Supporting Information

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SI Materials and Methods

Materials. We obtained Enzyme Immunoassay Kits for ghrelin measurements (unacylated, catalog no. A05117; acylated, catalog no. A05118) from Cayman Chemical Co. (Ann Arbor, MI); DMEM/F-12 50:50 medium (catalog no. 10-090-CV) from Mediatech, Inc. (Manassas, VA); insulin-transferrin-selenium-X (ITS-X) from Invitrogen (Carlsbad, CA); fatty acids, BSA (fatty acid-free), forskolin, epinephrine, norepinephrine, muscimol, carbachol, S(−)-atenolol, ICI118,551, reserpine, glucagon, insulin, secretin, somatostatin, and gastrin-releasing peptide from Sigma (St. Louis, MO); mouse recombinant IGF-1, FGF-21, and leptin from ProSpec-Tany Technogene, Ltd. (Rehovot, Israel); endothelin-1 from Peptides International (Louisville, KY); and growth hormone from the National Hormone and Peptide Program of National Institute of Diabetes and Digestive and Kidney Diseases (through A. F. Parlow, Torrance, CA). Delipidated FBS was prepared by solvent extraction with l-butanol and isopropyl ether as described (1). Stock solutions of fatty acids bound to BSA were prepared at a final concentration of 5 mM (palmitate) or 10 mM (octanoate and oleate) in 0.15 M sodium chloride containing 10% (wt/vol) BSA as described (1). A stock solution of reserpine (0.5 mg/mL) was made up in 0.1% (vol/vol) acetic acid. A stock solution of forskolin (10 mM) was made up in 100% (vol/vol) DMSO. Stock solutions of epinephrine and norepinephrine (5 mM) and of atenolol (5 mM for cell culture; 10 mg/mL for in vivo experiments) were made up in 0.01 M HCl. Stock solutions of all other compounds (5 mM) were made up in PBS or water.

Generation of Transgenic Ghrelin-SV40 T-Antigen Mice. The plasmid used to generate transgenic mice that express SV40 large T-antigen in ghrelin cells, hereafter referred to as TgGhrelin-SV40-T mice, was constructed in our laboratory by BAC engineering using reagents provided by Neal Copeland and Nancy Jenkins (Institute of Molecular and Cell Biology, Singapore). A BAC clone (RP23-62H1) containing the entire coding region of mouse *preproghrelin* plus a total of 164 kb of flanking genomic DNA (60 kb upstream of the start codon and 104 kb downstream of the stop codon) (2) was engineered as follows. The SV40 early region [which contains the T-antigen coding sequence and corresponds to the XbaI/BamHI fragment of the previously reported RIP1-tag plasmid (3); kindly provided by Douglas Hanahan, University of California, San Francisco, CA] was inserted immediately downstream of the start codon of *preproghrelin*. This insertion, which removed the first 29 bp of the preproghrelin coding sequence, allowed both the transcription and translation of SV40 T-antigen to be controlled by *preproghrelin* regulatory elements. The modified BAC was microinjected into pronuclei of fertilized one-cell stage embryos of C57BL/6J mice (by the Transgenic Core Facility at University of Texas Southwestern Medical Center at Dallas) to generate several lines of transgenic mice expressing SV40 T-antigen in ghrelin cells of the stomach and pancreas. All the TgGhrelin-SV40-T mice used in this study were on a pure C57BL/6J genetic background.

Mice were housed in colony cages with a 12-h light/12-h dark cycle and fed a standard chow diet containing 4% (wt/wt) fat (Teklad Mouse/Rat Diet 7001; Harlan Teklad Premier Laboratory Diets). All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center at Dallas.

