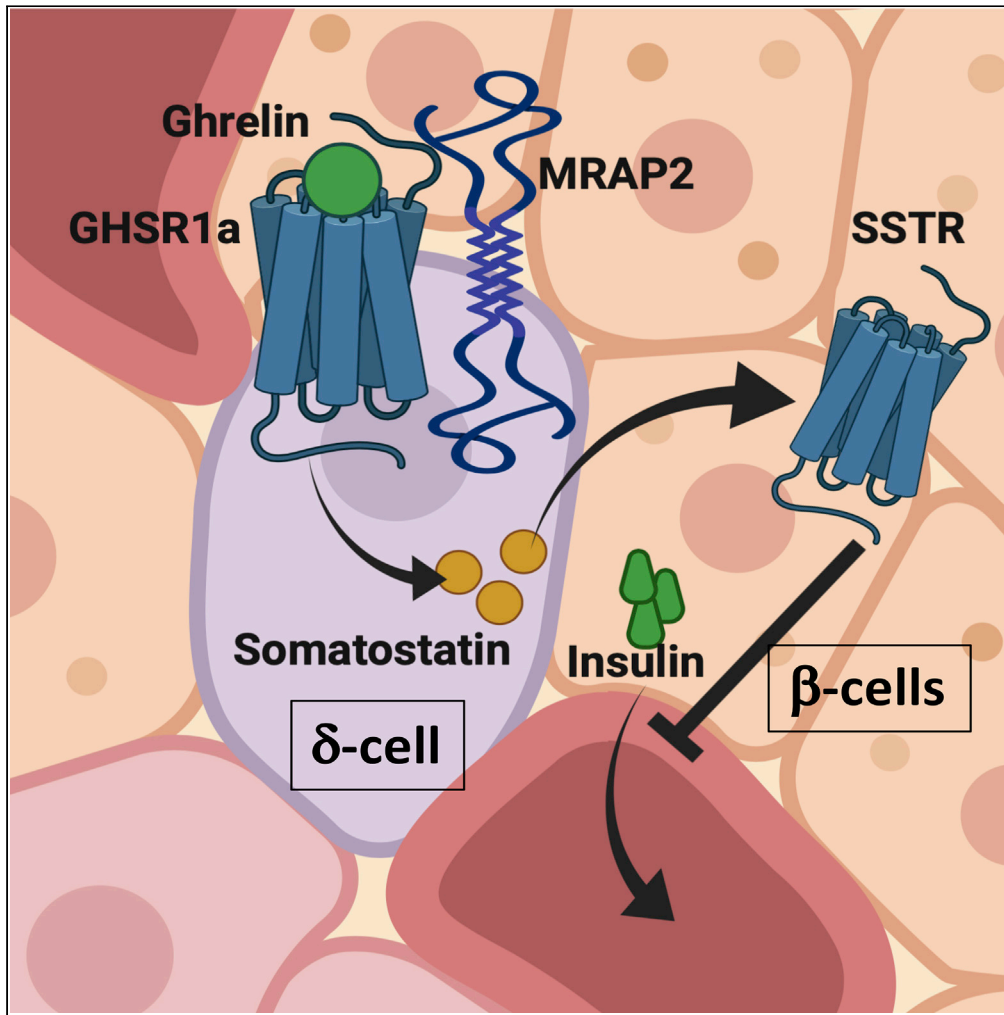


Article

The Insulinostatic Effect of Ghrelin Requires MRAP2 Expression in δ Cells



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HIGHLIGHTS

δ Cells are responsible for the action of ghrelin in the endocrine pancreas

MRAP2 is expressed in multiple cell types in the endocrine pancreas including δ cells

MRAP2 is required for GHSR1a signaling in δ cells

Deletion of MRAP2 results in loss of ghrelin-mediated inhibition of insulin secretion

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Article

The Insulinostatic Effect of Ghrelin Requires MRAP2 Expression in δ CellsTerry C. Yin,¹ Casey J. Bauchle,² Alix A.J. Rouault,¹ Samuel B. Stephens,² and Julien A. Sebag^{1,3,*}

SUMMARY

Ghrelin regulates both energy intake and glucose homeostasis. In the endocrine pancreas, ghrelin inhibits insulin release to prevent hypoglycemia during fasting. The mechanism through which this is accomplished is unclear, but recent studies suggest that ghrelin acts on δ cells to stimulate somatostatin release, which in turn inhibits insulin release from β cells. Recently, the Melanocortin Receptor Accessory Protein 2 (MRAP2) was identified as an essential partner of the ghrelin receptor (GHSR1a) in mediating the central orexigenic action of ghrelin. In this study we show that MRAP2 is expressed in islet δ cells and is required for ghrelin to elicit a calcium response in those cells. Additionally, we show that both global and δ cell targeted deletion of MRAP2 abrogates the insulinostatic effect of ghrelin. Together, these findings establish that ghrelin signaling within δ cells is essential for the inhibition of insulin release and identify MRAP2 as a regulator of insulin secretion.

