Supplemental Information

The Cancer-Associated *FGFR4-G388R* Polymorphism Enhances Pancreatic Insulin Secretion and Modifies the Risk of Diabetes

Shereen Ezzat, Lei Zheng, Jose C. Florez, Norbert Stefan, Thomas Mayr, Maw Maw Hliang, Kathleen Jablonski, Maegan Harden, Alena Stančáková, Markku Laakso, Hans-Ulrich Haring, Axel Ullrich, and Sylvia L. Asa

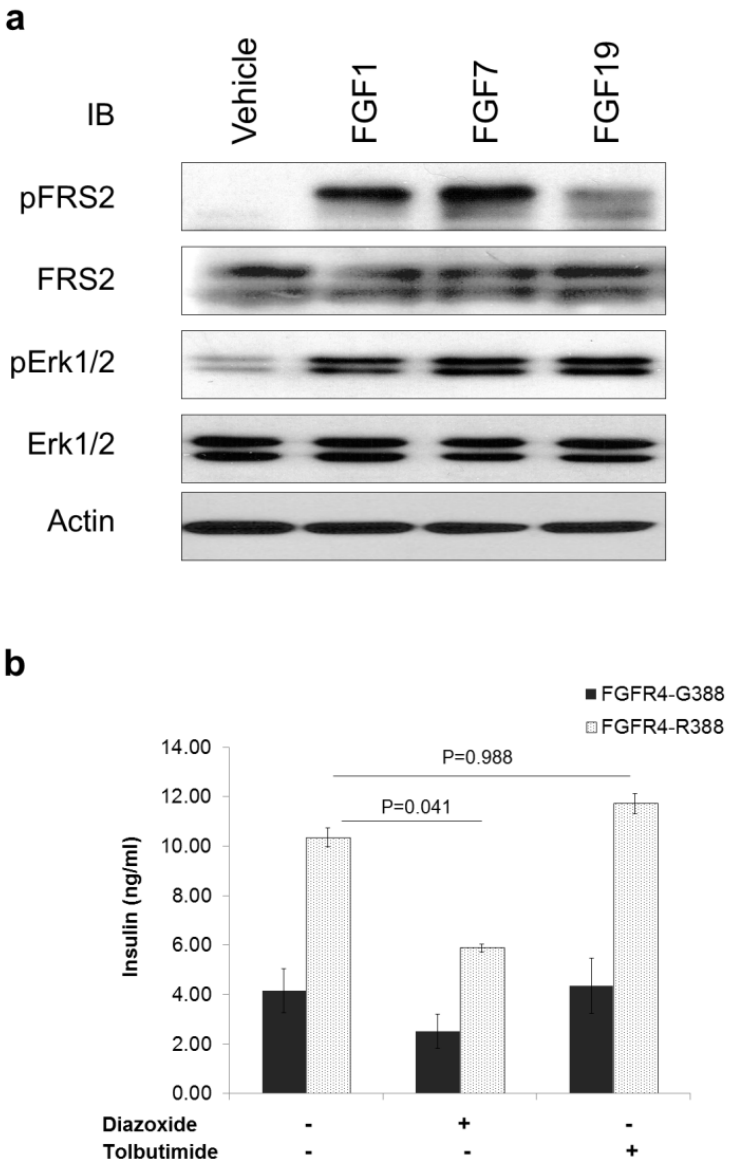

Figure S1. Pancreatic islet responses to stimulation, Related to Figure 1

(a) Pancreatic islet RINm5F cells response to FGF stimulation. After overnight serum-deprivation, cells were exposed for 15 minutes in serum-free media in the presence of heparin without or with the non-FGFR selective FGF1, the FGFR2-selective FGF7, or the FGFR4-selective FGF19 ligands.

Signaling responses were tracked using the immediate FGFR substrate (pFRS2) and with MAPK (pErk1/2). (b) RINm5F cells were treated with diazoxide (100 μM) or tolbutamide (100 μM) for 20 min. followed by 30 min. of glucose (20 mM) exposure as indicated. *FGFR4-R388* cells show greater inhibition to diazoxide but similar responses to tolbutamide compared to *FGFR4-G388* cells. Values represent mean+SEM from triplicate treatments in 2 separate experiments.

