GFAP-directed Inactivation of _Men1_ Exploits Glial Cell Plasticity in Favor of Neuroendocrine Reprogramming

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**SUMMARY**

Loss of the tumor suppressor protein menin in glial fibrillary acidic protein-expressing cells stimulates gastric neuroendocrine hyperplasia and neuroendocrine tumors in the pituitary and pancreas. Deletion of menin favors glial cell reprogramming toward a neuroendocrine phenotype with tumorigenic potential.

**BACKGROUND & AIMS:** Efforts to characterize the signaling mechanisms that underlie gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) are precluded by a lack of comprehensive models that recapitulate pathogenesis. Investigation into a potential cell-of-origin for gastrin-secreting NENs revealed a non-cell autonomous role for loss of menin in neuroendocrine cell specification, resulting in an induction of gastrin in enteric glia. Here, we investigated the hypothesis that cell autonomous _Men1_ inactivation in glial fibrillary acidic protein (GFAP)-expressing cells induced neuroendocrine differentiation and tumorigenesis.

**METHODS:** Transgenic _GFAP<sup>Men1<sup>−/−</sup></sup>_ mice were generated by conditional GFAP-directed _Men1_ deletion in GFAP-expressing cells. Cre specificity was confirmed using a tdTomato reporter. _GFAP<sup>Men1<sup>−/−</sup></sup>_ mice were evaluated for GEP-NEN development and neuroendocrine cell hyperplasia. Small interfering RNA-mediated _Men1_ silencing in a rat enteric glial cell line was performed in parallel.

**RESULTS:** _GFAP<sup>Men1<sup>−/−</sup></sup>_ mice developed pancreatic NENs, in addition to pituitary prolactinomas that phenocopied the human MEN1 syndrome. _GFAP<sup>Men1<sup>−/−</sup></sup>_ mice exhibited gastric neuroendocrine hyperplasia that coincided with a significant loss of GFAP expression. _Men1_ deletion induced loss of glial-restricted progenitor lineage markers and an increase in neuroendocrine genes, suggesting a reprogramming of GFAP<sup>+</sup> cells. Deleting _Kif3a_, a mediator of Hedgehog signaling, in GFAP-expressing cells attenuated neuroendocrine hyperplasia by restricting the neuroendocrine cell fate. Similar results in the pancreas were observed when _Sox10_ was used to delete _Men1_.

**CONCLUSIONS:** GFAP-directed _Men1_ inactivation exploits glial cell plasticity in favor of neuroendocrine differentiation. (Cell Mol Gastroenterol Hepatol 2022;14:1025–1051; https://doi.org/10.1016/j.jcmgh.2022.06.009)

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prevalence across the United States. GEP-NENs are comprised of hormone-producing cells and include gastric carcinoids, gastrinomas, and pancreatic neuroendocrine tumors (NETs). The development of GEP-NENs is associated with sporadic and inherited mutations in the Multiple Endocrine Neoplasia I (MEN1) gene. Inactivation of the MEN1 locus causes loss of the tumor suppressor protein menin and coincides with the development of endocrine tumors in the pancreas, pituitary, and upper gastrointestinal (GI) tract. Patients carrying a MEN1 mutation are predisposed to developing gastrinomas, a GI NET that produces excess levels of gastrin, a peptide hormone that stimulates acid secretion and parietal and enterochromaffin cell hyperplasia. Such MEN1-associated gastrinomas preferentially develop in Brunner’s glands (BGs) located within the duodenal submucosa, with an estimated >50% frequency of MEN1 gastrinomas exhibiting lymph node metastases at the time of diagnosis.

Loss of menin function is a critical event underlying the formation of MEN1 gastrinomas; however, the signaling cues that regulate menin-mediated suppression of gastrin remain elusive. Homozygous deletion of Men1 in utero is embryonic lethal in mice, whereas heterozygous inactivation promotes endocrine tumors of the pancreas and pituitary, but not in the luminal GI tract. Importantly, these studies did not identify potential cells-of-origin for tumor development. Subsequently, we reported the development of the first genetically engineered mouse model that displays gastric NET development and gastric neuroendocrine hyperplasia, hypergastrinemia, and gastric NETs. Systemic gastric acid suppression using a proton inhibitor accelerated gastric NET development and coincided with the emergence of hyperplastic gastrin-expressing enteric glial cells marked by glial fibrillary acidic protein (GFAP) expression. The plasticity of glial cells directed towards an endocrine phenotype coincided with a reversible and non-cell autonomous loss of menin. Taken together, these observations suggest that hyperplastic G-cells might emerge from reprogrammed neural crest-derived cells in addition to endoderm-derived enteroneuroendocrine cells. Indeed, prior studies have shown that multipotent glial cells marked by GFAP or SRY-box transcription factor 10 (SOX10) can generate neuroendocrine cells during normal development and upon overexpression of oncogenes such as MYCN. Therefore, we tested the hypothesis that cell autonomous deletion of Men1 in GFAP-expressing cells promotes neuroendocrine cell development. Here, we show that conditional deletion of Men1 in GFAP-expressing glial cells results in NET development in the pituitary and pancreas, in addition to neuroendocrine hyperplasia in the stomach. These events coincided with loss of the GFAP-restricted lineage and emergence of an endocrine phenotype with tissue-specific hormone expression, suggesting a direct role for glial cell reprogramming upon removal of menin. Directing Men1 deletion to SOX10-expressing cells recapitulated pancreatic NET development and gastric neuroendocrine hyperplasia, further supporting the premise that a neural crest-derived cell might be a cell-of-origin for GI NETs.

**Results**

**GFAPΔMen1 Mice Develop Pancreatic Neuroendocrine Neoplasms (PNENs)**

To determine whether loss of menin in GFAP-expressing cells was sufficient to drive the development of GEP-NENs, we conditionally deleted Men1 under the control of 2.2 kb of the human GFAP promoter (GFAPΔMen1). Human GFAP expression is restricted to cell types known to express GFAP, including enteric glial cells. Moreover, the specificity of GFAP for marking the glial lineage has been extensively reported previously. After 15 to 24 months of age, 11 of 22 GFAPΔMen1 mice developed macroscopic tumors throughout the pancreas (Table 1). Histological analysis of the pancreas identified significant islet hyperplasia and features characteristic of neuroendocrine neoplasms (Figure 1, A). Pancreatic lesions varied from poorly differentiated with a solid sheet-like morphology to highly differentiated with a rosette pattern (Figure 1, B–D). Consistent with their unique histology, highly differentiated neoplasms exhibited cytoplasmic or absent menin expression (Figure 1, E) and strong immunoreactivity for the neuroendocrine markers synaptophysin (SYP) and chromogranin A (CHGA) (Figure 1, E–F). In contrast, poorly differentiated tumors were negative for CHGA and exhibited low expression of SYP (Figure 1, G). To assess whether these tumors were indeed of pancreatic islet origin, we examined the expression of iroquois homeobox protein 2 (IRX-2), a tissue-restricted transcription factor required for alpha cell lineage specification. Both well-differentiated and poorly differentiated tumors exhibited strong nuclear expression of IRX-2, confirming an endocrine cell origin for both tumor types (Figure 1, F–G). Based on the proliferative index used to categorize human pancreatic NECs, we stained GFAPΔMen1 neoplasms for Ki-67 and classified the neoplasms as pancreatic neuroendocrine tumors (PNETs) (<2% Ki-67+ cells per high power field, HPF) (Figure 1, H–I) or...
pancreatic neuroendocrine carcinomas (PNECs) (>20% Ki-67 \(^\text{+}\) cells per HPF) (Figure 1, J). Classification of the Ki-67 \(^\text{HI}\)/CHGA \(^\text{NE}\)/SYP \(^\text{LO}\) neoplasms as PNECs is consistent with human NECs exhibiting reduced expression of the classical neuroendocrine biomarkers. \(^{25,26}\) Further characterization of highly differentiated PNETs revealed the presence of both hyperplastic INS1 (insulin)-expressing beta-cells and glucagon (GCG)-expressing alpha-cells (Figure 1, K–M). Consistent with positive staining for neuroendocrine markers, PNETs exhibited a 10-fold increase in Chga transcript and a trending but nonsignificant increase in Syp mRNA expression compared with normal pancreas (Figure 1, N).

PNETs were predominantly comprised of PNETs (approximately 83% of all PNEN-bearing mice), whereas PNECs were only observed in 2 mice (comprising 17% of all PNEN-bearing mice, one of which was heterozygous for \(Men1\) deletion). Notably, there was not a mixture of NETs and NECs in the same mice. As gender-related differences have been previously reported in patients with \(Men1\) syndrome, \(^{27,28}\) we further stratified the occurrence of \(GFAP^{\text{+}}Men1\) PNECs and PNETs by sex (Table 1; Figure 1, O). Both cases of PNECs occurred in female mice. The incidence of PNETs was slightly higher in female mice compared with male mice, occurring in 7 of 13 females vs 4 of 9 males over the age of 15 months (53% vs 44%, respectively). Interestingly, female \(GFAP^{\text{+}}Men1\) mice tended to present with multiple lesions compared with males that presented with a solitary tumor (Figure 1, P). Of the 7 female tumor-bearing mice, 6 (86%) had 2 or more PNETs compared with only 1 of 4 (25%) males. As anticipated, \(GFAP^{\text{+}}Men1\) mice exhibited significantly elevated levels of circulating GCG and INS1 hormones, with tumor-bearing mice generally exhibiting the highest hormone expression irrespective of sex (Figure 1, Q–R). Consistent with the opposing effects of GCG and INS1 on glucose regulation, serum glucose levels were unchanged in \(GFAP^{\text{+}}Men1\) mice regardless of sex and tumor status (Figure 1, S).