Tissue Preparation, H&E Staining, and Photomicrographs. Mice were anesthetized with i.p. injection of chloral hydrate (500 mg/kg) and subsequently perfused transcardially with diethylpyrocarbonate-treated 0.9% (wt/vol) saline followed by 10% (wt/vol) neutral buffered formalin. Tissues were removed, further fixed by immersion with gentle agitation in the same fixative for 16 h at 4 °C, and then submitted to the University of Texas Southwestern Medical Center Molecular Pathology Core, where routine histological procedures were conducted according to standard protocols. Briefly, formalin-fixed tissues were dehydrated with an ascending ethanol series, cleared with xylenes, and infused with paraffin. Five micrometer-thick sections of paraffin-embedded tissues were generated using a rotary microtome, mounted onto slides, air-dried before deparaffinization with xylenes, rehydrated with descending concentrations of ethanol, and then stained using H&E.

Tissue sections were viewed with a Zeiss Axioskop microscope using bright-field optics. Photomicrographs were produced with a Zeiss digital camera attached to the microscopes and a Dell desktop computer. An imaging editing software program, Adobe PhotoShop CS2, was used to combine the photomicrographs into plates and to adjust contrast and brightness.

Isolation of Enriched Pools of Gastric Ghrelin Cells. Gastric mucosal cells from mice harboring a transgene encoding GFP driven by the mouse *preproghrelin* promoter (2) were separated by FACS as previously described (4).

In Situ Hybridization. In situ hybridization histochemistry was performed on formalin-fixed 25-µm-thick tissue sections using antisense and sense riboprobes specific for mouse ghrelin as previously described (2).

Ghrelin Measurements in Plasma and in Cultured Cells. Plasma levels of ghrelin and des-acyl ghrelin were measured as previously described (5). For measurements in cultured cells, cell suspensions from each well were centrifuged at $1,000 \times g$ for 5 min at 4 °C. The resulting supernatant (medium) was removed and treated with HCl at a final concentration of 0.1 M and stored at −20 °C until assay. Each of the cell pellets was resuspended in 1 mL of boiling water, boiled for 10 min, and then treated with acetic acid and HCl at final concentrations of 1 and 0.02 M, respectively. The cell lysates were homogenized by passing through a 22-gauge needle 10 times and spun at $20,000 \times g$ for 10 min at 4 °C. Each of the resulting supernatants was then lyophilized for 6–12 h, resuspended in 200 µL of 0.1 M HCl, and assayed immediately thereafter, together with the medium from the same wells. The concentrations of ghrelin and des-acyl ghrelin in medium and cells were measured by Enzyme Immunoassay Kits (Cayman Chemical Co.). For each experiment, two wells of untreated cells were lysed in 0.1 M NaOH and assayed for cell protein concentration using a BCA kit (Pierce). Ghrelin levels are expressed as picograms per microgram of cell protein.

Quantitative Real-Time PCR. Extraction of total RNA from tissues and cells, and real-time PCR measurements were carried out as described (6, 7). Primer sequences are listed in Table S4. The relative amounts of all mRNAs were calculated using the comparative threshold cycle method. mRNAs for 36B4 and cyclophilin were used as invariant controls.

1. Hannah VC, Ou J, Luong A, Goldstein JL, Brown MS (2001) Unsaturated fatty acids down-regulate srebp isoforms 1a and 1c by two mechanisms in HEK-293 cells. *J Biol Chem* 276: 4365–4372.
2. Sakata I, et al. (2009) Characterization of a novel ghrelin cell reporter mouse. *Regul Pept* 155:91–98.

3. Hanahan D (1985) Heritable formation of pancreatic β-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature* 315:115–122.
4. Sakata I, et al. (2009) Colocalization of ghrelin *O*-acyltransferase and ghrelin in gastric mucosal cells. *Am J Physiol Endocrinol Metab* 297:E134–E141.
5. Zhao TJ, et al. (2010) Ghrelin *O*-acyltransferase (GOAT) is essential for growth hormone-mediated survival of calorie-restricted mice. *Proc Natl Acad Sci USA* 107:7467–7472.
6. Yang J, et al. (2001) Decreased lipid synthesis in livers of mice with disrupted Site-1 protease gene. *Proc Natl Acad Sci USA* 98:13607–13612.
7. Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL (2008) Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* 132:387–396.

