Supporting online information for Soranzo et al.
Supplementary Methods and Appendix

Common variants at ten genomic loci influence hemoglobin A\textsubscript{1c} levels via glycemic and non-glycemic pathways

Corresponding author(s):

Jose C. Florez MD, PhD
Diabetes Research Center (Diabetes Unit) and Center for Human Genetic Research
Simches Research Center – CPZN 5.250
Massachusetts General Hospital
185 Cambridge Street
Boston, MA 02114
Office: 617-643-3308
email: jcflorez@partners.org

Manuela Uda
Istituto di Neurogenetica e Neurofarmacologia
Consiglio Nazionale delle Ricerche
Cittadella Universitaria di Monserrato
Monserrato, Cagliari, Italy 09042
Office: +39 070675 4591
email: manuela.uda@inn.cnr.it

Nicholas J. Wareham
MRC Epidemiology Unit
Institute of Metabolic Science
Addenbrooke's Hospital
Hills Road
Cambridge, CB2 0QQ
Office: +44 (0)1223 330315
email: nick.wareham@mrc-epid.cam.ac.uk

Inês Barroso
Wellcome Trust Sanger Institute
Genome Campus
Hinxton, CB10 1SA
Office: +44 (0)1223 495341
email: ib1@sanger.ac.uk

James B. Meigs MD, MPH
General Medicine Division
Massachusetts General Hospital
50 Stanford St 9th Floor
Boston, MA 02114
Office: 617-724-3203
email: jmeigs@partners.org

Supplementary Methods
Genotyping, imputation and quality control

Study samples are described in Table 1. Study specific parameters and pre-imputation filters are specified in Table S1. Each study applied similar criteria for data calling. Prior to imputation, the criteria applied for exclusion of SNPs were: (i) minor allele frequency (MAF) <0.01, (ii) Hardy-Weinberg equilibrium P <10^{-4} or 10^{-6} and (iii) call-rate <0.90 or 0.95. Criteria applied for exclusion of samples were: (i) call-rate <0.95 or <0.97, (ii) sex mismatch between genotypes and reported sex, and (iii) outliers as assessed by population structure analysis. Imputation of additional autosomal SNPs from the HapMap CEU (1) reference panel was performed using the software MACH (2) or IMPUTE (3).

As standard for imputation, we excluded sex chromosome-linked SNPs from analyses given the difficulty of accurately imputing non-autosomal SNPs and the poor overlap of X-chromosome SNPs across different platforms. SNPs were also excluded if the cohort-specific imputation quality was particularly poor (observed-over-expected variance ratio (r^2.hat) <0.3 if MACH was used for imputation, or proper-info <0.4 if IMPUTE was used) or if MAF < 0.01. In total, up to 2.5 million genotyped or imputed autosomal SNPs were considered for meta-analysis. We only report on individual SNPs imputed or genotyped in ≥6,000 participants.

Statistical methods for primary analyses

In each cohort we fitted a linear regression model using measured HbA_1C (%) as the dependent variable to evaluate the additive effect of genotyped and imputed SNPs. The model was adjusted for age, sex and/or study site and family structure (Table S1). The association was tested taking genotype and imputation uncertainty into account, using a missing data likelihood test as implemented in SNPTEST (3) or by using allele dosages in the linear regression model as implemented in ProbABEL (4) or MACH2QTL (2) for unrelated samples or in Merlin (5) or using a linear mixed effects model implemented in the Imekein function of the R kinship package for family-based studies. Regression estimates for each SNP were combined across studies in a meta-analysis using a fixed effect inverse-variance approach, as implemented in METAL (http://www.sph.umich.edu/csg/abecasis/Metal/index.html). The individual cohort analysis results were corrected prior to performing the meta-analysis for residual inflation of the test statistic using the genomic control method if the lambda coefficient was > 1.0. Heterogeneity was assessed using the standard chi-square test implemented in METAL, Cochran’s Q statistic and the F statistics (6).

Conditional analyses

We used conditional analyses to infer whether the ten HbA_1C loci (Table 2) have associations with HbA_1C through glycemic or non-glycemic pathways by implementing a two-stage regression approach. First, we selected a subset of up to 23,654 non-diabetic participants from 15 cohorts having HbA_1C and fasting glucose (FG) levels measured, or up to 6,394 non-diabetic participants from 6 cohorts having HbA_1C and 2-hr post-challenge levels measured. In these participants we calculated separate regressions of HbA_1C and FG on each of the ten genome-wide significant SNPs; these estimates reflect the unadjusted effect of the genetic variants on HbA_1C and glucose. To identify the glucose-dependent and glucose-independent effects on HbA_1C, we adjusted the HbA_1C regressions on the genetic variants additionally for FG or 2-hr post challenge in models adjusted for sex, age and other study-specific covariates. We further meta-analyzed summary statistics using inverse-variance meta-analysis as in the primary analysis.