**Table 1. Summary of Phenotypes Observed in GFAP Cre; Men1\(^{FL/FL}\) Mice Aged 13 to 24 Months**

| Phenotypical Characterization | Pancreatic hyperplasia/tumor, n/N; average age | Pituitary adenoma, n/N | Antral hyperplasia/tumor, n/N | BG/duodenal polyp, n/N | SI lipoma, n/N |
|-----------------------------|-----------------------------------------------|-----------------------|-----------------------------|-----------------------|----------------|
| WT                          | 0/30; 17.5 mo                                 | 0/30                  | 0/30                        | 0/30                  | 0/30           |
| FL/+                        | 1/9; 17.9 mo                                  | 1/9                   | 1/9                         | 1/9                   | 0/9            |
| FL/FL                       | 11/36; 17.9 mo                                | 18/39                 | 11/36                       | 2/36                  | 2/36           |
| 11/22: 15 mo and older      | • 7/13 female                                 | • 2/7 female          | • 2/27 female               | • 2/27 female         | • 2/27 female  |
|                             | • 1/9 male                                    | • 3/9 male            |                             |                       |                |
|                             | 0/14; Under 14 mo                             |                       |                             |                       |                |

BG, Brunner’s glands; SI, small intestinal; WT, wild-type.

Development of PNETs in \(GFAP^{\text{+}}Men1\) Mice Coincides With Loss of \(GFAP\) Expression

Due to the low overall incidence of PNECs, we focused our subsequent investigation on PNETs only. To determine the effect of \(Men1\) loss on \(GFAP\) expression in PNETs, we stained normal pancreas and PNETs for \(GFAP\). Consistent with previous reports, \(GFAP\) expression was strongly localized to the periphery of SYP \(^\text{+}\) and hormone-expressing islets in both wild-type and \(GFAP^{\text{+}}Men1\) mice; \(^{19}\) however, \(GFAP\) expression was uniquely lost in cells encircling the PNETs (Figure 2, A–B). Consistent with this loss, the expression of glial lineage transcripts \(Gfap\) and \(SI00b\) were significantly lower in PNETs compared with the pancreas of wild-type (WT) mice, whereas \(Men1\) mRNA exhibited a trending decrease (Figure 2, C). The specificity of \(GFAP\) marking the glial lineage was confirmed by showing that \(GFAP\) did not colocalize with phospho-Neurofilament-H (NF-H)-expressing nerve fibers in the pancreas and in PNETs (Figure 2, A–B). To further evaluate the specificity of \(GFAP\) expression, we generated \(GFAP\) Cre-loxP-tdTomato mice and confirmed that fluorescent tdTomato protein expression associated with \(GFAP\) and the glial lineage markers \(S100b\) and \(p75^{\text{NTR}}\) in frozen mouse pancreas sections, as previously reported \(^{19}\) (Figure 2, D–E). As expected, GFAP-tdTomato expression did not co-localize with NF-H \(^\text{+}\) nerve fibers, further confirming the specificity of \(GFAP\) marking the glial lineage. In summary, development of well-differentiated PNETs in this model coincided with loss of \(GFAP\) expression from the periphery of normal pancreatic islets.

We next evaluated whether \(GFAP^{\text{+}}Men1\) PNETs carried the potential to propagate in 3-D organoid culture as a measure of stemness. Organoid lines were established from PNETs from 4 mice and cultured for 3 weeks prior to staining or cryopreservation (Figure 2, F). Similar to parent tumors, the organoids strongly expressed SYP, CHGA, GCG, and INS1, but only a few cells exhibited Ki-67 positivity (Figure 2, F). Therefore, PNET-derived organoids recapitulated the biological features of their parent tumors and exhibited stem-like features in the absence of Ki-67 expression.

**GFAP^{\text{+}}Men1** Mice Develop PitNETs That Phenocopy Human \(Men1\) Prolactinomas

Nine of 30 female \(GFAP^{\text{+}}Men1\) mice presented with clinical signs of hydrocephalus starting at 15 months of age, including presentation of a domed head appearance, loss of
muscle coordination, seizures, and repetitive motor movements. Following the onset of hydrocephalus, mice were necropsied, and brains were analyzed for potential effects of GFAP-directed Men1 inactivation. The overarching rationale for these studies was emphasized by the fact that GFAP is the main intermediate filament protein in brain astrocytes and other cells of the glial-restricted lineage. We discovered that GFAP<sup>Men1</sup> mice developed hemorrhagic pituitary adenomas (Figure 3, A–B). Subsequent immunostaining of GFAP<sup>Men1</sup> adenomas demonstrated diffuse immunoreactivity for prolactin and CHGA as expected (Figure 3, C). Strong staining for prolactin showed these tumors to be prolactinomas (Figure 3, D). As prolactin is produced by lactotropes of the PIT-1<sup>+</sup> lineage, we stained pituitary NETs for the cell-patterning transcription factor PIT-1. As expected, tumors exhibited strong expression of PIT-1, thus confirming that both pancreatic and pituitary NETs arise in the context of endocrine lineage-specific transcription factor expression (Figure 3, E). Additionally, Ki-67 inversely correlated with the expression of neuroendocrine markers, with stronger neuroendocrine status associated with lower Ki-67 staining (Figure 3, F). Because cells of the PIT-1<sup>+</sup> lineage also comprise growth hormone (GH)-expressing somatotrophs but not adrenocorticotrophic hormone (ACTH)-expressing corticotropes, we stained pituitary tumors for GH and ACTH. Tumors exhibited patchy but generally robust expression of GH, whereas the expression of ACTH was strongly reduced in prolactinomas (Figure 3, G). Strong expression of proliferative index (PI) is indicated by the percentage of Ki-67-positive tumor cells in each HPF. A proliferative index (PI) of 10% indicates that 10% of tumor cells in the field are proliferating. Because cells of the PIT-1<sup>+</sup> lineage appear to arise from neural stem cell lineages, we stained pituitary NETs for markers of the neural stem cell lineages, such as SOX2 and nestin, suggesting that these hormone-expressing tumor cells arose from a neural stem cell lineage (Figure 3, H). Like pitNETs that carry the potential to propagate as organoids, pitNETs also exhibited features of stemness that allowed them to be subcultured as 3-D neurospheres.

**Hyperplastic Reprogramming of the GI Epithelium in GFAP<sup>Men1</sup> Mice**

To determine whether loss of menin in enteroendocrine cells supports the development of NENs in the luminal GI tract, we evaluated the stomach and proximal small intestine of GFAP<sup>Men1</sup> mice for changes in enteroendocrine cell numbers and composition. Although no gastrinomas or small intestinal NENs were observed at 14 to 24 months of age, 11/36 GFAP<sup>Men1</sup> mice (30%) showed hyperplastic changes in the proximal and distal stomach (Table 1; Figure 4, A). We further observed the development of small intestinal lipomas and duodenal polyps emerging from the BSIs at a low incidence (2/36 mice; 5%). In contrast to the previous observations of sex differences in the occurrence of NETs, the incidence of gastric hyperplasia was similar between male and female mice (33% and 30%, respectively) (Figure 4, A).

Compared with WT mice, GFAP<sup>Men1</sup> mice exhibited histological evidence of hyperplasia and metaplasia marked by parietal cell atrophy (Figure 4, B–C). These events coincided with increased expression of CHGA and SYP in the corpus, and CHGA, SYP, and gastrin in the gastric antrum. Notably, CHGA and SYP
did not co-localize in all cells of the proximal and distal stomach, suggesting the presence of divergent endocrine cell populations. Whereas all gastrin$^+$ cells appeared to express SYP, not all G cells expressed CHGA (Figure 4, C). Increased expression of Chga, Syp, and Gast mRNA was confirmed by quantitative polymerase chain reaction (qPCR), and this was consistent with increased numbers of CHGA$^+$, SYP$^+$, and gastrin$^+$ cells in the gastric antra of GFAP$^{ΔMen1}$ mice.
No differences in endocrine mRNA levels were observed in the proximal duodenum, indicating that hyperplastic reprogramming of endocrine cells was limited to the stomach. Consistent with increased G cell numbers and tissue gastrin expression, GFApΔMen1 mice exhibited a small but significant increase in serum gastrin levels compared with littermate controls (Figure 4, F). Previous studies in transgenic mice identified hypergastrinemia-induced changes to the gastric epithelium, including parietal cell atrophy.39,40 Here, we also observed decreased gastric acid levels in the setting of hypergastrinemia in GFApΔMen1 mice, consistent with the presence of metaplasia (Figure 4, G). qPCR analysis of other gastric cell types further confirmed decreased expression of the parietal cell-specific marker Atp4b and significant upregulation of Gif, a marker of zymogenic chief cells (Figure 4, H–I). Therefore, conditional deletion of menin in enteric GFAP-expressing cells stimulated neuroendocrine hyperplasia that is specific to the corpus and gastric antrum. However, unlike the pancreatic islets and pituitary, glial-directed Men1 deletion was insufficient to convert the hyperplastic endocrine cells into NETs.

(Figure 4, D–E).
Loss of Menin Reduces GFAP Expression in GFAP<sup>ΔMen1</sup> Mice and In Vitro

Because NET development in the pituitary and pancreas coincided with loss of GFAP expression, we examined the impact of Men1 deletion on the fate of GFAP-expressing cells in vivo. We performed fluorescent imaging of both whole tissue mounts and frozen sections of GFAP Cre-tdTomato mice to confirm the specificity of GFAP Cre expression throughout the stomach (Figure 5, A–B). Consistent with previous characterization of this model, fluorescent tdTomato protein expression was strongly localized to the myenteric plexus in whole tissue mounts (Figure 5, A). Further, tdTomato expression co-localized at the cellular level with GFAP protein and the glial lineage markers S100B and p75<sup>NTR</sup>, but not with NF-H<sup>+</sup> nerve fibers, as expected (Figure 5, B). Surprisingly, ex vivo fluorescent imaging of tissues from GFAP<sup>ΔMen1</sup> mice revealed a ~4-fold reduction in the tdTomato signal across the upper GI tract compared with wild-type mice (Figure 5, C–D). Imaging of frozen tissue sections confirmed near-loss of tdTomato fluorescence in the mucosa and lamina propria, whereas some signal was still retained in the submucosal and myenteric plexi (Figure 5, E). Therefore, deletion of Men1 in GFAP<sup>+</sup> cells extinguished GFAP expression.

