

## SUPPLEMENTAL INFORMATION

This Supplemental Information file contains Supplemental Materials and Methods, Supplemental Figures S1-S4 and Supplemental Tables S1-S2 in a PDF file.

### Supplemental Material and Methods

#### *Cell culture*

BJ human foreskin fibroblasts were maintained in Minimum Essential medium supplemented with 10% fetal calf serum, non-essential amino acids, glutamine and antibiotics. WI38 human fibroblasts, U2OS-HR reporter cell line, 293T and LinX-A retroviral packaging cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, glutamine and antibiotics. HMEC Human Mammary Epithelial Cells were grown in HuMEC Ready Medium containing HuMEC Basal Medium and contents of HuMEC Supplement Kit (Thermo Fisher Scientific). The parental cell lines were all purchased from ATCC and authenticated, and tested for mycoplasma within 6 months before analysis.

#### *Plasmids*

The expression vectors for miRNAs and the lentiviral miRNA library were purchased from Biosettia, Inc (San Diego, CA). The lentiviral pLV-miR-30-Locker that inhibits the expression of all miR-30 isoforms was also purchased from Biosettia, Inc. It expresses an EF1 $\alpha$  promoter-driven GFP mRNA containing sponges for miR-30a-e and U6 promoter-driven hairpin inhibitors for miR-30a-e. pcDNA3-flag-CHD7 was kindly provided by Dr. Joanna Wysocka (Stanford

University). The Ha-RasV12 expression vector was kindly provided by Dr. Scott Lowe (Memorial Sloan Kettering Cancer Center). The TNRC6A cDNA was obtained from Addgene. To construct the pGL4-TNRC6A 3'-untranslated region (UTR) reporter, the TNRC6A 3'-UTR sequence was amplified by PCR from the cDNA of BJ cells using primers TNRC6A-UTR-f (5'-CCGTGTAATTCTAGA ACTCACCGACCGGGACCT-3') and TNRC6A-UTR-r (5'-CGCCCCGACTCTAGA CCCAAAGATGCTCAGAGAGG-3') and cloned into pGL4 (Promega) digested with XbaI. The mutant TNRC6A 3'UTR reporter in which the 4 miR-30 binding sites are mutated were generated with primers TNRC6A-3'UTR-mut1, 5'-CTGTCCCAGTGTCAATGTATACTCAAGGGTGTCTTA-3'; TNRC6A-3'UTR-mut2, 5'-AGGTTCTCACTTTGAACCTTCTTGTATACAGATTTTTTTGTTTGTTTTTTGA-3'; TNRC6A-3'UTR-mut3, 5'-ACAGATTTTTTTGTTTGTTTTTTGAGAAAAAAAAATGTATACTCTTCCATCATTTAAA AAAAATG-3'; TNRC6A-3'UTR-mut4, 5' -AAAAGAAGTCAGTGGCTTTTAACTGTATACAAATAGAATGTGATTGTAAAATG-3', using the QuikChange® Multi Site-Directed Mutagenesis Kit (Agilent Technologies), following the manufacturer's protocol.

To construct the pGL4-CHD7 3'-untranslated region (UTR) reporter, the CHD7 3'-UTR sequence was amplified by PCR from the cDNA of BJ cells using primers CHD7-UTR-f (5'-CCGTGTAATTCTAGA CCAGTACCAGTTCAGTTCAGT-3') and CHD7-UTR-r (5'-CGCCCCGACTCTAGA CTCTGCATATCATGGGTCACCT-3') and cloned into pGL4 (Promega) digested with Xba I. And the 2 miR-30 binding site mutant 3'UTR were generated with primers CHD7-3'UTR-mut1, 5'-ACAAGTGGTAGTCCTACTGTATACACTCACAGTTAATGTTC-3'; CHD7-3'UTR-mut2, 5'

-AGCTCTAAACTAGCCTTGAAGTATACAGACATGACTTTGTAAATG-3' using QuikChange® Multi Site-Directed Mutagenesis Kit (Agilent Technologies) following manufacturer's protocol.

The short hairpin RNA (shRNA) constructs for CHD7, TNRC6A, TNRC6B, Ago2, and Dicer were designed based on the single oligonucleotide RNA interference technology (Biosettia). The DNA oligonucleotide for each shRNA was cloned into the lentiviral pLV-H1-EF1 $\alpha$ -puro vector, following manufacturer's protocol, and verified by DNA sequencing. The sequences of oligos used to generate shRNAs are shCHD7-2, 5'-

AAAAGCTGATGATTGGAAGAAATTTGGATCCAAATTTCTTCCAATCATCAGC  
-3'; sh CHD7-3, 5'-

AAAAGCAAGAATTTGGTGATCTATTGGATCCAATAGATCACCAAATTCTTGC  
-3'; shTNRC6A-1, 5'-

AAAAGCAGTTTATGTCCAGTCAATTGGATCCAATGACTGGACATAAACTGC  
-3'; shTNRC6A-2, 5'-

AAAAGCAACAATCTCGTCAACTTTTGGATCCAAAAGTTGACGAGATTGTTGC-3';  
shTNRC6B-1293, 5'-

AAAAGCAGAGAACAGGCTCAAATTTGGATCCAAATTTGAGCCTGTTCTCTGC  
-3'; shTNRC6B-3206, 5'-

AAAAGGAGGAAACAATGATTCATTTGGATCCAAATGAATCATTGTTTCCTCC  
-3'; shAgo2-1, 5'-

AAAAGCACGGAAGTCCATCTGAATTGGATCCAATTCAGATGGACTTCCGTGC-3';  
shAgo2-2, 5'-

AAAAGCAGGACAAAGATGTATTATTGGATCCAATAATACATCTTTGTCCTGC

-3'; shAgo2-3, 5'-

AAAAGGGTCTGTGGTGATAAATATTGGATCCAATATTTATCACCACAGACCC

-3'; shDicer-1, 5'-

AAAAAAGGCTTACCTTCTCCAGGCTTTGGATCCAAAGCCTGGAGAAGGTAAGCCTT

-3'; shDicer-4, 5'-

AAAATTTGTTGCGAGGCTGATTCTTGGATCCAAGAATCAGCCTCGCAACAAA

-3'. A scramble sequence

(5'-AAAAGCTACACTATCGAGCAATTTTGGATCCAAAATTGCTCGATAGTGTAGC-

3') was used as a negative control.

For pGL2-p16-3570~+306 and pGL2-p16-891~+306, p16 promoter -3570~+306 or -891~+306

region was generated by PCR and cloned into pGL2-Basic vector (Promega). For pBabeBlast-

p16-3570~+306-Luc and pBabeBlast-p16-891~+306-Luc, p16 promoter -3570~+306 or -

891~+306 region was cloned into pBabeBlast-Luc vector (8). For pGL2-p16-891Δ-119~-12 and

pBabeBlast-p16-891Δ-119~-12-Luc, p16 promoter -119~-12 region was deleted from pGL2-

p16-891~+306 and pBabeBlast-p16-891~+306-Luc respectively, using QuikChange II XL Site-

Directed Mutagenesis Kit (Agilent Technologies) according to manufacturer's instruction.

For pLV-EF1α-Cas9-sgCHD7-396, pLV-EF1α-Cas9-sgCHD7-403, pLV-EF1α-Cas9-sgCHD7-

405, we selected hCHD7 sgRNA sequences from the GeCKOv2 Human Library ([http://genome-](http://genome-engineering.org/gecko/?page_id=15)

[engineering.org/gecko/?page\\_id=15](http://genome-engineering.org/gecko/?page_id=15)) (sgRNA sequence for 396<sup>#</sup>, 5'-

GAAGAGACGGTCCAGCAGAC-3'; for 403<sup>#</sup>, 5'-CTCCAATGAAGGATCTACCC-3'; and for

405<sup>#</sup>, 5'-TTCTCTAGAAAGGGCTCCGA-3'), and cloned these sgRNA sequences into pLV-

EF1α-Cas9-puro (Biosettia) following manufacturer's protocol.

To construct lentiviral I-Sce-I expression vector, I-SceI was amplified from I-SceI-IRES-dsRed (ISIR) (35) by PCR using primers I-SceI-f: 5'-GCCCGGGTTGGATCCGCCACCATGTTTCATGCCTTCTTCTTTTTCTTCTTTTTCCTACA-3' and I-SceI-r: 5'-GGGAGAGGGGCTAGCTTATTTTCAGGAAAGTTTCGGAGGAGATAG-3' and cloned into pLentivirus-MCS-IRES-Bsd (Biosettia).