For the ANK1 locus, an additional conditional analysis was carried out to test whether an independent association signal was present for SNP rs6474359. This signal appeared to be statistically independent from the lead SNP rs4737009, as shown by low linkage disequilibrium (pairwise r^2 with rs4737009 = 0.0001). In each study, the association at chromosome 8 was evaluated including SNP rs4737009 as an additional covariate to
the basic model, and then results were meta-analyzed as in the primary analysis.

Similarly, we used conditional analyses to assess whether the ten loci (Table 2) affect HbA1C through hematological mechanisms. First, we selected a subset of up to 7,500 samples from four cohorts (KORA F3, KORA F4, SardiNIA and NHANES III) with available data for hemoglobin levels (Hb), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), iron levels and transferrin. In these participants we calculated separate regressions of HbA1C and HbA1C adjusted for each of the hematological parameters on each of the ten genome-wide significant SNPs. All models were also adjusted for sex, age and other study-specific covariates. We further meta-analyzed summary statistics using inverse-variance meta-analysis as in the primary analysis.

**Calculation of explained variance**

To estimate the total variance in HbA1C explained by the ten lead SNPs (rs2779116, rs552976, rs1800562, rs1799884, rs4737009, rs16926246, rs1387153, rs7998202, rs1046896, rs855791), we fitted a regression model for each GWAS cohort including the ten SNPs, and calculated an estimate of the variance explained by the SNPs as sample size weighted average in the following samples: ARIC, B58C-T1DGC, B58C-WTCCC, BLSA, DESIR, EPIC cases, EPIC cohort, Fenland, FHS, GenomeEUtwin, KORA F3, KORA F4, Lollipop, NTR and SHIP. The cohort-specific total variance explained by the ten SNPs was calculated as the difference between the variance explained by the full model and the variance of a basic model including only sex, age and the study-specific covariates.

**Calculation of HbA1C genotype score**

We defined a risk score for the ten leading SNPs as a weighted sum of the number of expected risk alleles, where the sum of the weights was set to the number of SNPs and the weights were proportional to the estimate of the effect size for each SNP (beta coefficients from the association model). The same approach was taken for the seven non-glycemic loci. Mean HbA1C (%) levels according to the number of weighted risk alleles were computed in some of the largest population cohorts (FHS, ARIC, SardiNIA and KORA F4) with all seven or ten SNPs available (genotyped or imputed). For FHS and SardiNIA, a mixed effect model with a single variable with two groups (lower 10% versus upper 10%) was used to account for relatedness among participants, for the other studies, fixed-effects models were used. We carried out the same calculation using the seven non-glycemic loci (rs2779116, rs1800562, rs4737009, rs16926246, rs7998202, rs1046896, rs855791). For both ten and seven loci we calculated a weighted average difference in the HbA1c level between the 10% tails of the genotype score distribution (N=200 in FHS, N=335 in SardiNIA, N=149 in KORA).

**Association with intermediate and disease endpoints**

The top SNPs were additionally tested for association with other metabolic traits using available meta-analysis data from MAGIC (7). Associations with FG (n=40,934-46,184), fasting insulin (n=33,182-38,236), β-cell function by homeostasis model assessment (HOMA-B; n=31,434-36,464) and insulin resistance by homeostasis model assessment (HOMA-IR; n=31,884-37,035) were calculated as described previously (7). Associations with oral glucose tolerance tests (2-hr glucose, n=10,075-15,234 and 2-hr insulin, n=3,690-7,062) were calculated as described previously (8). Analyses of HbA1C conditional on FG were calculated in a subset of the samples as described above. Associations with hematologic traits were obtained from a meta-analysis carried out on the four populations (KORA F3, KORA F4, SardiNIA and NHANES III) with available data for Hb, MCH, MCV iron levels and transferrin. Associations of MTNR1B, GCK and G6PC2/ABCB11 with type 2 diabetes (T2D) were obtained from a total of 8,130 cases and 38,987 controls (or 6,206 cases and 36,049 controls for SNP rs1800562 (HFE)) from the DIAGRAM+ consortium (9).
For associations with coronary artery disease, we obtained summary statistics for 13,925 cases and 14,590 controls (in aggregate) from nine case-control collections. Sample characteristics and case/control definitions are given in Table S5, and study-specific association results are given in Table S6. Pooled summary statistics (odds ratios, 95% confidence intervals and $P$ values) were calculated under a fixed-effects model as there was no evidence for inter-cohort heterogeneity, using custom scripts implemented in the R environment (available from the authors on request).

