Supplement

Materials and methods

Chemicals

Nicotinamide, actinomycin D and p38 inhibitor SB203580 were purchased from Sigma. Doxorubicin was purchased from Enzo Life Sciences (Plymouth Meeting, PA). Nutlin-3 was purchased from Cayman Chemical (Ann Arbor, MI).

Antibodies for immunoblots

The antibodies against Hsp27, Hsp70 (SPA810) and Hsf1 were purchased from Enzo Life Science. SIRT1, β-actin, p-Hsp27, p-IκB, IκB, p-p38 and p38 were from Cell Signaling Technology (Danvers, MA). SIRT1 for mouse was from Millipore (Lake Placid, NY). p53 and HuR were from Santa Cruz Biotechnology (Santa Cruz, CA). p21 and p16 were from BD Biosciences (San Jose, CA).

Cell cultures

HEK293 cells were from American Type Culture Collection and maintained in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S); TIG-1 human fetal lung fibroblasts were obtained from the Coriell Cell Repository (CCR, Camden, NJ) and maintained in Minimum Essential Medium (MEM), supplemented with 10% FBS and Penicillin/Streptomycin, at 5% CO_2, 37°C incubator. For heat shock at 43°C a circulating water bath was used. Ionizing Radiation of cells was done using 137Cs source (GammaCell 40, Nordion International, Inc., Ontario, Canada) at 60 rad/min.

Hsf1 knockout mouse

Generation and characterization of Hsf1 knockout mouse has been described previously (Xiao *et al.* 1999). Hsf1 knockout mice and age-matched control littermates were maintained in compliance with the guidelines of the Institutional Animal Care and Use Committee.

Senescence-associated β-Galactosidase (SA-β-Gal) Staining

Cell staining of SA-β-Gal was performed using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, Danvers, MA).

Plasmids and shRNAs

Retroviral and lentiviral vectors, and infection - RNAi-Ready pSIREN-RetroQ vector from BD Biosciences retroviral delivery system was used for knockdown of Hsf1. The sequence of human Hsf1 gene was selected as reported before [33]: 5′-TATGGACTCCAACCTGGATAA-3′. pBABE HSF1-FLAG [34] was purchased from Addgene plasmid 1948 (Cambridge, MA). shRNA for SIRT1 (clones TRCN0000018979 and TRCN0000018983) and HuR (clones TRCN0000017274 and TRCN0000017276) were purchased from Open Biosystems (Huntsville, AL). p21 retroviral expression vector was constructed by cloning PCR product into pQCXIN vector at AgeI and PacI. pBABE-p16 was used as before [35]. As controls, retroviral or lentiviral vectors without an insert were used. Retroviruses were produced by transfection of 293T cells with plasmids expressing retroviral proteins Gag-Pol, G (VSVG pseudotype) and the constructs with Lipofectamin 2000. Lentiviruses were produced by transfection of 293T cells with plasmids psPAX2, pMD2.G and our constructs. At 48 hours after transfection, supernatants containing retrovirus were collected and frozen at -80°C. Cells were infected with twice diluted supernatant and 10μg/mL polybrene overnight and washed, and selected with puromycin, G-418 or blasticidin 1μg/mL 48 hours after infection.

Hsf1 reporter virus

HSE-luc lentiviral vector: six tandem repeats of heat shock elements (HSE) GAACCTTCGCGAATTTTCAAGAATGTTCAGGAATATTCTAGAACTTTCCTGAACCTT were inserted into pGreenFire lentiviral vector (System Biosciences, Mountain View, CA) at the ClaI and SpeI sites. Detailed description of the luciferase assay can be found in Supplementary

Immunoprecipitation

Immunoprecipitation was conducted using Direct IP Kit (Pierce) following manufacturer's instructions. Briefly, TIG-1 cells were first infected with Hsf1-FLAG retrovirus and then infected with shSIRT1 or empty vector, selected for 2 days post infection and grown for 5 days with or without 10μM nutlin-3. After heat shock at 42°C for 1 hour cells were immediately lysed, and Hsf1-FLAG was immunoprecipitated with anti-Flag M2-agarose (Sigma) according to the manufacturer's instructions. The pulled-down proteins were analyzed by immunoblotting for SIRT1 and FLAG.