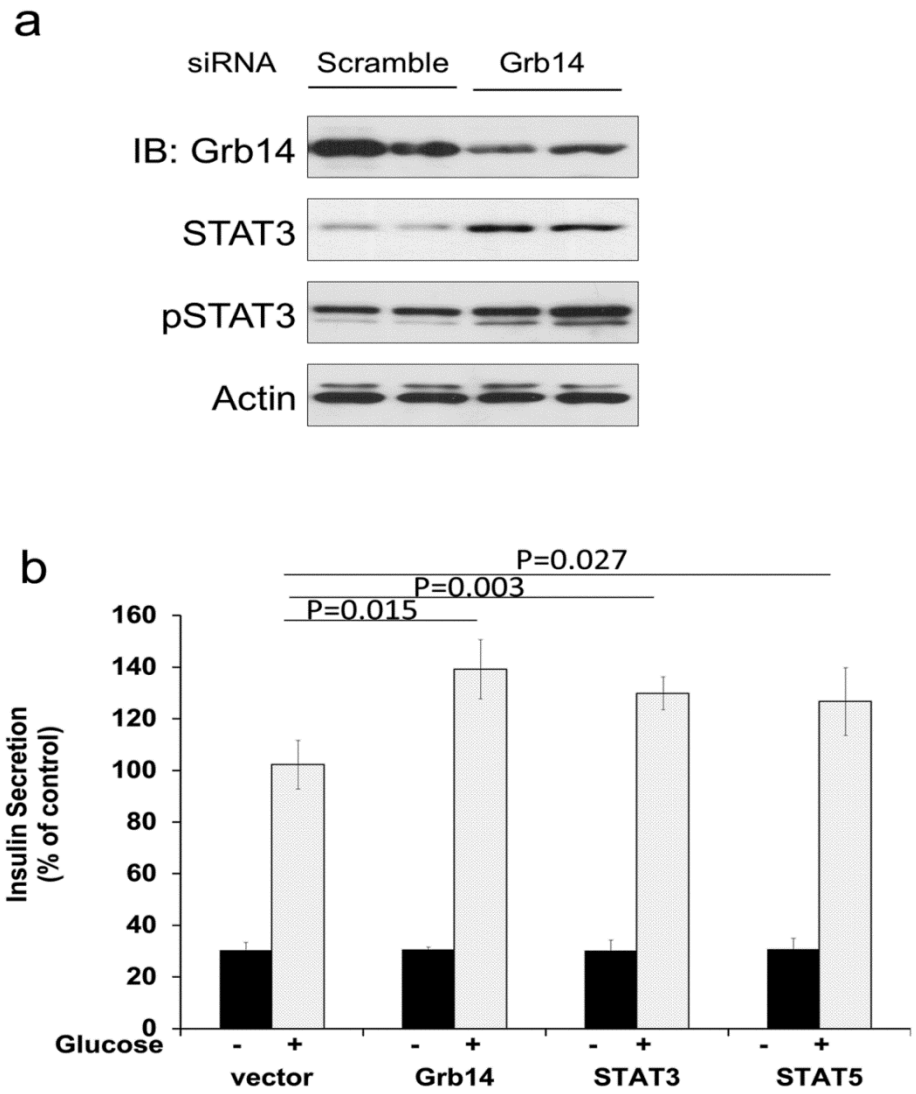

Figure S2. Impact of Grb14, STAT3, or STAT5 on pancreatic islet cells, Related to Figure 3
(a) Native pancreatic islet RIN cells were down-regulated for Grb14 expression using two independent siRNA oligonucleotides targeting the adaptor protein. Western blotting demonstrates effective Grb14 reduction (siRNA) compared with scrambled control (scramble). Each lane represents cells grown without and with glucose respectively. The reduction in Grb14 is associated with STAT3 up-regulation. (b) Pancreatic islet MIN6 cells were forced to express Grb14, STAT3, or STAT5 as indicated for assessment of insulin secretion. Cells were removed from glucose (-) for 3 hours for synchronization followed by another 3 hours of exposure to 25 mM (+) glucose as indicated. Values represent mean+SEM from 4-6 wells per plasmid performed in 2 separate experiments.