#### *Retrovirus-based and lentivirus-based gene transduction*

Recombinant retroviruses were packaged and transduced into cells as previously described(45). Recombinant lentiviruses were packaged and transduced into cells as previously described(16). Transduced cells were purified with 80 (BJ) or 50 (WI38) or 5 (HMEC)  $\mu\text{g}/\text{mL}$  of hygromycin B, 600  $\mu\text{g}/\text{mL}$  of G418, 1.2 (BJ) or 1 (U2OS, HMEC, WI38)  $\mu\text{g}/\text{mL}$  of puromycin, or 5  $\mu\text{g}/\text{mL}$  of blasticidin.

#### *Analysis of senescence*

For growth curves,  $10^4$  cells were seeded into each well in 12-well plates in duplicates. Every 3-5 days, cells were trypsinized from plates and cell numbers were counted with hemocytometer Counter. At each split,  $10^4$  cells were replated to each well in new plates, and allowed to grow until the next split. Population doublings (PD) were calculated with the formula  $\text{PD} = \log(\text{N}_2/\text{N}_1)/\log 2$ , where  $\text{N}_1$  is the number of cells seeded and  $\text{N}_2$  is the number of cells recovered. Cellular senescence was confirmed by SA- $\beta$ -Gal staining. Cells were washed with 1X PBS, fixed with 0.5% glutaraldehyde solution in 1X PBS for fifteen minutes at room temperature, washed again with 1X PBS containing 1 mM magnesium chloride, incubated in X-Gal staining solution (35mM potassium ferricyanide, 35mM potassium ferrocyanidetrihydrate

2mM magnesium chloride in 1X PBS (pH 6.0) and 1 mg/ml X-Gal) for 6-8 hours and washed with H<sub>2</sub>O. At least 200 cells were counted under microscope in randomly chosen fields from each well. Each experiment was performed in duplicates or triplicates.

#### *Western blot analysis*

Western blot was performed with lysates prepared 7-10 days after transduction of Ras or 1 day after ionizing radiation from sub-confluent cells as described(7). 40 µg total protein is loaded. Signals were detected using enhanced chemiluminescence and captured by the FluorChem-HD2 Imaging System (Proteinsimple).

#### *3'UTR Luciferase reporter assay*

For the 3'UTR Luciferase reporter assay,  $4 \times 10^4$  Grip Tite 293 cells were seeded the day before transfection and transfected with 180 ng of miRNA-expressing vector, 20 ng of Firefly luciferase reporter containing wild type or mutant 3'UTR region of the target gene, and 10 ng of Renilla-luciferase reporter in 96-well plates in triplicates using Lipofectamine 2000 (Invitrogen). Cells were lysed 24 hours after transfection in 100 µL/well luciferase assay lysis buffer. The dual luciferase activity was measured with Dual-Luciferase Assay System (Promega) and Renilla luciferase was used as an internal normalization control.

#### *RNA isolation and quantitative Real-Time PCR*

RNA was isolated from cells using TRIzol (Thermo Fisher Scientific) according to manufacturer's protocol. 200 ng of RNA was reverse transcribed to cDNA with iScript<sup>TM</sup> Reverse Transcription Supermix (Bio-Rad). Quantitative real-time PCR was performed in

duplicates with gene-specific primers and SsoAdvanced™ SYBR Green Supermix (Bio-Rad) in a Bio-Rad CFX96 REAL TIME SYSTEM following manufacturer's protocols. GAPDH was used as internal control to normalize the mRNA level for each gene.

Primer sequences for RT-PCR:

CHD7-rt-F1: 5'-ACCCACACCATTACCATTGAG-3'

CHD7-rt-R1: 5'-TCTTAGCCCACTTTTGCCAG-3'

CHD7-rt-F2: 5'-AAATGTGGAGGGACTTGATCTG-3'

CHD7-rt-R2: 5'-TCCGGGAGAAGGAATGTTTC-3'

TNRC6A-rt-F: 5'-ACTCCTGGCAGTGTCATAAAC-3'

TNRC6A-rt-R: 5'-TGTAGTTGGAGGCACGAATG-3'

TNRC6A-rt-F2: 5'-GTAAACAGCCAGTCAGAAAGC-3'

TNRC6A-rt-R2: 5'-CGCTGGGAATTTTCATAATGGG-3'

TNRC6B-rt-F: 5'-AGAAGGAGCAAGAAAGGGAAG-3'

TNRC6B-rt-R: 5'-ACTTGGTTTCGTCACTTCGG-3'

Ago2-rt-F: 5'-CCTCCCATGTTTACAAGTCG-3'

Ago2-rt-R: 5'-TCTTTGTCCTGCCACAATG-3'

Dicer-rt-F: 5'-CAAGTGTCAGCTGTCAGAACTC-3'

Dicer-rt-R: 5'-CAATCCACCACAATCTCACATG-3'

p16-rt-F4: 5'-CCCAACGCACCGAATAGTTA-3'

p16-rt-R4: 5'-ACCAGCGTGTCCAGGAAG-3'

Mouse CHD7: m-Chd7-rt-f: 5'-GAAGTACAATTCCATGCGAGC-3'

m-Chd7-rt-r: 5'-TCAAATTCTCCACCGTCACC-3'

Mouse TNRC6A: m-TNRC6A-rt-f: 5'-ACTCCTGGCAGTGTCATAAAC-3'

m- TNRC6A-rt-r:5'-TGTAGTTGGAGGCACGAATG-3'

GAPDH-rt-F: 5'-ACATCGCTCAGACACCATG-3'

GAPDH-rt-R: 5'-TGTAGTTGAGGTCAATGAAGGG-3'

To measure miRNA expression levels, 100 ng of total RNA was used for reverse transcription with iScript™ Select cDNA Synthesis Kit (Bio-Rad). Expression levels of the mature miRNA were measured by quantitative real-time PCR using 1:10 dilutions of reverse transcribed products and TaqMan miRNA Assays kit (Thermo Fisher Scientific) following the manufacturer's protocol. The RUN48 primers were used as a normalization control.

#### *Luciferase reporter assay*

The p16<sup>INK4A</sup> promoter activity was analyzed in 293T cells by transient transfection of luciferase reporter constructs and in BJ cells containing stably integrated luciferase reporters. For 293T cells,  $5 \times 10^5$ /well cells were seeded into 12-well plates on the 1st day. On the 2nd day, 293T cells were transfected with 0.15 μg pGL2 reporter vector, 1.5 μg pcDNA3/pcDNA3-flag-CHD7 WT vector with 4.0 μl lipo-2000 according to manufacturer's instruction. 48h after transfection, cell lysates were collected using Passive Lysis Buffer (E1941, Promega). Luciferase activity was detected using Luciferase Assay System (E1500, Promega) on GloMax®-Multi+ Microplate Multimode Reader (Promega), and normalized to protein concentrations. For BJ cells, BJ-p16-3570~+306-Luc, BJ-p16-891~+306-Luc and BJ- p16-891Δ-119~-12-Luc reporter cell lines were constructed using the retrovirus packaged with pBabeBlast-p16-3570~+306-Luc, pBabeBlast-p16-891~+306-Luc or pBabeBlast-p16-891Δ-119~-12-Luc. These BJ reporter cells were infected with WZL-Hygro or WZL-Hygro-HaRasV12 retrovirus. 7 days after infection, cell lysates were collected using Passive Lysis Buffer. Luciferase activity was detected using



Luciferase Assay System on GloMax<sup>®</sup>-Multi+ Microplate Multimode Reader, and normalized to protein concentrations. Each experiment was performed in triplicates.

The p53-dependent transcription was analyzed in BJ cells stably transduced with a retroviral luciferase reporter driven by a promoter containing multiple copies of a functional p53-binding site (PG-Luc)(8). These cells were transduced with lentiviruses encoding miR-30 or vector control, or shRNA for TNRC6A or Dicer or a scrambled sequence. Infected cells were selected, and either subsequently transduced with retroviruses encoding Ha-RasV12 or vector, or treated with 1 Gy of ionizing radiation. Cells were split into 12-well plates on day 7 post ras transduction and lysed on day 8, or lysed 10 hours after IR treatment, in Passive Lysis Buffer.

Luciferase activity was detected using Luciferase Assay System on GloMax<sup>®</sup>-Multi+ Microplate Multimode Reader, and normalized to protein concentrations. Each experiment was performed in triplicates.