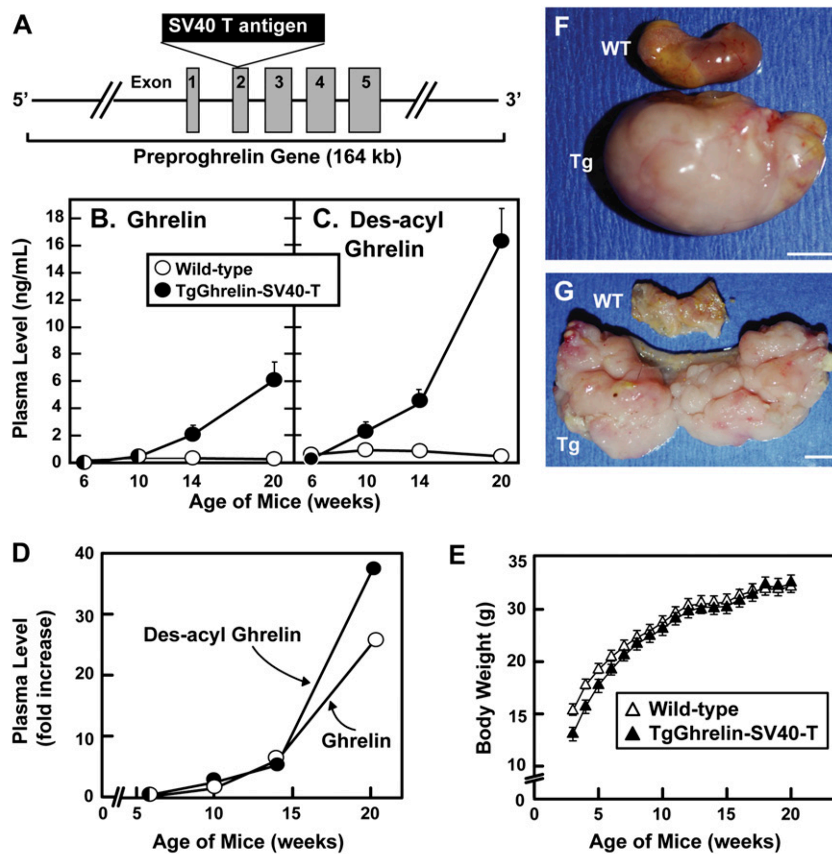

Fig. S1. Generation of transgenic TgGhrelin-SV40-T mice. (*A*) Schematic of the construct used to generate transgenic mice that express SV40 large T-antigen in ghrelin cells. This construct was made by BAC engineering as described in *Materials and Methods* using a BAC clone (RP23-62H1) that contains the entire coding region of the mouse *preproghrelin* gene plus a total of 164 kb of genomic DNA flanking its start and stop codons. The SV40 T-antigen coding region (3) was inserted immediately downstream of the start codon of preproghrelin, thereby allowing both the transcription and translation of SV40 T antigen to be controlled by *preproghrelin* regulatory elements. Plasma levels of ghrelin (*B*) and des-acyl ghrelin (*C*) from WT and TgGhrelin-SV40-T male littermates at the indicated time after birth. The same cohort of WT mice and TgGhrelin-SV40-T mice was fed ad libitum on a chow diet (4% fat) and followed serially from 4–20 wk of age. Plasma levels were measured during the light cycle (between noon and 4:00 PM). Each value represents the mean ± SEM of values from 13 WT mice and 17 TgGhrelin-SV40-T mice. (*D*) Increase in plasma levels of ghrelin (○) and des-acyl ghrelin (●) in TgGhrelin-SV40-T mice as compared with littermate WT mice. Each value represents the fold increase in TgGhrelin-SV40-T mice (n = 17) relative to WT mice (n = 13) as calculated from the data in Fig. 1 *B* and *C*. (*E*) Body weight in WT (△) and TgGhrelin-SV40-T (▲) mice measured weekly at 9:00 to 10:00 AM. These mice are the same as those studied in Fig. 1 *B–D*. Each value represents the mean ± SEM of values from 13 (△) and 17 (▲) mice. (*F*) Representative photographs of stomachs from 6-mo-old WT and TgGhrelin-SV40-T mice. (*G*) Same stomachs from *D* opened and photographed. (Scale bar: *F* and *G*, 0.5 cm.)

