Supplemental Information for:

Dysfunctional telomeres trigger cellular senescence mediated by cyclic GMP-AMP synthase

Salim Abdisalaam[#,1], Souparno Bhattacharya[#,1], Shibani Mukherjee[#,1], Debapriya Sinha[1], Kalayarasan Srinivasan[1], Mingrui Zhu[2], Esra A. Akbay[2], Hesham A. Sadek[3], Jerry W. Shay[4] and Aroumougame Asaithamby[*,1]

[1]Department of Radiation Oncology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

[2]Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

[3]Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

[4]Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

[#]Co-first authors

*Corresponding author: Aroumougame Asaithamby (aka Thambi)
E-mail: Asaithamby.Aroumougame@UTSouthwestern.edu

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Figure S1: **Telomeric DNA-targeted DSBs (T-DSBs) trigger innate immune signaling but not telomere shortening in response to T-DSBs. A-C**. Telomeric DSBs trigger innate immune signaling in U2OS cells. Bar graph shows frequency of micronuclei formation at 48 h after the induction of telomeric DSBs (**A**). Western blot shows increased activation of IRF3 (**B**), and the bar graph shows increased expression of immune pathway genes (**C**) in U2OS cells stably co-expressing DD-Cas9 and sgTelomere RNA at different time intervals. Bar graph presents the mean and STDEV from two independent experiments. w/d-withdrawal; M-mock. Statistical analyses were performed using student's t-test (A) and two-way ANOVA (C). **D.** Bar graph shows a major fraction of DSBs induced by sgTelomere+DD-Cas9 is localized in the telomeric region. U2OS cells stably co-expressing DD-Cas9 and sgTelomere RNA were treated with doxycycline and Shield1 for 24 hours, protein-DNA complex was cross-linked with 4% paraformaldehyde and the chromatin fraction was co-immunoprecipitated using anti-γH2AX antibodies. The purified genomic DNA was subjected to next-generation sequencing (NGS) and the NGS was then used to enumerate the percentage of telomeric DNA enrichment in in sgTelomere+DD-Cas9 cells. Briefly, Bowtie2's build command was used to index the latest release of human reference genome (Homo Sapiens Genome Reference Consortium Human Build 38; GRCh38), downloaded from the University of California, Santa Cruz (UCSC). The indexed reference genome was used to align the raw sequencing fastq reads using Bowtie2 to create an aligned .sam file. The .sam file was then binarized into .bam using Samtools view command. The .bam files were then sorted and indexed using Samtools sort and index command, respectively. The indexed bam files were created for ease of visualization. These steps were performed for all datasets, including T0, Mock and Input. Finally, Model-based analysis for ChIP-Sequence (MACS2) was used for calling significant peaks using Input as the control data. The commands used for the data analysis are: 1. Bowtie2-build hg38.fa Indices/hg38 (Indexing using Bowtie2); 2. Bowtie2 -x Indices/hg38 -U T0_24hrdoxy.fastq.gz -S T0_24hrdoxy.sam (aligning using Bowtie2); 3. Samtools view -bSo T0_24hrdoxy.bam T0_24hrdoxy.sam (Binarizing sam output file); 4. Samtools sort T0_24hrdoxy.bam -o T0_24hrdoxy.sorted.bam and Samtools index T0_24hrdoxy.sorted.bam (sorting and indexing); 5. MACS2 callpeak -t T0_24hrdoxy.sorted.bam -c input.sorted.bam --format=BAM --name=DoxyT0cas9 --gsize=2913022398 (Peak calling). The average signal enrichment scores above 750 in a given genomic region was used for calculating the percentage of telomere peaks in total number of peaks detected by the MACS2. **E**. Telomeric DSBs do not cause telomere shortening. Representative gel images show distribution of differently sized telomeres in HT1080 cells co-expressing DD-Cas9 and sgTelomere RNA in mock-treated and 1-7 days after DOX and Shield1 treatment (**Ei**). Bar graph shows mean length of telomeres in mock- and DOX+Shield1–treated HT1080+sgTelomere+DD-Cas9 cells at indicated time points (**Eii**). M-DNA marker.

Figure S2: **Cellular senescence caused by dysfunctional telomeres is different from normal physiological senescence. A.** Schematics of the experimental design used in panels B-E, illustrating treatment of hTERT immortalized human skin fibroblasts

(82-6) with 6-thio-dG; T-hours, D-days. **B.** Induction of dysfunctional telomeres in hTERT immortalized human skin fibroblasts generates micronuclei at 72 h after 10 μM 6-thio-dG withdrawal Bar graph presents the mean and STDEV from three independent experiments. **C**. Induction of dysfunctional telomeres in hTERT immortalized human skin fibroblasts activates IRF3 and STAT1 at different time intervals after 10 μM 6-thio-dG withdrawal. **D**. Induction of dysfunctional telomeres in hTERT immortalized human skin fibroblasts show elevated expression of immune signaling genes 72 h after 6-thio-dG withdrawal. Data in the bar graph present the mean and STDEV from two-three independent experiments. **E**. Fibroblasts show increased levels of premature senescence phenotype 15 days after the withdrawal of 10 μM 6-thio-dG. Data in the bar graph present the mean and STDEV from 10 fields from 3-6 independent experiments. **F-H.** Replicative senescence is independent of immune signaling activation. Graph shows frequency of β-gal staining in IMR90 cells undergoing replicative senescence (**F**). Data in the bar graph present the mean and STDEV from two independent experiments. Graph shows frequency of micronuclei formation in young and aged IMR90 cells (**G**). Data in the bar graph present the mean and STDEV from 20-30 fields from three independent experiments. Graph shows expression of innate immune pathway genes (**H**) in IMR90 cells undergoing replicative senescence. Data in the bar graph present the mean and STDEV from two independent experiments. Statistical analyses were performed using student's t-test (B, E, F, G and H) and two-way ANOVA (D).

Figure S3: **Dysfunctional telomeres cause marginal cell death. A.** Induction of telomeric DSBs does not cause apoptosis. Western blot shows expression of total and cleaved caspase 3 (a well-known marker for apoptosis) at different time intervals after Doxycycline and Shield1 treatment in HT1080 cells co-expressing DD-Cas9 and sgTelomere RNA. HT1080 cells treated with 2 μM camptothecin (CPT) continuously for 24 h were used as a positive control for apoptosis. U-untreated. **B.** 6-thio-dG treatment has a marginal effect on cell survival. Cell survival was analyzed by a colony formation assay. Bar graph shows the proportion of surviving HT1080 cells 7 days after 3 μM 6-thio-dG treatment relative to DMSO-treated cells. The relative survival efficiencies were plotted. The error bars represent the STDEV calculated from triplicate experimental setup.

