Supporting Information

Natarajan et al. 10.1073/pnas.1311945111

SI Materials and Methods

Cells. Rat embryo fibroblast (REF52) cells and 293FT (Invitrogen) cells were maintained in DMEM supplemented with 10% (vol/vol) FBS, 1 mM sodium pyruvate, 10 mM Hepes buffer, 55 nM β-mercaptoethanol, and antibiotics.

Lentiviral Transduction. Lentiviral stocks were prepared using the 293T packaging cell line. Cells were plated overnight without antibiotics so that they were 90% confluent at the time of transfection. Lentiviral packaging plasmids consisting of a mixture of pGag-pol, pRev, and VSVG (obtained from System Biosciences) were mixed with the desired target lentiviral construct and used to transfect 293FT cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Virus-containing supernatants were collected from cell cultures at 48 h and 72 h posttransfection, filtered, and stored at −70 °C. For lentiviral infection, target cells were incubated with viral supernatants supplemented with 4 μg/mL polybrene (Sigma), and media was replaced after 6 h.

Development of 293-NF-κB-GFP Reporter Cells. A basic single-color lentiviral transcriptional reporter (TR) vector, pRTR-mCMV-copGFP with destabilized copGFP reporter gene under the control of minimal CMV (mCMV) promoter, was used for construction of the NF-κB-293FT-TR cell line. The lentiviral NF-κB–responsive reporter construct pTR-NF-κB-mCMV-copGFP was constructed by cloning four repeats of the 10-bp NF-κB consensus response element upstream of the mCMV promoter and destabilized copGFP reporter gene in the TR vector pRTR-mCMV-copGFP (Fig. S1). This NF-κB reporter construct was packaged as lentiviral particles and used to infect 293T cells to establish NF-κB reporter cells. Functionality of the NF-κB GFP reporter in these cells was assessed by measuring their GFP expression level by FACS following TNF treatment. To reduce the impact of cell-to-cell variability in the intensity of reporter induction, we sorted single cells with "high" GFP expression (~300-fold after TNF induction) by FACS, propagated the clones, and then reanalyzed GFP expression by FACS with and without TNF induction. The resulting NF-κB TR cell clone demonstrated a very low basal level of reporter activity and robust, uniform reporter induction after treatment with TNF and IL-1 cytokines (5 ng/mL) (Peprotech, Inc.). This cell line was therefore used as the reporter cell line in this study (referred to as 293-NF-κB-GFP) (Fig. S2).

Development of Lentiviral Peptide Libraries. To have a source of genetic elements for selection of peptide modulators of NF-κB activity, we constructed pooled peptide-encoding libraries based on a high-throughput (HT) synthesis of oligonucleotides on microarray surfaces. We assembled coding sequences of ~5,000 human and mouse cell-surface, secreted, extracellular-matrix proteins using key-word search [Medical Subject Headings (MeSH) terms] in the National Library of Medicine database. For each coding region, we designed overlapping fragments of cDNA encoding either 20 aa or 50 aa flanked with BbsI sites. A pool of ~50,000 oligonucleotides was synthesized on the surface of glass slides (55,000 Agilent custom microarrays). We amplified the pool of oligonucleotides by PCR (12 cycles) using primers complementary to common flanking sequences. The amplified peptide cassettes were digested with BbsI, and each oligonucleotide pool was cloned into the set of three lentiviral extracellular peptide expression vectors, constructed as follows. A cassette comprising the signal sequence of alkaline phosphatase (SEAP) was cloned into the lentiviral vector pR-CMV to generate pR-BAP for expression of bioactive peptides. pR-BAP was designed to include internal BbsI sites to allow direct cloning of the peptide library and to introduce additional cassettes coding for the expression of peptides directing dimerization (RMKQLEDKIEELLSKIYHLENEIARLKKLIGER) or trimerization (RMKQIEDKIEEILSKIYHIENEIARIKKLIGER). The peptide expression cassette is flanked by Gex1, Gex2, and GexSeq primer-binding sites to allow PCR amplification and HT sequencing using the Solexa platform. Lentiviral peptide libraries were packaged using standard procedures (see *Lentiviral Transduction* section).