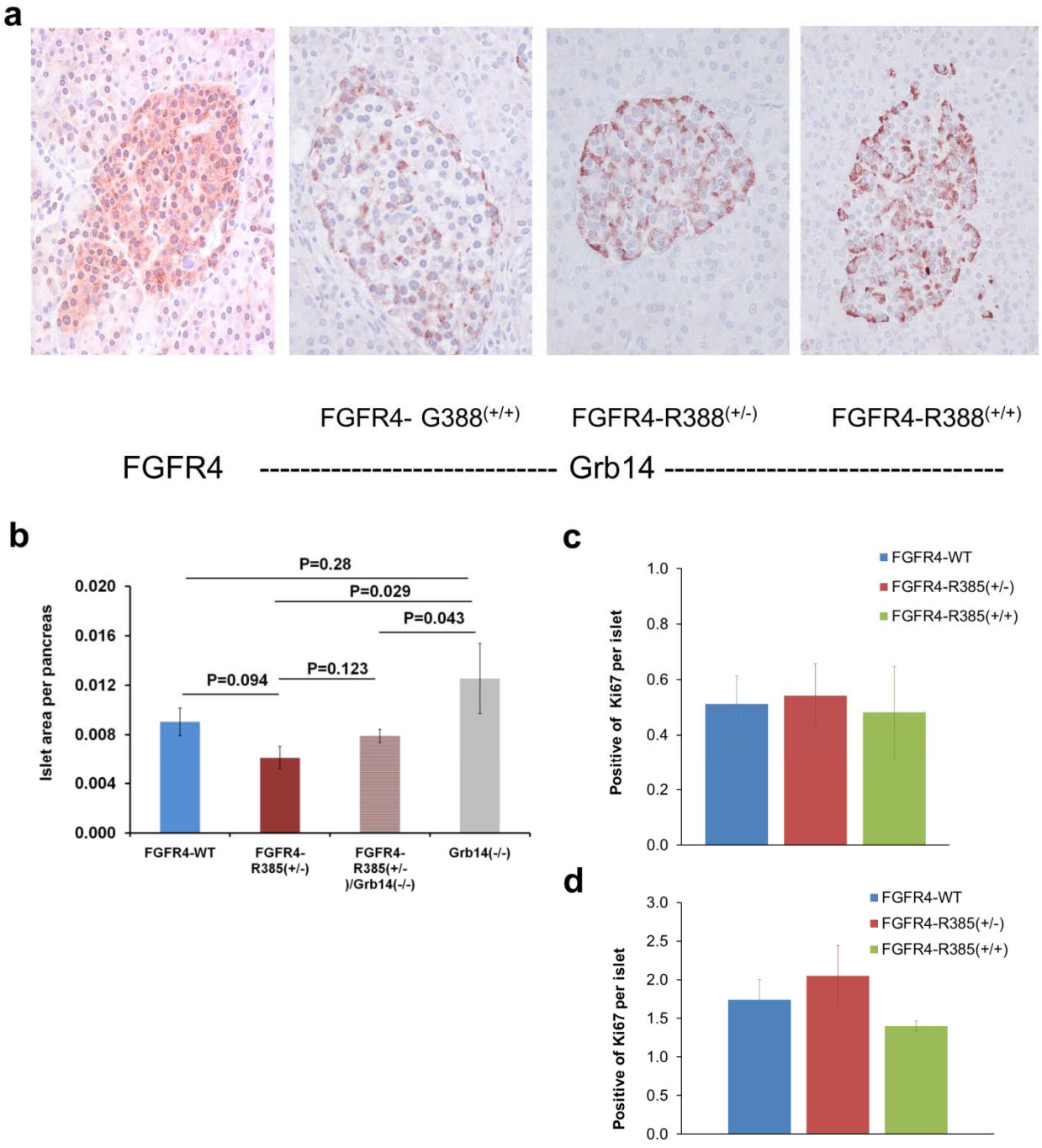

Figure S3. Primary human and mouse pancreatic islet morphology, Related to Figure 5
(a) Primary human pancreatic islet FGFR4 and Grb14 expression. FGFR4 staining was evident by immunostaining in human islets independent of codon 388 genotype. *FGFR4-WT* islets show Grb14 staining mainly in the periphery of the islets. *FGFR4-R388*$^{(+/-)}$ islets show enhanced Grb14 staining within the rest of the islets; a feature most evident in *FGFR4-R388*$^{(+/+)}$ islets. (b) Pancreatic islet morphology in *FGFR4* knock-in Mice. Six month old male mice (8-10/genotype) were subjected to quantitative morphologic assessments. For morphometric measures, total areas of pancreas and total islet areas on hematoxolyin & eosin stained slides were traced using the Aperio imagescope morphometry program and the relative amount of the endocrine component was calculated. (c) To assess cell proliferation animals were maintained on regular diet or (d) high fat (60%) diet for 6 weeks after which pancreatic tissues were harvested for Ki-67 staining and image analysis quantification. Values represent the mean+SEM with group comparisons tested by ANOVA.

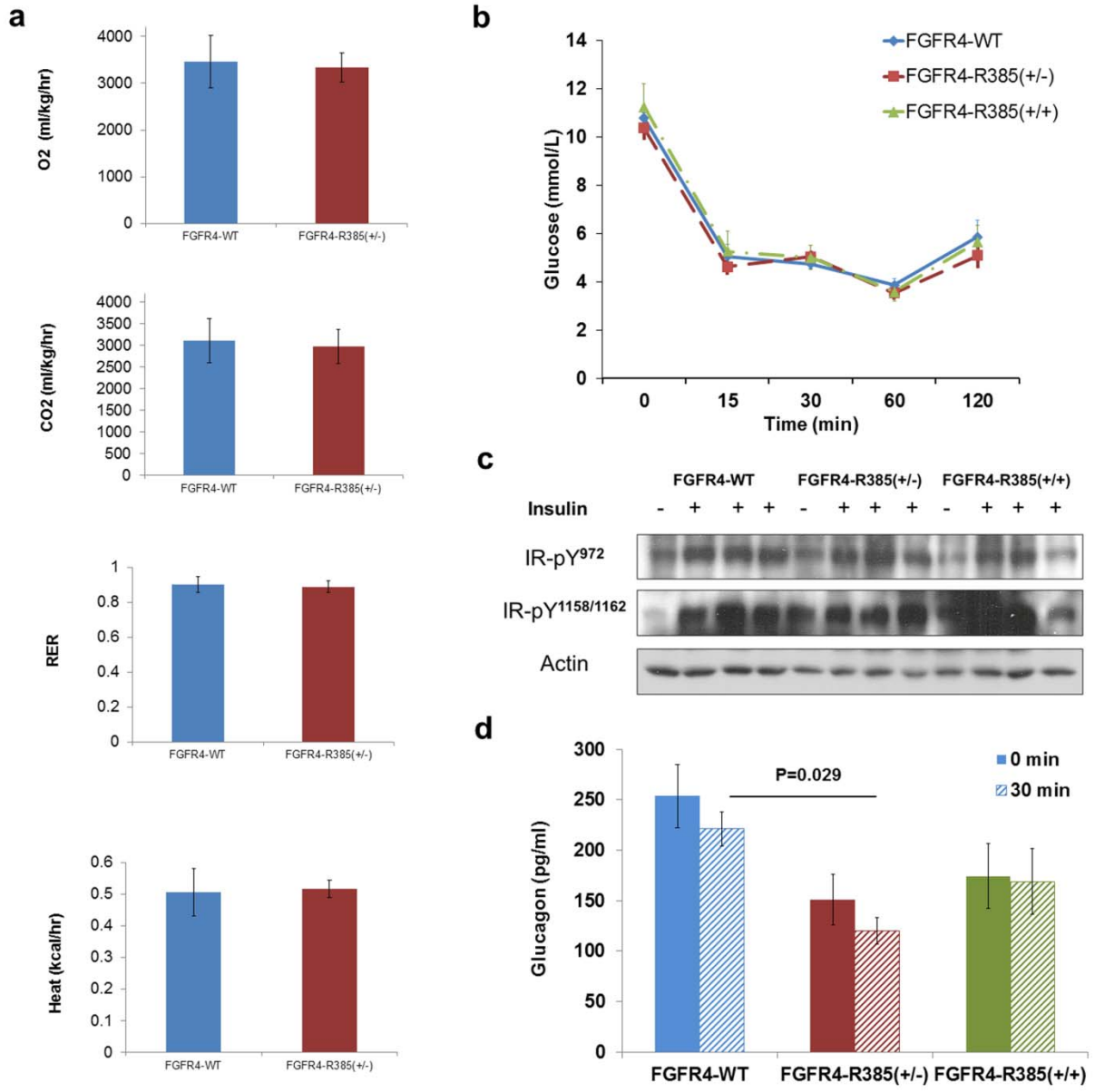

Figure S4. *FGFR4* knock-in mouse metabolic requirements and response to insulin, Related to Figure 6

(a) Metabolic requirements of *FGFR4* knock-in mice. To measure energy homeostasis, 6 month old *FGFR4* knock-in mice of the indicated genotypes were monitored for multiple parameters of energy expenditure over a 24 hour period in an indirect calorimeter chamber. Bar graphs represent mean+SEM of oxygen consumption, CO2 production, respiratory exchange rate (RER), and heat production, respectively from at least 6 mice per group. Representative tracing measurements from individual animals are shown immediately to the right. (b) Insulin tolerance testing in *FGFR4* knock-in mice. Insulin was administered at a dose of 0.075 IU/kg intraperitoneally in 6 month old male mice of the indicated *FGFR4* genotypes. At least 6 mice were included in each group. (c) Effect of insulin administration on IR phosphorylation in liver. *FGFR4* knock-in mice as in panel "b" were fasted overnight, administered insulin 0.075 IU/kg and tissue harvested within 7 minutes. Western blotting shows similar IR phosphorylation in absence (-) and presence of insulin (+) across *FGFR4* genotypes. Each lane represents an independent mouse. (d) Serum glucagon was obtained from 12 month old mice following an overnight fast and 30 minutes following glucose tolerance testing. Values represent the mean+SEM of 5 animals in each genotype.

