Supporting Information

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SI Materials and Methods

Cell Culture and Transfection. Young adult mouse colon (YAMC) cells expressing both $p53^{175H}$ and oncogenic HRasV12 (referred to as mp53/Ras) were cultured at 33 °C and with 5% (vol/vol) CO_2 in RPMI growth medium (Gibco), containing 10% (vol/vol) FBS and supplements, as previously described (1, 2). All mp53/Ras cell derivatives were cultured at 39 °C in RPMI medium supplemented with 10% (vol/vol) FBS, 1× insulin–selenium–transferrin–A (ITS-A), and 2.5 μg/mL gentamicin. YAMC cells and all of their derivatives were grown on dishes coated with 1 μg/cm^2 of collagen I (BD Biosciences). HeLa cells, HEK-293T cells, and human colon cancer DLD-1 and HT-29 cells were maintained at 37 °C and with 5% (vol/vol) CO_2 in water-jacketed, humidified incubators. Growth medium was DMEM (Gibco) supplemented with 10% (vol/vol) FBS, 2 μg/mL gentamicin, and 100 μg/mL kanamycin. HEK-293T cells were transfected using LipofectamineTM 2000 (Invitrogen) according to the manufacturer's protocol.

Genetically perturbed cells were derived by means of lentiviral infection with virus containing the appropriate cDNA or shRNA expression constructs. Lentivirus for infection was produced by transiently transfecting 293T cells. After 12 h of incubation, the transfection medium was removed from the cells and replaced with 4 mL standard growth medium; the cells were incubated for an additional 36 h. Freshly collected virus-containing media from transfected 293T cells were filtered through 0.45-μm syringe filters (Pall), and were used to infect target cells. Polyclonal cell populations stably expressing the indicated cDNAs and/or shRNAs were generated by selection in standard growth medium containing 5 μg/mL puromycin. All stable knockdown cell lines were generated using a ViraPower lentiviral expression system, whereas the FUG12-puro lentiviral vector system was used for generating the other stable cell lines.

Molecular Biology and DNA Constructs. Plasmid DNA constructs with Flag-tagged heterochromatin protein 1α (HP1α) cDNA and the variants with V21M, I165K, and W174A mutations were kindly provided by Mark S. Lechner (Simon Fraser University, Burnaby, BC, Canada). GFP-tagged HP1α was from Addgene. GFP-tagged STAT5A and truncated STAT5A shown in Fig. 1*A* were generous gifts from Nancy Reich (Stony Brook University, Stony Brook, NY). STAT5A variants encoding the V467A, Y694F, or the V467A, Y694F double mutations were generated from a wild-type human STAT5A cDNA using the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene). DNA sequence substitutions were verified.

Immunostaining, Immunoprecipitation, and Western Blotting. For coimmunoprecipitation, HEK-293T cells were harvested in lysis buffer (50 mM Tris·HCl, pH 8.0, 250 mM NaCl, 5 mM EDTA, 1 mM DTT, 5% glycerol) supplemented with complete protease inhibitor mixture and PMSF. Portions of lysate containing equal amounts of protein (200 μg) were then immunoprecipitated overnight at 4 °C with protein G-Agarose (Roche)-bound antibodies. The beads were then washed, and associated protein complexes were recovered in SDS sample buffer. Protein samples were resolved on a 10% SDS/PAGE. Rabbit anti-FLAG (1:500; Rockland) and rabbit anti-GFP (1:500; Rockland) primary antibodies were used to detect HP1α-Flag and STAT5A-GFP, respectively.

For chromatin immunoprecipitation (ChIP), HeLa cells stably overexpressing the vector control, HP1α, or STAT5A variants were collected and cross-linked at room temperature with formaldehyde. The cells were pelleted, lysed, and sonicated to shear the genomic DNA, and then centrifuged to remove the cell debris. A portion of the resulting chromatin lysate was prepared as the input chromatin using a QIAquick PCR Purification kit (Qiagen; 28106). The remaining chromatin lysate was incubated overnight at 4 °C with 4 μg of anti-H3K9me3 antibodies (Upstate; 07–442) or an equivalent volume of IgG for mock treatment. Agarose-A beads, preblocked with salmon sperm DNA, were added to each chromatin lysate and the samples were incubated overnight at 4 °C on a rocking platform. After elution and de-crosslinking, the chromatin was isolated using a QIAquick PCR Purification kit, and a 1-μL portion of the chromatin was used for each Sybr Green quantitative PCR (qPCR) reaction.

