Supporting Information

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SI Materials and Methods

Mice and Infection. All animal experiments were carried out according to the National Institutes of Health principles of laboratory animal care and approved by the University of Southern California Institutional Animal Care and Use Committee (IACUC). For the tumor xenograft experiments, 6- to 8-wk-old athymic (*nu/nu*) nude mice (Jackson Laboratory) were housed in appropriate sterile filter-capped cages with chow and water provided for ad libitum consumption. Viable cells were harvested, washed, resuspended in PBS, mixed with 4×10^5 SVEC cells (a simian virus 40-transformed mouse microvascular endothelial cell line), and injected s.c. into the flank of nude mice. The animals were monitored twice every week for tumor development. At 6–8 wk after inoculation, mice were killed and tumor weight was determined. The *TIE2-tva* transgenic mouse line (mouse expressing avian leukosis virus receptor TVA under the control of vascular endothelial cell-specific TIE2 promoter) was previously reported (1). Avian leukosis virus production and infection were performed as previously described (1). Briefly, DF-1 cells were transfected with RCAS [replication-competent avian sarcoma-leukosis virus long terminal repeat (LTR) with a splice acceptor] vectors avian leukosis virus (ALV)-derived retroviral vector encoding indicated genes to produce recombinant viruses. Viral stocks (10^5 IU/mouse) were isolated, titered, and then injected intraperitoneally into 5-d-old littermates.

Constructs, Cell Lines, and Transfection. If not specified, pcDNA5/FRT/TO (Invitrogen) and pCDH-CMV-EF-Puro (System Bioscience) were used for transient and stable expression of corresponding genes. For protein expression, the HA or Flag epitope was inserted upstream or downstream of protein coding sequence, respectively. The p65(NF-κB subunit RelA)-eGFP plasmid was a gift from Zhijian James Chen (University of Texas Southwestern Medical Center, Dallas, TX). HEK293T (293T) was obtained from the American Type Culture Collection and immortalized murine endothelial cells (SVECs) were gifts from Philip Thorpe (University of Texas Southwestern Medical Center, Dallas, TX). *Ikbke*$^{+/+}$ (IKKε wildtype), *Ikbke*$^{+/-}$, *Ikbke*$^{-/-}$ mouse embryonic fibroblast (MEF) cells and DF-1 chicken fibroblasts were gifts from Tom Maniatis and Julio Gutkind (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD). All cell lines were grown in DMEM supplemented with 10% (vol/vol) FBS and 100 U penicillin/streptomycin. Transfection was performed with Lipofectamine 2000 reagent (Invitrogen) or calcium phosphate reagent (Clontech.) according to the manufacturers' protocols. To establish stable cell lines, MEF cells and SVECs were infected with lentivirus containing indicated genes and selected with puromycin (1 μg/mL) as described previously (2, 3).

Luciferase Reporter Assay. HEK293T cells in 24-well plates were transiently transfected with a reporter mixture as previously described (3). The reporter mixture contained 50 ng of the plasmid expressing firefly luciferase under the control of response elements of NF-κB transcription factor and 150 ng of the plasmid expressing β-galactosidase. At 24 h posttransfection, cells were harvested and lysed on ice. Centrifuged supernatant was used to measure luciferase and β-galactosidase activity according to the manufacturer's protocol (Promega).