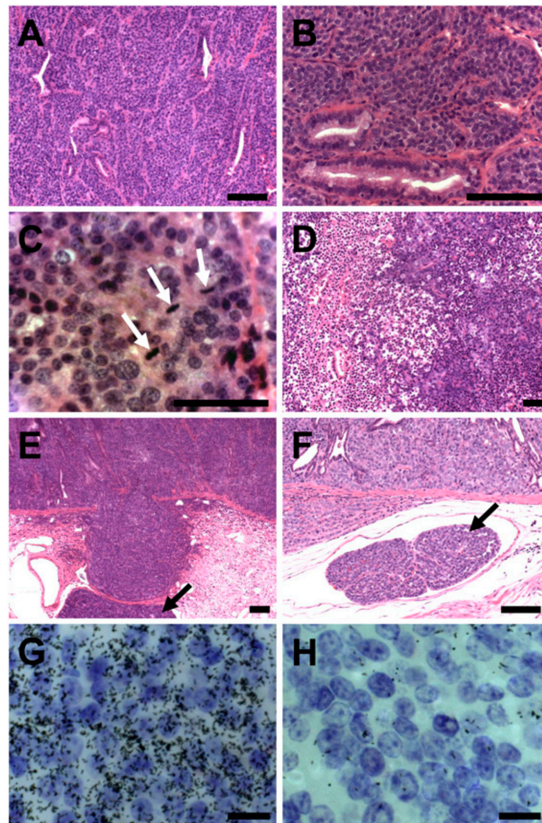

Fig. S2. Histological examination of gastric tumors from TgGhrelin-SV40-T mice. Paraffin-embedded sections of different regions of gastric tumors were prepared and stained with H&E as described in *SI Materials and Methods*. (*A* and *B*) Sheets of neoplastic cells with delicate fibrovascular stroma and few remaining gastric glands. (*C*) Pleomorphic nuclei with occasional mitoses (arrows). (*D*) Necrotic areas. (*E* and *F*) Invasion of neoplastic cells into submucosa and submucosal vasculature (arrows). In situ hybridization histochemistry, demonstrating binding to gastric tumor sections of ^{35}S-labeled antisense ghrelin riboprobe (*G*) but not of ^{35}S-labeled sense ghrelin riboprobe (*H*). (Scale bars: *A*–*F*, 100 μm; *G* and *H*, 12.5 m.)