INTRODUCTION

Pancreatic islets coordinate whole-animal nutrient status through release of glucoregulatory hormones such as insulin and glucagon, which have powerful actions in peripheral tissues to promote nutrient uptake during feeding or to signal hepatic glucose release during fasting to maintain euglycemia. Nutrients such as glucose, fatty acids, and amino acids serve as direct signals for islet hormone release through coupling of metabolic and electrical pathways and are generally considered the primary driver of islet cell functions. In addition to nutrient cues, endocrine and intra-islet paracrine hormones coordinate and refine islet cell functions to meet specific physiological demands (Caicedo, 2013). For example, the orexigenic hormone ghrelin is secreted by the stomach during fasting and plays an important role in preventing hypoglycemia by promoting hepatic glucose production (Zhao et al., 2010; Broglio et al., 2001) and inhibiting β cell insulin secretion (Mani and Zigman, 2017; Muller et al., 2015; Reimer et al., 2003; Broglio et al., 2001). The inhibitory effect of ghrelin on insulin release was originally hypothesized to be mediated through direct action of ghrelin on β cells (Dezaki et al., 2004, 2007); however, recent transcriptomic analysis of islet α , β , and δ cells revealed that the ghrelin receptor (GHSR1a) is primarily expressed in δ cells rather than in β cells (DiGrucchio et al., 2016; Adriaenssens et al., 2016). Based on this observation and studies using somatostatin receptor antagonists, the new proposed mechanism of ghrelin action in the pancreas involves the stimulation of somatostatin release from δ cells, which in turn directly inhibits β cell insulin secretion (DiGrucchio et al., 2016). These studies highlight emerging roles for intra-islet communication as a critical signaling network to coordinate physiological demands with the regulation of nutrient-dependent islet hormone responses.

Ghrelin acts through activation of its receptor, the Growth Hormone Secretagogue Receptor (GHSR1a), a GPCR that mainly couples to $G\alpha_{q/11}$ and promotes calcium release from the endoplasmic reticulum (Falls et al., 2006). We have recently demonstrated that the Melanocortin Receptor Accessory Protein 2 (MRAP2), a single transmembrane protein that regulates the activity of several GPCRs (Chaly et al., 2016; Rouault et al., 2017a, 2017b; Srisai et al., 2017; Sebag et al., 2013; Asai et al., 2013), is essential for GHSR1a signaling and function in hypothalamic neurons (Srisai et al., 2017). Ghrelin-stimulated $G\alpha_{q/11}$ signaling is strongly potentiated by MRAP2, and the deletion of MRAP2 blocks the ghrelin-mediated increase in food intake (Srisai et al., 2017). The importance of $G\alpha_{q/11}$ signaling downstream of GHSR1a was shown to be important for the regulation of food intake (Wettschureck et al., 2005), whereas the signaling pathway involved in ghrelin action in the pancreas and the requirement for MRAP2 are unclear. Based on our previous work in the hypothalamus, we hypothesized that MRAP2 may similarly regulate GHSR1a-ghrelin signaling in pancreatic islets. Here we demonstrate that MRAP2 is expressed in the pancreatic islet and show that

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loss of MRAP2 in δ cells abolishes ghrelin signaling in the pancreas. Furthermore, MRAP2 deletion abrogates the suppressive effect of ghrelin on insulin secretion, whereas the insulinostatic effect of somatostatin is retained. These data identify an important role for MRAP2 in the coupling of endocrine and paracrine ghrelin signaling in the pancreatic islet.

RESULTS

Production and Validation of anti-MRAP2 Antibodies

In order to verify the presence of MRAP2 in δ cells, we generated a polyclonal antibody directed against the recombinantly produced C-terminal tail of mouse MRAP2, which is well conserved between species, including mouse and humans. The polyclonal antibody was tested by western blot, in-cell ELISA, and immunofluorescence microscopy. For this, CHO cells were transfected with GFP, C-terminally 3XFLAG-tagged human, or mouse MRAP2, and lysates were separated by SDS-PAGE. Membranes were probed with monoclonal anti-FLAG antibody (Figure 1A) or with the polyclonal anti-MRAP2 antibody (Figure 1B). The MRAP2 antibody was able to detect the characteristic bands for both human and mouse MRAP2 (Figure 1B). Furthermore, the bands detected with the anti-MRAP2 antibody matched the bands detected with the anti-FLAG antibody, whereas no bands were detected in lysates from GFP-expressing control cells by either antibody (Figures 1A and B). This result demonstrates the successful generation of an anti-MRAP2 antibody and its ability to detect both human and mouse MRAP2 by western blot. To further validate the MRAP2 antibody, we tested its ability to quantitatively detect mouse and human MRAP2 in transfected CHO cells by in-cell ELISA. Although both the anti-FLAG (Figure 1C) and the anti-MRAP2 (Figure 1D) antibodies detected human and mouse MRAP2-3XFLAG with signal significantly higher than background (mock transfected cells), the anti-MRAP2 antibody showed higher sensitivity for detection of the mouse rather than the human MRAP2 isoform. This is likely due to the use of a mouse MRAP2 fragment as immunogen. We also tested the ability of the anti-MRAP2 antibody to detect MRAP2 by immunofluorescence. Although no specific signal was detected in cells transfected with empty vector (Figure 1E), fluorescent signal was readily detectable in cells expressing mouse (Figure 1F) and human (Figure 1G) MRAP2. To determine if the antibody was sensitive enough to detect endogenous MRAP2, detection of MRAP2 by immunofluorescence was carried out in brain slices from wild-type (WT) and *Mrap2* KO mice. Although fluorescence was readily detectable in the arcuate nucleus (ARC) of the hypothalamus of WT mice (Figure 1H), a brain region known to express MRAP2 (Srisai et al., 2017), no specific signal was detected in slices from *Mrap2* KO animals (Figure 1I). Finally, we used the antibody to immunoprecipitate and detect endogenous MRAP2 in hypothalamic lysates from WT and *Mrap2* KO mice. MRAP2 was once again detected in WT hypothalamic lysates but no MRAP2 band was present in lysates from *Mrap2* KO hypothalamus (Figure 1J). These experiments establish the specificity and sensitivity of the polyclonal anti-MRAP2 antibody and thus validate its use for detection of endogenous MRAP2 in tissues.