Immunoblotting and IKKε Kinase Assay. Cells were harvested, rinsed once with ice-cold PBS, and resuspended with Nonidet P-40 buffer supplemented with protease inhibitor mixture (Roche). Whole-cell lysates were resolved by SDS/PAGE and transferred to PVDF membrane. Membrane was incubated with corresponding primary antibodies at 4 °C overnight and then with IRDye 800CW or 680CW secondary antibody (Li-Cor) for 1 h. Proteins were visualized with an Odyssey infrared imaging system. In vitro [IκB kinases (IKKs) IKKε and IKKβ] kinase assays were performed as described in ref. 3. Briefly, 293T stable cells were harvested and resuspended with kinase lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 10% (vol/vol) glycerol, 0.5% Triton X-100, and 0.5 mM DTT], and lysates were precipitated with 10 μL of anti-Flag M2-conjugated agarose (Santa Cruz). After extensive washing with kinase lysis buffer and final washing with kinase reaction buffer (1 mM DTT, 5 mM KCl, 2mM $MgCl_2$, 2 mM $MnCl_2$, 10 mM NaF, and 25 mM Hepes, pH 7.5), precipitated IKKε was used to phosphorylate selected substrates in vitro. Reaction mixtures containing 0.5 μg of indicated substrate proteins and 10 μCi of [γ-^{32}P]ATP in 30 μL of total volume were incubated for 30 min at 25 °C. Reactions were stopped with SDS/PAGE sample buffer by boiling for 5 min at 100 °C, and samples were resolved by SDS/PAGE, transferred to PVDF membrane, and analyzed by autoradiography.

Immunofluorescence and Immunohistochemistry. Cells were fixed with paraformaldehyde [4% (vol/vol) in PBS] and permeabilized with Triton X-100 (0.2% in PBS). After staining with primary (monoclonal anti-HA antibody), secondary antibody (Alexa 568-conjugated antibody) and DAPI, cells were analyzed with a Nikon E800M microscope (Nikon) as previously described (4, 5). For immunohistochemistry staining, mouse or human tissue samples were fixed in the neutral buffered 10% (vol/vol) formalin solute (Sigma) overnight at 4 °C. Tissue specimens were dehydrated, embedded in paraffin, and cut into 3-μm sections. Tissue sections were analyzed by H&E, immunohistochemistry staining with antibodies against IKKε, S468 phosphorylated RelA (Bethyl Laboratories), or the HA epitope (Sigma), rabbit or mouse ABC staining system (Santa Cruz), and DAB substrate kit (Vector Laboratories). Images were visualized with a Nikon E800M microscope equipped with a Nikon DXM1200 digital camera and the Nikon ACT-1 imaging software system (Nikon Instruments Inc.).

RT-PCR and Quantitative RT-PCR. To determine the relative levels of cytokine transcripts, RT-PCR and quantitative (q)RT-PCR were performed as previously reported (6). Briefly, total RNA was extracted from cells using RNAeasy kit (Qiagen). cDNA was prepared with 1.5 μg of total RNA and reverse transcriptase (Invitrogen). The abundance of cytokine mRNAs was assessed by qRT-PCR using a StepONEPlus Real-Time PCR system (Applied Biosystems). Mouse β-actin was used as an internal control. All primers were designed by Primer Express 3.0 (Applied Biosystems) and validated individually, as shown in Table S2.

Nuclear Extraction and EMSA. Nuclear extraction was performed by NE-PERNuclear and Cytoplasmic Extraction Reagents (Thermo). Two microgram of nuclear extracts was incubated with a ^{32}P-labeled oligonucleotide (Promega) containing the NF-κB consensus site (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) for 15 min at room temperature in a binding reaction buffer [10 mM Hepes (pH 7.9), 1 mM EDTA, 40 mM NaCl, 0.5 mM DTT, and 0.2 mg/mL poly(dI:dC), and 4% (vol/vol) glycerol]. For the competition assay and the supershift assay, 50-fold molar

excess of cold probe or 20 mg/mL rabbit polyclonal anti-RelA (Santa Cruz Biotechnology) or anti–c-Rel (Cell Signaling) antibody was preincubated with nuclear extracts for 10 min before adding the ^{32}P-labeled probe. DNA-protein complexes were subjected to electrophoresis in 6% native polyacrylamide gels (0.5× Tris/borate/EDTA). Gels were dried and analyzed by FLA-7000 (Fuji Film) for autoradiography.