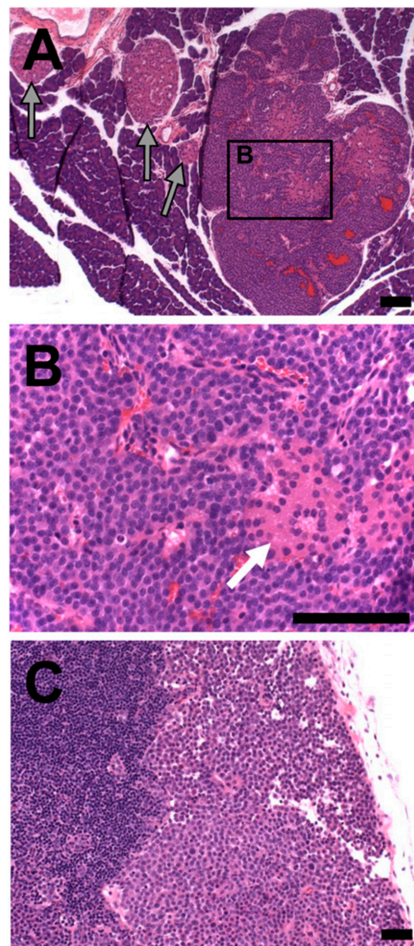

Fig. S3. Histological examination of pancreatic tumors from TgGhrelin-SV40-T mice. (*A*) H&E staining of paraffin-embedded 5-μm-thick sections of different regions of pancreas demonstrating variably-sized islet-based tumors surrounded by normal exocrine tissue and adjacent to occasional normal-appearing islets (arrows). (*B*) Enlarged view of boxed area in *A* showing surviving normal islet cells (arrow, surrounded by sheets of neoplastic cells). (*C*) Peripancreatic lymph node with subcapsular infiltrates of metastatic tumor cells. (Scale bars: 100 μm.)

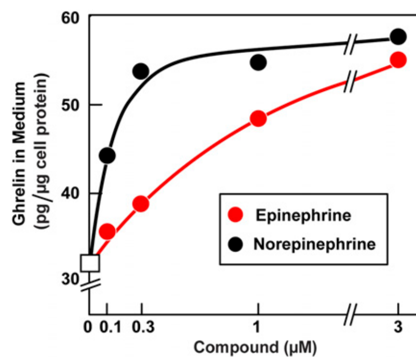

Fig. S4. Norepinephrine is more potent than epinephrine in stimulating ghrelin secretion. On day 0, PG-1 cells were set up in medium A with 10% FBS. On day 2, sodium octanoate was added to the medium at a final concentration of 50 μM. On day 3, cells were centrifuged, resuspended in serum-free medium B with 50 μM sodium octanoate, aliquoted into 24-well plates (5×10^4 cells per well), and treated with varying concentrations of norepinephrine and epinephrine as indicated. After incubation at 37 °C for 6 h, the medium from each well was harvested for measurement of ghrelin levels. Each value is the average of duplicate values. This experiment was repeated with virtually identical results.

