Supporting Information

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SI Materials and Methods

Generation of B-Raf$^{+/LSLV600E}$ Mice. To target the B-*Raf* locus, we generated a targeting vector by amplifying a 4.2-kbp *EcoR*I DNA fragment from the Roswell Park Cancer Institute-23 BAC clone and introducing it into the pBlueScript SKII plasmid (Fig. S1*A*). This DNA fragment contains 978 bp upstream of exons 14 and 15 and 2,352 bp downstream of exon 15. A GTG→GAG mutation in codon 600 and a NotI site 60 bp upstream of exon 15 were generated by site-directed mutagenesis. The NotI site was used to insert a phosphoglycerate kinase (PGK)-Hyg-STOP cassette (1) flanked by *loxP* sites (Fig. S1*A*). This targeting vector was electroporated into R1 ES cells as described (2). Detection of recombinant ES cell clones was carried out by Southern blot analysis using probes (b and c) derived from sequences located outside the targeting vector (Fig. S1). Probe b (341 bp) was generated by PCR amplification using primers b-F (5′-GTGACCTCTGATTTTC-TCTTG-3′, forward) and b-R (5′-TCTAA CAGTGACCACC-TTGAA-3′, reverse). Probe c (442 bp) was amplified using oligonucleotides c-F (5′-TCTCAAGAATGAATCTCTACT-3′, forward) and c-R (5′-ATTCCTTCCTAACAA CAC-3′, reverse). One Hyg^R/Gan^R clone (total of 1,008) carrying the desired homologous recombination event was used to generate chimeric mice by microinjection into C57BL/6J blastocysts. These mice were crossed with C57BL/6J females to obtain germ-line transmission of the targeted allele. Routine genotyping of WT B-*Raf*$^+$ and recombinant B-*Raf*^{LSLV600E} alleles was carried out by PCR analysis of tail DNA using forward B1 (5′-TTACACGCCA-AGTCAATCATC-3′) and B2 (5′-ACACCTCCCCCTGAACC-TGAA-3′) primers and reverse (5′- TGACCAACTGAGATA-CCTCCTCA-3′) primer. The sizes of diagnostic PCR products were 962 (B1:B2) and 564 bp (B2:B3) for the B-*Raf*$^+$ and the B-*Raf*^{LSLV600E} alleles, respectively. For RT-PCR analysis, RNAs were isolated using the RNeasy Mini Kit (Qiagen) and reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen) and random hexamer primers (Invitrogen) following the manufacturers' instructions. PCR amplification cycles were as described above. The diagnostic cDNA fragment for the WT transcript (361 bp) was obtained with primers 1 (5′- CAGC-ACCTACACCTCAGCAG-3′) and 2 (5′-ACCCACTCCACCG-AGATTTCA-3′). The diagnostic cDNA fragment for the mutated B-*Raf* transcript was obtained with primers 1 and 3 (5′-ACCCACTCCACCGAGATTTCT-3′) (Fig. S1*D*). All animal experiments were approved by the Centro Nacional de Investigaciones Oncológicas (CNIO) Ethical Committee and performed in accordance with the guidelines stated in The International Guiding Principles for Biomedical Research involving Animals developed by the Council for International Organizations of Medical Sciences.

Western Blot and Kinase Assay. Mouse monoclonal antibodies against B-Raf, phospho-Mek1 (pSer217 and pSer221), and GAPDH were obtained from Santa Cruz, Cell Signaling, and Sigma, respectively. Rabbit monoclonal antibody against phospho-Mek1 (pSer217 and pSer221) was from Cell Signaling, and rabbit polyclonal antibodies to Erk1/2 and Mek1 were from Santa Cruz. Rabbit polyclonal antibodies to phospho-Erk1/2 (pThr202 and pTyr204) were purchased from Cell Signaling. Primary antibodies were detected with appropriate goat secondary antibodies to mouse or rabbit IgGs (Alexa Fluor 680; Invitrogen) and visualized using a LI-COR system (Odyssey). For B-Raf kinase assays, protein extracts obtained from adult heart and adult brain (600 μg) were immunoprecipitated with B-Raf monoclonal antibody (Santa Cruz), and the resulting immunoprecipitates were used to determine B-Raf kinase activity using the B-Raf Kinase Assay kit (Milipore) according to the manufacturers' instructions.