The following primers were used for qPCR after ChIP:

Human *IGF*2_CpG1 forward–TGGAAAGAATGTTTGAGGGCAGGG

Human *IGF*2_CpG1 reverse–AGGCCAGAAACAATAGTGGGAGGA

Human *IGF*2_P3-a forward–TTGGGTTTCTTCCTTACACCCGGA

Human *IGF*2_P3-a reverse–TTCTTTGCCTTCTCCACACGGA

Human *IGF*2_P3-b forward–AAATTTGGGCATTGTTCCCGGCTC

Human *IGF*2_P3-b reverse–TGTGTTTGGGCAACGCTAGAGAGA

Human GAPDH forward–CATGGGTGTGAACCATGAGA

Human GAPDH reverse–CAGTGATGGCATGGACTGTG.

Fluorescence Recovery After Photobleaching. Stable HeLa cell lines expressing vector control, $STAT5A^{WT}$, $STAT5A^{V467A}$, $STAT5A^{Y694F}$, or $STAT5A^{V467A,Y694F}$ were generated by lentivirus infection. GFP-HP1α was transfected into these stable cell lines using LipofectamineTM 2000 (Invitrogen). Fluorescence recovery after photobleaching (FRAP) was done using an Olympus confocal microscope. A 405-nm laser was used to irreversibly bleach a defined spot and then measure the recovery of the fluorescence intensity in the bleached spot. Quantitative analysis was done using Excel (Microsoft), and the bleached fluorescence intensity of each bleached spot was normalized to its intensity before bleaching, which was set at 100%.

Xenograft Assays. Tumor formation was assayed by xenoimplantation of genetically perturbed cells. Before xenoimplantation, genetically perturbed cells were grown for 48 h under standard culture conditions without selective drugs. Next, 5×10^5 cells were s.c. injected into the left and right flanks of 4- to 6-mo-old female CD-1 nude mice (Crl:CD-1-$Foxn1^{nu}$, Charles River Laboratories). Tumor volumes were measured by caliper at indicated times and mice were euthanized following final tumor measurements. Tumor volume was calculated using the average of three measurements of the tumor radius and the formula volume = $(4/3)\pi r^3$. The statistical significance of differences in tumor size was determined by Student *t* test. All animal procedures conformed to American Association for the Accreditation of Laboratory Animal Care (AAALAC) regulations and were approved by the

University Committee on Animal Resources (UCAR) at the University of Rochester Medical Center.

Cell Proliferation Measurements and Flow Cytometry. Before measuring cell proliferation, all genetically perturbed mp53/Ras cells were grown under standard culture conditions for 48 h without selective drugs. To assess proliferation, mp53/Ras cell derivatives were seeded at 3,400 cells/cm^2 in standard RPMI growth medium. Cell samples were counted at the indicated time points. The following formula was used to calculate doubling. Cell doubling = (the number of live cells at harvest/the number of cells initially plated) − 1.

For measuring cell cycle profiles with Flow Cytometry, 2×10^6 cells were washed with 1× PBS and resuspended with 1 mL PBS in 15-mL tubes and fixed in 75% ethanol overnight at −20 °C. Fixed cells were washed with PBS and resuspended in 1 mL of DNA staining solution [20 μg/mL propidium iodide (PI) and 200 μg/mL DNase free RNase in PBS]. After 30 min, cells were analyzed by flow cytometry with the BD FACSCanto flow cytometer.

For detecting apoptosis, Alexa Fluor 488 annexin V/Dead Cell Apoptosis kit (Invitrogen; V13241) was used. Cells were stained with Alexa Fluor 488-conjugated annexin V according to the manufacturer's instruction. Briefly, 1×10^6 cells were resuspended in 100 μL of 1× annexin-binding buffer. A total of 5 μL of Alexa Fluor 488 annexin V and 1 μL of 100 μg/mL PI were added onto the cells. Cells were then incubated for 15 min at room temperature followed by addition of 400 μL annexin-binding buffer. The stained cells were analyzed with the BD FACSCanto flow cytometer. Emission was collected at 530 nm and 575 nm with 488-nm excitation as recommended by the manufacturer.