Paracrine Stimulation of Endothelial Cells. Human umbilical vein endothelial cells (HUVEC)/Vec and HUVEC/Kaposi sarcoma-associated herpesvirus G protein-coupled receptor (kGPCR) cells were cultured for 2 d, and cell-free supernatant was collected after centrifugation at 1,500 × *g* for 10 min, after which WT HUVEC cells were seeded at different densities (50,000 or 75,000 per well) and treated with conditioned medium. After incubating for 24 or 48 h, cells were harvested and RNA was extracted by using TRIzol (Life Technologies) according to manufacturer's instructions. cDNA was synthesized by reverse transcription. qRT- PCR was performed to determine the quantity of mRNA of interest that was normalized to β-actin.

For treatment with IKKε inhibitor (amlexanox), 50,000 WT HUVEC cells were seeded in vector or kGPCR-conditioned medium. Meanwhile, amlexanox was added at 0, 15, or 30 μM into the culture medium. Eighteen hours later, RNA was extracted, cDNA was synthesized, and qPCR was performed. For RelA phosphorylation, human endothelial cell line ECV-304 were seeded in 12-well plate for 16 h. After starvation for 6 h, cells were incubated in conditioned medium without or with amlexanox (15 μM). At indicated time points, whole-cell lysates were prepared for immunoblot analysis. The ECV/Vec and ECV/IKKεK38A were established by lentivirus infection and selection with puromycin (1 μg/mL). Cells were similarly processed with conditioned medium and whole-cell lysates were prepared and analyzed by immunoblot.

Statistical Analyses. Statistical analyses were performed using an unpaired, two-tailed Student *t* test. *P* values of less than 0.05 were considered to be statistically significant.

1. Montaner S, et al. (2003) Endothelial infection with KSHV genes in vivo reveals that vGPCR initiates Kaposi's sarcomagenesis and can promote the tumorigenic potential of viral latent genes. *Cancer Cell* 3(1):23–36.
2. Feng H, Sun Z, Farzan MR, Feng P (2010) Sulfotyrosines of the Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor promote tumorigenesis through autocrine activation. *J Virol* 84(7):3351–3361.
3. Feng H, Dong X, Negaard A, Feng P (2008) Kaposi's sarcoma-associated herpesvirus K7 induces viral G protein-coupled receptor degradation and reduces its tumorigenicity. *PLoS Pathog* 4(9):e1000157.
4. Ye Y, Shibata Y, Yun C, Ron D, Rapoport TA (2004) A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature* 429(6994): 841–847.
5. Feng P, et al. (2007) A novel inhibitory mechanism of mitochondrion-dependent apoptosis by a herpesviral protein. *PLoS Pathog* 3(12):e174.
6. Dong X, et al. (2010) Murine gamma-herpesvirus 68 hijacks MAVS and IKKbeta to initiate lytic replication. *PLoS Pathog* 6(7):e1001001.