Table S1. Summary of Microarray Gene Profiling of Pancreatic Islet Cells Expressing *FGFR4-R388* versus *FGFR4-G388*, Related to Figure 2

		FGFR4-R388 vs. FGFR4-G388			***FGFR4-R388* vs. pcDNA3.1**			***FGFR4-G388* vs. pcDNA3.1**		
Method	**Gene Symbol**	**Fold Change**	**regulation**	**p-value**	**Fold Change**	**regulation**	**p-value**	**Fold Change**	**regulation**	**p-value**
arrayassist-MASS	Grb14	2.863	up	0.0247	4.480	up	0.1085	NA		
	Grb14	2.633	up	0.0534	4.478	up	0.0937			
	FGFR1op2	2.100	down	0.131						
	FGFR1	2.908	down	0.0046	2.714	down	0.1240			
	FGFR2	5.356	down	0.296	2.501	down	0.5139	2.141	up	0.095
	IGF-1R				2.337	up	0.2680	2.024	down	0.248
	IGF-1R				2.036	up	0.1011			
arrayassist-PLIER	Grb14	2.84	up	0.0220	3.390	up	0.257			
	Fn1	2.38	up	0.143	2.170	up	0.18			
genesifter	Grb14	3.05	Up	0.040	5.180	up	NA	NA	NA	NA
	Grb14	2.8	Up	0.050	4.970	up	NA	NA	NA	NA
	FGFR1	2.75	down	0.009	2.380	down	0.028	NA	NA	NA
	FGFR2	4.96	down		6.400	up	0.0412	7.84	Up	NA
								2.3	Up	NA
genespring	Grb14	3.408	up	NA	5.736	up		NA	NA	NA
	Grb14	3.132	up	NA	5.506	up		NA	NA	NA
	FGFR1	2.46	down		2.150	down	NA	NA	NA	NA
	FGFR2	4.44	down					2.280	up	NA

Oligonucleotide microarray analysis. Total RNA from cultured pancreatic islet RIN cells was purified and hybridized to the Affymetrix rat 230_2.0 Array at The Centre of Applied Genomics Hospital for Sick Children, Toronto. RNA from 6 clones (two independent *FGFR4-R388* pancreatic islet RIN clones and two independent *FGFR4-G388*, and two control (pcDNA3.1) clones) were subjected to in vitro transcription, labeling and hybridization using standard Affymetrix protocols. Hybridized chips scanned on an Affymetrix GeneChip 3000 confocal scanner. Raw microarray data were analyzed using 4 independent alogorithms as indicated. Genes were considered to be differentially expressed if the signal consistently changed at least 2-fold (or signal log2 ratio 1). The six data sets were analyzed using gene expression and statistical tools in Spotfire's DecisionSite software package. Target genes were further restricted upon filtering for a minimum of 2-fold changes in signal log ratio. Gene targets were then characterized and graphically summarized based on similar expression profiles using Hierarchal and K-Means Clustering. These clustering algorithms were able to further elucidate intrinsic grouping of the significant genes based on two different statistical methods (Hierarchal and Exclusive Clustering).

Table S2. Relationships between the *FGFR4* rs351855 single nucleotide polymorphism with anthropometrics and metabolic traits in 6,915 non-diabetic male subjects in the Finnish METSIM study, Related to Figure 7

Genotype	G388G		G388R		R388R		Additive model		Dominant model	
N	3176		2998		741		P	$P_{adjusted}$	P	$P_{adjusted}$
Age	57	(51.0, 62.0)	56	(51.0, 62.0)	57	(51.0, 62.0)	0.655		0.869	
Body mass index (kg/m^2)	26.3	(24.3, 28.8)	26.3	(24.4, 28.7)	26.4	(24.5, 29.4)	0.178	0.265	0.731	0.731
Waist (cm)	96.5	(90.0, 103.5)	96	(90.0, 103.0)	96	(90.5, 104.0)	0.709	0.92	0.793	0.8
OGTT fasting plasma glucose (mmol/l)	5.7	(5.4, 6.0)	5.7	(5.4, 6.0)	5.7	(5.4, 6.0)	0.487	0.209	0.254	0.261
OGTT 120 min plasma glucose (mmol/l)	5.8	(4.9, 7.0)	5.8	(4.9, 7.0)	5.8	(4.9, 7.0)	0.604	0.55	0.876	0.743
OGTT fasting plasma insulin (mU/l)	6.4	(4.5, 10.2)	6.4	(4.5, 10.0)	6.3	(4.4, 10.6)	0.938	0.741	0.731	0.87
OGTT 120 min plasma insulin (mU/l)	34.7	(20.3, 62.7)	34.4	(20.2, 62.6)	34.3	(21.0, 66.5)	0.905	0.966	0.822	0.975
OGTT fasting plasma proinsulin (pmol/l)	12.1	(9.5, 15.7)	12.1	(9.6, 15.6)	12.1	(9.7, 16.1)	0.459	0.451	0.886	0.722
OGTT 120 min plasma proinsulin (pmol/l)	45.7	(31.3, 63.2)	45	(31.4, 62.5)	46.2	(31.1, 64.4)	0.792	0.672	0.908	0.75
Insulinogenic index (pmol, mmol)	96	(58.2, 156.8)	96.7	(58.3, 159.5)	96.3	(57.1, 158.1)	0.918	0.719	0.996	0.917
Matsuda ISI (mg/dl, mU/l)	6.1	(3.9, 9.1)	6.2	(3.9, 9.0)	6.2	(3.7, 9.3)	0.925	0.634	0.693	0.801
HOMA-B	60	(42.7, 90.0)	60.8	(42.9, 91.0)	61.1	(43.2, 91.6)	0.968	0.51	0.953	0.515

Data are presented as median (interquartile range). P-values were calculated using log10-transformed variables to correct for their skewed distribution. P-values are unadjusted (ANOVA for additive model, t-test for dominant model), $P_{adjusted}$-values are adjusted using linear regression as follows: BMI and waist are adjusted for age, glucose levels, insulin levels, proinsulin levels, Matsuda ISI and HOMA-IR are adjusted for age and waist, Insulinogenic index and HOMA-B are adjusted for age, waist, and insulin sensitivity index (Matsuda ISI).