Supplementary Tables

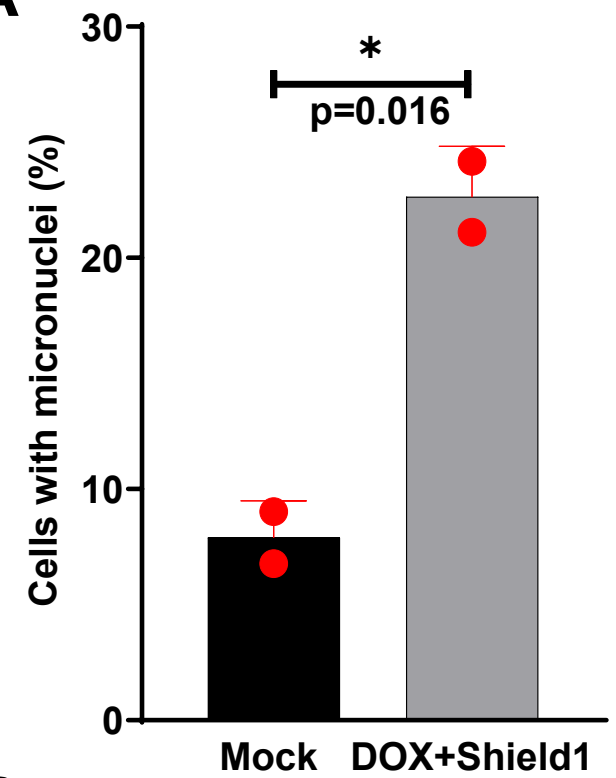
Table S1: List of primers used for cloning full length and truncated cDNAs reported in this study.

Table S2: List of primary antibodies and their respective dilutions used for both western blotting (WB) and immunofluorescence staining (IF) reported in this study.

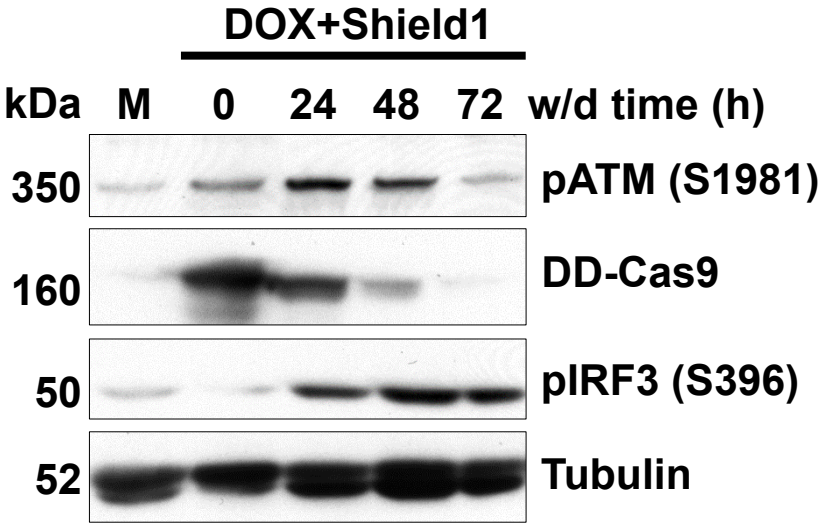
Table S3: List of primers used for quantitative real-time polymerase chain reaction reported in this study.

