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Supplementary Information for

Altered p53 functionality in cancer-associated fibroblasts contributes to their cancer-supporting features

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†Deceased 25.11.2017. This paper is dedicated to his memory.

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Supplementary text

Methods:

Cell culture: All cells were maintained at 37˚C in a 5% (Vol/Vol) CO_2 humidified incubator. H460, H1299 and Calu1 cells were cultured in RPMI-1640 (Biological Industries) supplemented with 5% heat inactivated Fetal Bovine Serum (FBS) (Invitrogen), 1% GlutaMAX (Thermo Fisher Scientific) and 1% penicillin and streptomycin (P/S) (Biological Industries, Israel). HEK293 Phoenix cells were cultured in DMEM (Biological Industries) supplemented with 10% FBS and 1% P/S. NFs and CAFs were cultured in RPMI-1640 supplemented with 10% FBS, 1% glutamine, 1% sodium pyruvate and 1% P/S. For co-culture experiments, H460 cells and fibroblasts were seeded together at a 1:1 ratio and co-cultured for 3 days.

Viral infections: NFs and CAFs (patient 4731) were immortalized using a lentivirus co-expressing hTERT and GFP (Lenti-hTERT-eGFP; Cat No- LG508, BioGenova). To further enhance GFP expression, the immortalized fibroblasts were transduced with a lentivirus derivative of the pLEX_TRC206 plasmid (generous gift of Dr. Ravid Straussman). Retroviral packaging was performed by jetPEI-mediated transfection of HEK293 Phoenix cells with the shLacZ or shp53 expression plasmids together with a plasmid encoding the VSVG envelope protein. Virus-containing supernatants were collected 24h and 36h after transfection, filtered and supplemented with 4μg/ml polybrene (Sigma). Puromycin selection was initiated 48 h after infection and continued for a week. To generate stable mCherry expressing H460, H1299 and Calu1 cells, cultures were infected with an amphotropic retrovirus based on plasmid pLPCmCherry (generous gift of Dr. Valery Krizhanovsky).

Conditioned media: To generate conditioned media (CM), fibroblasts were grown to confluency and the regular culture medium was replaced with serum-free medium. 48hrs later, the CM was collected and filtered through a 0.2μm filter.

Collagen gel contraction assay: Immortalized or primary NFs and CAFs (patient 4731) were embedded in 400 μl of rat tail collagen-I (Corning, Germany). This mixture was seeded in a 24-well plate and incubated for 24 hours in a humidified incubator at 37ºC. To calculate the area of gel contraction (in cm^2), scanned images were quantified with Fiji software.

Live cell imaging: Tissue culture dishes were pre-coated with fibronectin (10 μg/ml). CAF/NF-GFP shLacZ/shp53 and Calu1 mCherry cells were seeded in the two wells of a silicon insert (Ibidi, Germany). 16h later, inserts were removed and the area between the wells was subjected to live cell imaging to monitor gap closure. Migration velocity of the Calu1-mCherry cells was calculated by comparing the position of the migrating front of these cells immediately after insert removal (t=0) and 6 hours later (before complete gap closure).

Trans-well migration assay: NFs and CAFs were seeded in the upper chamber of a Trans-well device (8μm pore size) (Costar, cat no #3422, Germany). EGF (10ng/ml) was added to the bottom chamber as chemo-attractant. 16 hours later, filters were fixed with ice cold methanol (100%) for 5 min, and migrated cells were stained with crystal violet for 30 minutes; non migrated cells were removed with cotton plugs. Fixed and stained cells were imaged (Nikon eclipse Ti-E microscope). To quantify the extent of migration,

the percentage of each image stained with Crystal violet was calculated, using the Fiji/ImageJ software; 4 non-overlapping fields were quantified from each trans-well.

To calculate the relative migration areas in Fig. 1D and Fig. 5H, the percentages of Crystal violet-stained areas from the four different conditions in a given experiment were averaged, and the normalized relative migration area for each condition was calculated against that average. Values were then compiled from 3 independent experiments. p-value were determined using one-way ANOVA and Tukey post hoc test.

To measure migration of co-cultured tumor cells, mCherry-expressing H460 cells (3-5x10^4 cells) were seeded together with indicated immortalized fibroblasts (3x10^4) (Patient 4731) in the upper chamber of a Trans-well device as above, EGF (10ng/ml) was added to the bottom chamber, and 16-20 hours later non-migrated cells were removed, and migrated tumor cells were photographed under a fluorescent microscope (Nikon eclipse Ti-E microscope).