Total RNA isolation and qRT-PCR

Tissues from mice were harvested and preserved in RNAlater reagent (Qiagen). Total RNA from tissues or cells was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed with RetroScript Kit (Ambion), following manufacturer's instructions. Quantitative Real-Time PCR (qRT-PCR) was performed using SYBR Green Rox Master Mix (Qiagen). Primers for Hsf1 were purchased from SABiosciences. Primer sequences used in qRT-PCR are listed in the Supplementary Methods (Supporting information).

Other methods

The densitometric quantitations were done using the LAS-3000 and Image Gauge from Fuji or ImageJ. Statistical analysis was done using Student's t-test.

qRT-PCR primers

SIRT1: For 5’- TGGCAAAGGAGCAGATTAGTAGG-3’; Rev 5’-CTGCCACAAGAACTAGAGGATAAGA-3’

HuR: For 5’-CAGGAAACGCCTCCTCCGGC-3’; Rev 5’-ACGGCACCAAACGGCCCAAA-3’

GAPDH: For 5’- GGCCTCCAAGGAGTAAGACC-3’; Rev 5’-AGGGGAGAGATTCAGTGTGGTG-3’

IL-6: For 5’-CCAGGAGCCCAGCTATGAAC-3’; Rev 5’-CCCAGGGAGAAGGCAACTG-3’

IL-8: For 5’-TTGGCAGCCTTCCTGATTTC-3’; Rev 5’-TCTTTAGCACTCCTTGGCAAAAC-3’

Mouse SIRT1: For 5’-AGCAACATCTCATGATTGGCACCG-3’; Rev 5’-TCTGCCACAGCGTCATATCATCCA -3’

Mouse HuR: For 5’- GGATGACATTGGGAGAACGAAT-3’; Rev 5’-TGTCCTGCTACTTTATCCCGAA-3’

Mouse GAPDH: For 5’- AAATTCAACGGCACAGTCAAGG-3’; Rev 5’-GCCTCACCCCATTTGATGTTAGT-3’

Luciferase Assay

Cells were infected with HSE-luc lentivirus and treated with Nutlin-3, doxorubicin or γ-irradiation or infected with p21, p16, shSIRT1 or HuR constructs 24 hours later. 5 days later, cells were heat shocked at 43°C for 35 minutes. After 6 hour incubation, medium was aspirated; lysis buffer was added and was frozen at -80°C. Upon thawing, lysates were plated into 96 well plate. 50µl of luciferase assay reagent (Promega) was injected into each well and luminescence was read by luminometer. In parallel, lysates were plated into black 96 well plate and fluorescence was read by the same luminometer. All measurements were done in triplicates, and the assays were repeated three times.

Supporting information

S1. Werner Syndrome fibroblasts (passage 4) exhibited prematurely senescent phenotype when compared to age matched controls (passage 15).

S2. (A) Early passage TIG-1 fibroblasts were treated with 10Gy γ-irradiation and cultured for 6 days. Cells were fixed, stained with rabbit anti-Ki-67 antibody and anti-rabbit IgG conjugated with Texas-Red secondary antibody. Images were acquired with Axiovert 200 (Carl Zeiss, Oberkochen, Germany) microscope with an ×100 objective using the manufacturer's software. Radiation decreased % of cells with Ki-67 staining. (B) Early passage cells were treated with 100nM Dox or 10Gy Rad and cultured for 5 additional days. DNA damage caused increased SA- β-gal staining.

S3. Cells were infected with empty vector, retroviral shRNA for p53 or recombinant p21. Two days post infection, 10μM nutlin-3 was added for additional 5 days. (A) nutlin-3 treatment or expression of p21 caused increased staining with SA-β-gal, whereas p53 depletion prevented staining in nutlin-3 treated cells. (B) Nutlin-3 treatment led to accumulation p53 and p21 without directly causing DNA damage, and did not cause p16 upregulation. (C) Depletion of p53 prevented p21 accumulation after nutlin-3 or 6 Gy Rad treatment.

S4. Early passage TIG-1 was infected with empty vector or shRNA against p21 and two days later treated with 10μM nutlin-3. p21 depletion did not restore the level of Hsp70 accumulation after nutlin-3.

