

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

Donato Iacovazzo,^{1*} Sarah E. Flanagan,^{2*} Emily Walker,^{3*} Rosana Quezado,^{4*} Fernando Antonio de Sousa Barros,⁴ Richard Caswell,² Matthew Johnson,² Matthew Wakeling,² Michael Brändle,⁵ Min Guo,³ Mary N. Dang,¹ Plamena Gabrovska,¹ Bruno Niederle,⁶ Emanuel Christ,⁷ Stefan Jenni,⁸ Bence Sipos,⁹ Maike Nieser,⁹ Andrea Frilling,¹⁰ Ketan Dhatariya,¹¹ Philippe Chanson,^{12, 13} Wouter de Herder,¹⁴ Björn Konukiewitz,¹⁵ Günter Klöppel,^{15*} Roland Stein,^{3*} Márta Korbonits,^{1*} and Sian Ellard^{2*}

¹Centre for Endocrinology, Barts and The London School of Medicine, Queen Mary University of London, London, EC1M 6BQ, UK; ²Institute of Biomedical and Clinical Science, University of Exeter Medical School, Exeter, EX2 5DW, UK; ³Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee, 37232, USA; ⁴Serviço de Endocrinologia e Diabetes, Hospital Universitário Walter Cantídio, Universidade Federal do Ceará, Fortaleza, 60430-372, Brazil; ⁵Division of Endocrinology and Diabetes, Department of Internal Medicine, Kantonsspital St. Gallen, St. Gallen, CH-9007, Switzerland; ⁶Section of Endocrine Surgery, Division of General Surgery, Department of Surgery, University of Vienna, Vienna, A-1090, Austria; ⁷Division of Diabetes, Endocrinology and Metabolism, University Hospital of Basel, Basel, CH-4031, Switzerland; ⁸Division of Endocrinology, Diabetes and Clinical Nutrition, University Hospital of Bern, Inselspital, Bern, CH-3010, Switzerland; ⁹Department of Pathology, University of Tübingen, Tübingen, 72076, Germany; ¹⁰Department of Surgery and Cancer, Imperial College London, London, W12 0HS, UK; ¹¹Elsie Bertram Diabetes Centre, Norfolk and Norwich University Hospitals NHS Foundation Trust, Norwich, NR4 7UY, UK; ¹²Service d'Endocrinologie et des Maladies de la Reproduction, Assistance Publique-Hôpitaux de Paris, Hôpital de Bicêtre, Le Kremlin-Bicêtre, F-94275, France; ¹³Inserm 1185, Fac Med Paris Sud, Université Paris-Saclay, Le Kremlin-Bicêtre, F-94276, France; ¹⁴Department of Internal Medicine, Sector of Endocrinology, ENETS Centre of Excellence for Neuroendocrine Tumors, Erasmus MC, Rotterdam, 3015, The Netherlands; ¹⁵Department of Pathology, Consultation Center for Pancreatic and Endocrine Tumors, Technical University of Munich, Munich, 81675, Germany

*These authors contributed equally to this work

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Corresponding author and person to whom reprint requests should be addressed:

Márta Korbonits, MD, PhD

Professor of Endocrinology and Metabolism.

Centre for Endocrinology, William Harvey Research Institute,

Barts and the London School of Medicine,

Queen Mary University of London.

Charterhouse Square, London EC1M 6BQ, UK.

Tel: +44 20 7882 8284 – m.korbonits@qmul.ac.uk

43 **Abstract**

44

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

62

63 **Significance statement**

64

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

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

72

73

74 **Introduction**

75

76 The MAFA (V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog A) basic leucine zipper
77 containing protein is unique among the many distinct islet-enriched transcription factors, as it plays a pivotal role
78 in the regulation of insulin secretion *in vivo* while at the same time displaying oncogenic transformation potential
79 *in vitro* (1-3). MAFA belongs to the family of large Maf transcription factors, also including MAFB, MAF, and
80 NRL. MAFA and MAFB are both expressed in islet β cells, but only MAFA is required for their post-natal function
81 (4-6), acting as transactivator of insulin and several genes involved with glucose-stimulated insulin secretion (1,
82 7-9). The transformation potential of MAFA was shown by its ability to induce proliferation of quail neuroretina
83 cells (2) and chicken embryo fibroblasts (3) when overexpressed *in vitro*. Notably, the related human *MAF* gene
84 is upregulated in 50% of human multiple myelomas and 60% of angioimmunoblastic T-cell lymphomas (10, 11).
85 In addition, recurrent translocations involving *MAF*, *MAFB*, and *MAFA* are identified in 5-10% of multiple
86 myelomas (12-14), highlighting the significant role of these oncogenes in hematological malignancies.