Trans-well invasion assay: Trans-well upper chambers (8μm pore size) (Costar, cat no #3422, Germany) were coated with 40 μg of Matrigel Growth Factor Reduced (GFR) Corning, #356321) according to the manufacturer's instructions. Calu1 cells were seeded in the upper chamber of the Trans-well device. CM derived from different fibroblasts were added to the bottom chamber as chemo-attractant. 20 hours later, filters were fixed with ice-cold methanol (100%) for 5 min, and invading cells were stained with crystal violet for 30 minutes; non invading cells were removed with cotton plugs. Fixed and stained cells were imaged (Nikon eclipse Ti-E microscope). Image analysis and quantification of relative invasion area were done essentially as described above for trans-well migration.

FACS analysis: Co-cultured cells were harvested by using Accutase cell detachment solution (Millipore) and resuspended in PBS containing 2% FBS. GFP-expressing fibroblasts and mCherry-expressing H460 or H1299 cells were subjected to Fluorescent Activated Cell Sorting (FACSAria III, BD biosciences) using a 100μM nozzle. Sorted cells were immediately flash frozen in liquid nitrogen for further analysis.

Western blot analysis and immunoprecipitation: NFs and CAFs were lysed in NP-40 lysis buffer (150mM NaCl, 50mM Tris pH8.0, 1% NP-40 supplemented with phosphatase inhibitor cocktails 2 and 3 (Sigma) and protease inhibitor mix (Sigma)). Total protein concentration was estimated by Pierce BCA Protein Assay kit (Thermo Scientific) according to the manufacturer's protocol. For Western blot (WB) analysis of p53, membranes were probed with a mixture of DO-1 and PAb1801 antibodies. For IP, Immobilized protein A (IPA300, Repligen) was incubated with either PAb1620, PAb240, or PAb421 antibodies. Cell lysates were then incubated with the bound antibodies for 16 hours at 4^{o}C and then washed with NP-40 lysis buffer. Immunoprecipitates were resuspended in protein sample buffer and samples were resolved by SDS-PAGE followed by WB analysis with p53-HRP antibody. Imaging and quantification were performed using a ChemiDoc MP imaging 8 system (BioRad) with the Image Lab 4.1 program (BioRad). Phos-tag (Wako Pure Chemical Industries) gels were prepared and run according to the manufacturer's protocol.

RNA isolation and qRT-PCR analysis: Total RNA was isolated with the PerfectPure RNA kit (5 PRIME, USA). 1μg of total RNA was reverse transcribed with MMLV-RT (Promega), using random hexamers. RT-qPCR was performed using SYBER Green Master-mix (Applied Biosystems) in a StepOnePlus instrument (Applied Biosystems),

and analyzed with StepOne software. mRNA levels of tested genes were normalized to *GAPDH* mRNA. In Figure 3C, 6C and 6D the normalized expression of each gene was averaged across all conditions compared in the same experiment, and then expression values in each condition were calculated against that average.

RNA Sequencing: For RNA-seq analysis, two independent batches of cells served as biological replicates. Total RNA was processed using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina). Libraries were evaluated by Qubit fluorometric quantitation (Thermo Fisher) and Bioanalyzer (Agilent Genomics). Sequencing libraries were constructed with barcodes to allow multiplexing of multiple samples on one lane. Samples were sequenced on 2 lanes of an Illumina HiSeq machine, using the Single-Read 60 protocol; average output was ~26 million reads per sample. Reads were trimmed using cutadapt and mapped to GRCh38 using STAR v2.4.2a (default parameters). Counting proceeded over genes annotated in Ensembl release 83, using htseq-count (intersection-strict mode). Differential expression analysis was performed using DESeq2 with the betaPrior, cooksCutoff and independent filtering parameters set to False. Raw P values were adjusted for multiple testing, using the procedure of Benjamini and Hochberg. The pipeline was constructed using Snakemake. Clustering and dendrogram visualization were performed using Expander (EXPression ANalyzer and DisplayER)(3).

Gene expression matrices: The matrices in Figure 3 show genes differentially expressed between CAFs and NFs (fold change > 1.5 and adjusted p-value < 0.05, 1677 genes). Genes were defined as being p53-dependent if the fold change exceeded 1.5 in both experimental replicates. “p53 dependent educated genes” shown in the expression matrix in Figure 6 were defined as genes up-regulated upon co-culture of NFs with cancer cells,

relative to NFs in mono-culture (>1.5 fold in both replicates, 1193 genes), whose expression in co-cultured NFs shLacZ was at least 1.2 fold higher than in co-cultured NFs shp53, in both experimental replicates (250 genes). The Sorting Points Into Neighborhood (SPIN) method was used to analyze and visualize expression data.