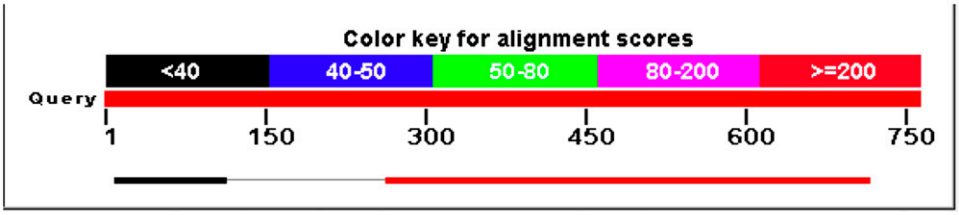
Reverse Transcription and qPCR. Total RNA was isolated from cells using an RNeasy Mini kit (Qiagen). cDNA was generated for qPCR analysis by using SuperScript II reverse transcriptase and oligo-dT as primers. SYBR Green-based quantitative PCR was performed using Bio-Rad iQ SYBR Green master mix, specific forward and reverse primers, and an iCycler machine (Bio-Rad). Gene expression was calculated using the ΔΔCt method, and RhoA or GAPDH expression was used for normalization.

Microarray Studies. Total RNA was purified from 1×10^6 cells expressing desired transgene using a RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Two hundred nanograms of total RNA was used to synthesize the first strand of cDNA using ArrayScript reverse transcriptase and an oligo(dT) primer bearing a T7 promoter. The single-stranded cDNA was then converted into a double-stranded DNA (dsDNA) using DNA polymerase I in the presence of *Escherichia coli* RNase H and DNA ligase. The dsDNA was used to make biotin-labeled antisense RNA (aRNA) by in vitro transcription using an Ambion MessageAmp Premier RNA Amplification kit (Applied Biosystems). A total of 750 ng of purified aRNA per sample was applied to an Illumina HumanHT-12 v4 chip at 58 °C for 18 h. After washing and staining with streptavidin-Cy3, the chip was scanned using an Illumina BeadArray Reader and standard DirectHyb Gene Expression protocol with the following settings: factor = 1, photomultiplier tube (*PMT*) = 587, filter = 100%. The raw data were extracted using Illumina BeadStudio software without normalization. The raw intensity data from the gene chips were normalized and further analyzed using computer software, which included Excel, Bioconductor, Illumina Gene Expression Module, and GenePattern (Broad Institute).

1. McMurray HR, et al. (2008) Synergistic response to oncogenic mutations defines gene class critical to cancer phenotype. *Nature* 453(7198):1112–1116.

2. Xia M, Land H (2007) Tumor suppressor p53 restricts Ras stimulation of RhoA and cancer cell motility. *Nat Struct Mol Biol* 14(3):215–223.

Graphic Summary



Score = 327 bits (837), Expect = 3e-103, Method: Compositional matrix adjust.
Identities = 195/470 (41%), Positives = 274/470 (58%), Gaps = 39/470 (8%)

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STAT92E,     265 ELKNWRHQQAQAGNGAPFNEGSLDDIQRCFEMLESFIAHMLAAVK--ELMRVRLVTEEPE
STAT5A,      251 ELIQWKRRQQLAGNGGP-PEGSLDVLQSWCEKLAEIIWQNRQQIRRAEHLCQQLPIPGPV
                 **  *   *  **** *  *****  *   * *   *          *     *    *
STAT92E,     323 LTHLLE---QVQNAQKNLVCSAFIVDKQPPQVMKTNTRFAASVRWLIGSQLGIHNNPPTV
STAT5A,      310 EEMLAEVNATITDIISALVTSTFIIEKQPPQVLKTQTKFAATVRLLVGGKLNVHMNPPQV
                   * *          ** * **  ****** ** * *** ** * *  *  * *** *
STAT92E,     380 ECIIMSEIQSQRFVTRNTQMDNSSLSGQSSGEIQNASSTMEYQQNNHVFSASFRNMQLKK
STAT5A,      370 KATIISEQQAKSL------LKNENTRNECSGEILNNCCVMEYHQATGTLSAHFRNMSLKR
                   * ** *           *       **** *    *** *     ** **** **
STAT92E,     440 IKRAEKKGTESVMDEKFALFFYTTTTV--NDFQIRVWTLSLPVVVIVHGNQEPQSWATIT
STAT5A,      424 IKRADRRGAESVTEEKFTVLFESQFSVGSNELVFQVKTLSLPVVVIVHGSQDHNATATVL
                 ****   * ***  ***   *     *  *     * ************ *     **
STAT92E,     498 WDNAFAEIVRDPFMITDRVTWAQLSVALNIKFGS--CTGRSLTIDNLDFLYEKLQREERS
STAT5A,      484 WDNAFAEPGRVPFAVPDKVLWPQLCEALNMKFKAEVQSNRGLTKENLVFLAQKLFNNSSS
                 *******  * **   * * * **  *** **       * **  ** **  **     *
STAT92E,     556 EY-------ITWNQFCKEPMPDRSFTFWEWFFAIMKLTKDHMLGMWKAGCIMGFINKTKA
STAT5A,      544 HLEDYSGLSVSWSQFNRENLPGWNYTFWQWFDGVMEVLKKHHKPHWNDGAILGFVNKQQA
                       * **  *  *    *** **   *   * *    *  * * ** **  *
STAT92E,     609 QTDLLRSVYGIGTFLLRFSDSELGGVTIAYVNENGLVTM--LAPWTARDFQVLNLADRIR
STAT5A,      604 HDLLINKPDG--TFLLRFSDSEIGGITIAWKFDSPERNLWNLKPFTTRDFSIRSLADRLG
                    *     *  ********** ** ***            * * * ***    ****
STAT92E,     667 DLDVLCWLHP
STAT5A,      662 DLSYLIYVFP
                 **  *    *
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Fig. S1. Alignment of STAT92E and STAT5A. Graphic summary and an alignment of homologous sequences found in STAT92E (*Drosophila*) and human STAT5A generated using BLASTP. Among the seven human STAT proteins, the one showing the most identity to *Drosophila* STAT92E is STAT5A. In a homologous domain of 470 residues in STAT5A, 42.8% are identical to the corresponding residues in STAT92E. Like STAT92E, STAT5A contains the potential HP1-binding motif PVVVI (red letters beginning at amino acid 465).

