Cell Metabolism
Resource

A Systems Genetics Approach Identifies Genes and Pathways for Type 2 Diabetes in Human Islets

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SUMMARY

Close to 50 genetic loci have been associated with type 2 diabetes (T2D), but they explain only 15% of the heritability. In an attempt to identify additional T2D genes, we analyzed global gene expression in human islets from 63 donors. Using 48 genes located near T2D risk variants, we identified gene coexpression and protein-protein interaction networks that were strongly associated with islet insulin secretion and HbA_{1c}. We integrated our data to form a rank list of putative T2D genes, of which *CHL1*, *LRFN2*, *RASGRP1*, and *PPM1K* were validated in INS-1 cells to influence insulin secretion, whereas *GPR120* affected apoptosis in islets. Expression variation of the top 20 genes explained 24% of the variance in HbA_{1c} with no claim of the direction. The data present a global map of genes associated with islet dysfunction and demonstrate the value of systems genetics for the identification of genes potentially involved in T2D.

INTRODUCTION

Type 2 diabetes (T2D) is one of the fastest increasing diseases worldwide, with an estimated prevalence of 280 million affected patients in 2011 (http://www.diabetesatlas.org/). This epidemic has been ascribed to an interaction between common genetic variants and environmental factors like obesity and a sedentary lifestyle. To date, genome-wide association studies (GWAS) have identified 47 common genetic variants associated with T2D or glucose/insulin levels (Dupuis et al., 2010; Saxena et al., 2007, 2010; Scott et al., 2007; Sladek et al., 2007; Steinthorsdottir et al., 2007; Voight et al., 2010; Zeggini et al., 2008). Despite this apparent success, these variants explain only about 10%–15% of the heritability of T2D, emphasizing the need for novel approaches to identify susceptibility genes. One alternative strategy is to use genetic loci associated with expression traits in disease-relevant tissues to identify previously unrecognized susceptibility variants (Schadt et al., 2008). Although T2D is characterized by both impaired insulin secretion and action in target tissues like muscle, fat, and liver (Defronzo, 2009), most of the known disease-associated variants seem to influence insulin secretion rather than action (Florez, 2008; Ingelsson et al., 2010; Lyssenko et al., 2008). In most cases the causal variant is not known, nor is it known how the identified variants may influence islet function in man. One of the obstacles in human diabetes research has been the inaccessibility of pancreatic islets. Recently, this hurdle has to some extent been circumvented by research using islets from cadaver donors intended for islet transplantation.

We have systematically characterized donated human islets by performing cDNA microarray and GWAS in addition to measuring insulin response to glucose and glycemic control (HbA_{1c}) from the same individuals. Here, we have combined data from human islet gene expression, genetics, and function to build a global map of genes associated with islet dysfunction in T2D. We form a rank list of potential T2D genes, highlighting several candidate genes that might affect islet function in man.

RESULTS

Expression Pattern of 48 Putative T2D-Associated Genes in Human Islets

Using microarray data from human islets from 63 cadaver donors (nine with T2D), we studied the expression of 48 genes (47 SNPs) located in the vicinity of the known single-nucleotide polymorphisms (SNPs) that to date have been associated with T2D or glycemic traits (hereafter referred to as T2D seeder genes). The expression of *IGF1*, *CHCHD9*, *PPARG*, and *HMGA2*