#### *Chromatin-immunoprecipitation (ChIP) assay*

BJ cells were seeded into 10cm dishes at 50% confluency on the 1st day, and infected with WZL-Hygro or WZL-Hygro-HaRasV12 retrovirus on the 2nd day. For ChIP analysis of the p16 promoter, 6×15cm dishes of BJ-WH or BJ-Ras cells at 60-70% confluency were harvested 7-8 days after infection. For H3K4me1 and H3K4me3, ChIP assays were performed as described before (9). For CHD7, ChIP assays were performed following dual-crosslinking protocol(46). For this dual crosslinking procedure, briefly, cell samples were incubated with 1.5mM long-arm cross-linker EGS in PBS for 30min at room temperature, and then formaldehyde was added at 1% final concentration and incubated for another 10 min.

For ChIP analysis of I-PpoI-induced DSB site, wild type U2OS cells or U2OS cells overexpressing miR-30 or with knockdown of TNRC6A were seeded into 10-cm dishes at  $2 \times 10^6$  cells per dish on the 1st day, and infected by retroviruses encoding HA-ER-I-Ppo1 (packaged with pBABE-HA-ER-I-PpoI, Addgene plasmid #32565)(38) on the 2nd day. 24 hours after infection, 4-hydroxytamoxifen (4-OHT) was added to cultures to a final concentration of  $1 \mu\text{M}$  for up to 6 hours.  $6 \times 10$ -cm dishes of U2OS cells were harvested for each condition after 4-OHT induction. For H4K20me3 and H4K16Ac, ChIP assay was performed as described(9). For TNRC6A and 53BP1, ChIP assays were performed following dual-crosslinking protocol as mentioned above(46).

The cross-linking was stopped with 50 mM glycine. After sonication, 10% of each sample was saved as total input. The amount of DNA in each sample was estimated by electrophoresis on agarose gel. 45% of each sample was incubated with 2  $\mu\text{g}/25 \mu\text{g}$  DNA of anti-H3K4me1 antibody (ab8895, Abcam), or 2  $\mu\text{g}/25 \mu\text{g}$  DNA of anti-H3K4me3 antibody (ab8580, Abcam), 2  $\mu\text{g}/10 \mu\text{g}$  DNA of anti-CHD7 antibody (ab176807, Abcam), 3  $\mu\text{g}/25 \mu\text{g}$  DNA of anti-H4K20me3 antibody (ab9053, Abcam), 3  $\mu\text{g}/25 \mu\text{g}$  DNA of anti-H4K16Ac antibody (07-329, EMD Millipore), 5  $\mu\text{g}/25 \mu\text{g}$  DNA of anti-TNRC6A antibody (ab114857, Abcam) or 10  $\mu\text{g}/25 \mu\text{g}$  DNA of anti-53BP1 antibody (NB100-304, Novus Biologicals), and the rest 45% of each sample was incubated with equal amount of normal rabbit IgG (sc-2027, Santa Cruz) at  $4^\circ\text{C}$  overnight and then with 50  $\mu\text{l}$  (bead volume)/10  $\mu\text{g}$  DNA of Pierce Protein A/G Agarose (20423, Thermo Fisher Scientific) at  $4^\circ\text{C}$  for 2h. After washing and reverse crosslinking, DNA was extracted with phenol-chloroform, precipitated with ethanol, and dissolved in 30-40  $\mu\text{l}$  of  $\text{H}_2\text{O}$ . 0.5  $\mu\text{l}$  of ChIP samples or 1:10 diluted input samples were quantified in a 10  $\mu\text{l}$  reaction volume by real-time PCR using primers amplifying -3523~+2917 region of the p16 promoter (primer sequences

available upon request), or a primer pair that amplify a 235-bp fragment whose proximal end is 51-bp 5' to the single I-Ppo1 cleavage site on chromosome 1 (in an intron of the DAB1 gene) (Forward: 5'-TGCTGCTTTTTCTTCTTCTCC-3'; Reverse: 5'-CTTCTTTCCCACCAAGTCTTC-3'). All the ChIP-qPCR data were normalized to the Input-qPCR data following Percent Input Method(48).

#### *RNase A treatment and DDR focus rescue experiments*

RNase A treatment and DDR focus rescue experiments were performed as previously described(18). 10<sup>5</sup> BJ Cells were seeded 7 days after Ras transduction or 1 day before ionizing radiation treatment on coverslips in 24-well plates. On day 8 post Ras transduction or 1 day after ionizing radiation (1 Gy), cells were permeabilized with 0.5% Tween-20 in PBS for 10 min at room temperature. After washing twice with PBS, 1 mg/mL of RNaseA (Sigma) in PBS was added for 30 min at room temperature. After RNaseA treatment, cells were washed twice with PBS, followed by incubation with 80 units of RNase inhibitor (NEB) and 20 µg/mL of  $\alpha$ -amanitin (Sigma) for 15 min. RNaseA-treated cells were then incubated with 50 ng of total RNA, small RNA, yeast total RNA, or vehicle control for another 15 min at room temperature, and processed for immune-staining for DDR foci as described below.

Total RNA was isolated from BJ cells 7 days after transduction with HaRasV12 or 6 hours after treatment with 1 Gy  $\gamma$ -radiation using TRIzol (Invitrogen) according to manufacturer's protocol. *mirVana* microRNA Isolation Kit (Ambion) was used to purify small ( $\leq 200$  nucleotides) RNA from total RNA preparations according to manufacturer's instructions, with two sequential filtrations with different ethanol concentrations.

### *Immuno-staining for DNA damage foci*

10<sup>5</sup> BJ Cells were seeded 7 days after Ras transduction or 1 day before ionizing radiation treatment on coverslips in 24-well plates. On day 8 post Ras transduction or 1 day after ionizing radiation (1 Gy), or after RNase A treatment, cells were fixed with 4% PFA for 10 min at room temperature. After washing twice with PBS, cells were blocked with blocking solution (1mg/ml BSA, 3% goat serum, 0.1% TritonX-100 and 1 mM EDTA pH 8.0 in PBS) for 30 min at room temperature. Primary antibodies diluted at 1:100 in blocking solution were incubated with cells for 1h at room temperature. After washing three times with PBS for 5 min each, cells were incubated with Alexa Fluor® 488 Goat Anti-Rabbit IgG 1:500 diluted in blocking solution for 1h at room temperature. After washing 3 times with PBS for 5 min each, cell was mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories). Coverslips were sealed in up-side-down direction onto slide glass. The cells were photographed and the percentage of cells with more than 5 foci was determined under a fluorescence microscope. Each experimental point was performed in triplicates. At least 200 cells were counted in at least 3 randomly chosen fields.

### *Apoptosis assay*

3-4x10<sup>5</sup>/well of BJ cells were seeded into 6-well plates and treated with proper dosage of ionizing radiation. 1 day or 4 days later cells were collected and stained for Annexin V using Annexin V Apoptosis detection kit FITC according to manufacturer's protocol (eBioscience). Percentage of apoptotic cells were determined by flow cytometry. As a positive control for apoptosis, cells were treated with 3 µM Thapsigargin (Sigma) for 12 h. Each experiment was performed in duplicates.

### *Generation of miR-30c-1 transgenic mice*

To generate miR-30c-1 transgenic mice, we cloned 4 tandem copies of a miR-30c-1-coding genomic DNA fragment (miR-30c-1 hairpin plus 150 bp flanking sequences on each side) into the CTV Rosa26 locus targeting vector (Addgene plasmid #15912) (20), downstream of a loxP-flanked STOP cassette containing 4 tandem copies of SV40 polyadenylation sites. The miR-30c-1 sequence was amplified by PCR from pLV-miR-30c-1-puro (Biosettia) using primers LV-to-CTV-f (5'-AGTTATGGGCGCGCCGCGCCGTCCAGGCACCTCGATTAGTTC-3') and LV-to-CTV-r (5'-CGGCCGCGGCGCGCCCCAACCTAAAGACGACTCTCCAAAAGC-3') and cloned into the AscI site in CTV. Gene targeting was conducted according to established protocols at the Scripps Research Institute Murine Genetics Core, using the C57BL/6N-derived C2 ES cells(49). The transgene (CTV30C) containing 4 copies of pre-miR-30c-1 sequence preceded by the CAG promoter and a loxP-flanked Neo-STOP cassette was targeted into the Rosa26 locus (Fig. S2A). CTV30C targeting vectors were transfected into C2(XY) ES cells. Targeted ES cells were selected by Southern blotting (Fig. S2B), and injected into B6 albino (white) blastocysts and maintained on a C57BL/6 background, to obtain chimera. Male chimeras were crossed with female B6 Albino to obtain black mice with germline transmission of the targeted allele. The presence of CTV30C allele was confirmed by PCR analysis of tail DNA (Fig. S2C). Animal protocols are approved by the IACUC committee of The Scripps Research Institute and Wake Forest University Medical Center.