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

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

101

102 **Results**

103

104 **Exome and targeted sequencing identify the p.Ser64Phe MAFA mutation.** The study population consisted of
105 an index family with autosomal dominant insulinomatosis and diabetes (Family 1; Figure 1A; 29 subjects, 17
106 females), a second family with the same phenotype, whose case was previously clinically described (16) (Family
107 2; Figure 1B; seven subjects, two females), and nine cases of sporadic insulinomatosis (eight females). All subjects
108 were of white Caucasian ethnic background.

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

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

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

129

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

143 Most patients diagnosed with diabetes or impaired fasting glucose were males (male-to-female ratio was 3:1), and
144 the mean age at diagnosis was 38.4±16.5 years. The mean BMI of patients with diabetes with available data was
145 25±3kg/m². There were no other clinical features of insulin resistance, no history of diabetic ketoacidosis and islet
146 auto-antibodies were negative, configuring a phenotype resembling maturity-onset diabetes of the young (MODY)
147 (17). Diabetes was managed with diet or oral medications (i.e. metformin and/or sulphonylureas) in most cases,
148 with current HbA1c levels ranging between 37 and 74 mmol/mol (5.5-8.9%). There was no history of clinically
149 significant micro- or macrovascular complications. Among the subjects with diabetes, two homozygous patients
150 from Family 1 born to consanguineous parents presented with congenital glaucoma (IV/2) and congenital cataract

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

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

161 The three unaffected heterozygotes had normal HbA1c and fasting glucose levels in the absence of clinical
162 symptoms of hypoglycemia. Subject IV/2 (Family 2) was prospectively diagnosed with impaired glucose tolerance
163 following an oral glucose tolerance test (oGTT) (Figure S2). The insulinogenic index calculated for this patient on
164 the basis of baseline and 30 minutes glucose and insulin levels was 37.6pmol/mmol, with a normal HOMA-IR of
165 1.7, in keeping with impaired insulin secretion. An oGTT in one of the unaffected heterozygotes from Family 1
166 (IV/3) showed normal glucose tolerance, with 120 minutes glucose levels of 5.4mmol/L.

167

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

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

181

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

193 The p.Ser64Phe mutation was found to have a profound effect on MAFA turnover. The stability of the mutant
194 protein was dramatically increased in both the human EndoC- β H1 β cell line (Figure 4) and MIN6 cells (Figure
195 S3) in the presence of cycloheximide, a protein synthesis inhibitor. Normally, MAFA is highly unstable in β cells
196 at low, non-stimulating glucose concentrations, while its stability is enhanced in the presence of high glucose
197 concentrations (19). However, the p.Ser64Phe mutant was stable and abundant regardless of glucose levels (Figure
198 4A-B and S3). No significant difference was observed between transfected wild type (WT) and mutant *MAFA*
199 mRNA levels (Figure 4C), confirming the post-transcriptional nature of the effect observed on protein turnover.
200 We next tested whether the p.Ser64Phe mutation affected stimulation of an insulin enhancer/promoter-driven
201 reporter. There appeared to be no difference in the transactivation capacity in HeLa cells, as no predictable change
202 in the specific activity pattern was observed between constructs (Figure S4). Notably, there was a non-linear

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

213

214 **Discussion**

215

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

222 MAFA regulates the expression of insulin and several genes involved in glucose-stimulated insulin secretion (1,
223 7-9), and serves as a glucose “barometer”, since its stability and activity in β cells are increased under high glucose
224 stimulating conditions and repressed in the presence of low glucose (19). The p.Ser64Phe mutation affects a highly
225 conserved residue within the N-terminal transactivation domain of MAFA, neighboring the priming kinase Ser65
226 phosphorylation site. Significantly, no missense variants have been reported in publicly available databases at any
227 of the N-terminal residues in MAFA subjected to sequential phosphorylation (Ser49, Thr53, Thr57, Ser61, or
228 Ser65) or at immediately neighboring residues, including Ser64. The identical mobility of the p.Ser64Phe and

