A *MAFA* missense mutation causes familial insulinomatosis and diabetes mellitus

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Abstract

The β cell-enriched MAFA transcription factor plays a central role in regulating glucose-stimulated insulin secretion while also demonstrating oncogenic transformation potential *in vitro*. No disease-causing *MAFA* variants have been previously described. We investigated a large pedigree with autosomal dominant inheritance of diabetes mellitus or insulinomatosis, an adult-onset condition of recurrent hyperinsulinemic hypoglycemia caused by multiple insulin-secreting neuroendocrine tumors of the pancreas. Using exome sequencing we identified a novel missense *MAFA* mutation (p.Ser64Phe, c.191C>T) segregating with both phenotypes of insulinomatosis and diabetes. This mutation was also found in a second unrelated family with the same clinical phenotype, while no germline or somatic *MAFA* mutations were identified in nine patients with sporadic insulinomatosis. In the two families, insulinomatosis presented more frequently in females (eight females/two males) and diabetes more often in males (12 males/four females). Four patients from the index family, including two homozygotes, had a history of congenital cataract and/or glaucoma. The p.Ser64Phe mutation was found to impair phosphorylation within the transactivation domain of MAFA, and profoundly increased MAFA protein stability under both high and low glucose concentrations in β cell lines. In addition, the transactivation potential of p.Ser64Phe MAFA in β cell lines was enhanced as compared with wild type MAFA. In summary, the p.Ser64Phe missense *MAFA* mutation leads to familial insulinomatosis or diabetes by impacting MAFA protein stability and transactivation ability. The human phenotypes associated with the p.Ser64Phe *MAFA* missense mutation reflect both the oncogenic capacity of MAFA and its key role in islet β cell activity.

Significance statement

We report the first instance of a disease-causing mutation in the β cell-enriched MAFA transcription factor. Strikingly, the missense p.Ser64Phe *MAFA* mutation was associated with either of two distinct phenotypes, multiple insulin-producing neuroendocrine tumors of the pancreas – a condition known as insulinomatosis – or diabetes mellitus, recapitulating the physiological properties of MAFA both as an oncogene and as a key islet β

cell transcription factor. The implication of MAFA in these human phenotypes will provide novel insights on how this transcription factor regulates human β cell activity as well as on the mechanisms of Maf-induced tumorigenesis.

Introduction

The MAFA (V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog A) basic leucine zipper containing protein is unique among the many distinct islet-enriched transcription factors, as it plays a pivotal role in the regulation of insulin secretion *in vivo* while at the same time displaying oncogenic transformation potential *in vitro* (1-3). MAFA belongs to the family of large Maf transcription factors, also including MAFB, MAF, and NRL. MAFA and MAFB are both expressed in islet β cells, but only MAFA is required for their post-natal function (4-6), acting as transactivator of insulin and several genes involved with glucose-stimulated insulin secretion (1, 7-9). The transformation potential of MAFA was shown by its ability to induce proliferation of quail neuroretina cells (2) and chicken embryo fibroblasts (3) when overexpressed *in vitro*. Notably, the related human *MAF* gene is upregulated in 50% of human multiple myelomas and 60% of angioimmunoblastic T-cell lymphomas (10, 11). In addition, recurrent translocations involving *MAF*, *MAFB*, and *MAFA* are identified in 5-10% of multiple myelomas (12-14), highlighting the significant role of these oncogenes in hematological malignancies.

In this study, we aimed to determine the genetic etiology of insulinomatosis, a condition characterized by the occurrence of multicentric insulinomas, pancreatic neuroendocrine tumors with β cell-like features causing hyperinsulinemic hypoglycemia. Insulinomatosis usually occurs sporadically (15), although had also been described to occur in a familial setting in one single kindred where hyperinsulinemic hypoglycemia was paradoxically associated with a strong family history of diabetes mellitus (16). Due to the multicentric nature of the disease, insulinomatosis patients have a significantly higher chance of persistent or recurrent disease following conservative surgery compared to patients with a single sporadic insulinoma, and their management is often challenging (15). By sequencing the exomes of multiple affected individuals from a large autosomal dominant pedigree with insulinomatosis and diabetes, we identified a novel missense p.Ser64Phe (c.191C>T) mutation in the *MAFA* gene segregating with both phenotypes. Targeted sequencing in a second independent family with an identical clinical phenotype revealed the same *MAFA* mutation, while no pathogenic variants were found in a series of insulinomatosis patients with sporadic clinical presentation. Functional analysis demonstrated that the