High-Throughput Sequencing. To identify peptide modulators, genomic DNA was isolated from control [NF-κB–activating selectable peptide (NASP) library-transduced cells] and GFP-positive cells after each round of FACS and used for the amplification of the peptide cassette using flanking Gex primers. HT Solexa sequencing was used to identify the amplified fragments. We have optimized the protocols for amplification and HT sequencing in the course of our preliminary studies and obtained about 5×10^6 reads from each sample, averaging about 100 reads for each peptide in the library. To assess the reproducibility of these data, each HTS screen was repeated with the specific 50,000 peptide NASP library thrice. Statistical analysis was performed using SPSS v15.0 for Windows and in-house software developed at Cellecta Inc., to identify a set of peptide candidates from the sequencing data. We identified ~50 NF-κB–activating candidate peptides that were enriched at least threefold in the sorted cell fractions in at least two duplicate screens. Of the designs tested, leucine zipper dimeric and trimeric NASP libraries produced the largest number of hits and demonstrated the highest potency in our assay. In addition, we found that NASP libraries comprising 20-aa-long peptides or 50-aa-long peptides demonstrated similar efficiencies of hit isolation.

Immunoprecipitation. For coimmunoprecipitation (co-IP) experiments, the cells expressing different flag-tagged versions of ApoF NASPs were lysed with radio immunoprecipitation assay (RIPA) buffer without SDS, and anti-FLAG agarose (Sigma) was used to pull down interacting proteins (Sigma; A4596, ANTI-FLAG M1 Agarose Affinity Gel). Immunoprecipitated ApoF-NASPs were detected on Western blot using anti-FLAG antibodies (Sigma; A8592, Monoclonal ANTI-FLAG M2-Peroxidase). Coimmunoprecipitated TRAF6 was detected using anti-TRAF6 antibodies (Santa Cruz Biotech; sc-7221, H-274).

Oncogene Cooperation Assays. Early passage wild-type mouse embryonic fibroblasts (MEFs) (passage 3) were plated in 60-cm dishes 1 d before transfection with lentiviruses encoding individual peptides or with the empty lentiviral vector used for library construction. After 48 h, each population was trypsinized, and the cells were divided equally between three plates. One plate served as a control to test the effect of peptide alone, and the other two plates were used to test the effect of the peptide in combination with oncogenic Ras expression. Thus, the second plate was transduced with the lentiviral PLV-rasV12-bleo construct (encoding H-Ras^{v12}), and the third plate was transduced with the empty vector PLV-bleo. For positive controls, $p53^{-/-}$ MEF cells were infected in parallel. Cells transduced with PLV-rasV12-bleo or PLV-bleo were subjected to selection in bleomycin

(40 μg/mL). Media were replaced twice a week, and cell morphology was monitored for 2–3 wk by microscopic examination.

Senescence-Associated β-Galactosidase Activity. Cells were fixed with 0.5% glutaraldehyde (Sigma), washed twice with PBS, and incubated at 37 °C for 18 h with staining solution [1 mM $MgCl_2$, 3.3 mM $K_4Fe(CN)_6$, 3.3 mM $K_3Fe(CN)_6$, 0.02% Nonidet P-40, 0.2% X-Gal] (Sigma) at pH 5.5. Plates were photographed, and the proportions of positively stained cells were counted in at least five fields of each plate.