Mass spectrometry: CM collected from different fibroblasts was subjected to in-solution tryptic digestion, followed by a desalting step. The resulting peptides were analyzed using liquid chromatography-mass spectrometry (LC-MS)**.** Each sample was analyzed on the instrument separately in a random order and discovery mode. Raw data was processed with MaxQuant v1.6.0.16 and searched with the Andromeda search engine against the human proteome database (SwissProt). Label-Free Quantification (LFQ) intensities were determined and used for further calculations using Perseus v1.6.0.7. LFQ values were used to plot the individual proteins in Figure 4C.

For Figure 4A, a heatmap was generated for secreted proteins differentially expressed between immortalized CAFshLacZ and CAFshp53 (fold change > 1.5 and adjusted p-value < 0.05 in all four repeats; total= 58 proteins). The heatmap was generated with a web-based tool, employing average linkage clustering and Pearson distance measure (4). Heat map was in Figure 4A show proteins differentially expressed between CAFshLacZ and CAFshp53 (fold change > 1.5 and adjusted p-value < 0.05, 58 proteins).

In vivo **tumor growth:** All animal experiments and methods were approved by the Weizmann Institutional Animal Care and Use Committee. mCherry expressing H460 cells were injected sub-cutaneously into the flank of male C.B-17/Icr-scid-bg mice (Harlan laboratories), either alone or together with a 3-fold excess of different GFP-expressing fibroblasts. Tumors were harvested and photographed either 9 or 12 days

post-injection. In Figure 7, relative tumor size was deduced from the photographic images with Fiji software. In Figure S8, tumors were weighed.

Statistical analysis: For qRT-PCR analysis (Figure 3) two-way *ANOVA* was conducted. The difference between cell types and between shRNA treatments were found significant, as well as the interaction between cell type and shRNA treatment (α=0.05). The significance of specific comparisons were assessed using Bonferroni`s correction. Figure 4C, 5H, 5I, 6 and 7 were analyzed with One-way ANOVA by using Graphpad PRISM5 software. In all other cases, p-values were determined by two-tailed t-tests.

Methods for viral infection, conditioned media, live cell imaging, Trans-well migration and invasion assays, Western blot analysis, immunoprecipitation, RNA isolation, qRT-PCR, Mass spectrometry and RNA sequencing are described in SI methods.

Figure S1

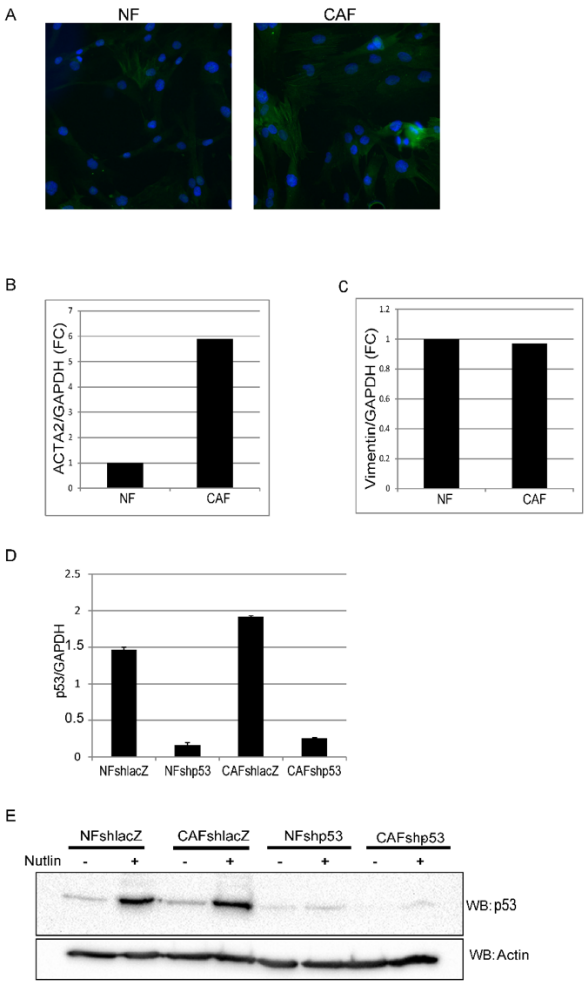

Figure S1 (related to Figure 1). Protein levels and gene expression in primary and immortalized NFs and CAFs from patient 4731. (A) Immortalized NFs and CAFs (patient 4731) were immunostained with an antibody against alpha smooth muscle actin (green). Nuclei were counter-stained with DAPI. (B) qRT-PCR analysis of alpha smooth muscle actin (ACTA2) mRNA expression in NFs and CAFs (patient 4731). Values were normalized to GAPDH mRNA in the same samples. (C) qRT-PCR analysis of vimentin mRNA expression in NFs and CAFs. Values were normalized to GAPDH mRNA. (D) qRT-PCR analysis of p53 mRNA in immortalized NFs and CAFs (patient 4731), stably expressing p53 shRNA or control LacZ shRNA. (E) NFs and CAFs were treated for 8hrs with Nutlin-3a (10μM) or DMSO as control. Cell lysates were subjected to Western blot analysis with antibodies specific for p53 (DO1 plus PAb1801) or beta actin as loading control.