Figure S1

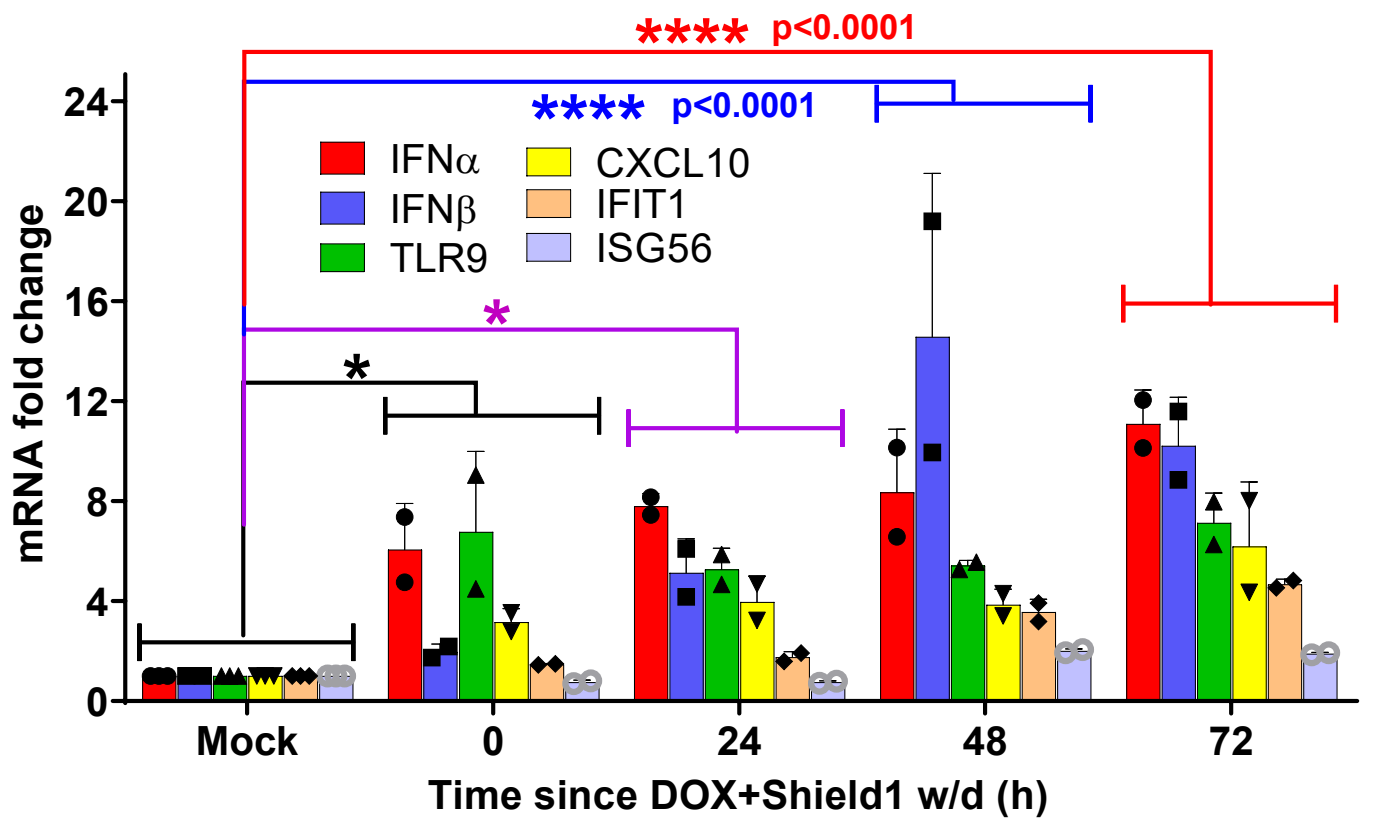
A



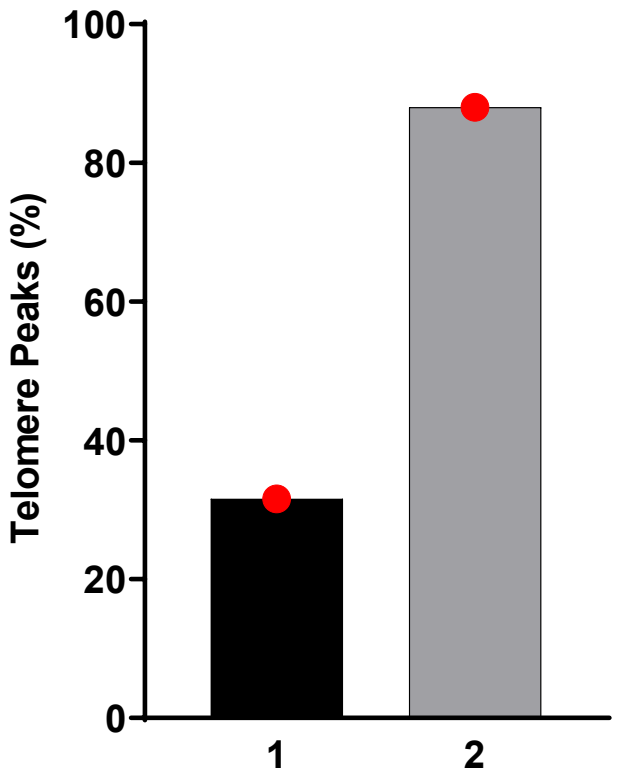
B



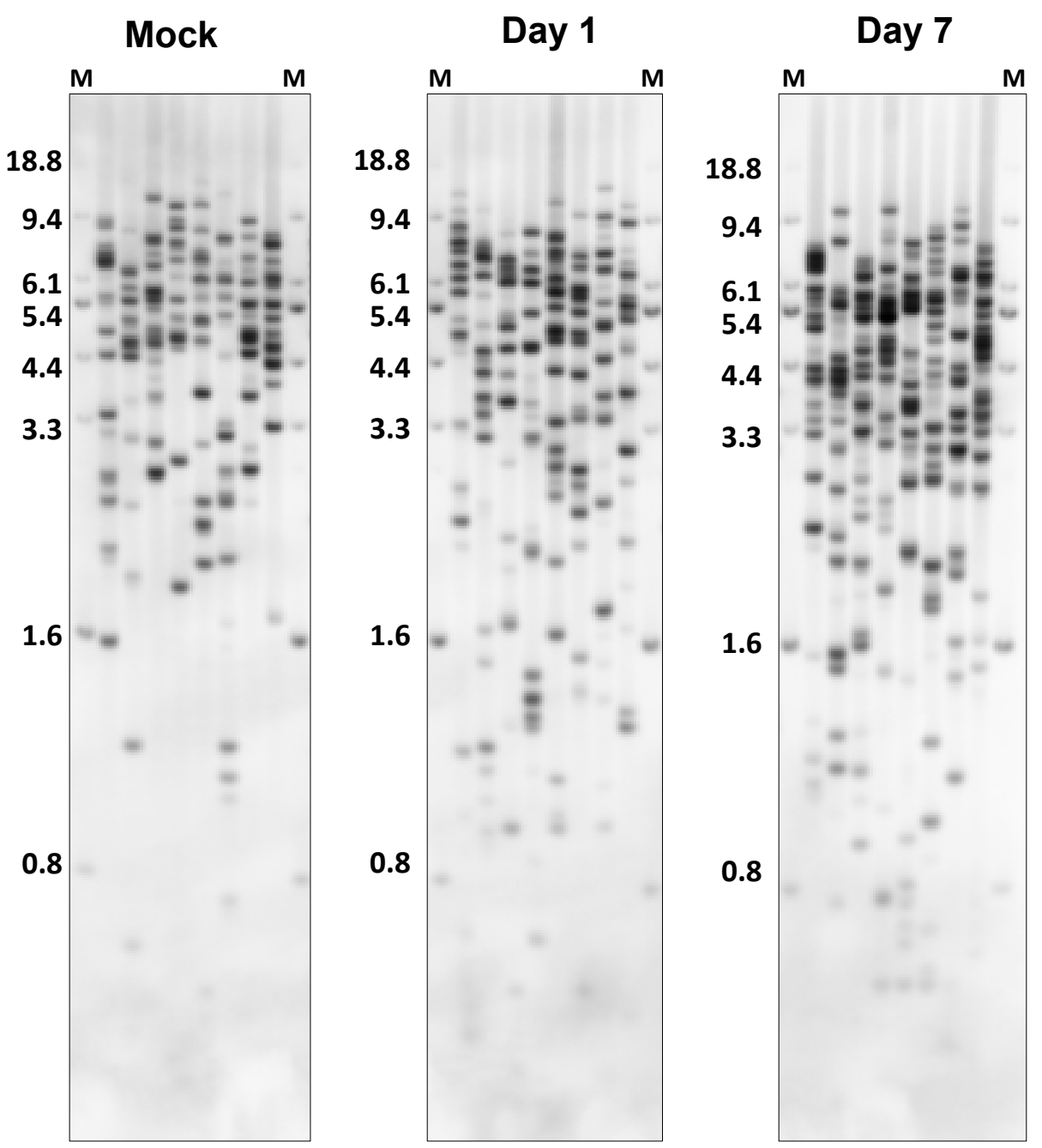
C



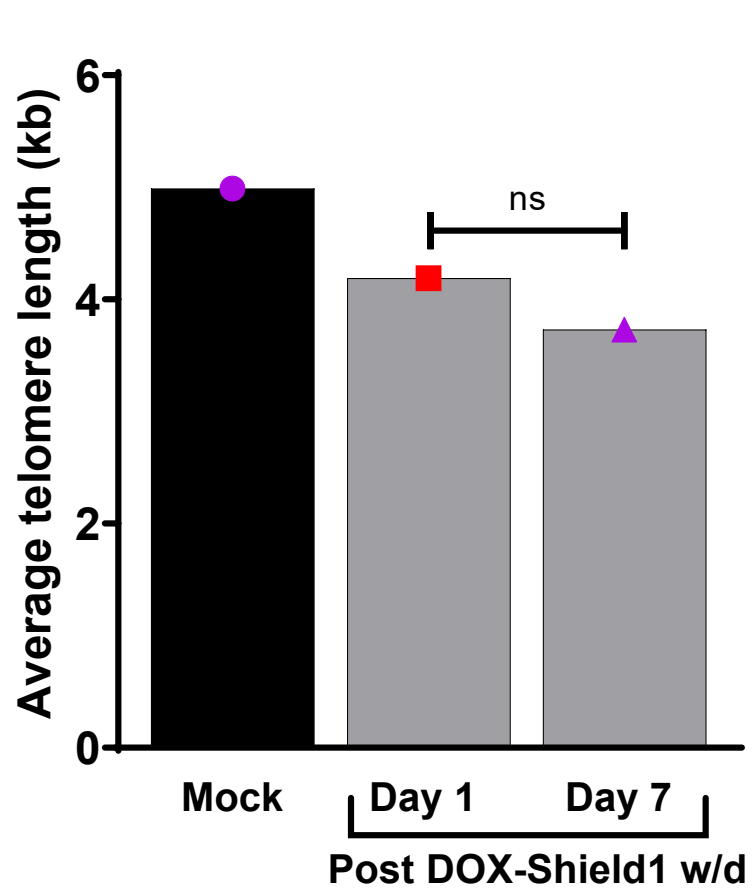
D