A prior study showed that GFAP and menin proteins interact in astrocytes, suggesting that the 2 proteins might be interdependent. To test whether this interaction also occurs in GFAP-expressing glial cells, we performed co-immunoprecipitation using cytoplasmic extracts from a rat enteric glial cell line (EGC) and confirmed interaction between GFAP isoforms and menin (Figure 4, F–G). Studies were performed in EGCs as they are known to express GFAP and menin, and these cells also exhibit robust growth in culture compared with primary glial cells. To test whether loss of menin in a GFAP-expressing cell type modulates the expression of GFAP, we used small interfering RNA (siRNA) to knockdown Men1 expression in GFAP<sup>+</sup> EGCs. Consistent with the in vivo observations, Men1 silencing decreased the expression of GFAP and S100B at the transcript and protein levels (Figure 5, H–J). Taken together, these results indicated a role for menin in regulating glial cell identity by modulating GFAP expression.

Transcriptome-wide Analysis of Gastric Neuroendocrine Hyperplasia and Pituitary NETs Supports Glial-to-neuroendocrine Reprogramming

Because GFAP-specific deletion of menin was sufficient to drive NET formation in the pituitary and pancreas but not in the stomach or intestine, we used RNA-Seq to identify the molecular pathways that might contribute to the transition from neuroendocrine hyperplasia to NET development. GFAP<sup>ΔMen1</sup> pitNETs, gastric antrum, and their respective WT counterparts were submitted for bulk RNA sequencing. Unsurprisingly, the number of significant differentially expressed genes (DEGs) was 10 times higher when comparing normal pituitary with pitNETs vs the antral tissues of both genotypes (Figure 6, A–B). As expected, a number of genes related to neuroendocrine differentiation were upregulated in both datasets (eg, Grp, Vip, Syp, and Ncam1/2 in antra and Prl, Chgb, Eno2, Cckbr, Vip, and Ascl2 in pitNETs) (Figure 6, C–D). Genes associated with the neuronal-restricted localization were significantly upregulated in GFAP<sup>ΔMen1</sup> tissues compared with WT controls (Figure 6, E). Furthermore, most upregulated antral genes were correlated with neural differentiation and acquisition of a neuronal cell fate, including Ngrf, Elav3/4, Nrtn1/2, Phox2b, Nos1, Tubb3, Uchl1, Ret, and Hoxb (Figure 6, E). PitNETs also exhibited increased expression of neural-specific transcripts (eg, Elav3/4, Nrtn1/2, and Tubb3) that coincided with significant downregulation of glial lineage-associated genes, including Gfap and others (Figure 6, F). Consistent with their ability to propagate in 3-D culture, pitNETs exhibited increased expression of neural crest cell transcripts related to stemness, including Vim, Fabp7, and Sox11 (Figure 6, F).

Enteric neurons express gastrin-releasing peptide (GRP), which was identified in the RNA-seq dataset as the...
most significantly upregulated transcript in GFAP\textsuperscript{Men1}\textsuperscript{−}\textsuperscript{−} gastric antra. Increased GRP provides a direct mechanism for increased antral gastrin levels in these mice. Hence, we evaluated whether GFAP-directed deletion of Men1 stimulates GRP expression leading to G cell expansion by staining the gastric antra of GFAP\textsuperscript{Men1}\textsuperscript{−}\textsuperscript{−} mice for GRP. As anticipated, GFAP\textsuperscript{Men1}\textsuperscript{−}\textsuperscript{−} mice exhibited increased antral expression of GRP, and its expression co-localized with SYP in the epithelial mucosa but did not overlap with NF-H\textsuperscript{+} nerve fibers (Figure 6, G–H). The specificity of GRP antibody binding...
Figure 5. Conditional deletion of menin in GFAP<sup>+</sup> cells stimulates reprogramming from a glial-restricted progenitor lineage. (A) Whole tissue mounts of proximal duodenum from Cre-negative and tdTomato-expressing mice showing tdTomato fluorescence localized to the myenteric plexus (MP). (B) Immunofluorescent images of frozen stomach sections from WT GFAP-tdTomato mice co-stained for the glial-restricted progenitor lineage markers GFAP, S100B, and p75<sub>NTR</sub>, showing strong localization to the same cell types. Negative localization with the nerve fiber marker NF-H serves as a control. Immunofluorescent images were merged on a transmitted light micrograph to distinguish submucosal layers. Widefield images (C) and quantitation (D) of tdTomato signal in the stomach and duodenum of WT and GFAP<sup>ΔMen1</sup> mice expressing tdTomato reporter. N = 4–5 mice per group; ***P < .001; ****P < .0001. (E) Representative images of cryosections of corpus (CP), gastric antrum (AT), and proximal duodenum (DUO) from WT and GFAP<sup>ΔMen1</sup> mice expressing tdTomato. (F) Co-immunoprecipitation (Co-IP) of menin from rat EGC lysate followed by Western blot for GFAP. Input is 5% of lysate used for IP. (G) Quantitation of band density in 3 Co-IP experiments comparing expression of GFAP with IP with IgG isotype control in cell extracts. (H) Expression of Men1 and the glial transcripts Gfap and S100b following siRNA-mediated Men1 silencing in cultured rat EGCs. N = 3 independent experiments; *P < .05; **P < .01; ***P < .001; ****P < .0001. (I) Western blot of menin and GFAP in whole cell lysates following 72-hour Men1 silencing in rat EGCs with (J) quantitation of band density normalized to loading control. N = 3 experiments; **P < .01; ***P < .001 by unpaired t test. All data are represented as mean ± standard deviation.
was further validated by preadsorption with recombinant GRP protein (Figure 6, I). Lastly, we assessed whether loss of menin in GFAP⁺ EGCs directly stimulates the expression of Grp and Syp to further explain a mechanism for the induction of Grp and other neuroendocrine transcripts in GFAPΔMen1 mice. Indeed, Men1 silencing in rat EGCs induced Grp and Syp mRNA expression (Figure 6, J). Taken together, these results demonstrate that Men1 deletion suppresses
the mature glial lineage in favor of a neuroendocrine phenotype, perhaps due to loss of GFAP.

**Transcriptome Analysis Identifies Molecular Targets in Neuroendocrine Differentiation Leading to Gastric Neuroendocrine Hyperplasia and NET Development in GFAP<sup>ΔMen1</sup> Mice**

To further home in on the molecular changes leading to gastric neuroendocrine hyperplasia and NET development, we identified significant DEGs associated with growth and developmental pathways. Consistent with the role of menin in regulating proliferation and endocrine cell development, GFAP<sup>ΔMen1</sup> pitNETs exhibited increased expression of genes involved in cell cycle inhibition, senescence, and differentiation (e.g., Cdkn1a, Cdkn2a, and Cend1), as well as tissue development (e.g., Shh, Nog, Bmp2/7, Bdnf, and Ezh2) (Figure 7, A–B). Increased expression of genes related to NET differentiation and cellular senescence identified by RNA-seq were further validated by qPCR of pituitary and pancreatic NETs and age-matched WT tissues (Figure 7, C–D). Datasets comparing GFAP<sup>ΔMen1</sup> gastric antra and pitNETs with their respective WT tissues showed an enrichment of genes involved in neuroactive ligand receptor interaction and synaptic signaling pathways known to be upregulated in NET development (Figure 7, E–F). Interestingly, GFAP<sup>ΔMen1</sup> pitNETs uniquely showed perturbations in the Hedgehog signaling pathway, which was consistent with increased expression of Shh mRNA and ciliary motor proteins known to facilitate transduction of Hedgehog signaling (Figure 7, B, C, and E). In contrast, increased expression of neuroendocrine and neural-lineage genes in GFAP<sup>ΔMen1</sup> antral extracts coincided with downregulation of cytokeratins and immune-related transcripts marking epithelial and immune cells, respectively (Figure 7, H–I). Collectively, transcriptome-wide sequencing identified the involvement of dysregulated cell cycle and developmental pathways prefiguring NET development and suggests a potential role for Hedgehog signaling in mediating the transition from neuroendocrine hyperplasia to tumorigenesis.