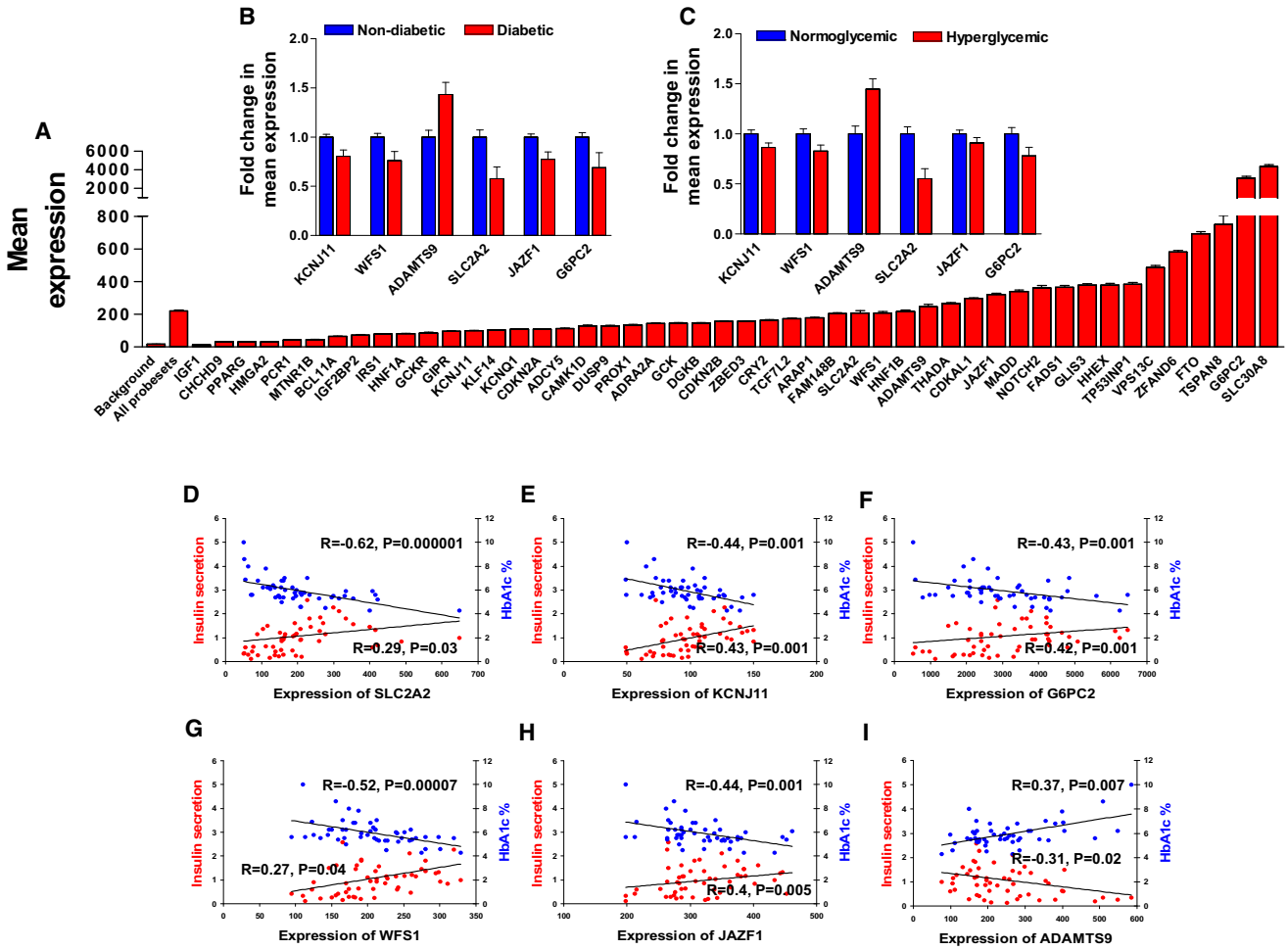

Figure 1. Expression of Putative T2D-Associated Genes in Human Pancreatic Islets

(A) Microarray gene expression profile showing mean expression of 48 putative T2D genes in human pancreatic islets. Background signal was estimated by calculating the mean value of all negative control probe sets (~2,900) represented on the array. All probe sets signal indicate mean expression of all probe sets on the array (~20,000). Bars represent mean ± SEM.

(B) Lower expression of *KCNJ11* (p = 0.01), *WFS1* (p = 0.03), *SLC2A2* (p = 0.008), *JAZF1* (p = 0.004), and *G6PC2* (p = 0.03) was observed in diabetic donors (n = 9; 5 male, 4 female) compared to nondiabetic (n = 54; 31 male, 23 female) donors. *ADAMTS9* (p = 0.01) showed higher expression level. Bars represent mean ± SEM.

(C) Lower expressions of *KCNJ11* (p = 0.03), *WFS1* (p = 0.01), *SLC2A2* (p = 0.0001), *JAZF1* (p = 0.02), and *G6PC2* (p = 0.0009) were observed in hyperglycemic donors (n = 20; 11 male, 9 female) compared to normoglycemic (n = 30; 18 male, 12 female). *ADAMTS9* (p = 0.01) was shown to have higher expression level. Bars represent mean ± SEM.