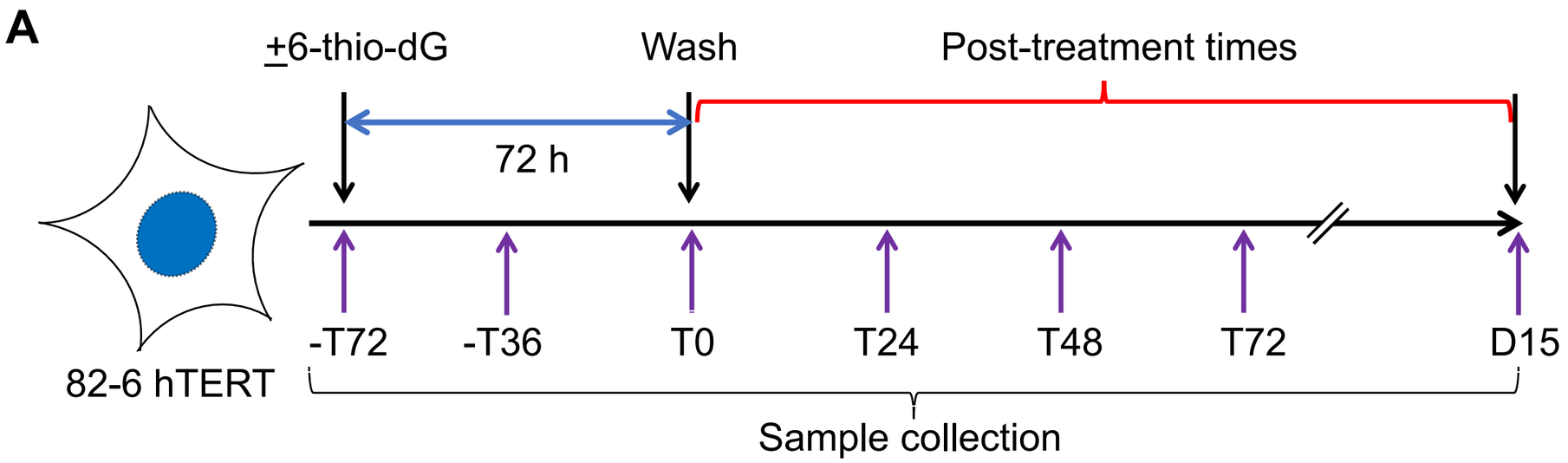


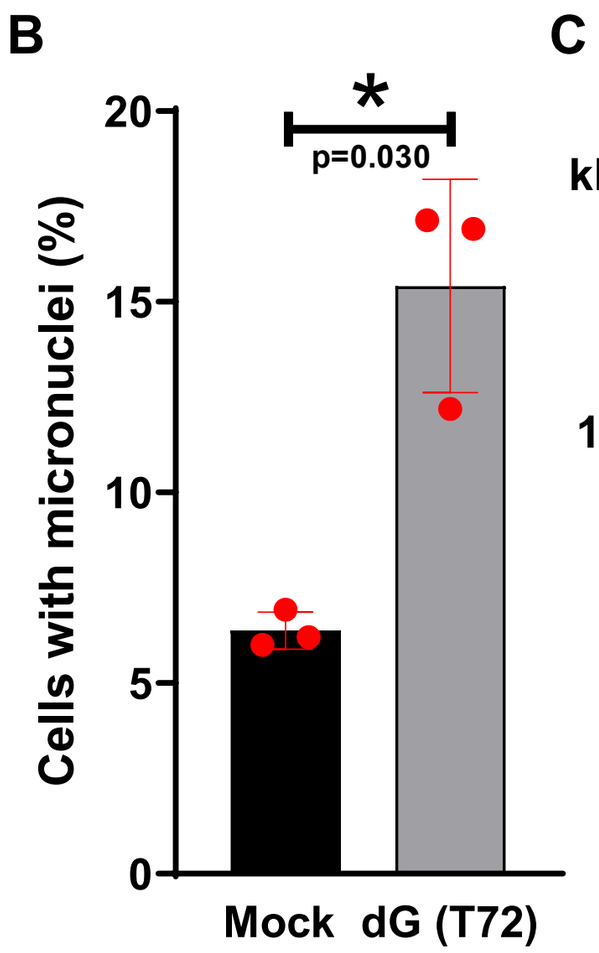
Ei

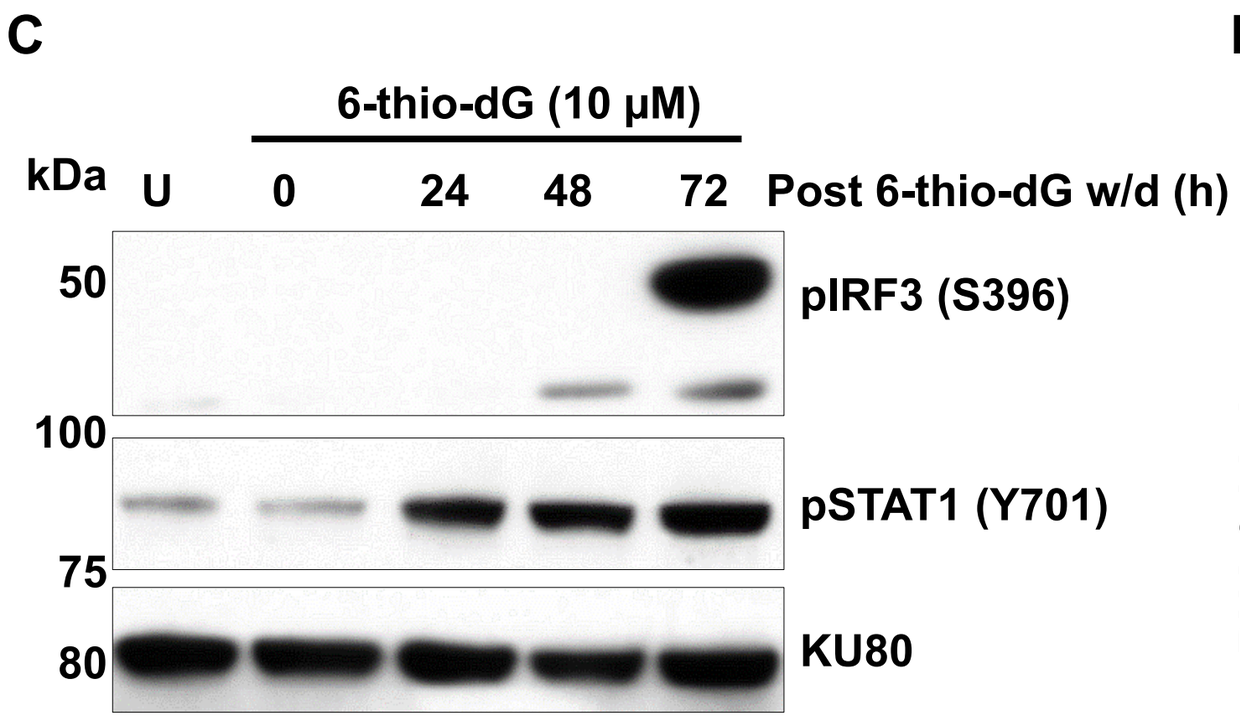


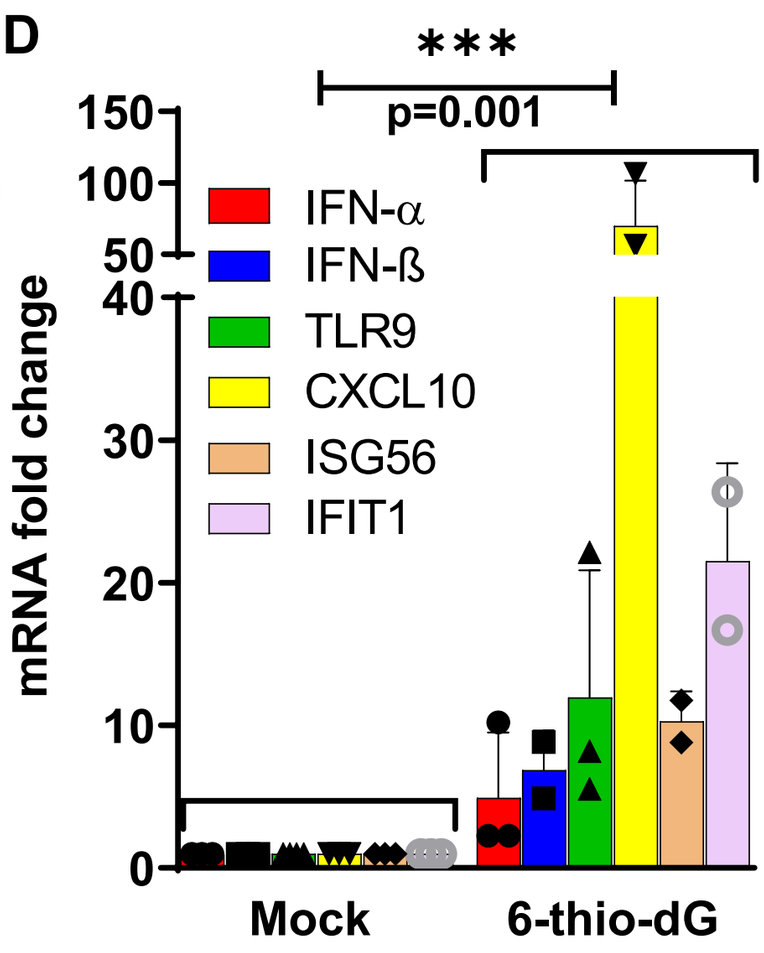
Eii

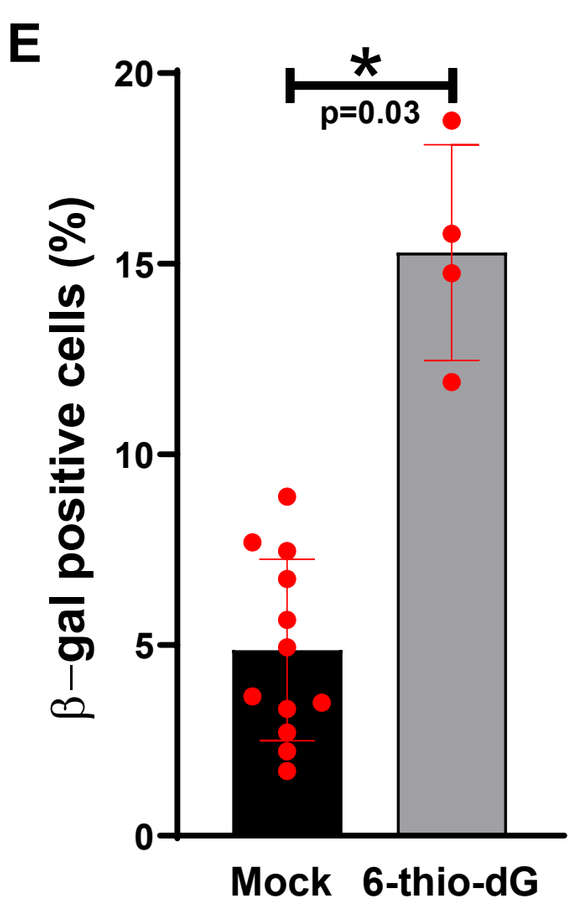


