Identification of a novel β-cell glucokinase (GCK) promoter mutation (-71G>C) which modulates GCK gene expression through loss of allele-specific Sp1 binding causing mild fasting hyperglycaemia in humans

Daniela Gašperíková1*, Nicolas D Tribble2*, Juraj Staník1,3, Miroslava Hučková1, Nadežda Mišovíková4, Martijn van de Bunt5, Lucia Valentínová1, Beryl A Barrow2,5, Lubomír Barák3, Radoslav Dobránsky6, Eva Bereczková7, Jozef Michálek8, Kate Wicks9, Kevin Colclough10, Julian C Knight9, Sian Ellard10,11, Iwar Klimeš1**, Anna L Gloyn2,5**,

* Joint first authors  ** Joint last authors

1. DIABGENE and Diabetes Laboratory, Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovak Republic
2. Diabetes Research Laboratories, Oxford Centre for Diabetes, Endocrinology & Metabolism, University of Oxford, UK
3. Children Diabetes Center at the First Pediatric Department, Comenius University School of Medicine, Bratislava, Slovak Republic
4. Jessenius School of Medicine, Department of Clinical Genetics, Martin, Slovak Republic
5. Oxford NIHR Biomedical Research Centre, Churchill Hospital, Oxford, UK
6. Diabetology Outpatient Department, Reimanus Hospital, Presov, Slovak Republic
7. Children Endocrinology Outpatient Department, Dunajska Streda, Slovak Republic
8. National Institute of Endocrinology and Diabetology, Lubochna, Slovak Republic
9. Wellcome Trust Centre for Human Genetics, University of Oxford, UK
10. Department of Molecular Genetics, Royal Devon & Exeter NHS Foundation Trust, Exeter, UK
11. Institute of Biomedical and Clinical Science, Peninsula Medical School, Exeter, UK

Corresponding Author
Dr Anna L Gloyn
Email: anna.gloyn@drl.ox.ac.uk

Additional information for this article can be found in an online appendix at
http://diabetes.diabetesjournals.org

Submitted 14 January 2009 and accepted 21 April 2009.

This is an uncopyedited electronic version of an article accepted for publication in Diabetes. The American Diabetes Association, publisher of Diabetes, is not responsible for any errors or omissions in this version of the manuscript or any version derived from it by third parties. The definitive publisher-authenticated version will be available in a future issue of Diabetes in print and online at http://diabetes.diabetesjournals.org.
Identification of a novel GCK promoter mutation

Objective: Inactivating mutations in glucokinase (GCK) cause mild fasting hyperglycaemia. Identification of a GCK mutation has implications for treatment and prognosis therefore it is important to identify these individuals. A significant number of patients have a phenotype suggesting a defect in glucokinase but no abnormality of GCK. We hypothesized that the GCK β-cell promoter region, which is currently not routinely screened, could contain pathogenic mutations and we therefore sequenced this region in 60 such probands.

Research Design & Methods: The β-cell GCK promoter was sequenced in patient DNA. The effect of the identified novel mutation on GCK promoter activity was assessed using a luciferase reporter gene expression system. Electrophoretic Mobility Shift Assays (EMSAs) were employed to determine the impact of the mutation on Sp1 binding.

Results: A novel -71G>C mutation was identified in a non-conserved region of the human promoter sequence in 6 apparently unrelated probands. Family testing established co-segregation with fasting hyperglycemia, (≥5.5mmol/L) in 39 affected individuals. Haplotype analysis in the UK family and 4 of the Slovakian families demonstrated that the mutation had arisen independently. The mutation maps to a potential transcriptional activator binding site for Sp1. Reporter assays demonstrated that the mutation reduces promoter activity by up to 4 fold. EMSAs demonstrated a dramatic reduction in Sp1 binding to the promoter sequence corresponding to the mutant allele.

Conclusion: A novel β-cell GCK promoter mutation was identified which significantly reduces gene expression in vitro through loss of regulation by Sp1. To ensure correct diagnosis of potential GCK-MODY cases analysis of the β-cell GCK promoter should be included.
Diagnostic molecular genetic testing is available for many different monogenic forms of diabetes (1). One of the remaining clinical and scientific challenges however are the patients who clearly have a monogenic subtype of diabetes but are negative on screening using existing genetic tests (2; 3). Genetic linkage analysis can be employed to demonstrate or exclude linkage to known genes if there are sufficient family members to reach statistical significance (4).