p.Ser64Phe mutation not only significantly increased the stability of MAFA, whose levels were unaffected by variable glucose concentrations in β cell lines, but also enhanced its transactivation activity.

Results

Exome and targeted sequencing identify the p.Ser64Phe *MAFA* mutation. The study population consisted of an index family with autosomal dominant insulinomatosis and diabetes (Family 1; Figure 1A; 29 subjects, 17 females), a second family with the same phenotype, whose case was previously clinically described (16) (Family 2; Figure 1B; seven subjects, two females), and nine cases of sporadic insulinomatosis (eight females). All subjects were of white Caucasian ethnic background.

Exome sequencing of subjects III/1, III/2, III/8, and IV/4 from Family 1 identified 59, 85, 80, and 84 novel heterozygous variants, respectively, annotated as missense, nonsense, frameshift, or splice site variants. Only one of these, *MAFA* p.Ser64Phe (c.191C>T; NM_201589.3), was shared by all four affected individuals (Table S1 and S2). This variant affects a highly conserved amino acid within the transactivation domain of MAFA, and has not been reported before (ExAC, GnomAD, ESP, dbSNP, and 1000 Genomes databases). *In silico* prediction supported a pathogenic role (Table S3). Testing for this *MAFA* missense variant in 25 additional members from Family 1 identified 14 further heterozygous individuals (18 in total) and two homozygotes (IV/2 and IV/5). Nine unaffected family members did not inherit the variant (Figure 1A). Seven of the 18 heterozygotes had insulinomatosis, 10 had diabetes, and one was clinically unaffected (IV/3, aged 35). No DNA was available from patient III/10, who was an obligate carrier of the *MAFA* variant (Figure 1A) and was known to have an impaired fasting glucose.

Targeted sequencing of *MAFA* in Family 2 identified the same heterozygous p.Ser64Phe *MAFA* mutation in the proband (III/1, with insulinomatosis) and in four additional family members, one currently affected with insulinomatosis (III/3), one with diabetes (IV/1), and two who were not known to be affected (IV/2, aged 47 and IV/3, aged 41). The three deceased affected subjects in Family 2 – two with diabetes (II/2 and III/5) and one with insulinomatosis (II/1) – were obligate carriers (Figure 1B). Disease penetrance for both phenotypes

(insulinomatosis or diabetes) was 90%. Haplotype analysis within the two families suggested that the mutations had arisen on separate alleles (Figure S1), although a recombination event within a 364kb region encompassing *MAFA* could not be excluded. DNA sequence analysis of the nine sporadic insulinomatosis cases did not detect germline or somatic *MAFA* pathogenic variants.

Individuals with the p.Ser64Phe *MAFA* mutation develop either hyperinsulinemic hypoglycemia or diabetes mellitus. In the two families we report, 10 subjects had hyperinsulinemic hypoglycemia secondary to insulinomatosis (Table S4), while 15 patients were diagnosed with diabetes mellitus (Table S5). Most subjects with hyperinsulinemic hypoglycemia were females (male-to-female patient ratio was 1:4), and the mean age at diagnosis was 39.4±13.1 years. There was no history of early-onset hypoglycemia suggestive of congenital hyperinsulinism. In four out of six patients that underwent imaging investigations, multicentric pancreatic neuroendocrine tumors (ranging in size between 0.4 and 1.1cm) were shown, while local or distant metastases were not observed. In one patient with hyperinsulinemic hypoglycemia from Family 2 (III/1) who was diagnosed before cross-sectional imaging investigations became available, a 5mm insulinoma was found in the resected sample following pancreatic surgery (16). Overall, six patients underwent surgery, with persistent or recurrent disease in all cases, and four patients underwent more than one operation. The subjects with persistent or recurrent disease, and those who did not undergo pancreatic surgery, were managed with medical treatment with generally poor results and recurrent symptomatic hypoglycemia.