Table S3. Effect of genotype at *FGFR4-R388* on diabetes incidence in the Diabetes Prevention Program, stratified by sex and treatment arm, Related to Figure 7

	Placebo		Metformin		Lifestyle	
	HR (95% CI)	*P* val	HR (95% CI)	*P* val	HR (95% CI)	*P* val
Men	1.11 (0.79-1.57)	0.546	0.59 (0.39-0.87)	0.008	0.78 (0.46-1.31)	0.340
Women	0.84 (0.65-1.10)	0.200	1.00 (0.74-1.34)	0.993	1.02 (0.73-1.42)	0.901

Cox proportional hazard models testing the effect of genotype at *FGFR4-R388* on diabetes incidence under an additive genetic model adjusted for treatment group, sex, age at randomization and self-reported ethnicity. There was a significant SNP × treatment interaction (*P*=0.03) for the placebo vs. metformin comparison in men only. HR, hazard ratio.

Table S4. Primers used in RT-PCR, Real-time PCR, and siRNA, Related to Figure 3

Target	Gene Accession	Sequence (5'-3')	Annealing temp. (^{0}C)	Product size (base pairs)
Abcc8	NM_013039	F: CCTGCAGCCAGACATAGACA	60	154
		R: CGAGAAAGGGTCATCCAAAA		
Grb14	NM_031623	F: CTGCTGTTCTCCACTTACATCG	60	203
		R: CAAAAAGGGTCCAGCTATTGTC		
Grb14, Real-time	NM_031623	ABI Taqman Gene Expression Assays Rn00581165_m1		
Grb14, siRNA	NM_031623	F: GGACAGTGGAGGACCATGATT		
		R: TCATGGTCCTCCACTGTCCTT		
Grb7	NM_053403	F: CTCTAAGGACCCGAGACACCTA	60	293
		R: GTGGAGACCCCAAATACGATAA		
Grb10	XM_001053718	F: GATGGGACAAGCAAAGTGGT	60	164
		R: ACGATCTCATGGTCCTCCAG		
HK1	NM_012734	F: CACCGGCAGATTGAGGAAAC	60	101
		R: CTCAGCCCCATTTCCATCTCT		
HK4	M25807.1	CCGAGTGGCTTACAGTTCTG	60	140
		ACCTGAGTGTTGGAGATGATTC		
KCNJ11	NM_031358.3	GCCATGCTGTCCCGAAAGGG	60	437
		GGCCAGGGGACATTCCTCTGT		
KCNJ15	NM_13321	CCGTTCCATCACAGAGGAGT	60	119
		GCTTTTTGGGTCTTGCAATC		
β-Klotho	NM_031180.2	F: ACCTGATCAAGGCACATTCG	60	146
		R: CTGGCAGTTGATCACGTCCT		
Insulin receptor siRNA	NM_017071	TGAGGAATGTGGGGACGTCTT		
		GACGTCCCCACATTCCTCATT		
Insulin	NM_0191129	F: GTACCTGGTGTGTGGGGAAC	60	200
		R: CCAGTTGGTAGAGGGAGCAG		
FGFR1	XM_346491	F: ATCGAGGTGAATGGGAGTAAGA	60	253
		R: TTTCCAGGTACAGAGGTGAGGT		
FGFR2	Z35139	F: GGGACGTAGAATTTGTCTGC	58	199
		R: TATTCCCCAGCATCCATCTC		
FGFR3	NM_053429	F:GAACTCCAACACACCTCTCGTC	59	259
		R:CTTGTCAGTCGCATCATCTTTC		
FGFR4	NM_001109904	F:CAGGAGACACCAGCCTTCTC	60	473
		R:GGACAGCGGAATTTGACAGT		
PGK1	BC063161	F: GCTGACAAGTTTGATGAGAAT	57	338
		R: AGGACTTTACCTTCCAGGAGC		

F, forward primer. R, reverse primer.

Table S5. List of antibodies, Related to Figure 4

Antibody Name	Source	Host	Working Dilution
Actin	Sigma	Mouse	1:1000
FGFR4	Santa Cruz Biotechnology	Rabbit	1:1000
FRS2	R&D systems	mouse	1:1000
Grb14	Chemicon	Rabbit	1:1000
HK1	ABCAM	mouse	1:1000
Insulin	Biomeda	Rabbit	1:1000
Insulin Receptor α subunit	Santa Cruz Biotechnology	Rabbit	1:1000
Insulin Receptor β subunit	Upstate	Rabbit	1:1000
IRS1	Upstate	Rabbit	1:1000
IRS2	Upstate	Rabbit	1:1000
KCNJ11	ABCAM	Rabbit	1:1000
β-Klotho (KLB)	Lifespan Biosciences	Rabbit	1:1000
MAPK	Sigma	Rabbit	1:40000
Phospho-FGFR (Y653/654)	Cell Signaling Technology	Rabbit	1:1000
Phospho-FRS2 (Y196)	Cell Signaling Technology	Rabbit	1:1000
Phospho-IR (Y1158/1162/1163)	Biosource	Rabbit	1:1000
Phospho-IR (Y972)	Biosource	Rabbit	1:1000
Phospho-IRS1(S312)	Biosource	Rabbit	1:1000
Phospho-IRS1(Y612)	Biosource	Rabbit	1:1000
Phospho-MAPK	Sigma	Mouse	1:4000
Phospho-PLCγ (Y783)	Cell Signaling Technology	Rabbit	1:1000
Phospho-STAT3 (Y705)	Cell Signaling Technology	Rabbit	1:1000
Phospho-STAT5 (Y694)	Cell Signaling Technology	Rabbit	1:1000
Phosphotyrosine, 4G10	Upstate	Mouse	1:1000
Phosphotyrosine, PY20	Upstate	Mouse	1:1000
PLCγ	Cell Signaling Technology	Rabbit	1:1000
STAT3	Cell Signaling Technology	Rabbit	1:1000
STAT5	Cell Signaling Technology	Rabbit	1:1000

