody for 1h at room temperature. After washing the cells with PBS 3 x 10min at room temperature, nuclei were stained with DRAQ5 (ab1084101, Abcam) and the slides were mounted (Fluoromount, Sigma, Aldrich). Images were produced by laser-scanning confocal microscopy (LSM510 META confocal microscope, Nikon Eclipse C1).