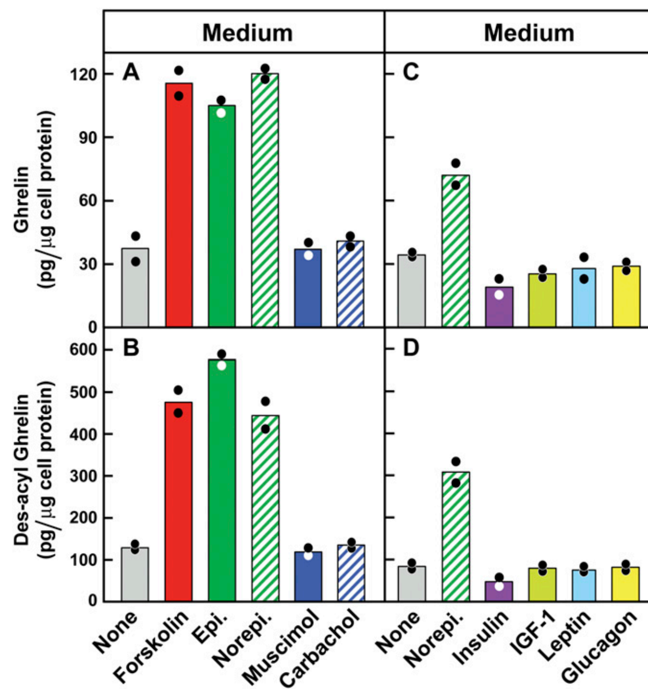

Fig. S5. Stimulation of ghrelin secretion by adrenergic compounds in SG-1 cells. On day 0, SG-1 cells were set up in medium A with 10% FBS. On day 2, octanoate was added to the medium at a final concentration of 50 μM. On day 3, cells were centrifuged, resuspended in serum-free medium B with 50 μM octanoate, aliquoted into 24-well plates (5×10^4 cells per well), and treated with the indicated compound at a final concentration of either 10 μM [forskolin, epinephrine (Epi.), norepinephrine (Norepi.), muscimol, and carbachol] or 100 nM (insulin, glucagon, IGF-1, and leptin). After incubation at 37 °C for 6 h, the medium and cells from each well were harvested for measurement of ghrelin (*A* and *C*) and des-acyl ghrelin (*B* and *D*). Each bar denotes the average of duplicate incubations, the individual values of which are shown in circles.

Table S1. Relative amount of mRNAs in PG-1 and SG-1 cells

Experiment A

mRNA	Stomach WT	Stomach ghrelinoma	PG-1 cells	SG-1 cells
Preproghrelin	1.0 (20.7 ± 0.3)*	7.1 ± 0.7	5.5 ± 0.7	7.7 ± 0.9
GOAT	1.0 (28.9 ± 0.6)	8.3 ± 1.7	13.8 ± 2.7	13.4 ± 3.0
Adrenergic receptors				
α_{1A}	1.0 (34.4 ± 0.2)	2.1 ± 0.6	1.5 ± 0.4	1.3 ± 0.8
α_{1B}	1.0 (32.2 ± 0.3)	1.5 ± 0.1	65 ± 8.1	37 ± 5.3
α_{1D}	1.0 (32.3 ± 0.3)	1.8 ± 0.1	1.2 ± 0.2	0.60 ± 0.20
α_{2A}	1.0 (29.0 ± 0.1)	0.46 ± 0.04	0.52 ± 0.04	0.05 ± 0.01
α_{2B}	1.0 (32.2 ± 0.3)	1.4 ± 0.14	1.2 ± 0.15	1.3 ± 0.15
α_{2C}	1.0 (33.9 ± 0.7)	2.4 ± 0.73	1.3 ± 0.41	0.61 ± 0.29
β_1	1.0 (33.2 ± 0.4)	138 ± 23	416 ± 46	138 ± 16
β_2	1.0 (28.8 ± 0.2)	1.1 ± 0.02	0.52 ± 0.03	0.07 ± 0.01
β_3	1.0 (32.2 ± 0.4)	0.49 ± 0.10	0.10 ± 0.02	0.09 ± 0.03
Neuroendocrine markers				
Chromogranin A	1.0 (23.6 ± 0.3)	13 ± 0.8	5.8 ± 0.6	5.4 ± 0.7
Secretogranin II	1.0 (29.5 ± 0.3)	438 ± 26	311 ± 3.0	220 ± 1.0
Secretogranin III	1.0 (29.6 ± 0.1)	73 ± 6.3	94 ± 9.1	77 ± 9.1
Secretogranin V	1.0 (28.0 ± 0.3)	189 ± 7.0	134 ± 9.0	115 ± 2.0
PC 1/3	1.0 (28.2 ± 0.6)	36 ± 3.5	231 ± 55	188 ± 49
PC 2	1.0 (28.3 ± 0.1)	80 ± 15	57 ± 12	75 ± 16
Hormone receptors				
GHSR	Not detected†	Not detected	Not detected	Not detected
Growth hormone	1.0 (24.7 ± 0.2)	1.3 ± 0.10	0.88 ± 0.04	0.41 ± 0.03
Glucagon	1.0 (33.3 ± 0.5)	1.7 ± 0.73	0.37 ± 0.08	1.1 ± 0.30
Leptin	1.0 (27.5 ± 0.2)	1.7 ± 0.03	0.01 ± 0.002	0.005 ± 0.003
IGF-1	1.0 (26.1 ± 0.3)	0.20 ± 0.01	0.04 ± 0.003	Not detected
Insulin	1.0 (25.6 ± 0.2)	0.91 ± 0.03	1.3 ± 0.08	0.69 ± 0.03