### *Keratinocytes Isolation:*

7-day-old mice were euthanized by cervical dislocation. A big piece of skin was surgically excised from the back, rinsed in PBS with 2X antibiotics/antimycotics cocktail (Thermo Fisher Scientific) on ice for 1 hour, and then transferred into a 15-ml Falcon tube containing 6 ml PBS with 2.5 unit/ml of dispase (Thermo Fisher Scientific) for overnight at 4°C. The skin was transferred into a 6-well plate, and incubated in 3 ml of 0.05 % Trypsin EDTA (Thermo Fisher Scientific) at 37°C for 10 mins. Keratinocytes were scraped off from the skin in the trypsin solution with a sterile, disposable cell scraper. 1 ml of Trypsin Neutralizing Solution (TNS, Lonza) was added to terminate trypsinization. Cells were collected into a 15-ml Falcon tube, spun down at 2000 rcf for 3 min, and washed once with PBS. The cell pellet was either used for RNA isolation, or resuspended in 2 ml of Defined keratinocyte serum-free medium (D-KSFM) supplemented with all the supplements and 5 µg/ml of Gentamicin (Thermo Fisher Scientific), plated into a 6-well plate and incubated at 37°C for one day followed by RNA isolation.

*Analysis of tumor formation and senescence in DMBA-induced skin carcinogenesis*

Mouse with the targeted miR-30c-1 allele (CTV30c) was crossed with the Tg(KRT14-cre)1Amc/J strain (the Jackson Laboratory) to generate animals with skin keratinocyte-specific expression of miR-30c-1. The 2-stage DMBA-induced skin carcinogenesis was carried out in CTV30c/CTV30c;K14-Cre/- (miR-30c-1 transgenic) and CTV30c/CTV30c;-/- (non-transgenic) littermates as previously described(47). Skin tumor appearance, size of the largest tumor on each mouse, and number of tumors on each mouse were monitored weekly. Analysis of senescence, DNA damage response, and p16<sup>INK4A</sup> and p53 status was performed in an independent group of DMBA/TPA-treated mice sacrificed 60-70 days after the initial TPA treatment using size-matched tumors. Frozen sections of tumors were stained for SA-β-gal (10 micron sections) or for

$\gamma$ -H2AX, p16<sup>INK4A</sup> or p53-pS15 by immunohistochemistry (5 micron sections) as previously described(12). Numbers of positive cells were quantified under microscope on each slide and normalized to the area to tumor tissues. Animal protocols are approved by the IACUC committee of The Scripps Research Institute and Wake Forest University Medical Center.

*Analysis of tumor progression and senescence in KrasG12D-driven mouse pancreatic cancer model.*

Pdx1-Cre (B6.FVB-Tg(Pdx1-cre)6Tuv/J) and LSL-KRASG12D (B6.129S4-Krastm4Tyj/J) transgenic strains were purchased from the Jackson Laboratory. CTV30c transgenic mice were crossed with Pdx1-Cre or LSL-KRASG12D mice respectively. The resulting Pdx1-Cre<sup>+/-</sup>; CTV30c<sup>+/-</sup> and LSL-KRASG12D<sup>+/-</sup>; CTV30c<sup>+/-</sup> mice were identified by PCR and crossed with each other to generate Pdx1-Cre<sup>+/-</sup>; LSL-KRASG12D<sup>+/-</sup>; CTV30c<sup>-/-</sup> and Pdx1-Cre<sup>+/-</sup>; LSL-KRASG12D<sup>+/-</sup>; CTV30c<sup>+/-</sup> mice for analysis.

The evaluation of PanIN progression was performed following an established method (23). Briefly, pancreata were obtained from sacrificed transgenic mice, and HE-staining was performed with 5 $\mu$ m paraffin sections. The total number of ductal lesions and their grade were scored in pancreatic sections of Pdx1-Cre<sup>+/-</sup>; LSL-KRASG12D<sup>+/-</sup>; CTV30c<sup>-/-</sup> and Pdx1-Cre<sup>+/-</sup>; LSL-KRASG12D<sup>+/-</sup>; CTV30c<sup>+/-</sup> mice according to the criteria for PanIN grades 1-3(23). In each pancreatic lobule, only the highest-grade lesion was evaluated to avoid scoring the same serpentine duct more than once. Lobule numbers of each grade were quantified under microscope and normalized to total lobule numbers.

For SA- $\beta$ -galactosidase staining, 10  $\mu$ m frozen pancreatic sections were fixed in 0.2% glutaraldehyde in PBS for 10 min at 4°C. After being washed with 2 mM MgCl<sub>2</sub> in PBS

(pH4.0), slides were stained in freshly prepared 1 g/L X-gal, 1 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 2 mM MgCl<sub>2</sub> in PBS (pH4.0) for 6 hours at 37°C. After being washed with ddH<sub>2</sub>O, slides were co-stained with nuclear fast red (Cat#N3020, Sigma) for 5 min, followed by washing in running tap water. Slides were then dehydrated and mounted. Numbers of positive cells were quantified under microscope and normalized to total PanIN epithelial cells. Here we stained the mouse pancreatic tissues for SA-β-gal under pH4.0, instead of pH6.0 for the skin tissues in the skin carcinogenesis model, because we found that pH4.0 allowed more efficient detection of senescent cells than pH6.0 in PanIN lesions from Pdx1-Cre;LSL-KRasG12D mice. However, both condition (pH4.0 and pH6.0) barely detected any SA-β-gal positive cells in pancreatic epithelial cells from LSL-KRasG12D mice in which KRasG12 is not activated, suggesting that the pH4.0 condition specifically detects SA-β-gal positive cells induced by KRasG12D. In addition, the SA-β-gal positive cells detected under pH4.0 in Pdx1-Cre;LSL-KRasG12D pancreas were greatly reduced by the miR-30c transgene in Pdx1-Cre;LSL-KRasG12D;CTV30c<sup>+/-</sup> mice (Fig. 1M, S2G). Taken together, these results demonstrate the specificity of SA-β-gal staining under pH4.0 in mouse pancreas.

For immunohistochemical staining of p16<sup>INK4A</sup>, 5 μm paraffin pancreatic sections were deparaffinized, and antigen retrieval was performed by boiling in microwave for 10 min in sodium citrate Buffer (10 mM sodium citrate, 0.05% Tween-20, pH6.0). After cooling down to room temperature, slides were quenched with 3% hydrogen peroxide for 10 min, followed by blocking with 5% BSA for 1h at room temperature. Slides were then incubated sequentially with a p16 antibody (Cat#1661, Santa Cruz, 1:100 diluted) O/N at 4°C, biotinylated anti-mouse immunoglobulins (Cat#E0433, Dako, 1:200 diluted) for 1h at RT, and Streptavidin, Horseradish Peroxidase (Cat#SA5704, Vector Laboratories) for 1h at RT. Labelling was detected with DAB