Reporter Assays. For screening of peptide libraries, 293-NF-κB-GFP reporter cells were transduced with the different lentiviral libraries, and, 2 d later, the GFP-positive population was FACS sorted (FACSAria sorter; BD Biosciences). Control reporter cells were used to normalize the sorting gate. For validation experiments, 293-NF-κB-GFP reporter cells were plated in 24-well plates and infected with individual peptide-expressing lentiviruses. Forty-eight hours after infection, GFP was visualized using fluorescence microscopy (plates photographed) and, in some experiments, was assessed by flow cytometry using the flow analyzer LSRII (BD Biosciences), and the percentage of cells expressing GFP was determined.

Mouse NIH 3T3-derived cells with wild-type p53 cells carrying the con A-p53-luc reporter were infected with lentiviral stocks of individual NASPs and, 2 d later, were plated in 96-well plates (5,000 cells in 100 μL per well) and then treated with 1 μM doxorubicin (Sigma). After 18 h, β-gal activity was measured using a standard kit (β-galatosidase Assay System; Promega).

Soft-Agar Assays. Cell suspension in 0.4% low-melting agarose prepared in growth medium was plated over a solidified bottom layer containing 0.5% agar in growth medium at a density of 50,000 cells per well in triplicates. Cells were fed weekly twice, and colonies were counted 3 wk postplating.

In Vivo Tumorigenicity Assays. Experiments with mice in this study were conducted under the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the Roswell Park Cancer Institute. Eight-week-old female, athymic nu/nu mice (The Jackson Laboratory) were injected s.c. with 5×10^6 REF52 cells stably transformed by transduction with different combinations of lentiviral expression vectors carrying cDNAs for $H\text{-}Ras^{V12}$ and isolated NASPs (or the corresponding empty vectors as controls). Cells were injected into the rear flanks of mice (two sites per mouse, eight mice per group) 2–3 wk after transduction. Mice were euthanized when tumors reached 1,000 mm^3 according to the IACUC guidelines.

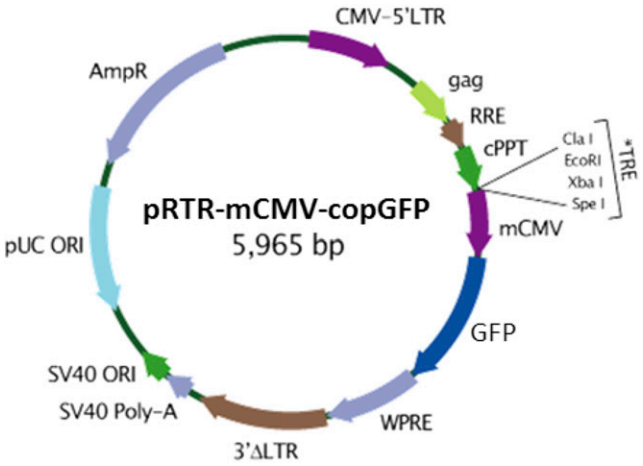

Fig. S1. Map of a single-color lentiviral TR vector. Transcription factor-specific transcriptional response elements (TREs) can be cloned into polylinker upstream of the mCMV promoter to provide transcriptional factor-dependent expression of the GFP reporter.

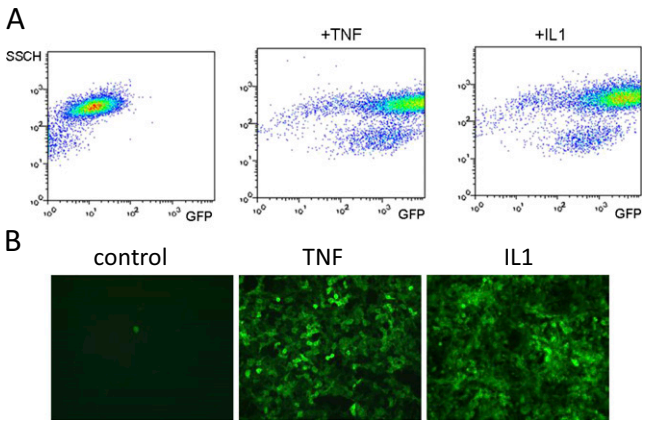

Fig. S2. Induction of GFP in NF-κB reporter by TNF and IL1. (*A*) The 293-NF-κB-GFP reporter cells were established by transduction of lentiviral reporter construct pTR-NF-κB-mCMV-copGFP followed by selection of a clonal population with uniform properties. They were induced with 5 ng/mL TNF or IL1 (Peprotech). After overnight, GFP expression was determined by flow cytometry. (*B*) Fluorescence micrographs showing GFP expression in reporter cells before and after overnight induction with TNF (5 ng/mL).