Figure S2

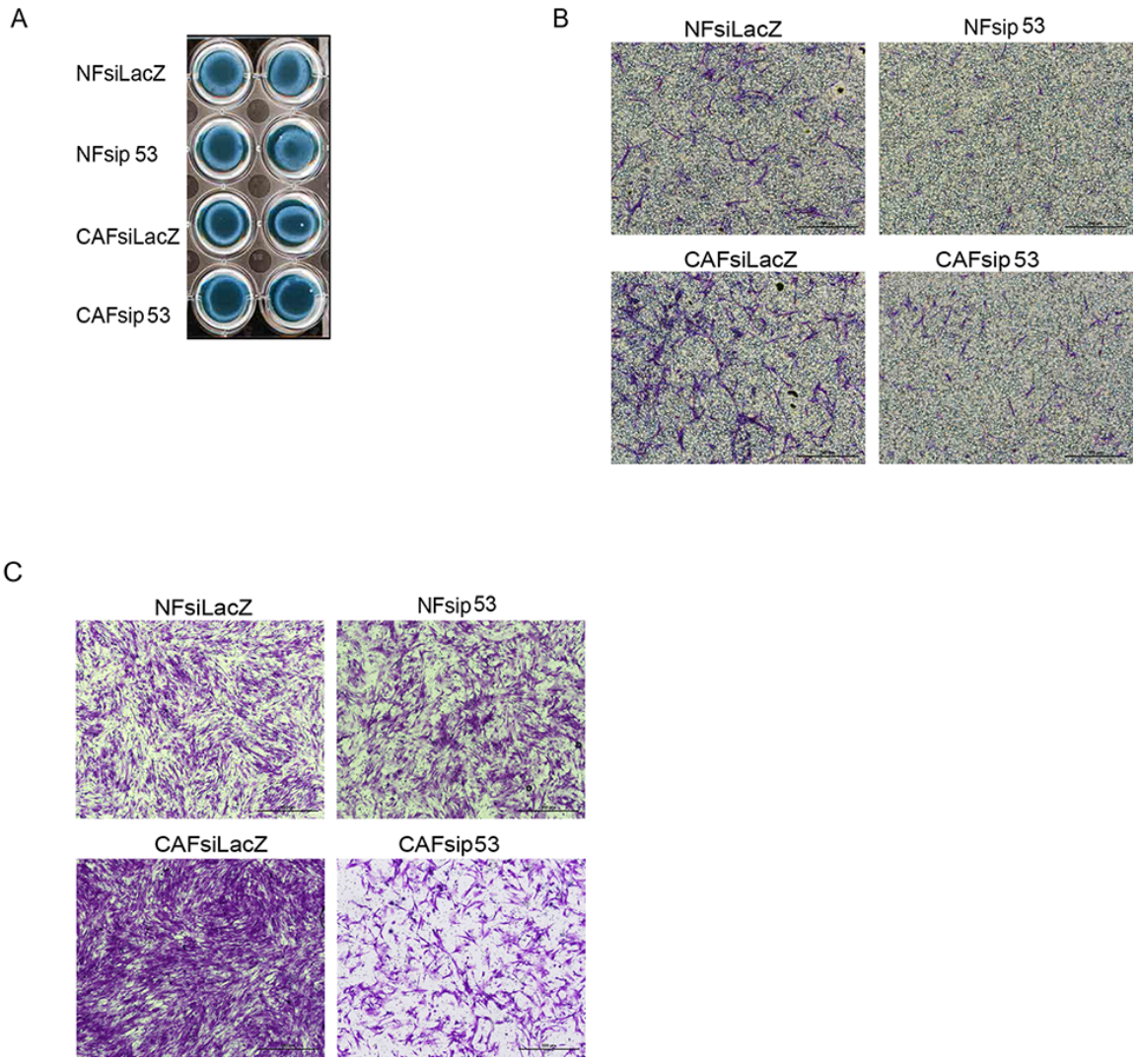

Figure S2 (related to Figure 1). Functional impact of p53 depletion in primary NF-CAF pairs. (A) Low passage NF and CAF cultures from patient 8163 were transiently transfected with siRNA oligonucleotides against p53 or control LacZ siRNA, and assayed for collagen gel contraction activity as in Figure. 1A. (B) Low passage NF and CAF cultures from patient 8163, transfected as in (A), were subjected to a transwell migration assay as in Figure. 1C. (Scale bar=500µm). (C) Low passage NF and CAF cultures from patient 9585, transfected as in (A), were subjected to a transwell migration assay as in Figure. 1C. (Scale bar=500µm).