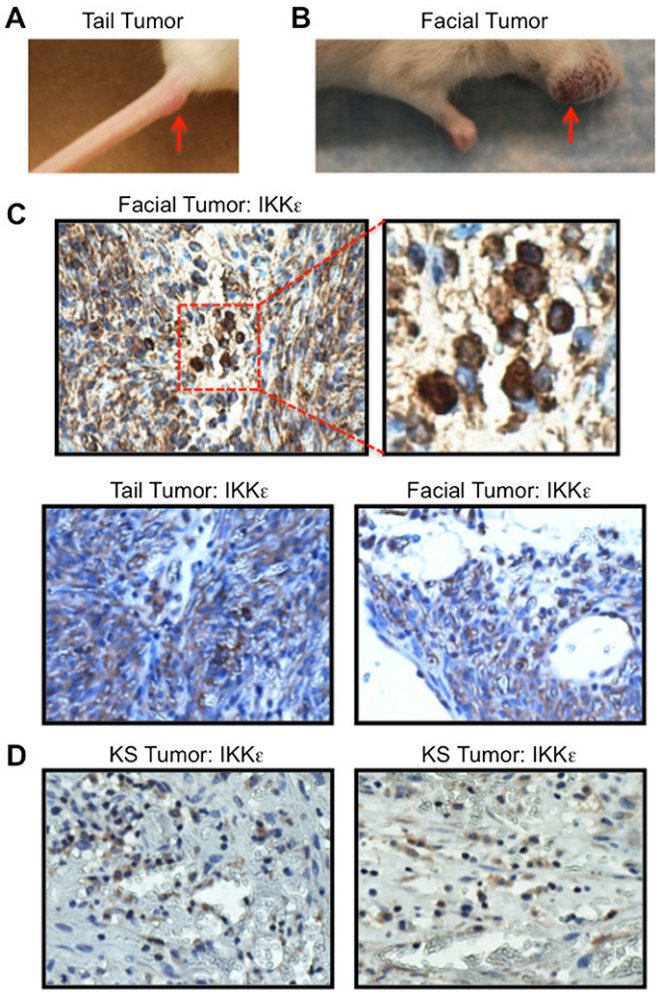

Fig. S1. IKKε expression in kGPCR-induced mouse lesions and human KS tumors. (*A* and *B*) Representative tail and facial tumors of kGPCR-expressing endothelium of *TIE2-tva* mouse. (*C*) Immunohistochemistry staining for IKKε in tail and facial tumors derived from kGPCR-expressing endothelium as in *A* and *B*. (*D*) Immunohistochemistry staining of IKKε in human Kaposi sarcoma (KS).

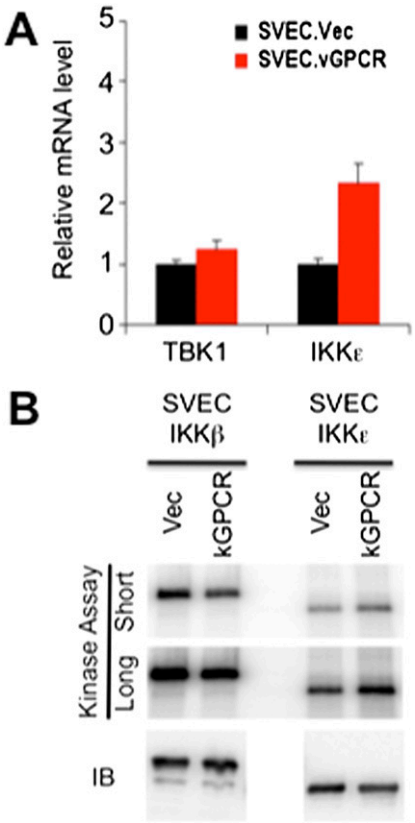

Fig. S2. kGPCR up-regulates IKKε expression and kinase activity. (*A*) Total RNA was extracted from control SVEC (SVEC.Vec) or SVEC stably expressing kGPCR. Real-time PCR was performed to determine the levels of TANK-binding kinase 1 (TBK1) and IKKε mRNA with sequence-specific primers. (*B*) IKKβ and IKKε were precipitated from SVEC stable cell lines. In vitro kinase assays for IKKβ and IKKε were performed using GST–IκBαNT and GST–IRF3C, respectively (*B*).