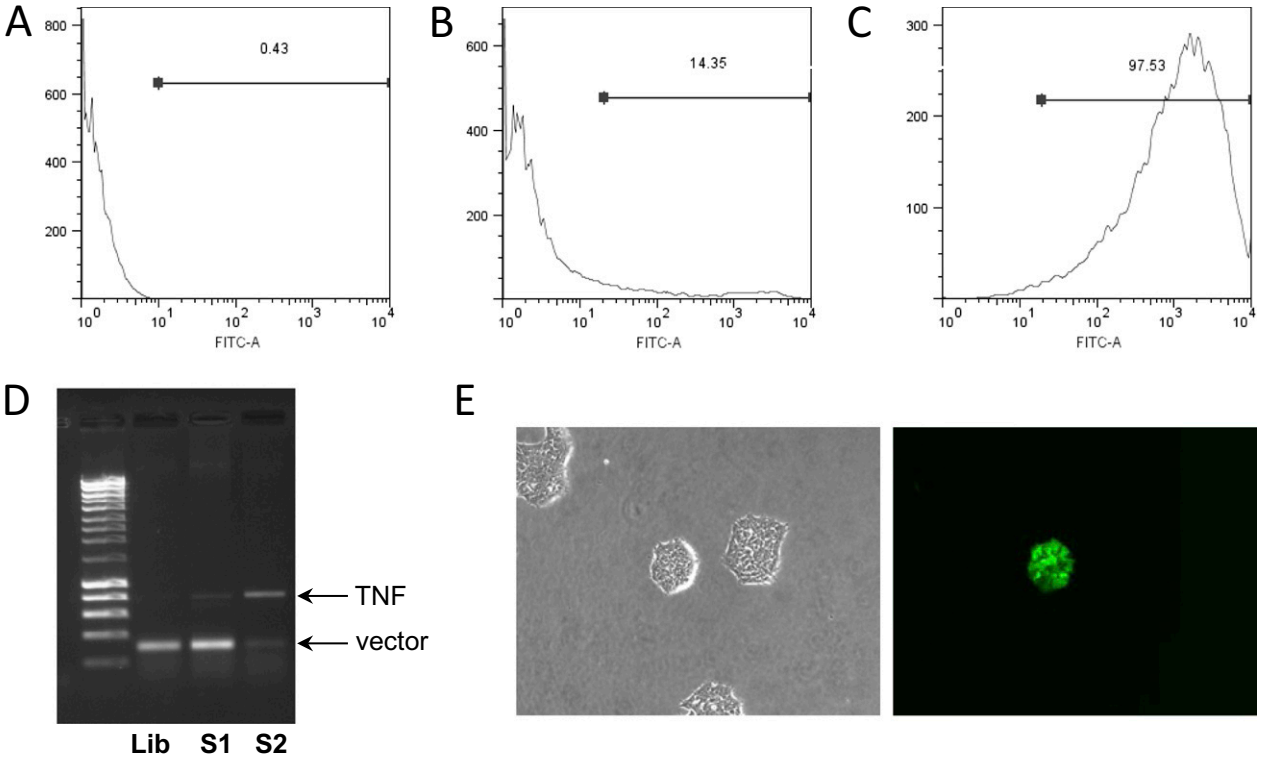

Fig. S3. Pilot experiment demonstrating the feasibility of isolating a functional NF-κB–activating peptide using the strategy outlined in Fig. 1*D*. The 293-NF-κB-GFP reporter cells transduced with a lentiviral TNFα expression construct were mixed with 293-NF-κB-GFP reporter cells transduced with the empty lentiviral vector at a ratio of 1:10,000 to generate a mock library. Forty-eight hours later, GFP-positive cells were sorted and plated, and then resorted 3 d later. The percentage of cells expressing GFP (horizontal line shows gate set for positive GFP fluorescence) is shown for (*A*) control reporter cells (no transduction), (*B* and *C*) the 1:10,000 mix of TNF-transduced and empty vector-transduced reporter cells at the time of (*B*) the first FACS sort or (*C*) the second FACS sort. (*D*) Genomic DNA was isolated 48 h after transduction of control reporter cells with mock library (Lib) or from the GFP-positive population of cells after the first (S1) or second (S2) round of sorting. One microgram of genomic DNA from each pool was used as a template to amplify inserts by PCR using primers specific for sequences flanking the insert site in the library vector. (*E*) The positive control 293-NF-κB-GFP reporter cells stably expressing the secreted form of TNF (GFP-positive due to TNF-induced NF-κB activity) were mixed with an excess of nontransduced 293-NF-κB-GFP reporter cells (GFP-negative) at a ratio of 1:10,000 and plated them at low density and photographed after 2 d of plating. Note the lack of paracrine activation of adjacent cell clusters by GFP-positive cells.