S5. Cells treated with 100nM Dox was incubated with or without 10μM SB for 5 days. Cell lysates were immunoblotted for phosphorylated forms of p38MAPK and Hsp27.

S6. (A) Cells with lentiviral NFκB luciferase reporter was treated with 10Gy γ-irraidation and 3 days later infected with either lentiviral empty vector or shRelA and selected. 6 days after Rad, cells were collected for luciferase assay. The means and ±SEM indicate 3 independent experiments. (B) Cells treated as (A) were collected for RNA. qRT-PCR was performed using IL-6, IL-8 and GAPDH mRNA. IL-6 and IL-8 mRNA dramatically increased in Rad treated cells and SB inhibited the induction. The mean and ±SEM were from triplicates of 2 independent experiments. Abbreviation: Rad – γ-irradiation, SB – SB203580.

S7. Cells treated with 10Gy Rad was lysed and immunoblotted for p16. Rad increased p16 levels. Abbreviation: Rad – γ-irradiation.

S8. (A) Cells were treated with 100nM Dox overnight and cultured for 6 days or retroviral expression of p21 or p16 for 6 days, and immunoblotted for SIRT1. SIRT1 decreased after DNA damage induced senescence. (B) Same set of cells as (A) were collected for RNA and qRT-PCR was performed using SIRT1 and GAPDH (housekeeping gene for control) mRNA specific primers. As in protein, SIRT1 mRNA was significantly decreased in senescent cells. The means

and ±SEM are from 3 independent experiments. Abbreviations: CT - control, Dox - doxorubicin, Vec - vector.

S9. Early passage TIG-1 was infected with lentiviral empty vector or shRNAs against HuR and SIRT1. Two days post infection, 10μM nutlin-3 was added for additional 5 days. Nutlin-3 treatment alone decreased SIRT1 and HuR. HuR depletion caused SIRT1 decrease, whereas SIRT1 depletion did not cause HuR decrease (not shown).

S10 (A) Cells were treated with 100nM Dox overnight and cultured for 6 days or retroviral expression of p21 or p16 for 6 days, and immunoblotted for HuR. HuR decreased after DNA damage induced senescence. (B) Same set of cells as (A) were collected for RNA and qRT-PCR was performed using HuR and GAPDH (housekeeping gene for control) mRNA specific primers. As in protein, HuR mRNA was significantly decreased in senescent cells. The means and ±SEM are from 3 independent experiments. Abbreviations: CT - control, Dox - doxorubicin, Vec - vector.

S11. HuR mRNA half-life was measured in control and nutlin-3 induced senescent cells by incubating with 5μg/ml of actinomycin D and collecting RNA after 45 minutes and 90 minutes. HuR and GAPDH mRNA were measured qRT-PCR and normalized by GAPDH mRNA. Data is represented by percentage of HuR mRNA measured at time 0 min (prior to adding actinomycin D), in semi-logorithmic scale. Half-life is calculated as time need for 50% reduction of mRNA. The means and ±SEM was calculated from triplicates of two independent experiments.

S12. Early passage TIG-1 was treated with 10μM nutlin-3 for 5 days and 5mM nicotinamide was added overnight before heat shock. Cell lysates were immunoblotted for Hsp70. Nicotinamide suppressed Hsp70 induction in early TIG-1 fibroblasts as nutlin-3 treatment.