However, many patients presenting with apparent monogenic forms of diabetes do not have large extended families to facilitate this process (4). Traditionally the coding region and exon-intron boundaries of the gene of interest have been screened for mutations, and in some cases high levels of conservation between species have been used to identify putative regulatory regions such as promoters and enhancers for additional mutational screening (5; 6).

Mutations in the gene encoding the key regulatory enzyme glucokinase (GCK) are one cause of Maturity-onset diabetes of the young (MODY) subtype glucokinase (GCK-MODY) previously termed MODY2, which is an autosomal dominantly inherited form of non-insulin dependent diabetes classified by the gene which is mutated (7). The phenotype of GCK-MODY has been well characterized and is distinct from other subtypes of MODY due to mutations in genes encoding transcription factors (8).

The heterozygous inactivating mutations seen in patients with GCK-MODY shift the set point for glucose-stimulated insulin secretion from ~5 to ~7 mmol/l, resulting in elevated fasting plasma glucose (FPG) levels (5.5–8.0 mmol/l) but normal (typically <4.6mmol/L) 2 hour plasma glucose increments (2HPGI) after a 75g oral glucose tolerance test (OGTT) (8). There are a number of patients who fulfill these criteria who have no abnormality of the GCK coding region (9). Tissue specific expression of GCK is governed by two promoters, initially described as specific for pancreatic β-cells and hepatocytes but now recognized to regulate expression in a wider range of tissues (10). The rodent hepatic GCK promoter has been extensively characterized but there is relatively little data on the transcriptional regulation of the human β-cell promoter (11).

The aim of this study was to extend our mutational screen in probands with a phenotype consistent with an abnormality of GCK who have no abnormality of the coding sequence, to the β-cell GCK promoter to identify mutations which could affect GCK expression.

MATERIALS & METHODS

Subjects: Unrelated probands of 60 families (30 Slovakian and 30 from the UK) with a clinical phenotype suggesting a defect in glucokinase but without a mutation in the GCK coding region were included in the study. Partial or entire deletions of the GCK gene were previously excluded by multiplex ligation-dependent probe amplification (MLPA) analysis in all UK probands (9). Selection criteria included a FPG levels ≥ 5.5mmol/l, treatment by diet, oral hypoglycaemic agents (OHAs), or very low doses of insulin (mean dose consistently <0.3IU/Kg/day) and detectable C-peptide levels. Eight-five blood relatives of the 6 probands with the GCK pancreatic promoter mutation were subsequently contacted and invited for blood sampling and mutation testing. This study was performed with full ethical approval of Ethical Committees in Bratislava and Lubochna (Slovakia) and in the UK and all subjects gave informed consent.

Genetic analysis of the human β-cell GCK promoter: DNA was isolated from peripheral blood using standard protocols. 324bp of the human pancreatic islet GCK promoter was amplified by PCR (primers
Identification of a novel GCK promoter mutation

The promoter region was examined by direct sequencing on an ABI 3130 Capillary sequencer (Applied Biosystems, Warrington, UK). Sequences were compared to the published sequence (NM_000162.2) using either SeqScape V2.1.1 (Applied Biosystems, Warrington, UK) or Mutation Surveyor V3.0 software (Softgenetics, Cambridge, UK). Haplotypes were constructed using the following microsatellites (D7S3043, D7S691, D7S2428, GCK1, GCK2, D7S667, D7S2506) which were run on an ABI 3130 analyzer. MLPA analysis was performed in the 5 Slovakian probands with the GCK promoter mutation using the SALSA MLPA Kit P241-Bi MODY/MRC-Holland (Amsterdam, Netherlands). Results were analyzed using GeneMarker V1.75 (Softgenetics). To circumvent computational constraints due to the pedigree size the pedigree SK R78 was divided into 40 two generation small pods using a facility in MEGA2 (http://portal.litbio.org/registered/help/mega2/inex/html) and the analyses were run in Merlin (12). The LOD score for family SK R78 was performed applying a rare autosomal dominant inheritance model using a disease allele frequency of 0.001 and a frequency of phenocopies of 0.1%.