Supplemental Experimental Procedures

Pharmacologic treatments

FGF stimulation was performed using the non-FGFR isotype selective FGF-1 (Sigma, 10 ng/ml; St. Louis MO), the FGFR2-selective FGF7 (10 ng/ml, Sigma), or the FGFR4-selective FGF19 (10 ng/ml; R&D Systems) in serum-free defined media containing 10 U/ml heparin. To probe ATP-sensitive K^+ channel activity, cells were treated with diazoxide (100 μM) or tolbutamide (100 μM)(Sigma) for 20 min. followed by glucose exposure (20 mM) for 30 min.

Pancreatic islet morphology

Cell pellets and tissue blocks were embedded for routine histology and immunohistochemistry. Immunoreaction of primary antibodies is listed in Suppl. table S5. Cell proliferation was examined by Ki-67 and BrdU staining, apoptosis was determined using the terminal deoxyncleotidyl transferase TDT-mediated deoxyuridine-triphosphate nick-end labeling (TUNEL) technique. For morphometric studies, total areas of pancreas and total islet areas on hematoxolyin & eosin (H&E) stained slides were traced using the Aperio imagescope morphometry program and the relative amount of the endocrine component was calculated.

Glucose tolerance, insulin tolerance, and energy homeostasis testing

Fasting blood glucose from tail veins was measured following an overnight fast using a glucose meter. For glucose tolerance 20%D-glucose was injected into the peritoneal cavity at a dose of 1.5 g/kg for 2 hour testing. For insulin tolerance testing, insulin (0.5-1 U/kg) was injected into the peritoneal cavity. For tissue phosphorylation studies, animals were sacrificed within 7 minutes following insulin administration. To measure energy homeostasis, animals were monitored for multiple parameters of energy expenditure over a 24 hour period in an indirect calorimeter chamber (Columbus Instruments, Columbus, OH). At least 6-12 mice were included in each experiment as indicated.

Human studies

Data from Southern German subjects without diabetes included participants in an ongoing study to reduce adiposity and prevent type 2 diabetes (Kantartzis et al., 2011). Informed written consent was obtained from all participants following local Medical Ethics Committees' approval. Results from the MAGIC meta-analyses of fasting glycemic traits have been published (Dupuis et al., 2010; Strawbridge et al., 2011) and are available at www.magicinvestigators.org. The design and results of the Diabetes Prevention Program (DPP) have also been published)(Knowler et al., 2002). Briefly, this clinical trial enrolled 3,819 overweight participants with elevated fasting glucose and impaired glucose tolerance, and randomized them to placebo, an intensive lifestyle modification program, metformin 850 mg twice daily or troglitazone 400 mg once daily (the troglitazone arm was discontinued after an average of 10 months follow-up due to hepatotoxicity; troglitazone participants are only analyzed for baseline characteristics here). The principal endpoint was the development of diabetes by ADA criteria. At baseline the participants' mean age was 51 years, mean BMI was 34.0 kg/m², 68% were women, and 45% belonged to U.S. ethnic minority groups (African American, Hispanic, Asian and American Indian). After a mean 2.8 years of follow-up, the lifestyle and metformin interventions reduced the incidence of diabetes by 58% and 31% respectively versus placebo. For the purposes of this study, 3,543 participants provided DNA and consented to genetic investigation. All procedures were approved by institutional review boards at the 27 study sites. We genotyped rs351855 on a Sequenom iPLEX platform as described (Florez et al., 2006); genotyping success rate was 94%.

Statistical analyses
Data are shown as mean+standard error (SEM) with comparisons using t-tests. Multiple group comparisons were performed by ANOVA with a statistical threshold of 0.05. For the human studies statistical comparison of continuous variables was performed using logarithmically transformed data (for non-normally distributed parameters). Multivariate linear and logistic regression models were used to determine relationships of the SNP with the parameters of interest. We evaluated the effects of genotype on diabetes incidence by Cox proportional hazard models under additive genetic coding, and tested for genotype × treatment interaction. We used general linear models to test log-transformed quantitative glycemic traits at 1 year.

Acknowledgements for the DPP

The Investigators gratefully acknowledge the commitment and dedication of the participants of the DPP (see appendix for list of participants). The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) of the National Institutes of Health provided funding to the clinical centers and the Coordinating Center for the design and conduct of the study; collection, management, analysis, and interpretation of the data. The Southwestern American Indian Centers were supported directly by the NIDDK and the Indian Health Service. The General Clinical Research Center Program, National Center for Research Resources, and the Department of Veterans Affairs supported data collection at many of the clinical centers. Funding for data collection and participant support was also provided by the Office of Research on Minority Health, the National Institute of Child Health and Human Development, the National Institute on Aging, the Office of Research on Women's Health, the Centers for Disease Control and Prevention, and the American Diabetes Association. Bristol-Myers Squibb and Parke-Davis provided medication. This research was also supported, in part, by the intramural research program of the NIDDK. LifeScan Inc., Health O Meter, Hoechst Marion Roussel, Inc., Merck-Medco Managed Care, Inc., Merck and Co., Nike Sports Marketing, Slim Fast Foods Co., and Quaker Oats Co. donated materials, equipment, or medicines for concomitant conditions. McKesson BioServices Corp., Matthews Media Group, Inc., and the Henry M. Jackson Foundation provided support services under subcontract with the Coordinating Center. The opinions expressed are those of the investigators and do not necessarily reflect the views of the Indian Health Service or other funding agencies. A complete list of Centers, investigators, and staff can be found in the Appendix.