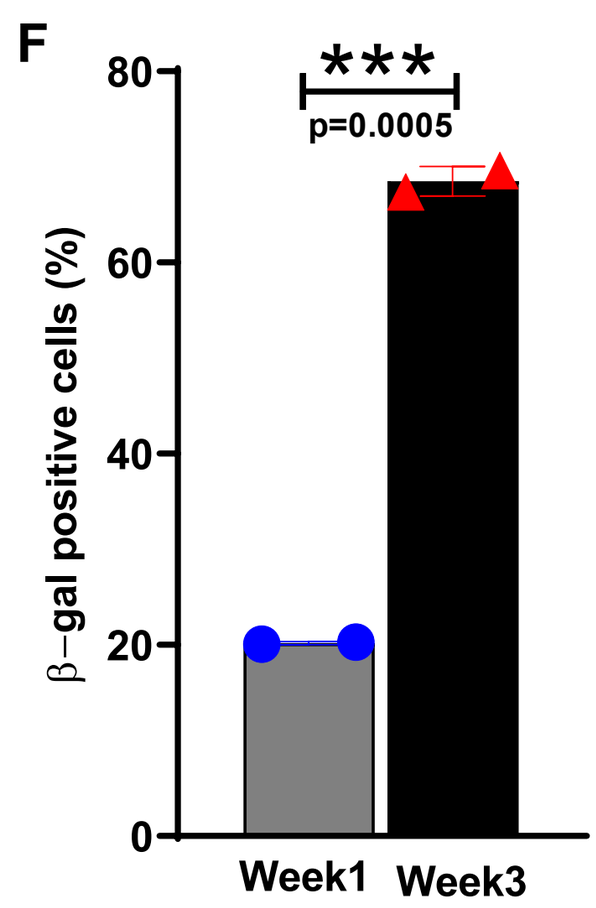

A
±6-thio-dG
Wash
Post-treatment times
72 h
82-6 hTERT
-T72
-T36
T0
T24
T48
T72
D15
Sample collection

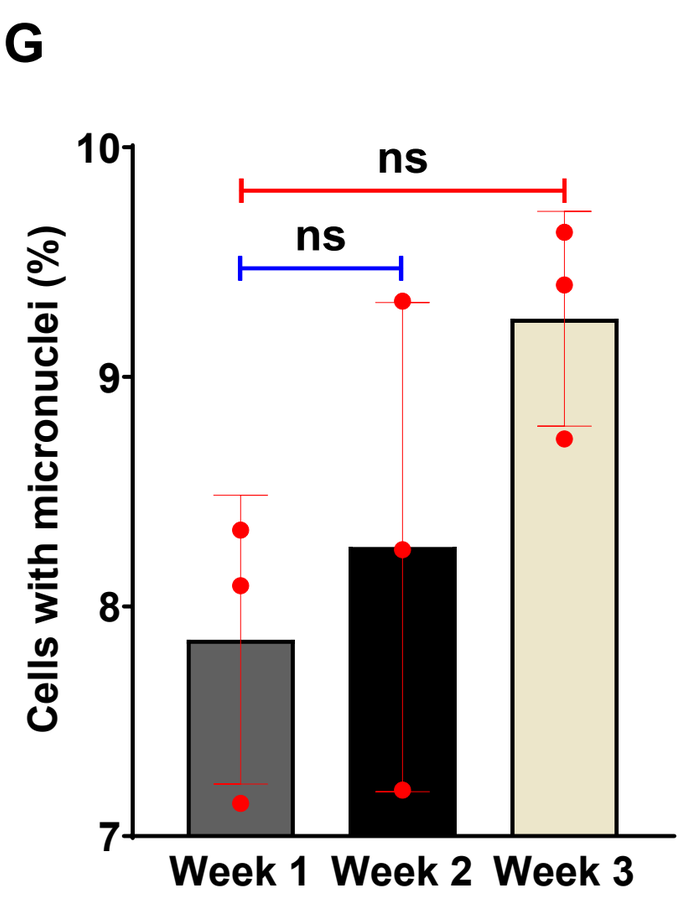

B
Cells with micronuclei (%)
*
p=0.030
Mock
dG (T72)


C
6-thio-dG (10 µM)
kDa
U
0
24
48
72
Post 6-thio-dG w/d (h)
pIRF3 (S396)
pSTAT1 (Y701)
KU80


D
***
p=0.001
mRNA fold change
IFN-α
IFN-ß
TLR9
CXCL10
ISG56
IFIT1
Mock
6-thio-dG