Most patients diagnosed with diabetes or impaired fasting glucose were males (male-to-female ratio was 3:1), and the mean age at diagnosis was 38.4±16.5 years. The mean BMI of patients with diabetes with available data was 25±3kg/m^2. There were no other clinical features of insulin resistance, no history of diabetic ketoacidosis and islet auto-antibodies were negative, configuring a phenotype resembling maturity-onset diabetes of the young (MODY) (17). Diabetes was managed with diet or oral medications (i.e. metformin and/or sulphonylureas) in most cases, with current HbA1c levels ranging between 37 and 74 mmol/mol (5.5-8.9%). There was no history of clinically significant micro- or macrovascular complications. Among the subjects with diabetes, two homozygous patients from Family 1 born to consanguineous parents presented with congenital glaucoma (IV/2) and congenital cataract

(IV/5), while two heterozygous subjects (III/16 and III/20) from the same family had congenital cataract associated, in one of these (III/16), with congenital glaucoma. There was no history of congenital eye disorders in Family 2.

Insulinomatosis and diabetes seemed to be mutually exclusive diagnoses in most patients. However, one subject from Family 2 (III/3) might have developed the two phenotypes in a sequential manner. This subject was diagnosed with gestational diabetes at the age of 27. After giving birth, she had impaired glucose tolerance and was treated with sulphonylureas between age 33 and 35. An oral glucose tolerance test whilst off treatment at the age of 39 years was reported normal. This patient started to show symptoms of hypoglycemia at the age of 55, and was later diagnosed with hyperinsulinemic hypoglycemia and multiple pancreatic neuroendocrine tumors on ^{18}F-DOPA PET imaging (Figure 2A-B).

The three unaffected heterozygotes had normal HbA1c and fasting glucose levels in the absence of clinical symptoms of hypoglycemia. Subject IV/2 (Family 2) was prospectively diagnosed with impaired glucose tolerance following an oral glucose tolerance test (oGTT) (Figure S2). The insulinogenic index calculated for this patient on the basis of baseline and 30 minutes glucose and insulin levels was 37.6pmol/mmol, with a normal HOMA-IR of 1.7, in keeping with impaired insulin secretion. An oGTT in one of the unaffected heterozygotes from Family 1 (IV/3) showed normal glucose tolerance, with 120 minutes glucose levels of 5.4mmol/L.

Hyperinsulinemic hypoglycemia in patients with the p.Ser64Phe *MAFA* mutation is due to multiple insulinomas. In the subjects with hyperinsulinemic hypoglycemia that underwent surgery, histopathology showed the presence of small (microadenomas, <5mm) and larger (macrotumors, >5mm) multifocal well-differentiated neuroendocrine tumors (Ki-67 <2%) with a trabecular tissue architecture (Figure 2C-D). The number of lesions was variable, depending on the type of surgery and extension of sampling. Over 100 lesions were identified in a patient from Family 2 (III/1) whose surgical specimen was fully sampled (15). Islets with β cell hyperplasia transforming into microadenomas were not observed. None of the tumors exceeded 2cm in size. All tumors expressed insulin, while immunostaining for the other pancreatic hormones was negative. MAFA immunostaining revealed a diffuse positivity in one case from Family 1 (III/19), which was less intense as compared to the

neighboring normal islets (Figure 2E), and a patchy positivity in the index case from Family 2 (III/1). Notwithstanding the limitations due to the small number of tissue samples available for further analysis, the MAFA staining intensity did not appear to be different in *MAFA* mutation-positive tumor cells compared with *MAFA* mutation-negative sporadic insulinomatosis or sporadic insulinomas (Table S6).

