Supporting Information

Stewart et al. 10.1073/pnas.1418795112

SI Materials and Methods

Materials. We generated the antibodies to RGS6 (immunohistochemistry) and RGS6L (immunoblotting) in rabbit. The RGS7 and $G\beta_5$ antibodies were provided by Ching Kang (Jason) Chen, Baylor College of Medicine, Houston. α-Tubulin and TH antibodies were from EMD Millipore, the antibody for phospho-MAPK from Cell Signaling Technology, and the antibody for total MAPK from Santa Cruz Biotechnology. Baclofen, saccharin, quinine, DPI, and actin antibody were from Sigma Chemical, and SCH-50911, raclopride, and GBR-12909 were from Tocris Biosciences. EtOH was from Pharmco AAPER.

Short-Term Alcohol Consumption. Short-term alcohol consumption was measured in WT and *RGS6*$^{-/-}$ mice essentially as previously described (1). First, mice were individually housed for 2 d before the experiment. Each cage was then outfitted with two drinking bottles made from graduated serological pipettes sealed to prevent evaporation. Both bottles were initially filled with tap water for 3 d, then one each with water or a solution containing 8% (vol/vol) EtOH, and finally 8% (vol/vol) EtOH in both bottles for 2 d (Fig. S1*A*). The position of the bottles was alternated to avoid place preference bias. Volume levels were recorded daily, tubes refilled, and mice weighed. Alcohol consumption by volume or weight was reported normalized to individual mouse body weight. In a follow-up experiment, mice received intraperitoneal injections of SCH-50911 (12.5 mg/kg), the $GABA_BR$ antagonist; raclopride (3 mg/kg), the D2R antagonist; or GBR-12909 (10 mg/kg), the DAT inhibitor daily on days when given access (either free or forced) to alcohol. One week after completion of the alcohol-drinking procedure, taste preference was examined by measuring saccharin (sweet, no caloric value) fluid intake by presenting 0.04% followed by 0.08% saccharin solutions and quinine (bitter, no caloric value) fluid intake by presenting 20 mM and 40 mM quinine containing solutions for 1 wk each. Alcohol (or saccharin/quinine) preference was calculated by dividing the EtOH volume consumed by the total fluid intake.

Two-Bottle Choice Test. Alcohol consumption and preference were assessed in mice using the two-bottle choice test essentially as previously described (2). Mice were individually housed and given 24-h access to both bottles, one containing standard tap water and the other containing an alcohol solution increasing in concentration every week from 3% to 6%, 10%, and finally 20% (vol/vol) alcohol. EtOH consumption was measured each week along with animal weight. Bottle positions were changed periodically to avoid side preference. For the alcohol-drinking experiment, average alcohol consumption per day was calculated and normalized to body weight.

Acute Withdrawal Severity. EtOH withdrawal-induced seizure activity was evaluated by scoring handling-induced convulsions as previously described (2). Handling-induced convulsion scoring was performed hourly for 12 h following a single EtOH dose (4 g/kg, i.p.). Each mouse was picked up by the tail and behavior observed. The scoring scale ranged from a score of 0 (no convulsion) to 7 (full tonic-clonic convulsion). As previously reported, scores rarely exceeded a value of 4.

Conditioned Place Preference. Before conditioning, mice were habituated to the testing apparatus. During this phase, mice were placed in the center of the chamber after a saline injection (10 mL/kg, i.p.) and allowed to freely explore for 15 min. The conditioning phase involved a series of 12 5-min sessions in which saline or EtOH (2 g/kg, i.p.) doses were administered and the mouse restricted to the associated side of the chamber (ethanol: white walls, textured floor; saline: dark walls, smooth floor). Each animal received six conditioning trials with each treatment/chamber pairing. Following the conditioning phase, animals were again given a single saline injection and allowed free access to both compartments after placement in the center of the cage. The amount of time spent in each chamber during this testing phase was recorded during a 15-min trial.

Loss of Righting Reflex. Mice were given a sedative dose of EtOH (3.2 g/kg i.p.) and placed in a supine position in a V-shaped trough. Loss of righting reflex was defined as the total sleep time and the time required for recovery (reacquisition of the mouse's ability to right itself three times in a 30-s period after placement on its back).