Figure S3

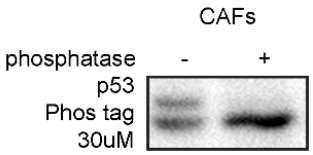

Figure S3 (related to Figure 2). Validation of phosphorylated p53 by phosphatase treatment. Lysates of immortalized CAFs (patient 4731) were incubated at 30^0C for 1h, either with (+) or without (-) Lambda phosphatase, and then subjected to Phos-tag SDS-PAGE and Western blot analysis as in Figure. 2.

Figure S4

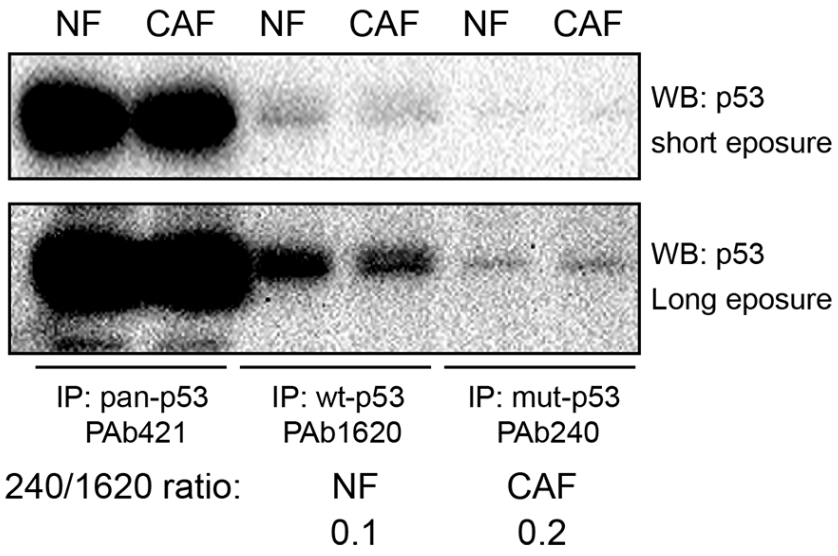

Figure S4 (related to Figure 2). Analysis of NF and CAF p53 with conformation-specific monoclonal antibodies. Immortalized NF and CAF (patient 4731) protein lysates were immunoprecipitated (IP) with the indicated antibodies, followed by Western blot (WB) analysis with p53-HRP antibody. PAb1620 and PAb240 detect the WT p53 conformation and a mutant p53 conformation, respectively, whereas PAb421 detects all conformations.