MRAP2 Is Expressed in δ Cells of the Pancreatic Islet

Our previous work has demonstrated the importance of MRAP2 for the regulation of the ghrelin receptor, GHSR1a, in the hypothalamus (Srisai et al., 2017). Based on transcriptomic studies in the endocrine pancreas, which demonstrated that GHSR1a is exclusively expressed in islet δ cells (DiGrucchio et al., 2016), we hypothesized that MRAP2 is also present in δ cells. To test this, we stained mouse pancreas slices with anti-insulin antibody to identify β cells (Figures 2A and 2E), anti-MRAP2 (Figures 2B and 2F), and anti-somatostatin antibody to identify δ cells (Figures 2C and 2G). As shown in Figures 2B and 2F, MRAP2 was detected in a subset of cells within the islet including cells expressing somatostatin (Figures 2C, 2D, 2G, and 2H), suggesting that, like GHSR1a, MRAP2 is expressed in δ cells. MRAP2 appears to be enriched in δ cells since quantitation in 35 islets (6–8 islets from 5 mice) show that 92% of δ cells express MRAP2. MRAP2 is also expressed in other cell types in the islet since quantitation in the same islets shows that only 7% of MRAP2-expressing cells express somatostatin. This result is in agreement with the published single-cell transcriptome analysis of human pancreatic islets, which shows MRAP2 mRNA expression in α , β , γ , and δ cells (Segerstolpe et al., 2016). To verify that the MRAP2 staining was specific, the same experiment was conducted in pancreas slices from *Mrap2* KO mice. In this case, both insulin (Figure 2I) and somatostatin (Figures 2K and 2L) staining were detectable, whereas no MRAP2 staining was detected (Figures 2J and 2L). To determine whether MRAP2 is also expressed in human δ cells, the same staining was conducted on human pancreas slices (Figures 2M–2P). Similar to our observation in mouse pancreas, MRAP2 staining was enriched in somatostatin-positive cells (Figures 2N–2P), thus establishing that MRAP2 is expressed in both human and mouse endocrine pancreas, including in δ cells.