Rotarod Performance Test. WT and *RGS6*$^{-/-}$ mice were tested on a motorized rotarod apparatus (Columbus Instruments). Tests were performed at fixed speeds of 5, 10, or 15 rpm and with acceleration (3 rpm/s) from 5 rpm. A maximum of 120 s was allowed per mouse for fixed speed tests. One cohort of mice was given a single dose of EtOH (1.75 g/kg, i.p.) 30 min before testing.

Balance Beam Test. The balance beam consisted of a plastic cylinder (50-cm long with a diameter of 1 cm) elevated 25 cm above the floor. Mice were trained to run the length of the beam twice before testing. As expected, based on the known ataxic phenotype of *RGS6*$^{-/-}$ mice (3), these animals struggled to complete the training. On the third beam cross the number of footslips and falls and total time required to traverse the beam were recorded by an observed blinded to genotype. If an animal stopped on the beam, its tail was gently pressed to encourage movement and, in the event an animal fell from the beam, it was replaced on the beam at the location from which it fell and allowed to finish the test. One cohort of mice was given a single dose of EtOH (1.75 g/kg, i.p.) 30 min before testing.

Immunohistochemistry. Formaldehyde [4% (wt/vol)]-perfused frozen brain sections from WT and *RGS6*$^{-/-}$ mice were processed to examine protein expression and localization. Briefly, cryosections were washed in PBS, blocked with 5% (wt/vol) BSA and incubated overnight at 4 °C with primary antibodies. Following washing four times in PBS (10 min each), sections were incubated for 1 h at room temperature with Alexa Fluor secondary antibodies (Life Technologies). Sections were visualized using confocal microscopy (Zeiss LSM710) as previously described (4).

Formalin-fixed, paraffin-embedded sections of gastrointestinal tissue from WT and *RGS6*$^{-/-}$ mice were obtained from University of Iowa Central Microscopy Facility and were processed to examine expression of different key signaling proteins, as previously described (5). Briefly, sections were de-waxed in xylene, treated with a graded series of alcohol solutions, immersed in 3% (vol/vol) hydrogen peroxide to block endogenous peroxide activity, blocked with 5% (wt/vol) BSA, and then incubated overnight at 4 °C with specific antibody. Following washing (3 × 10 min) in PBS, sections were incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies. The sections were counterstained in Harris hematoxylin and observed under the microscope.

Immunoblotting. Tissues were rapidly dissected from WT and *RGS6*$^{-/-}$ mice and flash-frozen in liquid nitrogen. Tissue homogenates and cell lysates were prepared in RIPA buffer containing protease (p8340) and phosphatase (#3) inhibitor mixtures (Sigma), quantified, and probed as we previously described (3). Twenty micrograms of protein per sample was subjected to SDS/PAGE and immunoblotting using standard techniques. Immunoblots were visualized using the Odyssey Imaging System with appropriate fluorescently labeled secondary antibodies (LI-COR Biosciences). Densitometric quantification of Western blots was performed using ImageJ software (NIH). Protein expression was normalized to loading controls and expressed relative to control conditions.

Tissue Biogenic Amine Measurements. Neurochemical measures were obtained from the Neurochemistry Core Facility at Vanderbilt University operated by the Vanderbilt Brain Institute. Brain regions, harvested and dissected as noted above, were flash-frozen in liquid nitrogen, and stored at −80 °C before shipment to the core facility.

Real-Time PCR. Tissue mRNA was extracted using the Qiagen RNeasy Mini kit and first stand cDNA synthesis was performed with SuperScript III (Life Technologies). Real-time PCR was carried out using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's protocol. PCR primers sequences are listed in Table S1. *GAPDH* was used as an internal control to normalize RNA levels.

Activity Measurement in Home Cage. The activity of WT and *RGS6*$^{-/-}$ mice was observed in the home cage following administration of a single dose of baclofen (10 mg/kg, i.p.). After drug treatment, mice were placed back in their home cage and behavior videotaped for 20 min. Videos were scored for total immobility time (no discernable ambulation, grooming, social, or other behaviors) by an observer blinded to mouse genotype.