(D–I) Correlations between expression of the differential gene expressions with HbA_{1c} level (n = 51) and insulin secretion (ng/islet/hr) measured at 16.7 mM glucose (n = 53): *SLC2A2* (D), *KCNJ11* (E), *G6PC2* (F), *WFS1* (G), *JAZF1* (H), and *ADAMTS9* (I).

did not differ significantly from background (after Bonferroni correction). Twenty-one genes starting from *ARAP1* showed higher expression than the mean expression of all genes on the array ($p < 0.05$). The highest transcript levels were seen for *G6PC2* and *SLC30A8* (Figure 1A), whose expression was restricted to islets and absent in peripheral blood leukocytes (Figure S1). At genome-wide level, 72 genes showed higher expression than *SLC30A8* in pancreatic islets. We then studied transcript abundance in donors with known T2D or hyperglycemia ($HbA_{1c} \geq 6\%$) compared with normoglycemic donors (no known T2D and $HbA_{1c} < 6\%$) (Figures 1B and 1C). The expression of *KCNJ11*, *WFS1*, *SLCA2A*, *JAZF1*, and *G6PC2* was decreased in islets from T2D donors. Furthermore, increased expression of these genes in islets was associated with higher insulin secretion and lower HbA_{1c} (Figures 1D–1I).

cis and *trans* Effects of T2D-Associated SNPs on Gene Expression in Human Islets

The majority of known risk variants for T2D are located in intronic or intergenic regions, suggesting that they may influence gene expression. We therefore analyzed whether any of the 47 T2D-associated SNPs would influence gene expression in *cis* (within 1 Mb of the SNP) or in *trans* (further than 1 Mb away or on a different chromosome) by applying a linear model with additive effects. For *cis* eQTLs a significance threshold of $p < 0.001$ was defined based upon 1,000 permutations. For *trans* eQTLs with

a very large number of tests (901,863), we applied a p value threshold of $p < 0.00019$ using the best p values of 1,500 random SNPs, referring to the 5% showing the strongest *trans* eQTLs. We observed 5 *cis* and 176 *trans* eQTLs (Figure S2 and Table S1). Of the *trans* eQTLs, the highest numbers were seen for the *G6PC2* (rs560887) (n = 66) and the *GCKR* (rs780094) (n = 31) variants (Table S1). None of the five *cis* eQTLs showed different expression in islets from normo- and hyperglycemic donors. By slightly relaxing the p values for *cis* effects, we observed that risk T allele carriers of rs5912 in the *KCJN11* gene were associated with decreased expression of *KCJN11* ($p = 0.005$) in human islets. We also confirmed the previously shown elevated expression of the *TCF7L2* gene in carriers of risk genotype (T) for rs7903146 ($p = 0.02$).

Pathways Based upon Coexpression Analysis with Putative T2D Associated Genes

Genes with similar expression patterns are hypothesized to share functional relationships and may represent pathways of interest for the pathogenesis of islet dysfunction in T2D (Inouye et al., 2010). Thus, we correlated the expression of the 48 T2D seeder genes with the ~20,000 genes on the array. Genes showing strong correlation with the T2D seeder genes were considered coexpressed ($r^s > 0.8$ or < -0.8; genome-wide $p = 4.73 \times 10^{-8}$). Of individual genes, *SLC30A8*, *G6PC2*, and *GCK* showed the largest number of coexpressed genes (130, 142, and 167) (Figure 2A and Table S2), while for 26 genes expression did not correlate with any gene. By restricting the analysis to genes coexpressed with at least two of the 48 T2D seeder genes, we observed a strongly connected network of 248 genes (including 14 seeder genes), which we called T2DNet (Figure 2B). To investigate whether this network represented a relevant collection of T2D-associated genes in human islets, we analyzed coexpression of 48 randomly selected genes in a set of 10,000 randomly selected networks of the same size. As shown in Figure S3, the T2DNet showed significantly more coexpressed genes than the randomly selected networks ($p < 0.01$).