ApoF : ref|NP_001629.1| apolipoprotein F precursor [Homo sapiens]

```
aa120                                                                    aa200
   QVLIQHLRGLQKGRSTERNVSVEALASALQLLAREQQSTGRVGRSLPTEDCENEKEQAVHNVVQLLPGVGTFYNLGTALY
                          (ApoF-T20) RVGRSLPTEDCENEKEQAVH
                     (ApoF-T50)LLAREQQSTGRVGRSLPTEDCENEKEQAVHNVVQLLPGVGTFYNLGTALY
   QVLIQHLRGLQKGRSTERNVSVEALASALQLLAREQQSTGRVGRSLPTED (ApoF-D50)
   QVLIQHLRGLQKGRSTERNVS------------------GRVGRSLPTED (ApoF-D50-del)
```

COMP: ref|NP_000086.2| cartilage oligomeric matrix protein precursor [Homo sapiens]

```
aa431                                                                    aa517
DSDQDQDGDGHQDSRDNCPTVPNSAQEDSDHDGQGDACDDDDDNDGVPDSRDNCRLVPNPGQEDADRDGVGDVCQDDFDADKVVDK
         HQDSRDNCPTVPNSAQEDSD (COMP T20)
DSDQDQDGDGHQDSRDNCPTVPNSAQEDSDHDGQGDACDDDDDNDGVPDS (COMP-T50)
```

PLP: |gb|AAH25784.1| Pancreatic lipase-related protein 1 [Homosapiens]

```
aa171                                                                    aa257
SLGAHVAGEAGSKTPGLSRITGLDPVEASFESTPEEVRLDPSDADFVDVIHTDAAPLIPFLGFGTNQQMGHLDFFPNGGESMPGCK
SLGAHVAGEAGSKTPGLSRITGLDPVEASFESTPEEVRLDPSDADFVDVI (PLP-T50)
          GSKTPGLSRITGLDPVEASFESTPEEVRLDPSDADFVDVIHTDAAPLIP(PLP-D50)
```

Fig. S4. Sequence alignment of overlapping NASPs of apoliprotein F (ApoF), pancreatic lipase-related protein (PLP), and cartilage oligomeric matrix protein (COMP) with a fragment of corresponding protein.