Author Contributions:


Acknowledgments

**ARIC**
The Atherosclerosis Risk in Communities Study is carried out as a collaborative study supported by National Heart, Lung, and Blood Institute contracts N01-HC-55015, N01-HC-55016, N01-HC-55018, N01-HC-55019, N01-HC-55020, N01-HC-55021, N01-HC-55022, R01HL087641, R01HL59367 and R01HL086694; National Human Genome Research Institute contract U01HG004402; and National Institutes of Health contract HHSN268200625226C. The authors thank the staff and participants of the ARIC study for their important contributions. Infrastructure was partly supported by Grant Number UL1RR025005, a component of the National Institutes of Health and NIH Roadmap for Medical Research. This research was supported in part by NIH/NIDDK grants R21 DK080294 and K01 DK076595 (Dr. Selvin) and K01067207 (Dr. Kao).

**B58C-T1DGC**
This research utilizes resources provided by the Type 1 Diabetes Genetics Consortium, a collaborative clinical study sponsored by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institute of Allergy and Infectious Diseases (NIAID), National Human Genome Research Institute (NHGRI), National Institute of Child Health and Human Development (NICHD), and Juvenile Diabetes Research Foundation International (JDRF) and supported by U01 DK062418. T1DGC GWAS data were deposited by the Diabetes and Inflammation Laboratory, Cambridge Institute for Medical Research (CIMR), University of Cambridge (John Todd, Helen Stevens and Neil Walker), which is funded by Juvenile Diabetes Research Foundation International, the Wellcome Trust and the National Institute for Health Research Cambridge Biomedical Research Centre; the CIMR is in receipt of a Wellcome Trust Strategic Award (079895).

**B58C-WTCCC**
We acknowledge use of genotype data from the British 1958 Birth Cohort DNA collection, funded by the Medical Research Council grant G0000934 and the Wellcome Trust grant 068545/Z/02. Genotyping for the Wellcome Trust Case Control Consortium was funded by the Wellcome Trust grant 076113/B/04/Z.

**BLSA**
This research was supported in part by the Intramural Research Program of the NIH, National Institute on Aging. A portion of that support was through a R&D contract with MedStar Research Institute.

**Croatia**
The VIS study in the Croatian island of Vis was supported through the grants from the Medical Research Council UK to H.C., A.F.W. and I.R.; and Ministry of Science, Education and Sport of the Republic of Croatia to I.R. (number 216-1080315-0302). The authors collectively thank a large number of individuals for their individual help in organizing, planning and carrying out the field work related to the project and data management: Professor Pavao Rudan and the staff of the Institute for Anthropological Research in Zagreb, Croatia (organization of the field work, anthropometric and physiological measurements, and DNA extraction); Professor Ariana Vorko-Jovic and the staff and medical students of the Andrija Stampar School of Public Health of the Faculty of Medicine, University of Zagreb, Croatia (questionnaires, genealogical reconstruction and data entry); Dr Branka Salzer from the biochemistry lab "Salzer", Croatia (measurements of biochemical traits); local general practitioners and nurses (recruitment and communication with the study population); and the employees of several other Croatian institutions who participated in the field work, including but not limited to the University of Rijeka, Croatia; Croatian Institute of Public Health. SNP Genotyping of the samples was carried out by the Genetics Core Laboratory at the Wellcome Trust Clinical Research Facility, WGH, Edinburgh.
We thank the Icelandic study participants whose contribution made this work possible. We would also like to acknowledge the staff at the Clinical Research Centre (Iceland) and the deCODE Genetics biological materials and genotyping facilities for their work. The research performed at deCODE Genetics was part funded through the European Community’s Seventh Framework Programme (FP7/2007-2013), ENGAGE project, grant agreement HEALTH-F4-2007-201413.

**DESIR**

French GWA study was supported in part by the “Conseil Regional Nord-Pas-de-Calais: Fonds européen de développement économique et regional”, Genome Quebec-Genome Canada and the British Medical Research Council. NB-N’s position is supported by a grant from the ANR (Agence Nationale pour la Recherche: ANR-06 PHYSIO - 037 -02). We acknowledge funding to PF by the European Union (Integrated Project EURODIA LSHM-CT-2006-518153 in the Framework Programme 6 [FP06] of the European-Community). We thank Christian Dina for statistical support, Jean Tichet and Michel Marre from the DESIR study group.