**Impairment of Sonic Hedgehog Signaling (SHH) in Enteric GFAP<sup>+</sup> Cells Attenuated Neuroendocrine Cell Hyperplasia in GFAP<sup>ΔMen1</sup> Mice**

Hedgehog signaling mediated by primary cilia is known to promote premature differentiation of neural progenitor cells toward the neuronal lineage.<sup>42</sup> As GFAP<sup>ΔMen1</sup> pitNETs exhibited significantly elevated SHH transcript levels, we investigated whether loss of Kif3a, a ciliary motor protein required for SHH signal transduction, blocks GFAP<sup>ΔMen1</sup> glia from developing a neuroendocrine phenotype. To further stimulate gastric neuroendocrine hyperplasia, GFAP<sup>ΔMen1</sup> mice were crossed onto a somatostatin null (Sst<sup>–/–</sup>) background. Somatostatin (SST) suppresses hormone secretion and gene expression, including gastrin, thus removal of Sst-mediated feedback inhibition increases gastrin levels. The effect of Kif3a deletion on neuroendocrine hyperplasia was accomplished by breeding GFAP<sup>ΔMen1</sup>;Sst<sup>–/–</sup> mice onto a Kif3a<sup>fl/fl</sup> genetic background (GFAP<sup>ΔMen1</sup>;Kif3a<sup>−/−</sup>). We next investigated whether Kif3a deletion could reverse the gastric hyperplasia observed in GFAP<sup>ΔMen1</sup> mice. Compared with littermate controls, GFAP<sup>ΔMen1</sup>;Kif3a<sup>−/−</sup> mice more frequently presented with severe dermatitis and polycystic livers, and thus were euthanized and necropsied at 12 months of age. GFAP<sup>ΔMen1</sup>;Kif3a<sup>−/−</sup> mice exhibited reduced hyperplasia in the corpus and gastric antrum compared with age-matched GFAP<sup>ΔMen1</sup> mice (Figure 8, A). To determine the effect of Kif3a deletion on enteroendocrine cell composition, we analyzed the expression of CHGA and gastrin in the stomach after sequential deletion of Sst, Men1, and Kif3a genes. Whereas GFAP<sup>ΔMen1</sup> mice exhibited hyperplasia of CHGA<sup>+</sup> and gastrin<sup>+</sup> cells, additive deletion of Kif3a appeared to reverse these events (Figure 8, A). These observations were supported by associated fluctuations in Chga and Gast transcript levels. Whereas deleting Men1 significantly increased the expression of Chga and Gast mRNA in the gastric antra, loss of Kif3a in the presence of Sst and Men1 deletions inhibited this induction (Figure 8, B–C). Similarly, analysis of gastric antral extracts from these mice showed reduced gastrin peptide levels in GFAP<sup>ΔMen1</sup>;Kif3a<sup>−/−</sup> mice compared with those carrying the Men1 deletion alone (Figure 8, D). Thus, blockade of SHH-mediated signaling in enteric GFAP<sup>+</sup> cells attenuated neuroendocrine cell hyperplasia in the stomach.

**SHH Signaling Through Primary Cilia Modulates GFAP Expression**

Because disruption of SHH signaling on GFAP<sup>+</sup>/menin-deficient cells resulted in reduced neuroendocrine cell hyperplasia, we further investigated a potential mechanism to bridge these events. We first confirmed that deletion of Kif3a resulted in the impairment of primary cilia on GFAP<sup>+</sup>...
SOX10-driven Deletion of Men1 Recapitulates Hyperplastic Reprogramming of the Gastric Epithelium and Leads to Accelerated Development of PNETs

GFAP is expressed at low levels in nestin+ neural stem cells and turns on more strongly in terminally differentiated cells along the glial lineage in addition to other cell types, including stellate cells. Therefore, we postulated that targeting Men1 deletion by a more specific driver of glial cell specification might potentiate NET development. The nuclear transcription factor Sry-related HMG Box gene 10 (Sox10) is expressed by neural crest cells and its expression is maintained in glial and melanocyte cell lineages, but not in other neural crest cell derivatives. Thus, we conditionally deleted Men1 in Sox10-expressing cells using the Sox10 Cre system expressed on the same background described previously. Unlike GFAPΔMen1 mice that showed no apparent adverse health effects, Sox10ΔMen1 mice initially presented with reduced body weight and increased morbidity by 10 weeks of age; however, no significant fluctuations in gastric pH were observed (Figure 10, A–C). These events were later circumvented by delaying the age of weaning by 1 week and providing mice with moistened chow. Following these interventions, Sox10ΔMen1 mice showed similar survival to littermate controls with no signs of adverse health or weight loss.

We next assessed whether surviving Sox10ΔMen1 mice aged 10 to 12 months developed endocrine hyperplasia akin to GFAPΔMen1 mice. Four of 7 Sox10ΔMen1 mice (57%) presented with PNETs, with the incidence being slightly higher in females compared with male mice when age-matched (60% vs 50%) (Table 2; Figure 10, D). Like GFAPΔMen1 mice, Sox10ΔMen1 mice also exhibited significantly elevated serum GCG levels compared with Sst−/− littermate controls (Figure 10, E). Intriguingly, serum INS1 levels were too low for detection using the same high range standard curve for INS1 determination in GFAPΔMen1 sera (1 ng/mL as the limit of detection). Surprisingly, glucose levels also trended lower in Sox10ΔMen1 mice (Figure 10, F). Although no pituitary tumors were observed in this strain at 10 to 12 months of age, Sox10ΔMen1 mice exhibited a trending but nonsignificant increase in circulating prolactin levels (Figure 10, G). Subsequent histological analysis of PNETs revealed rosette-like features typical of well-differentiated NETs (Figure 10, H). PNETs exhibited strong immunoreactivity for SYP; however, CHGA expression was restricted to a discreet cell population. Robust SYP expression overlapped with GCC, whereas CHGA+ cells appeared to overlap with INS1-expressing cells within tumors (Figure 9, E). Moreover, SYP+ tumor cells did not overlap with NF-H+ nerve fibers, indicating that tumor cells neither arise from, nor associate with nerve fibers (Figure 9, E).

An additional 21-month-old male mouse heterozygous for Men1 deletion (Men1+/–) presented with PNETs, severe corpus hyperplasia, and multiple macroscopic antral tumors (Table 2; Figure 9, F). Subsequent staining for endocrine markers identified the presence of a CHGA+ /SYP+ gastric NET in the fundic submucosa (Figure 9, G). Moreover, the gastric antrum was characterized by hyperplasia of CHGA+, SYP+, and gastrin+ cells, consistent with increased serum gastrin levels in these mice (Figure 9, G–H). Similar to GFAPΔMen1 mice, all G cells expressed SYP but not CHGA. Thus, cell autonomous inactivation of Men1 using Sox10 Cre as a more selective driver of the glial lineage accelerated the development of PNETs and recapitulated the hyperplastic gastric phenotype observed in GFAPΔMen1 mice.

Discussion

Progress in understanding GEP-NET pathogenesis is impeded by a lack of comprehensive in vivo models, in part due to tissue heterogeneity from which the neoplasms arise, and the paucity of driver mutations identified that precede

Figure 7. (See previous page). Transcriptome analysis identifies potential pathways in neuroendocrine differentiation leading to gastric neuroendocrine hyperplasia and NET development in GFAPΔMen1 mice. Heat maps of significant DEGs mapped to cell cycle (A) and development (B) in GFAPΔMen1 pitNETs compared with WT pituitaries. (C) Validation of select DEGs in pitNETs vs WT pituitary by qPCR. N = 4–5 mice per group. WT pituitary group represents 4 samples of 3 pooled pituitaries, for a total of 12 tissues in this group. *P < .05; ***P < .001 by 2-way analysis of variance. (D) Cross-validation of select pitNET-enriched transcripts in GFAPΔMen1 PNETs and WT pancreas. Data are represented as mean ± standard deviation. (E) Kyoto Encyclopedia of Genes and Genomes (KEGG) Ontology Pathway analysis of wild type pituitary and GFAPΔMen1 pitNETs showing the number of genes mapped to enriched pathways and their level of statistical significance. (F) KEGG Ontology Pathway analysis of gastric antrum from WT and GFAPΔMen1 mice showing the number of genes mapped to enriched pathways and their level of statistical significance. (G) Heat map showing enrichment of kinesin motor protein transcripts in GFAPΔMen1 pitNETs. Heat maps depicting downregulation of cytokeratins (H) and immune-related transcripts (I) in GFAPΔMen1 antral extracts.
We report here that conditional deletion of the tumor suppressor protein Men1 (menin) in GFAP+ cells induced neuroendocrine cell hyperplasia in the gastric antrum and the development of neuroendocrine tumors in the pituitary and pancreas.

Neuroendocrine cell hyperplasia and NET development coincided with an unexpected loss of GFAP expression and induction of neuronal and neuroendocrine-related genes in the stomach, pancreas, and pituitary. In contrast to pituitary and pancreatic NETs, which exhibited global reduction in GFAP protein and mRNA expression, the gastric antra of GFAPΔMen1;Kif3a;Sst−/− mice showed reduced GFAP-reporter expression but did not exhibit significant loss of GFAP at the transcript level. This observation may be explained in part by the fact that menin interacts and colocalizes with GFAP. Hence, it remains a possibility that conditional deletion of menin in GFAP+ cells disrupts proper localization of GFAP, leading to altered glial cell identity and creating a microenvironment.

Figure 8. Blockade of SHH signaling in GFAP+ cells attenuates gastric neuroendocrine differentiation in GFAPΔMen1 mice. (A) Representative hematoxylin and eosin (H&E) and immunofluorescent images of corpus and gastric antrum of GFAPΔMen1;Sst−/−, GFAPΔMen1;Kif3a;Sst−/−, and littermate control mice showing reduced numbers of CHGA- and gastrin-positive cells with additive Kif3a deletion. CHGA (B) and gastrin (C) mRNA expression is reduced in the gastric antra following Kif3a deletion. (D) Expression of antral gastrin peptide among the different genotypes as evaluated by enzyme immunoassay. N = 7–8 mice per group; **P < .01; ***P < .001. All data are represented as mean ± standard deviation.
sufficient for tumor formation in the pancreatic islet and pituitary but not in the gastric antrum or intestine.

