Supplemental Information

Materials and methods

Generation of AEG-1KO mouse:

To generate AEG-1KO mice, an AEG-1KO targeting vector was constructed. Contiguous AEG-1 genomic regions were generated by high-fidelity PCR from 129/SvEv embryonic stem (ES) cell DNA and sequentially cloned into the NDTV targeting vector, which contains both a floxed neomycin phosphotransferase (*neo*) cassette for positive selection of homologous recombinants with the neomycin analog G418, and a diphtheria toxin A (DT-A) cassette for negative selection of non-homologous recombinants. The final AEG-1KO targeting vector possesses a 5′ homology arm containing both the AEG-1promoter and exon 1, and a 3′ homology arm containing part of intron 1. The 5′ arm also has a *loxP* site inserted just 5′ of a 257-bp region of homology (71%) between the human and mouse promoters.

The AEG-1KO targeting vector was linearized with *Nhe* I and electroporated into 129/SvEv ES cells. ES cell clones resistant to G418 were screened for homologous recombination by Southern blot analysis using both 3′-flanking genomic and *neo* cassette probes. Retention of the introduced *loxP* site in homologous recombinants was verified by PCR using a 5′-flanking primer in combination with a unique *loxP* primer. Two ES cell clones possessing the targeted AEG-1KO allele were injected into C57BL/6 blastocysts, which were then implanted into pseudopregnant CD-1 recipients. To generate the final conventional AEG-1KO alleles, chimeric males were bred to homozygous EIIa-*cre* females (Jackson Laboratory, stock number 003724). Agouti offspring were screened for the conventional KO allele with a 3-primer PCR protocol using a common anti-sense primer (5′-CTTGAGATAGACATCTCTCTCTAC-3′) in combination with two sense primers: (5′-GAAGAATCTCAGAAGCGAGGAGC-3′) and (5′-GCTTAGCAAGAGTAGCTTATTACG-3′),

specific for the wild-type and conventional KO alleles and generating PCR products of 397 bp and 577 bp, respectively.

All animal studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University, and were conducted in accordance with the Animal Welfare Act, the PHS Policy on Humane Care and Use of Laboratory Animals, and the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training.

AEG-1KO mice were viable and fertile, although litter sizes were very small (1-2 pups per litter). Further, even litters generated by crossing AEG-1+/- breeding pairs were very small (2-3 pups per litter), which precluded generating large numbers of WT and AEG-1KO mice as littermates. Therefore, the majority of the experiments were carried out with age-matched mice generated by breeding WT and AEG-1KO mice separately. However, it should be noted that the same phenotypes were observed in AEG-1KO mice generated from AEG-1+/- X AEG-1+/- matings as from AEG-1KO X AEG-1KO matings. Additionally, the experiments described here were performed with mice on a C57BL/6:129/Sv background. However, we have backcrossed the line to C57BL/6 for 10 generations and obtained similar results for both the WT and AEG-1KO mice on the C57BL/6 background as on the C57BL/6:129/Sv background. Thus our findings are not restricted to strains or littermates status.

Induction of chemical carcinogenesis:

For induction of chemical carcinogenesis, a single i.p. injection of 30 μg/gm body weight of N-nitrosodiethylamine (DEN) was given at 14 days of age to male WT and Alb/AEG-1 mice (1). The animals were sacrificed at 32 weeks of age. For DEN/ phenobarbital (PB) model, 14 days old male mice were injected with DEN (10 μg/gm) and then PB (0.05%) was given daily in drinking water. The animals were sacrificed at 28 weeks of age. At the end of the experiment,

liver, internal organs and blood were collected. Serum liver enzymes were analyzed in the Molecular Diagnostic Laboratory, Department of Pathology, VCU using standard procedures.

Treatment of cells:

Hepatocytes were treated with LPS (200 ng/ml) for 6 h, washed with media twice and fresh media was added. Conditioned media (CM) was collected after 12 h. Macrophages were treated with CM for 4 h before harvesting for extraction of total RNA.

Western blotting:

Western blotting was performed as described previously (2). The list of the antibodies is provided in Table S7.

Immunohistochemistry (IHC) and immunofluorescence (IF):

IHC on formalin-fixed paraffin-embedded (FFPE) sections and IF on cultured hepatocytes and macrophages were performed as described previously (2). For IHC images were analyzed using an Olympus microscope. For IF images were analyzed using a Zeiss confocal laser scanning microscope.

Enzyme-linked immunosorbent assay (ELISA)

ELISA for IL-6 in DEN-treated liver homogenates was performed using a kit from Affymetrix/eBioscience (San Diego, CA) according to the manufacturer's instruction.

Bone marrow analysis

Bone marrow was isolated from femur of adult (8-12 weeks old) WT and AEG-1KO mice and bone marrow smears were prepared on a slide which was stained using Easy III Step Stain Set (Azer Scientific; Catalog# ES902).

Analysis of spleen cells by flow cytometry

Fluorochrome-conjugated monoclonal antibodies (mAbs), including PE-CD3 (17A2), PE/Cy5-CD8a (53-6.7), FITC-CD4 (GK1.5), PE-B220 (RA3-6B2), APC-NK1.1 (PK136), PE-CD11b (M1/70), APC-CD11b (M1/70), APC-CD11c (N418), PE-F4/80(BM8), FITC-Ly6G (1A8), APC-Ly6C (HK1.4), isotype control rat IgG2b (RTK4530) and IgG1(RTK2071), were purchased from BioLegend (San Diego, CA). Single cell suspensions from spleens (10^6 cells per sample) were incubated with anti-CD16/CD32 Abs for 20 min on ice to block non-specific binding to Fc receptors. Cells were then stained with Abs specific for various surface markers on ice in the dark for 20 min. Cells were washed twice and fixed with 1% paraformaldehyde, and run on a BD FACSCalibur (BD Bioscience). Data were analyzed using FlowJo (v7.6) software (Tree Star, Inc., Ashland, OR).

Reference

1. Murakami H, Sanderson ND, Nagy P, Marino PA, Merlino G, Thorgeirsson SS. Transgenic mouse model for synergistic effects of nuclear oncogenes and growth factors in tumorigenesis: interaction of c-myc and transforming growth factor alpha in hepatic oncogenesis. Cancer Res. 1993;53:1719-23.

2. Srivastava J, Siddiq A, Emdad L, Santhekadur P, Chen D, Gredler R, et al. Astrocyte elevated gene-1 (AEG-1) promotes hepatocarcinogenesis: novel insights from a mouse model. Hepatology. 2012;56:1782-91.