Supporting Information

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SI Materials and Methods

Materials. Senescence Beta-Galactosidase Staining Kit #9860 was purchased from Cell Signaling, and the standard protocol was performed. Nutlin-3 and SB431542 were obtained from Calbiochem, and TGF-β was obtained from PeproTech.

Retroviral Constructs. LV-RasV12, pBabebleo-RasV12, pBabe-puro-V12T, LVTHM-shp53, and shGFP were described previously (1). pBabebleo-RasV12, pBabebleo-RasV12-Y40C, pBabe-puro-RasV12-T55S, pBabe-puro-RasV12-E37G, pBabe-puro-Large T, pBabe-puro-Large T KI, pBabe-puro-Large T ΔE34-44 (Addgene plasmids 1768, 12276, 12274, 12275, 1780, 8581, and 8582) were obtained from Addgene, deposited by Dr. Robert Weinberg (Whitehead Institute, Cambridge, MA), pRetroSUPER-shCH2, pRetroSUPER-shATM, and pRetroSUPER-shGFP were obtained from Dr. Yosef Shilooh (Department of Neurobiology, Tel Aviv University, Tel Aviv, Israel) (2). LNCX2-DNTGFβRII was created by subcloning DNTGFβRII from pMFG-DNR-IREs-Neo and recloning it into the LNCX2 vector (3). SINpuro-shp16 and SINhygro-shp16 were provided by Dr. Scott Cowart (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). LXSN-GSE56 and pLVbleo-shp21 were provided by Andrei Gudkov (Roswell Park Cancer Institute, Buffalo, NY), pLKO.1-shMYC-1 was provided by Mikhail Nikiforov (Roswell Park Cancer Institute, Buffalo, NY), LNCX2-GFP, LNCX2-GFP-IREs-RasV12 (Gir), LNCX2-MYC-IREs-GFP (Mg), and LNCX2-MYC-IREs-RasV12 (MiR) were created by subcloning RasV12 from pBabebleo-RasV12, IRES and GFP from pIREs-GFP, and MYC from pzw-MYC (Addgene plasmid 10674). After successful cloning all four constructs were sequence verified.

Virus Production and Infection. Retroviruses were produced as previously described (4). Briefly, retroviral vectors were transfected into Phoenix-Ampho cells together with a packaging plasmid encoding the MLV-gag-pol and env genes. Lentiviruses were packaged in 293T cells using the second-generation packaging constructs pCMV-dR8.74 and pMD2G, kind gifts from Didier Trono (University of Geneva, Switzerland). Supernatant media containing virus, collected at 24–48 h, were supplemented with 4 μg/mL polybrene before being frozen in aliquots or used to infect cells for 6–24 h.

Immunoblot Analysis. Whole-cell extracts were prepared by incubating cell pellets in lysis buffer containing 50 mmol/L of Tris (pH 8.0), 150 mmol/L of NaCl, 1.0% Nonidet P-40, 10 μg/mL of aprotinin, 100 μg/mL of phenylmethylsulfonyl fluoride, 5 μg/mL of leupeptin, 5 μg/mL of pepstatin, and 1 mmol/L of NaVO₃. Cell extracts containing equal quantities of proteins, determined by the Bradford method, were separated by SDS-PAGE (8–12.5% acrylamide) and transferred to polyvinylidene difluoride membranes (Millipore). Antibodies to MYC (sc-40), RAS (sc-520), RB (sc-50), p21(sc-397), p53 (sc-126), p16 (sc-9968), and TGFβ2 (sc-90) were from Santa Cruz Biotechnology, antibodies to β-actin (pan Ab-5) and p14ARF (Ab-1) were from Neomarkers, antibodies to glyceraldehyde-3-phosphate dehydrogenase were from Calbiochem, antibodies to ATM were from Bethyl Laboratories, antibodies to HDME2 were from Chemicon, antibodies to p-RB (Ser-249/Thr-252) were from Biosource, and antibodies for ERK1/2, P-ERK1/2 (Thr202/Tyr204), P-p53 (Ser-15), CHK2, P-STAT3 (Ser-217/221), AKT, and P-AKT (Ser473) were from Cell Signaling. Primary antibodies were detected with goat antimouse or goat anti-rabbit conjugated to horseradish peroxidase (Hoffman-La Roche), using enhanced chemiluminescence (Perkin-Elmer).

Confocal Microscopy. Shp53/RAS-G12V-HMEC (HMEC, human mammary epithelial cells) or shp53/Vector-HMEC were fixed in 4% paraformaldehyde in PBS for 15 min, permeabilized with 1% Triton X-100 in PBS for 15 min, 4 d after infection. The cells were treated with DAPI (1 μg/mL) for 5 min and mounted with Gel Mount (Biomeda) The cells were analyzed by confocal fluorescence microscopy for senescence-associated heterochromatin foci (SAHF) and multinucleated cells.