**DGI**

We thank study participants, the Botnia and Skara research teams for clinical contributions, and colleagues at MGH, Broad, Novartis and Lund for data collection, genotyping and analysis. The initial GWAS genotyping was supported by Novartis (to DA). DA was a Burroughs Wellcome Fund Clinical Scholar in Translational Research, and is a Distinguished Clinical Scholar of the Doris Duke Charitable Foundation. LG, VL and the Botnia Study are principally supported by the Sigrid Juselius Foundation, the Finnish Diabetes Research Foundation, The Folkhalsan Research Foundation and Clinical Research Institute HUCH Ltd; work in Malmö, Sweden was also funded by grants from the Swedish Research Council and the Wallenberg Foundation.

**DIAGEN**

We are grateful to all of the patients who cooperated in this study and to their referring physicians and diabetologists in Saxony. This study was supported by the Dresden University of Technology funding grant, Med Drive.

**EPIC-Norfolk**

EPIC-Norfolk is supported by programme grants from the Medical Research Council UK and Cancer Research UK and with additional support from the European Union, Stroke Association, British Heart Foundation, Research into Ageing, Department of Health, The Wellcome Trust and the Food Standards Agency. SLR is funded by the BHF.

**Fenland**

The Fenland Study is funded by the Wellcome Trust and the Medical Research Council. We are grateful to the volunteers, and to the General Practitioners and practice staff for help with recruitment. We thank the Fenland Study Co-ordination team, the Field Epidemiology team and the Fenland Study Investigators. HbA1c measurement was performed by the Cambridge University Hospitals NHS Foundation Trust Department of Clinical Biochemistry.

**FHS**

This research was conducted in part using data and resources from the Framingham Heart Study of the National Heart Lung and Blood Institute of the National Institutes of Health and Boston University School of Medicine. The analyses reflect intellectual input and resource development from the Framingham Heart Study investigators participating in the SNP Health Association Resource (SHARe) project. This work was partially supported by the National Heart, Lung and Blood Institute’s Framingham Heart Study (Contract No. N01-HC-25195) and its contract with Affymetrix, Inc for genotyping services (Contract No. N02-HL-6-4278). A portion of this research utilized the Linux Cluster for Genetic Analysis.
(LinGA-II) funded by the Robert Dawson Evans Endowment of the Department of Medicine at Boston University School of Medicine and Boston Medical Center. Also supported by National Institute for Diabetes and Digestive and Kidney Diseases (NIDDK) R01 DK078616 to Drs. Meigs, Dupuis and Florez, NIDDK K24 DK080140 to Dr. Meigs, and a Massachusetts General Hospital Physician Scientist Development Award and a Doris Duke Charitable Foundation Clinical Scientist Development Award to Dr. Florez.

**GenMETS/HEALTH2000**

This research was partly funded through the European Community's Seventh Framework Programme (FP7/2007-2013), ENGAGE project, grant agreement HEALTH-F4-2007-201413, by the Academy of Finland SALVE program PUBGENSENSE; grants no. 129322, 129494, and Academy of Finland - Finnish Centre of Excellence in Complex Disease Genetics, grant number 129680, by the Sigrid Juselius Foundation and by the Finnish Foundation for Cardiovascular Research. Mirkka Perkkalainen, Verner Anttila and Michael Inouye are thanked for their help in data analyses.

**GenomeEUtwin**

We acknowledge support from the European Commision (Genomewide analyses of European twin and population cohorts EU/QLRT-2001-01254); Twins UK are also funded by the Wellcome Trust; NIHR fellowship, NIHR Biomedical Research Centre grant to Guys' and St. Thomas' Hospitals and King's College London.

**KORA**

The KORA Augsburg studies were financed by the Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany and supported by grants from the German Federal Ministry of Education and Research (BMBF). Part of this work was financed by the German National Genome Research Network (NGFN). Our research was supported within the Munich Center of Health Sciences (MC Health) as part of LMUinnovativ. Work on diabetes traits within KORA S4/F4 was funded by a German Research Foundation project grant (DFG; RA 459/2-1) and was supported by the German Diabetes Center which is funded by the German Federal Ministry of Health and the Ministry of Innovation, Science, Research and Technology of the State of North-Rhine Westphalia. We gratefully acknowledge the contribution of P. Lichtner, G. Eckstein, N. Klopp and all other members of the Helmholtz Zentrum München genotyping staff in generating the SNP data. We thank all members of field staffs who were involved in the planning and conduct of the KORA Augsburg studies. Finally, we express our appreciation to all study participants.

**LURIC**

We extend appreciation to the participants of the LURIC study and thank the LURIC study team either temporarily or permanently involved in patient recruitment, sample and data handling, and the laboratory staff at the Ludwigshafen General Hospital and the Universities of Freiburg and Ulm, Germany. This research was partly funded by the 6th Framework Programme of the European Union (LSHM-CT-2004-503485)

**METSIM**

Support was provided by grant 124243 from the Academy of Finland.