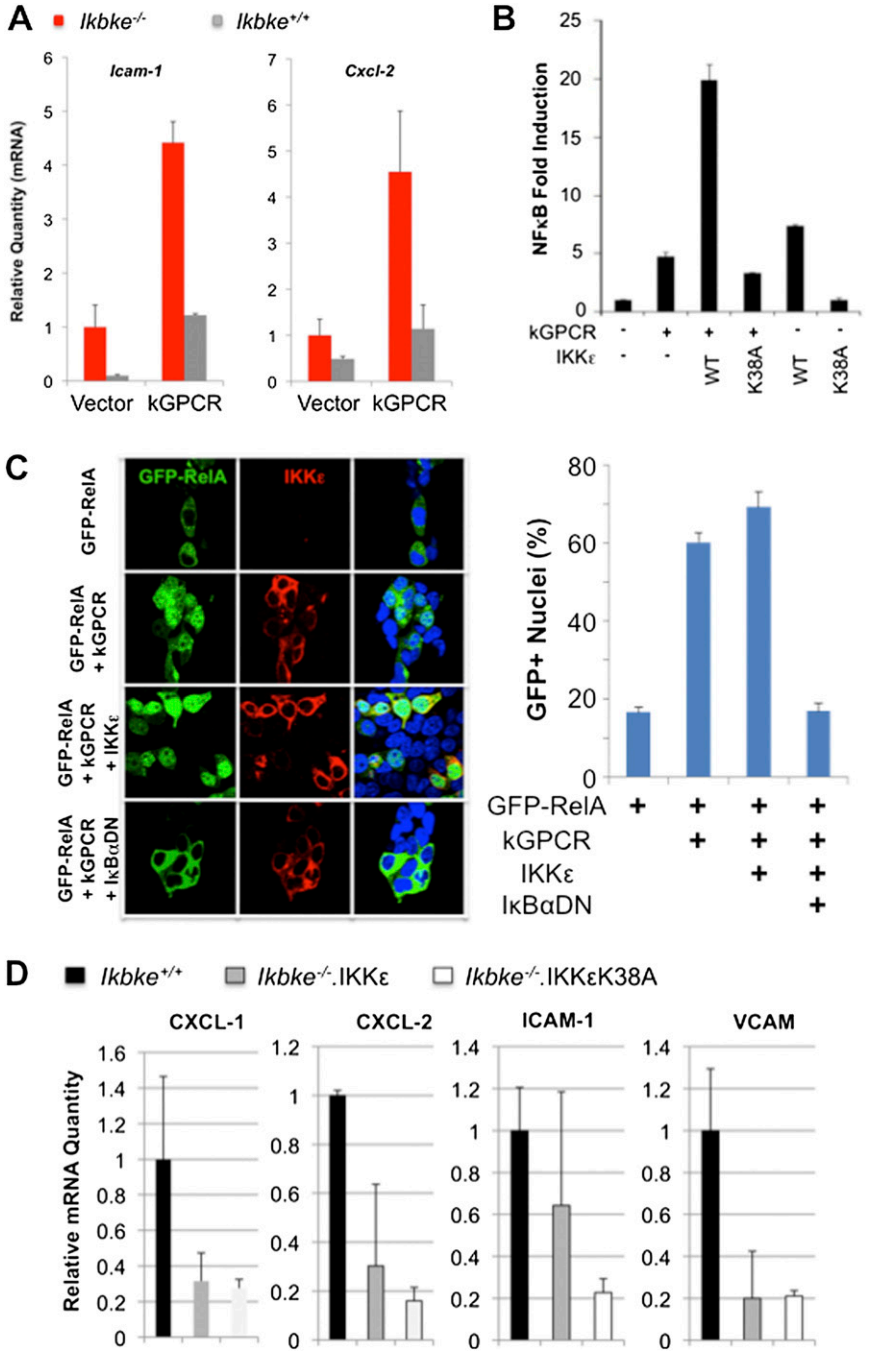

Fig. S3. IKKε is crucial for kGPCR-induced NF-κB activation. (*A*) Intercellular adhesion molecule 1 (Icam-1) and chemokine C-X-C motif ligand 2 (Cxcl-2) expression was determined by real-time PCR analysis from *Ikbke*$^{+/+}$ and *Ikbke*$^{-/-}$ MEFs expressing kGPCR. (*B*) The 293T cells were transfected with an NF-κB luciferase reporter plasmid mixture and plasmids containing kGPCR, IKKε, or the kinase-dead IKKεK38A mutant. Luciferase activity, normalized against β-galactosidase activity, was calculated for NF-κB activation. (*C*) The 293T cells were transfected with plasmids containing GFP–RelA, kGPCR, IKKε, and IκBα dominant negative mutant (IκBαDN). Cells were fixed, stained, and analyzed by immunofluorescence microscopy. Representative images are shown