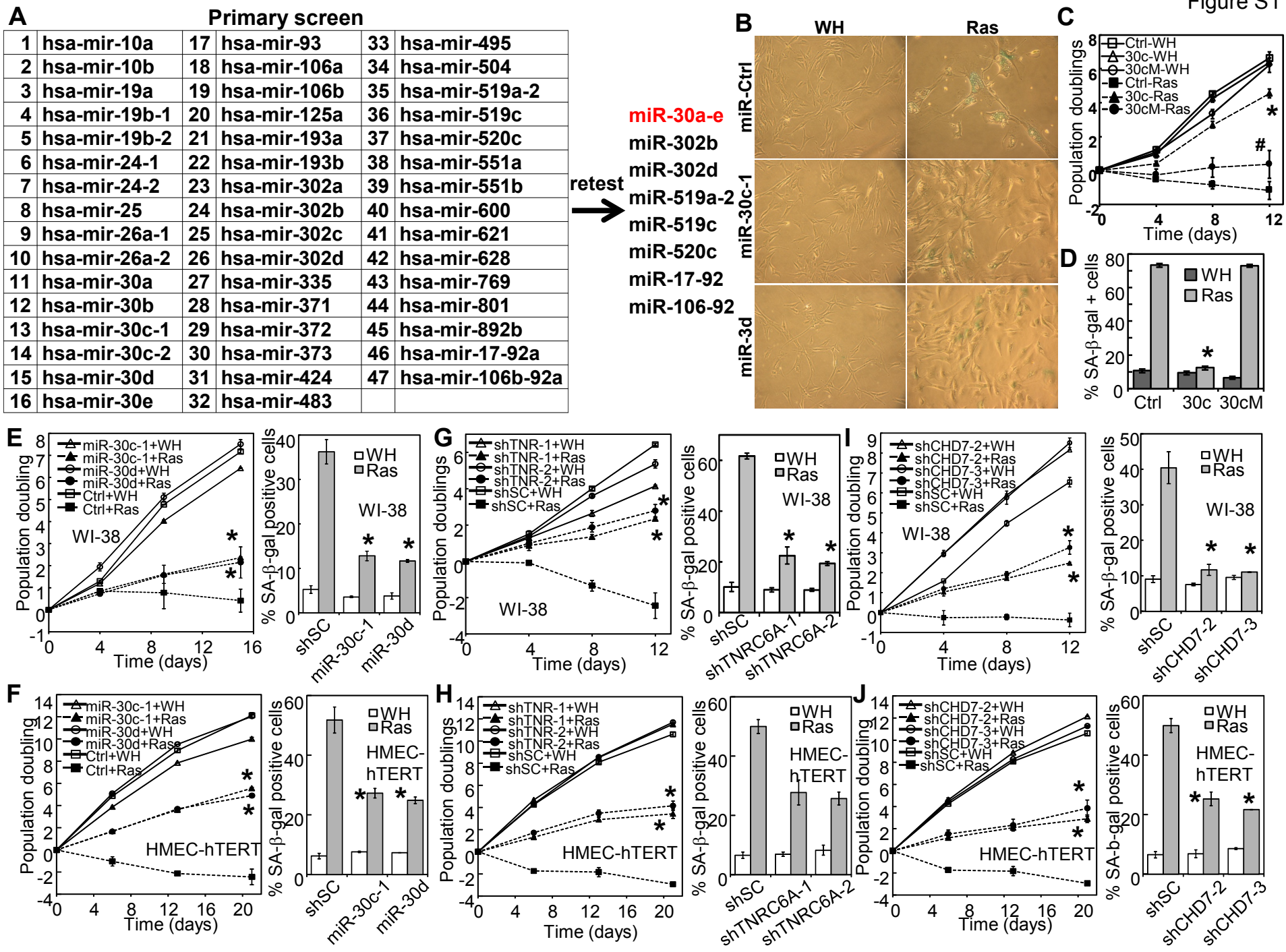


Peroxidase (HRP) Substrate Kit (Cat#SK4100, Vector Laboratories). Slides were then dehydrated and mounted. Numbers of positive cells were quantified under microscope and normalized to total PanIN epithelial cells.

Animal protocols are approved by the IACUC committee of The Scripps Research Institute and Wake Forest University Medical Center.

#### *NaCl solubility assay*

BJ cells overexpressing miR-30 or with TNRC6A knockdown were seeded into 10-cm dishes at 50% confluency on the 1st day, and infected with WZL-Hygro or WZL-Hygro-HaRasV12 retroviruses on the 2nd day. 9 days after infection, cells were harvested and washed twice with ice-cold PBS. Cell pellet was re-suspended in ice-cold extraction buffer (20 mM HEPES, 0.5 mM DTT pH7.9, 1 mM PMSF, 1.5 mM MgCl<sub>2</sub>, 0.1% Triton and 1×Roche EDTA-free Protease Inhibitor Cocktail) containing 0.5 M or 1.0 M NaCl. Samples were agitated for 1h at 4°C, and centrifuged at 14,000 rpm for another 1h at 4°C. Supernatant from 0.5 M NaCl extraction buffer were retained for Western Blots analysis of H3 (anti-H3, 4499, Cell Signaling) and H2AX (anti-H2AX, 7631, Cell Signaling). Supernatant from 1.0 M NaCl extraction buffer were retained for Western Blots analysis of  $\gamma$ -H2AX [anti-phospho H2AX (S139), 05-636, EMD Millipore].



**Fig. S1.** Identification of miR-30 as an inhibitor of oncogenic *ras*-induced senescence in multiple primary cell lines.

(A) List of 47 miRNAs identified from the primary senescence-disrupting screen and 8 hits identified after retest.

(B) Photograph of SA- $\beta$ -gal staining of BJ cells transduced with miR-30c-1, miR-30d or vector (Ctrl) and HaRasV12 (Ras) or vector (WH).

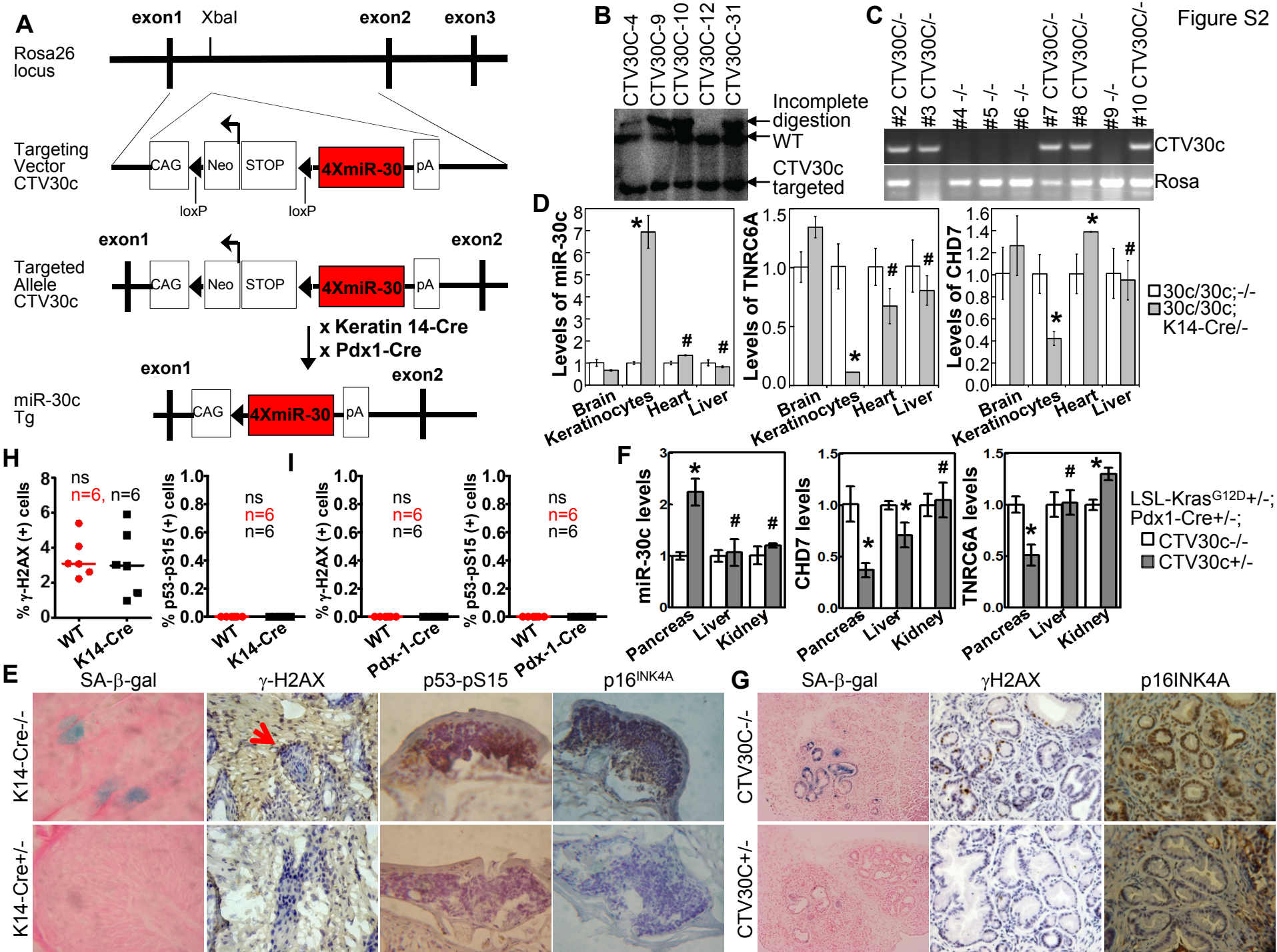
(C, D) miR-30c-1, but not its mutant with a scrambled seed sequence, disrupts oncogenic *ras*-induced senescence. BJ cells transduced with miR-30c-1 (30c), miR-30c-1 mutant (30cM) or vector (Ctrl) and HaRasV12 (Ras) or vector (WH) were analyzed for their growth curves (C), and % of SA- $\beta$ -gal positive cells were quantified after staining on day 9 post *ras* transduction (D).

