LEAP2 Is an Endogenous Antagonist of the Ghrelin Receptor

Graphical Abstract

Highlights
- LEAP2 identified as an endogenous peptide antagonist of the ghrelin receptor
- LEAP2 modulates ghrelin function in response to physiological conditions
- Overexpressing LEAP2 in mice phenocopies ghrelin deficiency
- Suppressing LEAP2 using function-blocking antibodies enhances ghrelin action

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In Brief
Ghrelin maintains blood glucose levels in the face of starvation. Ge et al. identify LEAP2 as an endogenous antagonist of the ghrelin receptor that modulates ghrelin function in response to nutrient status, such as fasting. Increasing or suppressing LEAP2 leads to corresponding counter-regulation of ghrelin action in vivo.
LEAP2 Is an Endogenous Antagonist of the Ghrelin Receptor

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SUMMARY

Ghrelin, an appetite-stimulatory hormone secreted by the stomach, was discovered as a ligand for the growth hormone secretagogue receptor (GHSR). Through GHSR, ghrelin stimulates growth hormone (GH) secretion, a function that evolved to protect against starvation-induced hypoglycemia. Though the biology mediated by ghrelin has been described in great detail, regulation of ghrelin action is poorly understood. Here, we report the discovery of liver-expressed antimicrobial peptide 2 (LEAP2) as an endogenous antagonist of GHSR. LEAP2 is produced in the liver and small intestine, and its secretion is suppressed by fasting. LEAP2 fully inhibits GHSR activation by ghrelin and blocks the major effects of ghrelin in vivo, including food intake, GH release, and maintenance of viable glucose levels during chronic caloric restriction. In contrast, neutralizing antibodies that block endogenous LEAP2 function enhance ghrelin action in vivo. Our findings reveal a mechanism for fine-tuning ghrelin action in response to changing environmental conditions.

INTRODUCTION

Ghrelin is a 28-amino-acid peptide hormone produced by the stomach (Kojima and Kangawa, 2005) that plays critical roles in controlling growth hormone (GH) release and energy homeostasis. Active ghrelin is octanoylated at Ser3 (Gutierrez et al., 2008; Yang et al., 2008), and its actions are mediated through binding to the growth hormone secretagogue receptor (GHSR), a G protein-coupled receptor (GPCR) enriched in the pituitary gland and hypothalamus (Cruz and Smith, 2008). Upon ghrelin binding, GHSR is activated in pituitary cells and stimulates GH release (Kojima et al., 1999). The activation of GHSR in the hypothalamic arcuate nucleus stimulates appetite and promotes food intake (Cowley et al., 2003; Sun et al., 2004).

Recent studies have indicated that an essential physiological role of ghrelin is to maintain viable blood glucose levels during chronic caloric restriction (CR) (Zhang et al., 2015; Zhao et al., 2010). During CR, maintaining sufficient blood glucose levels to enable brain function is critical to survival (Cahill, 2006). Ghrelin is secreted in response to CR and promotes survival by stimulating GH release, which functions to promote gluconeogenesis and maintains viable glucose levels (Li et al., 2012; Zhang et al., 2015). Under chronic CR, subtle changes in GH levels may determine whether blood glucose is maintained at a level consistent with survival. In such conditions, fine-tuning of ghrelin action is critical. Regulation of ghrelin production has been well studied—serum levels peak before meals and fall shortly afterward (Cummings et al., 2001; Nass et al., 2008). Some forms of bariatric surgery also reduce ghrelin levels (Cummings et al., 2002a). However, regulation of ghrelin action at the receptor level has not been described.

Here, we report the discovery of a peptide hormone, liver-expressed antimicrobial peptide 2 (LEAP2), as an endogenous antagonist of the ghrelin receptor. We identified LEAP2 as a peptide with profound differentially regulated expression following bariatric surgery. The physiological role of LEAP2 has not been identified. In both gain- and loss-of-function experiments, we found that LEAP2 is a potent inhibitor of ghrelin action in vivo. Our findings reveal a hitherto-unknown mechanism regulating ghrelin action.

RESULTS

LEAP2, a Bariatric Surgery-Regulated Peptide, Antagonizes the Ghrelin Receptor

Bariatric surgery, an effective treatment for obesity and diabetes, leads to profound remodeling of whole-body energy homeostasis (Miras and le Roux, 2013; Seeley et al., 2015). We utilized a mouse model of vertical sleeve gastrectomy (VSG), a common bariatric surgery (Figure 1A), as a tool to identify novel secreted proteins and peptides that might act as important metabolic regulators. We analyzed gene expression in the stomach and intestines following VSG or sham surgery in diet-induced obese mice...
and sought to identify differentially regulated genes encoding secreted proteins and peptides. One set of genes exhibited a striking pattern of inverse regulation between the stomach and duodenum, tissues that are profoundly remodeled by VSG surgery (Figure 1B). Among these genes is *Leap2*, whose expression level increased 52-fold in the stomach and decreased by 94% in the duodenum following VSG surgery, as compared to sham surgery controls (Figure 1C). These expression changes were confirmed by quantitative PCR (Figure S1A).

LEAP2 is a secreted peptide originally purified from human blood ultrafiltrate (Krause et al., 2003). The mature form of LEAP2 is a 40-amino-acid bicyclic peptide containing two disulfide bridges (Henriques et al., 2010) (Figures S1B and S1D). The amino acid sequence of mature LEAP2 is identical in mouse and human, with four highly conserved cysteine residues forming the two disulfide linkages (Figure S1C). Although the bicyclic structure of LEAP2 is reminiscent of many peptide hormones (Joo, 2012), the biological function of LEAP2 is not well understood.

To gain insight regarding the function of LEAP2, we set out to identify potential receptors through which it might act. Given that many peptide hormones act through GPCRs (Krumm and Grisshammer, 2015), we tested the activity of LEAP2 against LEAP2 is a 40-amino-acid bicyclic peptide containing two disulfide bridges (Henriques et al., 2010) (Figures S1B and S1D). The amino acid sequence of mature LEAP2 is identical in mouse and human, with four highly conserved cysteine residues forming the two disulfide linkages (Figure S1C). Although the bicyclic structure of LEAP2 is reminiscent of many peptide hormones (Joo, 2012), the biological function of LEAP2 is not well understood.

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LEAP2 Is a Non-competitive Antagonist for Ghrelin

To identify the mode of antagonism employed by LEAP2, we measured GHSR activation by ghrelin in the presence of increasing concentrations of LEAP2. We found that LEAP2 reduced the magnitude of maximal GHSR activation by ghrelin (Figures 1H and S2F) and that this inhibition could not be overcome by increasing concentrations of ghrelin. In a parallel experiment, we evaluated the ability of ghrelin to activate GHSR in the presence of [D-Arg^1, D-Phe^3, D-Trp^9, Leu^11]-Substance P, a competitive GHSR antagonist (Holst et al., 2003). This competitive antagonist shifted the ghrelin concentration-response curve to the right but had no impact on maximal GHSR activation by ghrelin (Figure S2G). These data demonstrate that LEAP2 is a noncompetitive antagonist of GHSR, likely modulating the receptor through binding to an allosteric site (Christopoulos et al., 2014).

LEAP2 Binds to GHSR

To examine binding of LEAP2 to GHSR, we transfected COS7 cells with GHSR and then evaluated ligand binding to these cells using Alexa-647-labeled LEAP2 peptide. After fixation with parafomaldehyde, GHSR was detected by immunofluorescence under nonpermeabilized conditions. We found that LEAP2 binds to GHSR-expressing cells (arrows in Figure 2H), but not non-expressing cells (arrow heads in Figure 2H), consistent with direct binding of LEAP2 to GHSR.

LEAP2 Is Produced in Small Intestine and Liver and Is Regulated by Food Intake

Ghrelin is notable for its highly localized expression in the stomach and its regulation in response to feeding status and to surgical procedures that remodel the gastrointestinal tract (Miras and le Roux, 2013). Given our discovery of interplay between ghrelin and LEAP2, we set out to examine the localization of LEAP2 expression and its regulation in response to feeding and bariatric surgery. We extracted total RNA from 24 tissues of adult mice and detected Leap2 expression by quantitative PCR. The highest expression was found in jejunum, Duodenum, ileum, and liver exhibited less than one-fifth of the jejunal expression level, whereas all other tissues showed minimal expression (Figure S3A). The predominant form of LEAP2 produced in each of these tissues is the mature 40-amino-acid peptide (see Figure S1D).

We then set out to identify the cell types that produce LEAP2. Since LEAP2 is a secreted protein, we focused on the localization of LEAP2 transcript using in situ hybridization (Wang et al., 2012). Consistent with the qPCR results, Leap2 mRNA was enriched in jejunum and expressed at a lower level in liver (Figure S3B). In liver, LEAP2 showed moderate expression in hepatocytes (Figure S3C), whereas within the jejunum, Leap2 was highly and specifically expressed in enterocytes along the luminal surface of the villi, but not in lamina propria or crypts (Figure S3D).

The enriched expression of Leap2 in enterocytes indicates its potential for regulation in response to nutrient status. To measure endogenous LEAP2 levels, we developed a “sandwich ELISA” (Figures S3E–S3G). We measured the baseline level of LEAP2 in the serum and its response to fasting and refeeding. After a 24 hr fast, LEAP2 levels decreased by 67% (from 11.6 ng/mL to 3.8 ng/mL). One hour after refeeding, LEAP2 levels were partially restored to 6.6 ng/mL (Figure S3H). Serum ghrelin levels exhibited an inverse regulatory pattern, increasing from 1.1 ng/mL to 2.6 ng/mL after fasting and decreasing to pre-fast levels after refeeding (Figure S3H). This opposing regulation of LEAP2 and ghrelin in response to food intake aligns with the action of LEAP2 in opposing ghrelin signaling through GHSR. Interestingly, a similar pattern of counter-regulation between LEAP2 and ghrelin was observed in their expression in the stomach following VSG surgery (Figure S1A).

LEAP2 Inhibits Ghrelin Action In Vivo

To investigate whether LEAP2 antagonizes ghrelin in vivo, we focused on two well-established actions of ghrelin in the mouse, GH release and food intake (Kojima et al., 1999; McFarlane et al., 2014; Sun et al., 2004; Thomas et al., 2016). Activation of GHSR by ghrelin in pituitary cells leads to robust GH release (Kojima et al., 1999). As previously described, a low dose of ghrelin (6 nmol/kg BW) administered intravenously (i.v.) induced a pulse of GH release (Figure 2B). To determine the impact of LEAP2 on ghrelin action, we administered LEAP2 intraperitoneally (i.p.) 10 min before ghrelin (Figure 2A). LEAP2 attenuated ghrelin-induced GH release in a dose-dependent manner (Figure 2B). Starting from 7.2 nmol/kg BW, LEAP2 significantly reduced the peak of GH excursion (Figure 2C) and the total amount of GH released as determined by the area under curve (AUC) (Figure 2D). Notably, this dose of LEAP2 is similar to the dose of ghrelin used in the experiment (6 nmol/kg BW), consistent with the in vitro pharmacology demonstrating that the IC50 of LEAP2 at GHSR is similar to the EC50 of ghrelin (see Figure 1).

We speculate that the binding of LEAP2 to GHSR on pituitary cells blocks ghrelin-induced GH release from these cells. Activation of GHSR in hypothalamic neurons stimulates an orexigenic neural pathway, resulting in increased food intake (Nakazato et al., 2001). This appetite-stimulatory effect is induced by supra-physiological levels of ghrelin (Sun et al., 2004; McFarlane et al., 2014; Lippl et al., 2012). Consistent
with previous reports, we found that subcutaneous administration of ghrelin at 0.15 ρmol/kg BW promoted food intake in mice (Figure 2E). When mice were pretreated with a high dose of LEAP2 (3 ρmol/kg BW; Figure 2F), ghrelin-induced food intake was completely abolished, reverting to the level observed in vehicle-treated mice (red line in Figure 2G). Remarkably, mice treated with this same dose of LEAP2 alone consumed significantly less food than vehicle-treated mice (orange line in
Figure 2G), indicating antagonism of endogenous ghrelin by LEAP2. Low-dose LEAP2 (0.15 μmol/kg BW) had no effect on ghrelin-induced food intake (compare green and light blue lines in Figure 2G), nor did it impact food intake when administered alone (dark blue line in Figure 2G). Taken together, these results indicate that LEAP2 blocks ghrelin-induced food intake in mice.

**Blocking Endogenous LEAP2 Enhances Fasting-Induced GH Release**

To evaluate loss of endogenous LEAP2 function in adult mice, we developed LEAP2-neutralizing monoclonal antibodies. Individual antibodies were mixed with LEAP2 and assayed in an antagonist mode using the GHSR-stable cell line (Figure 3A). While a control antibody had no impact on LEAP2-mediated inhibition of GHSR, LEAP2 antibodies reversed this inhibition (Figure 3B). We chose the two most potent anti-LEAP2 antibodies, M2 and M18, for further study.

We then investigated the effect of blocking endogenous LEAP2 on ghrelin’s action in vivo. To study this biology under the most physiological conditions possible, we challenged mice through fasting, which stimulates ghrelin secretion in rodents (Tschöp et al., 2000; Figure S3H). We measured GH release as a readout, as it is the physiological response that is most sensitive to changes in ghrelin levels. Following a 24 hr fast, LEAP2 antibodies were administered by i.p. injection. GH levels were then determined at the time points indicated (Figure 3C). Remarkably, M2 and M18 increased the peak of GH secretion (Figure 3D) and the total amount of GH released (as determined by the AUC; Figure 3E). These data indicate that blocking LEAP2 function exaggerates fasting-induced ghrelin responses that lead to increased GH release. In conclusion, blocking endogenous LEAP2 promotes ghrelin-mediated GHSR activation in vivo.

**LEAP2 Over-Expression Antagonizes the Function of the Ghrelin-GH Axis in Maintaining Viability during Chronic Caloric Restriction**

Recent studies in ghrelin-deficient mice revealed that a key physiological role of ghrelin is to maintain viable blood glucose levels during chronic CR (Li et al., 2012; Zhao et al., 2010). To evaluate the impact of LEAP2-GHSR interaction during such a
physiological challenge, we set up a mouse model of chronic CR (Figure 4A). Individually housed mice were given 40% of their normal daily food intake for 15 days (CR group). Control groups had *ad libitum* (AL) access to food. BW and blood glucose were measured daily.

LEAP2 is rapidly cleared from the circulation (Figure S3G), which is generally the case for unmodified peptides (Fosgerau and Hoffmann, 2015). Thus, we used an adeno-associated virus (AAV) “minigene” system to drive lasting systemic expression of LEAP2 or a control (secreted GFP) (Galon-Tilleman et al., 2017) in

Figure 4. LEAP2 Exacerbates Viability during Chronic Caloric Restriction

(A) Experimental design of chronic CR assay.
(B) Serum LEAP2 levels following introduction of AAV-LEAP2 into mice by tail-vein injection.
(C) Body weight (BW) in mice after dietary intervention.
(D) Blood glucose levels in mice after dietary intervention.
(E) Circulating levels of ghrelin and GH at the end of the chronic CR experiment. *p < 0.05 versus GFP-CR. Student’s t test. n = 6 mice/group.
(F) Experimental design for GH infusion during chronic CR.
(G) Blood glucose levels in CR mice with GH or vehicle osmotic pumps. *p < 0.05 versus LEAP2-GH. Student’s t test.
(H) Kaplan-Meier survival curve of mice during chronic CR. *p < 0.05 versus LEAP2-GH. Log-rank (Mantel-Cox) test. n = 7 mice/group. Data are from one experiment that is representative of three separate experiments and are represented as mean ± SEM.

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the CR and AL mice, AAV-LEAP2 led to a 3-fold increase in circulating LEAP2 levels 2 weeks after injection (Figure 4B).

After 1 week of CR, both GFP-CR (GFP-expressing mice undergoing CR) and LEAP2-CR (LEAP2-expressing mice undergoing CR) mice lost about 28% of their BW (Figure 4C). In both CR groups, blood glucose declined within the first week. After day 8, blood glucose in GFP-CR mice remained relatively constant at approximately 50 mg/dL. In contrast, blood glucose in LEAP2-CR mice never stabilized, but continued to decline until it dropped below 30 mg/dL (Figure 4D). While GFP-CR mice appeared active and healthy throughout the course of the experiment, LEAP2-CR mice were too moribund and lethargic to consume their food on day 8. At this point, the LEAP2-CR mice had to be euthanized in accordance with institutional animal care and use guidelines.

Ghrelin levels increased in both GFP-CR and LEAP2-CR mice as compared to AL mice (Figure 4E). Surprisingly, the absolute level of ghrelin was much lower in LEAP2-CR mice as compared to GFP-CR mice (Figure 4E). CR also increased serum GH levels, but the GH secretion was significantly lower in LEAP2-CR mice than in GFP-CR mice (Figure 4F). CR depleted body fat in all groups, as indicated by magnetic resonance imaging (Figure S4A). Consequently, the levels of free fatty acids and ketone bodies dramatically decreased both in the GFP-CR and LEAP2-CR mice (Figure S4B).

Previous studies have indicated that after body-fat depletion due to chronic CR, the main source of blood glucose is gluconeogenesis, a process dependent on GH (Goldstein et al., 2010). In our study, the observed hypoglycemia and compromised survival in LEAP2-CR mice may be due to the inhibition of ghrelin, which in turn prevents maximal release of GH. To test this, we delivered GH by osmotic mini-pumps to CR mice (Figure 4G). We confirmed GH delivery in a separate group of mice kept in AL conditions over 2 weeks (Figure S4D). Osmotic pumps were implanted 3 days before initiation of CR. In all groups, glucose levels decreased as before during CR. In LEAP2-CR mice implanted with GH pumps, 60% were able to maintain stable levels of blood glucose and survived to the end of the experiment. In contrast, LEAP2-CR mice implanted with vehicle pumps failed to maintain viable glucose levels, and the entire group had to be euthanized on day 12 (Figures 4H and 4I). These data indicate that delivery of GH bypasses LEAP2-mediated antagonism of ghrelin at GHSR, thus rescuing survival of LEAP2-expressing mice in the face of CR.

**DISCUSSION**

Since the discovery of ghrelin, regulated secretion has been the sole mechanism described for controlling the action of this hormone. Our current studies reveal a hitherto-unknown mechanism of ghrelin regulation. We have discovered that LEAP2 is an intestinally derived regulatory hormone that acts as an endogenous antagonist of ghrelin action. LEAP2 inhibits ghrelin binding to GHSR in a noncompetitive manner (see Figure 1) and blocks ghrelin-mediated GH release and food intake (see Figure 2). Neutralizing LEAP2 with function-blocking antibodies enhanced ghrelin action in vivo (see Figure 3). Further, LEAP2 antagonizes the function of the ghrelin-GH axis in maintaining viability during chronic CR (see Figure 4). This discovery reveals a novel regulatory mechanism modulating ghrelin action; additionally, this study identifies LEAP2 as a potential therapeutic target for ghrelin-related diseases, including obesity and diabetes, anorexia, cachexia, alcohol abuse, and Prader-Willi Syndrome (Cummings et al., 2002b; Leggio et al., 2014; Müller et al., 2015).

Though regulation of ghrelin interaction with GHSR has not been described previously, a precedent for a similar regulatory mechanism exists in the form of the interaction between Agouti-Related Protein (AgRP) and melanocortin receptor subtypes MC3R and MC4R (Ollmann, 1997). AgRP is produced by hypothalamic neurons and acts as an inverse agonist of MC3R and MC4R. AgRP stimulates food intake by antagonizing the satiety-promoting activity of these receptors (Barsh and Schwartz, 2002). Such endogenous counter-regulatory mechanisms may be more widespread than previously appreciated, as exemplified by the role we describe here for LEAP2.

Previous studies indicated that at high concentrations (>6.6 μM), LEAP2 exhibits antimicrobial activity in vitro (Howard et al., 2010; Krause et al., 2003). The effective antimicrobial concentration reported in these studies is much higher than the physiological levels of LEAP2 (~2 nM) (Figure S3H). In our current study, we found that physiological levels of LEAP2 antagonize GHSR (IC50 = 6.0 nM; Figure 1G). Relatively small fluctuations in LEAP2 levels have a dramatic impact on ghrelin biology, as a 3-fold increase of LEAP2 concentration significantly exacerbated survival during chronic CR (Figure 4). Thus, our current discovery identifies a role for LEAP2 that occurs at physiological levels of the hormone.

Another notable finding is that LEAP2 may suppress ghrelin action through additional mechanisms beyond inhibiting ghrelin binding with GHSR. An inverse relationship is found between circulating levels of LEAP2 and ghrelin in response to changes in nutritional status. After a fast, serum LEAP2 decreased while serum ghrelin increased, whereas upon refeeding, serum LEAP2 increased while serum ghrelin decreased (Figure S3H). After chronic CR, ghrelin levels increased less in LEAP2-expressing mice as compared to the control group (see Figures 4E and S4G). We speculate that LEAP2 may therefore also act to inhibit the production or secretion of ghrelin. This dual mechanism of inhibition (antagonizing the ghrelin receptor and inhibiting ghrelin production) makes LEAP2 a particularly strong regulator of ghrelin actions in vivo and may explain how small fluctuations in LEAP2 can exert a powerful effect on ghrelin biology. Determining whether GHSR-independent actions of LEAP2 contribute to these dual regulatory mechanisms will be a fruitful area for future study. For example, evaluating the action of LEAP2 in GHSR-knockout mice could help to determine whether LEAP2’s regulation of ghrelin production occurs through GHSR antagonism or a distinct mechanism.

Our discovery adds LEAP2 to the list of hormones that connect the gut, brain, and metabolic control. Leap2 expression in the stomach is nearly undetectable under normal physiological conditions but is dramatically increased following VSG surgery (see Figures 1B and 1C). This increase of LEAP2 may contribute to appetite suppression following bariatric surgery. Elucidation of the neuroendocrine changes that occur following the profound
metabolic remodeling of bariatric surgeries is revealing molecular mechanisms that link the gastrointestinal tract with metabolic control (Seeley et al., 2015). Harnessing these mechanisms may provide new and less invasive therapeutic strategies for treating neuroendocrinological and metabolic diseases.

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures and can be found with this article online at https://doi.org/10.1016/j.cmet.2017.10.016.

**AUTHOR CONTRIBUTIONS**

X.G. designed and performed experiments, analyzed data, and wrote the manuscript. H.Y., H.G.-T., P.C., M.C., J.S.L., Y.Y., and O.D. performed experiments, analyzed data, and reviewed the manuscript. D.D.K. designed experiments, performed data analysis and interpretation, and wrote the manuscript. M.A.B., J.G., C.M.R., and A.K. contributed with discussion and data interpretation and reviewed the manuscript.

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**REFERENCES**


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KEY RESOURCES TABLE

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**Deposited Data**

- VSG versus sham surgery RNA-seq data: This study, GEO: GSE104956

**Experimental Models: Cell Lines**

- PathHunter U2OS GHSR β-Arrestin cell line (female, osteosarcoma): DiscoverX, Cat# 93-0242C3
- COS7 cell line (male, fibroblast): ATCC, Cat# CRL-1651
- LEAP2 antibody-producing cells (female, hybridoma): This study, N/A
- GPCR stable cell line screening (female, Chinese hamster ovary): DiscoverX, gpcrMAX, orphanMAX
- AAV-293 cell line (female, embryonic kidney): Agilent Technologies, Cat# 240073

**Experimental Models: Organisms/Strains**

- C57BL/6, male: Jackson Laboratory, Cat# 000664
- NZB/NZW, female: Jackson Laboratory, Cat# 100008
- BALB/c, female: Jackson Laboratory, Cat# 000651

**Oligonucleotides**

- RNAScope in situ hybridization probe for LEAP2: This study, N/A

**Recombinant DNA**

- Human GHSR: This study, N/A
- LEAP2 for AAV packaging: This study, N/A
- Secreted EGFP for AAV packaging: This study, N/A

**Software and Algorithms**

- Prism: GraphPad software, N/A
- Xcalibur, version 2.2: Thermo Fisher, N/A
- GSNAP/CUFFLINKS: Wu and Nacu, 2010; Trapnell et al., 2012
- CUFFCOMPARE: Trapnell et al., 2012
CONTACT FOR REAGENT AND RESOURCE SHARING

More information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Daniel D. Kaplan (dkaplan@ngmbio.com).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse Model
C57BL/6 male mice were obtained at 2-3 months of age from The Jackson Laboratory (Bar Harbor, ME). Mice were kept in accordance with welfare guidelines under controlled light (12 hr light and 12 hr dark cycle, dark 6:30 pm-6:30 am), temperature (22 ± 4 °C) and humidity (50% ± 20%) conditions. They had free access to water (autoclaved distilled water) and were fed ad libitum on a commercial diet (Harlan Laboratories, Irradiated Teklad Global 18% Protein Rodent Diet) containing 18 kcal% fat, 24 kcal% protein (18% protein by weight) and 58 kcal% carbohydrate. Alternatively, mice were maintained on a high-fat diet (D12492, Research Diets, New Brunswick, NJ, USA) containing 60 kcal% fat, 20 kcal% protein and 20 kcal% carbohydrate. All animal studies were approved by the NGM Institutional Animal Care and Use Committee under the protocol NGM-5-2008 entitled “Characterization of Biologics, Compounds and Viral Vectors for Treatment of Diabetes Using Rodent Models.”

Cell Lines
COS7 cells were obtained from ATCC, and were cultured in DMEM supplemented with 10% FBS and 1000 U/ml penicillin/streptomycin. GHSR-β-arrestin engineered stable U2OS cell line was purchased from DiscoverX. AAV-293 cells were purchased from Agilent technologies. All cells were maintained at 37 °C and 5% CO2.

METHOD DETAILS

Vertical Sleeve Gastrectomy
Seventeen week old male C57BL/6J mice were maintained on high-fat diet for 13 weeks and then fasted overnight prior to the surgery. VSG and sham surgeries were performed under isoflurane anesthesia as described previously (Wilson-Pérez et al., 2013). Briefly, using sterile 7 French polyethylene tubing to size the gastric pouch remnant from the esophagus to the pylorus, the lateral 80% of the stomach was excised. Then, using a 6-0 PDS suture, the stomach was closed at the edge of the sizing using a single interrupted suture through both layers of stomach wall. The remaining stomach pouch has a lumen diameter sized to 6 French and approximating 20% of the original stomach area. The sham procedure involved the opening of the peritoneal cavity and applying a gentle pressure using a pair of hemostats at a location that is approximately 2/3 cranial to the greater curvature, along a vertical line between the esophageal sphincter and the pylorus. Mice consumed Osmolite 1 Cal liquid diet (Abbott Laboratories, Abbott Park, IL) for the first 3 postoperative days and were gradually reintroduced to high-fat diet on day 3. The surgery survival rate was 100%.

RNA-seq of Tissues after Vertical Sleeve Gastrectomy
Four weeks after surgery, tissues were harvested from three VSG mice and three sham surgery mice. Total RNA was prepared by homogenizing tissues in TRIzol Reagent (Thermo-Fisher, Waltham, MA) according to the manufacturer’s protocol. RNA quality was assessed using an Agilent Bioanalyzer (Agilent, Santa Clara, CA). RNA from the three animals in each group were pooled for RNA sequencing, but individual RNA from each animal was retained for qPCR follow-up. Library construction was completed using the TruSeq RNA Preparation Kit (Illumina, San Diego, CA). RNA was sequenced using 50 bp paired-end reads on a HiSeq2000 platform (Illumina). Raw sequence data were aligned against the mouse reference genome using GSNAPE/CUFFLINKS. The assembled dataset was annotated using CUFFCOMPARE. Represented in the Figure 1B heatmap are genes encoding secreted proteins that exhibit expression levels > 5 RPKM, expression changes > 2-fold in stomach, and that are regulated in opposite directions in stomach and duodenum. The gene expression dataset has been deposited at GEO with accession number GSE104956.

Quantitative Real-time PCR
Total RNA was isolated from indicated tissues as described above. RNA concentrations were normalized and qPCR was run using a QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher). The relative amount of mRNA was calculated by the comparative threshold cycle (ΔCt) method. GAPDH mRNA was used as the internal control.

IP-MS of LEAP2 from Tissues
Stomach, small intestine (duodenum, jejunum, and ileum), and liver tissues were harvested from C57BL/6 mice, then flash-frozen in liquid nitrogen, and ground in liquid nitrogen into a powder using a mortar-and-pestle. 1 mL water with protease inhibitors (Roche) was added to 20 mg of tissue and lysed using a dounce homogenizer. 50 μL Protein G Dynabeads (Invitrogen) were washed once with PBS and coated with 200 μL of 25 μg/ml anti-LEAP2 antibody (Santa Cruz Biotech, SC-164845) for 10 min at room temperature (RT). Beads were washed once with PBS, then incubated with the tissue lysate for 1 hr at RT. The beads were subsequently washed 3 times in PBS and eluted in 20 μL 0.5M NaCl. Samples were diluted with 20 μL of 0.1% formic acid and then filtered through 0.2 μm cellulose acetate spin filters (Costar, 8160). 2 μL of sample was loaded onto a 50 μm x 150 mm EASY-Spray PepMap C18 column (Thermo
suspension was frozen in an alcohol dry ice bath and was then thawed in a 37°C water bath. The freeze and thaw cycles were repeated for a total of three times; benzonase (Sigma-Aldrich, St. Louis, MO) was added to 50 units/ml; deoxycholate was added to the Wako Total Ketone Bodies assay kit (Wako Diagnostics). Total ketone bodies were measured using the Wako NEFA assay kit (HR Series NEFA-HR(2), Wako Diagnostics, Mountain View, CA). Free fatty acids in serum was measured using the following assays: total ghrelin (EZRGRA-90K, Millipore, Hayward, CA), growth hormone (EZRMGH-45K, Millipore). Blood was sampled in mice by nicking the tail vein and blood glucose levels were measured using ACCU-CHEK Active test strips read by an ACCU-CHEK Active meter (Roche Diagnostics, Indianapolis, IN) following the manufacturer’s instructions. All readings were performed in duplicate and averaged.

**Hormone Measurement**

All hormone assays were performed in mature 3–4 month old male mice. When indicated, fasting was performed for 24 hr, from 4 pm to 4 pm. For measurement of LEAP2 levels after AAV injection and growth hormone levels after osmotic pump implantation, blood samples were obtained through the tail vein. For ghrelin-induced GH release, blood samples were collected through retro-orbital bleeding. Sera were prepared and levels of hormones in sera were determined using the following assays: total ghrelin (EZRGRA-90K, Millipore, Hayward, CA), growth hormone (EZRMGH-45K, Millipore). Free fatty acids in serum was measured using the Wako NEFA assay kit (HR Series NEFA-HR(2), Wako Diagnostics, Mountain View, CA). Total ketone bodies were measured using the Wako Total Ketone Bodies assay kit (Wako Diagnostics).

**Detection of Endogenous LEAP2 by ELISA**

96-well plates (Maxisorp, Thermo Fisher, 442404) were coated with goat-anti-LEAP2 (sc164847, Santa Cruz Biotechnology, Santa Cruz, CA) for 72 hr at 4°C. Plates were then washed 3 times with PBST (0.05% TWEEN 20 in PBS), and blocked in 5% BSA in PBST. 10 μL of LEAP2 standard (synthetic LEAP2 peptide) or serum was mixed with 90 μL assay buffer (2% BSA in PBS), and added to appropriate wells. Samples were incubated at RT for 15 min on an orbital shaker, then for 2 hr at 37°C. Plates were washed 3 times in PBST, capture antibody (rabbit anti-LEAP2, ab122294, AbCam, Cambridge, UK) was added and incubated at RT for 1 hr. After 3 washes, secondary antibody (HRP-goat-anti-rabbit) was added to the wells and incubated for 1 hr at RT. Excess secondary antibody was washed away, followed by addition of KPL peroxidase substrate solution, which was incubated until the development of blue color was apparent. Stop solution (2N H2SO4) was added to the wells, and the plates were read using an enSpire2000 multimode plate reader (PerkinElmer). Standard curves were fitted using a 2nd order polynomial and LEAP2 levels were interpolated from the standard curve.

**LEAP2 Antibody Generation**

Anti-LEAP2 antibodies were generated using mouse hybridoma technology (Antibody Solutions, Sunnyvale, CA). Briefly, synthetic LEAP2 peptide was conjugated to bovine serum albumin carrier protein and used to immunize NZB/NZW and BALB/c mice. Following measurement of positive antibody titers, spleens were harvested from immunized mice and spleen cells were fused with myeloma cells to generate hybridomas. Individual hybridoma clones were isolated and hybridoma supernatants were screened for binding to LEAP2 peptide by ELISA. Positive hybridoma clones were scaled up and antibodies were purified from hybridoma culture supernatant using two dimensional chromatography implemented on an AKTA Pure (GE Healthcare Life Sciences, Pittsburgh, PA).
PA) chromatography system. Briefly, the exhausted hybridoma media was clarified by centrifugation (6000 g, 15 min), filtered through a 0.22 μm filter, and pH adjusted by addition of 1/20 volume of a neutralizing stock solution (500 mM Tris (pH 8.0), 400 mM NaCl, 20 mM EDTA). The antibodies were captured on a 5 mL MabSelect SuRe column (GE Healthcare Life Sciences), washed in PBS (20 column volumes) and back eluted with 100 mM acetic acid (pH 3.5), 100 mM NaCl, onto a 10 mL sample collection loop. Immediately after elution, the eluted protein was reinfected onto a Superdex 200 pg 26/600 size-exclusion column (GE Healthcare Life Sciences) equilibrated in PBS buffer. For each antibody, the fractions corresponding to the monomeric peak were pooled together. Protein purity was assessed by SDS-PAGE. The hydrodynamic properties of the purified antibodies were analyzed by HPLC (Agilent 1200) equipped with a Yarra 3000 column. Samples were compared to molecular weight standards (Bio-Rad, Hercules, CA).

**GPCR Screening**

Synthetic LEAP2 peptide was screened against a panel of 168 engineered stable cell lines, each expressing a single human GPCR, in both agonist and antagonist formats. Each GPCR-expressing cell line was engineered using the PathHunter beta-arrestin expression fragment complementation technology (DiscoverX, Fremont, CA), which monitors GPCR activation through measurement of reconstitution of a beta-galactosidase enzyme pair split into complementary enzyme acceptor and enzyme donor fragments (see Figure S2A). The enzyme acceptor is fused to beta-arrestin and the enzyme donor to the GPCR. GPCR activation leads to beta-arrestin recruitment to the active receptor, resulting in beta-galactosidase complementation, which is measured using chemiluminescent detection reagents. Cells were seeded in white walled, 384-well microplates and incubated at 37°C. For evaluation in agonist format, cells were incubated with 3 μM synthetic LEAP2 peptide or DMSO vehicle control for 90 or 180 min and responses were measured through chemiluminescent signal detection using the PathHunter Detection reagent cocktail (DiscoverX), which was read using an Envision instrument (PerkinElmer, Waltham, MA). For evaluation in antagonist format, cells were pre-incubated with 3 μM synthetic LEAP2 peptide or DMSO vehicle for 30 min, followed by addition of known agonist for the given receptor at the EC_{90} concentration and incubation for an additional 90 or 180 min and signal detection as described above.

**GHSR Activity Assay**

GHSR activity assays were performed using the PathHunter U2OS GHSR β-Arrestin Cell Line (DiscoverX, 93-0242C3) according to the manufacturer’s instructions. Briefly, cells (5000 cells per well) were plated in 384-well plates. 24 hr later, cells were used for GHSR assays in agonist or antagonist modes. In agonist mode, cells were incubated with a dilution series of human ghrelin (AbCam 1463) for 90 min. At the end of the incubation, PathHunter detection reagent cocktail was added to the cells, followed by a 1 hr incubation at RT. Chemiluminescence signals were read using an enSpire2000 multimode plate reader (PerkinElmer). For evaluation in antagonist mode, cells were first incubated with a dilution series of LEAP2 for 30 min, followed by incubation with ghrelin at 13 nM (EC_{80}) for 90 min, then processed for chemiluminescence detection. For LEAP2 antibody screening, LEAP2 (0.3 μM) was first mixed with a dilution series of anti-LEAP2 antibody. The mixture was added to the cells, and the GHSR assay was performed in antagonist mode, as described above.

**GHSR Activity Data Analysis**

For agonist mode assays, percentage activity was calculated using the following formula:

\[
\% \text{Activity} = 100 \% \times \left( \frac{\text{mean RLU of test sample} - \text{mean RLU of vehicle control}}{\text{mean MAX control ligand} - \text{mean RLU of vehicle control}} \right)
\]

For antagonist mode assays, percentage inhibition was calculated using the following formula:

\[
\% \text{Inhibition} = 100 \% \times \left( 1 - \frac{\text{mean RLU of test sample} - \text{mean RLU of vehicle control}}{\text{mean RLU of EC}_{90} \text{ control} - \text{mean RLU of vehicle control}} \right)
\]

**LEAP2 Binding to GHSR and GHSR Immunofluorescence in COS7 cells**

LEAP2 peptide was conjugated to Alexa647 (Molecular Probes A30009) following the procedure provided by the vendor. Labeled LEAP2 peptide was separated from the dye with the Biorad P2 gel (cat #1504118). COS7 cells were transfected with human GHSR using Lipofectamine 2000. Twenty-four hours after transfection, cells were washed in DMEM and incubated with Alexa647-labeled LEAP2 (3 μg/ml) for 30 min on ice. Sodium azide and Pitstop 2 (ab120687) were added in the incubation to block receptor internalization. Cells were then washed with PBS 3 times, and fixed with 4% paraformaldehyde for 10 min at RT. After paraformaldehyde was washed away, cells were blocked under non-permeabilized conditions (0.2% donkey serum in PBS) and incubated in primary antibody (rabbit-anti-GHSR, AbCam, ab85104, 1:200) diluted in blocking solution for 2 hr at RT. Cells were then washed three times with PBS and incubated with secondary antibody against rabbit IgG conjugated to Alexa488 (1:500, Jackson ImmunoResearch). Cells were counterstained with Hoechst 33342 (Sigma), and mounted with AntiFade reagent (Invitrogen) for confocal fluorescence microscopy. Images were taken using a Leica SP8 confocal microscope. Z sections were acquired and super-imposed into one image.
Ghrelin-induced Growth Hormone Release

To measure ghrelin-induced growth hormone release, 3-4 month old male mice were anesthetized with isoflurane, and blood samples were obtained through retro-orbital bleeding. Following a baseline blood collection (time 0), mice were injected with rat ghrelin (1465, R&D Systems, Minneapolis, MN) or vehicle (PBS) through tail vein, and blood samples were obtained at 5, 10, 15, 30, and 60 min. Mice were euthanized at the end of the experiment.

To test the effect of LEAP2 on growth hormone release, LEAP2 or vehicle (10% DMSO in PBS) were injected intraperitoneally immediately after the blood samples were taken at time 0. Ten minutes after LEAP2 injection, ghrelin (6 nmol/kg body weight) was administered through the tail vein, and blood samples were obtained at 5, 10, 15, 30, and 60 min. Serum was prepared and used for measurement of growth hormone. The effect of the anti-LEAP2 antibody on GH release was evaluated as follows: mice were fasted for 20 hr and then anesthetized with isoflurane, and baseline (time 0) blood samples were obtained through retro-orbital bleeding. Mice were then injected i.p. with anti-LEAP2 antibody at 10 mg/kg body weight. Blood samples were collected at 5, 10, 30, 60, 90, and 120 min. Serum was prepared and used for measurement of growth hormone by ELISA.

Acute Effects of Ghrelin on Food Intake

Mice were anesthetized with isoflurane, then injected subcutaneously with ghrelin (0.03, or 0.15 μmol/kg body weight) or vehicle (PBS). Mice were housed in individual cages and food intake was measured at 0.5, 1, and 2 hr. To test the effect of LEAP2 on food intake, anesthetized mice were first injected with LEAP2 subcutaneously. 20 min later, ghrelin (0.15 μmol/kg body weight) was injected subcutaneously and food intake was measured at 0.5, 1, and 2 hr.

Chronic Caloric Restriction and Growth Hormone Infusion

Two weeks before initiation of caloric restriction, 2-3 month old male mice were administered AAV virus expressing LEAP2 or secreted GFP through tail vein injection. Mice were kept in individual cages and fed chow diet ad libitum. Food intake was monitored daily to determine baseline food intake. Thereafter, the mice were separated into four groups. The GFP-AL and LEAP2-AL groups continued to receive the chow diet ad libitum. Mice in the GFP-CR and LEAP2-CR groups were fed 40% of the daily food intake consumed by the same mouse during the baseline period. Body weight and blood glucose were measured daily, immediately before food was reintroduced. Body composition was measured by NMR at the end of the experiment, or just before the mice were euthanized. Terminal bleeds were collected for analysis of hormone levels. For GH infusion studies, Alzet osmotic mini-pumps (Cat #1002, DURECT Corp., Cupertino, CA) filled with recombinant rat GH (GroPep Bioreagents, Thebarton, Australia) or vehicle were implanted subcutaneously in the interscapular region 3 days before the initiation of caloric restriction. GH concentration in the pumps was 2 mg/ml, and the GH was delivered at a rate of 0.25 μl/hr (0.5 μg/hr).

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analysis was performed using Graphpad Prism 7.0 Software (Graphpad Software; La Jolla, CA, USA). Statistical significance was determined by unpaired Student’s t test or ANOVA as indicated in the figure legends.
Supplemental Information

LEAP2 Is an Endogenous Antagonist of the Ghrelin Receptor

Xuecai Ge, Hong Yang, Maria A. Bednarek, Hadas Galon-Tilleman, Peirong Chen, Michael Chen, Joshua S. Lichtman, Yan Wang, Olivier Dalmas, Yiyuan Yin, Hui Tian, Lutz Jermutus, Joseph Grimsby, Cristina M. Rondinone, Anish Konkar, and Daniel D. Kaplan
**Ge et al. Supplementary Figure 1. LEAP2 is a secreted peptide with two conserved disulfide bonds**
Supplementary Figure 1, related to Figure 1. LEAP2 is a secreted peptide with two conserved disulfide bonds

(A) Expression level of Ghrl and Leap2 detected by RNA-seq or qPCR before and after vertical sleeve gastrectomy (VSG) surgery. **, p<0.01. ***, p<0.001 vs. sham. Data are from one experiment that is representative of three separate experiments. Data are represented as mean ± SEM. (B) Amino acid sequence of LEAP2 - disulfide bonds are indicated. (C) Multiple species alignment of LEAP2 showing conserved cysteine residues (highlighted). (D) To examine endogenous forms of LEAP2, LEAP2 was immunoprecipitated from stomach, intestine, and liver extracts using an antibody recognizing the C-terminus of the peptide, followed by a search for LEAP2 isoforms using LC-MS. The search included all likely charge states of the 76 aa prepro-peptide precursor, the 54 aa pro-peptide, the mature 40 aa peptide, and smaller potential LEAP2 forms that could be generated by cleavage following basic residues and that would leave the disulfide-bonded core of the peptide intact. The peptide forms that were examined are as follows:

```
MLQLKLFAVL LTCLLLLGQV NSSPVEVSS AKRSRRMTPF WRGVSLRPIG ASCRDDDSECI TRLCRKRRCS LSVAQE
SPVEVSS AKRSRRMTPF WRGVSLRPIG ASCRDDDSECI TRLCRKRRCS LSVAQE
MTPF WRGVSLRPIG ASCRDDDSECI TRLCRKRRCS LSVAQE
GVSLRPIG ASCRDDDSECI TRLCRKRRCS LSVAQE
PIG ASCRDDDSECI TRLCRKRRCS LSVAQE
```

From all three tissues, the mature, 40 aa form of LEAP2 was detected, but none of the other forms were found. The extracted ion chromatogram for the z=6 charge state representing mature LEAP2 peptide from the indicated samples is shown in the top panel. A representative isotopic distribution of the predominant (+6 charge state) peak representing the mature form of LEAP2 (4581.29 Da) is shown in the bottom panel.
**Ge et al. Supplementary Figure 2. LEAP2 Antagonizes Ghrelin-induced Activation of Growth Hormone Secretagogue Receptor (GHSR)**

**A** GHSR tagged with enzyme donor

Activating ligand binds to GHSR

β-arrestin & Enzyme Acceptor

Substrate

Luminescence

**B** Activating ligand binds to GHSR

Gq

Calcium dye

Dye chelated to calcium

**C**

**Agonist mode**

agonist

90 min

Read GPCR activity

**Antagonist mode**

LEAP2

agonist

90 min

Read GPCR activity

**D** Ghrelin-induced GHSR activation in calcium mobilization assay

**E** LEAP2 inhibits ghrelin-induced GHSR activity in calcium mobilization assay

**F** LEAP2 (nM)

0

1.23

3.70

11.1

33.3

100

**G**

[D-Arg¹⁸, D-Phe⁶, D-Trp⁷,⁹, Leu¹¹]-Substance P (nM)

0

1.23

3.70

11.1

33.3

100

**H** LEAP2

GHSR

LEAP2/GHSR/Hoechst

10 µm

---

**Notes:**

- GHSR tagged with enzyme donor.
- Activating ligand binds to GHSR.
- β-arrestin & Enzyme Acceptor.
- Substrate leads to luminescence.
- GHSR binds to Gq, which activates PLC.
- Calcium dye is chelated to calcium.
- LEAP2 competes with calcium mobilization.
- LEAP2 inhibits ghrelin-induced GHSR activity.
- LEAP2/GHSR/Hoechst imaging shows the localization of LEAP2 and GHSR.

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**Ge et al.**
Supplementary Figure 2, related to Figure 2. LEAP2 Antagonizes Ghrelin-induced Activation of Growth Hormone Secretagogue Receptor (GHSR)

(A) Diagram showing the detection of β-arrestin recruitment following GHSR activation in PathHunter cells. GHSR is tagged with a small enzyme donor, and is stably co-expressed with a fusion protein of β-arrestin and truncated β-galactosidase. Activation of GHSR leads to β-arrestin recruitment, which then allows complementation of the two enzyme fragments, generating active β-galactosidase. The active enzyme converts non-luminescent substrate to a chemiluminescent product. (B) Diagram showing the detection of calcium mobilization following GHSR activation. Cells stably-express wild-type GHSR. Activation of GHSR triggers Gαq activation, resulting in phospholipase C (PLC) activation, and ultimately, mobilization of calcium from intracellular stores. The calcium concentration is detected using the calcium-sensitive dye FLUO-3. (C) Experimental procedure for evaluating GHSR activation in agonist and antagonist modes. (D) Concentration-response curve showing ghrelin-induced GHSR activation, detected using calcium mobilization assay (EC50 = 4.25 ± 0.67 nM). (E) Concentration-response curve of LEAP2 on GHSR activation in the presence of ghrelin (at EC80) detected by calcium mobilization (LEAP2 IC50 = 11.6 ± 1.03 nM). (F) Concentration-response curve of ghrelin-induced GHSR activation (using calcium mobilization assay) in the presence of LEAP2, demonstrating that LEAP2 is a non-competitive antagonist of GHSR. (G) Concentration-response curve of ghrelin-induced GHSR activation (using calcium mobilization assay) in the presence of a known competitive antagonist, [D-Arg1, D-Phe6, D-Trp7,9, Leu11]-Substance P. (H) Binding of LEAP2 to GHSR in COS7 cells transiently transfected with GHSR. LEAP2 binds to transfected cells (arrows), but not non-transfected cells (arrow heads). Data are from one experiment that is representative of three separate experiments. Data are represented as mean ± SEM.
**A**

Relative Leap2 mRNA level (normalized to GAPDH) across various tissues:

- cortex
- cerebellum
- brain stem
- hippocampus
- hypothalamus
- esophagus
- stomach
- duodenum
- jejunum
- ileum
- colon
- liver
- ventricle
- atrium
- lung
- spleen
- pancreas
- adrenal
- testis
- kidney
- bone
- muscle
- skin

**B**

Leap2 mRNA expression is greater in jejunum than liver.

**C**

In situ hybridization of Leap2 in liver.

**D**

In situ hybridization of Leap2 in jejunum.

**E**

Development of LEAP2 “Sandwich ELISA”:

- Coating: goat-anti-LEAP2
- Capture: rabbit-anti-LEAP2
- Detection: HRP-goat-anti-rabbit

**F**

LEAP2 ELISA Standard Curve:

- Wide range of LEAP2 concentrations: $R^2 = 0.9951$
- Narrow range of LEAP2 concentrations: $R^2 = 0.9995$

**G**

LEAP2 levels in serum after single i.v. injection (LEAP2: 400 µg/kg BW).

**H**

Fasting reduces serum LEAP2 levels, increases Ghrelin levels.

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Supplementary Figure 3. LEAP2 is produced in enterocytes and hepatocytes; detection of endogenous LEAP2
Supplementary Figure 3, related to Figure 3. LEAP2 is produced in enterocytes and hepatocytes; detection of endogenous LEAP2

(A) Quantitative PCR showing that Leap2 is expressed in the small intestine and the liver, but not other tissues. Leap2 expression level is the highest in jejunum. Not shown: Separately, Leap2 expression was measured in RNA isolated from cerebral cortex, hypothalamus, and pituitary using RNA-seq. Leap2 was undetectable in cerebral cortex and hypothalamus, and was detected at a very low level in the pituitary (0.1 FPKM). (B) In situ hybridization showing expression levels of Leap2 in jejunum and liver. (C-D) Expression pattern of Leap2 in liver and jejunum. In liver, Leap2 is ubiquitously expressed in all hepatocytes. Note that in jejunum, Leap2 is highly expressed in the outer layer of villi (arrows in D), but not in crypts. Leap2 is absent from the lamina propria layer of the villus. (E) Diagram showing the development of LEAP2 “Sandwich ELISA”. (F) Detection of synthetic LEAP2. The wide range LEAP2 concentration curve is fitted with sigmoidal four parameter logistic (4PL) regression. The narrow range LEAP2 concentration curve is fitted with a second order polynomial. (G) LEAP2 ELISA used to detect the levels of LEAP2 in serum after a single dose of peptide was administered to mice intravenously. (H) Effects of fasting and refeeding on serum levels of LEAP2 and ghrelin. LEAP2 and ghrelin levels are shown in ng/ml (left axes) and nM (right axes) **, p<0.01, ***, p<0.001. Student’s t-test. N=5 mice/group. Data in (A) and (F-H) are from one experiment that is representative of three separate experiments. Data are represented as mean ± SEM.
Supplementary Figure 4. Hormone levels and body composition after chronic caloric restriction
Supplementary Figure 4, related to Figure 4. Hormone levels and body composition after chronic caloric restriction

(A) Ratio of fat or lean mass to body weight at the end of the chronic caloric restriction experiment, as measured by MRI. Caloric restriction caused reduction of fat mass, but had minimal effect on lean mass. (B) Serum levels of free fatty acids and total ketone bodies at the end of the chronic calorie restriction experiment. Both free fatty acid and total ketone body levels are dramatically decreased by chronic caloric restriction, indicating depletion of body fat and reduced ketogenesis. *, p<0.05; **, p<0.01; ***, p<0.001. Student's t-test. N=5 mice/group. (C) Serum levels of LEAP2 following chronic caloric restriction. (D) Serum levels of GH after osmotic pump implantation in naïve mice. The osmotic pumps stably delivered GH over the entire experimental period. (E-G) Serum levels of LEAP2, GH, and Ghrelin at the end of the experiment. (H) Ratio of fat or lean mass to body weight at the end of the experiment, as measured by MRI. Caloric restriction caused reduction of fat mass. (I) Levels of free fatty acids and total ketone bodies at the end of the assay, both of which are dramatically decreased by chronic caloric restriction. The normal levels are shown in panel (B). *, p<0.05; **, p<0.01; ***, p<0.001. Student’s t-test. N=7 mice/group. *, p<0.05; **, p<0.01; ***, p<0.001. Student t-test. N=5-7. Figure S4: Data are from one experiment that is representative of three separate experiments. Data are represented as mean ± SEM.