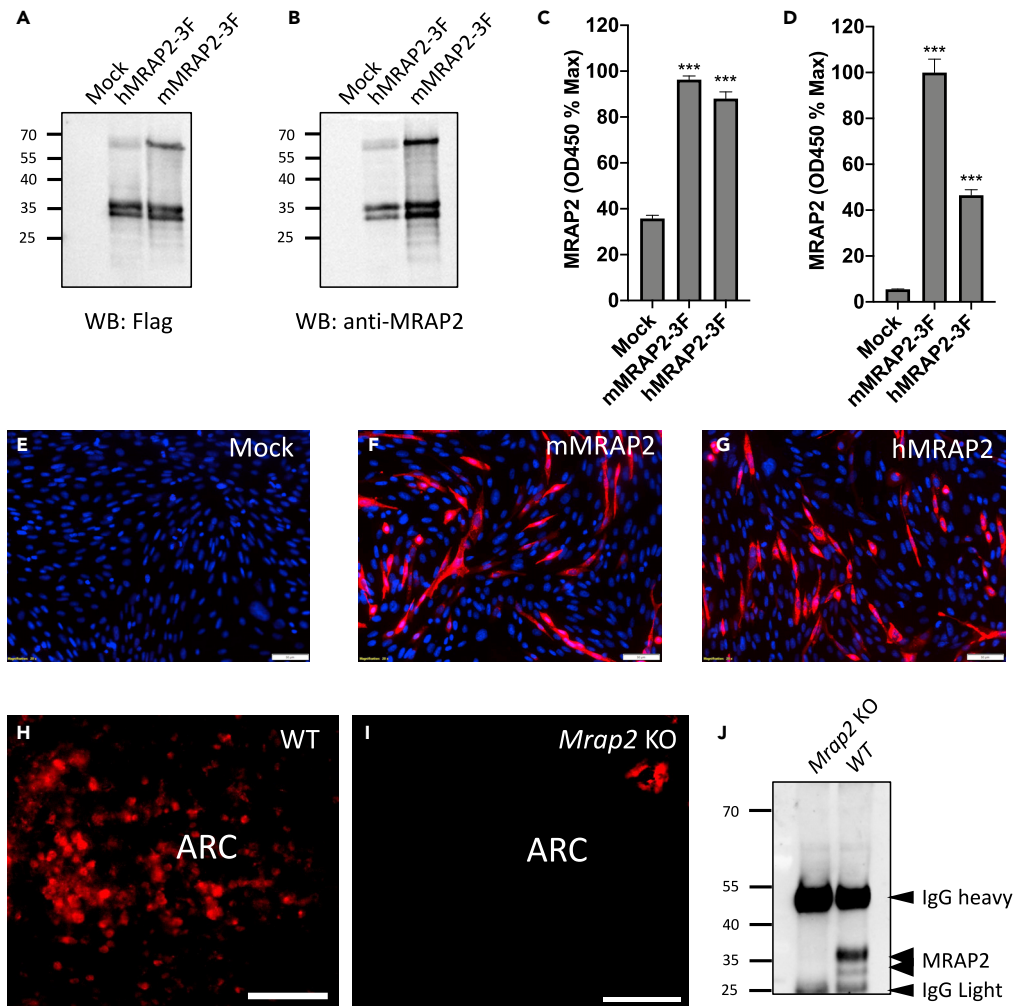


Figure 1. Validation of a New Anti-MRAP2 Antibody

(A and B) Western blot detection of MRAP2 in lysates from CHO cells transfected with empty vector, human or mouse 3XFLAG-tagged MRAP2 using anti-FLAG (A) or anti-MRAP2 (B) antibody. (C and D) In-cell ELISA detection of MRAP2 in non-permeabilized cells transfected with empty vector, human or mouse 3XFLAG-tagged MRAP2 using anti-FLAG (C) or anti-MRAP2 (D) antibody. (E–G) Immunofluorescence detection of MRAP2 in CHO cells transfected with empty vector (E), mouse MRAP2 (F), or human MRAP2 (G) using the anti-MRAP2 antibody. Nuclei are in blue. Scale bars are 50 μ m. (H and I) Immunofluorescence staining of MRAP2 using the anti-MRAP2 antibody in brain slices from WT (H) and *Mrap2* KO (I) mice. Scale bars are 100 μ m. ARC indicates the arcuate nucleus of the hypothalamus. (J) Western blot detection of MRAP2 immunoprecipitated from lysates of hypothalami harvested from WT and *Mrap2* KO mice using the anti-MRAP2 antibody. Error bars are mean \pm SEM, *** p < 0.001 one-way ANOVA.

MRAP2 Is Essential for Ghrelin- but Not Somatostatin-Mediated Inhibition of Glucose-Stimulated Insulin Secretion

Ghrelin is a potent inhibitor of insulin secretion and is suggested to work indirectly via stimulating somatostatin release from δ cells (DiGruccio et al., 2016). Given our previous work demonstrating that MRAP2 strongly potentiates ghrelin-mediated activation of GHSR1a in the hypothalamus, we tested whether MRAP2 is also required for the suppressive effect of ghrelin on glucose-stimulated insulin secretion (GSIS) in isolated islets. As expected, stimulatory glucose (16.7 mM) elicited insulin release from both WT and *Mrap2* KO islets with characteristic first and second phase secretion. Ghrelin significantly lowered GSIS from WT islets (Figure 3A), whereas it had no inhibitory effect on GSIS from *Mrap2* KO islets (Figure 3B). Since the suppressive effect of ghrelin on insulin secretion is thought to occur via paracrine release of somatostatin from δ cells, we tested the requirement of MRAP2 for somatostatin-mediated inhibition of