(E, F) miR-30 disrupts *ras*-induced senescence in WI-38 (E) and hTERT-immortalized HMEC (F) cells. Left, growth curves of WI-38 (E) and HMEC-hTERT (F) cells transduced with miR-30c-1, miR-30d or vector (Ctrl) and HaRasV12 (Ras) or vector (WH). Right, % of SA- $\beta$ -gal positive cells in WI-38 (E) and HMEC-hTERT (F) cell populations transduced with miR-30c-1, miR-30d or vector (Ctrl) and HaRasV12 (Ras) or vector (WH) on day 9 post *ras* transduction.

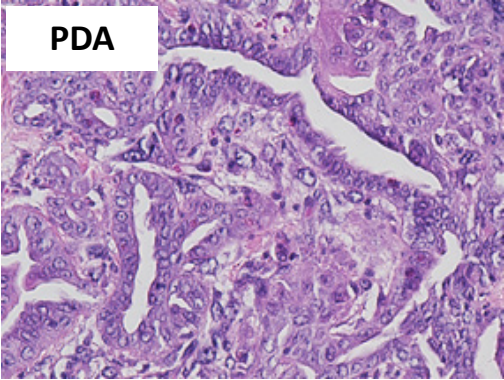
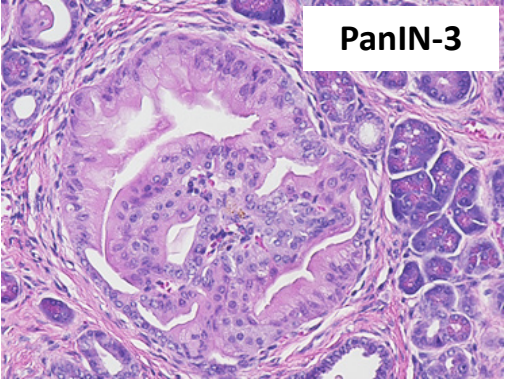
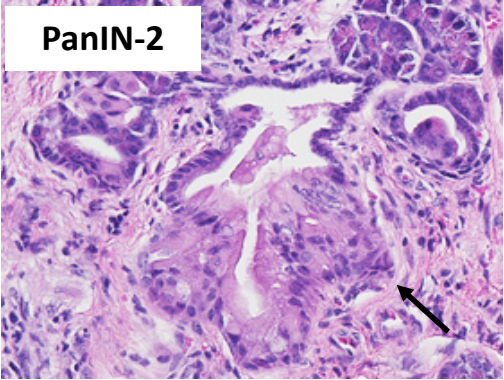
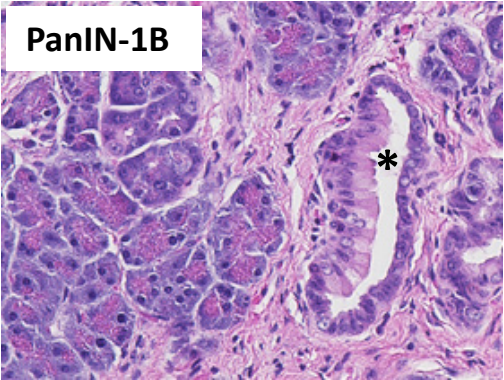
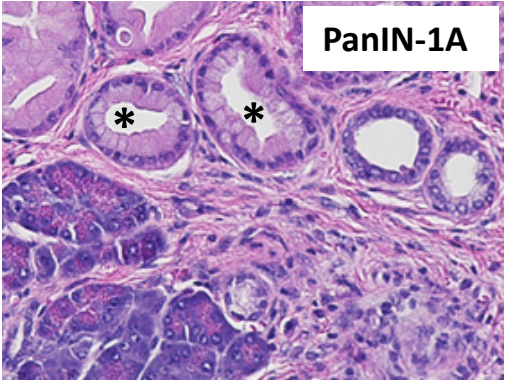
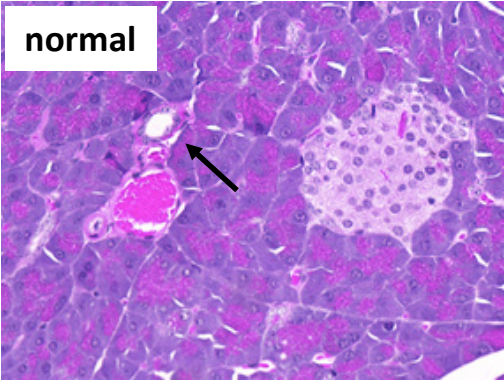
(G, H) shRNA for TNRC6A disrupts *ras*-induced senescence in WI-38 (G) and hTERT-immortalized HMEC (H) cells. Left, growth curves of WI-38 (G) and HMEC-hTERT (H) cells transduced with shRNA for TNRC6A (shTNR-1, 2) or a scrambled sequence (shSC) and HaRasV12 (Ras) or vector (WH). Right, % of SA- $\beta$ -gal positive cells in WI-38 (G) and HMEC-hTERT (H) cell populations transduced with shRNA for TNRC6A (shTNR-1, 2) or a scrambled sequence (shSC) and HaRasV12 (Ras) or vector (WH) on day 9 post *ras* transduction.

(I, J) shRNA for CHD7 disrupts *ras*-induced senescence in WI-38 (I) and hTERT-immortalized HMEC (J) cells. Left, growth curves of WI-38 (I) and HMEC-hTERT (J) cells transduced with shRNA for CHD7 (shCHD7-2, 3) or a scrambled sequence (shSC) and HaRasV12 (Ras) or vector (WH). Right, % of SA- $\beta$ -gal positive cells in WI-38 (I) and HMEC-hTERT (J) cell populations transduced with shRNA for CHD7 (shCHD7-2, 3) or a scrambled sequence (shSC) and HaRasV12 (Ras) or vector (WH) on day 9 post *ras* transduction.

(C-J) Values are mean  $\pm$  SD for triplicates. \*  $p < 0.05$  vs Ctrl or shSC in Student t test. # no statistical difference vs Ctrl in Student t test.



J



**Fig. S2.** Generation of miR-30c-1 transgenic mice and effect of miR-30c on senescence and cancer development in a DMBA-induced skin carcinogenesis model and a KrasG12D-driven pancreatic cancer model.

(A) Outline of the strategy for generation of miR-30c-1 transgenic mice.

4 tandem copies of a miR-30c-1-coding genomic DNA fragment (miR-30c-1 hairpin plus 150 bp flanking sequences on each side) was cloned into the CTV Rosa26 locus targeting vector downstream of a loxP-flanked Neo-STOP cassette containing 4 tandem copies of SV40 polyadenylation sites. The transgenic miR-30c-1 allele (CTV30c) was targeted into the Rosa26 locus in C57BL/6N-derived C2 ES cells. Mice were generated by injecting targeted ES cells into B6 albino blastocysts and maintained on a C57BL/6 background. Mouse with the targeted miR-30c-1 allele (CTV30c) was crossed with the Tg(KRT14-cre)1Amc/J strain to generate animals with skin keratinocyte-specific expression of miR-30c-1.

(B) Southern blot analysis of the ES cell clones containing the targeted allele of miR-30c-1 (CTV30c). Genomic DNA isolated from ES cell clones was digested with EcoRI, and Southern blot was hybridized to a probe derived from Rosa26 locus, yielding a 15.6 kb fragment for the wild type allele (WT) and a 5.9 kb fragment for the targeted allele (CTV30c targeted).

(C) Genotyping of the CTV30c and the wild type (Rosa) alleles by PCR using tail genomic DNA. The genotype of each mouse (either CTV30c/- or -/-) was indicated.

(D) Expression of miR-30c-1 is increased (left panel) and expression of TNRC6A (middle panel) and CHD7 (right panel) are suppressed specifically in skin keratinocytes from miR-30c-1 transgenic mice (30c/30c;K14-Cre/-) in comparison with the non-transgenic littermates (30c/30c;-/-), as determined by quantitative real time PCR. Values are mean  $\pm$  SD for triplicates. \*  $p < 0.05$ ; # no statistical difference, vs 30c/30c;-/- in Student t test.