**NHANES III**

The data are from the NHANES III Genetic Data Sets. We thank Dr. Sekar Kathiresan at the Massachusetts General Hospital and Broad Institute of Harvard and MIT for his collaboration in assembling the NHANES DNA samples, and Jody E. McLean at the Division of Health and Nutrition Examination Surveys, National Center for Health Statistics, Centers for Disease Control and Prevention, for her assistance with the analysis of NHANES genetic data. The findings and conclusions in this paper are those of the author(s) and do not necessarily represent the views of the Research Data Center, National Center for Health Statistics, Centers for Disease Control and Prevention.
The NTR acknowledges support from NWO/ZonMW: 904-61-090, 480-04-004, 400-05-717), SPI 56-464-14192; Center for Medical Systems Biology (NWO Genomics); Centre for Neurogenomics and Cognitive Research (CNCR-VU); Genotyping was funded by the Genetic Association Information Network (GAIN) of the Foundation for the US National Institutes of Health, and analysis was supported by grants from GAIN and the NIMH (MH081802).

The Ottawa Heart Genomic Study was supported by Canadian Institutes for Health Research grants #MOP82810 (RR), #NA6650 (RM), #MOP77682 (AFRS), Canada Foundation for Innovation #11966 (R. R.), and Heart and Stroke Foundation of Ontario #NA6001 (RM).

ORCADES

ORCADES was supported by the Scottish Executive Health Department (Chief Scientist Office), the Royal Society and the European Union framework program 6 EUROSPAN project (contract no. LSHG-CT-2006-018947). DNA extractions were performed at the Wellcome Trust Clinical Research Facility in Edinburgh. We would like to acknowledge the invaluable contributions of Lorraine Anderson and the research nurses in Orkney, the administrative team in Edinburgh and the people of Orkney.

Partners / Roche

We thank Roche Pharmaceuticals for its support and collaboration in assembling the Partners/Roche cohort. Supported by the Mallinckrodt General Clinical Research Program, National Center for Research Resources, General Clinical Research Centers Program grant no. M01-RR-01066.

PennCATH / MedStar

Recruitment of PennCATH was supported by the Cardiovascular Institute of the University of Pennsylvania. Recruitment of the MedStar sample was supported in part by the MedStar Research Institute and the Washington Hospital Center as well as a research grant from GlaxoSmithKline. Genotyping of PennCATH and MedStar was performed at the Center for Applied Genomics at the Children’s Hospital of Philadelphia.

PROCARDIS

Procardis is grateful to the participants and to the medical and nursing staff who assisted in this project. The work was funded EC Sixth Framework Programme (LSHM-CT-2007-037273) and AstraZeneca AB. RC acknowledges support from the MRC; HW acknowledges support from the Wellcome Trust and BHF; AH obtained support for this project from the Swedish Heart-Lung Foundation, the Swedish Medical Research Council (8691), the Knut and Alice Wallenberg Foundation, the Karolinska Institute and the Stockholm County Council (560183). We are indebted for the technical contributions of Ivo Gut and Diana Zelenika.

SardiNIA

We thank all of the volunteers who participated in this study, Monsignore Piseddu, Bishop of Ogliastra, the mayors and citizens of the Sardinian towns (Lanusei, Ilbono, Arzana, and Elini), the head of the Public Health Unit ASL4 for their volunteerism and cooperation, and team of physicians, nurses and the recruitment personnel. Funding was provided by the National Institute on Aging, NIH (contracts NO1-AG-1-2109 to the SardiNIA (“ProgeNIA”) team and 263-MA-410953 to the University of Michigan (G.R.A).

Segovia

Supported by grant FISS 03/1618 from Fondo de Investigaciones Sanitarias, and a grant from Instituto de Salud Carlos III - RETIC RD06 (RD06/0015/0012) and CIBER de Diabetes y Enfermedades Metabólicas Asociadas is an initiative of ISCIII (Ministerio de Ciencia e Innovacion),Madrid, Spain. We thank Milagros Pérez-Barba and Carmen
Obiang-Ansue for dedicated and careful technical assistance.

SHIP
SHIP is part of the Community Medicine Research net of the University of Greifswald, Germany, which is funded by the Federal Ministry of Education and Research (grants 01ZZ9603, 01ZZ0103 and 01ZZ0403), the Ministry of Cultural Affairs as well as the Social Ministry of the Federal State of Mecklenburg- West Pomerania. Generation of genome-wide data has been supported by Grants from the German Federal Ministry of Education and Research for "Zentren für Innovationskompetenz“ (ZIK; BMBF grant 03ZIK012) and a joint grant from Siemens Healthcare, Erlangen, Germany and the Federal State of Mecklenburg-West Pomerania. The University of Greifswald is a member of the "Center of Knowledge Interchange" program of the Siemens AG. The SHIP authors are grateful to the contribution of Ravi Kumar Chilukoti, Florian Ernst, Anja Hoffmann, and Astrid Petersmann in generating the SNP data. The contributions of the SHIP staff and participants are gratefully acknowledged.