Computed Tomography. Acquisition of computed tomography (CT) images was performed with an eXplore Vista PET CT (GE Healthcare) using an amperage of 130 μA and a voltage of 50 kV; 2.5-mo-old mice were anesthetized with ketamine (75 mg/kg body weight; Imalgene500) and xylazine (1 mg/kg body weight; Ronpum) during image acquisition. Morphological analysis was done using geometric morfometrics techniques. Ten 2D homologous landmarks were collected in the midsagittal plane of the skull of each specimen. Landmark configurations were aligned for translation, rotation, and scaling by a Generalized Procrustes Analysis (3), a least-squares fitting of all 2D configurations to the average. Coordinate data were acquired and processed using tpsDig and tpsSuper software (Rohlf FJ, Stony Brook University, Stony Brook, NY, http://life.bio.sunysb.edu/morph/). Differences between groups in the spatial position of these landmarks after Generalized Procrustes fit were checked by Hotteling's T^2 tests of each pair of x and y coordinates.

Positron Emission Tomography. Two-month-old animals were anesthetized with diazepam (3 mg/kg) and 2-deoxy-2-[^{18}F]fluoro-D-glucose (FDG; 18 Mbq) administered through tail-vein injection. After the uptake period (40 min), animals were reanesthetized with isoflurane (5% of isoflurane for induction and 2% for maintenance in an oxygen flow of 0.5 L/min). To obtain heart synchronization, heart electrocardiogram and respiration were monitored [gated positron emission tomography (PET)]. Tomographic images were reconstructed using a 2D-ordered subsets expectation maximization algorithm.

1. Lakso M, et al. (1992) Targeted oncogene activation by site-specific recombination in transgenic mice. *Proc Natl Acad Sci USA* 89:6232–6236.
2. Ortega S, et al. (2003) Cyclin-dependent kinase 2 is essential for meiosis but not for mitotic cell division in mice. *Nat Genet* 35:25–31.
3. Rohlf FJ, Slice D (1990) Extensions of the procrustes method for the optimal superimposition of landmarks. *Syst Biol* 39:40–59.