Similar to previous reports of conditional Men1KO mouse models, we did not observe the development of small intestinal gastrinomas, suggesting that additional genetic or microenvironmental factors are required to stimulate neoplastic transformation in these tissues. To identify molecular features that might inform the transition from neuroendocrine hyperplasia to NET development, we compared the transcriptomes of hyperplastic antral tissues with well-differentiated NETs arising in this model. Although GFAP-directed Men1 deletion induced neural differentiation pathways at both tissue sites, NETs exhibited loss of more glial-restricted progenitor lineage-associated genes (e.g., Gfap, Aldh1l1, Slc1a3, Aqp4, and Olig1) and downregulation of factors involved in directing gliogenesis (e.g., Nfia, Nfib, Zfp36l1, Id3, Sox9). Thus, the transition from a glial-to-neuronal cell phenotype appears to promote...
Figure 10. SOX10-driven deletion of Men1 recapitulates hyperplastic reprogramming of the gastric epithelium and leads to accelerated development of PNETs. (A) Survival curve for Sox10ΔMen1 mice prior to delaying the age of weaning from 3 weeks of age to 4 weeks. Body weight (B) and gastric pH (C) of 10-week-old Sox10ΔMen1 mice prior to adjusting the age of weaning from 3 weeks to 4 weeks of age. Blue triangles = male mice; Red circles = female mice. (D) Number of Sox10ΔMen1 mice presenting with PNETs by 11 months of age, as stratified by sex. Levels of serum GCG (E) glucose (F) and prolactin (G) in Sox10ΔMen1 mice compared with littermate controls, with symbols indicating male and female mice with and without the tissue-involved tumors. N = 6–7 mice per group; **P < .01 by the unpaired Student t test. (H) Representative hematoxylin and eosin and immunofluorescent images of a well-differentiated Sox10ΔMen1 PNET stained for neuroendocrine and hormone markers. Hormone-expressing tumor cells are negative for expression of the nerve fiber marker NF-H. (I) Macroscopic images of tissues from a 21-month-old Sox10ΔMen1 mouse presenting with corpus hyperplasia, multiple antral adenocarcinomas, and a PNET. (J) Hematoxylin and eosin and immunofluorescent staining of the previous stomach tissues indicating the presence of a gastric NET and neuroendocrine cell hyperplasia. Colored arrows indicate the degree of co-localization of CHGA (green), SYP (red), and gastrin (white), with several gastrin⁺ cells expressing SYP but not CHGA. (K) Serum gastrin peptide levels in 6–11-month-old Sox10ΔMen1 mice as evaluated by enzyme immunoassay. N = 9–12 mice per group; *P < .01 by the unpaired Student t test. All data are represented as mean ± standard deviation.
the progression from neuroendocrine cell hyperplasia to tumor development. In support of this, NETs exhibit increased expression of genes associated with neural stem and progenitor cell status (e.g., Fabp7, Vim, Sox11, and Hoxb genes) and upregulation of neural crest-secreted factors that favor neurogenesis and restrict a glial cell fate (e.g., Bmp2, Bmp7, and Shh). A number of these signaling factors are components of the Hedgehog signaling pathway, known to play a role in directing neuronal differentiation at the expense of restraining gliogenesis and glial cell maturation. A role for menin in this process is highlighted by the fact that menin is a known epigenetic repressor of both canonical and non-canonical Hedgehog signaling. Moreover, these studies demonstrate that blockade of Hedgehog signaling-attenuated islet cell proliferation in a Men1-mediated insulinoma mouse model. Consistent with these reports, we found that attenuation of SHH signaling by disrupting primary cilia on GFAP cells reduced neuroendocrine hyperplasia by restricting neural differentiation of GFAP/menin-deficient cells toward the glial-restricted progenitor lineage.

Enteric glia exhibit a high degree of cellular plasticity within the context of their specific microenvironment. Indeed, they have been identified as a source of neural progenitor cells and are able to de-differentiate and transdifferentiate to other cell lineages under explicit physiological and in vitro conditions. However, it remains unknown whether neural crest cells or their progeny can undergo context-specific reprogramming (eg, by acquiring MEN1 mutations) and give rise to neuroendocrine cells with hormone-secreting capabilities. Evidence to support glial or astrocytic reprogramming would challenge the longstanding paradigm that neuroendocrine tumors in the GI tract must develop from enteroendocrine cells that originate from Lgr5 stem cells of endodermal origin. Prior work by our group using Villin4Men1 mice demonstrated that non-cell autonomous loss of menin protein in enteric GFAP glial cells induces gastrin hormone expression. Moreover, the loss of menin suggested crosstalk between enteroctyes and enteric glia. In the current study, cell autonomous deletion of Men1 in GFAP cells was insufficient to drive gastric NET formation. Thus, gastric NET development in Villin4Men1 mice likely requires non-glial cell autonomous signaling from the epithelium.

Furthermore, human duodenal NETs are known to express glial cell markers, with some cells within the tumor exhibiting expression of both the neuroendocrine marker SYP and the glial-specific protein S100B. We recently used digital spatial profiling to characterize neuroglial features in a small subset of human duodenal NETs. Tumors exhibited reduced expression of mature neuronal and glial cell markers compared with the adjacent BGs from which 60% of these tumors are reported to originate. Collectively, these observations raise the potential for the presence of a potential hybrid transition state, in which reprogrammed enteric neural crest-derived cells escape a glial-restricted lineage and acquire a neuroendocrine phenotype.

Transcriptome-wide sequencing of the hyperplastic gastric antra of GFAPlabMen1 mice identified neural lineage genes previously reported to be upregulated in human distal duodenal gastrinomas emerging from the BGs, including Hap1, Mn1, and Uchl1. This suggests a potential concordance between the human BGs and the pyloric antra of mice. Indeed, a recent study by Wells and colleagues reported that transplantation of human antral organoids co-cultured with human ENCCs induced the formation of highly differentiated epithelium analogous to the BGs. Subsequent analysis of ENCCs identified elevated expression of the SHH-induced posteriorizing factors BMP4 and BMP7, and that inhibition of these factors in ENCCs attenuates BG formation in organoid co-cultures. Consistent with our observations, ENCCs and their progeny carry the potential to direct epithelial differentiation in favor of an endocrine phenotype, and these events are strongly influenced by SHH-mediated signaling. Importantly, our observations prompt further investigation into both glial cell autonomous and non-cell autonomous mechanisms presaging these outcomes.

In summary, we report the development of 2 glial-directed mouse models of human MEN1 syndrome with the aim of defining the contribution of neural crest cell reprogramming in neuroendocrine cell differentiation and tumorigenesis. Because it remains unknown at what point during the cell’s lifespan it transitions to a hormone-producing endocrine cell upon loss of menin, we used a constitutive Cre as reported here. Future work requires lineage tracing coincident with inducible deletion of Men1 using glial-restricted promoters to directly track GFAP+ and SOX10+ cells in NET development. By addressing a previously uncharacterized compartment of GEP-NETs, this study carries the potential to identify a unique cell-of-origin for these neoplasms. The importance of these discoveries is underscored by the fact that GEP-NETs comprise remarkably diverse neoplasms that vary in location, mutational profile, and response to therapy. Such heterogeneity may in part be explained by divergent cellular origins for NETs as a function of different tissue sites. Previous work centered on mapping their unique transcriptional signatures suggests the potential for neuroglial reprogramming in the development of human duodenal gastrinomas. Defining the cells-of-origin and the events preceding neoplastic transformation will be critical to informing molecular signaling pathways that can then be targeted therapeutically.

Materials and Methods

Animal Studies

All animal studies received approval by the University of Arizona Institutional Animal Care and Use Committee and conform to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Mice were housed in individually ventilated caging, with food and water ad lib. All mice were fasted overnight with water ad lib prior to blood collection. Mouse strains, Research Resource Identifiers, and vendors are listed in Table 3. GFAPlab-Cre transgenic mice on a C57BL/6J genetic background were purchased from Jackson Laboratories and bred onto a Men1FL/FL background to conditionally delete Men1 in GFAP-expressing cells (named GFAPlabMen1). A subset of wild type and GFAPlabMen1 mice
were bred onto a somatostatin-null background (Sst−/−). GFAPΔMen1;Sst−/− mice were further bred onto a Kif3aΔFL/FL background to conditionally delete Kif3a under the control of the Gfap promoter (named GFAPΔMen1;ΔKif3a;Sst−/−). WT, GFAPΔMen1, GFAPΔMen1;Sst−/−, and GFAPΔMen1;ΔKif3a;Sst−/− mice were also bred to mice carrying a lox-Stop-lox-TdTomato sequence to selectively express the tdTomato fluorescent reporter in GFAP+ cells. GFAPΔMen1 strains were necropsied at 8 to 9 months of age and between 13 and 24 months of age. All histological characterization and downstream comparisons were made using littermate controls that geno-

Table 2. Summary of Phenotypes Observed in Sox10 Cre; Men1FL/FL Mice Aged 10 to 21 Months

| Men1 | Pancreatic hyperplasia/tumor, n/N; average age | Pituitary adenoma, n/N | Antral hyperplasia/tumor, n/N |
|------|-----------------------------------------------|------------------------|-------------------------------|
| WT   | 0/6; 12.6 mo                                  | 0/6                    | 0/6                           |
| FL/+ | 1/1; 21 mo                                    | 0/1                    | 1/1 (gastric carcinoid and adenocarcinoma) |
| FL/FL| 4/7; 11 mo                                    | 0/7                    | 1/7                           |

WT, Wild-type.