Sorbs/SUSOD
Grants from the German Research Council (KFO-152 to Michael Stumvoll), from the IZKF (B27 to Michael Stumvoll, Peter Kovacs and Anke Tönjes) and from the German Diabetes Association (to Yvonne Böttcher, Peter Kovacs and Anke Tönjes).

Inês Barroso further acknowledges the Wellcome Trust grant 077016/Z/05/Z for funding; Nicole Soranzo further acknowledges the Wellcome Trust grant 091746/Z/10/Z for funding. Replication genotyping was supported in part by Diabetes UK grant RD08/0003704.

Disclosures
J.B.Me. currently has a research grant from GlaxoSmithKline, and serves on a consultancy board for Interleukin Genetics. J.C.F. has received consulting honoraria from Merck, Pfizer, bioStrategies, XOMA and Publicis Healthcare Communications Group, a global advertising agency engaged by Amylin Pharmaceuticals. Inês Barroso and spouse own stock in Incyte Ltd and Glaxosmithkline. Genotyping of PennCATH and MedStar was supported by GlaxoSmithKline through an Alternate Drug Discovery Initiative research alliance award (to MPR and DJR) with the University of Pennsylvania School of Medicine.

APPENDIX - Biological function of candidate genes in associated regions

G6PC2 (glucose-6-phosphatase, catalytic, 2) /ABCB11 (ATP-binding cassette, sub-family B, member 11)

GCK (glucokinase)

Common genetic variants in G6PC2 (10; 11), GCK (12), GCKR (glucokinase regulator) (13) and MTNR1B (melatonin receptor 1B) (14-16) have recently been identified as loci regulating FG in genome-wide association studies of diabetes-free adults. GCK and G6PC2 are expressed in the pancreas and code for proteins that are key regulators of the provision of FG. Glucokinases/hexokinases phosphorylate glucose to produce glucose-6-phosphate, the first step in most glucose metabolism pathways. Alternative splicing of GCK results in three tissue-specific forms of glucokinase, one found in pancreatic islet β cells and two found in liver. The protein localizes to the outer membrane of mitochondria. In contrast to other hexokinases, this enzyme is not inhibited by its product glucose-6-phosphate but remains active while glucose is abundant. Rare inactivating mutations in GCK cause maturity-onset diabetes of the young, type 2, a disorder characterized by mild, stable fasting hyperglycemia (17). In contrast, activating mutations in GCK cause persistent hyperinsulinemic hypoglycemia of infancy, in which the threshold for glucose-stimulated insulin release is reduced. Thus, both disorders highlight the role of glucokinase.
in regulating insulin secretion as the glucose sensor of β cells (18). *G6PC2* encodes an enzyme belonging to the glucose-6-phosphatase catalytic subunit family that is involved in the hydrolysis of glucose-6-phosphate, the terminal step in gluconeogenic and glycogenolytic pathways; in this manner it is directly linked to the availability of glucose for release into the bloodstream. The protein product encoded by this gene is found in pancreatic islets and does not exhibit phosphohydrolase activity, but it is a major target of cell-mediated autoimmunity in diabetes. *G6PC2* and *GCK* are likely candidates for altering an individual's physiologic glucostat set point in the absence of progressive hyperglycemia and symptomatic disease. Lead HbA1c SNPs for the *G6PC2/ABCB11* (rs552976) and *GCK* loci (rs1799884) are in high linkage disequilibrium with the most significant SNPs previously identified for FG (r²=0.69 between rs552976 and rs560887 and r²=1 between rs1799884 and rs4607517).

Within the same recombination hot spot (Hapmap II data) of *GCK*: CAMK2B (calcium/calmodulin-dependent protein kinase II beta)
The product of this gene belongs to the serine/threonine protein kinase family and to the Ca^{2+}/calmodulin-dependent protein kinase subfamily. It is possible that distinct isoforms of this chain have different cellular localizations and interact differently with calmodulin. Eight transcript variants encoding eight distinct isoforms have been identified for this gene, some of them are expressed in β cells.