Biochemistry: Fasting venous blood samples for glucose and hormonal analyses were collected into EDTA tubes (Sarstedt, Nümbrecht, Germany). Plasma glucose concentrations were measured with the glucose oxidase method (Hitachi 911, Hitachinaka, Japan). Insulin and C-peptide were determined using the Elecsys (Roche, Switzerland) chemiluminiscence automatic analyzer.

β-cell GCK promoter analysis: Alignment of the human β-cell GCK promoter was performed using the USCS bioinformatics Blat tool. The human β-cell promoter was analyzed for potential transcription factor binding sites by running a Matrix Search for Transcription Factor Binding Sites (MATCH) using TRANSFAC® Professional 12.1.

Transfection constructs: PCR primers were designed to generate five different lengths of the human GCK upstream promoter sequence (Supplemental tables and figures available in the online appendix at http://diabetes.diabetesjournal.org). The promoter fragments were amplified from human genomic DNA and subcloned into the pCR®2.1 TOPO® vector (Invitrogen, Paisley, UK). Prior to cloning DNA sequencing was performed to ensure the β-cell GCK promoter haplotype associated with variation in fasting plasma glucose (FPG) levels was not included (13). The fragments were subcloned into a pre-digested pGL3-basic luciferase reporter vector (Promega, Southampton, UK). The -71 G>C mutation was introduced using a Quick change mutagenesis kit (Stratagene, La Jolla, CA, USA) (Online supplementary Table 2). All mutations were confirmed by bidirectional sequencing.

Luciferase assay: INS-1 cells were cultured as previously described to a density of 1 x 10⁴ per well in 24-well tissue culture dishes 24 hours before transfection (14). Co-transfection was performed using Lipofectamine reagent (Invitrogen) with 580ng pGL3 promoter construct DNA plus 20ng pRL-TK per well, as according to the manufacturers protocol. Transfected cells were harvested after 24 hours and assayed for luciferase activity in a Veritas™ microplate luminometer using the Dual Luciferase Reporter Assay System (Promega). The Renilla luciferase (pRL-TK) was used as a recovery marker for data normalization. Promoter data are reported as the ratio of the Firefly/Renilla arbitrary units for each sample minus the value gained by the promoter-less pGL3-Basic vector. Each transfection experiment was carried out in triplicate on at least three independent occasions. Statistical
Electrophoretic Mobility Shift Assays (EMSA): Both wild type and -71 mutant GCK promoter oligonucleotides were generated spanning the potential Sp1 binding region predicted from TRANSFAC analysis and corresponding to sequences -53 to -88bp from the transcriptional start site. An additional oligonucleotide corresponding to a known Sp1 binding site was also used to serve as a positive control for Sp1 binding (Promega). The oligonucleotide probe sequences are given in Online supplementary Table 3. Binding reactions and EMSAs were conducted as previously described (15; 16). Briefly, 200ng Sp1 protein (Promega) was incubated with 0.2-0.5ng of α32P labeled probe (1-5 × 10⁴ cpm) at room temperature for 30 minutes. For supershift or competition analysis, the reaction mixture was pre-incubated with 1µg Sp1 or USF1 antiserum (Santa Cruz Biotechnology, Heidelberg, Germany) or with 7.5pmol (100X) unlabeled oligonucleotide probe at room temperature for 20 min prior to addition of the labeled probe.

RESULTS
Identification of a novel -71G>C GCK β- promoter mutation: Screening of 60 probands with a GCK-MODY phenotype and no abnormality of the GCK coding sequence identified 6 (5 from Slovakia and 1 from UK) apparently unrelated individuals with a novel -71G>C mutation. This mutation was not identified in > 400 ethnically matched normal chromosomes. Family testing demonstrated that the mutation co-segregated with fasting hyperglycemia (≥5.5mmol/L) in a total of 39 affected individuals and 52 unaffected individuals across the 6 pedigrees (Figure 1a). Using a rare autosomal dominant model the LOD score in family SKR78 alone was 5.00 which is well above the proposed guidelines for claiming statistically significant linkage with a LOD score of > 3.0 (17). MLPA analysis excluded partial or entire GCK gene deletions in all 6 probands. Haplotype analysis in the UK family and 4 of the Slovakian families demonstrated that the mutation had arisen independently and there was no evidence for a founder effect (Figure 1b). The β-cell -71 G>C promoter mutation is responsible for approximately 30% (5/17 families) of known cases of GCK-MODY in Slovakia (Gasperiková, Staník & Klimes unpublished data) whilst in the UK only one family has been identified and over 160 GCK-MODY cases with coding mutations have been identified (Colclough, Hattersley & Ellard unpublished data).