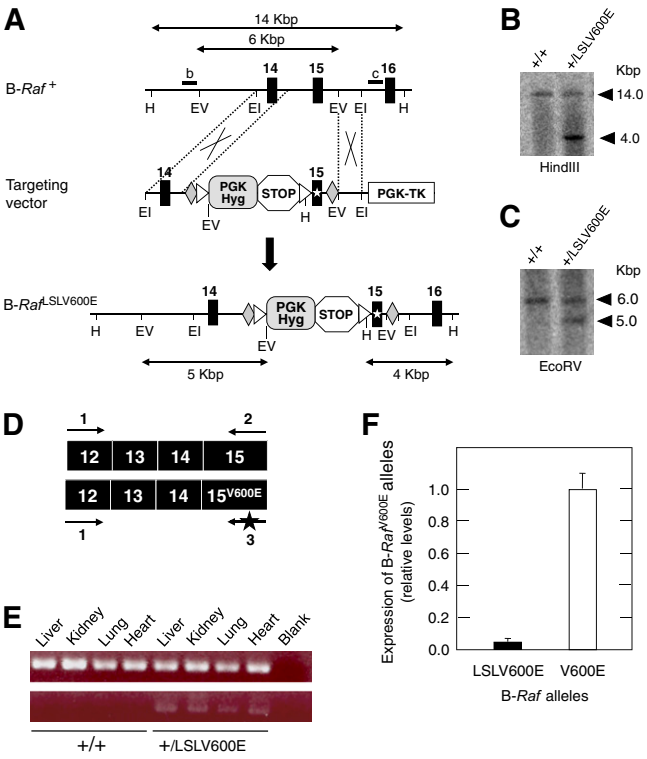

Fig. S1. Gene-targeting strategy to generate the hypomorphic B-*Raf*^{V600E} allele. (*A*) Schematic representation of (*Top*) the WT B-*Raf*$^{+}$ allele, (*Middle*) targeting vector, and (*Bottom*) targeted B-*Raf*^{LSLV600E} allele. Exons (solid boxes), PGK-Hygromycin (PGK-Hyg) cassette (gray box), STOP transcriptional sequences (octagonal box), PGK-thymidine kinase (PGK-TK) cassette (open box), *lox*P sequences (open triangles), *Frt* sequences (gray diamonds), and the V600E mutation (asterisk) are indicated. Cleavage sites for diagnostic restriction enzymes (H, *Hind*III; EV, *Eco*RV; EI, *Eco*RI) and the probes (b and c) used to identify the homologous recombinational events are also indicated. (*B* and *C*) Southern blot analysis of DNA isolated from recombinant ES cell clones. The migration and size of the WT B-*Raf*$^{+}$ and recombinant B-*Raf*^{LSLV600E} alleles are indicated. We also indicate the expected size of each diagnostic DNA fragment. (*D*) RT-PCR strategy to detect expression of WT B-*Raf*$^{+}$ and recombinant B-*Raf*^{LSLV600E} alleles. Primers 1–3 used for the PCR amplification are indicated. Primers 2 and 3 have similar sequences, except for the T1799A mutation responsible for the V600E miscoding mutation (indicated by a star). (*E*) RT-PCR analysis of transcripts isolated from the indicated tissues obtained from B-*Raf*$^{+/+}$ and B-*Raf*$^{+/LSLV600E}$ mice. (*F*) Quantitative RT-PCR analysis using primers 1 and 3 described in *D* of RNA isolated from fibroblasts derived from B-*Raf*$^{+/LSLV600E}$ embryos infected with either empty adenoviral particles (B-*Raf*$^{+/LSLV600E}$ cells; solid column) or adenoviral particles expressing the Cre recombinase to eliminate the floxed *lox*-STOP-*lox* cassette and allow normal levels of expression of the B-*Raf*^{V600E} allele (B-*Raf*$^{+/V600E}$ cells; open column).

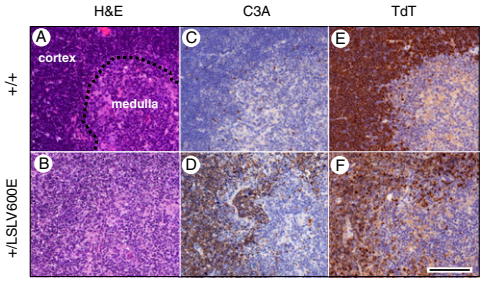

Fig. S2. Thymic atrophy in B-*Raf*$^{+/LSLV600E}$ mice. (*A* and *B*) H&E staining, (*C* and *D*) immunohistochemistry (IHC) staining with antibody for active caspase 3 (C3A), and (*E* and *F*) IHC staining with antibodies against terminal deoxynucleotidyl transferase (TdT) in thymic sections from 3-wk-old (*A*, *C*, and *E*) B-*Raf*$^{+/+}$ and (*B*, *D*, and *F*) B-*Raf*$^{+/LSLV600E}$ mice. Note increased apoptosis (*C* and *D*) and decreased number of immature thymocytes (*E* and *F*) in sections obtained from B-*Raf*$^{+/LSLV600E}$ mice. (Scale bar, 100 μm.)