**MTNR1B (MELATONIN RECEPTOR 1B)**
*MTNR1A* and *MTNR1B* encode two of the known human melatonin receptors (19). *MTNR1B* is a G-protein coupled cell surface receptor that is highly expressed in the brain and retina. It is also transcribed in human pancreatic islets and rodent insulinoma cell lines (20). Insulin secretion demonstrates a circadian rhythm which is disrupted in T2D (21). Human pancreatic melatonin receptor expression is elevated in T2D based on elevated mRNA levels as well as more intense immunostaining (22). In previous work we and others have established that the common variants near *MTNR1B* modulate of FG and increase T2D risk, suggesting a link between circadian rhythm regulation and glucose homeostasis (14; 16). Lyssenko and colleagues demonstrated that the risk variant of SNP rs10830963 is associated with impaired early insulin secretion (15). Ronn et al. found that the SNP identified in Europeans was associated with an increased risk of T2D and increased FG in a Han Chinese population (23). Increased expression of *MTNR1B* in pancreatic β-cells and melatonin-mediated impaired insulin secretion in risk allele carriers (14-16; 23-25) suggest mechanisms for *MTNR1B* variants to alter glucose levels in healthy individuals (14-16). No other known gene is located in the same recombination hotspot as *MTNR1B*.

**SPTA1 (spectrin, alpha, erythrocytic 1 (elliptocytosis 2))**
Spectrin is an actin crosslinking and molecular scaffold protein that links the plasma membrane to the actin cytoskeleton, and functions in the determination of cell shape, arrangement of transmembrane proteins, and organization of organelles. It is a tetramer made up of α-β dimers linked in a head-to-head arrangement. This gene is one member of a family of α-spectrin genes. The encoded protein is primarily composed of 22 spectrin repeats that are involved in dimer formation. It forms weaker tetramer interactions than non-erythrocytic α- spectrin, which may increase the plasma membrane elasticity and deformability of red blood cells. Rare mutations in SPTA1 are responsible for elliptocytosis type 2 [MIM 130600], pyropoikilocytosis [MIM 266140], and spherocytic hemolytic anemia [MIM 270970] (26).
**ANK1** (ankyrin 1, erythrocytic)

**ANK1** encodes erythrocytic ankyrin 1, an integral membrane protein linked to the underlying spectrin-actin cytoskeleton, and plays a role in cell motility and maintenance of specialized membrane domains. Multiple isoforms of ankyrin with different affinities for various target proteins are expressed in a tissue-specific, developmentally regulated manner. Most ankyrins are typically composed of three structural domains: an amino-terminal domain containing multiple ankyrin repeats; a central region with a highly conserved spectrin binding domain; and a carboxy-terminal regulatory domain which is the least conserved and subject to variation. Ankyrin 1, the prototype of this family, was first discovered in the erythrocytes, but since has also been found in brain and muscle. Mutations in **ANK1** are found in approximately half of all patients with hereditary spherocytosis (26-28). Complex patterns of alternative splicing in the regulatory domain, giving rise to different isoforms of ankyrin 1 have been described. Truncated muscle-specific isoforms of ankyrin 1 resulting from usage of an alternate promoter have also been identified.

**5' region of ANK1: NKX6-3** (NK6 homeobox 3)

The NKX family of homeodomain proteins controls numerous developmental processes. Members of the NKX6 subfamily, including **NKX6-3**, are involved in development of the central nervous system (CNS), gastrointestinal tract and pancreas (29).

**HK1** (HEXOKINASE 1)

Mammalian hexokinase comprises four isozymes that vary in properties and tissue distribution (**HK1**, **HK2**, **HK3**, and **GCK**) (30). Hexokinase catalyzes the first step in glucose metabolism, converting glucose to glucose-6-phosphate via phosphorylation. Hexokinase is normally found in the cytoplasm of the cell and **HK1** is the predominant isoform found in erythrocytes (30). It is also expressed in other tissues such as muscle and brain (31). Rare mutations in **HK1** have been described to cause non-spherocytic hemolytic anemia (31-33). Pare et al. reported that two intronic SNPs (rs2305198 and rs7072268) are associated with HbA1c (31).

**3' region of HK1: TACR2** tachykinin receptor 2. This gene belongs to a family of genes that encode receptors for tachykinins, characterized by interactions with G proteins and 7 hydrophobic transmembrane regions. This gene encodes the receptor for the tachykinin neuropeptide substance K, also referred to as neurokinin A.