Clinical characteristics of patients with novel promoter mutation: The clinical characteristics of the individuals with the novel promoter mutation are summarized in Table 1. In line with other cases of GCK-MODY all patients had fasting hyperglycaemia (8). The age of diagnosis ranged from 6-81 years with 15 cases being recognized following the identification of the GCK mutation in one of the 6 probands. In affected individuals BMI ranged from 18.2-40.8 Kg/m² with a mean value of 24.6 ± 5.3 Kg/m². The majority of patients (23/39, 59%) with the mutation are registered with and seen by a diabetologist.

Of these 83% (19/23) are managed by diet alone with 3 patients on oral hypoglycaemic agents (one metformin [1000mg/day], two sulphonylureas [Diaprel MR, twice daily]) and one patient is on insulin treatment (NPH insulin 0.22 U / kg / day). The decision to treat these 4 patients was made by their referring physicians. None of these patients have had HbA1c values >7%, whilst HbA1c values on treatment are all < 6.5%. FPG levels in these patients are independent of insulin dose and/or oral hypoglycaemic agents used.

There were 2 individuals without the novel promoter mutation with diabetes. One
Identification of a novel GCK promoter mutation

Patient, a 50 year old female (Figure 1a, Pedigree SK R78, subject V:9) with a BMI of 28.0Kg/m² has several features consistent with the metabolic syndrome including hypertension (blood pressure 140/90mmHg) and dyslipidemia (total cholesterol 5.79mmol/L). She was diagnosed with hyperglycaemia at the age of 47 years and is currently treated with metformin. The second female patient (Figure 1a, patient SK R25 I:2) was diagnosed with diabetes at 49 years of age and is insulin treated. No further clinical or laboratory data are available on this subject as following blood withdrawal for genetic testing the patient refused to co-operate further in the study. Based on these clinical characteristics both of these patients have a phenotype consistent with classical type 2 diabetes and can be considered as phenocopies.

Activity of β-cell GCK promoter in INS-1 cells and effects of the -71G>C mutation: In order to establish the appropriate promoter construct in which to study the effect of the -71G>C mutation, reporter gene constructs containing different GCK promoter fragment lengths were prepared ranging from -263bp to -1031bp with respect to the ATG start site. Luciferase activity was detectable with all GCK promoter constructs (Figure 2a). Decreased expression levels seen with fragments -618bp and -826bp suggesting the presence of repressor elements within this region. The effect of the -71G>C mutation was then analyzed using the -430bp promoter fragment which had near maximal reporter gene expression indicating necessary binding sites for expression were contained in this fragment; site directed mutagenesis was also carried out for the full length -1031bp fragment. When these constructs were analyzed, the GCK -71 G>C promoter variant causes a dramatic reduction in promoter activity in the presence of the C vs the G allele for both promoter lengths (P=3.6x10⁻⁶ and 1.9x10⁻⁶ for the -430bp and -1031bp fragments respectively) (Figure 2b). This provides robust evidence that the point mutation has a repressive effect on gene expression, suggesting either allele-specific recruitment of a transcriptional repressor or loss of activator binding.

Identification of potential transcription factor binding sites in the β-cell GCK promoter: Using the USCS Blat tool, no sequence could be identified corresponding to the -64bp to -89bp region of the human GCK promoter in either the mouse or rat GCK promoters due to either a deletion in the rodent or an insertion in the human DNA (Supplementary Figure 1). MATCH analysis on the wild type GCK promoter identified a number of potential activator and repressor regions within the -71bp region of interest (Online supplementary Table 4). Identical MATCH analysis conducted on the -71 variant GCK promoter demonstrated the potential loss of the majority of these transcription factor binding sites including the transcriptional activator Sp1 (Online supplementary Table 4).