Experiment B

mRNA	Stomach WT	PG-1 cells	SG-1 cells	Pancreatic islets WT
Preproghrelin	1.0 (20.9 ± 0.1)	7.2 ± 0.8	3.6 ± 0.3	Not detected†
GOAT	1.0 (31.2 ± 0.2)	26 ± 2.0	36 ± 3.7	Not detected
Neuroendocrine markers				
Chromogranin A	1.0 (23.8 ± 0.4)	7.0 ± 1.0	14 ± 4.5	17 ± 0.5
PC 1/3	1.0 (30.5 ± 0.3)	751 ± 102	577 ± 37	265 ± 7
PC 2	1.0 (31.9 ± 0.1)	319 ± 44	581 ± 66	2,737 ± 155
Pancreatic hormones				
Insulin	Not detected†	Not detected	Not detected	1.0 (14.4 ± 0.02)
Preproglucagon	Not detected	Not detected	Not detected	1.0 (16.8 ± 0.2)
Pancreatic polypeptide	Not detected	Not detected	Not detected	1.0 (18.6 ± 0.1)
Somatostatin	Not detected	Not detected	Not detected	1.0 (19.0 ± 0.1)

Total mRNA from the indicated tissues or cells was extracted and quantified by quantitative real-time PCR as described in *Materials and Methods*. 36B4 mRNA (*A*) and cyclophilin mRNA (*B*) were used as invariant controls. Each value represents the amount of mRNA relative to that in WT stomach or WT pancreatic islets, which is arbitrarily defined as 1.0 and shown as the mean ± SEM of three different experiments. Each determination in each experiment was done in duplicate (*B*) or triplicate (*A*). GHSR, growth hormone secretagogue receptor; PC, prohormone convertase.

*Values in parentheses denote the mean ± SEM of threshold cycle values.

†Not detected, threshold cycle value ≥35.

Table S2. Relative amount of mRNAs for adrenergic receptors in FACS-separated pools of ghrelin-enriched gastric mucosal cells

	Gastric cells expressing ghrelin	
mRNA	Nonenriched	Enriched
Preproghrelin	1.0 (26.5 ± 0.4)*	8,870 ± 2,346
GOAT	1.0 (31.5 ± 0.9)	90 ± 39
Adrenergic receptors		
α_{1A}	Not detected[†]	Not detected
α_{1B}	1.0 (30.4 ± 0.4)	Not detected
α_{1D}	1.0 (29.3 ± 0.3)	Not detected
α_{2A}	1.0 (28.9 ± 0.1)	Not detected
α_{2B}	Not detected	Not detected
α_{2C}	Not detected	Not detected
β_1	1.0 (32.2 ± 0.3)	59 ± 15
β_2	1.0 (29.8 ± 0.4)	0.3 ± 0.1
β_3	Not detected	Not detected

Ghrelin-enriched (GFP^+) and nonenriched (GFP^-) gastric mucosal cells were FACS-separated, and total mRNA from the indicated cells was extracted and quantified by quantitative real-time PCR as described in *SI Materials and Methods*. Cyclophilin mRNA was used as an invariant control. Each value represents the amount of mRNA relative to that in GFP^- cells, which is arbitrarily defined as 1.0 and is shown as the mean ± SEM of three different experiments. Each determination was done in duplicate.

*Values in parentheses denote the mean ± SEM of threshold cycle values.

[†]Not detected, threshold cycle value ≥35.