E
β−gal positive cells (%)
*
p=0.03
Mock
6-thio-dG


F
β−gal positive cells (%)
***
p=0.0005
Week1
Week3


G
Cells with micronuclei (%)
ns
ns
Week 1
Week 2
Week 3

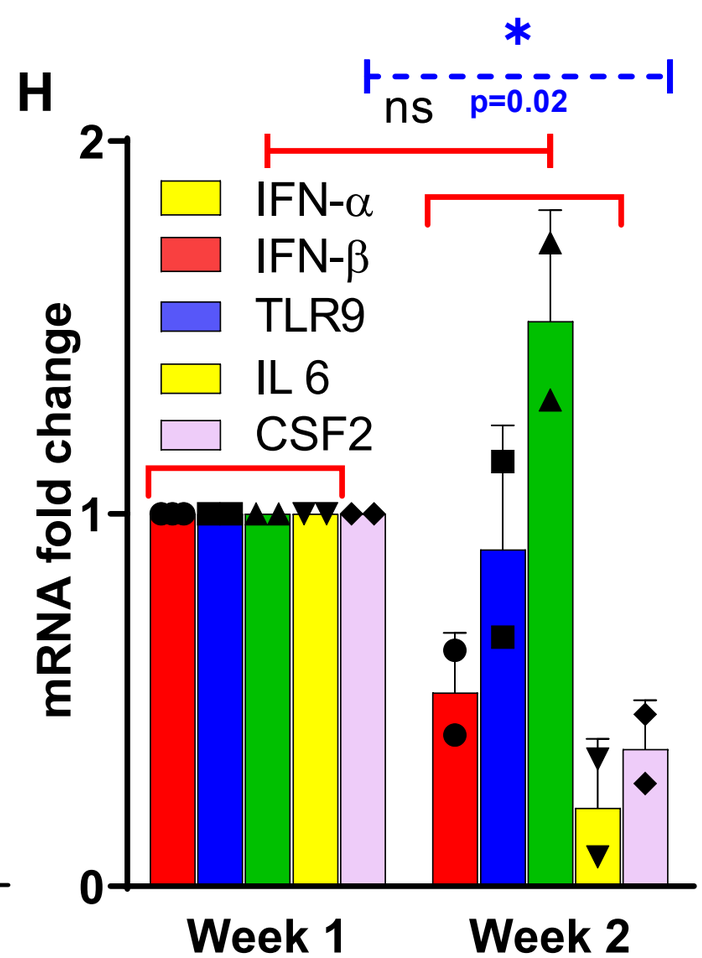

H
*
ns
p=0.02
mRNA fold change
IFN-α
IFN-β
TLR9
IL 6
CSF2
Week 1
Week 2

Figure S3

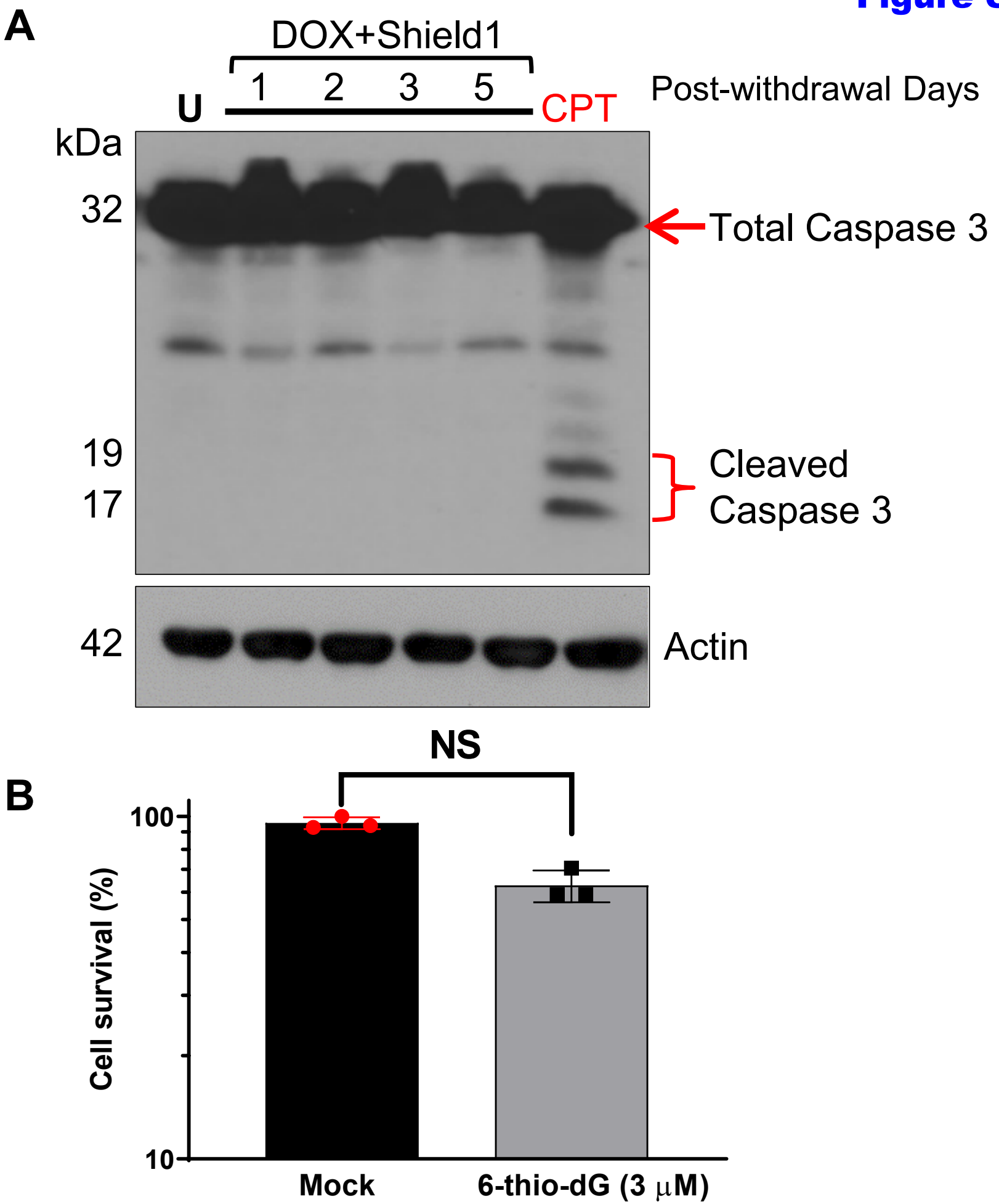

A
DOX+Shield1
1 2 3 5
U
CPT
Post-withdrawal Days
kDa
32
19
17
Total Caspase 3
Cleaved
Caspase 3
42
Actin
B
NS
100
10
Cell survival (%)
Mock
6-thio-dG (3 μM)