APPENDIX: List of DPP investigators

Pennington Biomedical Research Center **(Baton Rouge, LA)**
George A. Bray, MD*
Iris W. Culbert, BSN, RN, CCRC**
Catherine M. Champagne, PhD, RD
Barbara Eberhardt, RD, LDN
Frank Greenway, MD
Fonda G. Guillory, LPN
April A. Herbert, RD
Michael L. Jeffirs, LPN
Betty M. Kennedy, MPA
Jennifer C. Lovejoy, PhD
Laura H. Morris, BS
Lee E. Melancon, BA, BS
Donna Ryan, MD

Deborah A. Sanford, LPN
Kenneth G. Smith, BS, MT
Lisa L. Smith, BS
Julia A. St.Amant, RTR
Richard T. Tulley, PhD
Paula C. Vicknair, MS, RD
Donald Williamson, PhD
Jeffery J. Zachwieja, PhD

***University of Chicago* (Chicago, IL)**
Kenneth S. Polonsky, MD*
Janet Tobian, MD, PhD*
David Ehrmann, MD*
Margaret J. Matulik, RN, BSN**
Bart Clark, MD
Kirsten Czech, MS
Catherine DeSandre, BA
Ruthanne Hilbrich, RD
Wylie McNabb, EdD
Ann R. Semenske, MS, RD

***Jefferson Medical College* (Philadelphia, PA)**
Jose F. Caro, MD*
Pamela G. Watson, RN, ScD*
Barry J. Goldstein, MD, PhD*
Kellie A. Smith, RN, MSN**
Jewel Mendoza, RN, BSN**
Renee Liberoni, MPH
Constance Pepe, MS, RD
John Spandorfer, MD

***University of Miami* (Miami, FL)**
Richard P. Donahue, PhD*
Ronald B. Goldberg, MD*
Ronald Prineas, MD, PhD*
Patricia Rowe, MPA**
Jeanette Calles, MSEd
Paul Cassanova-Romero, MD
Hermes J. Florez, MD
Anna Giannella, RD, MS
Lascelles Kirby, MS
Carmen Larreal
Valerie McLymont, RN
Jadell Mendez
Juliet Ojito, RN
Arlette Perry, PhD
Patrice Saab, PhD

***The University of Texas Health Science Center* (San Antonio, TX)**
Steven M. Haffner, MD, MPH*
Maria G. Montez, RN, MSHP, CDE**
Carlos Lorenzo, MD, PhD
Arlene Martinez, RN, BSN, CDE

***University of Colorado* (Denver, CO)**
Richard F. Hamman, MD, DrPH*
Patricia V. Nash, MS**
Lisa Testaverde, MS**
Denise R. Anderson, RN, BSN
Larry B. Ballonoff, MD

Alexis Bouffard, MA,
B. Ned Calonge, MD, MPH
Lynne Delve
Martha Farago, RN
James O. Hill, PhD
Shelley R. Hoyer, BS
Bonnie T. Jortberg, MS, RD, CDE
Dione Lenz, RN, BSN
Marsha Miller, MS, RD
David W. Price, MD
Judith G. Regensteiner, PhD
Helen Seagle, MS, RD
Carissa M. Smith, BS
Sheila C. Steinke, MS
Brent VanDorsten, PhD

***Joslin Diabetes Center* (Boston, MA)**
Edward S. Horton, MD*
Kathleen E. Lawton, RN**
Ronald A. Arky, MD
Marybeth Bryant
Jacqueline P. Burke, BSN
Enrique Caballero, MD
Karen M. Callaphan, BA
Om P. Ganda, MD
Therese Franklin
Sharon D. Jackson, MS, RD, CDE
Alan M. Jacobsen, MD
Lyn M. Kula, RD
Margaret Kocal, RN, CDE
Maureen A. Malloy, BS
Maryanne Nicosia, MS, RD
Cathryn F. Oldmixon, RN
Jocelyn Pan, BS, MPH
Marizel Quitingon
Stacy Rubtchinsky, BS
Ellen W. Seely, MD
Dana Schweizer, BSN
Donald Simonson, MD
Fannie Smith, MD
Caren G. Solomon, MD, MPH
James Warram, MD

***VA Puget Sound Health Care System and University of Washington* (Seattle, WA)**
Steven E. Kahn, MB, ChB*
Brenda K. Montgomery, RN, BSN, CDE**
Wilfred Fujimoto, MD
Robert H. Knopp, MD
Edward W. Lipkin, MD
Michelle Marr, BA
Dace Trence, MD

***University of Tennessee* (Memphis, TN)**
Abbas E. Kitabchi, PhD, MD, FACP*
Mary E. Murphy, RN, MS, CDE, MBA**
William B. Applegate, MD, MPH
Michael Bryer-Ash, MD
Sandra L. Frieson, RN
Raed Imseis, MD
Helen Lambeth, RN, BSN

Lynne C. Lichtermann, RN, BSN
Hooman Oktaei, MD
Lily M.K. Rutledge, RN, BSN
Amy R. Sherman, RD, LD
Clara M. Smith, RD, MHP, LDN
Judith E. Soberman, MD
Beverly Williams-Cleaves, MD

Northwestern University's Feinberg School of Medicine **(Chicago, IL)**
Boyd E. Metzger, MD*
Mariana K. Johnson, MS, RN**
Catherine Behrends
Michelle Cook, MS
Marian Fitzgibbon, PhD
Mimi M. Giles, MS, RD
Deloris Heard, MA
Cheryl K.H. Johnson, MS, RN
Diane Larsen, BS
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Greg Strylewicz, PhD**
F. Alan Aldrich

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Charles Campbell, MS
Sharon Hall
Yabing Li, MD
Margaret Mills
Nancy Pemberton, MS
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Zhuming Zhang, MD

NIH/NIDDK **(Bethesda, MD)**
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Judith Fradkin, MD
Sanford Garfield, PhD

Nutrition Coding Center **(Columbia, SC)**
Elizabeth Mayer-Davis, PhD*
Robert R. Moran, PhD**

Quality of Well-Being Center **(La Jolla, CA)**
Ted Ganiats, MD*
Kristin David, MHP*
Andrew J. Sarkin, PhD*