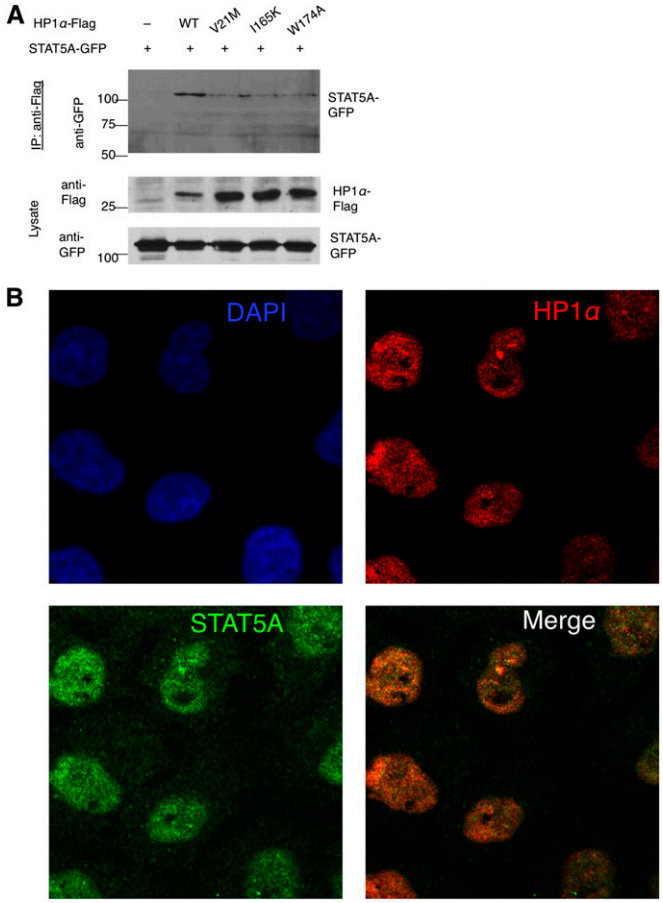

Fig. S2. Physical interaction and colocalization of STAT5A and HP1α. (*A*) 293T cells were cotransfected with wild-type STAT5A-GFP and with HP1α-FLAG or a mutant HP1α-FLAG with the V21M, I165K, or W174A mutation. Coimmunoprecipitation assays using anti-Flag antibodies were carried out to examine the interaction between HP1α and STAT5A. The presence of STAT5A-GFP was detected with anti-GFP antibodies. (*B*) HeLa cells cultured at 70% confluency were fixed and immunostained with anti-STAT5A (green) and anti-HP1α (red) antibodies and then with fluorescent secondary antibodies, and imaged with a laser scanning confocal microscope. Note the colocalization of green and red signals in the nuclei in the representative images shown.