Soft Agar and Relative Growth Assays. For soft agar assays, the medium was changed every 3 d until cells were harvested after 3 wk. To quantify colonies, each plate was scanned using an automated multipanel scanning microscope, and the digital images analyzed using MetaMorph image quantification software. SB431542 (5 μM and 10 μM) was obtained from Calbiochem and was added to the medium during feeding for indicated experiments, TGF-β (10 ng/mL) was obtained from PeproTech and was added to the medium during feedings for indicated experiments. Multiple independent experiments were performed in triplicate. For relative growth assays, SB431542 (10 μM) and Nutlin-3 (10 μM) were obtained from Calbiochem and added to the medium during feeding for indicated experiments. TGF-β (10 ng/mL) was obtained from PeproTech and was added to the medium during feedings for indicated experiments. Multiple independent experiments were performed in triplicate.

Fig. S1. HMEC expressing an shRNA targeting p53 are resistant to Nutlin-3. (A) HMEC expressing a control shRNA (shGFP) or an shRNA targeting p53 (shp53) were treated with Nutlin-3. Western analysis confirmed the knockdown of p53 protein levels and the ablation of p53-dependent transactivation of target genes HDM2 and p21. (B) Treatment of control HMEC with Nutlin-3 resulted in p53-mediated growth arrest, whereas cells expressing a shRNA targeting p53 remained unaffected.

Fig. S2. Cells undergoing RAS-induced senescence are frequently multinucleated. Shp53-HMEC were infected with a RAS-G12V–expressing retrovirus or control retrovirus (Vector). Four days after infection, fluorescence microscopy was performed to examine the presence of multinucleated cells by DAPI staining.

Fig. S3. GSE56 efficiently inactivates p53. Western analysis of Vector-HMEC and GSE56-HMEC confirms the stabilization of inactive p53 and a decrease in the basal levels of p21. Vector-HMEC and GSE56-HMEC were treated with Nutlin-3 for 5 d and relative growth assessed.

Fig. S4. Multiple RAS effectors are required for RAS-mediated oncogene-induced senescence in HMEC. Various RAS-G12V point mutants capable of activating specific effector pathways, including RAF (T35S), RAL-GEF (E37G), or PI3K (Y40C), were expressed in shp53-HMEC. Each of these point mutations abolished the ability of RAS-G12V to induce p21 expression and suppress the growth of shp53-expressing HMEC, as determined by Western analysis, and cell number was quantified 5 d after infection.
Fig. S5. RAS effectors remain active during RAS-mediated oncogene-induced senescence in HMEC. GSE56-HMEC or Vector-HMEC was infected with a RAS-G12V–expressing retrovirus or control retrovirus (Vector). Western analysis was performed 4 d after infection to examine the level of phosphorylated ERK1/2 and AKT.

Fig. S6. RAS-mediated oncogene-induced senescence occurs independently of the RB family of proteins. HMEC expressing SV40 large T proteins (wild-type large T, a K1 mutant that specifically inactivates p53, and a Δ434-444 mutant) were infected with a RAS-G12V–expressing retrovirus or control retrovirus (Vector). Infected cells were plated, grown 5 d, and counted. Western analysis and Nutlin-3 treatment confirmed that p53 was efficiently inactivated (stabilized in Large T- and Large T K1-expressing HMEC).

Fig. S7. HMEC undergoing RAS-mediated oncogene-induced senescence lack SAHF. Shp53-HMEC were infected with a RAS-G12V–expressing retrovirus or control retrovirus (Vector). Four days after infection, confocal fluorescence microscopy was performed to examine the presence of SAHF by DAPI staining.

Fig. S8. RAS-mediated oncogene-induced senescence occurs independently of p21. Shp53/shp21-HMEC were infected with a RAS-G12V–expressing retrovirus or control retrovirus (Vector). Infected cells were plated, grown, and counted 5 d later. Western analysis confirmed the efficient knockdown of p21 expression.
**Fig. S9.** MYC and RAS-G12V expression does not confer resistance to Nutlin-3. HMEC and shp53-HMEC expressing c-MYC and RAS-G12V were treated with Nutlin-3, and relative growth was assessed after 5 d of continuous treatment.

**Fig. S10.** Ablation of MYC engages senescence in cancer cells harboring RAS mutations. A549, H1299, and LoVo cells were infected with retroviruses encoding shRNAs targeting MYC or GFP. After infection, $5 \times 10^4$ cells were plated, grown for 5 d, and counted. The knockdown of MYC was confirmed by Western analysis. A549, H1299, and LoVo cells expressing shGFP (G) or shMYC (M) were stained for the presence of senescence-associated β-galactosidase activity.