on the left. Statistics was obtained by counting more than 300 cells from eight randomly selected fields (diagram on the right). (*D*) Total RNA was extracted from indicated cell lines and cDNA was synthesized and analyzed by qRT-PCR.

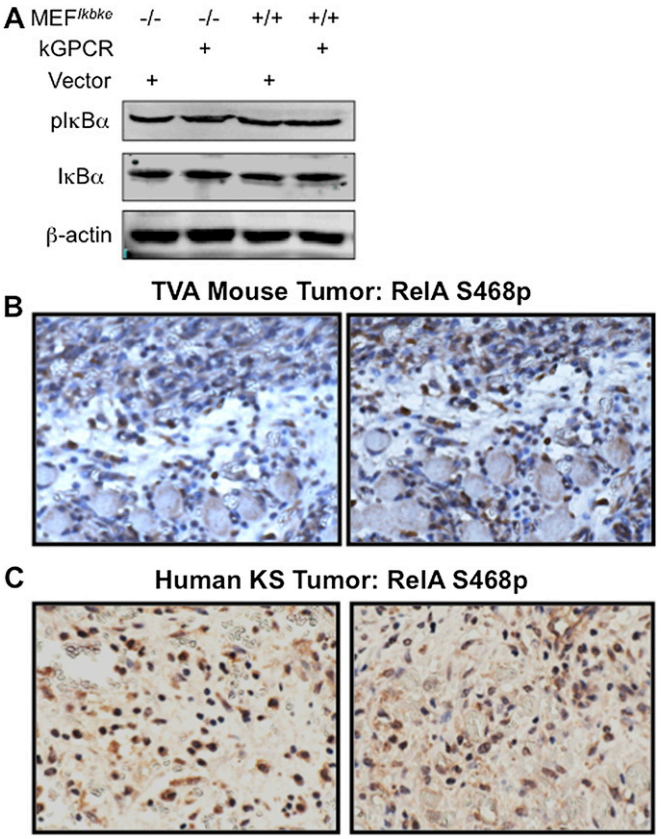

Fig. S4. Up-regulation of S468 phosphorylated RelA in kGPCR-induced tumor and human KS. (*A*) *Ikbke*$^{+/+}$ and *Ikbke*$^{-/-}$ MEFs expressing kGPCR or transduced with control lentivirus were analyzed by immunoblot with antibodies to phosphorylated IκBα, total IκBα, and β-actin. (*B*) Immunohistochemistry staining with antibody against Ser468 phosphorylated RelA of kGPCR-expressing tumors derived from *TIE2-tva* mouse. (*C*) Representative immunohistochemistry stainings of Ser468 phosphorylated RelA in human KS tumors.