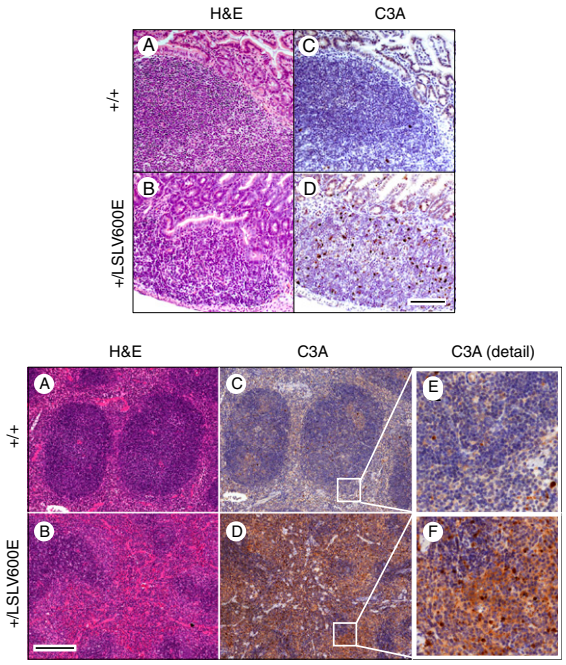

Fig. S3. Atrophy of Peyer's patches and spleen in B-*Raf*$^{+/LSLV600E}$ mice. (*Upper*) Atrophy of Peyer's patches. (*A* and *B*) H&E staining and (*C* and *D*) IHC staining with antibody for active caspase 3 (C3A) in small intestine sections from 3-wk-old (*A* and *C*) B-*Raf*$^{+/+}$ and (*B* and *D*) B-*Raf*$^{+/LSLV600E}$ mice. (Scale bar, 100 μm.) (*Lower*) Spleen atrophy. (*A* and *B*) H&E staining and (*C* and *D*) IHC staining with antibody for active caspase 3 (C3A) in spleen sections from 3-wk-old (*A*, *C*, and *E*) B-*Raf*$^{+/+}$ and (*B*, *D*, and *F*) B-*Raf*$^{+/LSLV600E}$ mice. *E* and *F* are amplified images of the quadrant indicated in *C* and *D*. Note increased apoptosis and atrophic white pulp in sections obtained from B-*Raf*$^{+/LSLV600E}$ mice. (Scale bar, 200 μm.)

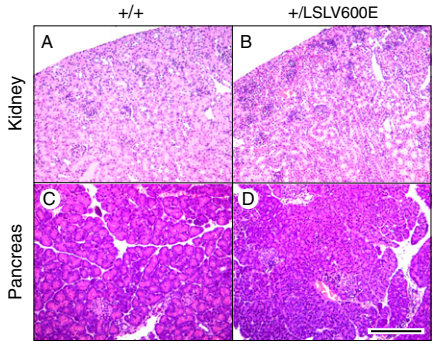

Fig. S4. Reduced cytoplasm to nuclear ratio in B-*Raf*$^{+/LSLV600E}$ mice. H&E staining of (*A* and *B*) kidney and (*C* and *D*) pancreas of 3-wk-old B-*Raf*$^{+/+}$ and B-*Raf*$^{+/LSLV600E}$ littermates (B6 background). (Scale bar, 100 μm.)