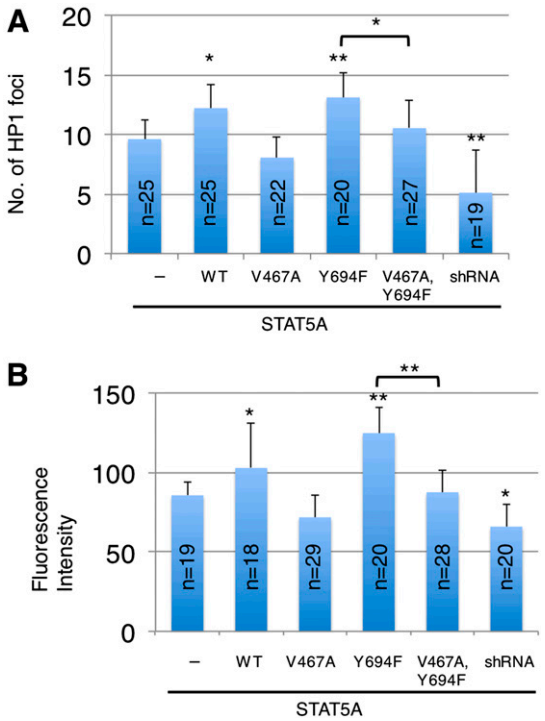

Fig. S3. Quantification of HP1α-GFP foci and DAPI fluorescence. The number of HP1α-GFP foci (*A*) and the intensity of DAPI fluorescence (in arbitrary units) (*B*) were quantified. *n* represents the number of cells counted for the indicated cell line. Error bars are SDs. $*P < 0.05$ and $**P < 0.01$ (Student *t* test), respectively, compared with the empty-vector control or with the indicated cell type.

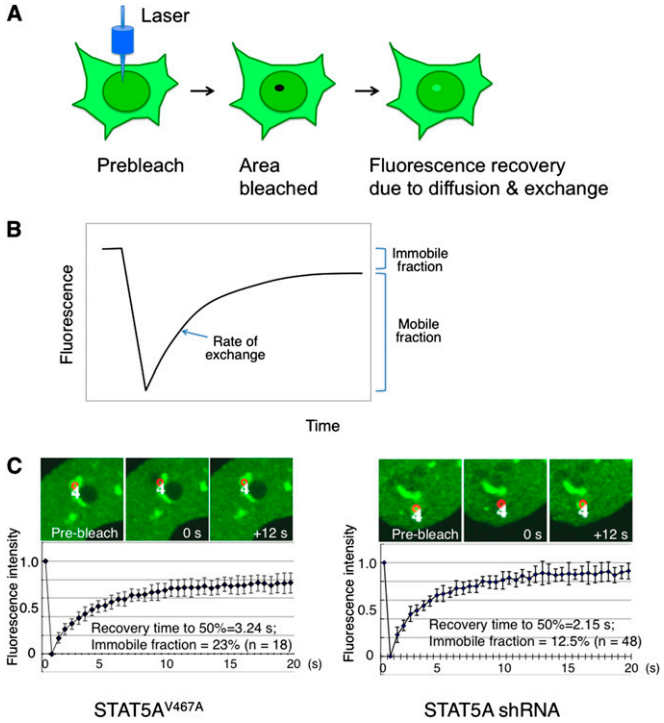

Fig. S4. FRAP. (*A*) Schematic illustration of FRAP. Briefly, in FRAP, a specific protein or cellular component is labeled with a fluorescent molecule. The live cell is imaged and a small, defined region of the cell is photobleached with a laser beam. Further imaging of the cell is done to determine the rate at which fluorescence returns to the area that was photobleached. This rate is governed by both the diffusion rate of the fluorescently tagged molecule and its affinity for cellular structures. Thus, quantitative estimates of the association and dissociation rates of binding can be extracted from the FRAP curve. In addition to determining the rate of fluorescence recovery, reflecting exchange of molecules, we can also determine the immobile fraction, which represents photobleached molecules that were not exchanged with the surrounding unbleached molecules, i.e., permanently bound molecules. In general, a slower exchange rate indicates that the target molecule is less mobile, and an increase in the immobile fraction indicates that the target molecule is more stably associated with cellular structures. (*B*) Typical FRAP curve with the parameters indicated. (*C*) HeLa cells stably expressing a vector control or the indicated STAT5A variants were transfected with HP1α-GFP and were analyzed for FRAP using a confocal microscope. The red circle delineates the area in heterochromatin (HP1α-GFP focus) that was bleached with a laser beam. White number 4 indicates sample number. The recovery of fluorescence in the bleached area was imaged over time. The indicated number of FRAP experiments was averaged and the mean FRAP curves are shown. Error bars indicated SDs. Two representative FRAP experiments are shown.