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

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

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

256 The mechanisms explaining how the same gene mutation can lead to diabetes or insulinomatosis remains to be
257 fully elucidated, and *in vivo* models will have to be developed to further investigate the effects of the p.Ser64Phe
258 mutation. A similarly paradoxical phenotype has been described for mutations in the transcription factor *HNF4A*
259 (27-29) and the potassium channel gene *ABCC8* (30), where diabetes can be preceded, in some patients, by
260 transient congenital hyperinsulinism. Insulinomatosis is, however, a very different disease, as it only manifests in
261 adults, and is a neoplastic condition defined by the occurrence of multicentric insulin-producing neuroendocrine
262 tumors, as opposed to congenital hyperinsulinism, characterized by islet cell hypertrophy in the absence of
263 neoplastic changes (31). Although we cannot exclude the possibility that insulinomatosis patients had diabetes
264 prior to developing symptoms of hyperinsulinemic hypoglycemia, in most cases the two phenotypes seemed
265 mutually exclusive, and inter-individual factors might determine the development of either insulinomatosis or
266 diabetes. Interestingly, in our two families, patients with insulinomatosis were mostly females and those with
267 diabetes were more frequently males. The reasons for this gender difference are not known, although sporadic
268 insulinomas also occur more frequently in females, with a male-to-female ratio of 1:1.4 (32). Treatment with
269 estrogens has been shown to promote proliferation (33) and increase insulin release in human β cells and human
270 insulinomas *in vitro* (34, 35). Moreover, the expansion of β cell mass observed during pregnancy is thought to be
271 induced by prolactin and placental lactogen signaling mediated by the prolactin receptor (PRLR) (36-38). Notably,
272 *Prlr* was significantly downregulated in *Mafa* knockout islets and in MIN6 β cells following siRNA-mediated
273 knockdown of *Mafa* (39), and, in the same study, the *Prlr* promoter was shown to be directly activated by MAFA
274 in luciferase reporter assays. Estrogens and prolactin could potentially promote β cell proliferation, predisposing
275 female carriers of the p.Ser64Phe *MAFA* mutation to develop insulinomatosis – remarkably all insulinomatosis
276 female patients manifested symptoms of the disease after puberty and most of them displayed the first
277 hypoglycemic symptoms either during (16) or after pregnancy – although we cannot exclude that additional factors
278 might influence the development of either phenotype.

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

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

292

293 **Materials and Methods**

294

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

300

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

305 sequencing on an Illumina HiSeq2500 system (Illumina, San Diego, CA, USA). Sequencing metrics for the four
306 samples are reported in Table S8. We assumed a rare autosomal dominant model of inheritance to filter novel (not
307 previously reported in the ExAC, ESP, dbSNP, and 1000 Genomes databases) heterozygous variants annotated as
308 missense, nonsense, frameshift, or splice site variants. The effect of the identified *MAFA* missense variant was
309 investigated *in silico* using SIFT (<http://sift.jcvi.org/>), PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>), and
310 Align GVCD (http://agvgd.iarc.fr/agvgd_input.php/) prediction tools. Sanger sequencing was used for validation
311 and co-segregation studies in Family 1, and for the sequencing of the whole coding sequence of *MAFA* in Family
312 2 and in sporadic insulinomatosis patients. Primer sequences are provided in Table S9. Methods for haplotype
313 analysis are reported in the Supporting Information.

314

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

321

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

327

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

331 along with (Gal4)₅E1bLuc and phRL-TK. Cellular extracts were collected 48 hours post-transfection, and the
332 Dual-Luciferase Reporter Assay (Promega) was performed according to the manufacturer's directions. MAFA
333 protein levels were normalized to endogenous β -actin by immunoblotting with anti-MAFA (Bethyl Laboratories;
334 A300-611A) and anti- β -actin (Cell Signaling, Danvers, MA, USA; 4967S) antibodies.

335

336 **Cycloheximide chase experiments.** WT and p.Ser64Phe MAFA-Myc were introduced into EndoC- β H1 cells (44)
337 using the Amaxa Nucleofector 2 (Program G-016; Lonza Walkersville, MD, USA). The medium was changed 48
338 hours following nucleofection to either 1.1 or 15.5mM glucose for 12 hours, and cycloheximide (Sigma, St. Louis,
339 MO, USA) was then added at a concentration of 25 μ g/mL for the time indicated. Nuclear extracts were prepared
340 for immunoblotting and probed with anti-Myc (Roche, Penzberg, Germany; clone 9E10) and anti- β -actin (Cell
341 Signaling; 4967S) antibodies. RNA from EndoC- β H1 cells was collected 72 hours post-nucleofection using the
342 Trizol reagent (Life Technologies), and the iScript cDNA synthesis kit (Biorad, Hercules, CA, USA) was used for
343 cDNA synthesis. The qPCR reactions were performed with *MAFA-Myc*, *MAFA* (endogenous), and *GAPDH* gene
344 primers on a LightCycler 480 II (Roche), and analyzed by the $\Delta\Delta$ CT method. Cycloheximide chase experiments
345 were also performed in MIN6 cells transfected with WT and p.Ser64Phe MAFA-Myc using the Lipofectamine
346 protocol (Life Technologies). Each experiment was repeated at least three times.