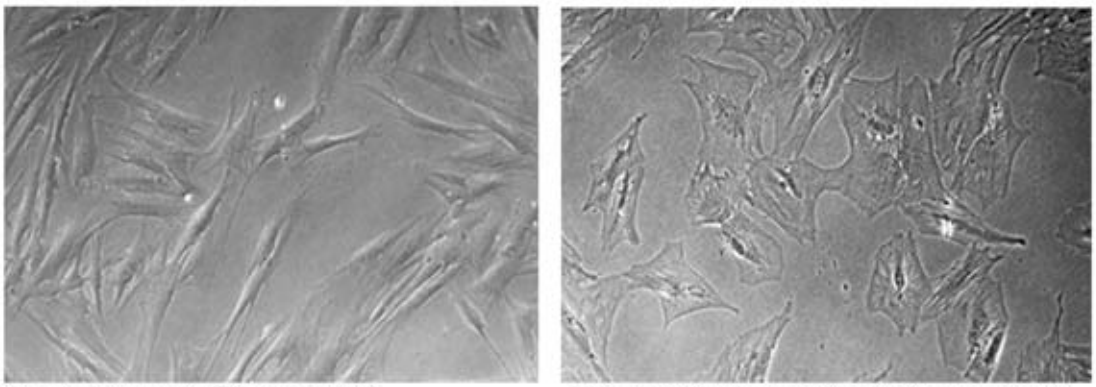
S13. (A) Cells from S12 were immunoblotted for HuR, SIRT1, p53 and p21. Overnight nicotinamide treatment did not cause decrease HuR and SIRT1, and did not induce senescence by p53-p21. (B) Early passage TIG-1 was infected with lentiviral shRNA against SIRT1 and two days later incubated with 10μM nutlin-3 for 5 additional days. SIRT1 depletion alone did not cause increase in p53 and p21

S14. SIRT1 depletion alone did not cause increased staining of SA-β-gal.

S15. TIG-1 cells were infected with retroviral empty vector or shRNA against p53 and treated with 6Gy Rad. After 5 days post irradiation, cells were lysed and immunoblotted for HuR, SIRT1 and Hsf1. p53 depletion increased basal level of HuR, SIRT1 and Hsf1, and prevented senescence associated decrease of those proteins.

S16. (A) Early passage cells were pre-incubated with 10μM SB for 6 hours then co-incubated with 100nM Dox for 2 days. After removing Dox, SB was added for the additional 4 days. Administration of SB to Dox treated cells led to partial restoration of HuR, SIRT1 and Hsf1. (B) Same set of cells as (A) were heat shocked and incubated for 6 hours before collection for

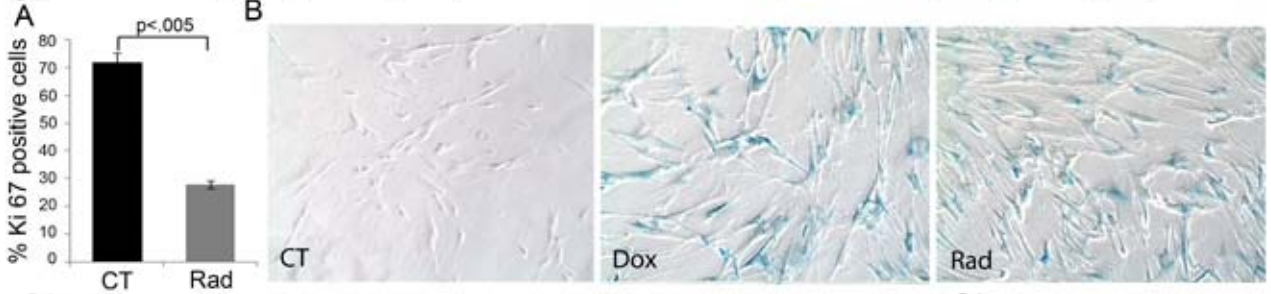
S1



Control Age 22 (Passage 15)

Werner Syndrome Age 19(Passage 4)