The p.Ser64Phe *MAFA* mutation affects MAFA protein stability and transactivation activity. Ser64 is found within the N-terminal transactivation domain of MAFA (Figure 3A). The neighboring Ser65 residue was previously shown to act as a priming phosphorylation site, as phosphorylation at Ser65 enables glycogen synthase kinase-3 (GSK3) to phosphorylate Ser61, Thr57, Thr53, and Ser49 in a sequential manner (Figure 3A) (18, 19). We found the mobility of the p.Ser64Phe mutant protein to be indistinguishable from the kinase priming defective mutant, p.Ser65Ala (Figure 3B), suggesting that the substitution of serine with a phenylalanine at residue 64 prevents phosphorylation at the priming Ser65 site, and the subsequent GSK3-mediated phosphorylation within the transactivation domain of MAFA. However, both p.Ser65Ala and p.Ser64Phe MAFA were still heavily phosphorylated proteins at the many other phosphorylation sites (Figure 3A), as shown by the ability of an endogenous phosphatase(s) to alter protein mobility when incubated in the presence of NaCl, but not the phosphatase inhibitor, sodium orthovanadate (Na_3VO_4) (Figure 3B) (20).

The p.Ser64Phe mutation was found to have a profound effect on MAFA turnover. The stability of the mutant protein was dramatically increased in both the human EndoC-βH1 β cell line (Figure 4) and MIN6 cells (Figure S3) in the presence of cycloheximide, a protein synthesis inhibitor. Normally, MAFA is highly unstable in β cells at low, non-stimulating glucose concentrations, while its stability is enhanced in the presence of high glucose concentrations (19). However, the p.Ser64Phe mutant was stable and abundant regardless of glucose levels (Figure 4A-B and S3). No significant difference was observed between transfected wild type (WT) and mutant *MAFA* mRNA levels (Figure 4C), confirming the post-transcriptional nature of the effect observed on protein turnover.

We next tested whether the p.Ser64Phe mutation affected stimulation of an insulin enhancer/promoter-driven reporter. There appeared to be no difference in the transactivation capacity in HeLa cells, as no predictable change in the specific activity pattern was observed between constructs (Figure S4). Notably, there was a non-linear

relationship in WT or mutant construct activity in relation to increasing amounts of protein, presumably due to the inability to properly phosphorylate the protein at its many phosphorylation sites (21) under these conditions. To focus on the impact of the p.Ser64Phe mutation on transactivation activity, chimeric proteins containing the N-terminal transactivation domain fused to the yeast Gal4 DNA-binding domain were produced. When analyzed in Gal4 binding site-driven reporter assays, the Gal4-Ser64Phe MAFA chimera was found to be more active than the WT chimera in INS-1 β cells compared with HeLa cells (Figure S5A). Importantly, the chimeric WT and p.Ser64Phe mutant proteins were expressed at equal levels (Figure S5B), as they both lack the lysine residues targeted for ubiquitination in the C-terminal DNA-binding/dimerization region (20). Collectively, these results suggest that the activity of p.Ser64Phe MAFA would be enhanced due to both increased transactivation capacity and increased protein stability.

Discussion

We report the first disease-causing mutation in the β cell-enriched MAFA transcription factor. A p.Ser64Phe *MAFA* missense mutation was identified in 25 individuals from two unrelated families who were affected with either insulinomatosis or non-insulin dependent diabetes resembling maturity-onset diabetes of the young (MODY). Our results are in keeping with previous evidence highlighting the role of MAFA in glucose-stimulated insulin secretion, and at the same time suggest that the p.Ser64Phe missense mutation can allow the oncogenic potential of MAFA – previously described in different cell contexts – to be manifested in the β cell.