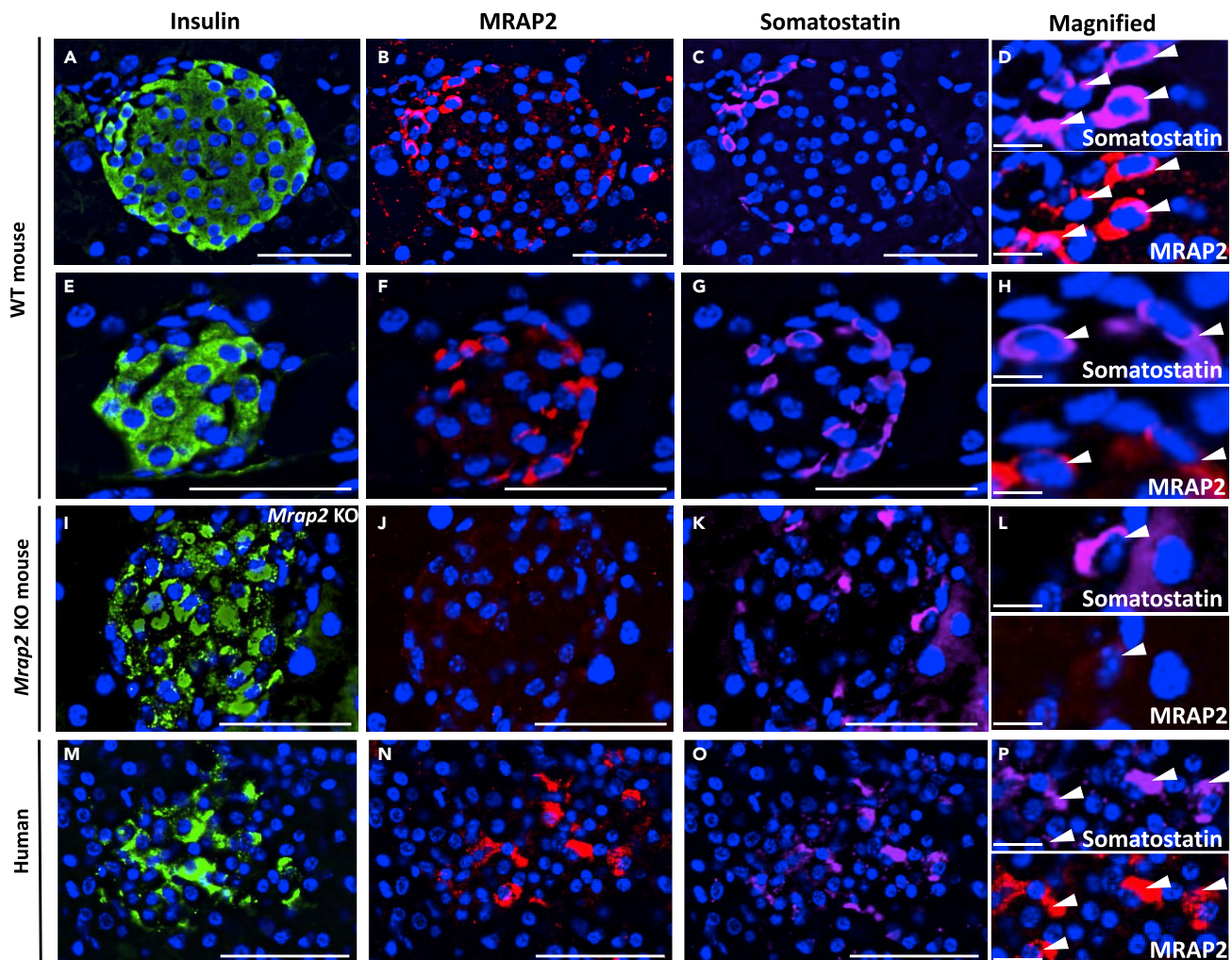


Figure 2. MRAP2 Is Expressed in δ cells of the Pancreatic Islets

(A–H) Immunofluorescence detection of insulin (A and E), MRAP2 (B and F), and somatostatin (C and G) in WT mouse pancreas slices. (D) and (H) are magnified images of (B), (C), (F), and (G).

(I–L) Immunofluorescence detection of insulin (I), MRAP2 (J), and somatostatin (K) in *Mrap2* KO mouse pancreas slices. (L) Magnified images of (F) and (G).

(M–P) Immunofluorescence detection of insulin (M), MRAP2 (N), and somatostatin (O) in human pancreas slices. (P) Magnified images of (N) and (O). Scale bars are 50 μ m. Scale bars for magnified images (D), (H), (L), and (P) are 10 μ m. Arrowheads indicate the localization of cells of interest in pannels D, H, L, and P.

GSIS. For this, we examined GSIS in islets isolated from WT and *Mrap2* KO mice in response to somatostatin treatment. In contrast to the results obtained with ghrelin, somatostatin potently inhibited GSIS from both WT and *Mrap2* KO islets (Figures 3C and 3D). These findings demonstrate that MRAP2 is required for ghrelin-mediated suppression of GSIS but not for the action of somatostatin on β cells.

Ghrelin-Mediated Inhibition of GSIS Requires MRAP2 Expression in δ Cells

Since in the pancreatic islet GHSR1a expression is restricted to δ cells, we hypothesized that the targeted deletion of MRAP2 in those cells would be sufficient to lose the insulinostatic effect of ghrelin. To test this hypothesis, we isolated islets from *SST^{CRE/+}/MRAP2^{FL/FL}* mice, in which MRAP2 was only deleted in cells expressing somatostatin, and control *MRAP2^{FL/FL}* mice. We found that ghrelin strongly inhibited GSIS from control *MRAP2^{FL/FL}* islets (Figure 3E) but failed to block GSIS from *SST^{CRE/+}/MRAP2^{FL/FL}* (Figure 3F). Since previous studies have shown that the expression of somatostatin was decreased in *SST^{CRE/+}* mice compared with WT animals (Viollet et al., 2017), we verified that, like in WT islets (Figure 3G), ghrelin inhibits GSIS from *SST^{CRE/+}* islets (Figure 3H). Additionally, no significant difference was observed in the peak of