Sp1 binding is reduced due to the -71 G>C mutation: To demonstrate that Sp1 can indeed bind to the putative binding site located at the -71bp region of the wild-type promoter, gel shift experiments were performed. Using the wild-type oligonucleotide two clear bands were observed which were specific on competition with molar excess of unlabelled self or an unlabelled probe corresponding to a consensus Sp1 binding site (Figure 3a). The mobility of these bands matched those seen when the consensus Sp1 binding site probe was radiolabelled and used in the binding reaction (Figure 3b). The complexes were lost when a radiolabelled probe corresponding to the C allele of the point mutation was used in the binding reaction; this probe also competed much less effectively when used as an unlabelled
Identification of a novel GCK promoter mutation

DISCUSSION

In this study we have identified the first mutation in the GCK β-cell promoter which causes elevated glucose levels due to decreased GCK expression in 6 probands; 5 from Slovakia and 1 from the UK. The mutation co-segregated with fasting hyperglycemia in all 6 families with a total of 39 affected individuals (Figure 1a). Haplotype analysis demonstrated that the mutation had arisen independently between the UK and Slovakian families with no evidence for a founder effect in the Slovakian families (Figure 1b). The clinical phenotype of patients with GCK-MODY attributable to a promoter mutation is indistinguishable from that caused by a mutation in the coding region of the gene [Table 1 and Stride et al (8)]. This is entirely expected as compensation will be provided by the wild type allele (18; 19).

Functional characterization of the GCK promoter clearly demonstrates that the GCK -71 C>G promoter variant causes a dramatic reduction in promoter activity. Bioinformatic analysis predicted that this mutation could potentially result in the loss of transcription factor binding within the -71bp region. Allele-specific modulation of Sp1 binding was then demonstrated by EMSA. Sp1 has previously been demonstrated to enhance GCK transcription in gilthead seabream (Sparus aurata) (20). In rodent studies Sp1 has also been shown to play an integral part of the mechanisms for the transcription of several glycolytic enzymes, such as pyruvate kinase, aldolase A and acetyl-CoA (21-23). The results clearly show an allele specific difference in Sp1 binding with the -71 G>C mutation losing Sp1 binding capacity. This provides a clear explanation of the reduced gene expression levels associated with this novel mutation and the first evidence in humans for an essential role of Sp1 regulation in GCK expression.

The significance of this finding is that routine molecular diagnostic testing for GCK-MODY does not currently include the promoter region. Given the implications for prognosis, treatment decisions and inheritance it is important to identify these individuals (24). Interestingly, a comparison of the promoter sequences across species demonstrated that the sequence containing the novel mutation was absent from both the rat and mouse β-cell promoters. This is unusual as normally pathogenic mutations occur in regions which are conserved (and therefore supposed to be functionally important) across species. This observation has implications for the design of future studies evaluating the role of non-coding regions in pathogenesis of both monogenic and multifactorial diabetes.

CONCLUSIONS

We have identified the first mutation in the GCK β-cell promoter in a total of 39 individuals with mild fasting hyperglycemia. This mutation has been demonstrated at the genetic and functional level to cause GCK-MODY. Our results support the inclusion of the GCK β-promoter region in routine diagnostic testing for GCK-MODY.