Cell and Tissue Culture

**PNET organoids.** Organoids were generated from four 17 to 18 month old GFAPΔMen1 mice presenting with pancreatic neuroendocrine tumors. Tumors were carefully removed from adjacent pancreas tissue and washed in ice-old Dulbecco’s phosphate buffered saline (PBS) (minus Ca2+/Mg2+) containing RNase inhibitor. Tumors were minced using a razor blade, then collected in 10 mL of collagenase solution consisting of 1.5 mg/ml Type VIII collagenase, 40 μg/ml DNase I, 10 mM HEPES, 5% fetal bovine serum (FBS) dissolved in prewarmed Dulbecco’s PBS (minus Ca2+/Mg2+). Tissue was digested at 200 rpm for 40 minutes at 37 °C. The suspension was shaken 5 to 7 times prior to filtering through a 100-μm cell strainer and centrifuging at 400 × g for 5 minutes at 4 °C. The pellet was gently washed once with prewarmed media and re-pelleted. The pellet was resuspended in ice-cold Matrigel (Corning, Corning, NY), and 25 μL was pipetted into each well of a prewarmed 24-well plate. The plate was inverted and incubated at 37 °C for 15 minutes before supplementing with complete PNET organoid growth media consisting of a 1:1 solution of L-WRN conditioned media and Basal Growth Media (advanced DMEM/F12, 1 × B-27 supplement, 1 × N-2 supplement, 10 mM HEPES, 2 mM GlutaMAX, 1 × penicillin-streptomycin, 1 mM N-acetylcysteine). Growth media was supplemented with ascorbic acid (284 μM, Sigma), INS1 (20 μg/mL, Tocris), hydrocortisone (0.25 μg/mL, Sigma), retinoic acid (100 nM, Sigma), FGF-10 (100 ng/mL, Biolegend), Y-27632 (10 μM, Tocris), and A8301 (500 nM, Tocris).

**PitNET neurospheres.** Primary tumor neurospheres were generated from three 17- to 18-month-old female GFAPΔMen1 mice presenting with pituitary adenomas. A 4 × 2 mm piece of tissue was cut from the tumor and minced into 1-mm pieces on ice. The tissue was transferred to a 10 mL solution of Dispase/DNase solution warmed to 37 °C (0.1% dispase, 0.01% DNase, 10 mM HEPES, DPBS without calcium and magnesium) and incubated in a shaking water bath (60 rpm) at 37 °C for 80 minutes. Halfway through the incubation, the tissue was shaken manually 3 times to facilitate dissociation. The tissue was triturated 10 times with a 10 mL serological pipette, then filtered through a 40-μm cell strainer before centrifuging at 123 × g for 10 minutes at 4 °C. The cell pellet was resuspended in Neurosphere Media consisting of DMEM/F12 (ThermoFisher, Waltham, MA), 1 × GlutaMAX (Invitrogen, Carlsbad, CA), 1 × N2 (Gibco, Waltham, MA), 1 × B-27 (Gibco), 100 U penicillin-streptomycin (Invitrogen), 10 mM HEPES (Invitrogen), 30 ng/mL mouse recombinant FGF-10 (Biolegend, San Diego, CA), and 30 ng/mL recombinant human EGF (R&D Systems, Minneapolis, MN). Cells were plated in a non-tissue culture treated 6-well plate and incubated overnight at 37 °C. Media was changed every 2 to 3 days by collecting neurospheres, centrifuging, and reseeding the pellet in fresh medium. Tumor neurosphere generation was repeated with 3 prolactinoma-bearing mice.

A rat enteric glial cell line was purchased from ATCC (#CRL-2690) and grown in DMEM supplemented with 10% FBS and 100 U penicillin-streptomycin (Egg/PK060399egfr, ATCC, Manassas, VA). Upon reaching 30% to 50% confluence, glial cells were switched to 5% FBS-containing media and used for siRNA experiments. Both the non-targeting siRNA and Men1 siRNA consisted of a SMARTpool of 4 ON-TARGETplus siRNA constructs (#L-090784-02-0005 for Men1 pool and #D-001810-10-05 for non-targeting pool, Dharmacon Horizon Discovery, Lafayette, CO). Cells were transfected with 25 nM siRNA using Lipofectamine 3000 without p3000 reagent according to manufacturer
instructions (ThermoFisher). Following 48 to 72 hours post-transfection, cells were harvested for downstream RNA or protein extraction.

**Immunohistochemistry and Immunofluorescent Staining**

Mouse tissues were fixed overnight at 4 °C in 4% paraformaldehyde. Tissues were paraffin-embedded, cut into 5-μm sections, and placed on frosted glass slides. Slides were deparaffinized in 3 xylene washes, then rehydrated in 100%, 90%, and 70% ethanol washes. Slides were washed in TBS with 0.05% Tween-20 (TBST) before proceeding with antigen retrieval. Slides were boiled in 1 x sodium citrate buffer pH 6.0 for 30 minutes and allowed to cool to room temperature (RT) for 20 minutes prior to washing with TBST. Slides were incubated for 1 hour at RT in blocking buffer consisting of 10% donkey serum, 1% bovine serum albumin (BSA), 0.1% Triton X-100 in TBST. In some instances, tissues were permeabilized with 0.5% Triton X-100 for 5 minutes at RT prior to blocking. Tissues were incubated in primary antibody overnight at 4 °C. Primary antibodies were prepared in blocking solution according to the dilutions listed in Table 4. Following overnight incubation, slides were washed in TBST and incubated in Alexa Fluor-conjugated secondary antibodies (Molecular Probes, Eugene, OR; Invitrogen) diluted 1:500 in 1% BSA and TBST for 1 hour at RT in the dark. Slides were washed in TBST before mounting with #1.5 coverslips using Prolong Gold anti-fade mounting medium with DAPI (Life Technologies, Rockville, MD). Immunofluorescence was visualized at 200 x, 400 x, and 1000 x magnification using the Olympus BX53F epifluorescence microscope (Center Valley, PA).

For immunofluorescent staining of PNET organoids and PitNET neurospheres, organoids and neurospheres were collected and centrifuged. The pellet was resuspended in 3.7% paraformaldehyde and transferred to a 24-well 0.17-mm glass-bottom plate. Organoids and neurospheres were fixed for 20 minutes at RT, then washed with 1 x TBST. Samples were permeabilized with 0.5% triton X-100 for 10 minutes at RT, washed, then blocked in 10% donkey serum, 1% BSA, and 0.2% triton in TBST for 1 hour at RT. Primary antibodies were diluted in 5% donkey serum 1% BSA, 0.1% triton in TBST and added to wells for overnight incubation at 4 °C. Samples were washed thoroughly with TBST and incubated for 1 hour at RT in Alexa Fluor-conjugated secondary antibodies diluted 1:500 in 1% BSA and TBST. Organoids and neurospheres were washed with TBS, then stored in TBS containing Prolong gold anti-fade mounting medium with DAPI. Samples were imaged at 600 x magnification using a Nikon Ti2-E inverted microscope with a CREST X-Light V2 L-FOV Spinning Disk Confocal and a Photometrics Prime 95B-25MM Back-illuminated sCMOS Camera (Nikon, Tokyo, Japan).

Mouse tissues with endogenous tdTomato reporter expression were fixed in 4% paraformaldehyde for 1 hour on ice before dehydrating overnight at 4 °C in a solution of 30% sucrose-PBS. Tissues were then embedded in Tissue-Tek optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA) and frozen on dry ice. OCT blocks were cryosectioned to a 10-μm thickness and melted onto frosted glass slides prior to mounting with Prolong Gold anti-fade mounting medium containing DAPI. TdToma signal was visualized at 200 x (whole mount) and 1000 x magnification (co-stained images) using the Olympus BX53F epifluorescence microscope. Images drawing comparisons between groups were acquired with identical laser acquisition settings (Center Valley, PA).

**Antibody Pre-adsorption**

Selective antibodies were tested for specificity by pre-adsorption with recombinant proteins. Briefly, human recombinant protein was mixed with respective antibody at a 5-fold excess by weight. Antibody-antigen solution was diluted to a working concentration in 10% donkey serum, 1% BSA, 0.1% triton in TBST and incubated overnight at 4 °C on a rotator. The antibody-antigen solution was then used for immunohistochemistry/immunofluorescence staining as previously described and compared with staining with antibody only. Images were acquired using identical acquisition settings for comparison of fluorescence signal.

**qPCR**

RNA was extracted from frozen mouse tissues using the Purelink RNA Extraction Kit (Invitrogen). Tissues were homogenized with a rotor-stator homogenizer in RNA lysis buffer containing 1% β-mercaptoethanol, and RNA was isolated following manufacturer’s instructions. Extracted RNA was treated with ezDNase to remove any residual genomic DNA and then prepared for cDNA synthesis using Superscript VILO IV Master Mix according to manufacturer’s instructions (Invitrogen). Real-time qPCR was performed using PowerUp SYBR Green Master Mix (Invitrogen) with 20 ng cDNA added to each reaction. Predesigned forward and reverse primer sets were purchased from Integrated DNA Technologies (IDT, Coralville, IA) and used at a final 500 nM concentration. qPCR was performed using the QuantStudio 3 Real-Time PCR System (Applied Biosystems, Waltham, MA) with the following cycling conditions: 2 minutes at 50 °C, 2 minutes at 95 °C, denaturing step for 1 second at 95 °C, extension and annealing for 1 minute at 60 °C, followed by a dissociation melt curve stage to confirm primer specificity. All forward and reverse primers were purchased as validated predesigned PrimeTime qPCR Assay primer sets used for SYBR Green dye (Integrated DNA Technologies, Coralville, IA). qPCR data was expressed as fold-change using the established 2^-ΔΔCt method and log-transformed to fit a normal distribution prior to performing statistical analysis.75,76

**Ex Vivo Fluorescence Imaging**

At 8 to 9 months of age, GFAP Cre-tdTomato mice were euthanized following overnight fasting, and the stomach and proximal duodenum were collected for ex vivo imaging prior to fixation for OCT embedding. The stomach and proximal duodenum were opened along the greater
Table 3. List of Mouse Strains and Vendors

| Mouse strain name | Strain # | RRID # | Vendor/provider |
|-------------------|----------|--------|-----------------|
| FVB-Tg(GFAP-cre)25Mes/J | 004600 | IMSR_JAX:004600 | Jackson Laboratories |
| 129S(FVB)-Men1tm1.2Cwe/J | 005109 | IMSR_JAX:005109 | Jackson Laboratories |
| B6.Cg-Gf(TOSA)26Socpm14(CAG-tdTomato)Kzs/J | 007914 | IMSR_JAX:007914 | Jackson Laboratories |
| B6:CCBA-Tg(Sox10-cre)1Wdr/J | 025807 | IMSR_JAX:025807 | Jackson Laboratories |