MAFA regulates the expression of insulin and several genes involved in glucose-stimulated insulin secretion (1, 7-9), and serves as a glucose "barometer", since its stability and activity in β cells are increased under high glucose stimulating conditions and repressed in the presence of low glucose (19). The p.Ser64Phe mutation affects a highly conserved residue within the N-terminal transactivation domain of MAFA, neighboring the priming kinase Ser65 phosphorylation site. Significantly, no missense variants have been reported in publicly available databases at any of the N-terminal residues in MAFA subjected to sequential phosphorylation (Ser49, Thr53, Thr57, Ser61, or Ser65) or at immediately neighboring residues, including Ser64. The identical mobility of the p.Ser64Phe and

p.Ser65Ala mutants strongly suggests that the p.Ser64Phe mutation impairs phosphorylation at Ser65, and the consequent GSK3-mediated phosphorylation within the transactivation domain of MAFA. These phosphorylation events in the N-terminal transactivation domain of MAFA induce both transactivation capacity (22) and protein degradation (18-20), the latter resulting from ubiquitination in the C-terminal domain. Consistently with the impaired phosphorylation within the transactivation domain, the p.Ser64Phe MAFA protein was strikingly more stable compared to WT MAFA, and its turnover was unaffected by different glucose concentrations in β cell lines. Moreover, the activity of the Gal4-Ser64Phe chimeric protein was found to be greater than Gal4-WT MAFA in INS-1 β cells compared with non-β HeLa cells. Previous studies have shown that the transactivation activity of the Gal4-Ser65Ala protein was reduced in non-β cells (20), while the activity of chimeric proteins lacking the priming phosphorylation and the GSK3 phosphorylation sites was found to be enhanced in an insulinoma cell line (19). This suggests that phosphorylation within the transactivation domain may affect MAFA function in a cell context dependent way, likely through interactions with other β cell-specific transcription factors and/or co-regulators. Together, our results suggest that the p.Ser64Phe mutation increases the activity of endogenous MAFA in β cells by impacting both protein stability and transactivation potential.

The family of Maf transcription factors derives its name from *v-maf*, which is transduced as a viral oncogene capable of inducing muscoloaponeurotic fibrosarcoma in chickens (23, 24). MAF, MAFB, and MAFA all display oncogenic activity (25), with MAFA having the greatest transformation potential *in vitro* (3). Notably, only high copy number *Maf* expressing transgenic mice develop T-cell lymphomas (10), and the translocations occurring in human multiple myelomas (12, 13) determine the ectopic overexpression of large Maf proteins, suggesting that cell transformation is dependent on the overexpression of these transcription factors. Both the higher protein levels and the increased activity of the p.Ser64Phe mutant are predicted to induce the expression of genes involved with cell cycle regulation, including *CCND2*, a known target of MAFA (6) and key regulator of β cell proliferation (26), presumably causing β cell transformation and occurrence of insulinomatosis. Our data also suggest that the p.Ser64Phe mutation alters the tight regulation of MAFA stability in response to changes in glucose concentration. The lack of up-regulation of MAFA in response to hyperglycemia is expected to impair glucose-stimulated insulin

secretion, consistent with the results of the oGTT in one of the prospectively identified mutation carriers, and this mechanism presumably underlies the diabetes phenotype.