ACKNOWLEDGEMENTS

The authors thank Cecilia Lindgren for assistance with calculating the LOD score in family SK R78 and Ms. Barbora Gavenciakova, Alica Mitkova and Miroslava Rabajdova for technical support. ALG is a Medical Research Council (MRC) New Investigator. This work was supported in Oxford by grants to ALG from the MRC (81696), Diabetes UK, the Nuffield Department of Clinical Medicine, University of Oxford and the Oxford NIHR Biomedical Research Centre Programme. This work was
supported in Slovakia by research grants to IK from MZ.2005/150NEDU-01, APVV-51-014205, BITCET, CENDO.
REFERENCES
1. Ellard S, Bellanne-Chantelot C, Hattersley AT: Best practice guidelines for the molecular genetic diagnosis of maturity-onset diabetes of the young. *Diabetologia* 51:546-553, 2008
2. Pearson ER, Pruhova S, Tack CJ, Johansen A, Castleden HA, Lumb PJ, Wierzbicki AS, Clark PM, Lebl J, Pedersen O, Ellard S, Hansen T, Hattersley AT: Molecular genetics and phenotypic characteristics of MODY caused by hepatocyte nuclear factor 4alpha mutations in a large European collection. *Diabetologia* 48:878-885, 2005
3. Frayling TM, Evans JC, Bulman MP, Pearson E, Allen L, Owen K, Bingham C, Hannemann M, Shepherd M, Ellard S, Hattersley AT: beta-cell genes and diabetes: molecular and clinical characterization of mutations in transcription factors. *Diabetes* 50:S94-100., 2001
4. Frayling TM, Lindgren CM, Chevre JC, Menzel S, Wishart M, Benmezroua Y, Brown A, Evans JC, Rao PS, Dina C, Lecoeur C, Kanninen T, Almgren P, Bulman MP, Wang Y, Mills J, Wright-Pascoe R, Mahtani MM, Prisco F, Costa A, Cognet I, Hansen T, Pedersen O, Ellard S, Tuomi T, Groop LC, Froguel P, Hattersley AT, Vaxillaire M: A genome-wide scan in families with maturity-onset diabetes of the young: evidence for further genetic heterogeneity. *Diabetes* 52:872-881, 2003
5. Thomas H, Jaschkowitz K, Bulman M, Frayling TM, Mitchell SMS, Roosen S, Lingott-Frig A, Tack CJ, Ellard S, Ryffel GU, Hattersley AT: A distant upstream promoter of the HNF-4alpha gene connects the transcription factors involved in maturity-onset diabetes of the young. *Hum Mol Genet* 10:2089-2097., 2001
6. Mitchell SM, Gloyn AL, Owen KR, Hattersley AT, Frayling TM: The role of the HNF4alpha enhancer in type 2 diabetes. *Mol Genet Metab* 76:148-151, 2002
7. Gloyn AL: Glucokinase (*GCK*) mutations in hyper- and hypoglycemia: Maturity-onset diabetes of the young, permanent neonatal diabetes, and hyperinsulinemia of infancy. *Hum Mutat* 22:353-362, 2003
8. Stride A, Vaxillaire M, Tuomi T, Barbetti F, Njolstad PR, Hansen T, Costa A, Congent I, Pedersen O, Sovik O, Lorini R, Groop L, Froguel P, Hattersley AT: The genetic abnormality in the beta cell determines the response to an oral glucose load. *Diabetologia* 45:427-435, 2002
9. Ellard S, Thomas K, Edghill EL, Owens M, Ambye L, Cropper J, Little J, Strachan M, Stride A, Ersay B, Eiberg H, Pedersen O, Shepherd MH, Hansen T, Harries LW, Hattersley AT: Partial and whole gene deletion mutations of the *GCK* and *HNF1A* genes in maturity-onset diabetes of the young. *Diabetologia* 50:2313-2317, 2007
10. Matschinsky FM: Regulation of pancreatic beta-cell glucokinase: from basics to therapeutics. *Diabetes* 51 Suppl 3:S394-404, 2002
11. Dussoix P, Vaxillaire M, Iyendedjan PB, Tiercy JM, Ruiz J, Spinas GA, Berger W, Zahnd G, Froguel P, Philippe J: Diagnostic heterogeneity of diabetes in lean young adults: classification based on immunological and genetic parameters. *Diabetes* 46:622-631, 1997
12. Abecasis GR, Cherny SS, Cookson WO, Cardon LR: Merlin--rapid analysis of dense genetic maps using sparse gene flow trees. *Nat Genet* 30:97-101, 2002
13. Weedon MN, Clark VJ, Qian Y, Ben-Shlomo Y, Timpson N, Ebrahim S, Lawlor DA, Pembrey ME, Ring S, Wilkin TJ, Voss LD, Jeffery AN, Metcalf B, Ferrucci L, Corsi AM, Murray A, Melzer D, Knight B, Shields B, Smith GD, Hattersley AT, Di Rienzo A, Frayling
Identification of a novel GCK promoter mutation

TM: A common haplotype of the glucokinase gene alters fasting glucose and birth weight: association in six studies and population-genetics analyses. *Am J Hum Genet* 79:991-1001, 2006

14. Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB: Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 130:167-178, 1992

15. Pugh BF, Tjian R: Mechanism of transcriptional activation by Sp1: evidence for coactivators. *Cell* 61:1187-1197, 1990

16. Udalova IA, Knight JC, Vidal V, Nedospasov SA, Kwiatkowski D: Complex NF-kappaB interactions at the distal tumor necrosis factor promoter region in human monocytes. *J Biol Chem* 273:21178-21186, 1998