We next plotted the centroid (mean expression of the coregulated genes) of T2DNet gene expression against insulin secretion and HbA_{1c} levels (Figure 2C). The mean centroid showed a positive correlation with insulin release and an inverse correlation with HbA_{1c}. These correlations could not be reproduced in a set of 1,000 randomly selected gene networks of the same size. Also, the mean centroid of the T2DNet gene expression was markedly decreased in islets from T2D compared with nondiabetic donors (Figure 2D). Next, we restricted the network to genes that were coexpressed with at least four known T2D seeder genes. This generated a more condensed network (Figure 2E), which we call T2DNet core. Of the 23 genes in this restricted network, 14 candidate genes for T2D were identified (*KLHDC5*, *SNAP91*, *MAFB*, *PPM1K*, *RGAG4*, *SH3GL2*, *FAM46C*, *RPS6KA6*, *MAPRE3*, *CLCN4*, *NMNAT2*, *TMEM63C*, *ELAVL4*, and *NOL4*). Expression of these genes differed significantly between normoglycemic and hyperglycemic donors (Figure 2F and Table S3). Furthermore, they correlated positively with insulin secretion and negatively with HbA_{1c} (Figures 2G and S3) (permuted 1,000 times by randomly selected lists of 14 genes at a $p < 0.01$). These 14 genes were included in the ranking of T2D-associated genes (see below). The mean centroid of the T2DNet core was significantly decreased in islets from diabetic versus nondiabetic donors (Figure 2H). Finally, we analyzed whether SNPs within a region spanning ± 50 kb up and downstream of 234 T2DNet genes (excluding the 14 seeder genes, in total 44,319 SNPs) were associated with T2D in the DIAGRAM+ database (Voight et al., 2010) using an arbitrary p value of > 0.001. Of the 133 SNPs showing an association at this significance level, we selected 12 SNPs with the lowest p values in or around *KLHDC5*, *LRFN2*, *ACVR1C*, *RASGRF1*, *PARD3*, *MTSS1*, *RAB3C*, *DACH1*, *NF1B*, *RIN2*, *PLCB4*, and *PAM* (Table S4). These 12 genes were included in the ranking of T2D-associated genes (see below).

Molecular Interaction Networks

To obtain information on functional pathways that may characterize the T2DNet, we subdivided the network into modules using the EAGLE algorithm (Shen et al., 2009). Four functional modules were identified. Module 1 consisted of *WFS1*, *KCNJ11*, *GCK*, and *MADD*, while module 2 included *G6PC2*, *SLC30A8*, *FADS1*, and *SLC2A2*. The other two modules were smaller; module 3 included *IGFBP2* and *JAZF1* and module 4 *NOTCH2* and *GCKR* (Figure 3A). These modules were enriched for genes regulating β cell function ($p = 1.4 \times 10^{-6}$) in the Reactome database (http://david.abcc.ncifcrf.gov/). To verify the functional connectivity of these modules, we interrogated the public STRING database compiling predicted protein-protein interactions (PPI). T2DNet genes were found to interact with each other more often than expected by chance alone in the PPI database ($p = 0.000012$) (Figure S5). Expansion of the four modules to the global PPI network indicated a 4- to 10-fold enrichment of T2D seeder genes (Table S5). In general, disease genes tend to be tissue-specific and functionally located in the periphery of the network (Barabási et al., 2011), which was also seen here for the T2D seeder genes. In the PPI network, 2,607 genes were first-order neighbors of the T2DNet seeder genes. Using network topology to prioritize these 2,607 genes, we identified 162 genes showing significant connectivity within the PPI network (hypergeometric distribution $p < 0.05$). Of them, 142 genes had significant connection with only one T2D seeder gene, 11 with 2 genes, and 9 with ≥ 3 genes. The nine genes showing connection with ≥ 3 T2D genes (*LGR5*, *PDX1*, *CDC123*, *NEUROD1*, *INS*, *FOXA2*, *ABCC8*, *PAX6*, and *GCG*) were included in the ranking of T2D associated genes (see below). Of note, expression of *PDX1*, *FOXA2*, *ABCC8*, and *PAX6* differed between islets from hyperglycemic and normoglycemic donors (Figure 3B) and correlated with insulin secretion and HbA_{1c} levels (Figure S4).

Genes Differentially Expressed in Pancreatic Islets from Hyperglycemic and Normoglycemic Donors

Next we analyzed differential expression of genes in islets by stratifying the donors on T2D status or HbA_{1c} (normoglycemic < 6% and hyperglycemic ≥ 6%). Using a cutoff of $p < 0.001$, we observed 118 genes that were differentially expressed in donors with known diabetes and 129 in hyperglycemic donors (Table S6). Of them, 18 genes were differentially expressed in both comparisons and were included in the subsequent ranking (Figure 4A). To exclude that these differences were due to differences in purity between diabetic and nondiabetic islets, we also