Genetics Working Group
Jose C. Florez, MD, PhD[1, 2]
David Altshuler, MD, PhD[1, 2]
Paul I.W. de Bakker, PhD[2]
Paul W. Franks, PhD, Mphil, MS[3, 6]
Robert L. Hanson, MD, MPH[3]
Kathleen Jablonski, PhD[5]
William C. Knowler, MD, DrPH[3]
Jarred B. McAteer, AB[1, 2]
Toni I. Pollin, PhD[4]
Alan R. Shuldiner, MD[4]

[1] Massachusetts General Hospital; [2] Broad Institute; [3] NIDDK; [4] University of Maryland; [5] Coordinating Center; [6] Umea University
* Principal Investigator; ** Program Coordinator

Supplemental References

Dupuis,J., Langenberg,C., Prokopenko,I., Saxena,R., Soranzo,N., Jackson,A.U., Wheeler,E., Glazer,N.L., Bouatia-Naji,N., Gloyn,A.L., Lindgren,C.M., Magi,R., Morris,A.P., Randall,J., Johnson,T., Elliott,P., Rybin,D., Thorleifsson,G., Steinthorsdottir,V., Henneman,P., Grallert,H., Dehghan,A., Hottenga,J.J., Franklin,C.S., Navarro,P., Song,K., Goel,A., Perry,J.R., Egan,J.M., Lajunen,T., Grarup,N., Sparso,T., Doney,A., Voight,B.F., Stringham,H.M., Li,M., Kanoni,S., Shrader,P., Cavalcanti-Proenca,C., Kumari,M., Qi,L., Timpson,N.J., Gieger,C., Zabena,C., Rocheleau,G., Ingelsson,E., An,P., O'Connell,J., Luan,J., Elliott,A., McCarroll,S.A., Payne,F., Roccasecca,R.M., Pattou,F., Sethupathy,P., Ardlie,K., Ariyurek,Y., Balkau,B., Barter,P., Beilby,J.P., Ben Shlomo,Y., Benediktsson,R., Bennett,A.J., Bergmann,S., Bochud,M., Boerwinkle,E., Bonnefond,A., Bonnycastle,L.L., Borch-Johnsen,K., Bottcher,Y., Brunner,E., Bumpstead,S.J., Charpentier,G., Chen,Y.D., Chines,P., Clarke,R., Coin,L.J., Cooper,M.N., Cornelis,M., Crawford,G., Crisponi,L., Day,I.N., de Geus,E.J., Delplanque,J., Dina,C., Erdos,M.R., Fedson,A.C., Fischer-Rosinsky,A., Forouhi,N.G., Fox,C.S., Frants,R., Franzosi,M.G., Galan,P., Goodarzi,M.O., Graessler,J., Groves,C.J., Grundy,S., Gwilliam,R., Gyllensten,U., Hadjadj,S., Hallmans,G., Hammond,N., Han,X., Hartikainen,A.L., Hassanali,N., Hayward,C., Heath,S.C., Hercberg,S., Herder,C., Hicks,A.A., Hillman,D.R., Hingorani,A.D., Hofman,A., Hui,J., Hung,J., Isomaa,B., Johnson,P.R., Jorgensen,T., Jula,A., Kaakinen,M., Kaprio,J., Kesaniemi,Y.A., Kivimaki,M., Knight,B., Koskinen,S., Kovacs,P., Kyvik,K.O., Lathrop,G.M., Lawlor,D.A., Le Bacquer,O., Lecoeur,C., Li,Y., Lyssenko,V., Mahley,R., Mangino,M., Manning,A.K., Martinez-Larrad,M.T., McAteer,J.B., McCulloch,L.J., McPherson,R., Meisinger,C., Melzer,D., Meyre,D., Mitchell,B.D., Morken,M.A., Mukherjee,S., Naitza,S., Narisu,N., Neville,M.J., Oostra,B.A., Orru,M., Pakyz,R., Palmer,C.N., Paolisso,G., Pattaro,C., Pearson,D., Peden,J.F., Pedersen,N.L., Perola,M., Pfeiffer,A.F., Pichler,I., Polasek,O., Posthuma,D., Potter,S.C., Pouta,A., Province,M.A., Psaty,B.M., Rathmann,W., Rayner,N.W., Rice,K., Ripatti,S., Rivadeneira,F., Roden,M., Rolandsson,O., Sandbaek,A., Sandhu,M., Sanna,S., Sayer,A.A., Scheet,P., Scott,L.J., Seedorf,U., Sharp,S.J., Shields,B., Sigurethsson,G., Sijbrands,E.J., Silveira,A., Simpson,L., Singleton,A., Smith,N.L., Sovio,U., Swift,A., Syddall,H., Syvanen,A.C., Tanaka,T., Thorand,B., Tichet,J., Tonjes,A., Tuomi,T., Uitterlinden,A.G., van Dijk,K.W., van Hoek,M., Varma,D., Visvikis-Siest,S., Vitart,V., Vogelzangs,N., Waeber,G., Wagner,P.J., Walley,A., Walters,G.B., Ward,K.L., Watkins,H., Weedon,M.N., Wild,S.H., Willemsen,G., Witteman,J.C., Yarnell,J.W., Zeggini,E., Zelenika,D., Zethelius,B., Zhai,G., Zhao,J.H., Zillikens,M.C., Borecki,I.B., Loos,R.J., Meneton,P., Magnusson,P.K., Nathan,D.M., Williams,G.H., Hattersley,A.T., Silander,K., Salomaa,V., Smith,G.D., Bornstein,S.R., Schwarz,P., Spranger,J., Karpe,F., Shuldiner,A.R., Cooper,C., Dedoussis,G.V., Serrano-Rios,M., Morris,A.D., Lind,L., Palmer,L.J., Hu,F.B., Franks,P.W., Ebrahim,S., Marmot,M., Kao,W.H., Pankow,J.S., Sampson,M.J., Kuusisto,J., Laakso,M., Hansen,T., Pedersen,O., and Pramstaller,P.P. (2010). New genetic loci implicated in fasting glucose homeostasis and their impact on type 2 diabetes risk. Nat. Genet. *42*, 105-116.

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