17. Ott J: *Analysis of Human Genetic Linkage*. Baltimore, MD, Johns Hopkins University Press, 1999

18. Liang Y, Najafi H, Smith RM, Zimmerman EC, Magnuson MA, Tal M, Matschinsky FM: Concordant glucose induction of glucokinase, glucose usage, and glucose-stimulated insulin release in pancreatic islets maintained in organ culture. *Diabetes* 41:792-806, 1992

19. Gloyn A, Odili S, Buettger C, Njolstad PR, Shiota C, Magnuson M, Matschinsky F: Glucokinase and the regulation of blood sugar: A mathematical model predicts the threshold for glucose stimulated insulin release for GCK gene mutations that cause hyper- and hypoglycaemia. In *Glucokinase and Glycemic Diseases: from the basics to novel therapeutics* Magnuson M, Matschinsky F, Eds., Karger, 2004, p. 92-109

20. Egea M, Meton I, Cordoba M, Fernandez F, Baanante IV: Role of Sp1 and SREBP-1a in the insulin-mediated regulation of glucokinase transcription in the liver of gilthead sea bream (Sparus aurata). *Gen Comp Endocrinol* 155:359-367, 2008

21. Hermfisse U, Schafer D, Netzker R, Brand K: The aldolase A promoter in proliferating rat thymocytes is regulated by a cluster of SP1 sites and a distal modulator. *Biochem Biophys Res Commun* 225:997-1005, 1996

22. Netzker R, Weigert C, Brand K: Role of the stimulatory proteins Sp1 and Sp3 in the regulation of transcription of the rat pyruvate kinase M gene. *Eur J Biochem* 245:174-181, 1997

23. Daniel S, Kim KH: Sp1 mediates glucose activation of the acetyl-CoA carboxylase promoter. *J Biol Chem* 271:1385-1392, 1996

24. Gloyn AL, Ellard S: Defining the genetic aetiology of monogenic diabetes can improve treatment. *Expert Opin Pharmacother* 7:1759-1767, 2006
Identification of a novel GCK promoter mutation

Figure Legends

Figure 1a: Extended pedigrees of the six probands identified with the novel GCK -71 G>C promoter mutation

Extended pedigrees of the six probands with the novel -71G>C GCK promoter mutation showing 5 Slovakian (SK R78, SK R25, SK R71, SK R94, SK R124) and 1 UK (UK MY180SC) family. Arrows indicate the probands. Filled icons indicate individuals with fasting hyperglycemia (≥5.5 mmol/L), empty icons indicate normoglycemic subjects. The GCK mutation status is shown under each symbol. NM – denotes heterozygous for the -71G>C GCK promoter mutation, NN – denotes wild type. Individuals V: 9 (SK R78 family) and I: 2 (SK R25 family) are diabetic subjects (phenocopies) with the NN genotype (for explanation see the text in the result section).

Figure 1b: Haplotype analysis performed in 5 of the 6 families with the novel GCK promoter mutation.

Squares denote males, circles females and solid symbols show individuals affected with fasting hyperglycemia (≥5.5 mmol/L). Genotype is shown underneath each symbol, with NM = heterozygous c.-71G>C mutation and NN = mutation not present. Solid bars indicate the haplotype co-segregating with fasting hyperglycemia on which the GCK c.-71G>C mutation has arisen. The marker order, location of the GCK gene and location on chromosome 7 are shown in the box.

Figure 2a: Comparison of luciferase activity generated with varying lengths of upstream GCK promoter.

The promoter fragment lengths incorporated into the PGL3-basic vector are shown on the left of the figure and ranged from -263bp to -1031bp. The bar chart on the right shows mean luciferase activity ± SE in co-transfected INS-1 cells with the different promoter lengths. The reported luciferase activity is normalized to renilla and PGL3 null expression levels.

Figure 2b: Functional characterization of novel GCK -71 G>C promoter mutation demonstrates reduced promoter activity.

Mean luciferase activity of wild type promoter fragments of -430bp and -1031bp are represented by the black bars whereas the luciferase activity of their counterparts containing a -71 G>C mutation are represented by the white bars ± SE. Statistical significance was determined by two-tailed t-test. The reduction in activity was significant (P values *3.6x10^{-6} and **1.9x10^{-6}). Expression is in INS-1 cells and reported luciferase activity is normalized to renilla and PGL3 null expression levels.

