

MAFA missense mutation causes familial insulinomatosis and diabetes mellitus

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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 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 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.

MAFA | insulinoma | insulinomatosis | diabetes | MODY

The V-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog A (MAFA) 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 postnatal function of murine β -cells (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 MAF gene is up-regulated 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 it had

Significance

We report 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 insights into how this transcription factor regulates human β -cell activity as well as into the mechanisms of Maf-induced tumorigenesis.

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phosphatase inhibitor, sodium orthovanadate (Na_3VO_4) (Fig. 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- β H1 β -cell line (Fig. 4) and MIN6 cells (SI Appendix, Fig. S3) in the presence of cycloheximide, a protein synthesis inhibitor. Normally, MAFA is highly unstable in β -cells at low, nonstimulating 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 (Fig. 4 A and B and SI Appendix, Fig. S3). No significant difference was observed between transfected wild-type (WT) and mutant MAFA mRNA levels (Fig. 4C), confirming the posttranscriptional 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 (SI Appendix, Fig. S4). Notably, there was a nonlinear 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 (SI Appendix, Fig. S5A). Importantly, the chimeric WT and p.Ser64Phe mutant proteins were expressed at equal levels (SI Appendix, Fig. 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 a disease-causing mutation in the β -cell-enriched MAFA transcription factor. A p.Ser64Phe MAFA missense

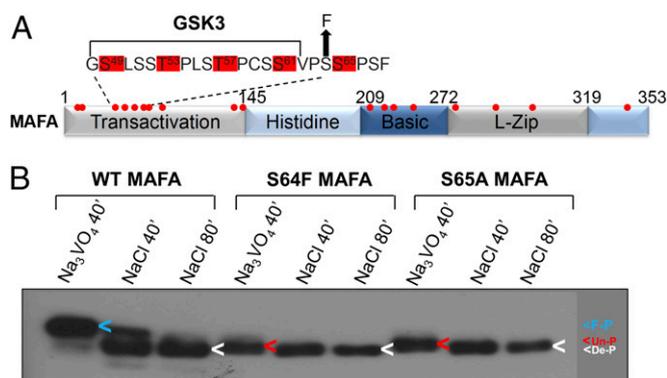


Fig. 3. The mobility of p.Ser64Phe (S64F) MAFA is indistinguishable from the p.Ser65Ala (S65A) kinase mutant. (A) Schematic of MAFA showing sites of phosphorylation (red dots) within the transactivation, DNA-binding (basic), and dimerization region (leucine zipper, L-Zip). (B) Wild type (WT) and mutant MAFA transfected HeLa nuclear extracts were incubated at 37 °C for 40 or 80 min (40' or 80') in the presence of the phosphatase inhibitor, sodium orthovanadate (Na_3VO_4 , 10 mM), or NaCl (10 mM). 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).