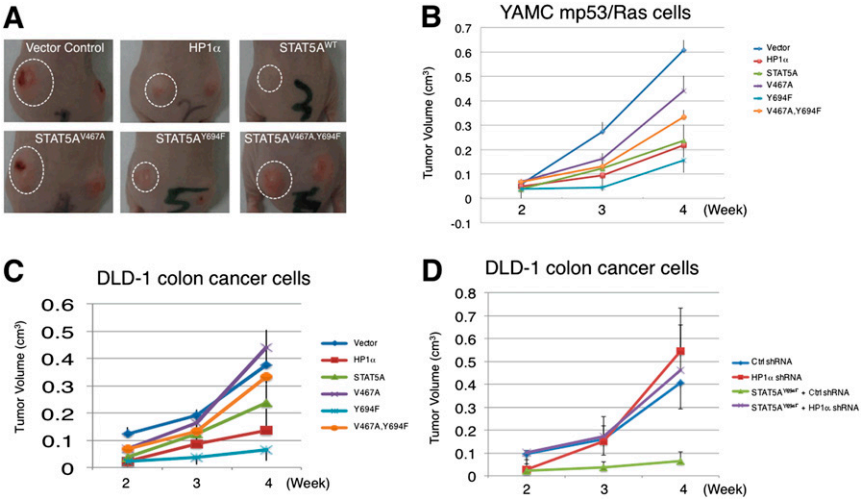

Fig. S5. Unphosphorylated STAT5A and HP1α inhibit tumor growth in vivo. (*A*) Representative images of recipient mice 4 wk after s.c. implantation of tumor cells expressing the indicated transgene. A total of 10^6 cells were injected into each flank. Dashed white circle indicates the left-side tumor. (*B*–*D*) Tumor volumes over time (*Left*; mean and SE) of the indicated derivatives of YAMC mp53/Ras cells (*B*) or human colon cancer DLD-1 cells (*C* and *D*) expressing the vector control (vector), HP1α, STAT5A^{WT}, STAT5A^{V467A}, STAT5A^{Y694F}, or STAT5A$^{V467A\&Y694F}$ (*B* and *C*), or expressing shRNA-HP1α, scramble shRNA, or shRNA-HP1α along with STAT5A^{Y694F} (*D*) are shown. Six injections ($n = 6$) were done for each of the indicated transgene and cell line combination. *n* represents the number of injections for the indicated cell line. $*P < 0.05$ and $**P < 0.01$ (Student *t* test), respectively, compared with the vector control or with the indicated cell type.