Figure 3. DNA mobility shift assay with recombinant human Sp1 nuclear protein. Sp1 binds both the wild-type and Sp1 oligonucleotides whereas the -71 G>C mutation causes a dramatic reduction in Sp1 binding. (A) Labeled GCK promoter (-53 to -88) and Sp1 consensus oligonucleotides were incubated in the presence or absence of Sp1 nuclear protein. Competition experiments were conducted using 100-fold molar excess of unlabeled oligonucleotides. (B) Specific Sp1 and non-specific USF antibodies were used to confirm the specificity of Sp1 binding which was visualized as a super-shifted band of reduced mobility.
**Table 1** Clinical Characteristics of individuals with the novel -71 C>G mutation

|                      | GCK promoter mutation carriers | Non-mutation carriers | Published data on GCK-mutation carriers (8) |
|----------------------|-------------------------------|-----------------------|--------------------------------------------|
| n                    | 39                            | 52                    | 245                                        |
| Gender (M/F)         | (19/20)                       | (17/35)               | (125/120)                                  |
| Mean age yrs (range) | 37.0 (8-86)                   | 31.0 (1-80)           | 26.7 (2-79)                                |
| Mean BMI Kg/m² (range)| 24.6 (18.2-40.8)              | 25.3 (19.3-36.9)      | 21.1 (13.8-40.9)                           |
| Mean FPG mmol/L (SD) | 7.0 (1.0)                     | 5.1 (0.5)             | 6.8 (0.8)                                  |
Extended pedigrees of the six probands with the novel -71G>C GCK promoter mutation showing 5 Slovakian (SK R78, SK R25, SK R71, SK R94, SK R124) and 1 UK (UK MY180SC) family. Arrows indicate the probands. Filled icons indicate individuals with fasting hyperglycemia (≥5.5 mmol/L), empty icons indicate normoglycemic subjects. The GCK mutation status is shown under each symbol. NM – denotes heterozygous for the -71G>C GCK promoter mutation, NN – denotes wild type. Individuals V: 9 (SK R78 family) and I: 2 (SK R25 family) are diabetic subjects (phenocopies) with the NN genotype (for explanation see the text in the result section).
Figure 1b: Haplotype analysis performed in 5 of the 6 families with the novel GCK promoter mutation.

Squares denote males, circles females and solid symbols show individuals affected with fasting hyperglycemia (≥5.5 mmol/L). Genotype is shown underneath each symbol, with NM = heterozygous c.-71G>C mutation and NN = mutation not present. Solid bars indicate the haplotype co-segregating with fasting hyperglycemia on which the GCK c.-71G>C mutation has arisen. The marker order, location of the GCK gene and location on chromosome 7 are shown in the box.
Figure 2a: Comparison of luciferase activity generated with varying lengths of upstream GCK promoter. The promoter fragment lengths incorporated into the PGL3-basic vector are shown on the left of the figure and ranged from -263bp to -1031bp. The bar chart on the right shows mean luciferase activity ± SE in co-transfected INS-1 cells with the different promoter lengths. The reported luciferase activity is normalized to renilla and PGL3 null expression levels.
Identification of a novel GCK promoter mutation

Figure 2b: Functional characterization of novel GCK -71 G>C promoter mutation demonstrates reduced promoter activity. Mean luciferase activity of wild type promoter fragments of -430bp and -1031bp are represented by the black bars whereas the luciferase activity of their counterparts containing a -71 G>C mutation are represented by the white bars ± SE. Statistical significance was determined by two-tailed t-test. The reduction in activity was significant (P values *3.6x10^{-6} and **1.9x10^{-6}). Expression is in INS-1 cells and reported luciferase activity is normalized to renilla and PGL3 null expression levels.
Figure 3. DNA mobility shift assay with recombinant human Sp1 nuclear protein. Sp1 binds both the wild-type and Sp1 oligonucleotides whereas the -71 G>C mutation causes a dramatic reduction in Sp1 binding. (A) Labeled GCK promoter (-53 to -88) and Sp1 consensus oligonucleotides were incubated in the presence or absence of Sp1 nuclear protein. Competition experiments were conducted using 100-fold molar excess of unlabeled oligonucleotides. (B) Specific Sp1 and non-specific USF antibodies were used to confirm the specificity of Sp1 binding which was visualized as a super-shifted band of reduced mobility.