The mechanisms explaining how the same gene mutation can lead to diabetes or insulinomatosis remains to be fully elucidated, and *in vivo* models will have to be developed to further investigate the effects of the p.Ser64Phe mutation. A similarly paradoxical phenotype has been described for mutations in the transcription factor *HNF4A* (27-29) and the potassium channel gene *ABCC8* (30), where diabetes can be preceded, in some patients, by transient congenital hyperinsulinism. Insulinomatosis is, however, a very different disease, as it only manifests in adults, and is a neoplastic condition defined by the occurrence of multicentric insulin-producing neuroendocrine tumors, as opposed to congenital hyperinsulinism, characterized by islet cell hypertrophy in the absence of neoplastic changes (31). Although we cannot exclude the possibility that insulinomatosis patients had diabetes prior to developing symptoms of hyperinsulinemic hypoglycemia, in most cases the two phenotypes seemed mutually exclusive, and inter-individual factors might determine the development of either insulinomatosis or diabetes. Interestingly, in our two families, patients with insulinomatosis were mostly females and those with diabetes were more frequently males. The reasons for this gender difference are not known, although sporadic insulinomas also occur more frequently in females, with a male-to-female ratio of 1:1.4 (32). Treatment with estrogens has been shown to promote proliferation (33) and increase insulin release in human β cells and human insulinomas *in vitro* (34, 35). Moreover, the expansion of β cell mass observed during pregnancy is thought to be induced by prolactin and placental lactogen signaling mediated by the prolactin receptor (PRLR) (36-38). Notably, *Prlr* was significantly downregulated in *Mafa* knockout islets and in MIN6 β cells following siRNA-mediated knockdown of *Mafa* (39), and, in the same study, the *Prlr* promoter was shown to be directly activated by MAFA in luciferase reporter assays. Estrogens and prolactin could potentially promote β cell proliferation, predisposing female carriers of the p.Ser64Phe *MAFA* mutation to develop insulinomatosis – remarkably all insulinomatosis female patients manifested symptoms of the disease after puberty and most of them displayed the first hypoglycemic symptoms either during (16) or after pregnancy – although we cannot exclude that additional factors might influence the development of either phenotype.

Four subjects, including the only two homozygotes, presented with congenital cataract and/or glaucoma. *MAFA* is expressed in the developing lens (40), and mutations in the *MAF* gene have been previously linked with congenital cataract and disorders of the anterior segment (41), supporting a role for the p.Ser64Phe *MAFA* mutation in the pathogenesis of the ocular phenotype. Moreover, no *MAFA* mutations, either at the germline or somatic level, were detected in insulinomatosis patients with sporadic clinical presentation, implying that MAFA-independent mechanisms are involved in the pathogenesis of sporadic insulinomatosis. Similarly, no *MAFA* pathogenic variants were previously identified in a series of patients with genetically undetermined MODY (42), indicating that *MAFA* mutations are specifically linked to the association of diabetes and familial insulinomatosis.

In conclusion, we identified a *MAFA* missense mutation as the cause of a dual familial condition of diabetes mellitus or hyperinsulinemic hypoglycemia secondary to insulinomatosis. Our data show that the p.Ser64Phe mutation impairs phosphorylation in the transactivation domain of MAFA, leading to significantly enhanced protein stability and activity in β cell lines. The implication of a *MAFA* mutation in human disease is expected to provide further insights on the role of this transcription factor in the β cell.

Materials and Methods

Patient samples. We recruited two families with autosomal dominant insulinomatosis and diabetes mellitus (36 subjects, 19 females), and nine patients with sporadic insulinomatosis (eight females; clinical features are summarized in Table S7). All patients and family members agreed to take part in our multicenter study approved by the National Research Ethics Service Committee East of England – Cambridge East by providing signed informed consent.

Genetic analyses. Genomic DNA was extracted from peripheral blood leukocytes, saliva, or formalin-fixed archival tissue using commercially available kits (further details are provided in the Supporting Information). Exome sequencing was performed in four individuals affected with insulinomatosis from Family 1 (III/1, III/2, III/8, and IV/4) using the Agilent's SureSelect Human All Exon Kit (v5) (Agilent, Santa Clara, CA, USA) with

sequencing on an Illumina HiSeq2500 system (Illumina, San Diego, CA, USA). Sequencing metrics for the four samples are reported in Table S8. We assumed a rare autosomal dominant model of inheritance to filter novel (not previously reported in the ExAC, ESP, dbSNP, and 1000 Genomes databases) heterozygous variants annotated as missense, nonsense, frameshift, or splice site variants. The effect of the identified *MAFA* missense variant was investigated *in silico* using SIFT (http://sift.jcvi.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), and Align GVCD (http://agvgd.iarc.fr/agvgd_input.php/) prediction tools. Sanger sequencing was used for validation and co-segregation studies in Family 1, and for the sequencing of the whole coding sequence of *MAFA* in Family 2 and in sporadic insulinomatosis patients. Primer sequences are provided in Table S9. Methods for haplotype analysis are reported in the Supporting Information.