(E) DMBA-induced skin papilloma from miR-30c-1 transgenic (CTV30c;K14-Cre+/-) (K14-Cre+/-) and non-transgenic (CTV30c;K14-Cre/-) (K14-Cre/-) littermates were stained for SA- $\beta$ -gal and for  $\gamma$ -H2AX, p53-pS15 and p16INK4A by IHC. Photos are taken under 20X (SA- $\beta$ -gal and  $\gamma$ -H2AX) or 10X (p53-pS15 and p16INK4A) magnification.

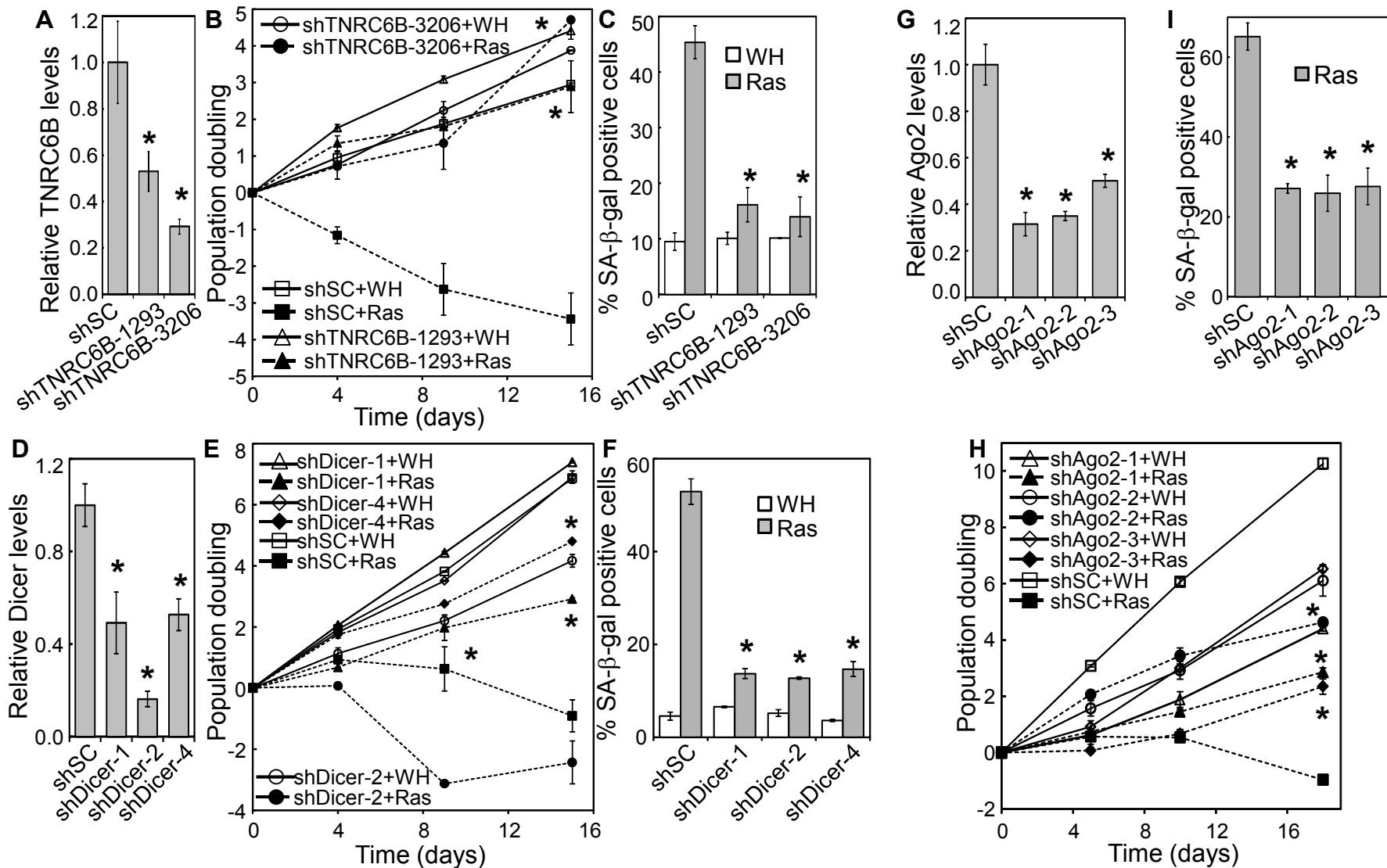
(F) Expression of miR-30c-1 is increased (left panel) and expression of TNRC6A (middle panel) and CHD7 (right panel) are suppressed specifically in pancreas from LSL-Kras<sup>G12D</sup>+/-;Pdx1-Cre+/-;CTV30c/- and LSL-Kras<sup>G12D</sup>+/-;Pdx1-Cre+/-;CTV30c+/- littermates, as detected by quantitative real time PCR. Values are mean  $\pm$  SD for triplicates. \*  $p < 0.05$ ; # no statistical difference, vs CTV30c/- in Student t test.

(G) Photographic representation of SA- $\beta$ -gal- (top),  $\gamma$ H2AX- (middle), and 16<sup>INK4A</sup>- (bottom) positive epithelial cells in PanIN lesions in LSL-Kras<sup>G12D</sup>+/-;Pdx1-Cre+/-;CTV30c/- and LSL-Kras<sup>G12D</sup>+/-;Pdx1-Cre+/-;CTV30c+/- pancreata.  $p < 0.01$  or 0.05 in unpaired t test.

(H, I) Cre did not induce DNA damage in skin keratinocytes or pancreas of mice. Skin from wild-type (WT) and K14-Cre+/- (K14-Cre) mice (H) and pancreas from wild-type (WT) and Pdx1-Cre+/- (Pdx1-Cre) mice (I) were stained for  $\gamma$ H2AX (left) or p-p53S15 (right) by IHC. % of  $\gamma$ H2AX- or p-p53S15-positive skin basal cells (H) and pancreatic epithelial cells (I) were quantified. Values are mean  $\pm$  SD. ns, no significant difference in unpaired t test.

(J) Representative images of PanIN grades and PDA. **Normal:** Arrow refers to a normal duct composed of cuboidal cells with uniform round nuclei. **PanIN-1A:** Asterisks refer to 2 typical PanIN-1A lesion. Suffering epithelial cells are with abundant mucin-containing supranuclear cytoplasm. **PanIN-1B:** Asterisk refers to a typical PanIN-1B lesion. Papillary structures restricted in intralobular ducts are formed in this stage. There is no significant loss of polarity. **PanIN-2:** Arrow shows loss of polarity in the cells of papillary structures. **PanIN-3:** Clusters of cells with significant nuclear atypia and loss of polarity bud off into the lumen. **PDA:** Well-differentiated infiltrative adenocarcinoma with prominent glandular architecture.