Blood Alcohol Concentration. Mice were treated with EtOH (3.5 g/kg) by oral gavage and blood collected 45 min postinjection. Blood samples were transferred to cold EDTA tubes and centrifuged at 2,000 × *g* for 10 min. Plasma samples were stored at −80 °C until blood alcohol concentrations could be determined at the University of Iowa Hospitals.

1. Lockridge A, et al. (2012) Timing-dependent reduction in ethanol sedation and drinking preference by NMDA receptor co-agonist D-serine. *Alcohol* 46(4):389–400.
2. Bahi A (2011) The pre-synaptic metabotropic glutamate receptor 7 "mGluR7" is a critical modulator of ethanol sensitivity in mice. *Neuroscience* 199:13–23.
3. Maity B, et al. (2012) Regulator of G protein signaling 6 (RGS6) protein ensures coordination of motor movement by modulating GABAB receptor signaling. *J Biol Chem* 287(7):4972–4981.
4. Yang J, et al. (2010) RGS6, a modulator of parasympathetic activation in heart. *Circ Res* 107(11):1345–1349.
5. Maity B, et al. (2011) Regulator of G protein signaling 6 (RGS6) induces apoptosis via a mitochondrial-dependent pathway not involving its GTPase-activating protein activity. *J Biol Chem* 286(2):1409–1419.

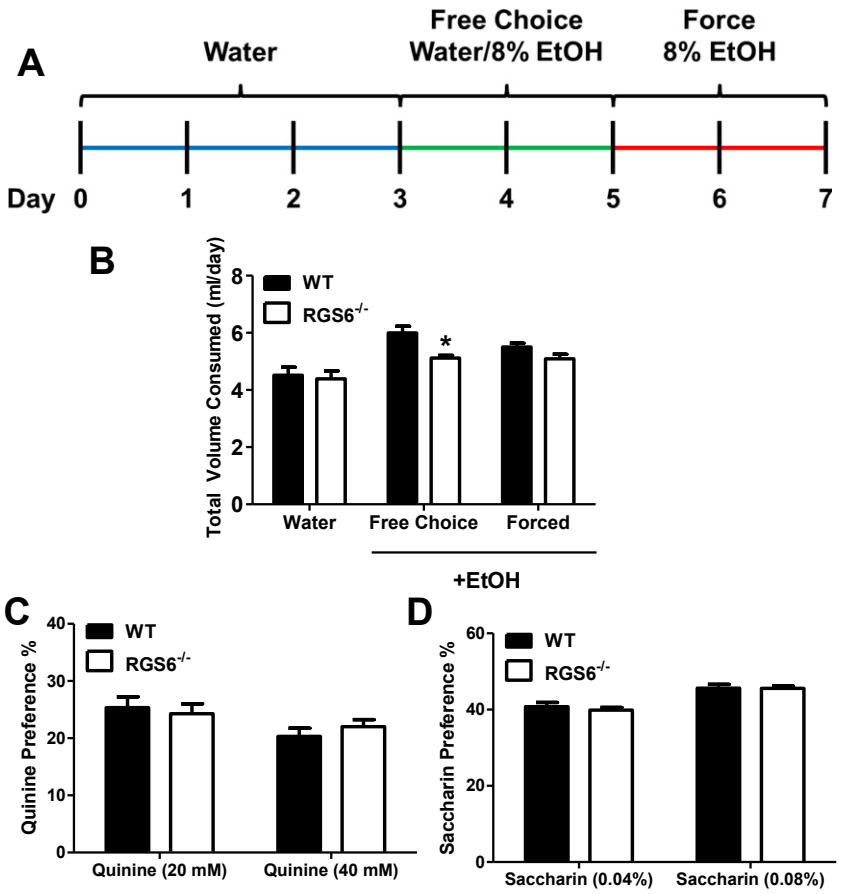

Fig. S1. Solution consumption and preference in the short-term consumption test. (*A*) Schematic depicting the experimental design for the short-term alcohol consumption test. (*B*) Total liquid volume consumed by WT ($n = 12$) and RGS6$^{-/-}$ ($n = 11$) mice during the water, free-choice, and forced EtOH consumption portions of the treatment paradigm. WT ($n = 5$) and RGS6$^{-/-}$ ($n = 5$) mice taste preference for (*C*) quinine and (*D*) saccharin containing solutions. *$P < 0.05$ vs. WT by the Student's *t* test. Data are presented as mean ± SEM.