Table S1

Serial #	Primer Name	Sequence (5'-3')
1	TRF2-Nhe1-F	GCTAGC **GCTAGC** CCACC ATG GCG GGA GGA GGCGG
2	TRF2-Flag-SalI-R	GTCGAC GTCGAC TTA AGATCCCTTGTCGTCATCGTCCTTGTAGTCCTTGTCGTCATCGTCCTTGTAGTC GTTCATGCCAAGTCTTTTCATGG
3	TRF2-45AA_Nhe1-F	**GCTAGCGCTAGC**CCACC**ATG**GCACGGCTGGAAGAGG
4	TRF2-453AA_Flag-SalI-R	GTCGACGTCGACTTA AGATCCCTTGTCGTCATCGTCCTTGTAGTCCTTGTCGTCATCGTCCTTGTAGTCGTTTTCTACAGTCCACTTCTGCTTTTTTG
7	DD-Nhe1-F	CGATGT GCTAGC ATGGGAGTGCAGG
8	DD-Nhe1--XhoI-R	CGCGCG GCTAGC CTC GAG TTC CGG TTT TAGAAGCTCC

Table S2

Sl. No.	Primary Antibodies	Catalog #	Vendor	Application (dilution)
1	Mouse monoclonal anti-TRF2	100-56506	Novus Bio	WB (1:500)
2	Rabbit polyclonal anti-STING	24683	Novus Bio	WB (1:500)
3	Rabbit polyclonal anti-TRF2	NA	gift from Karlsensder lab	IF (1:100)
4	Mouse monoclonal anti-FLAG	F3165	Sigma	WB (1:2000); IF (1:500)
5	Mouse monoclonal anti-γ-Tubulin (GTu88)	T6557	Sigma	WB (1:50000)
6	Rabbit polyclonal anti-phospho STING (Ser366)	85735	Cell Signaling	WB (1:700)
7	Rabbit monoclonal anti-phospho-IRF3 (Ser396;4D4G)	4947	Cell Signaling	WB (1:500)
8	Rabbit monoclonal anti-phospho-TBK1 (Ser172;D52C2)	5483	Cell Signaling	WB (1:500)
9	Rabbit polyclonal anti-phospho-CHK1 (Ser317)	2344	Cell Signaling	WB (1:1000)
10	Rabbit monoclonal anti-phospho-Histone H2AX (Ser139;20E3)	9718	Cell Signaling	IF (1:1000)
11	Rabbit polyclonal anti-phospho-CHK2 (Thr68)	2661	Cell Signaling	WB (1:1000)
12	Rabbit monoclonal anti-cGAS (D1D3G)	15102	Cell Signaling	WB (1:200); IF (1:100)
13	Rabbit monoclonal anti-caspase-3 (8G10)	9665	Cell Signaling	WB (1:500)
14	Rabbit polyclonal anti-phospho-ATM (Ser1981; EP1890Y)	81292	Abcam	WB (1:10000); IF (1:500)
15	Mouse monoclonal anti-phospho Stat1 p84/p91 (PSM1)	51700	Santacruz	WB (1:200)
16	Mouse monoclonal anti-Lamin A/C (636)	7292	Santacruz	IF (1:300)
17	Mouse monoclonal anti-ATM (2C1)	70103	Gentex	WB (1:5000)
18	Mouse monoclonal anti-phospho-Histone H2AX (Ser139; JBW30	05-636	Millipore	IF (1:1000)
19	Rabbit monoclonal anti-DNA-PKcs (Phospho S2056)	18192	Abcam	IF (1:400)
20	Mouse monoclonal anti-KU80	NA	homemade	WB (1:1000)
21	Rabbit polyclonal anti-phospho-Histone H3 (Ser10)	06-570	Millipore	IF (1:100)
22	Rabbit polyclonal anti-phospho-KAP1 (Ser824)	A300-767A	Bethyl	WB (1:5000)

Table S3

Serial #	Primer Name	Sequence (5'-3')
1	human IFNα Fwd (5'-3')	AACTCCCCTGATGAATGCGG
2	human IFNα Rev (5'-3')	TAGCAGGGGTGAGAGTCTTTG
3	human IFNβ Fwd (5'-3')	CAACTTGCTTGGATTCCTACAAAG
4	human IFNβ Rev (5'-3')	TATTCAAGCCTCCCATTCAATTG
5	human TLR9 Fwd (5'-3')	CGCCCTGCACCCGCTGTCTCT
6	human TLR9 Rev (5'-3')	CGGGGTGCTGCCATGGAGAAG
7	human CCL5 Fwd (5'-3')	CCAGCAGTCGTCTTTGTCAC
8	human CCL5 Rev (5'-3')	CTCTGGGTTGGCACACACTT
9	human CXCL10 Fwd (5'-3')	AGGAACCTCCAGTCTCAGCA
10	human CXCL10 Rev (5'-3')	CAAAATTGGCTTGCAGGAAT
11	human IFIT1 Fwd (5'-3')	CCTCCTTGGGTTCGTCTACA
12	human IFIT1 Rev (5'-3')	GGCTGATATCTGGGTGCCTA
13	human IFIT3 Fwd (5'-3')	GAAGGAACTGGGCCGCCTGCTAAG
14	human IFIT3 Rev (5'-3')	GCCCTGGCCCATTTCCTCACTACC
15	human ISG56 Fwd (5'-3')	TTGATGACGATGAAATGCCTGA
16	human ISG56 Rev (5'-3')	CAGGTCACCAGACTCCTCAC
17	human ISG54 Fwd (5'-3')	AAGCACCTCAAAGGGCAAAAC
18	human ISG54 Rev (5'-3')	TCGGCCCATGTGATAGTAGAC
19	human IL6 Fwd (5'-3')	CCTTCGGTCCAGTTGCCTTCT
20	human IL6 Rev (5'-3')	GCATTTGTGGTTGGGTCA
21	human CSF2 Fwd (5'-3')	AGCATGTGAATGCCATCCAG
22	human CSF2 Rev (5'-3')	AGGGGATGACAAGCAGAAAG
23	human β-Actin Fwd (5'-3')	TCGTCGACAACGGCTCCGGCATGT
24	human β-Actin Rev (5'-3')	CCAGCCAGGTCCAGACGCAGGAT