Figure S3



**Fig. S3.** The miRNA pathway components are essential for oncogenic *ras*-induced senescence.

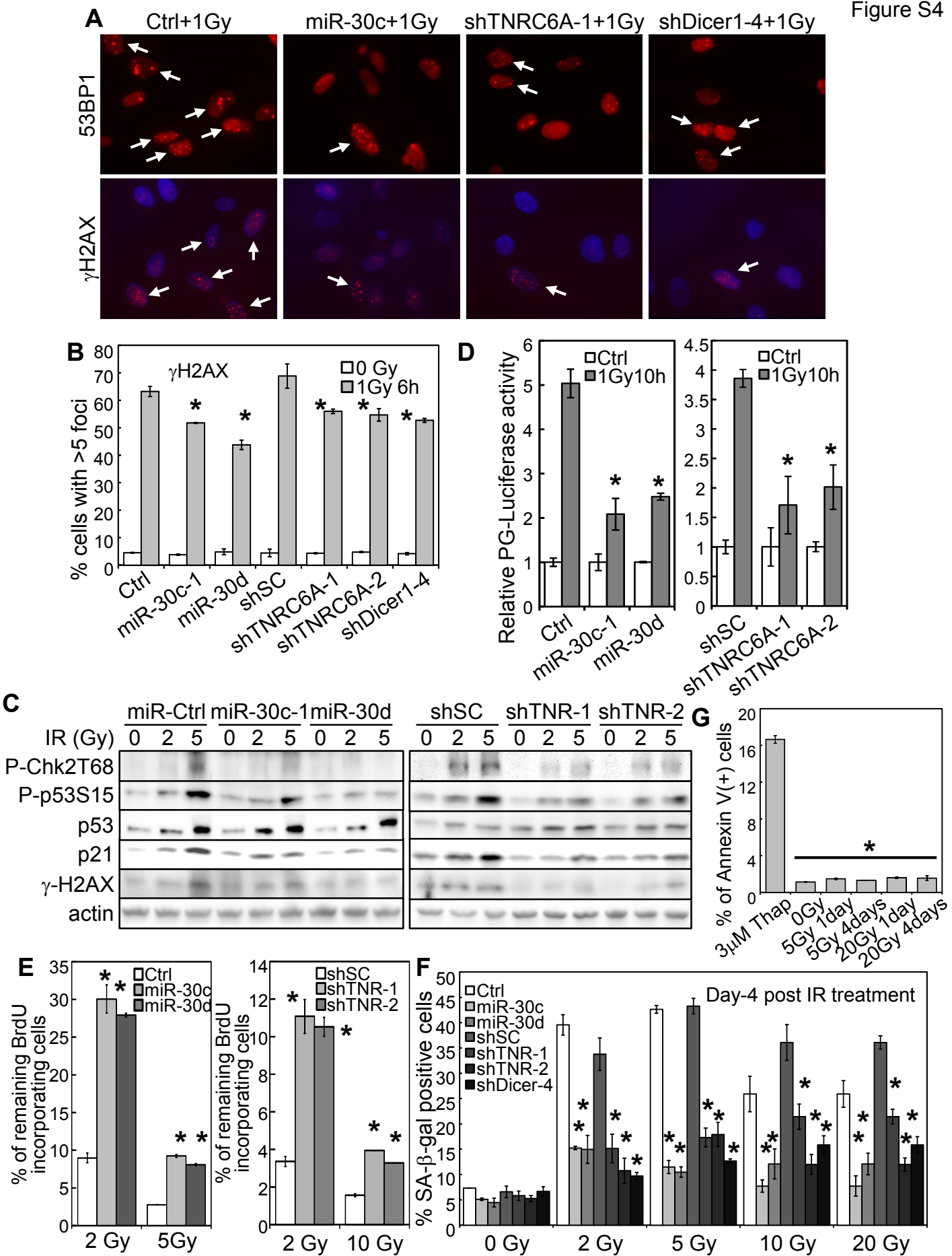
(A, D, G) Quantitative real time PCR analysis showing that the expression of TNRC6B (D), Dicer (D) and Ago2 (G) was knockdown by shRNAs. Values are mean  $\pm$  SD for triplicates.

(B, E, H) Growth curve of BJ cells transduced with shRNA for TNRC6B (B), Dicer (E) or Ago2 (H) or a scrambled sequence (shSC) and with HaRasV12 (Ras) or vector (WH). Values are mean  $\pm$  SD for triplicates.

(C, F, I) % of SA- $\beta$ -gal positive cells in BJ cell populations transduced with shRNA for TNRC6B (C), Dicer (F) or Ago2 (I) or a scrambled sequence (shSC) and with HaRasV12 (Ras) or vector (WH) on day 9 post *ras* transduction. Values are mean  $\pm$  SD for triplicates.

(A-I) \*  $p < 0.05$  vs shSC in Student t test.





**Fig. S4.** TNRC6A is essential for  $\gamma$ -radiation induced DNA damage responses, p53 activation, proliferative arrest and senescence.

(A) BJ cells transduced with miR-30 or vector (Ctrl) or shRNA for TNRC6A or Dicer or a scrambled shRNA (shSC) were treated with  $\gamma$ -radiation (1 Gy) and stained for 53BP1- or  $\gamma$ -H2AX-DNA damage foci. Arrows, cells with > 5 foci.

(B) Quantification of % of cells with  $\gamma$ -H2AX foci in BJ cells transduced with miR-30 or vector (Ctrl) or shRNA for TNRC6A or Dicer or a scrambled sequence (shSC) and treated with  $\gamma$ -radiation (1 Gy) or not (0 Gy). At least 200 cells were counted in randomly chosen fields.

(C) Western blot analysis of BJ cells transduced with miR-30 or vector (miR-Ctrl) or shRNA for TNRC6A (shTNR-1, -2) or a scrambled sequence (shSC) and then treated with  $\gamma$ -radiation.

(D) BJ cells with a stable p53-dependent luciferase reporter (PG-Luc) were transduced with miR-30 or vector (Ctrl) (left) or shRNA for TNRC6A or a scrambled sequence (shSC) (right), and treated with 1 Gy of  $\gamma$ -radiation followed by 10 h of incubation (1Gy10h) or left untreated (Ctrl). Luciferase activity was then determined and normalized to protein concentrations.

(E) BJ cells transduced with miR-30c-1, miR-30d or vector (Ctrl) (left) or shRNA for TNRC6A (shTNR-1, 2) or a scrambled sequence (shSC) were treated with  $\gamma$ -radiation and labeled with BrdU. BrdU incorporating cells were detected by flow cytometry. % of remaining BrdU incorporating cells were calculated by dividing the % of BrdU incorporating cells in treated cells by that in untreated cells.

(F) BJ cells transduced with miR-30c-1, miR-30d or vector (Ctrl) or shRNA for TNRC6A (shTNR-1, 2) or Dicer (shDicer-4) or a scrambled sequence (shSC) were treated with  $\gamma$ -radiation and stained for SA- $\beta$ -gal 4 days later.

(G)  $\gamma$ -radiation does not induce significant apoptosis in BJ cells. BJ cells were treated with indicated dosages of  $\gamma$ -radiation and then incubated for 1 or 4 days, before stained for Annexin V and analyzed by flow cytometry. Cells treated with Thapsigargin (Thap) were used as positive control for apoptosis.

(B, D-G) Values are mean  $\pm$  SD for triplicates.. \*  $p < 0.05$  vs Ctrl or shSC (B, D, E, F) or Thap (G) in Student t test.

**Table S1** miR-30 targets predicted by TargetScan and miRbase, and tested for the involvement in senescence in this study.

<b>1</b>	CELSR3
<b>2</b>	ABI3BP
<b>3</b>	RASA1
<b>4</b>	RFX6
<b>5</b>	MKRN3
<b>6</b>	STIM2
<b>7</b>	MTDH
<b>8</b>	TLL2
<b>9</b>	MIER3
<b>10</b>	KLHL28
<b>11</b>	TNRC6A
<b>12</b>	LHX8
<b>13</b>	PRDM1
<b>14</b>	CHD7
<b>15</b>	BRWD1

**Table S2** miR-30 binding sites in the 3'UTRs of CHD7 and TNRC6A genes. The 2 miR-30 binding sites in the hCHD7 3'UTR and the 4 binding sites in the TNRC6A 3'UTR are shaded, and their locations are indicated by numbers in the parentheses. The 3'UTR mutants lacking the miR-30 binding sites used in Fig. 2A are shown under each binding site. The mutations are in red.

### CHD7

hCHD7 3' UTR (55-62)	5'.. AAGUGGUAGUCCUAC UGUUUACA ... 3'
hCHD7 3' UTR mutant	5'.. AAGUGGUAGUCCUAC UGU <u>A</u> UACA ... 3'
hCHD7 3' UTR (813-819)	5'.. UAAACUAGCCUUGAA GUUUACA G ... 3'
hCHD7 3' UTR mutant	5'.. UAAACUAGCCUUGAA GU <u>A</u> UACA G ... 3'

### TNRC6A

hTNRC6A 3' UTR (455-461)	5'.. CUGUCCCAGUGUCA UGUUUAC U ... 3'
hTNRC6A 3' UTR mutant	5'.. CUGUCCCAGUGUCA UGU <u>A</u> UAC U ... 3'
hTNRC6A 3' UTR (746-753)	5'.. CACUUUGAACCUUCU UGUUUACA ... 3'
hTNRC6A 3' UTR mutant	5'.. CACUUUGAACCUUCU UGU <u>A</u> UACA ... 3'
hTNRC6A 3' UTR (786-792)	5'.. UUUGAGAAAAAAAAA UGUUUAC U ... 3'
hTNRC6A 3' UTR mutant	5'.. UUUGAGAAAAAAAAA UGU <u>A</u> UAC U ... 3'
hTNRC6A 3' UTR (1552-1559)	5'.. UUUGAGAAAAAAAAA UGUUUAC U ... 3'
hTNRC6A 3' UTR mutant	5'.. UUUGAGAAAAAAAAA UGU <u>A</u> UAC U ... 3'