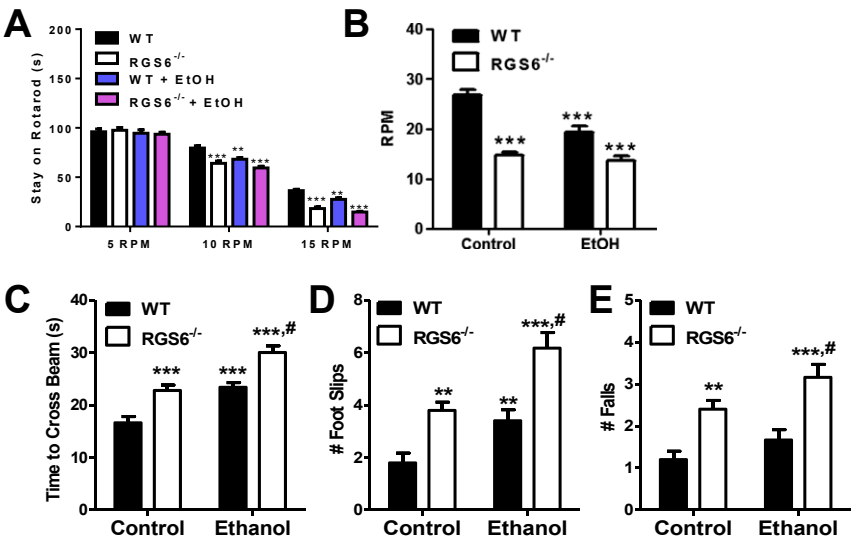

Fig. S2. Performance of WT and RGS6$^{-/-}$ mice on the rotarod and balance beam. Rotarod performance of WT ($n = 8$) and RGS6$^{-/-}$ ($n = 8$) mice treated with EtOH at (*A*) fixed speed and (*B*) during acceleration from 5 rpm. Ataxia induced by EtOH (1.75 g/kg, i.p.) was measured as (*C*) time to cross, (*D*) number of footslips, and (*E*) number of falls on the balance beam in WT ($n = 12$) and RGS6$^{-/-}$ ($n = 11$) mice. **$P < 0.01$ and ***$P < 0.001$ vs. WT controls and $^{\#}P < 0.01$ vs. WT ethanol-treated mice via ANOVA with the Bonferroni post hoc adjustment. Data are presented as mean ± SEM.