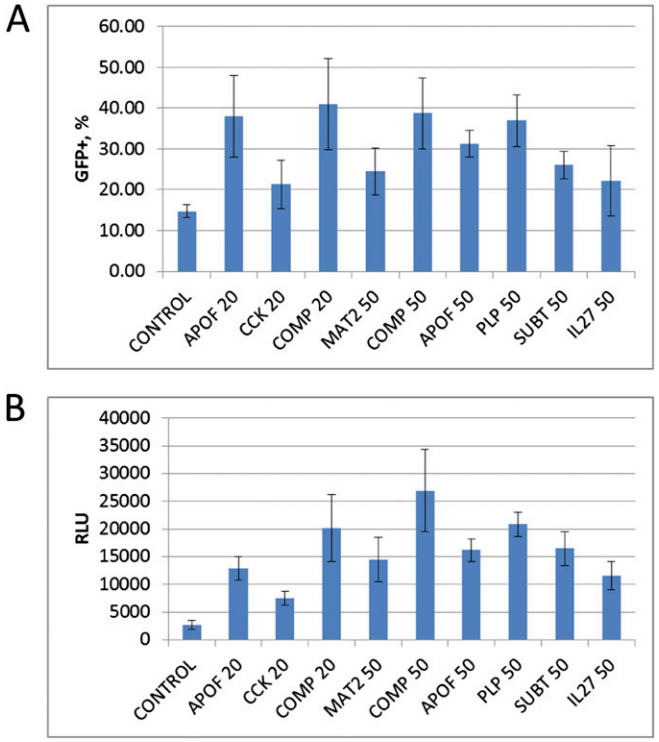

Fig. S5. Validation of individual NASPs in NF-κB reporter assays. (*A*) Individual NASPs induce GFP expression in HeLa-NF-κB-GFP reporter cells. HeLa-NF-κB-GFP cells were transduced with individual NASP lentiviral constructs, and the percentage of GFP-positive cells in each population was determined by flow cytometry 48 h after transduction. (*B*) Luciferase induction in MEF-NF-κB-Luc reporter cells transduced with individual NASP lentiviral constructs. MEF-NF-κB-Luc cells were transduced with individual NASPs and subjected to Luciferase assay 48 h after transduction.

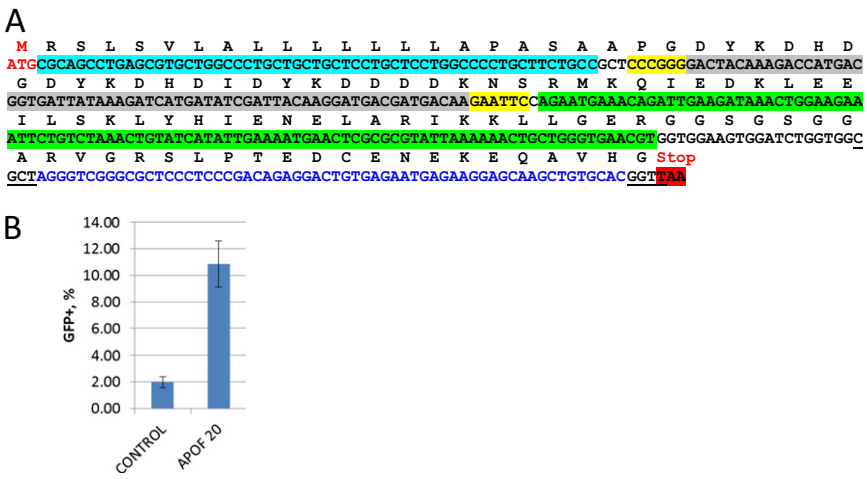

Fig. S6. (*A*) 3XFLAG sequence (gray) was inserted between the leader sequence (blue) and the Leu Zipper sequence (green) for two peptides: scrambled ApoF (control) and ApoF NASP. The diagram shows ApoF-T20 peptide with 3XFLAG scaffold peptide. (*B*) FLAG-tagged ApoF-T20 NASP demonstrates activation of the reporter in 293-NF-κB reporter cells.