Figure S5

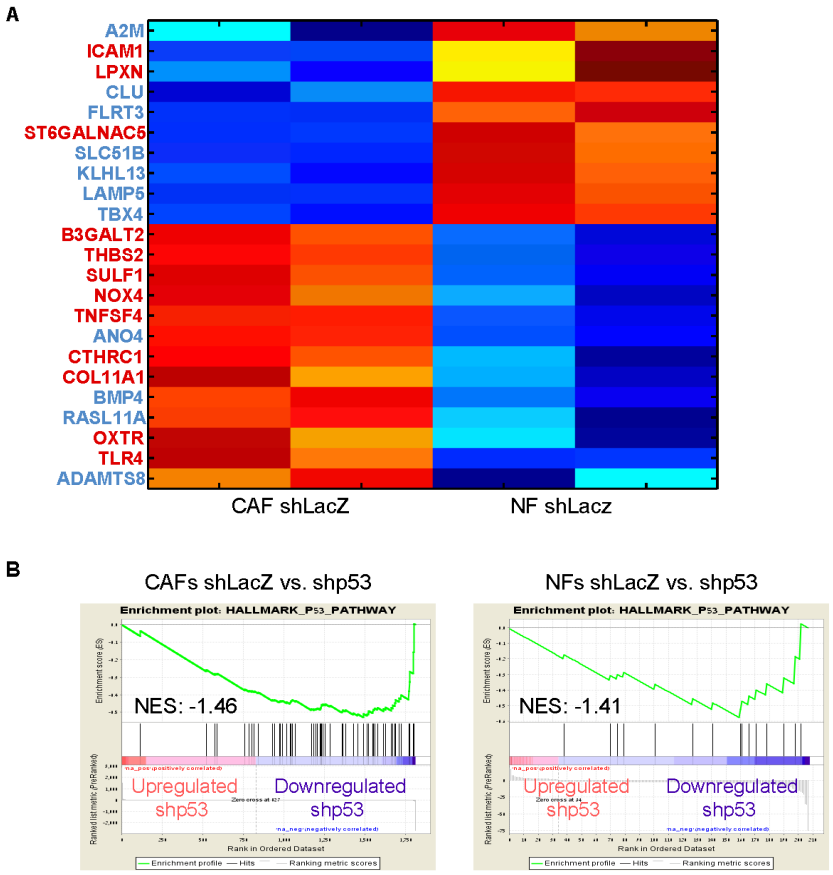

Figure S5 (related to Figure 3). Analysis of genes expressed differentially between immortalized CAFs and NFs. (A) Out of 46 genes identified by Navab et al. (5) as differentially expressed between CAFs and NFs of non-small cell lung cancer patients, 23 genes were significantly differentially expressed between immortalized CAFs and NFs (patient 4731) in our transcriptome analysis (fold change > 1.5 and adjusted p-value < 0.05). SPIN-ordered expression matrix of these 23 genes is shown. Heatmap colors indicate expression levels after standardizing each gene. Genes symbols are colored according to directionality reported by Navab et al.: blue = downregulated in CAFs, red = upregulated in CAFs. (B) Genes significantly differentially expressed (fold change > 1.5 and adjusted p-value < 0.05) between shLacZ and shp53 expressing cells, in either CAFs (1811 genes, left) or NFs (208 genes, right), were ranked according to fold changes and analyzed by Gene Set Enrichment Analysis tool (6) In both cases, differentially expressed genes are enriched for p53 pathway genes (HALLMARK_P53_PATHWAY gene set).