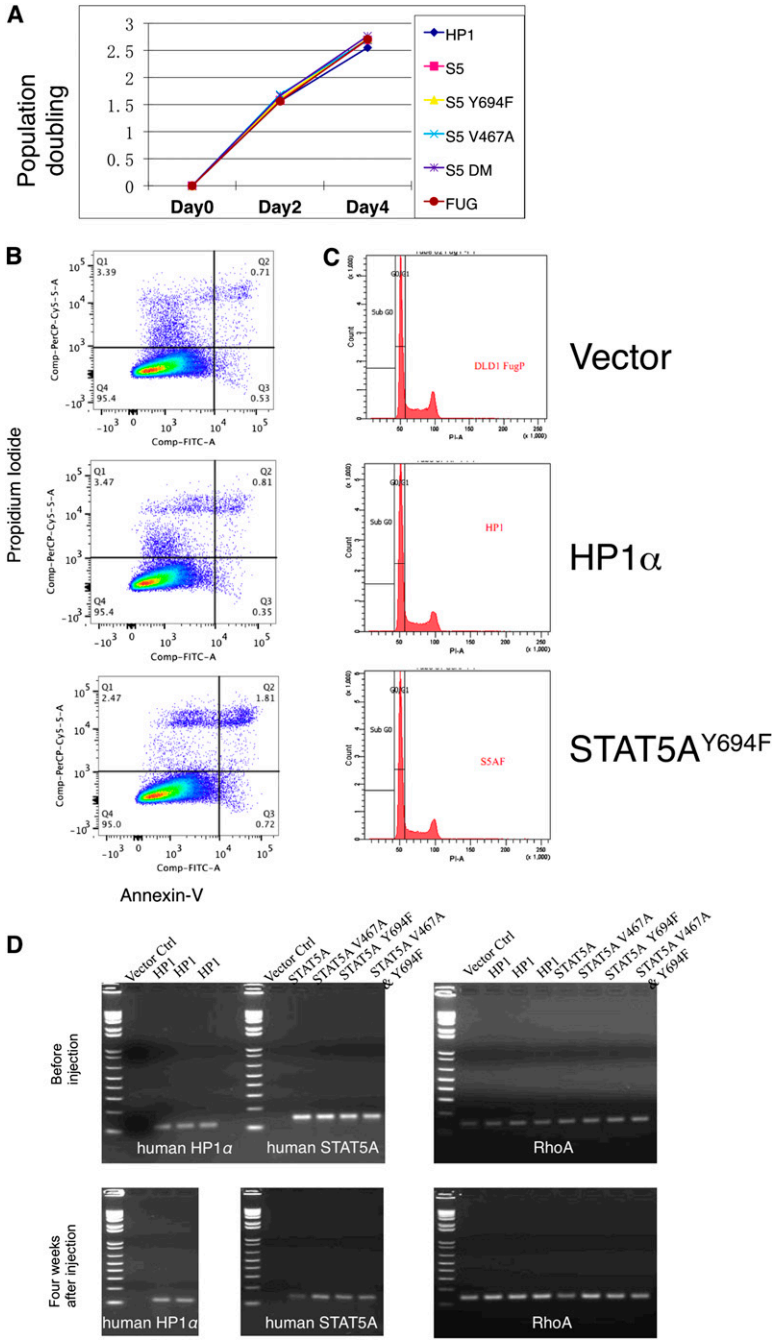

Fig. S6. Effects of overexpression of human HP1α, STAT5A^{WT}, or different STAT5A variants, on proliferation and apoptosis of YAMC mp53/Ras and DLD-1 cells in vitro. (*A*) Proliferation of mp53/Ras cells overexpressing vector control (vector), HP1α, STAT5A^{WT}, STAT5A^{V467A}, STAT5A^{Y694F}, or STAT5A$^{V467A\&Y694F}$ (double mutant) was measured by counting live cells at the indicated times after plating. Values represent the average of three independent experiments. (*B*) DLD-1 cells stably expressing empty vector (control), HP1a, or STAT5AY694F were grown to 70% confluency and were stained with Alexa Fluor 488-conjugated annexin V and propidium iodide (PI) and then subjected to flow cytometry to detect apoptotic or necrotic cells. (*C*) DLD-1 cells stably expressing empty vector (control), HP1a, or STAT5AY694F were grown to 70% and fixed. Cells were then stained with PI and subjected to flow cytometry to examine DNA content. Note that no significant differences were found in these conditions. (*D*) Transgene expression is maintained in YAMC mp53/Ras cells during tumor growth. Total RNA was extracted from YAMC mp53/Ras cells carrying vector control (vector), or the HP1α, STAT5A^{WT}, STAT5A^{V467A}, STAT5A^{Y694F}, or STAT5A^{V467A,Y694F} transgene, and expression of HP1α and STAT5A was measured via reverse transcription followed by real-time PCR using human gene-specific primers. Total RNA was isolated from a portion of cells before injection or of tumors excised from the mice 4 wk after injection. For cells carrying the HP1α transgene, HP1α mRNA levels before injection were measured on triplicate samples (*Top Left*) and after injection on duplicate samples (*Bottom Left*). For cells carrying the STAT5A^{WT} or a STAT5A variant transgene, STAT5A mRNA levels were measured on a single sample before and after injection (*Middle*). Mouse RhoA mRNA was used as a control and was measured before and after injection on triplicate samples of cells carrying the HP1α transgene, and on a single sample before and after injection, of cells carrying the STAT5A^{WT} or a STAT5A variant transgene (*Right Top* and *Bottom*).