RRID, Research Resource Identification.

curvature, and contents were flushed with cold PBS. Both tissues were placed in cold PBS with mucosa facing up and imaged in a dark room using a wide field fluorescence imaging system. Illumination was provided by a 300W xenon arc lamp source (LS-OF30, Sutter Instruments, Novato CA). Illumination light was filtered with a 554 nm excitation filter with 23 nm bandwidth (FF01-554/23-25, Semrock Inc., Rochester NY) and delivered using a fiber bundle (LLG, Sutter Instruments). Images were collected with an 0.5" CCD Monochrome Camera (EO-1312M, Edmund Optics, Barrington NJ) with a 35 mm fixed focal length lens (#59-872, Edmund Optics) and a 594 nm long-pass filter (BLP01-594R-25, Semrock) mounted on a rigid arm above the sample plane. Images were collected with an exposure time of 60 ms and saved in 16 bit .tif format. Each day, a flat-field image was collected by imaging a white diffuse reflectance target (#58-872, Edmund Optics) to correct for spatial non-uniformity using unfiltered illumination. Additionally, a power measurement was collected for the filtered illumination light at the sample plane using a power meter (S120C, Thorlabs, Newton NJ) to adjust for day-to-day variations of light source intensity. The collected images were first normalized through division by the flat-field image and the light source power measured on the day of sample acquisition. Regions of interest were then manually drawn around the forestomach, corpus, antrum and duodenum using ImageJ. Fluorescence intensity was calculated in the region of interest and averaged over the number of valid pixels. Any pixels with 5% of saturation (eg, digital value of 65535) were discarded from the analysis.

RNA Sequencing

RNA was extracted from 3 GFAPΔMen1 PNETs, pitNETs, and the pyloric antrum using the Purelink RNA Extraction kit as previously described (Invitrogen). Pancreas, pituitary, and antra of age-matched WT mice were used as controls. Due to its small size, 3 normal pituitaries were pooled for each sample, and a total of 3 samples were submitted for sequencing (ie, 9 WT pituitaries total). RNA was processed on the bioanalyzer for quality control prior to proceeding with mRNA library construction and bulk RNA sequencing. Due to degradation of pancreas tissues by pancreatic enzymes, only antral extracts and pituitary samples were usable for downstream library construction and sequencing. Samples were sequenced by Novogene using Illumina sequencing platforms and the resulting calculated fragments per kilobase of transcript sequence per millions base pairs sequenced values were analyzed for differential gene expression, principal component analysis, and Gene Ontology enrichment analysis. Differential expression between WT and GFAPΔMen1 groups was determined using the DESeq2 R package (1.20.0) using a negative binomial distribution model. P values were adjusted for multiple testing using the Benjamini and Hochberg’s method for controlling false discovery rate and genes with adjusted P value < .05 were considered significantly differentially expressed. The statistical enrichment of DEGs in Kyoto Encyclopedia of Genes and Genomes pathways was tested using the clusterProfiler R package with correction for gene length bias. Gene Ontology terms with a corrected P value < .05 were considered significantly enriched. Heatmaps of significant DEGs assigned to specific signatures of interest were generated using Python 3 using the open-source packages Seaborn and Pandas. For each gene, the Z-score was calculated and plotted as a function of color, with the color white being fixed to a Z-score of 0.

Western Blot Analysis

Seventy-two hours following siRNA-mediated Men1 silencing, rat enteric glial cells were harvested by washing twice in cold DPBS and lysing in cold RIPA buffer containing phosphatase and protease inhibitors. Cells were collected by scraping, then homogenized by passing through a 20G needle syringe 5 times. The resulting lysate was centrifuged at 15,000 × g for 20 minutes at 4 °C. The supernatant was collected and used for Western blot analysis as follows. Lysates were prepared in reducing conditions in 1× SDS buffer with 5% β-mercaptoethanol and denatured by boiling at 95 °C for 5 minutes. Fifteen µg of protein was loaded into a NuPage 4% to 12% Bis-Tris Mini Protein Gel and allowed to migrate for 1 hour at 140 V in 1× MOPS Gel Electrophoresis Buffer. Proteins were transferred onto a pre-wetted polyvinylidene fluoride membrane using the iBlot2 transfer system (P0 setting, 7 minutes), then blocked in 5% BSA in TBST for 1 hour at RT. Membranes were incubated overnight at 4 °C in primary antibody diluted in blocking buffer at the dilutions listed in Table 4. Membranes were washed in TBST, then incubated for 1 hour at RT in a corresponding host IgG horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:3000 in blocking buffer. Membranes were washed again in TBST and incubated in responding host IgG horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:3000 in blocking buffer.
| Primary antibodies | Vendor | Catalog # | RRID # | Dilution/application |
|--------------------|--------|-----------|--------|----------------------|
| Menin (Rabbit pAb) | Bethyl Laboratories | A300-105A | AB_2143306 | 1:2,000 (IHC/IF TSA) 1:10,000 (WB) |
| Chromogranin A | Abcam | 15160 | AB_301704 | 1:100 (IHC/IF) |
| Synaptophysin | Cell Signaling Technologies | 9020 | AB_2631095 | 1:200 (IHC/IF) |
| Irx-2 | Abcam | 196755 | AB_222302 | 1:100 (IHC/IF) |
| Ki-67 | Cell Signaling Technologies | 9449 | AB_2797703 | 1:400 (IHC/IF) |
| α-smooth muscle actin | Abcam | 5694 | AB_659831 | 1:100 (IHC/IF) |
| Glucagon | Cell Signaling Technologies | 2760 | AB_2126503 | 1:1000 (IHC/IF) |
| Insulin | Cell Signaling Technologies | 3014 | AB_10013382 | 1:400 (IHC/IF) 1:2000 (WB) |
| GFAP (Rabbit pAb) | Dako Agilent Technologies | Z0334 | AB_196755 | 1:100 (IHC/IF) |
| GFAP (Rat mAb) | Invitrogen | 13-0300 | AB_2532994 | 4 μg/mg lysate (IP) |
| GFAP (Chicken pAb) | Invitrogen | PA1-10004 | AB_1074620 | 1:1000 (IHC/IF) |
| Neurofilament-H | Novus Biologicals | NBP1-05209 | AB_1556328 | 1:100 (IHC/IF) |
| S100B | Abcam | ab52642 | AB_882426 | 1:200 (IHC/IF) |
| p75NTR | Abcam | ab52897 | AB_881682 | 1:1000 (IHC/IF) |
| ACTH | Novus Biologicals | NBP2-32911 | AB_1069955 | 1:100 (IHC/IF) |
| Growth hormone | R&D Systems | AF1067 | AB_354573 | 1:100 (IHC/IF) |
| Nestin | Novus Biologicals | NB100-1604 | AB_2282642 | 1:500 (IHC/IF) 1:100 (ICC/IF) |
| Sox2 | Novus Biologicals | NB110-37235 | AB_792070 | 1:200 (ICC/IF) |
| Pit-1 | Novus Biologicals | NB100-1604 | AB_11030310 | 1:500 (IHC/IF) |
| Prolactin | Abcam | 183967 | AB_2814839 | 1:200 (IHC/ICC/IF) |
| Gastrin (Rabbit mAb) | DAKO Agilent Technologies | A0568 | AB_2757270 | 1:1000 (IHC/IF) |
| Gastrin (Goat pAb) | Santa Cruz Technologies | Sc-7783 | AB_2108261 | 1:1000 (IHC/IF) |
| Acetylated-tubulin | Sigma | MABT868 | AB_2819178 | 1:200 (IHC/IF) |
| Gastrin-releasing peptide | Invitrogen | PA5-115272 | AB_2899908 | 1:200 (IHC/IF) |
| GAPDH | Cell Signaling Technologies | 5174S | AB_10622025 | 1:2000 (WB) |
| Gli2 | Santa Cruz Technologies | 271786 | AB_10708124 | 1:1000 (WB) |

ICC, immunocytochemistry; IF, immunofluorescence; IHC, immunohistochemistry; IP, immunoprecipitation; RRID, Research Resource Identification; TSA, tyramide signal amplification; WB, Western blot.
Pierce ECL reagent (ThermoFisher) for 1 minute prior to developing on film in a dark room.

For analysis of tissue extracts, gastric antra of 8- to 9-month-old mice were homogenized in ice-cold RIPA buffer using a rotor-stator homogenizer. Samples were centrifuged at 15,000 × g for 15 minutes, and the supernatant (20 μg) was evaluated for Gli2 protein expression (Table 5).

**Immunoprecipitation Assay**

Immunoprecipitation of menin was performed in rat enteric glial cells. Prior to lysis, cells were collected in ice-cold DPBS, centrifuged, and washed once with cold DPBS. The cell pellet was resuspended in cytoplasmic lysis buffer to generate a cytoplasmic extract. Buffer was supplemented with 1× HALT protease and phosphatase inhibitor. Cytoplasmic extracts were pipetted up and down 20×, incubated on ice for 10 minutes, then vortexed for 15 seconds prior to centrifuging at 15,000 × g for 5 minutes at 4°C. The supernatant was saved as the cytoplasmic fraction. Protein concentration in freshly prepared lysates was evaluated by bicinchoninic assay.