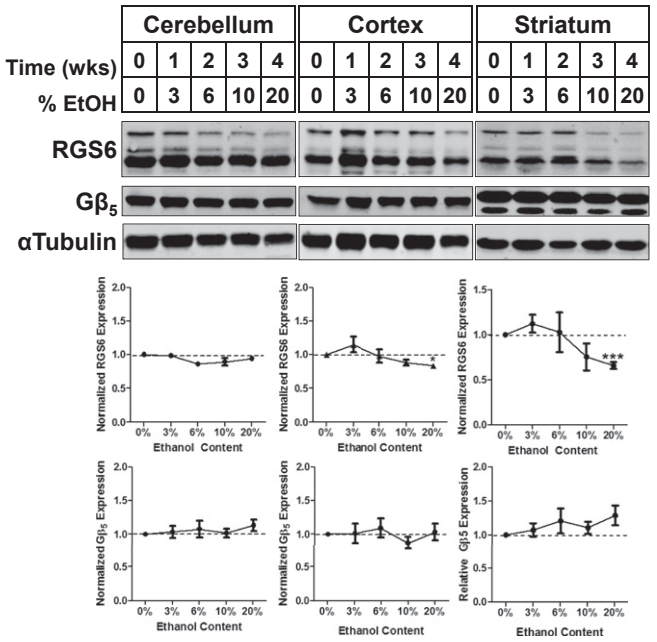

Fig. S3. RGS6 and Gβ5 expression in the cerebellum, striatum, and cortex of WT mice from the two-bottle choice test. Immunoblotting was performed probing for expression of RGS6 and Gβ5 in the cortex, cerebellum, and striatum of WT mice given free access to graduated alcohol containing solutions [0–20% (vol/vol)] for increasing periods of time (*Upper*). Densitometric quantification was performed with protein levels (n = 3) normalized to tubulin loading control and expressed relative to control conditions (*Lower*). $*P < 0.05$ and $***P < 0.001$ vs. control by ANOVA with the Bonferroni post hoc adjustment. Data are presented as mean ± SEM.

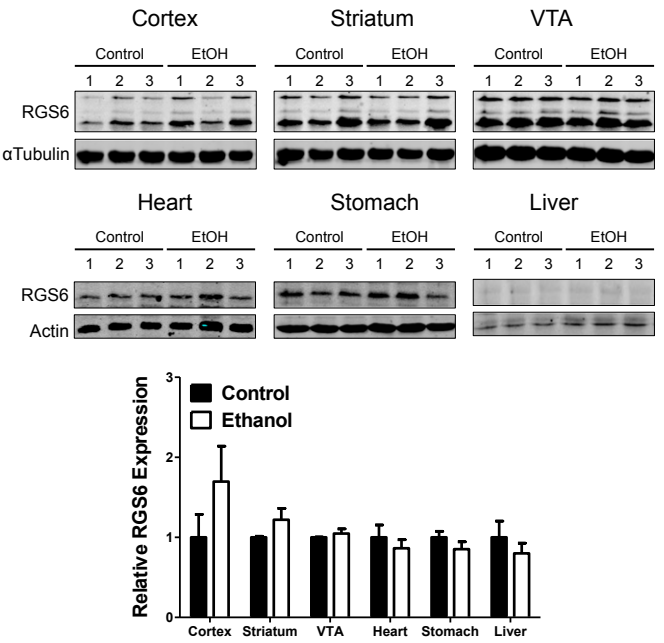

Fig. S4. Acute EtOH exposure does not alter RGS6 expression. Immunoblotting was performed probing for expression of RGS6 in the cortex, striatum, VTA, stomach, intestine, liver, and heart of WT mice treated with EtOH (3.5 g/kg by oral gavage) (*Upper*). Densitometric quantification was performed with protein levels (n = 3) normalized to tubulin or actin loading controls and expressed relative to control conditions (*Lower*). Data are presented as mean ± SEM.

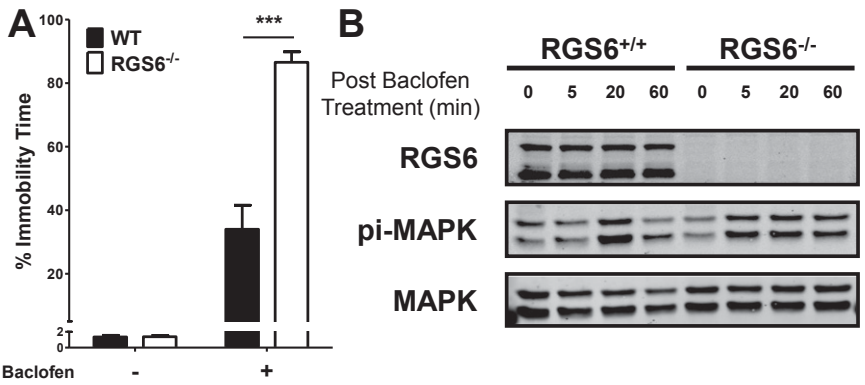

Fig. S5. RGS6 regulates noncerebellar $GABA_BR$ signaling. (*A*) Baclofen-induced sedation as depicted by immobility following drug treatment (WT, $n = 8$; $RGS6^{-/-}$, $n = 8$). (*B*) Immunoblotting for RGS6L, MAPK, and pi-MAPK in the cortex of WT and $RGS6^{-/-}$ mice treated with baclofen (10 mg/kg, i.p.) for 0, 5, 20, and 60 min. ***$P < 0.001$ via ANOVA with the Bonferroni post hoc adjustment. Data are presented as mean ± SEM.