Table S3. Effects of adrenergic agonists and antagonists on ghrelin secretion in PG-1 cells

	Medium, pg/μg of protein		
Addition to medium	Ghrelin	Des-acyl ghrelin	Total ghrelin
None	39 ± 1.5	99 ± 7.7	137 ± 8.8
Norepinephrine, 1 μM	81 ± 5.7 (2.1)*	214 ± 9.8 (2.2)	295 ± 15 (2.1)
+ Prazosin, 10 μM	80 ± 6.4 (2.1)	199 ± 7.9 (2.0)	279 ± 13 (2.0)
+ Atenolol, 10 μM	53 ± 1.5 (1.4)	115 ± 2.3 (1.2)	168 ± 2.6 (1.2)
Methoxamine, 30 μM	41 ± 1.7 (1.1)	107 ± 3.8 (1.1)	148 ± 4.3 (1.1)

On day 0, PG-1 cells were set up in medium A with 10% FBS. On day 2, octanoate was added to a final concentration of 50 μM. On day 3, cells were centrifuged, resuspended in serum-free medium B with 50 μM octanoate, aliquoted into 24-well plates (5×10^4 cells per well), and treated with the indicated compounds at the indicated final concentration. After incubation at 37 °C for 6 h, the medium from each well was harvested for measurement of ghrelin and des-acyl ghrelin levels. Each value represents the mean ± SEM of four incubations. Prazosin is a α_1-adrenergic antagonist, atenolol is a β_1-adrenergic antagonist, and methoxamine is a α_1-adrenergic agonist.

*Denotes fold increase relative to no addition.

Table S4. Real-time PCR primer sources

Mouse gene	Source of primer sequences
Growth hormone receptor	PrimerBank (6857795a1)*
Glucagon receptor	PrimerBank (6679965a1)*
Leptin receptor	PrimerBank (1139593a1)*
Secretogranin II	PrimerBank (6677865a1)*
Secretogranin III	PrimerBank (6677867a1)*
Secretogranin V	PrimerBank (31982002a1)*
Adrenergic receptor α_{1A}	PrimerBank (31542114a1)*
Adrenergic receptor α_{1B}	PrimerBank (6680660a1)*
Adrenergic receptor α_{1D}	PrimerBank (34328059a1)*
Adrenergic receptor α_{2A}	PrimerBank (6680662a1)*
Adrenergic receptor α_{2B}	PrimerBank (6752994a1)*
Adrenergic receptor α_{2C}	PrimerBank (6680664a1)*
Adrenergic receptor β_1	Mouse Genome Informatics (MGI:1204525)
Adrenergic receptor β_2	PrimerBank (34328092a1)*
Adrenergic receptor β_3	PrimerBank (298113a1)*
Growth hormone secretagogue receptor	5′-TGGAGATCGCGCAGATCAG-3′
	5′-CCGGGAACTCTCATCCTTCAG-3′
Insulin receptor	5′-CGAGTGCCCGTCTGGCTATA-3′
	5′-GGCAGGGTCCCAGACATG-3′
IGF-1 receptor	5′-AGCGCAGCTGATGTGTACGT-3′
	5′-GCTCCCGGTTCATGGTGAT-3′
Chromogranin A	5′-GCAGGCTACAAAGCGATCCA-3′
	5′-CTCTGTCTTTCCATCTCCATCCA-3′
Prohormone convertase 1/3	5′-GGCACCTGGACATTGAAAATTAC-3′
	5′-TTCATGTGCTCTGGTTGAGAAGA-3′
Prohormone convertase 2	5′-CAAGCGGAACCAGCTTCA-3′
	5′-ATTCCAGGCCAACCCCA-3′
Insulin	5′-TGAAGTGGAGGACCCACAAGT-3′
	5′-AGATGCTGGTGCAGCACTGAT-3′
Preproglucagon	5′-ATTCACCAGCGACTACAGCAA-3′
	5′-TCATCAACCACTGCACAAAATC-3′
Pancreatic polypeptide	5′-GAAACTCAGCTCCGCAGATACA-3′
	5′- TGTTCTCCTCTTCGGCTCTCT-3′
Somatostatin	5′-CCCAGACTCCGTCAGTTTCT-3′
	5′-GGGCATCATTCTCTGTCTGG-3′

*Available at http://pga.mgh.harvard.edu/primerbank.