S2



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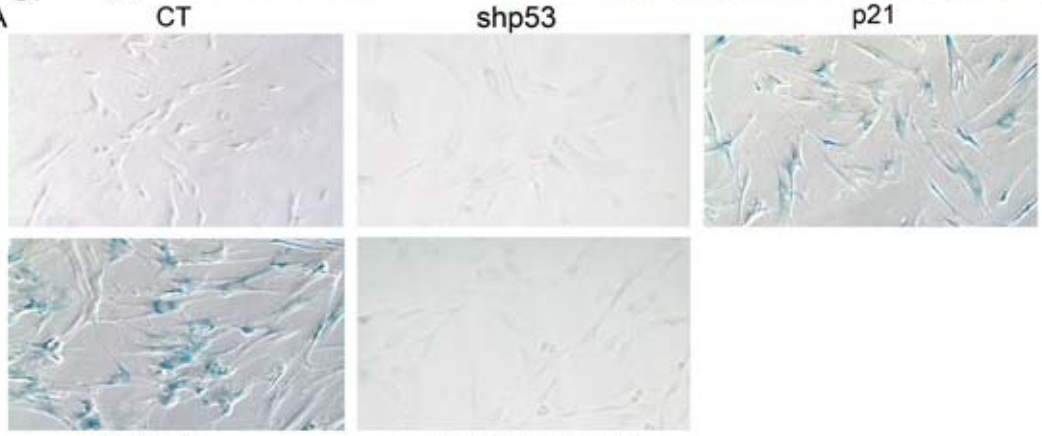
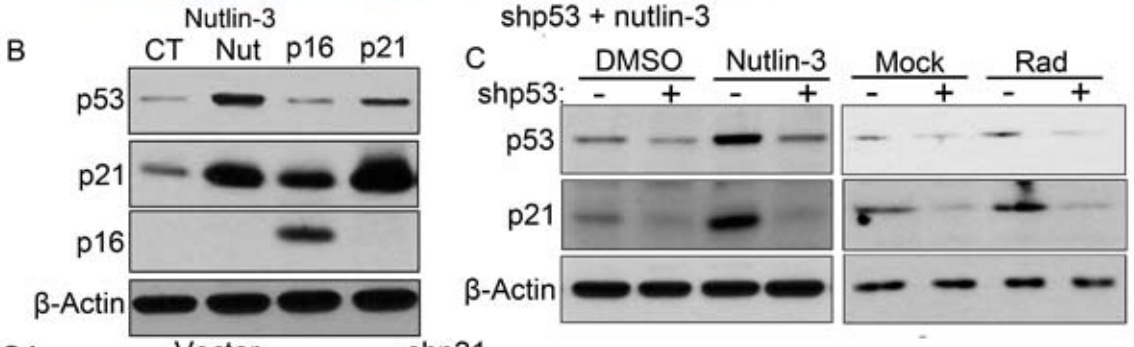