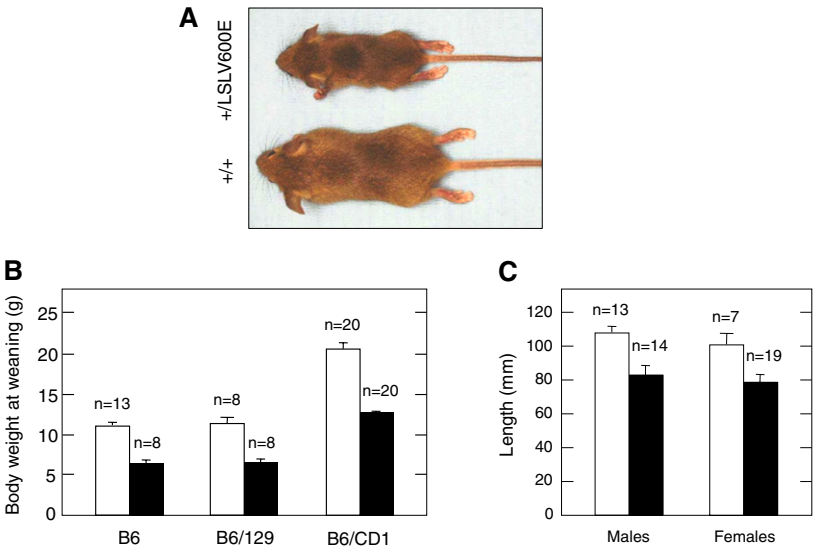

Fig. S5. Growth defects of B-*Raf*$^{+/LSLV600E}$ mice. (*A*) Representative image of B-*Raf*$^{+/+}$ and B-*Raf*$^{+/LSLV600E}$ mice at weaning. (*B*) Body weights of B-*Raf*$^{+/+}$ (open bars) and B-*Raf*$^{+/LSLV600E}$ (solid bars) mice of the indicated genetic backgrounds at weaning. (*C*) Sizes (length from head to tail) of B-*Raf*$^{+/+}$ (open bars) and B-*Raf*$^{+/LSLV600E}$ (solid bars) male and female mice of various genetic backgrounds. The number of mice (*n*) used is indicated. Error bars represent SEMs.

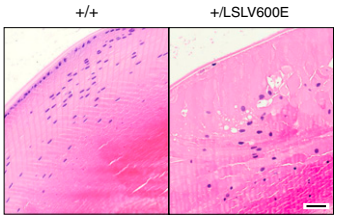

Fig. S6. B-*Raf*$^{+/LSLV600E}$ mice develop cataracts. H&E staining of representative crystalline lens obtained from B-*Raf*$^{+/+}$ and B-*Raf*$^{+/LSLV600E}$ mice. (Scale bar, 50 μm.)

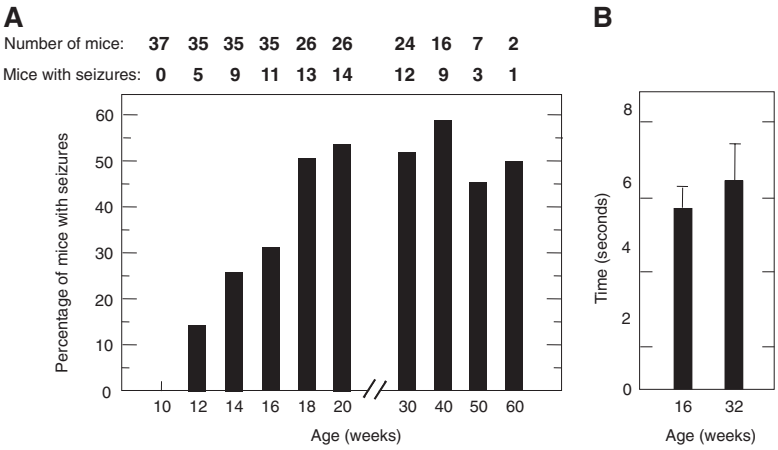

Fig. S7. B-*Raf*$^{+/LSLV600E}$ mice develop epileptic seizures. (*A*) Percentage of B-*Raf*$^{+/LSLV600E}$ mice (B6/CD1 background) that developed epileptic seizures at the indicated ages. The number of mice analyzed is indicated for each age group. (*B*) Duration of seizures in 16- (*n* = 5) and 32-wk-old (*n* = 5) B-*Raf*$^{+/LSLV600E}$ mice (B6/CD background). Error bars represent the SEMs.