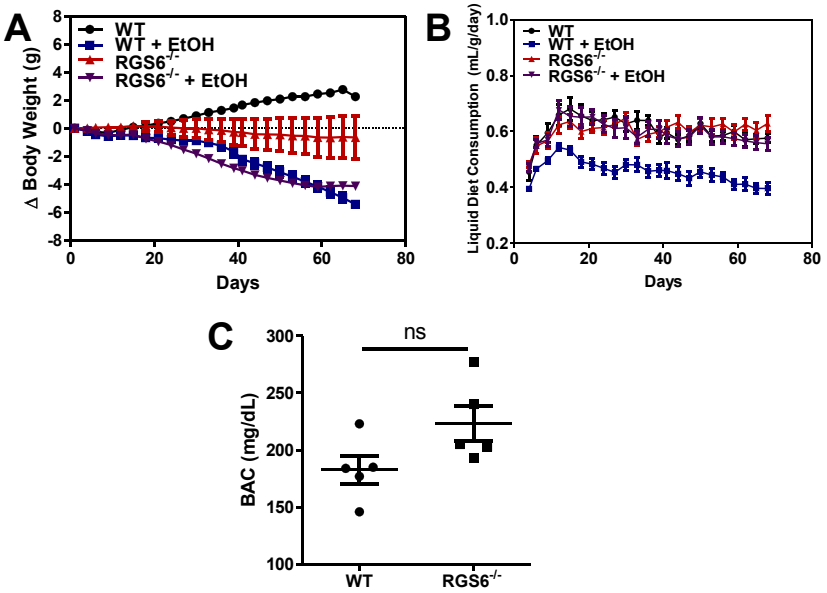

Fig. S6. Body weight change, alcohol consumption, and blood alcohol levels in WT and $RGS6^{-/-}$ mice. Mice of both genotypes were allowed ad libitum access to EtOH [5% (vol/vol)] containing or isocaloric Lieber deCarli liquid diets. (*A*) The net change in body weight and (*B*) alcohol consumption were monitored every 3 d for the entire 2-mo treatment course. (*C*) Blood alcohol concentrations 45 min following a single EtOH dose via oral gavage. Data are presented as mean ± SEM.

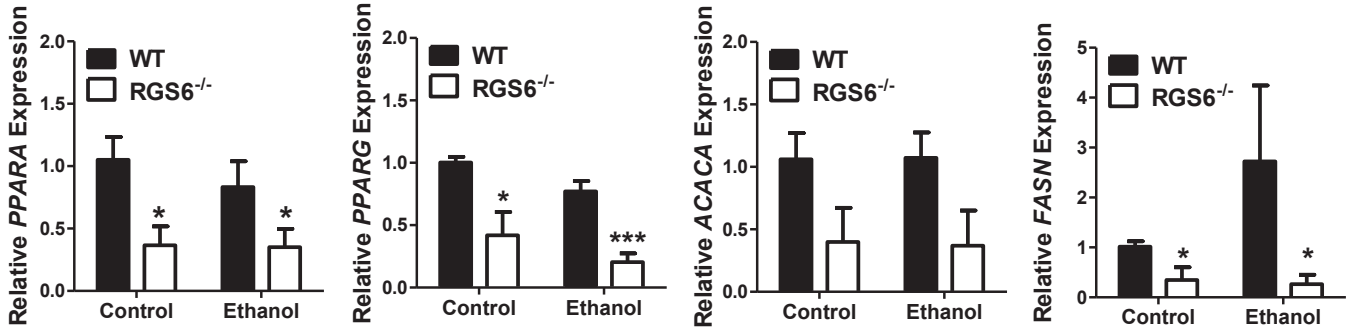

Fig. S7. RGS6 loss impacts gene expression in the liver. *PPARα*, *PPARγ*, *ACACA*, and *FASN* mRNA expression in the liver of WT ($n = 4$–5) and $RGS6^{-/-}$ mice ($n = 5$–6) fed on an isocaloric control or EtOH [5% (vol/vol)] containing Lieber deCarli liquid diet for 2 mo. *$P < 0.05$; ***$P < 0.001$ vs. WT controls by ANOVA with the Bonferroni post hoc adjustment. Data are presented as mean ± SEM.