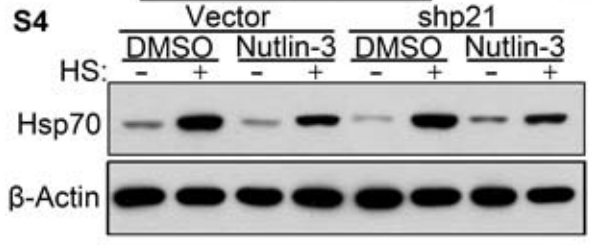
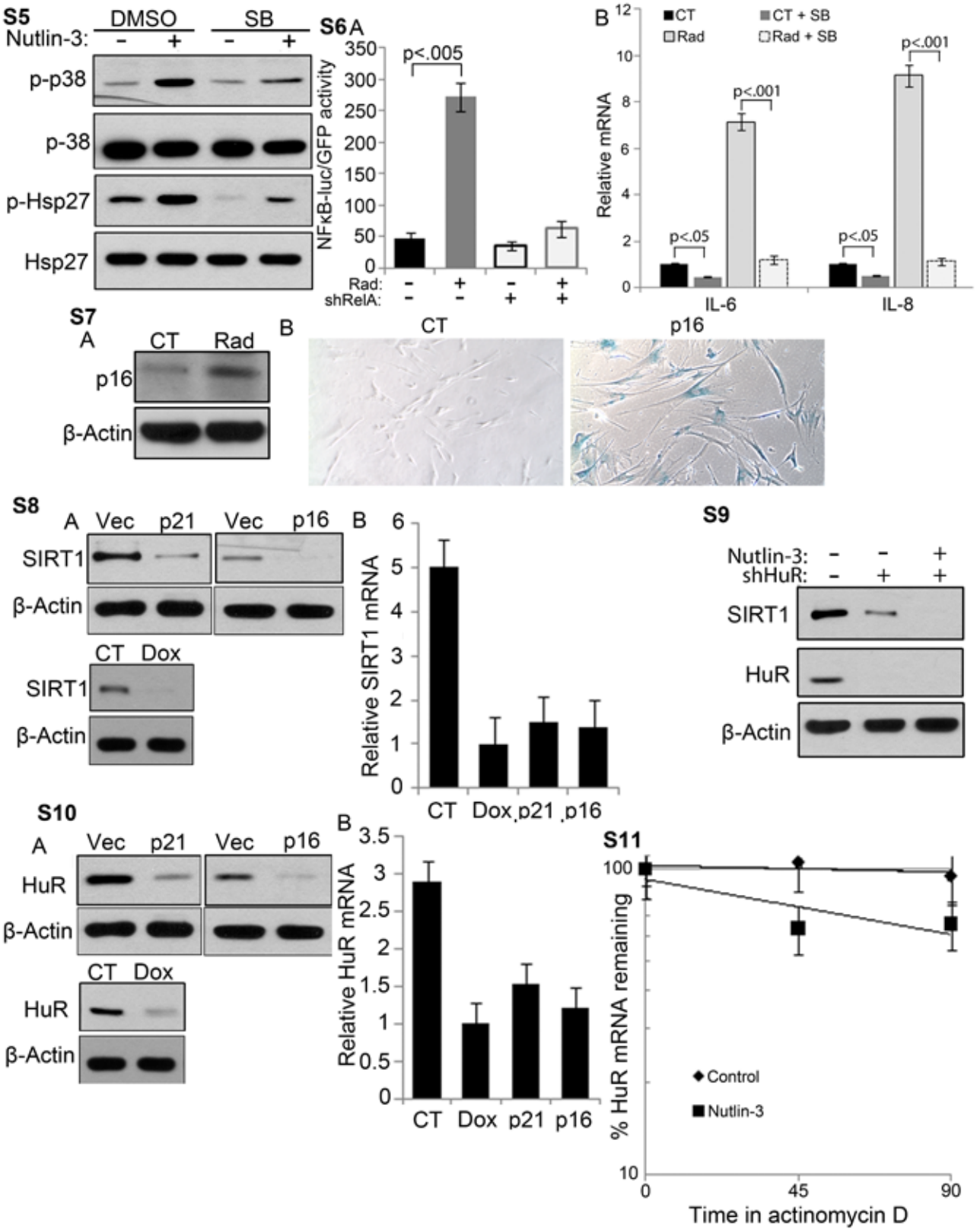
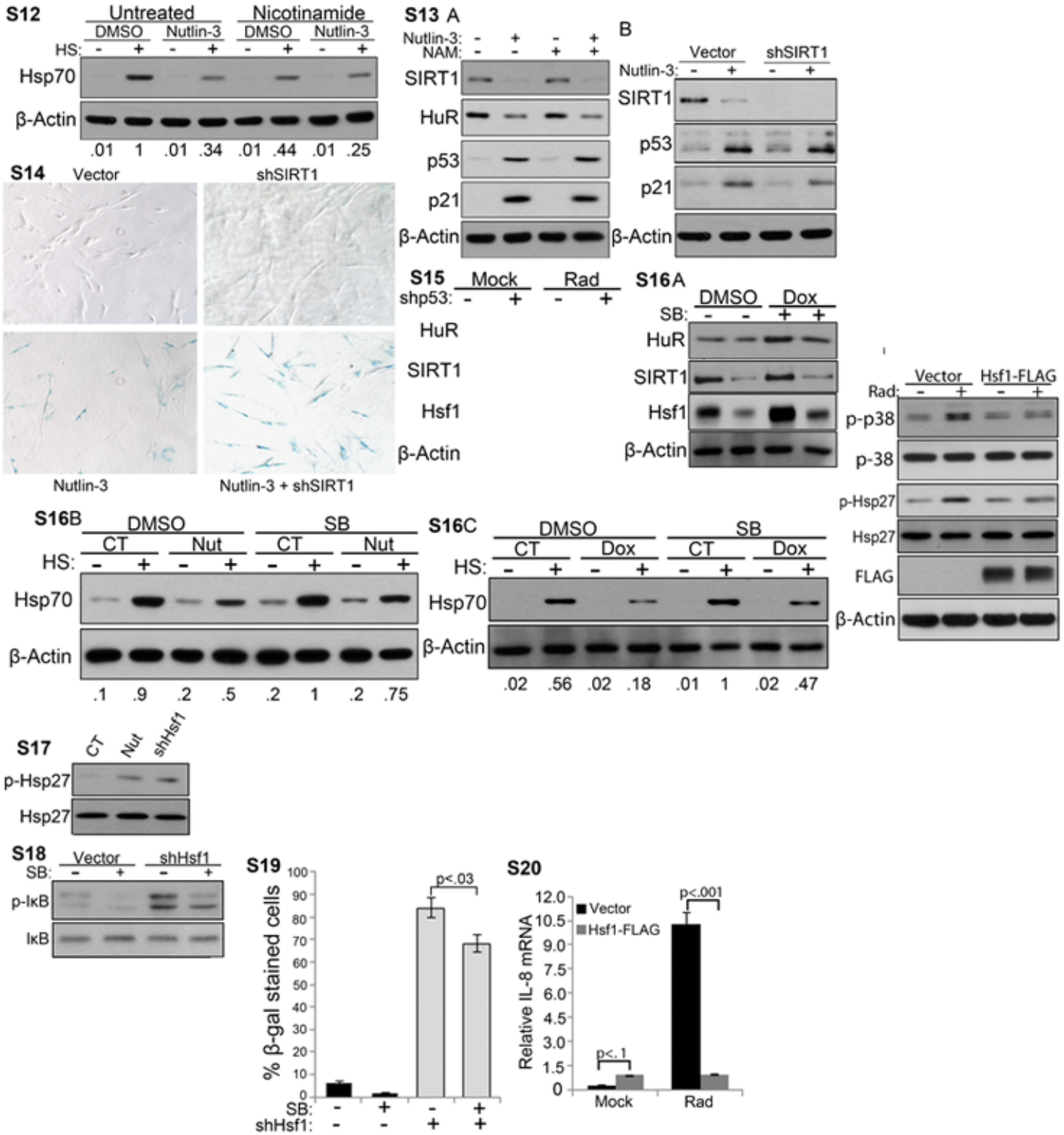