347

348 **Statistical analysis.** Parametric data are presented as mean \pm standard deviation (SD). Normal distribution was
349 assessed using the Shapiro-Wilk test. Experimental data (luciferase and qPCR experiments) were analyzed through
350 the Student's t-test using the software Prism v5 (GraphPad Software Inc, La Jolla, CA, USA). Cycloheximide
351 chase experiments were analyzed using a one-phase decay equation, and the degradation speed (K) was compared
352 between the mutant and the WT protein using the extra sum-of-squares F test. Significance was set for *P* values
353 <0.05.

354

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

357 functional *in vitro* studies. S.E.F. undertook the exome sequencing variant data analysis. E.W. undertook most of
358 the functional *in vitro* studies. R.Q. and F.A.S.B. provided samples and clinical data from the index family. R.C.
359 undertook the exome sequencing and *in silico* analysis of the *MAFA* variant effect on the protein. M.W. performed
360 the bioinformatic analysis of the exome sequencing data. M.J. performed the Sanger sequencing testing for *MAFA*.
361 M.G. contributed to the *in vitro* studies. M.N.G. and P.G. collected data, managed the patient database, and
362 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
363 clinical data from the insulinomatosis patients. M.N. and B.K. extracted DNA from archival tissues. G.K. provided
364 tissue samples, reviewed the histopathology and the immunohistochemistry for *MAFA*. R.S. participated in the
365 study design and supervised the *in vitro* studies. M.K. had the original idea, collected samples, and participated in
366 the study design. S.E. participated in the study design and supervised the genetic analyses. S.E.F., E.W., R.S.,
367 M.K., and S.E. contributed to the writing of the manuscript. All authors reviewed the manuscript.

368

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378

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469 inducible insulin secretion. *J Clin Invest* 121(9):3589-3597.

470

471

472 **Figure Legends**

473

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

477

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

485

486 **Figure 3. The mobility of p.Ser64Phe (S64F) *MAFA* is indistinguishable from the p.Ser65Ala (S65A) kinase
487 mutant.** A) Schematic of *MAFA* showing sites of phosphorylation (red dots) within the transactivation, DNA-
488 binding (basic), and dimerization region (leucine zipper, L-Zip). B) Wild type (WT) and mutant *MAFA* transfected
489 HeLa nuclear extracts were incubated at 37°C for 40 or 80 minutes (40' or 80') in the presence of the phosphatase
490 inhibitor, sodium orthovanadate (Na₃VO₄, 10mM), or NaCl (10mM). The arrowheads denote the location of fully
491 phosphorylated *MAFA* (F-P, blue), the form lacking Ser65 and GSK3-mediated phosphorylation (Un-P, red), and
492 the completely dephosphorylated protein produced by incubating in the presence of NaCl (De-P, white).

493

494 **Figure 4. The p.Ser64Phe (S64F) mutation greatly stabilizes *MAFA* in human EndoC-βH1 cells grown in
495 1.1 or 15.5mM glucose.** A) EndoC-βH1 cells were transfected with wild type (WT) and p.Ser64Phe (S64F)
496 *MAFA*-Myc and, after 48 hours, incubated with medium containing 1.1mM or 15.5mM glucose for an additional
497 12 hours. The transfected cells were then incubated with 25μg/mL cycloheximide (CHX) for the indicated time.

498 Transfected MAFA-Myc and endogenous β -actin protein levels were determined by immunoblotting (IB) using
499 anti-Myc and anti- β -actin antibodies, respectively. B) The Myc protein band intensity was measured in the
500 15.5mM glucose sample, normalized to β -actin, and plotted as a percentage of the initial band intensity. C) No
501 significant difference was found between WT and p.Ser64Phe (S64F) *MAFA* mRNA levels in transfected cells
502 grown in 1.1mM glucose. Endogenous *MAFA* mRNA levels also did not change under these conditions. Student's
503 two-tailed t-test. n.s., not significant. n = 3. Error bars represent SEM.

504