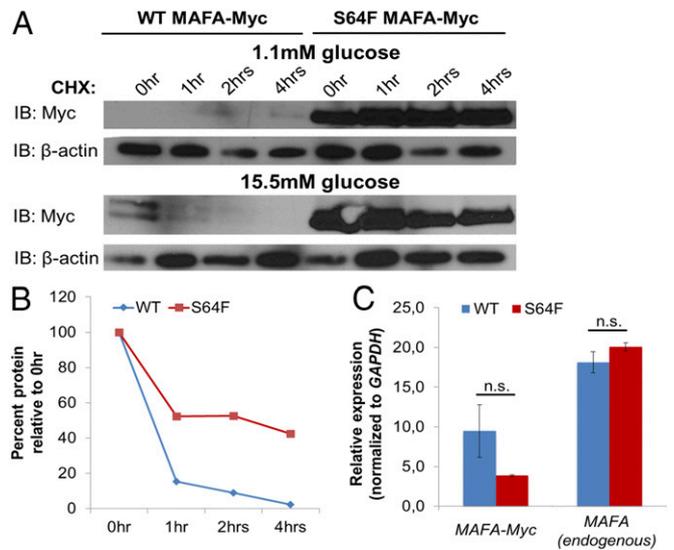


Fig. 4. The p.Ser64Phe (S64F) mutation greatly stabilizes MAFA in human EndoC- β H1 cells grown in 1.1 or 15.5 mM glucose. (A) EndoC- β H1 cells were transfected with wild type (WT) and p.Ser64Phe (S64F) MAFA-Myc and, after 48 h, incubated with medium containing 1.1 mM or 15.5 mM glucose for an additional 12 h. The transfected cells were then incubated with 25 $\mu\text{g}/\text{mL}$ 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 15.5 mM 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 1.1 mM 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.

mutation was identified in 25 individuals from two unrelated families who were affected with either insulinomatosis or noninsulin-dependent diabetes resembling 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 with 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 coregulators. 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 musculoaponeurotic 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 patients with insulinomatosis had diabetes before developing symptoms of hyperinsulinemic hypoglycemia, in most cases the two phenotypes seemed mutually exclusive, and interindividual 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 down-regulated 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 the possibility 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 patients with insulinomatosis 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 *SI Appendix, 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 *SI Appendix*). Exome sequencing was performed in four individuals affected with insulinomatosis from family 1 (III/1, III/2, III/8, and IV/4) using the SureSelect Human All Exon Kit (v5) (Agilent) with sequencing on an HiSeq2500 system (Illumina). Sequencing metrics for the four samples are reported in *SI Appendix, Table S8*. We assumed a rare autosomal dominant model of inheritance to filter heterozygous variants (not previously reported in the ExAC, ESP, dbSNP, and 1,000 Genomes databases) annotated as missense, nonsense, frameshift, or splice site variants. The effect of the identified MAFA missense variant was investigated in silico using SIFT (sift.jcvi.org/), PolyPhen-2 (genetics.bwh.harvard.edu/pph2/), and Align GVGD (agvgd.hci.utah.edu/) prediction tools. Sanger sequencing was used for validation and cosegregation studies in family 1, and for the sequencing of the whole coding sequence of MAFA in family 2 and in patients with sporadic insulinomatosis. Primer sequences are provided in *SI Appendix, Table S9*. Methods for haplotype analysis are reported in *SI Appendix*.

Pathological Assessment and MAFA Immunohistochemistry. Immunohistochemistry on archival pancreatic tissue for neuroendocrine markers, Ki-67, and pancreatic hormones (insulin, gastrin, glucagon, and 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.). Further details are reported in *SI Appendix*.

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

Luciferase Assays. The rat insulin II enhancer/promoter driven –238 firefly luciferase plasmid (Promega) 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)₅E1bLuc and pHRL-TK. Cellular extracts were collected 48 h posttransfection, 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 (A300-611A, Bethyl Laboratories) and anti- β -actin (4967S, Cell Signaling) antibodies.

Cycloheximide Chase Experiments. WT and p.Ser64Phe MAFA-Myc were introduced into EndoC- β H1 cells (44) using the Amara Nucleofector 2 (program G-016, Lonza). The medium was changed 48 h following nucleofection to either 1.1 or 15.5 mM glucose for 12 h, and cycloheximide (Sigma) was then added at a concentration of 25 μ g/mL for the time indicated. Nuclear extracts were prepared for immunoblotting and probed with anti-Myc (clone 9E10, Roche) and anti- β -actin (4967S, Cell Signaling) antibodies. RNA from EndoC- β H1 cells was collected 72 h postnucleofection using the TRIzol reagent (Life Technologies), and the iScript cDNA synthesis kit (Bio-Rad) 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 $\Delta\Delta$ CT method. Cycloheximide chase experiments were also performed in MIN6 cells transfected with WT and p.Ser64Phe MAFA-Myc

using the Lipofectamine protocol. Each experiment was repeated at least three times.

Statistical Analysis. Parametric data are presented as mean \pm SD or SEM in the figures. 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). Cycloheximide chase experiments were analyzed using a one-phase decay equation, and the degradation rate constant (*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.

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