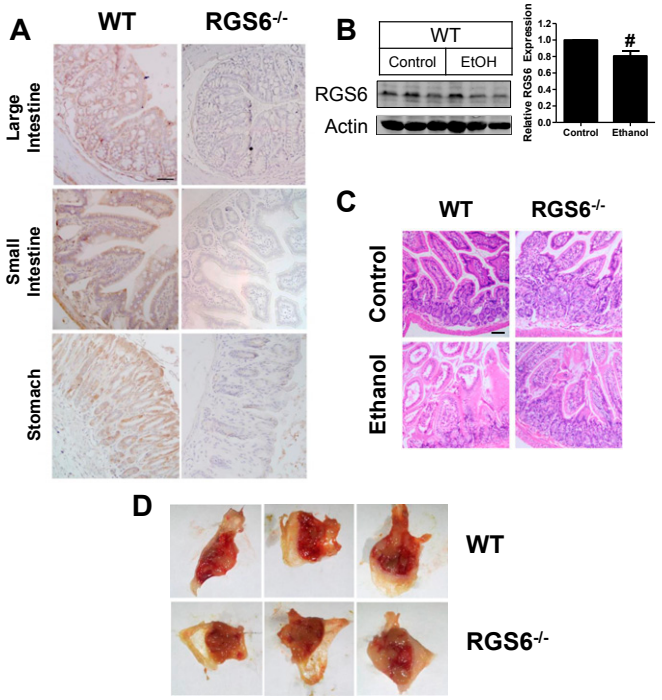

Fig. S8. RGS6 expression and impact in the gastrointestinal tract. (*A*) Immunohistochemistry staining of RGS6 expression (brown) in the stomach, small intestine, and large intestine of WT and RGS6$^{-/-}$ mice. (Scale bar, 50 μm.) (*B*) RGS6 expression in the stomach of alcohol-treated WT and RGS6$^{-/-}$ mice (*Left*). Densitometric quantification was performed with protein levels ($n = 3$) normalized to actin loading control and expressed relative to control conditions (*Right*). Animals (WT, $n = 8$; RGS6$^{-/-}$, $n = 8$) were treated EtOH according to a three-dose acute protocol. At the end of the treatment regimen (*C*) H&E staining was performed in intestine. (Scale bar, 100 μm.) (*D*) Stomach gross anatomy following acute EtOH treatment. Representative images ($n = 3$) of the stomachs of WT and RGS6$^{-/-}$ mice after a three dose EtOH-treatment regimen. $^{\#}P < 0.05$ vs. WT control via Student's *t* test. Data are presented as mean ± SEM.

Table S1. Primers used for RT-PCR analyses

Gene		Sequence
GAPDH	Forward	5′-CTACACTGAGGACCAGGTTGTCT-3′
	Reverse	5′-GGTCTGGGATGGAAATTGTG-3′
DAT	Forward	5′-GGCCTATGCCATCACACCTGAG-3′
	Reverse	5′-TGTCCCCGCTGTTGTGAGATG-3′
TH	Forward	5′-GAAGGGCCTCTATGCTACCCA-3′
	Reverse	5′-TGGGCGCTGGATACGAGA-3′
VMAT	Forward	5′-TTGCTCATCTGTGGCTGGG-3′
	Reverse	5′-TGGCGTTACCCCTCTCTTCAT-3′
PPARα	Forward	5′-ACGATGCTGTCCTCCTTGATG-3′
	Reverse	5′-GTGTGATAAAGCCATTGCCGT-3′
PPARγ	Forward	5′-AAGAGCTGACCCAATGGTTG-3′
	Reverse	5′-ACCCTTGCATCCTTCACAAG-3′
ACACA	Forward	5′-GCCTCTTCCTGACAAACGAG-3′
	Reverse	5′-TGACTGCCGAAACATCTCTG-3′
FASN	Forward	5′-TGGGTTCTAGCCAGCAGAGT-3′
	Reverse	5′-ACCACCAGAGACCGTTATGC-3′