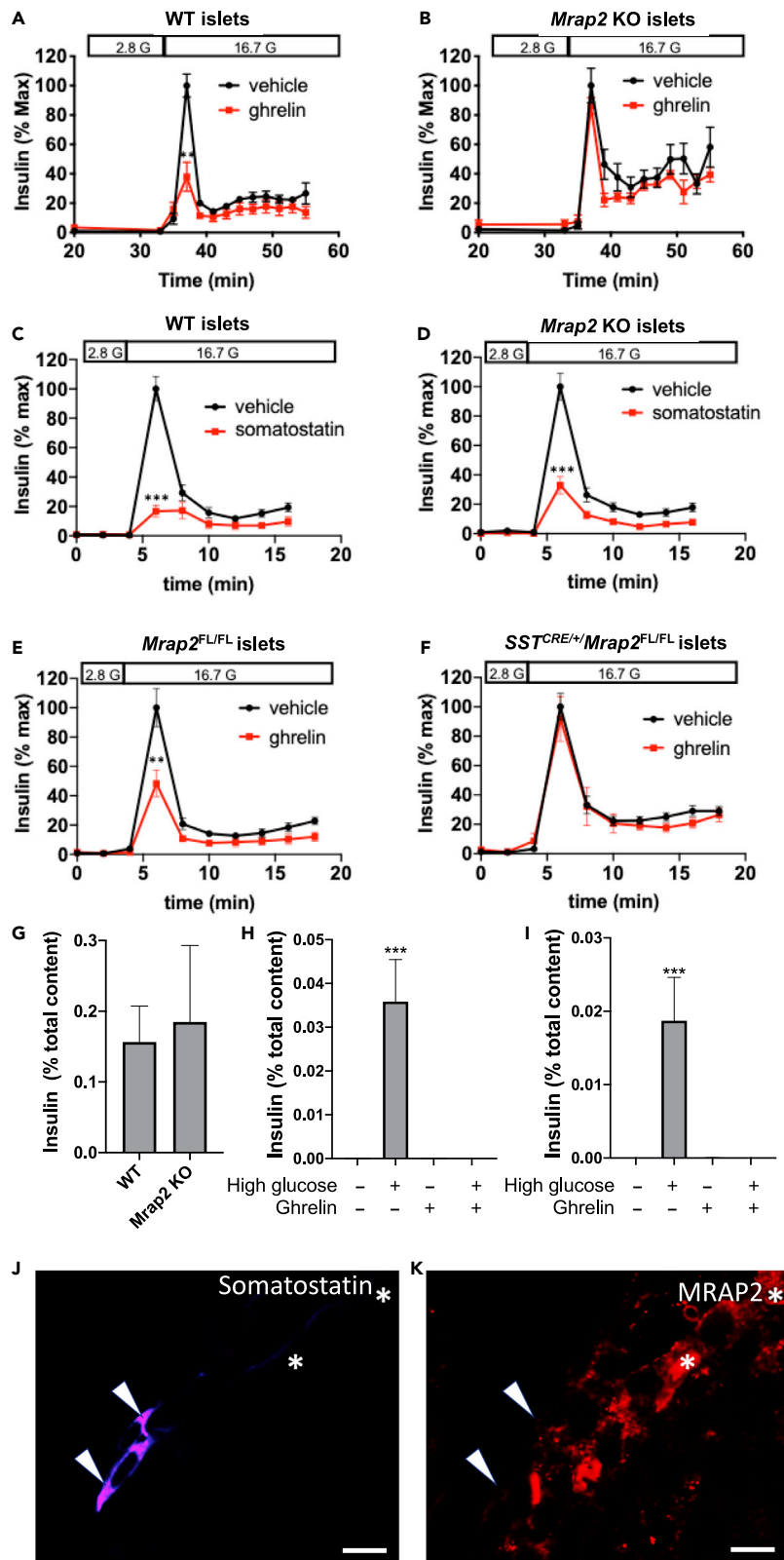


Figure 3. Inhibition of Glucose-Stimulated Insulin Secretion by Ghrelin Requires MRAP2 Expression in δ cells

(A and B) GSIS from islets (30–40/chamber) isolated from WT (A) and *Mrap2* KO (B) mice in the absence (black) or presence (red) of ghrelin.

(C and D) GSIS from islets (30–40/chamber) isolated from WT (C) and *Mrap2* KO (D) mice in the absence (black) or presence (red) of somatostatin.

(E and F) GSIS from islets (30–40/chamber) isolated from MRAP2^{FL/FL} (E) and SST^{CRE/+}/MRAP2^{FL/FL} (F) mice in the absence (black) or presence (red) of ghrelin.

(G and H) Static measurement of accumulated insulin release from WT (G) or SST^{CRE/+} (H) islets following indicated treatment.

(I) Histogram depicting the peak GSIS from WT and *Mrap2* KO islets normalized to total insulin content.

(J and K) Immunofluorescence microscopic detection of somatostatin and MRAP2 in pancreas slices from MRAP2^{FL/FL} (J) and SST^{CRE/+}/MRAP2^{FL/FL} (K) mice. Images are representative of results obtained from four mice per genotype and analysis of four to seven islets per mouse. Scale bars are 10 μ m. Results are mean \pm SEM of three independent experiments performed in duplicates or triplicates. **p < 0.01, ***p < 0.001. Arrowheads point to δ -cells, asterisks indicate non- δ -cells expressing MRAP2.

insulin secretion between WT and *Mrap2* KO islets (Figure 3I). We also verified the specific deletion of MRAP2 from δ cells in SST^{CRE/+}/MRAP2^{FL/FL} mice using immunofluorescence detection of MRAP2 in pancreas slices. MRAP2 was detectable in δ cells of MRAP2^{FL/FL} control mice (Figure 3J); however, MRAP2 was only detectable in a subset of somatostatin-negative cells but absent from somatostatin-positive δ cells in SST^{CRE}/MRAP2^{FL/FL} mice (Figure 3K). Together those results demonstrate that MRAP2 expression in δ cells is required for ghrelin to inhibit insulin secretion.