Figure S6

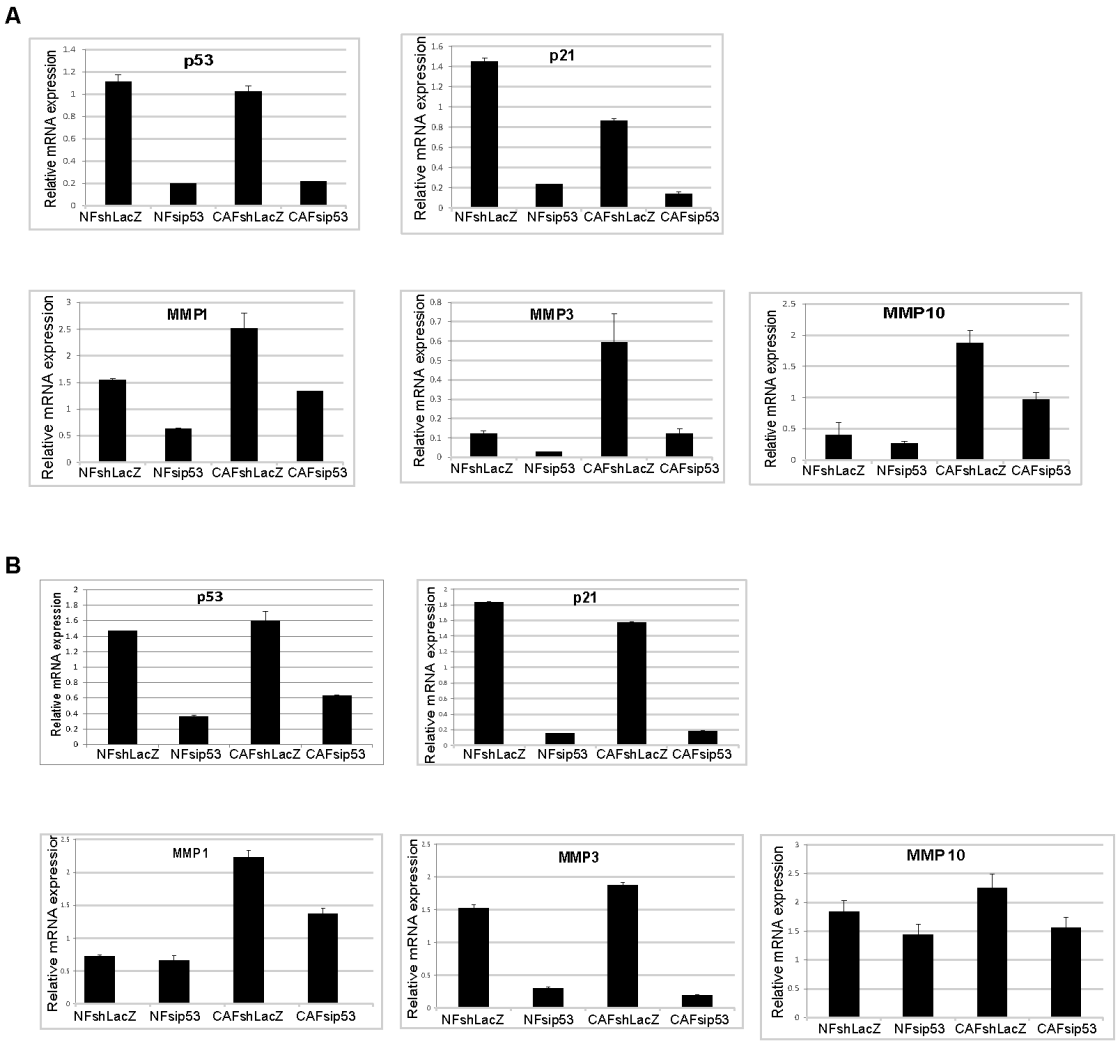

Figure S6 (related to Figure 3). p53-dependent gene expression in primary NF-CAF pairs. (A) Low passage primary NF and CAF cultures from patient 8163 were transiently transfected with siRNA oligonucleotides against p53 or control LacZ siRNA. RNA was extracted and subjected to qRT-PCR analysis of the indicated mRNAs. Values were normalized for GAPDH mRNA in the same samples. (B) Low passage primary NF and CAF cultures from patient 9585 was treated and analyzed as in (A).