Pathological assessment and MAFA immunohistochemistry. Immunohistochemistry on archival pancreatic tissue for neuroendocrine markers, Ki-67, and pancreatic hormones (insulin, gastrin, glucagon, pancreatic polypeptide) was performed as previously described (43). MAFA expression was assessed using immunohistochemistry in two familial insulinomatosis samples, eight sporadic insulinomatosis, and six sporadic insulinoma controls and classified as negative, weak, moderate, strong, or patchy. All cases were reviewed by an experienced endocrine pathologist (G.K.).

Protein mobility analysis. Details for plasmid preparation are reported in the Supporting Information. Nuclear extracts of WT, p.Ser64Phe, and p.Ser65Ala MAFA-transfected HeLa cells were incubated at 37°C for 40 or 80 minutes in the presence of sodium orthovanadate (Na_3VO_4, 10mM) or NaCl (10mM). The samples were analyzed by SDS polyacrylamide gel electrophoresis and immunoblotting with an anti-MAFA antibody (Bethyl Laboratories, Montgomery, TX, USA; A300-611A).

Luciferase assays. The rat insulin II enhancer/promoter driven -238 firefly luciferase plasmid (Promega, Madison, WI, USA) was transfected in HeLa cells along with pCMV4-MAFA and phRL-TK (Promega) using the Lipofectamine protocol (Life Technologies). Gal4-MAFA(1-167) was transfected in HeLa and INS-1 832/13 cells

along with $(Gal4)_5$E1bLuc and phRL-TK. Cellular extracts were collected 48 hours post-transfection, and the Dual-Luciferase Reporter Assay (Promega) was performed according to the manufacturer's directions. MAFA protein levels were normalized to endogenous β-actin by immunoblotting with anti-MAFA (Bethyl Laboratories; A300-611A) and anti-β-actin (Cell Signaling, Danvers, MA, USA; 4967S) antibodies.

Cycloheximide chase experiments. WT and p.Ser64Phe MAFA-Myc were introduced into EndoC-βH1 cells (44) using the Amaxa Nucleofector 2 (Program G-016; Lonza Walkersville, MD, USA). The medium was changed 48 hours following nucleofection to either 1.1 or 15.5mM glucose for 12 hours, and cycloheximide (Sigma, St. Louis, MO, USA) was then added at a concentration of 25μg/mL for the time indicated. Nuclear extracts were prepared for immunoblotting and probed with anti-Myc (Roche, Penzberg, Germany; clone 9E10) and anti-β-actin (Cell Signaling; 4967S) antibodies. RNA from EndoC-βH1 cells was collected 72 hours post-nucleofection using the Trizol reagent (Life Technologies), and the iScript cDNA synthesis kit (Biorad, Hercules, CA, USA) was used for cDNA synthesis. The qPCR reactions were performed with *MAFA-Myc*, *MAFA* (endogenous), and *GAPDH* gene primers on a LightCycler 480 II (Roche), and analyzed by the ΔΔCT method. Cycloheximide chase experiments were also performed in MIN6 cells transfected with WT and p.Ser64Phe MAFA-Myc using the Lipofectamine protocol (Life Technologies). Each experiment was repeated at least three times.

Statistical analysis. Parametric data are presented as mean ± standard deviation (SD). Normal distribution was assessed using the Shapiro-Wilk test. Experimental data (luciferase and qPCR experiments) were analyzed through the Student's t-test using the software Prism v5 (GraphPad Software Inc, La Jolla, CA, USA). Cycloheximide chase experiments were analyzed using a one-phase decay equation, and the degradation speed (K) was compared between the mutant and the WT protein using the extra sum-of-squares F test. Significance was set for *P* values <0.05.