S4




S5
DMSO
SB
Nutlin-3:
p-p38
p-38
p-Hsp27
Hsp27
S6 A
NFκB-luc/GFP activity
p<.005
Rad:
shRelA:
B
CT
CT + SB
Rad
Rad + SB
Relative mRNA
p<.001
p<.05
IL-6
IL-8
S7
A
CT
Rad
p16
β-Actin
B
CT
p16
S8
A
Vec
p21
Vec
p16
SIRT1
β-Actin
CT
Dox
B
Relative SIRT1 mRNA
S9
Nutlin-3:
shHuR:
SIRT1
HuR
β-Actin
S10
A
Vec
p21
Vec
p16
HuR
β-Actin
CT
Dox
B
Relative HuR mRNA
S11
% HuR mRNA remaining
Control
Nutlin-3
Time in actinomycin D


S12
Untreated
Nicotinamide
DMSO
Nutlin-3
DMSO
Nutlin-3
HS: - + - + - + - +
Hsp70
β-Actin
.01 1 .01 .34 .01 .44 .01 .25
S13 A
Nutlin-3: - + - +
NAM: - - + +
SIRT1
HuR
p53
p21
β-Actin
B
Vector
shSIRT1
Nutlin-3: - + - +
SIRT1
p53
p21
β-Actin
S14
Vector
shSIRT1
Nutlin-3
Nutlin-3 + shSIRT1
S15
Mock
Rad
shp53: - + - +
HuR
SIRT1
Hsf1
β-Actin
S16A
DMSO
Dox
SB: - - + +
HuR
SIRT1
Hsf1
β-Actin
Vector
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Rad: - + - +
p-p38
p-38
p-Hsp27
Hsp27
FLAG
β-Actin
S16B
DMSO
SB
CT
Nut
CT
Nut
HS: - + - + - + - +
Hsp70
β-Actin
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S16C
DMSO
SB
CT
Dox
CT
Dox
HS: - + - + - + - +
Hsp70
β-Actin
.02 .56 .02 .18 .01 1 .02 .47
S17
CT
Nut
shHsf1
p-Hsp27
Hsp27
S18
Vector
shHsf1
SB: - + - +
p-IκB
IκB
S19
% β-gal stained cells
100
90
80
70
60
50
40
30
20
10
0
p<.03
SB: - + - +
shHsf1: - - + +
S20
Relative IL-8 mRNA
12.0
10.5
9.0
7.5
6.0
4.5
3.0
1.5
0
Vector
Hsf1-FLAG
p<.001
p<.1
Mock
Rad