Figure S7

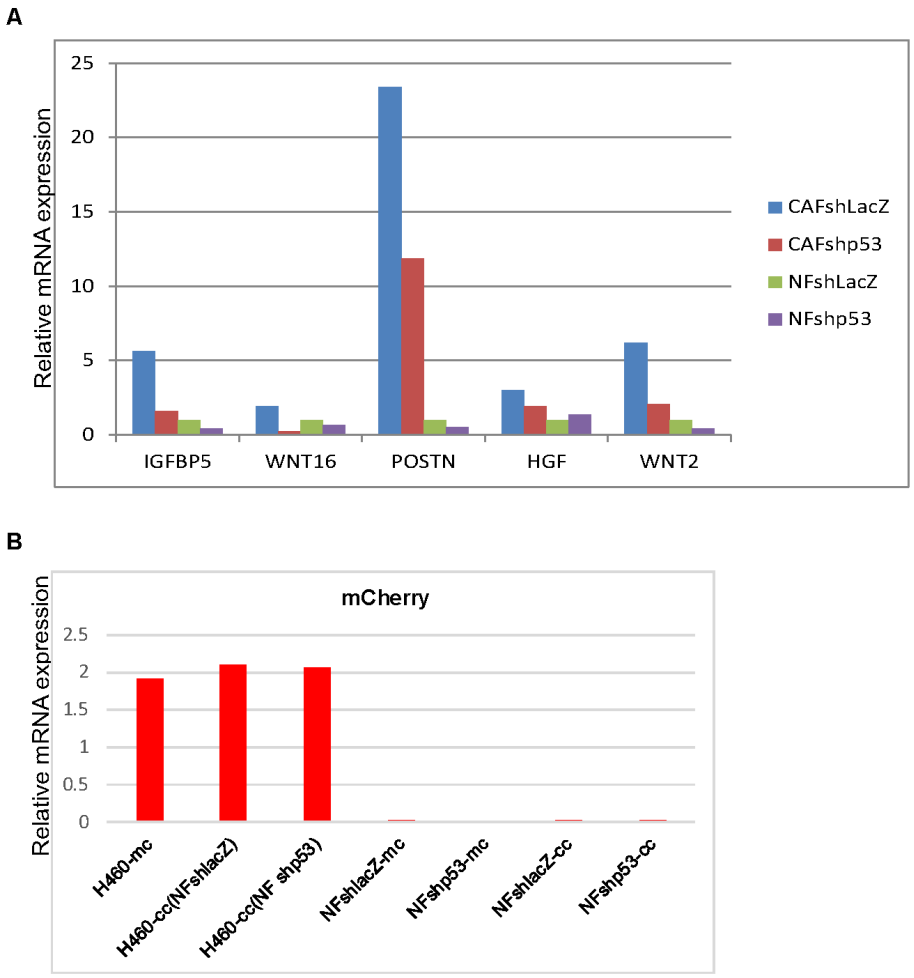

Figure S7 (related to Figure 4,6). CAFs display elevated p53-dependent expression of genes encoding secreted proteins. (A) Expression levels of the indicated gene transcripts in RNA from immortalized CAFs and NFs (patient 4731), with and without p53 silencing, were deduced from the RNA-seq analysis. Bars represent normalized RPKM values, depicted as fold change relative to control NFs (NFshLacZ). (B) immortalized normal fibroblasts (NF) were either cultured alone, or co-cultured with mCherry-labeled H460 cells and isolated by FACS sorting prior to RNA extraction. The figure shows qRT-PCR analysis of mCherry mRNA levels in the indicated fibroblast populations as well as in the cancer cells, either growing as monoculture (first column) or after co-cultivation with the indicated fibroblasts and subsequent FACS sorting. Values were normalized to GAPDH mRNA in the same samples.

Figure S8

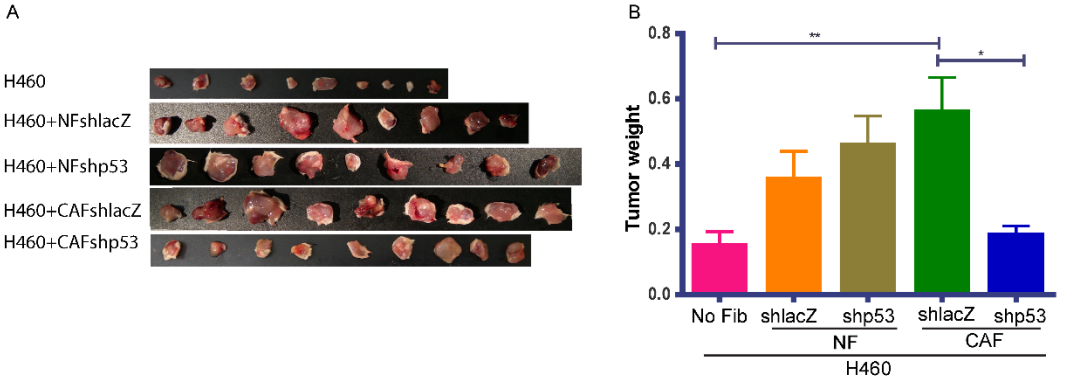

Figure S8 (related to Figure 7). CAF p53 promotes tumor growth in SCID mice. mCherry-expressing H460 cells ($5x10^5$) were injected either alone or together with $1.5x10^6$ immortalized fibroblasts, exactly as in Figure 7. Each group comprised 9 mice. Tumors were excised and weighed 12 days post-injection. (B) Graphical representation of tumor weight of each group (mean ± SEM).* p-value ≤, 0.05, ** p-value < 0.01 ≤, ***p ≤, 0.001 using one-way ANOVA and Tukey post hoc test.

Supplementary videos (related to Figure 5):

Supplementary video 1. Time lapse movie of GFP-expressing immortalized NFshLacZ cells from patient 4731 (Yellow) and mCherry-expressing Calu1 cells (magenta). Cells were seeded in 12-well plates containing ibidi culture inserts. The next day, inserts were removed and cells were allowed to migrate. Images were captured every 15 min, for a total of 6 h.

Supplementary video 2. Time lapse movie of GFP-expressing immortalized NFshp53 from patient 4731 (Yellow) and mCherry expressing Calu1 cells. Cells were processed as in video 1.

Supplementary video 3. Time lapse movie of GFP-expressing immortalized CAFshLacZ from patient 4731 (Yellow) and mCherry-expressing Calu1 cells. Cells were processed as in video 1.

Supplementary video 4. Time lapse movie of GFP-expressing immortalized CAFshp53 from patient 4731 (Yellow) and mCherry-expressing Calu1. Cells were processed as in video 1.

Supplementary video 5. Time lapse movie of GFP-expressing immortalized CAFshLacZ from patient 4731 (Yellow) and mCherry-expressing Calu1 cells. Cells were processed as in video 1, except that the culture was supplemented with CM from CAFs.

Supplementary video 6. Time lapse movie of GFP-expressing immortalized CAFshp53 from patient 4731 (Yellow) and mCherry-expressing Calu1 cells. Cells were processed as in video 1, except that the culture was supplemented with CM from CAFs.

Supplementary video 7. Time lapse movie of GFP-expressing immortalized CAFshp53 from patient 4731 (Yellow) and mCherry-expressing Calu1 cells. Cells were processed as in video 1, except that the culture was supplemented with CM from CAFshp53

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