Authors' contributions. D.I. prepared the first draft of the manuscript, collected and analyzed the clinical data, undertook the Sanger sequencing in tissue samples, performed the immunohistochemistry, and part of the

functional *in vitro* studies. S.E.F. undertook the exome sequencing variant data analysis. E.W. undertook most of the functional *in vitro* studies. R.Q. and F.A.S.B. provided samples and clinical data from the index family. R.C. undertook the exome sequencing and *in silico* analysis of the *MAFA* variant effect on the protein. M.W. performed the bioinformatic analysis of the exome sequencing data. M.J. performed the Sanger sequencing testing for *MAFA*. M.G. contributed to the *in vitro* studies. M.N.G. and P.G. collected data, managed the patient database, and contributed to the DNA extractions. M.B., B.N., E.C., S.J., B.S., A.F., K.D., P.C., and W.H. provided samples and clinical data from the insulinomatosis patients. M.N. and B.K. extracted DNA from archival tissues. G.K. provided tissue samples, reviewed the histopathology and the immunohistochemistry for MAFA. R.S. participated in the study design and supervised the *in vitro* studies. M.K. had the original idea, collected samples, and participated in the study design. S.E. participated in the study design and supervised the genetic analyses. S.E.F., E.W., R.S., M.K., and S.E. contributed to the writing of the manuscript. All authors reviewed the manuscript.

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Figure Legends

Figure 1. Family trees of the two families with insulinomatosis and diabetes mellitus. Different colors mark the *MAFA* genotypes. Unmarked subjects were not tested. A simplified version of the family tree was used for Family 1 to improve readability.

Figure 2. Features of *MAFA* mutation-positive insulinomatosis. A-B. ^{18}F-DOPA PET in a patient with *MAFA* mutation-positive insulinomatosis (Family 2, subject III/3) showing two pancreatic neuroendocrine tumors (red arrow) (A, tail; B, body of the pancreas). C. Chromogranin A immunohistochemistry in subject III/19 (Family 1) shows a macrotumor (>5mm) (asterisk) and multiple small (microadenomas, <5mm) neuroendocrine tumors (black arrows). D. H&E staining showing the trabecular pattern of *MAFA* mutation-positive insulinomas. E. Immunostaining shows diffuse MAFA expression in the tumor, at lower levels as compared to the neighboring normal islets strongly expressing MAFA (insert).

Figure 3. The mobility of p.Ser64Phe (S64F) MAFA is indistinguishable from the p.Ser65Ala (S65A) kinase mutant. A) Schematic of MAFA showing sites of phosphorylation (red dots) within the transactivation, DNA-binding (basic), and dimerization region (leucine zipper, L-Zip). B) Wild type (WT) and mutant *MAFA* transfected HeLa nuclear extracts were incubated at 37°C for 40 or 80 minutes (40' or 80') in the presence of the phosphatase inhibitor, sodium orthovanadate (Na_3VO_4, 10mM), or NaCl (10mM). The arrowheads denote the location of fully phosphorylated MAFA (F-P, blue), the form lacking Ser65 and GSK3-mediated phosphorylation (Un-P, red), and the completely dephosphorylated protein produced by incubating in the presence of NaCl (De-P, white).

Figure 4. The p.Ser64Phe (S64F) mutation greatly stabilizes MAFA in human EndoC-βH1 cells grown in 1.1 or 15.5mM glucose. A) EndoC-βH1 cells were transfected with wild type (WT) and p.Ser64Phe (S64F) MAFA-Myc and, after 48 hours, incubated with medium containing 1.1mM or 15.5mM glucose for an additional 12 hours. The transfected cells were then incubated with 25μg/mL cycloheximide (CHX) for the indicated time.

Transfected MAFA-Myc and endogenous β-actin protein levels were determined by immunoblotting (IB) using anti-Myc and anti-β-actin antibodies, respectively. B) The Myc protein band intensity was measured in the 15.5mM glucose sample, normalized to β-actin, and plotted as a percentage of the initial band intensity. C) No significant difference was found between WT and p.Ser64Phe (S64F) *MAFA* mRNA levels in transfected cells grown in 1.1mM glucose. Endogenous *MAFA* mRNA levels also did not change under these conditions. Student's two-tailed t-test. n.s., not significant. n = 3. Error bars represent SEM.