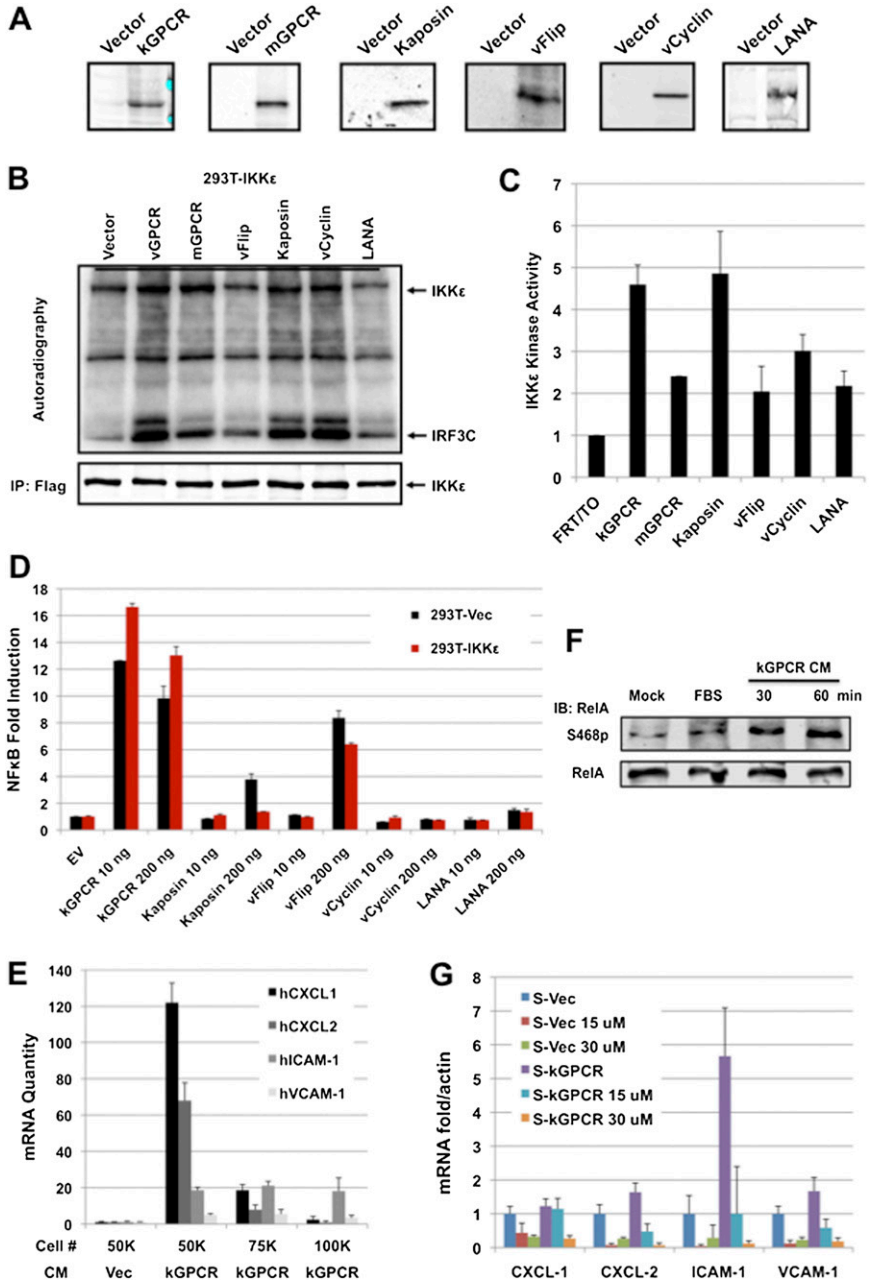

Fig. S5. IKKε activation by latent gene products and kGPCR. (*A*) Expression of kGPCR, gamma herpesvirus 68 vGPCR (mGPCR), Kaposin B, viral FLICE-inhibitory protein (vFLIP), viral cyclin (vCyclin), and latent nuclear antigen (LANA) in 293T/IKKε cells was analyzed by immunoblot. (*B* and *C*) The 293T/IKKε cells were transfected with plasmids carrying indicated genes. At 36 h posttransfection, cells were harvested and IKKε was precipitated for in vitro kinase assay to phosphorylate GST–IRF3C. Phosphorylation was analyzed by autoradiography (*B*) and quantified (*C*). (*D*) NF-κB activation was determined by luciferase reporter assay in 293T and 293T/IKKε cells with plasmids carrying indicated genes. (*E*) HUVEC cells of indicated number were seeded in conditioned medium of HUVEC/Vec (Vec) or HUVEC/kGPCR (kGPCR) for 48 h, harvested, and RNA was extracted and used for cDNA synthesis, followed by qPCR analysis. (*F*) Human ECV endothelial cells were incubated with HUVEC/kGPCR-conditioned medium and whole-cell lysates were analyzed by immunoblot for RelA Ser468p and total RelA. FBS, 10% FBS-containing complete DMEM. (*G*) SVEC cells were incubated with conditioned medium from HUVEC/kGPCR, without or with amlexanox of indicated concentration for 18 h. RNA was extracted and analyzed by cDNA synthesis, followed by qPCR.