MRAP2 Expression in δ Cells Is Required for GHSR1a Signaling

Our previous studies in the hypothalamus demonstrated that ghrelin signaling through GHSR1a is strongly potentiated by MRAP2. To determine if MRAP2 is similarly required for ghrelin to stimulate GHSR1a signaling in δ cells, we took advantage of the GCaMP6 fluorescent calcium reporter. We bred SST^{CRE/+}/MRAP2^{FL/+} male mice with GCaMP6^{+/-}/MRAP2^{FL/+} female mice (GCaMP6 allele is downstream of Flox-stop-Flox sequence so that expression is dependent on CRE) to produce control SST^{CRE/GCaMP6}/MRAP2^{+/+} (WT expressing δ cell specific GCaMP6) and SST^{CRE/GCaMP6}/MRAP2^{FL/FL} mice with targeted expression of GCaMP6 and deletion of MRAP2 exclusively in somatostatin-positive δ cells. Using isolated islets from littermates of both genotypes, we examined their Ca²⁺ responses to ghrelin. As expected, GCaMP6 fluorescence was only detectable in a small number of islet cells consistent with the low abundance of δ cells (Figure 4A). Calcium concentration in δ cells, as measured by the fluorescence intensity of GCaMP6, was recorded in the absence of ghrelin to acquire baseline and then for 6 min after addition of 10 nM ghrelin. Examples of GCaMP6 fluorescence in δ cells from SST^{CRE/GCaMP6}/MRAP2^{+/+} and SST^{CRE/GCaMP6}/MRAP2^{FL/FL} before and after addition of ghrelin are depicted in Figures 4B and 4C. In islets from SST^{CRE/GCaMP6}/MRAP2^{+/+} mice, ghrelin induced a significant increase in intracellular calcium in δ cells owing to the activation of GHSR1a (Figures 4D and 4E). The targeted deletion of MRAP2 in δ cells resulted in a loss of ghrelin-stimulated calcium response (Figures 4D and 4E). This result establishes that MRAP2 is essential for ghrelin-mediated stimulation of GHSR1a in δ cells and explains the inability of ghrelin to inhibit GSIS in *Mrap2* KO and SST^{CRE/+}/MRAP2^{FL/FL} mice.

DISCUSSION

Ghrelin is an essential survival hormone, especially during starvation periods in which it is secreted by the stomach (Mani and Zigman, 2017). It acts on the hypothalamus to promote food intake (Asakawa et al., 2001; Shintani et al., 2001; Wren et al., 2001) and on the pituitary to stimulate the release of growth hormone (Howard et al., 1996; Date et al., 2000). Ghrelin also acts on the pancreas to inhibit insulin secretion (Broglia et al., 2001; Dezaki et al., 2004, 2007; Reimer et al., 2003; DiGruccio et al., 2016). The increase in growth hormone and decrease in insulin both act to prevent hypoglycemia during fasting. The mechanism of action of ghrelin on hypothalamic neurons is well understood, whereas the mechanism through which ghrelin inhibits insulin secretion has been controversial. It was hypothesized that GHSR1a, which is known to preferentially couple to G $\alpha_{q/11}$, instead couples to G α_i in β cells and thus elicits an inhibitory signal on insulin secretion (Dezaki et al., 2004, 2007); however, recent studies have demonstrated that GHSR1a is not expressed in β cells but rather exclusively expressed in δ cells (Adriaenssens et al., 2016; DiGruccio et al., 2016). This raised the possibility that, through G $\alpha_{q/11}$ coupling in δ cells, ghrelin could stimulate somatostatin secretion, which in turn would act in a paracrine manner on δ cells to decrease insulin release. This hypothesis was supported by the finding that ghrelin directly stimulates somatostatin release and the inhibitory effect of ghrelin on GSIS can be blocked by a somatostatin receptor antagonist (DiGruccio et al., 2016).