To form an antigen-antibody complex, 1 mg lysate was incubated with either mouse anti-menin monoclonal antibody (Bethyl Laboratories, #A500-003A, 3 μg per mg lysate) or mouse IgG1 Isotype control (Cell Signaling Technologies, 3 μg per mg lysate) overnight at 4°C. Fifty μL of prewashed Protein A/G agarose beads (Pierce, #20423) was added to each antigen-antibody complex and incubated for 2 hours at 4°C on a rotator. Bead-complexes were pelleted at 3000 × g for 2 minutes and washed 6 times with lysis buffer. Bound proteins were eluted by mixing with 2× SDS sample buffer. Samples were incubated at 50°C for 10 minutes, vortexed briefly, then centrifuged to remove beads. Dithiothreitol was added to the protein suspension at a final concentration of 10 mM prior to boiling at 95°C for 5 minutes. Samples were then evaluated by Western blot as previously described. Extracts were probed using rabbit anti-GFAP antibody (Dako Agilent Technologies, #Z033429, 1:3000). Immunoprecipitation was repeated 3 times in independent experiments and quantified via densitometry analysis using Image J Fiji software.

**Hormone Immunoassays**

GCG hormone was evaluated in mouse serum using the Mouse Glucagon enzyme-linked immunosorbent assay.

### Table 5. List of RT-qPCR Primers From Integrated DNA Technologies

| Gene    | Primer sequence 1 (5’ to 3’)                     | Primer sequence 2 (5’ to 3’)                     | IDT PrimeTime Id          |
|---------|--------------------------------------------------|--------------------------------------------------|---------------------------|
| Chga    | CCAAGGTGATGATGAAHTGAGGTGTG                      | TGTTTCGACAGGATAGAGGA                            | Mm.PT.58.41352258         |
| Syp     | CAGACAGAAACACTAAGGAA                            | TCCTCTGAAACAGAAGGATAGA                          | Mm.PT.58.29275406         |
| Glpo    | GCATCTCCAGCTTCTTTACA                             | AACCGCATACAGCTTCTTTG                            | Mm.PT.58.31927710         |
| Glpo    | GCATCTCCAGCTTCTTTACA                             | AACCGCATACAGCTTCTTTG                            | Mm.PT.58.31927710         |
| S100b   | TCATCTCTCCACCTTACTTC                             | GCTCTCTTCAGGATCCACTTTCT                         | Rn.PT.58.36785742         |
| Men1    | CCCAAGCATGATCCTTCCAGACA                         | CCCAAGCATGATCCTTCCAGAC                         | Rn.PT.58.30625021         |
| Men1    | CATCTCTCCACCTTACTTC                             | CATCTCTCCACCTTACTTC                             | Rn.PT.58.9524749          |
| Chgb    | CTTCTCTCCACCTTACTTC                             | CTTCTCTCCACCTTACTTC                             | Rn.PT.58.11605373         |
| En2o    | ACATCTCCATCAGCATCACCATC                         | CAACTCAATGAGTCCAGAAAGA                         | Rn.PT.58.31414881         |
| Prl     | ATATCAGCAACAGAAGGAGGTG                         | GTCTCTCCAGGACATCTTTG                            | Mm.PT.58.9092137          |
| Vip     | GAGATGAGAATGACAGGAA                             | GAATCTACAGGACATCTTTG                            | Mm.PT.58.32346790         |
| Gast    | CCAAATCATCCATCACCAGT                             | TCCTCAACTCAGCAGCAGC                            | Mm.PT.58.9580738          |
| Grp     | CTTCTCGTCTCGTCCTTCC                             | CTTCTCGTCTCGTCCTTCC                            | Mm.PT.58.41177438         |
| Grp     | CTTCTCGTCTCGTCCTTCC                             | CTTCTCGTCTCGTCCTTCC                            | Rn.PT.58.45266321         |
| Gl2     | ATATCGTCTCGTCTCGTCT                              | CATCTCTCCACCTTACTTC                             | Mm.PT.58.11001473         |
| Cdkn2a  | CTTCTGCTCTTGGAGATGTTGGC                         | GTGGCATATTGAGGGTTCG                             | Mm.PT.58.43961185         |
| Cdkn2b  | GTGCAGAGGACTTGGACTTGGG                          | AGATCCACAGCCCTG                                | Mm.PT.58.7138437          |
| Cend1   | CTTACCTCTGGGCAATATGAGG                          | GAATCTACAGGACATCTTTG                            | Mm.PT.58.8515727          |
| Cckr    | CAGAAAGATACAGCAGGAA                             | TCCCTCTCCAGGACATCTTTG                          | Mm.PT.58.45922274         |
| Shh     | CGTAAAGTCTGCACACTAGTTG                          | GAATATCTACAGGACATCTTTG                          | Mm.PT.58.14105875         |
| Atp4b   | CTTCTCTCCATCAGCATC                              | ATCTATTGAGCTTCAAGTAGC                          | Mm.PT.58.58927711         |
| Glf     | CTTAGTCTTGGCTTACAGTTG                           | ATCTATTGAGCTTCAAGTAGC                          | Mm.PT.58.13185585         |
| Lgr5    | GTCAAAGATCAGTACAGGAA                            | CTTCACTCAGTACAGGAA                             | Mm.PT.58.12492947         |
| Muc5ac  | CTTGTTGTTGGTCTTGGTTGTTG                        | CTTCACTCAGTACAGGAA                             | Mm.PT.58.42279692         |
| Muc6    | CGATGGGAGCAAGAAGGATG                            | CTCACTCAGTACAGGAA                              | Mm.PT.58.3323120          |
| Hprt1   | AAACAAGTCTGGCTCAGTGATCC                        | CCTCAGTCTCTCAGGATCC                            | Mm.PT.39a.22214828        |
| Hprt1   | GCCTTTCCACTTCCGCTGATG                           | GTGGAAGAGGACTCCTCTGAGA                         | Rn.PT.39a.22214832        |

RT-qPCR, Real-time quantitative polymerase chain reaction.
(ELISA) Kit according to manufacturer’s instructions (Crystal Chem, #81518). Serum was diluted 1:10 (10 μL serum per well) and incubated with anti-mouse GCG and HRP-secondary antibody overnight at 4 °C. Glucagon concentration was determined by HRP enzymatic activity, and the absorbance at 450 nm was averaged across duplicate wells. INS1 hormone was measured in mouse serum using the Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem, #90080). Five μL serum (1:20) was evaluated in duplicate and incubated with anti-mouse INS1 antibody overnight at 4 °C. INS1 concentration was interpolated from a high range standard curve of 1–64 ng/mL. Serum glucose was measured using a Mouse Glucose Assay Kit (Crystal Chem, #81692). Two μL serum (1:50) was incubated with an oxidizing enzyme solution for 10 minutes at 37 °C to yield hydrogen peroxide and generate a red dye. Absorbance at 505 nm was measured and averaged across technical duplicates. Glucose concentration was interpolated from a standard curve ranging from 10.5 to 675 mg/dL. Serum prolactin levels were measuring using the Mouse Serum Prolactin ELISA Kit (Invitrogen, #EMPRL). Serum was diluted 1:20 (5 μL serum per well) and incubated with anti-mouse prolactin antibody overnight at 4 °C. HRP-secondary antibody activity was evaluated by measuring absorbance at 450 nm, and optical density values were averaged prior to interpolating concentrations from a standard curve of 27.43 to 20,000 pg/mL.

Gastrin peptide was evaluated in mouse serum using the Gastrin Enzyme Immunoassay according to manufacturer’s instructions (Sigma, #RAB0200). Serum was diluted 1:4 (25 μL serum per well) and incubated with biotinylated gastrin peptide anti-gastrin-1 antibody overnight at 4 °C. Gastrin concentration was determined by HRP enzymatic activity, and the average absorbance at 450 nm was calculated across duplicate wells. Gastrin concentration was interpolated from a standard curve of 0.1 to 1000 pg/mL gastrin-1 peptide that was plotted using a 4-parameter logistic regression. For tissue, gastrin peptide was extracted from the antra of 8- to 9-month-old mice by boiling tissues in 200 μL of the antra of 8- to 9-month-old mice by boiling tissues in 200 μL of the supernatant was evaluated in duplicate.

**Gastric Acid Determination**

Following overnight fasting and necropsy, whole stomachs were removed, opened, and flushed with 5 mL of 0.9% saline solution. The stomach acid-solution was centrifuged to pellet debris, and 50 μL serum (1:20) was evaluated in duplicate and incubated with anti-mouse prolactin antibody overnight at 4 °C. HRP-secondary antibody activity was evaluated by measuring absorbance at 450 nm, and optical density values were averaged prior to interpolating concentrations from a standard curve of 1–64 ng/mL. Serum glucose was measured using a Mouse Glucose Assay Kit (Crystal Chem, #81692). Two μL serum (1:50) was incubated with an oxidizing enzyme solution for 10 minutes at 37 °C to yield hydrogen peroxide and generate a red dye. Absorbance at 505 nm was measured and averaged across technical duplicates. Glucose concentration was interpolated from a standard curve ranging from 10.5 to 675 mg/dL. Serum prolactin levels were measuring using the Mouse Serum Prolactin ELISA Kit (Invitrogen, #EMPRL). Serum was diluted 1:20 (5 μL serum per well) and incubated with anti-mouse prolactin antibody overnight at 4 °C. HRP-secondary antibody activity was evaluated by measuring absorbance at 450 nm, and optical density values were averaged prior to interpolating concentrations from a standard curve of 27.43 to 20,000 pg/mL.

**Statistical Analysis**

Assays comparing one parameter (eg, gastrin hormone) across 2 genotypes were evaluated for statistical significance by applying the unpaired Student t test. All qPCR data expressed as fold-change (2^−ΔΔCt) were log-transformed to fit a normal distribution prior to performing statistical analysis. Comparisons consisting of 3 or more genotypes or groups were evaluated for significance using 1-way analysis of variance, with Tukey post-test. Group comparisons of 2 or more parameters (eg, multiple genes) across genotypes and tissues were evaluated for significance using 2-way analysis of variance with Sidak post-test. Significance was determined as *P < .05, **P < .01, ***P < .001, ****P < .0001 using Graphpad Prism (v9) software.

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