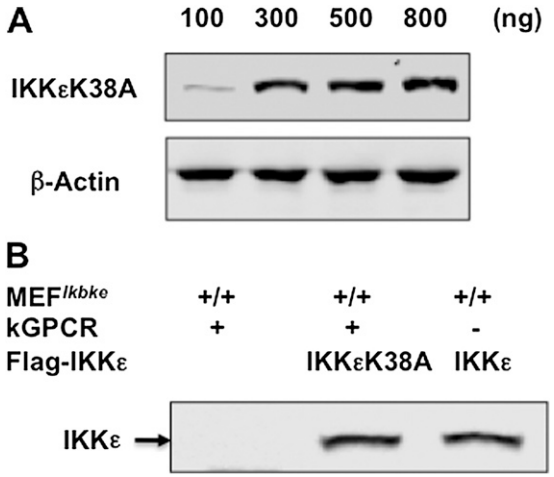

Fig. S6. Transient and stable expression of the IKKεK38A mutant. (*A*) The 293T cells were transfected with an NF-κB reporter plasmid mixture and plasmids containing kGPCR and the kinase-dead IKKεK38A mutant. Whole-cell lysates were analyzed by immunoblot for IKKεK38A and β-actin. (*B*) *Ikbke*$^{+/+}$ MEFs with or without kGPCR expression were transduced with lentivirus expressing the IKKεK38A mutant and WT IKKε. Whole-cell lysates were analyzed for the expression of the IKKεK38A mutant and WT IKKε.

Table S1. shRNA sequences targeting IKKε

shRNA number	Sequence
77–1	CCGGCTGGACGATGATGAGAAGTTTCTCGAGAAACTTCTCATCATCGTCCAGTTTTT
77–2	CCGGCGACTCATCGAACGGTTACATCTCGAGATGTAACCGTTCGATGAGTCGTTTTTG
77–3	CCGGTTGGCCAACAAACTAGCATTACTCGAGTAATGCTAGTTTGTTGGCCAATTTTTG
77–4	CCGGGCAGGAGCTTTGTAATGATATCTCGAGATATCATTACAAAGCTCCTGCTTTTT
77–5	CCGGGGTTGACCTACAGGCCGATTACTCGAGTAATCGGCCTGTAGGTCAACCTTTTTG

Table S2. Primer sequences for qRT-PCR

Genes	Sequence
mCxcl1-F	ACTGCACCCAAACCGAAGTC
mCxcl1-R	TGGGGACACCTTTTAGCATCTT
mCxcl2-F	AGTGAACTGCGCTGTCAATG
mCxcl2-R	CTTCAGGGTCAAGGCAAACT
mIcam1-F	GGAGACGCAGAGGACCTTAAC
mIcam1-R	CGCTCAGAAGAACCACCTTC
mCcl5-F	TTTGCCTACCTCTCCCTCG
mCcl5-R	CGACTGCAAGATTGGAGCACT
mIl6-F	TCCATCCAGTTGCCTTCTTG
mIl6-R	GGTCTGTTGGGAGTGGTATC

mCxcl1, chemokine C-X-C motif ligand 1; mCxcl2, chemokine C-X-C motif ligand 2; mIcam1, intercellular adhesion molecule 1; mCcl5, chemokine C-C motif ligand 5; mIl6, interleukin-6.