**ATP11A** (ATPase TYPE 11A)

Altered membrane integrity may cause a potassium leak in erythrocytes, leading to higher sodium/potassium ATPase activity, increased glycolytic activity, and lower intracellular glucose concentrations. In this regard, the association of ATPase TYPE 11A (**ATP11A**) with HbA1c is particularly intriguing. **ATP11A** is a P-type ATPase that is involved in the transport of ions across membranes through phosphorylation and de-phosphorylation. Kikuno et al. isolated a partial cDNA encoding **ATP11A** and RT-PCR analysis detected widespread but moderate expression with lowest levels in spleen, pancreas, and testis (34). Resistance to farnesyltransferase inhibitors in Bcr/Abl positive lymphoblastic leukemia cells has been associated with overexpression of **ATP11A** (35). Altered membrane permeability and monovalent ion leak is also a cause of erythrocyte over- or under-hydration and several hereditary stomatocytoses (for instance, MIM %185000, MIM %194380).

**FN3K** (FRUCTOSAMINE 3-KINASE)

Fructosamine 3-kinase is an intracellular enzyme that catalyzes the phosphorylation of...
fructosamines formed by glycation. The fructosamine 3-phosphates that are formed are unstable and they undergo spontaneous decomposition (36). FN3K is therefore involved in de-glycation. Inhibition of FN3K in erythrocytes leads to an increase in HbA1C (37). Delpierre et al. demonstrated that purified FN3K catalyzed ATP-dependent phosphorylation of a synthetic fructosamine (38). Fructosamine 3-kinase is active in erythrocytes and in the lens, which are characterized by slow protein turnover, and may be more susceptible to protein glycation (37). An RNA analysis from 11 different human tissues demonstrates that FN3K is widely expressed in a variety of cell types (39). There is wide inter-individual variability in FN3K activity but little correlation between FN3K activity and the levels of HbA1C (36). HbA1C variability has been associated with one SNP in the promoter region and one SNP in the exon 6 of the FN3K gene (P <0.0001) (36).

**HFE (HEMOCHROMATOSIS)**

HFE encodes a membrane protein that appears involved in iron sensing through the interaction with the transferrin receptor (40). Defects in this gene can cause hereditary hemochromatosis (MIM 235200), a recessive iron storage disorder due to inappropriately low hepcidin levels. The A allele at rs1800562 codes for the pathological C→Y mutation at position 262, for the most common cause of hereditary hemochromatosis. The prevalence of the HFE mutation is higher in patients with T2D than those without diabetes, and iron overload associated with hemochromatosis is a risk factor for T2D (41; 42); however, our data show that the hemochromatosis risk A allele is associated with lower levels of HbA1C as described in the main manuscript.

**TMPRSS6 (TRANSMEMBRANE PROTEASE, SERINE 6)**

TMPRSS6 (also referred to as matriptase-2) is a type II transmembrane serine protease enzyme that hydrolyzes a variety of synthetic substrates as well as endogenous proteins, such as fibronectin, fibrinogen, and type I collagen (43). TMPRSS6 is involved in regulation of iron homeostasis through the control of hepcidin expression (44). The T allele at SNP rs855791 encodes a missense mutation [Val736Ala] that has been detected, together with other mutations, in families with iron-refractory iron deficiency anemia (IRIDA, MIM 206200). This mutation leads to the overproduction of hepcidin and, in turn, to defective iron absorption and utilization (45). Similar to HFE above, mutations in TMPRSS6 ‘uncouple’ iron stores sensing from the regulation of iron absorption; however in IRIDA, TMPRSS6 mutations result in inappropriately elevated hepcidin and an opposite phenotype from hemochromatosis. Northern blot analysis of multiple human tissues revealed expression of the TMPRSS6 in the fetal and adult liver (43). In our data, the IRIDA risk T allele is associated with lower MCH and higher HbA1C levels, as one would predict in a state of iron deficiency and disproportionately lower hemoglobin concentrations, thereby raising the measured percentage of glycated hemoglobin. Thus, our association results suggest the presence of two complementary and directionally consistent pathways that through deficiency or excess make iron metabolism a key determinant of measured levels of hemoglobin glycation in erythrocytes.

5' region of TMPRSS6: KCTD17 (potassium channel tetramerisation domain containing 17), also known as REN. KCTD17 has unknown function. Overexpression of Ren in mice induced neuronal differentiation, growth arrest, and p27(KIP1) expression in central and peripheral neural progenitor cell lines. Inhibition of Ren impaired retinoic acid induction of neurogenin-1 coded by NEUROG1 and NeuroD coded by NEUROD1 expression. NEUROD1 harbors known rare mutations of maturity onset diabetes of the young (46) and regulates expression of the insulin gene, whereas p27 is encoded by CDKN1B, a T2D-associated locus (9).
3’ region of **TMPRSS6**: **IL2RB** (interleukin 2 receptor B). The interleukin 2 receptor is involved in T cell-mediated immune responses. Both the intermediate and high affinity forms of the receptor are involved in receptor-mediated endocytosis and transduction of mitogenic signals from interleukin 2.

**Supplementary references**


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