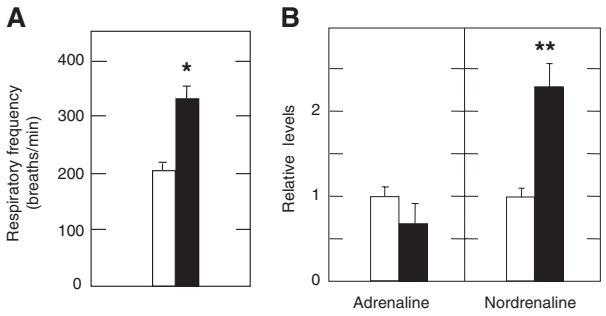

Fig. S8. Increased respiratory rate and noradrenaline plasma levels in B-*Raf*$^{+/LSLV600E}$ mice. (*A*) Increased respiratory frequency in 4-mo-old B-*Raf*$^{+/LSLV600E}$ mice (solid bar; $n = 4$) compared with WT B-*Raf*$^{+/+}$ (open bar; $n = 4$) littermates (B6/CD1 background). *$P < 0.05$. (*B*) Adrenaline and noradrenaline plasma levels in 5-mo-old B-*Raf*$^{+/+}$ (open bars; $n = 7$) and B6/129 B-*Raf*$^{+/LSLV600E}$ (solid bars; $n = 5$) mice. **$P < 0.01$. Error bars represent SEMs.

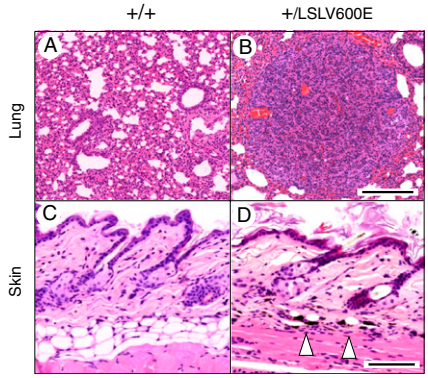

Fig. S9. Lung adenomas and melanocytic hyperplasia in B6/CD1 B-*Raf*$^{+/LSLV600E}$ mice. (*A* and *B*) H&E staining of lungs of adult (*A*) B-*Raf*$^{+/+}$ and (*B*) B-*Raf*$^{+/LSLV600E}$ mice. The latter depicts a low-grade adenoma. (Scale bar, 200 μm.) (*C* and *D*) H&E staining of skin of adult (*C*) B-*Raf*$^{+/+}$ and (*D*) B-*Raf*$^{+/LSLV600E}$ mice. Arrowheads indicate foci of melanocytic hyperproliferation. (Scale bar, 100 μm.)

Table S1. Physiological parameters of 2-mo-old mice*

Parameter	B-*Raf*$^{+/+}$ ($n = 9$)	B-*Raf*$^{+/LSLV600E}$ ($n = 6$)	Change (%)
Body weight (g)	21.8 ± 1.2	13.6 ± 0.5	−37.6
Kidney weight/body weight	0.0081 ± 0.0008	0.0091 ± 0.0120	NS†
Liver weight/body weight	0.086 ± 0.003	0.071 ± 0.016	NS
Heart weight/body weight	0.0072 ± 0.0001	0.0099 ± 0.0001	+37.5
Aorta media wall thickness (μm)	34.1 ± 2.1	32.1 ± 2.8	NS
Systolic arterial pressure (conscious; mmHg)	112.1 ± 2.3	112.7 ± 3.7	NS
Urine production (μL/d body weight)	45.72 ± 8.89	20.18 ± 5.20	−55.8
Creatinine clearance (mL/min)	1.81 ± 0.18	0.73 ± 0.08	−59.6

*B6 genetic background.
†Not significant.

Movie S1. Representative epileptic seizure of a 3-mo-old B-*Raf*$^{+/LSLV600E}$ female.

Movie S1