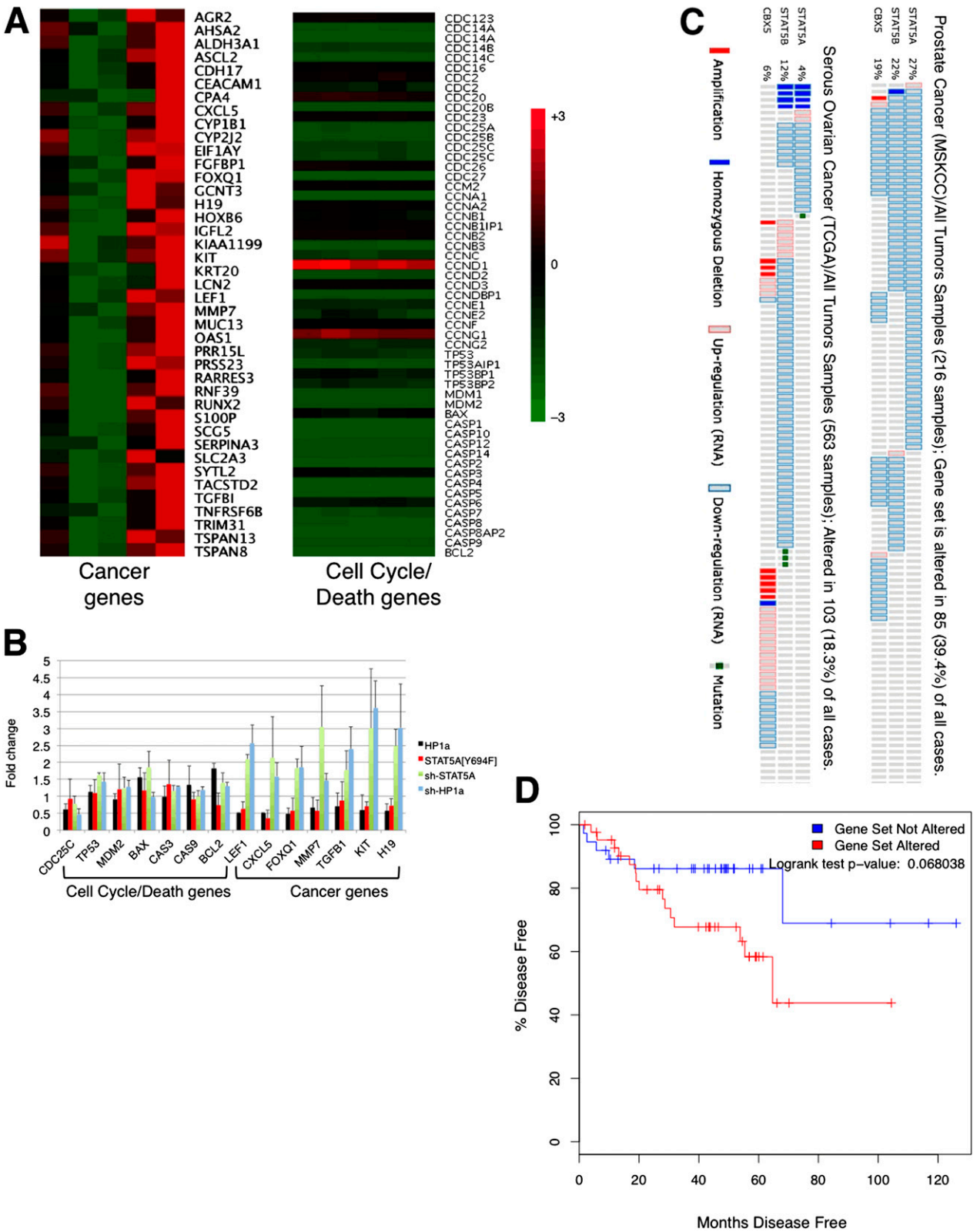

Fig. S7. Gene expression in DLD-1 cells and human patients. (*A*) Microarray studies of DLD-1 cells overexpressing different STAT5A and HP1α transgenes. Total RNA isolated from DLD-1 cells stably expressing the vector control, HP1α, STAT5A^{Y694F}, shRNA-GFP, shRNA-STAT5A, or shRNA-HP1α were subjected to microarray analysis (*A*) or were reverse transcribed into cDNA and subjected to qPCR analysis (*B*, *Left*) Heat map representation of transcript expression levels of subgroup of genes shown in Fig. 4*C*, which have previously been shown to be up-regulated or important in colon cancers (referred to as "cancer genes"; also see Table S2). (*Right*) Heat map representation of transcript expression levels of genes implicated in cell cycle progression or apoptosis is shown. Note that expression levels of these genes were not significantly different between all of the difference cell types. (*B*) Total RNA were isolated from DLD-1 cells

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stably expressing indicated transgenes and elected genes from "cancer genes" and "cell cycle/apoptosis genes" as listed in *A* were reverse transcribed into cDNA and subjected to qPCR analyses. GAPDH transcripts were used as control. Relative expression levels are shown as fold changed compared with empty vector transfected control DLD-1 cells. Error bars indicate SDs of two independent RNA isolation, each assayed in triplicates. (*C* and *D*) Down-regulation of STAT5 and HP1α in human cancer samples. Genomic alterations in STAT5A, STAT5B, and HP1α (also known as CBX5) across the indicated sets of tumor samples are shown as OncoPrints, which were generated using cBio Cancer Genomics Portal (1). Each column represents an individual case or patient sample. Those cases without alteration of genomic data (gray columns) are only partially shown. Expression changes are based on microarray data with a z-score threshold of ±2. (*D*) Disease-free survival data for the completed studies for the Memorial Sloan-Kettering Cancer Center patients with prostate cancer.

1. Cerami E, et al. (2012) The cBio cancer genomics portal: An open platform for exploring multidimensional cancer genomics data. *Cancer Discov* 2(5):401–404.

Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOCX)