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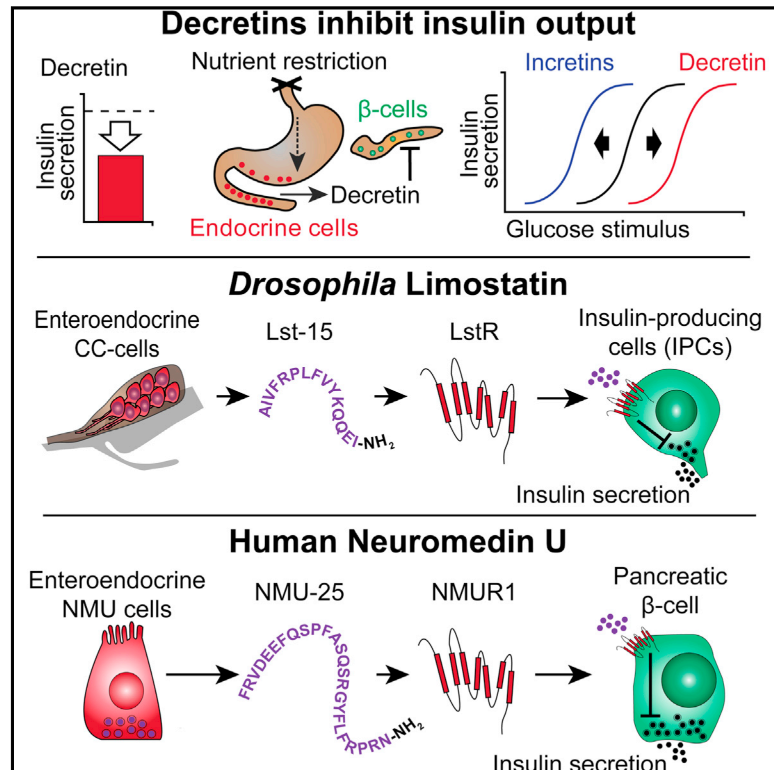
Cell Metabolism

Suppression of Insulin Production and Secretion by a Decretin Hormone

Graphical Abstract



Highlights

- Lst hormone is induced in gut-associated CC cells by carbohydrate restriction
- Lst suppresses insulin output by fly insulin-producing cells (IPCs)
- Lst signals IPCs through a conserved GPCR encoded by CG9918
- NMU inhibits human islet insulin secretion and is a candidate mammalian decretin

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In Brief

Classical studies in mammals suggest that fasting induces circulating hormones that actively suppress insulin production and secretion. Alfa et al. identify a nutrient-responsive “decretin” hormone pathway that suppresses insulin output in Drosophila and describe a cognate enteroendocrine-derived hormone signaling pathway in humans that suppresses insulin secretion by pancreatic β cells.

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Suppression of Insulin Production and Secretion by a Decretin Hormone

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SUMMARY

Decretins, hormones induced by fasting that suppress insulin production and secretion, have been postulated from classical human metabolic studies. From genetic screens, we identified *Drosophila* Limostatin (Lst), a peptide hormone that suppresses insulin secretion. Lst is induced by nutrient restriction in gut-associated endocrine cells. *limostatin* deficiency led to hyperinsulinemia, hypoglycemia, and excess adiposity. A conserved 15-residue polypeptide encoded by *limostatin* suppressed secretion by insulin-producing cells. Targeted knockdown of *CG9918*, a *Drosophila* ortholog of Neuromedin U receptors (NMURs), in insulin-producing cells phenocopied *limostatin* deficiency and attenuated insulin suppression by purified Lst, suggesting *CG9918* encodes an Lst receptor. NMUR1 is expressed in islet β cells, and purified NMU suppresses insulin secretion from human islets. A human mutant NMU variant that co-segregates with familial early-onset obesity and hyperinsulinemia fails to suppress insulin secretion. We propose Lst as an index member of an ancient hormone class called decretins, which suppress insulin output.

INTRODUCTION

The coupling of hormonal responses to nutrient availability is fundamental for metabolic control. In mammals, regulated secretion of insulin from pancreatic β cells is a principal hormonal response orchestrating metabolic homeostasis. Circulating insulin levels constitute a dynamic metabolic switch, signaling the fed state and nutrient storage (anabolic pathways) when elevated, or starvation and nutrient mobilization (catabolic pathways) when decreased (Cahill, 1971; Saltiel and Kahn, 2001). Thus, insulin secretion must be precisely tuned to the nutritional state of the animal. Increased circulating glucose stimulates β cell depolarization and insulin secretion (Rorsman and Braun, 2013). In concert with glucose, gut-derived incretin hormones amplify glucose-stimulated insulin secretion (GSIS) in response to ingested carbohydrates, thereby tuning insulin output to the feeding state of the host (La Barre, 1932; Campbell and Drucker, 2013; Creutzfeldt, 2005).

While the incretin effect on insulin secretion during feeding is well-documented, counter-regulatory mechanisms that suppress insulin secretion during or after starvation are incompletely understood (Campbell and Drucker, 2013; Longo and Mattson, 2014). Classical starvation experiments in humans and other mammals revealed that sustained fasting profoundly alters the dynamics of insulin production and secretion, resulting in impaired glucose tolerance, relative insulin deficits, and "starvation diabetes" (Cahill et al., 1966; Fery et al., 1990; Fink et al., 1974; Hofmeister, 1890; Lilavivathana et al., 1978; Unger et al., 1963). Remarkably, starvation-induced suppression of GSIS was not reverted by normalizing circulating glucose levels, suggesting that the dampening effect of starvation on insulin secretion perdures and is uncoupled from blood glucose and macronutrient concentrations (Lilavivathana et al., 1978). Based on these observations, it has been postulated that hormonal signals induced by fasting may actively attenuate insulin secretion (Lilavivathana et al., 1978; Unger et al., 1963). Ensinck et al. (1997) suggested that enteroendocrine "decretin" hormones may constrain the release of insulin to prevent hypoglycemia. This concept is further supported by recent studies identifying a G protein that suppresses insulin secretion from pancreatic β cells (Wang et al., 2011). Thus, after nutrient restriction, decretin hormones could signal through G protein-coupled receptors (GPCRs) to attenuate GSIS from β cells.

The discovery of hormonal pathways regulating metabolism in mammals presents a formidable challenge. However, progress has revealed conserved mechanisms of metabolic regulation by insulin and glucagon-like peptides in *Drosophila*, providing

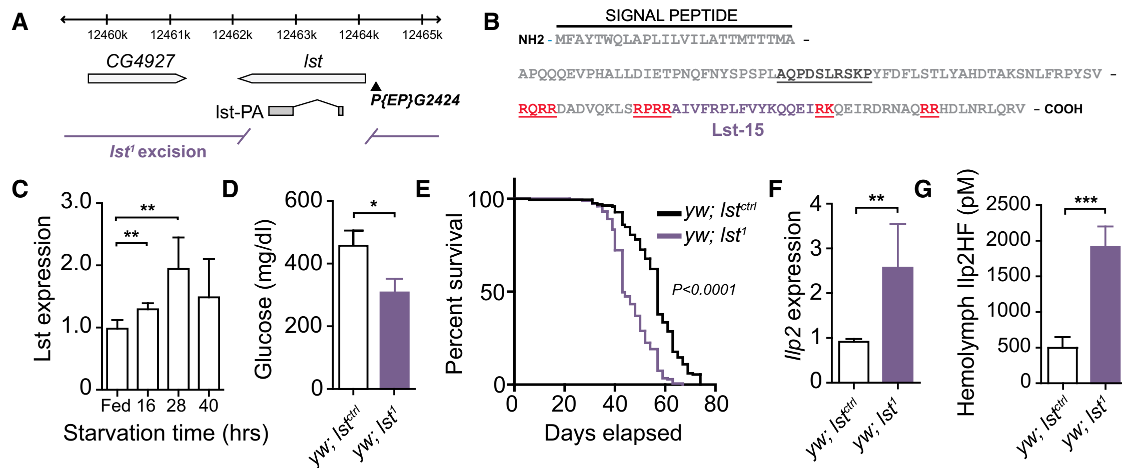

Figure 1. Loss of Lst, a Starvation-Regulated Prepropeptide, Causes Hyperinsulinemia

(A) Genomic organization of *lst* locus (previously *CG8317*) with location of *P{EP}G424* and breakpoints of *lst*^{1} deletion.

(B) Schematic of preprolimostatin with predicted signal peptide and dibasic cleavage sites (red, underline). The highly conserved region used to generate Lst-15 is indicated between cleavage sites #2 and #3 (magenta, bold). Lst antibodies and control peptide were generated using a 9-aa peptide as indicated (underline).

(C) Time-course of *lst* expression during starvation in wild-type adult flies, normalized to fed condition.

(D) Glucose levels in control and *lst* mutant flies.

(E) Lifespan of *yw; lst*^{ctrl} (n = 164) and *yw; lst*^{1} (n = 173) male flies. Median survival times are 57 and 43 days for *yw; lst*^{ctrl} and *yw; lst*^{1} flies, respectively. $p < 0.0001$ (log rank test).

(F) *Ilp2* expression in adult *yw; lst*^{1} flies compared to isogenic controls (*yw; lst*^{ctrl}).

(G) Hemolymph levels of Ilp2HF in *yw; lst*^{ctrl}*; Ilp2HF* and *yw; lst*^{1}*; Ilp2HF* flies (Ilp2HF homozygous). All data displayed as mean + SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ ($n \geq 5$ for all conditions). See also Figure S1.

a powerful genetic model to address unresolved questions relevant to mammalian metabolism (Baker and Thummel, 2007; Erion and Sehgal, 2013; Leopold and Perrimon, 2007). Similar to mammals, secretion of *Drosophila* insulin-like peptides (Ilps) from neuroendocrine cells in the brain regulates glucose homeostasis and nutrient stores in the fly (Broughton et al., 2005; Géminard et al., 2009; Ikeya et al., 2002; Rulifson et al., 2002). Ilp secretion from insulin-producing cells (IPCs) is responsive to circulating glucose and macronutrients and is suppressed upon nutrient withdrawal (Géminard et al., 2009; Kréneisz et al., 2010). Notably, recent studies have identified hormonal and GPCR-linked mechanisms regulating the secretion of Ilps from IPCs, suggesting further conservation of pathways regulating insulin secretion in the fly (Géminard et al., 2009; Kwak et al., 2013; Owusu-Ansah et al., 2013; Rajan and Perrimon, 2012).

In mammals, the incretin hormones gastric inhibitory peptide (GIP) and glucagon-like peptide-1 (GLP-1) are secreted by enteroendocrine cells following a meal and enhance glucose-stimulated insulin production and secretion from pancreatic β cells (Campbell and Drucker, 2013; Creutzfeldt, 2005). Thus, we postulated that a *decretin* hormone would have the "opposite" hallmarks of incretins. Specifically, a decretin (1) derives from an enteroendocrine source that is sensitive to nutrient availability, (2) is responsive to fasting or carbohydrate deficiency, and (3) suppresses insulin production and secretion from insulin-producing cells. However, like incretins, the action of decretins on insulin secretion would be manifest during feeding, when a stimulus for secretion is present.

Here, we identify a secreted hormone, Limostatin (Lst), that suppresses insulin secretion following starvation in *Drosophila.* We show that *lst* is regulated by starvation, and flies deficient for *lst* display phenotypes consistent with hyperinsulinemia. We localize Lst production to glucose-sensing, endocrine corpora cardiaca (CC) cells associated with the gut and show that *lst* is suppressed by carbohydrate feeding. Using calcium imaging and in vitro insulin secretion assays, we identify a 15-aa Lst peptide (Lst-15) sufficient to suppress activity of IPCs and Ilp secretion. We identify an orphan GPCR in IPCs as a candidate Lst receptor. Moreover, we show that Neuromedin U (NMU) is likely a functional mammalian ortholog of Lst that inhibits islet β cell insulin secretion. These results establish a decretin signaling pathway that suppresses insulin output in *Drosophila.*

RESULTS

CG8317 Encodes Limostatin, a Polypeptide That Suppresses Insulin Production

To identify a hormone that may function as a decretin, we performed an ectopic misexpression screen of selected starvation-regulated *Drosophila* genes (Palanker et al., 2009; Zinke et al., 2002) predicted to encode peptide hormones. Expression of *CG8317* in the fat body, a tissue capable of humoral secretion (Géminard et al., 2009; Rajan and Perrimon, 2012), induced phenotypes consistent with insulin deficiency in *Drosophila* (Rulifson et al., 2002), including hyperglycemia and developmental delay (Figures S1A and S1B). *CG8317* encodes a predicted primary translation product of 139 amino acids with a signal peptide and four putative dibasic cleavage sites, characteristic features of metazoan pre-prohormones (Figures 1A and 1B). Quantitative RT-PCR (qPCR) in fasting adult flies showed induction of

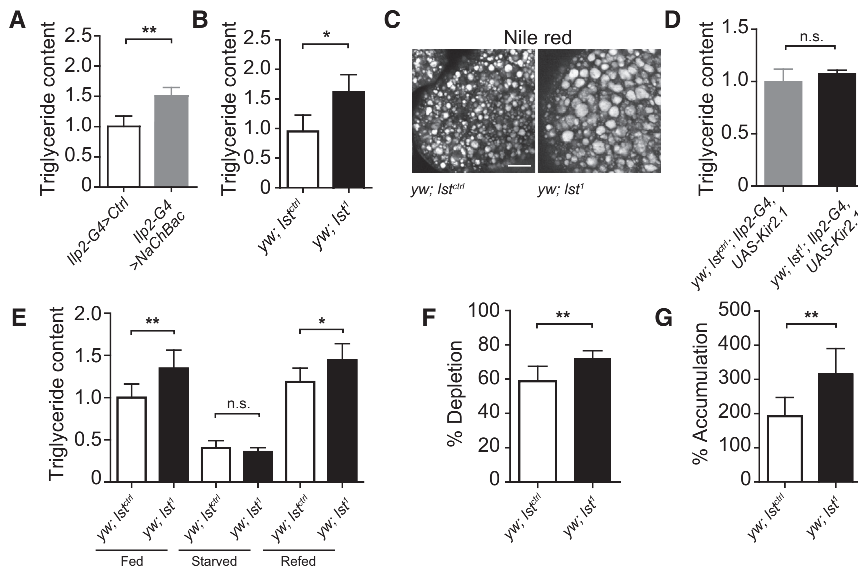

Figure 2. Obesity in *lst* Mutants

(A) Triglyceride content of control *Ilp2-GAL4* and *Ilp2-GAL4>NaChBac* flies.

(B and C) Whole-fly triglyceride content and nile red staining of abdominal lipid droplets in adult lst^1 flies and controls.

(D) Triglyceride content after silencing of IPCs using *Ilp2-GAL4* to drive *UAS-Kir2.1* in *yw;* lst^{ctrl} and *yw;* lst^1 background, normalized to *yw;* lst^{ctrl}*; Ilp2-GAL4 > UAS-Kir2.1*.

(E) Triglyceride levels in *yw*; lst^1 and controls in random fed, starved, and starved then overnight re-fed flies. Data are normalized to *yw;* lst^{ctrl} fed condition.

(F and G) Quantification of triglyceride depletion after starvation and triglyceride accumulation following refeeding after starvation; data from experiment in (E). Scale bar 15 μm in (C). Data displayed as mean + SD. *p < 0.05, **p < 0.01, and ***p < 0.001, (n = 5–8 per condition). See also Figure S2.

CG8317 by 16 hr with peak mRNA levels after 24–28 hr of nutrient deprivation (Figure 1C), confirming results from whole-genome expression analysis (Palanker et al., 2009; Zinke et al., 2002). *CG8317* and its predicted products appear to be conserved in *Drosophila* species, including a 15-aa region flanked by cleavage sites also conserved in mosquitoes and other *Insecta* (Figure S1D). Inactive pre-prohormones undergo post-translational processing, including cleavage at dibasic residues, prior to secretion as bioactive peptides (Duckert et al., 2004). To identify dibasic cleavage sites that are critical for *CG8317* function, we generated misexpression lines with arginine/lysine to alanine substitutions at each of the four sites and screened for phenotypes (see Experimental Procedures). Substitution of arginines 101 and 102, immediately N-terminal to the most highly conserved region, eliminated *CG8317* gain-of-function phenotypes, suggesting that this dibasic cleavage site is necessary for pro-hormone processing and function (Figures 1B, S1C, and S1D). Thus, *CG8317* encodes a starvation-regulated gene likely encoding a processed pre-propeptide that can inhibit insulin production and secretion (see below). We named *CG8317 limostatin* (*lst*) after Limos, the Greek goddess of starvation.

To investigate *lst* function, we mobilized a P element (Bellen et al., 2004) near *lst* and identified an imprecise excision that deleted 2 kb encompassing the entire coding sequence (lst^1 allele; Figure 1A). *lst* mRNA was undetectable in *lst* mutant flies by qPCR, indicating that lst^1 is a null allele. Expression of an *lst* transgene rescued *lst* mutant phenotypes (see below). Insulin deficiency in *Drosophila* produces hyperglycemia, starvation resistance, and lifespan extension (Broughton et al., 2008, 2005; Rulifson et al., 2002). Thus, we hypothesized that *lst*-deficient flies would display phenotypes consistent with insulin excess. As expected, *lst* mutants were hypoglycemic and short-lived compared to isogenic controls (Figures 1D and 1E). We next assessed insulin production in *lst* mutants. *Drosophila Ilps-2*, *-3*, and *-5* are produced by IPCs, median neurosecretory cells of the pars intercerebralis, and are essential regulators of growth and metabolism (Grönke et al., 2010; Ikeya et al., 2002; Rulifson et al., 2002). We measured transcript levels of *Ilp2*, *Ilp3*, *and Ilp5* by qPCR and found elevated mRNAs encoding all brain-derived Ilps in lst^1 flies during ad libitum feeding (Figures 1F, S1E, and S1F). While expression and IPC accumulation of Ilp protein have been used to assess insulin signaling in *Drosophila* (Buch et al., 2008; Géminard et al., 2009), we sought to directly measure circulating picomolar levels of Ilp2 in the hemolymph. To do this, we generated mutant flies and isogenic controls harboring a bioactive form of dual-epitope tagged Ilp2, Ilp2HF, in place of the endogenous locus (Park et al., 2014). If Lst functioned as a decretin, we hypothesized that circulating Ilp2 levels would be elevated in *lst* mutants. ELISA measurement of Ilp2HF revealed a significant increase in circulating Ilp2HF in lst^1 flies compared to controls (Figure 1G). Collectively, these results demonstrate that *lst* is produced during fasting and is required to suppress insulin production by *Drosophila* IPCs.

Obesity in Hyperinsulinemic *limostatin*-Deficient Flies

Elevated insulin signaling can stimulate obesity in flies by increasing both adipocyte number and lipid accumulation (DiAngelo and Birnbaum, 2009). Consistent with this precedent, increased IPC excitability by targeted expression of a bacterial sodium channel (*NaChBac*) was sufficient to increase triglyceride stores in adult flies compared to age-matched controls (Figure 2A). Likewise, in hyperinsulinemic lst^1 flies we found triglyceride content was elevated to 150% of control values, using standard assays including colorimetry, nile-red staining, and thin-layer chromatography (Figures 2B, 2C, and S2). To assess whether IPC activity is necessary for obesity in *lst* mutants, we generated lines that permit electrical silencing of IPCs through targeted expression of the inward rectifying potassium channel *Kir2.1*. Upon silencing of IPCs, we detected no difference in triglyceride content of *lst* mutants and controls (Figure 2D). Thus, *lst* mutant flies are obese and display phenotypes associated with insulin excess.

To further evaluate the balance between catabolic and anabolic activity in *lst* mutants, we fasted flies for 24 hr to deplete

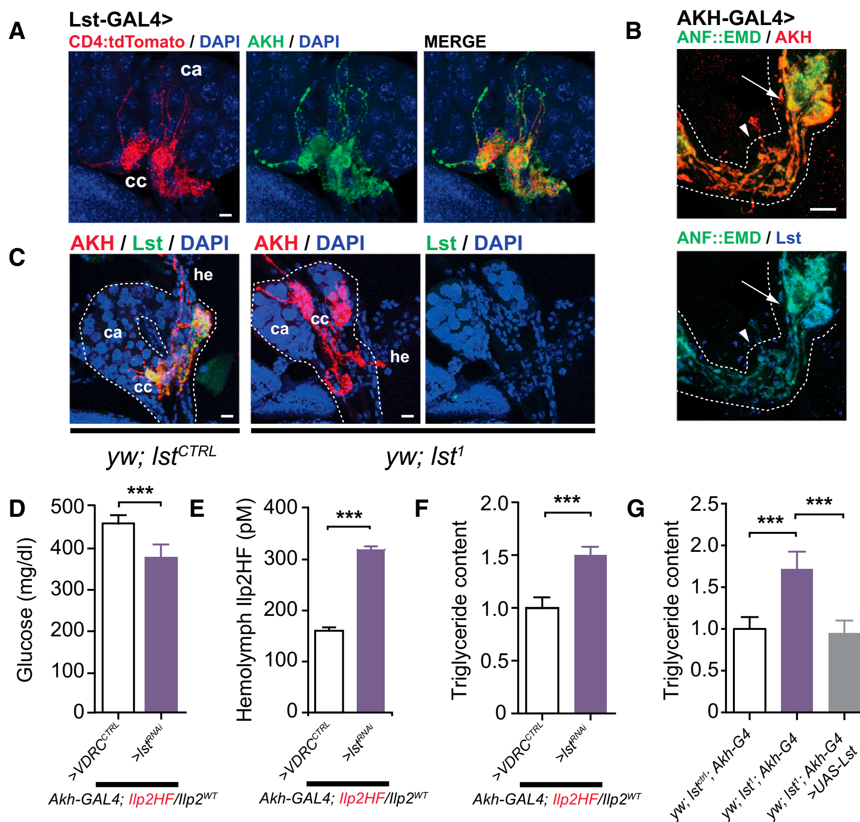

Figure 3. Lst is Produced in Gut-Associated CC cells

(A) Expression of *Lst-GAL4 > CD4::tdTomato* and AKH immunoreactivity in CC cells. Labels here and below: cc, corpora cardiaca; ca, corpus allatum; he, heart/dorsal vessel.

(B) Dense-core vesicle marker preproANF::EMD (GFP) and Lst antibody staining of CC cells. Outline marks ring gland. Arrow indicates CC cell soma, arrowhead marks path of dorsal vessel.

(C) AKH and Lst immunoreactivity in ring gland of control and *lst* mutant flies. Labels as above, hatched lines denote boundary of ring gland.

(D–F) Glucose, circulating Ilp2HF (heterozygous Ilp2HF flies), and triglyceride levels following knockdown of *lst* in CC cells using *Akh-GAL4* (lst^{RNAi}) compared to isogenic controls ($VDRC^{ctrl}$).

(G) Triglyceride content in controls (*yw;* lst^{ctrl}*; Akh-GAL4*), *lst* mutants (*yw;* lst^{1}*; Akh-GAL4*) and following rescue with *UAS-lst* (*yw;* lst^{1}*; Akh-GAL4/ UAS-lst*). Scale bars 10 μm in (A)–(C). Data displayed as mean + SD. $^{**}p < 0.01$,and $^{***}p < 0.001$. See also Figure S3.

lipid stores, then re-fed flies for 24 hr to promote lipid accumulation (Figure 2E). Starvation-induced lipid depletion remained fully intact in lst^{1} mutants and was even slightly elevated (Figures 2E and 2F). Remarkably, *lst* mutants rapidly accumulated triglycerides upon re-feeding and displayed significant obesity after only 24 hr (Figures 2E and 2G). These results indicate catabolic defects are not the principal basis for obesity in *lst* mutants.

Lst Is Regulated by Carbohydrate Feeding in Gut-Associated CC Cells

To identify the tissue source(s) of Lst, we generated an *lst* reporter line (*Lst-GAL4 > mCD4::tdTomato)* and a monoclonal antibody against the pre-propeptide (see Experimental Procedures). Lst-GAL4-mediated expression of mCD4::tdTomato co-localized with Adipokinetic hormone (AKH) in CC cells (Figure 3A). The CC cells comprise 14 gut-associated endocrine cells that send projections to the midgut and secrete hormones into the circulation from projections to the dorsal vessel (Cognigni et al., 2011; Kim and Rulifson, 2004; Park et al., 2011). CC cells secrete AKH, a hormone thought to be a functional ortholog of mammalian glucagon, indicating that CC cells have roles analogous to preproglucagon-expressing cells in the mammalian pancreas and gastrointestinal tract (Kim and Rulifson, 2004; Park et al., 2011). Lst protein co-localized with AKH, and with the dense-core vesicle marker ANF-EMD (Rao et al., 2001) in CC cell neurites ramifying on heart, consistent with the postulated role of Lst as a secreted hormone (Figures 3B and 3C).

Following specific knockdown of *lst* using RNAi (lst^{RNAi}) in CC cells, Lst immunoreactivity was reduced or undetectable, as in homozygous *lst* mutants (Figures 3C and S3A). Lst knockdown in CC cells, but not the fat body (Figure S3B), recapitulated the hypoglycemia, elevated circulating Ilp2HF levels, and obesity observed in lst^{1} mutant flies (Figures 3D–3F). Thus, CC cells are a crucial physiological source of Lst. To confirm that *lst* loss-of-function causes obesity in *lst* mutants, we expressed UAS-*lst* specifically in the CC cells of *lst* mutant flies. We observed reversion of lst^{1} obesity, with triglyceride levels indistinguishable from those in controls (Figure 3G). Thus, Lst is principally produced and secreted by CC cells, and phenotypes in *lst* mutants derive from Lst loss in CC cells.

A critical feature of incretin hormones is their regulation by carbohydrate feeding (Creutzfeldt, 2005). Thus, if Lst functioned as a decretin, we postulated that elevated *lst* expression after fasting should be reduced upon refeeding with carbohydrates. Refeeding fasted flies with carbohydrates rapidly suppressed *lst* mRNA expression (Figure 4A). By contrast, refeeding with protein did not detectably affect *lst* expression (Figure 4B). Hence, *lst* expression is increased by dietary carbohydrate restriction. Carbohydrate refeeding after fasting led to significant increases of circulating Ilp2HF (Figure 4C). Consistent with our finding that *lst* is required to suppress insulin, we found that this post-prandial increase of circulating Ilp2HF was significantly greater in *lst* mutants compared to controls (Figure 4C). In summary, *lst* is regulated by dietary carbohydrate and is required to regulate insulin output in post-prandial settings.

A Peptide Derived from Lst Inhibits IPC Activity and Insulin Secretion

Drosophila IPCs share electrophysiological properties with mammalian pancreatic β cells, including coupling of electrical

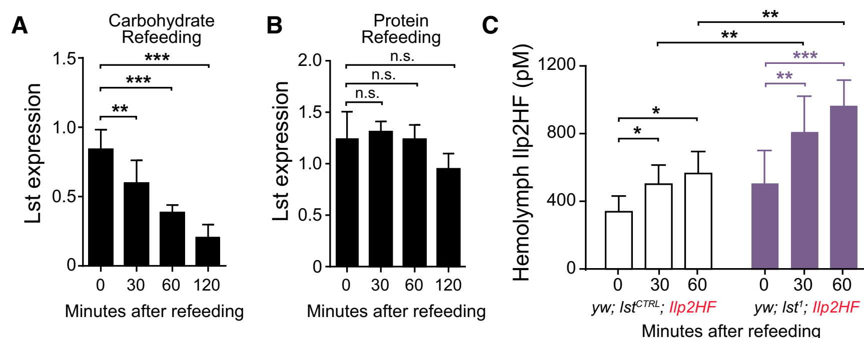

Figure 4. Lst Regulates Insulin Secretion in Response to Dietary Sugar

(A and B) qPCR analysis of *lst* expression in wild-type adult flies starved then refed for 30, 60, or 120 min with carbohydrate-only (A) or protein-only (B) food. 0 time point indicates flies starved and not refed.

(C) Hemolymph Ilp2HF levels in *lst* mutants (purple bars) and controls (open bars) refed for 0, 30, or 60 min following starvation. 0 time point indicates starved. Flies here *homozygous* for Ilp2HF. Data displayed as mean + SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

excitation to induction of calcium transients (Kréneisz et al., 2010). Incretins such as GLP-1 augment insulin secretion from β cells by increasing the frequency and amplitude of intracellular calcium transients (MacDonald et al., 2002). If Lst functioned as a decretin, we hypothesized it should decrease the excitability of IPCs. To monitor IPC activity, we generated flies that produce the genetically encoded calcium indicator GCaMP3 (Tian et al., 2009) specifically in IPCs. We quantified in vivo GCaMP3 fluorescence of IPCs by confocal microscopy in brains of immobilized live adult flies (see Experimental Procedures). GCaMP3 fluorescence was dose-dependently attenuated (Figure 5A) by exposure to a 15-aa peptide with carboxy-terminal amidation (Lst-15) corresponding to the highly conserved Lst region (Figures 1B and S1D). In contrast, exposure to a control peptide derived from an alternate domain in the pre-propeptide (Figure 1B) did not detectably affect GCaMP3 signal (Figure 5A). These results illustrate that a conserved region of the Lst peptide can regulate calcium signaling in IPCs.

To directly assess the effects of Limostatin on Ilp secretion, we developed an in vitro assay to measure Ilp2HF secretion from brain IPCs following exogenous application of purified Lst-15 peptide. Heads from *Ilp2^1 gd2HF* flies were cultured in artificial hemolymph-like (AHL) solution for 30 min, and Ilp2HF concentration in supernatants was measured by ELISA (Park et al., 2014). Exposure to Lst-15 significantly depressed Ilp2HF secretion under basal conditions (Figure 5B). Secretion remained modestly depressed following addition of high-KCl AHL solution to depolarize IPCs (Figure 5B), and a control peptide had no effect on Ilp2HF secretion (Figure 5B). Taken together, these results further support classification of Lst as a peptide hormone and suggest that Lst acutely regulates insulin secretion from IPCs (and see below). Furthermore, we have identified a minimal amidated peptide that is sufficient for the insulinostatic effect of Lst. Our demonstration that Lst is a hormone (1) produced by gut-associated endocrine cells, (2) regulated by carbohydrate restriction that (3) inhibits Ilp production, and secretion from IPCs supports classification of Lst as a *Drosophila* decretin.

Knockdown of the GPCR *CG9918* in IPCs Phenocopies *lst* Loss of Function

Many neuropeptides signal through GPCRs (Taghert and Nitabach, 2012), and receptor activity or expression is often modulated to balance signaling strength (Gardner and Nissenson, 2004). To identify a candidate receptor for Lst, we designed a qPCR-based screen to reveal GPCRs encoded by mRNAs that were both (1) reduced upon *lst* overexpression and (2) elevated in *lst* mutants. As proof of principle, ectopic expression of *Akh* in fat body reduced expression of the G protein-coupled Akh receptor *AkhR* (data not shown). We identified three candidate receptors with mRNA levels that appropriately and reciprocally varied in this manner upon *lst* gain- or loss-of-function (Figure 6A).

Based on our findings that excitatory activity of the IPCs is crucial to *lst* loss of function phenotypes (Figure 2D), we reasoned that knockdown of a candidate Lst receptor specifically in IPCs should phenocopy the *lst^1* mutation. Only IPC-directed knockdown of the receptor encoded by *CG9918* (*CG9918^{RNAi}*) produced increased adiposity, accompanied by elevated *Ilp2* mRNA levels (Figures 6B and 6C), phenotypes observed in *lst^1* flies. *CG9918* has been called *Pyrokinin 1 receptor* (*PK1r*), based on its reported affinity for *Drosophila* pyrokinin Drm-PK-1 (Cazzamali et al., 2005), but other studies failed to activate *CG9918* with Drm-PK-1 or pyrokinins (Park et al., 2002), a peptide class thought to regulate sex pheromone production (Choi and Vander Meer, 2012). Consistent with a role in *lst* signaling, *CG9918^{RNAi}* in IPCs also increased hemolymph Ilp2HF levels and endogenous *lst* expression (Figures 6D and 6E). To confirm expression of *CG9918* in IPCs, we performed fluorescent in situ hybridization (FISH) combined with immunohistochemistry (for Ilp2) in *CG9918^{RNAi}* and controls. Hybridization signal for *CG9918* co-localized with Ilp2 protein in IPCs, was reduced with *CG9918^{RNAi}* and undetectable with sense probes (Figures 6F and S4). Thus, we have identified *CG9918* as a GPCR expressed in IPCs that negatively regulates insulin expression and secretion in the adult fly.

To test further if CG9918 encodes a GPCR required for Lst signaling, we reasoned that *CG9918^{RNAi}* in IPCs should alter effects of purified Lst-15 peptide on insulin secretion. Compared to controls, we observed that *CG9918* knockdown prevented the effects of Lst-15 on attenuating Ilp2HF secretion (Figure 6G). These pharmacogenetic findings indicate that Lst regulates insulin secretion directly in IPCs and support the view that *CG9918* encodes an Lst receptor.

NMU Is a Functional Ortholog of Lst That Inhibits Insulin Secretion by Human Islets

The GCPR encoded by *CG9918* is conserved among a cluster of receptors within a phylogenetic group of peptide receptors that includes human Neuromedin U receptors (NMURs) (Metpally and Sowdhamini, 2005). Among these receptors, CG9918 is most closely related to human NMURs, displaying 32% conservation with NMUR1 and 30% conservation with NMUR2 (Figures S5A and S5B; Experimental Procedures). In humans and other

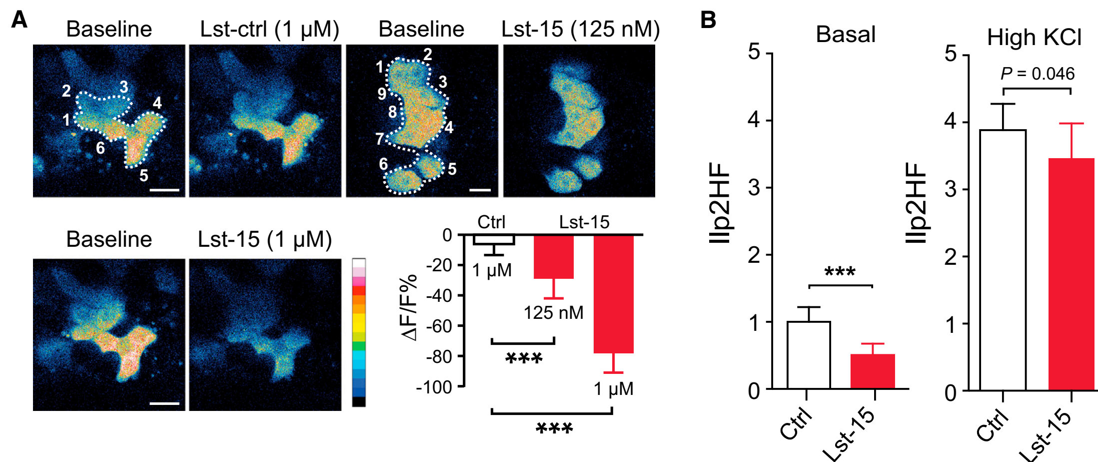

Figure 5. Lst-15 Inhibits Electrical Activity and Insulin Secretion from IPCs

(A) GCaMP3 fluorescence in head-fixed adult flies expressing GCaMP3 in IPCs under the control of the Ilp2 promoter. Baseline images from IPCs in standard AHL (3 mM glucose) before treatment. Treatment panel images were obtained 30 s after treatment with control peptide or Lst-15 (125 nM and 1 μM) diluted in standard AHL and displayed with 16-color look-up table. IPC cell clusters are indicated by hatched outline, and individual cells in the imaging plane are numbered around perimeter of the cluster. Average ΔF/F% from baseline for each condition are plotted (bottom, right).

(B) Normalized Ilp2HF protein secreted into supernatant from *Drosophila* heads incubated for 30 min in standard AHL with control peptide or Lst-15 peptide (1 μM) under basal and high-KCl conditions as indicated. Data are normalized to basal control condition. Scale bars 10 μm in (A). Data displayed as mean + SD. ***$p < 0.001$.

mammals, peripheral effects of NMU are mediated by NMUR1, while NMUR2 is primarily expressed in the CNS (Mitchell et al., 2009). NMUR1 immunoreactivity and mRNA were detected in human pancreatic islets in insulin$^+$ β cells (Figures 7A, S5C, and S5F). By contrast, little to no protein or mRNA were detected in glucagon$^+$ α cells, somatostatin$^+$ δ cells, exocrine acinar cells, or pancreatic ducts (Figures 7B, 7C, and S5D–S5F). In addition, qPCR revealed expression of *NMUR1* mRNA in human gastrointestinal tissues with enrichment in pancreatic islets (Figure 7D), consistent with prior reports (Howard et al., 2000). Lst is produced in CC cells that contact the *Drosophila* foregut. Thus, we assessed *NMU* expression in human gastrointestinal organs. *NMU* mRNA expression was enriched in human foregut organs (stomach and duodenum) (Figure 7E). Immunofluorescence localized NMU to ChgB$^+$ duodenal cells with an "open-type" morphology adjacent to the lumen of intestinal glands (Figures 7F and 7G). These results support the view that NMU from a gastrointestinal source might impact insulin output by pancreatic β cells.

To test directly if NMU can suppress insulin secretion, we purified human islets and assessed GSIS at a concentration of NMU reported to elicit physiological responses (Kaczmarek et al., 2006). NMU-25 potently suppressed GSIS from human islets in static batch culture assays (Figure 7H, $p < 0.001$ for GSIS) and islet perifusion experiments (Figures 7I–7K). An *NMU* R165W allele that encodes a mutant peptide was previously found to co-segregate in an autosomal dominant pattern with early-onset obesity (Hainerová et al., 2006). In that study, a subset of carriers displayed elevated insulin C-peptide levels; based on these findings and mutation of the highly conserved NMU C-terminal pentapeptide in this family, we reasoned that suppression of insulin secretion might be impaired by the mutant NMU R165W variant. In human islet perifusion assays, the R165W NMU variant failed to suppress insulin secretion (Figures 7K and S5G) compared to wild-type NMU. These data suggest that the human *NMU* R165W mutation represents a hypomorphic loss-of-function allele and that impaired regulation of insulin secretion by NMU could underlie metabolic changes in carriers of this allele.

DISCUSSION

Limostatin is a peptide hormone induced by carbohydrate restriction from endocrine cells associated with the gut that suppresses insulin production and release by insulin-producing cells. Thus, *Drosophila* Lst fulfills the functional criteria for a decretin and serves as an index member of this hormone class in metazoans. Results here also show that Lst signaling from corpora cardica cells may be mediated by the GPCR encoded by *CG9918* in insulin-producing cells. In addition, our results reveal cellular and molecular features of a cell-cell signaling system in *Drosophila* with likely homologies to a mammalian entero-insular axis.

Reduction of nutrient-derived secretogogues, like glucose, is a primary mechanism for attenuating insulin output during starvation in humans (Cahill et al., 1966) and flies (Colombani et al., 2003; Géminard et al., 2009). Consistent with this, we found that circulating Ilp2HF levels were reduced to a similar degree in *lst* mutant or control flies during prolonged fasting (Figure 4C). Therefore, *lst* was dispensable for Ilp2 reduction during fasting. However, *lst* mutants upon re-feeding or during subsequent ad libitum feeding had enhanced circulating Ilp2HF levels compared to controls, findings that demonstrate a requirement for Lst to restrict insulin output in fed flies. Thus, while induced by nutrient restriction, Lst decretin function was revealed by nutrient challenge. This linkage of feeding to decretin regulation

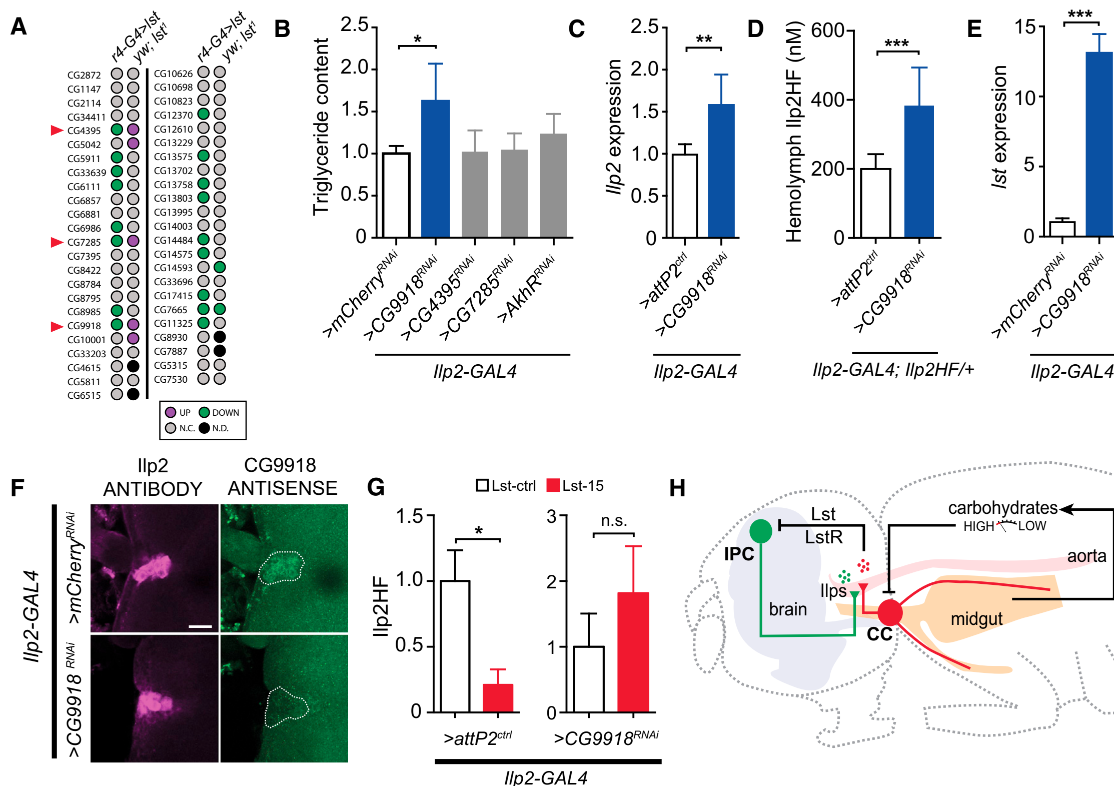

Figure 6. *CG9918* Is a Candidate Lst Receptor

(A) qPCR for expression of *Drosophila* GPCRs in *lst* overexpression (*r4-G4 > UAS-lst)* or *lst*[1] loss of function. Expression changes in comparison to control are indicated by gray (no change), green (decreased), purple (elevated), and black (not determined). Red arrowheads denote transcripts reciprocally regulated and assessed in (B).

(B) Triglyceride levels following IPC specific knockdown of receptors identified in (A) (red arrowheads). Receptors encoded by *CG9918*, *CG4395*, and *CG7285* were knocked down in IPCs using *Ilp2-GAL4; UAS-Dcr2. AKHR* was included as a negative control.

(C and D) Ilp2 expression and hemolymph Ilp2HF levels in *CG9918*^{RNAi} flies and controls (Ilp2HF heterozygous here).

(E) *lst* expression in *CG9918*^{RNAi} flies. Data in (B), (C), and (E) normalized to control condition.

(F) FISH for *CG9918* mRNA with immunohistochemistry (IHC) using antibodies against Ilp2 in control and *CG9918*^{RNAi} flies.

(G) Normalized Ilp2HF levels in supernatant from *CG9918*^{RNAi} and control heads incubated with 1 μM Lst-15 (red bars) or Lst control peptide (open bars).

(H) Summary model for Lst signaling. Ingested carbohydrates levels are monitored by CC cells. Under carbohydrate-poor conditions, secreted Lst hormonally suppresses activity and secretion of Ilps from insulin-producing cells (IPCs). Scale bar 25 μm in (F). Data displayed as mean + SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. See also Figure S4.

of insulin output is reminiscent of incretin regulation and action (Campbell and Drucker, 2013).

Recent studies have demonstrated functional conservation in *Drosophila* of fundamental hormonal systems for metabolic regulation in mammals, including insulin (Ikeya et al., 2002; Rulifson et al., 2002), glucagon (Kim and Rulifson, 2004; Lee and Park, 2004), and leptin (Rajan and Perrimon, 2012). Here we used *Drosophila* to identify a hormonal regulator of insulin output, glucose, and lipid metabolism without an identified antecedent mammalian ortholog—emphasizing the possibility for work on flies to presage endocrine hormone discovery in mammals. Gain of Lst function in our studies led to reduced insulin signaling, and hyperglycemia, consistent with prior work by our group and others (Broughton et al., 2005; Kim and Rulifson, 2004). By contrast, loss of Lst function led to excessive insulin production and secretion, hypoglycemia, and elevated triglycerides, phenotypes consistent with the recognized anabolic functions of insulin signaling in metazoans, and with the few prior metabolic studies of flies with insulin excess (Erion et al., 2012; Rajan and Perrimon, 2012).

Prior studies show that somatostatin and galanin are mammalian gastrointestinal hormones that can suppress insulin secretion. Somatostatin-28 (SST-28) is a peptide derivative of the pro-somatostatin gene that is expressed widely, including in gastrointestinal cells and pancreatic islet cells. Islet somatostatin signaling is thought to be principally paracrine, rather than endocrine, and serum SST-28 concentrations increase post-prandially (D'Alessio et al., 1989; Strowski and Blake, 2008). Galanin is an orexigenic neuropeptide produced throughout the CNS and in peripheral neurons and has been reported to inhibit insulin secretion (Fehmann et al., 1995). Unlike enteroendocrine-derived hormones that act systemically, galanin is secreted from intrapancreatic autonomic nerve terminals and is thought to exert local effects (Dunning et al., 1986; Dupré, 1988; Tang et al., 2012). In addition, Galanin synthesis and secretion are increased by feeding and dietary fat (Leibowitz et al., 2004;

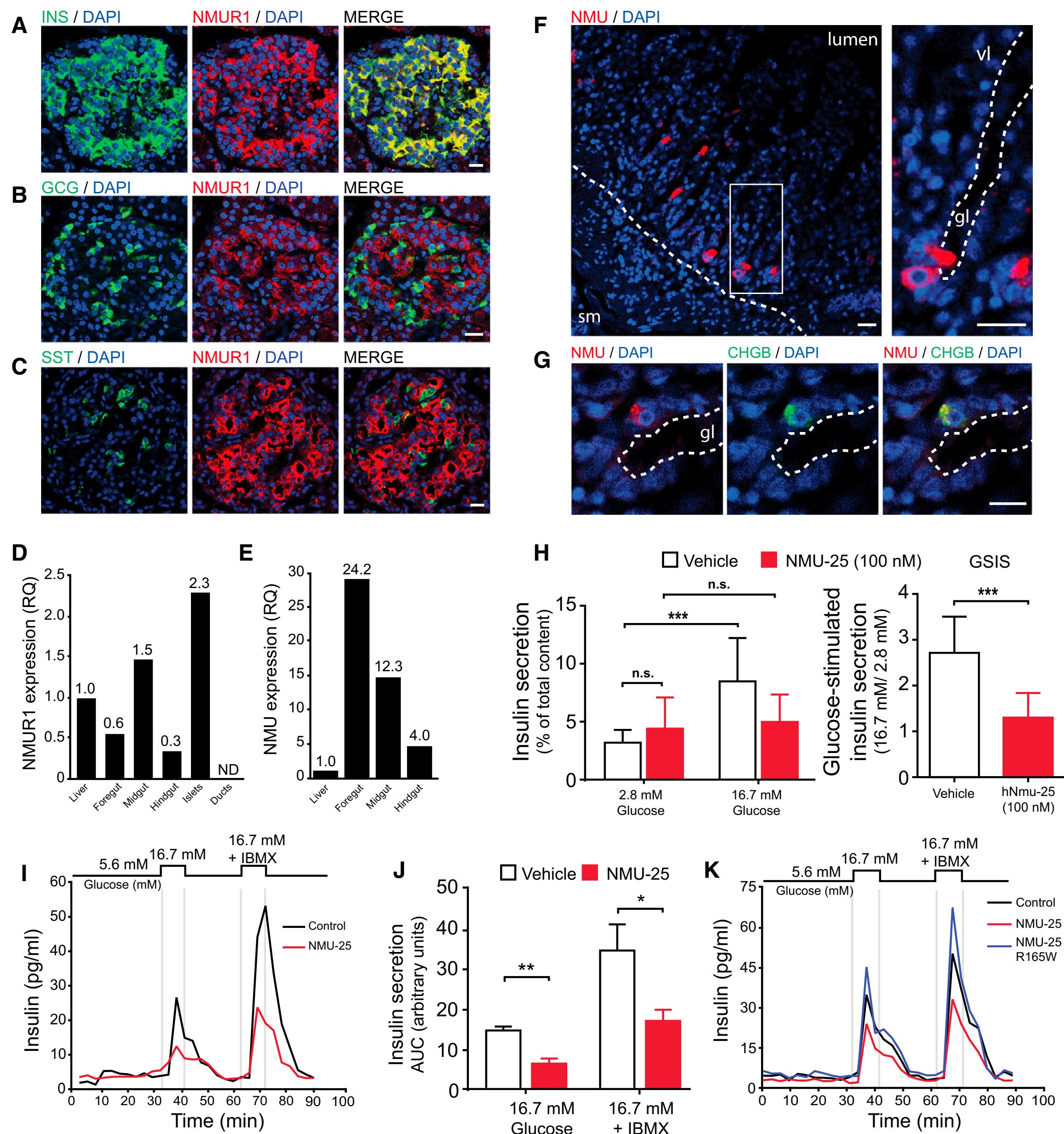

Figure 7. NMU Signaling Suppresses Insulin Secretion from Human β cells

(A–C) Immunoreactivity of NMUR1 and insulin (β cells), glucagon (α cells), or somatostatin (δ cells) in adult human pancreas.

(D) qPCR analysis of *NMUR1* expression in human gastrointestinal tissues, including purified human islets and pancreatic ductal cells. Data expressed as relative quantification (RQ) and normalized to liver sample. N.D., not detected.

(E) qPCR analysis of *NMU* expression in human gastrointestinal tissues. Data expressed as relative quantification (RQ) and normalized to liver sample.

(F) NMU immunoreactivity in villous mucosa of human duodenum. Mucosal villi (vl) oriented toward lumen at upper right of image. Hatched line in left panel denotes boundary of mucosal layer. Magnification of boxed region in right panel shows base of duodenal gland (gl), with lumen outlined by hatched line and open-type enteroendocrine cell immunoreactive for NMU. sm, submucosa.

(G) NMU and Chromogranin B immunoreactivity in open-type enteroendocrine cell. Lumenal edge of duodenal gland (gl) is marked by hatched line. Scale bars 10 μM in (A)–(C) and 20 μM in (D) and (E).

(H) Insulin secretion from human islets from 59-year-old male donor assayed in static batch assay with vehicle or 100 nM NMU-25. Data normalized to insulin content and expressed as percent of total content. GSIS panel (right) displays ratio of stimulated (16.7 mM) to basal (2.8 mM) secretion. Data displayed as mean + SD.

(I) Insulin secretion from human islet perifusion assay using islets from 40-year-old male donor. NMU-25 (red trace) was applied at 100 nM and included in all incubation solutions. Top diagram depicts stimulation protocol. IBMX, 3-isobutyl-1-methylxanthine.

(J) Quantification of insulin secretion area under the curve (AUC) from independent perifusion experiments (in Figures 7I, 7K, and S5I) using islets from three human donors under stimulation conditions (16.7 mM glucose or 16.7 mM + IBMX). Islets were treated with vehicle (open bars) or 100 nM NMU-25 (red bars). Data displayed as mean + SEM.

(K) Insulin secretion in human islet perifusion assay using islets from a 49-year-old male donor. NMU-25 (red trace) and mutant NMU-25 R165W (blue) were applied at 100 nM and included in all incubation solutions. *p < 0.05, **p < 0.01, and ***p < 0.001. See also Figure S5.

Wang and Leibowitz, 1997). Thus, like incretins, output of SST-28 and galanin are induced by feeding, but in contrast to incretins, these peptides suppress insulin secretion. Further studies are needed to assess the roles of these peptide regulators in the modulation of insulin secretion during fasting.

While sequence-based searches did not identify vertebrate orthologs of Lst, we found that the postulated Lst receptor in IPCs, encoded by *CG9918*, is most similar to the GPCRs NMUR1 and NMUR2. In rodents, *NMU* signaling may be a central regulator of satiety and feeding behavior (Hanada et al., 2004; Howard et al., 2000), and this role may be conserved in other organisms (Pang and Curran, 2014; Schoofs et al., 2014). In addition, *NMU* mutant mice have increased adiposity and hyperinsulinemia (Hanada et al., 2004), but a direct role for NMU in regulating insulin secretion by insulin-producing cells was not identified. In rodents, the central effects of NMU on satiety are thought to be mediated by the receptor NMUR2; however, hyperphagia, hyperinsulinemia, and obesity were not reported in *NMUR2* mutant mice (Bechtold et al., 2009). Together, these studies suggest that a subset of phenotypes observed in *NMU* mutant mice may instead reflect the activity of NMU on peripheral tissues like pancreatic islets, but this has not been previously shown. Notably, humans harboring the *NMU* R165W allele displayed obesity and elevated insulin C-peptide levels, without evident hyperphagia—further suggesting that the central and peripheral effects of NMU reflect distinct pathways that may be uncoupled (Hainerová et al., 2006). Here we showed that *NMU* is produced abundantly in human foregut organs and suppresses insulin secretion from pancreatic β cells, supporting the view that NMU has important functions outside the CNS in regulating metabolism. Thus, like the incretin GLP-1 (Drucker, 2006), NMU may have dual central and peripheral signaling functions in the regulating metabolism. Demonstration that NMU is a mammalian decretin will require further studies on NMU regulation and robust methods to measure circulating NMU levels in fasting and re-feeding. In summary, our findings should invigorate searches for mammalian decretins with possible roles in both physiological and pathological settings.

EXPERIMENTAL PROCEDURES

Drosophila Methods

Experimental crosses were maintained at 25°C under 12 hr:12 hr light/dark conditions and provided fresh food every 2 to 3 days. Unless otherwise indicated, standard molasses (6% molasses, 5% corn meal, 2.5% baker's yeast, and 0.7% agar) food was used for all experiments. Adult flies were collected 2 days after eclosion and aged for 8–12 days on standard molasses food for all experiments. Carbohydrate-only food was comprised of 15% W/V dextrose or sucrose and 1% agar. Protein-only food was comprised of 10% W/V bacto-peptone (BD). For starvation experiments, flies were tipped to fresh vials or bottles containing 1% agar or wetted cotton plugs and fasted for 20–24 hr unless otherwise indicated. For re-feeding experiments, agar-starved flies were tipped to foods prepared with food coloring and feeding was verified by visualization of pigment in gut.

The lst^1 allele was generated by imprecise excision of a P element upstream of the *lst* gene in the *yw; P{EP}G424* line (Bellen et al., 2004) using standard methods. The extent of the deletion was assessed by PCR and sequencing. The deletion spans 1,946 bp fragment (2R: 12462183…12464128 in the genome assembly release r5.52), only removing the *lst* gene, including the entire coding region. A control *yw* stock and the *yw*; lst^1 line were then backcrossed into the original *yw; P{EP}G424* line to generate isogenic *yw*; lst^{ctrl} and *yw*;lst^1 stocks. To generate lines for epistasis experiments, *yw*; lst^{ctrl} and *yw*; lst^1 (located on chromosome II) were combined with transgenes or deficiencies located on chromosome III by standard methods to generate isogenic flies. Thus, stocks were *yw*; lst^{ctrl} or *yw*; lst^1 chromosomes I & II, and isogenic for indicated transgenic or mutant chromosome III.

Drosophila metabolic assays were performed using protocols described in detail (Tennessen et al., 2014). Insulin measurements in *Drosophila* were performed using flies homozygous or heterozygous for the Ilp2HF transgene, as indicated. Hemolymph Ilp2HF levels were quantified using custom made ELISA assays as described in Park et al. (2014).

Human Tissues

Institutional review board approval for research use of tissue was obtained from Stanford University School of Medicine and Vanderbilt University. Human pancreata and islets were obtained from previously healthy, non-diabetic organ donors by the Integrated Islet Distribution Program (IIDP). For histology studies, fresh human pancreata and gastro-intestinal organs were fixed and processed for sectioning by standard histology protocols. Pancreata from donors aged 3, 23, and 30 years old were used in immunofluorescence studies. Islets used in static batch incubation and perifusion assays were from donors aged 3, 40, 49, 51, and 59 years old. Human gastrointestinal cDNA was obtained from Clontech (Human Digestive System MTC panel, cat: 636746) and derived from multiple donors. Human islet and pancreatic ductal cell RNA for qPCR was obtained as described previously (Lee et al., 2013). Adult human stomach slides used for histology were obtained from Abcam (cat: ab4371). Adult human pyloric stomach and duodenum specimens used in histology were procured by the National Disease Research Interchange (NDRI). Human pancreas sections used for RNAscope 2.0 assays were obtained from the Stanford Tissue Bank.

Peptides

Drosophila peptides used in this study were supplied by LifeTein (South Plainfield). Peptide sequences are as follows: Limostatin-control peptide (Lst-ctrl), AQPDSLRSKP; Limostatin-15 (Lst-15) AIVFRPLFVYKQQEI-amide. Human NMU-25 was obtained from Sigma (N4284) and LifeTein (Hillsborough), human NMU-R165W was obtained from LifeTein.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2015.01.006.

AUTHOR CONTRIBUTIONS

R.W.A. and S.K.K. designed experiments and wrote the manuscript. R.W.A., K.-R.S., N.J., X.G., and J.W. performed the experiments. R.W.A. and S.P. carried out the initial screen. S.P. and K.-R.S. generated the *limostatin* null allele. S.P. generated transgenic lines and ELISA methods. L.K. performed experiments and assisted with experimental design. G.P. and A.C.P. designed and performed human islet perifusion experiments.

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