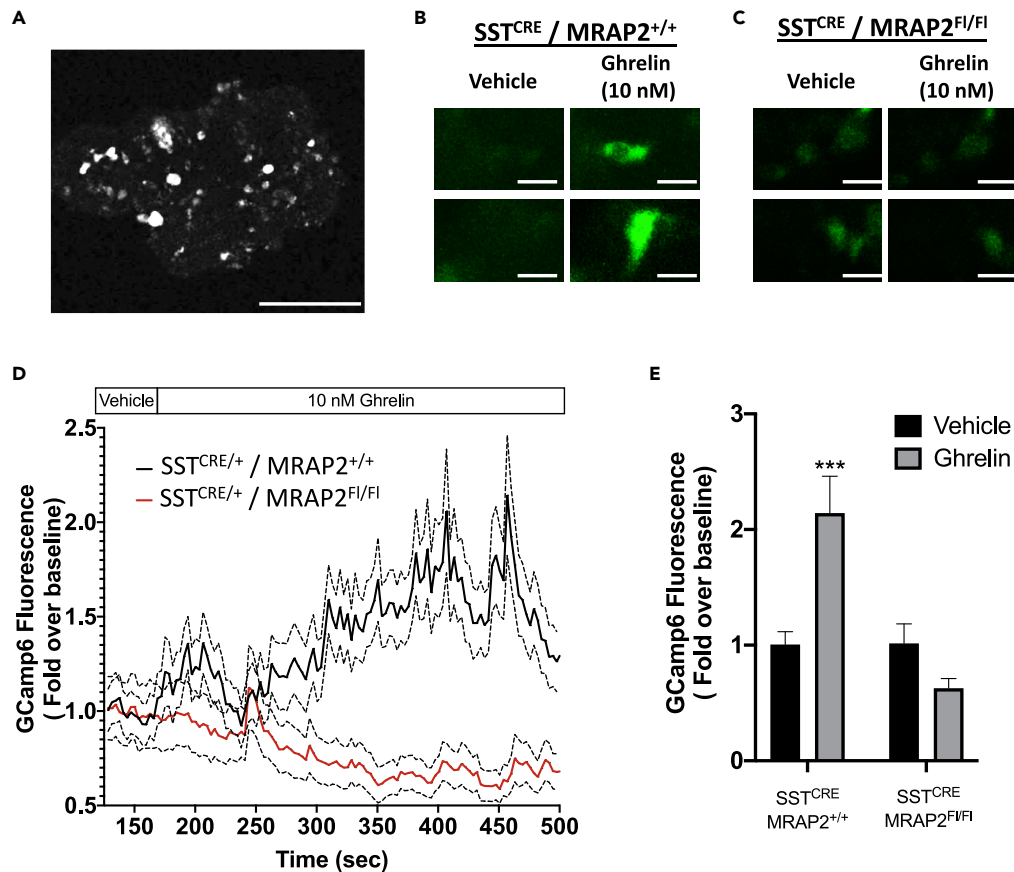


Figure 4. Ghrelin-Stimulated Calcium Signal in δ cells Requires MRAP2

(A) Confocal image of a mouse islet isolated from $SST^{CRE/GCamp6}$ mouse. Scale bar is 50 μm .
 (B and C) Examples of ghrelin-elicited calcium responses, as monitored by GCamp6 fluorescence, in δ cells of islets isolated from $SST^{CRE/GCamp6}/MRAP2^{+/+}$ (B) and $SST^{CRE/GCamp6}/MRAP2^{FL/FL}$ (C). Scale bars are 10 μm .
 (D) Confocal recording of GCamp6 fluorescence in δ cells from $SST^{CRE/GCamp6}/MRAP2^{+/+}$ (black) and $SST^{CRE/GCamp6}/MRAP2^{FL/FL}$ (red) perfused with 10 nM Ghrelin. Traces represent mean \pm SEM of 126 δ cells from $SST^{CRE/GCamp6}/MRAP2^{+/+}$ islets and 76 δ cells from $SST^{CRE/GCamp6}/MRAP2^{FL/FL}$ islets.
 (E) Mean GCamp6 fluorescence intensity in δ cells of $SST^{CRE/GCamp6}/MRAP2^{+/+}$ and $SST^{CRE/GCamp6}/MRAP2^{FL/FL}$ after 5 min treatment with vehicle or 10 nM ghrelin compared with baseline. *** $p < 0.001$.

Our data provide additional support for the mechanism of ghrelin action on the β cell to occur indirectly through δ cell activation. In this report, we have identified the GPCR accessory protein, MRAP2, as a key regulator of ghrelin signaling in the pancreatic islet. We demonstrate that, in the pancreatic islet, MRAP2 is expressed in δ cells and is required for the insulinostatic effect of ghrelin. We show that deletion of MRAP2 impairs ghrelin-induced Ca^{2+} transients in δ cells, which is consistent with GHSR1a signaling via $G\alpha_{q/11}$, rather than $G\alpha_i$. Importantly, the suppressive effect of somatostatin on β cell function was retained, thus further suggesting that MRAP2 regulation of ghrelin action is primarily mediated upstream in the δ cell through GHSR1a. The requirement of MRAP2 for the insulinostatic effect of ghrelin reveals that the importance of MRAP2 for GHSR1a signaling is not limited to AGRP neurons but extends to pancreatic cells and thus further establishes MRAP2 as an important partner of the receptor.

Although the insulinostatic action of somatostatin is well documented, the physiological role of δ cell function has been elusive (Rorsman and Huisig, 2018). Given the incredibly short half-life (~ 1 min) of somatostatins (SST-14 or SST-28) in circulation, the primary role of islet-derived somatostatin is likely to be paracrine, rather than endocrine, acting to attenuate α and β cell responses (Caicedo, 2013; DiGrucchio et al., 2016). Our studies on ghrelin signaling as well as others highlight an important role for endocrine to paracrine regulation within the islet as a critical physiological rheostat to temper islet hormonal responses and support a central role for the δ cell in this process.

Limitations of the Study

Although our results demonstrate the importance of MRAP2 for ghrelin signaling in δ cells, this study was performed on isolated islets. Consequently, the overall impact of ghrelin action on the pancreas for the regulation of insulin secretion in a live animal remains to be identified.

Resource Availability

Lead Contact

Further information and reagent request should be directed to the lead contact, Julien A. Sebag (julien-sebag@uiowa.edu).

Material Availability

This study has not generated new material.

Data and Code Availability

All data generated for this study are included in the manuscript.

METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2020.101216>.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.C.Y., S.B.S., and J.A.S.; Methodology, T.C.Y., S.B.S., and J.A.S.; Investigation, T.C.Y., A.A.J.R., C.J.B., and J.A.S.; Formal Analysis, T.C.Y. and J.A.S.; Writing – Original Draft, J.A.S.; Writing – Review and Editing, J.A.S. and S.B.S.; Project Administration, J.A.S.; Funding Acquisition, J.A.S.

DECLARATION OF INTERESTS

The authors declare no competing interest.

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