Methods

See Methods in Supplementary Materials for further details.

Cells

YAMC cells[31] and derivation of YAMC cells with multiple oncogenic lesions[17] are described in the supplementary materials. Briefly, four polyclonal cell populations, control (Bleo/Neo), mp53 ($p53^{175H}$/Neo), Ras (Bleo/Ras^{V12}) and mp53/Ras ($p53^{175H}$/Ras^{V12}) were derived by retroviral infection of low-passage polyclonal young adult mouse colon (YAMC) cells. Human colon cancer cells HT-29 were obtained from the ATCC; DLD-1 cells were provided by J. Filmus.

Microarray Experiments

Polysomal RNA was harvested from YAMC, bleo/neo, mp53/neo, bleo/Ras and mp53/Ras cells to obtain gene expression profiles reflective of protein synthesis rates. RNA was harvested from ten replicates for each cell population grown in non-permissive conditions for 48 hr, followed by 24 hr in media with 0% FBS to maximize the contribution of oncogenic signaling to gene expression. RNA was collected while cells were sub-confluent and all cell populations were actively cycling. Cells were lysed in Extraction Buffer (50 mM MOPS, 15 mM MgCl, 150 mM NaCl, 0.5% Triton X-100 with 100 μg/mL cycloheximide, 1 mg/mL heparin, 200U RNAsin (2 μL/mL of buffer), 2mM PMSF). Supernatants were applied to 10-50% sucrose gradients, centrifuged at 36,000 rpm for 2 hr at 4°C and fractions were collected using an ISCO gradient fractionator reading absorbance

at 254 nm. Polysome containing fractions were pooled and RNA was purified using the RNeasy Mini Kit (Qiagen) following the standard protocol for animal cells, except that sucrose fractions were mixed with 3.5 volumes Buffer RLT before binding to the RNeasy column. RNA was on-column DNase digested as part of the RNeasy RNA extraction protocol.

Five micrograms of RNA was reverse transcribed and labeled using the mAMP kit (Ambion), with the 1x amplification protocol. The cRNA yield was fragmented and hybridization cocktails were prepared using Affymetrix standard protocol for eukaryotic target hybridization. Targets were hybridized to Affymetrix Mouse Genome 430 2.0 Expression Arrays at 45°C for 16 hours, washed and stained using Affymetrix Fluidics protocol EukGE-WS2v4_450 in the Fluidics Station 450. Arrays were scanned with the Affymetrix GeneChip Scanner 3000.

Statistical Analysis and CRG Identification

Expression values from the 50 microarrays processed were obtained using the RMA procedure with background correction in Bioconductor (http://www.bioconductor.org). Differentially expressed genes were identified by the step-down Westfall-Young procedure[32] in conjunction with the permutation N-test[33]. The latter test is non-parametric and does not require log-expression levels to be normally distributed. The family-wise error rate (FWER) was controlled at a level of 0.01. Gene expression values derived from mp53/Ras RNA samples were compared to those from two control cell populations, YAMC and bleo/neo cells, and differentially expressed genes within the intersection of

both comparisons were selected for further analysis, {p value of mp53/Ras vs. YAMC < 0.01} AND {p value of mp53/Ras vs. Bleo/Neo < 0.01}. This selection process was executed in parallel using both raw and quantile normalized expression values, with the genes forming the union of both procedures being selected for further analysis, {Raw} OR {Normalized}. ESTs and "Transcribed loci" were rejected from the set of genes thus selected.

Genes that respond synergistically to the combination of mutant p53 and activated Ras, i.e. with a fold-change larger than the sum of fold-changes induced by mutant p53 and activated Ras individually, were termed CRGs. The following procedure was applied in parallel to mean values of raw and quantile normalized expression measurements, with the genes forming the union of both procedures being selected as CRGs for further analysis, {CRG Raw} OR {CRG Normalized}. Let *a* be the mean expression value for a given gene in mp53 cells, *b* represent the mean expression value for the same gene in Ras cells and *d* represent the mean expression value for this gene in mp53/Ras cells. Then, the selection criterion defines CRGs as $\frac{a+b}{d} \leq 0.9$ for genes over-expressed in mp53/Ras cells and as $\frac{d}{a}+\frac{d}{b} \leq 0.9$ for genes under-expressed in mp53/Ras cells, as compared to controls. Unlike a similar criterion based on the general isobol equation[34], this criterion has no rigorous theoretical justification. However, this formulation is heuristically appealing and served well for the purposes of our study. In order to assess robustness of synergy scores, jackknife sub-sampling was used to generate estimated p values for these scores.

Gene-associated biological processes for CRGs were assigned according to Gene Ontology (GO) data base[35].

TLDA QPCR

The TaqMan Low-Density Array (Applied Biosystems) consists of TaqMan qPCR reactions targeting the cooperation response genes available (Supplementary Table 2) and control genes (18S rRNA, GAPDH) in a microfluidic card. TLDAs were used (four replicates/sample) to independently test gene expression differences observed by Affymetrix arrays.

Expression of CRGs in human colon cancer

Co-regulation of CRGs in mp53/Ras cells and colon human cancer was assessed by comparing the t-statistics of CRG expression data reported here with those of two independent analyses of primary human colon cancers using cDNA or oligonucleotide arrays[36, 37], respectively.

Genetic Perturbation of Gene Expression

Re-expression of down-regulated genes: For stable gene re-expression, cDNA for each gene was cloned into the pBabe retroviral vector, which was used to produce ecotropic or pseudotyped retrovirus for infection of mp53/Ras, HT-29 or DLD-1 cells. Cells were drug selected to derive polyclonal cell populations for xenograft assays.

Knock down of up-regulated genes: For stable gene knock-down, shRNA targeting each gene was cloned into the pSuper-retro retroviral vector, which was used as pBabe vectors above. The specificity of Plac8 knock-down was independently confirmed by expression of Plac8 cDNA rendered shRNA-resistant by introduction of appropriate silent mutations. This shRNA resistant cDNA was cloned into the pBabe-hygro retroviral vector and introduced into mp53/Ras cells harboring Plac8sh240 shRNA.

Quantitation of gene perturbation: The efficiency of gene perturbations was tested by comparison of RNA expression levels in empty vector-infected mp53/Ras cells and cells subjected to gene perturbation via SYBR Green qPCR with gene-specific primers. Re-expression or knock-down was also compared with the respective levels of RNA expression in YAMC control cells.

References for Methods

31. Whitehead, R. H., VanEeden, P. E., Noble, M. D., Ataliotis, P. & Jat, P. S. Establishment of conditionally immortalized epithelial cell lines from both colon and small intestine of adult H-2Kb-tsA58 transgenic mice. Proc Natl Acad Sci U S A 90, 587-91 (1993).
32. Westfall, P. H. & Young, S. S. Resampling-based multiple testing : examples and methods for P-value adjustment (Wiley, New York, 1993).
33. Klebanov, L., Gordon, A., Xiao, Y., Land, H. & Yakovlev, A. A permutation test motivated by microarray data analysis. Computational Statistics & Data Analysis 50, 3619-3628 (2006).
34. Berenbaum, M. C. What is synergy? Pharmacol Rev 41, 93-141 (1989).
35. Ashburner, M. et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25, 25-9 (2000).
36. Saaf, A. M. et al. Parallels between global transcriptional programs of polarizing Caco-2 intestinal epithelial cells in vitro and gene expression programs in normal colon and colon cancer. Mol Biol Cell 18, 4245-60 (2007).
37. Ramaswamy, S. et al. Multiclass cancer diagnosis using tumor gene expression signatures. Proc Natl Acad Sci U S A 98, 15149-54 (2001).