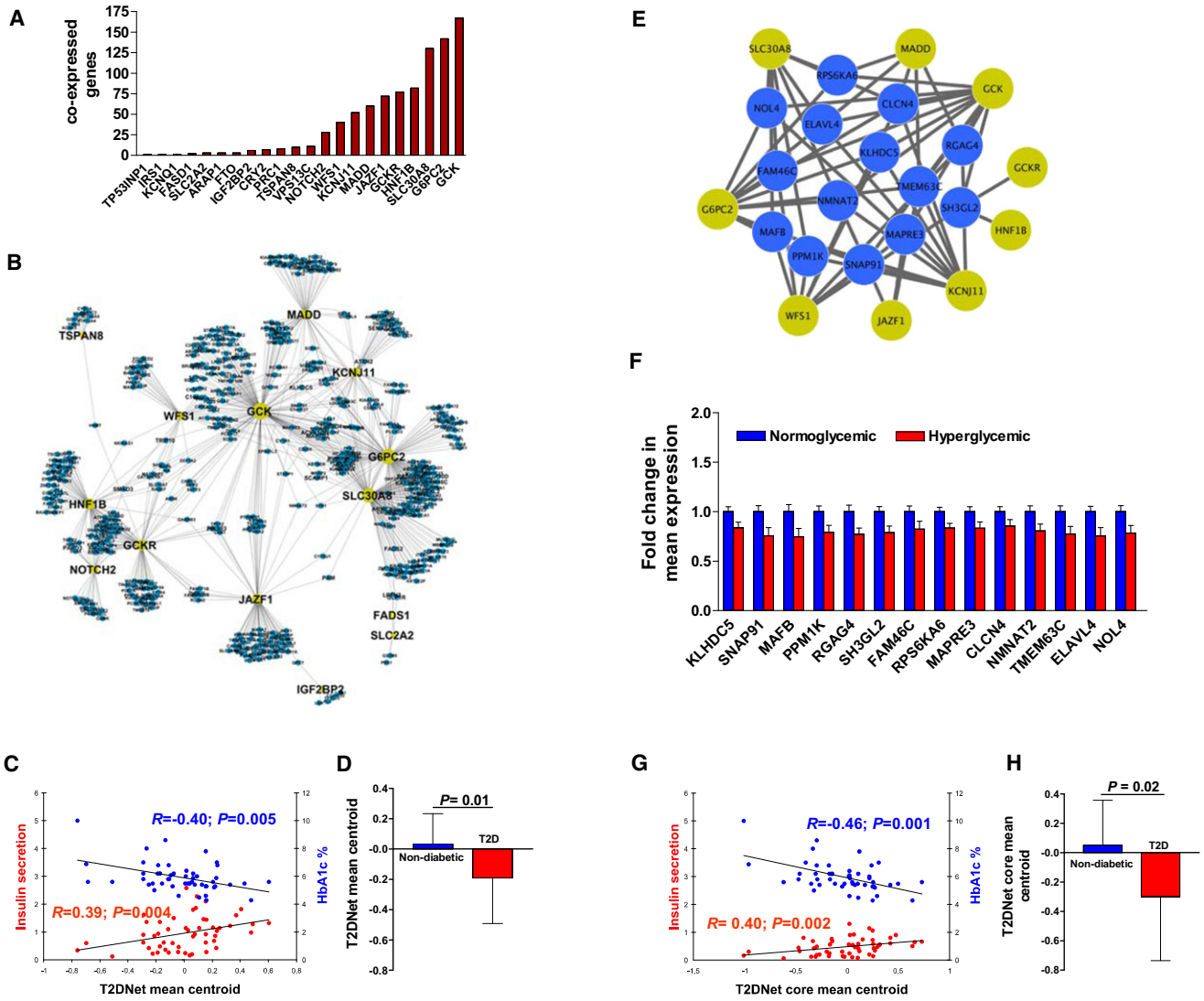

Figure 2. Pathways Based upon Coexpression Analysis of T2D-Associated Genes

(A) Number of coexpressed genes in the arrays that correlated with each of the 48 T2D genes using cutoff values of $r > 0.8$ or < -0.8.

(B) Illustration of the coexpression network (T2DNet) in Cytoscape. T2DNet was created by identifying genes in the arrays that correlated with at least 2 genes of the 48 seeder T2D-associated with a cutoff value of $r > 0.8$ or < -0.8. The T2DNet includes 14 T2D genes (known T2D-associated genes shown as yellow nodes), which were correlated with 234 genes (blue nodes).

(C) Correlations of mean centroid expression of T2DNet genes (248) with HbA_{1c} level and insulin secretion (ng/islet/hr). A negative correlation was observed to HbA_{1c} level and positive to insulin secretion.

(D) Low expression of T2DNet mean centroid in diabetic compared to nondiabetic pancreatic islets ($p = 0.01$). Bars represent mean ± SEM.

(E) Illustration of T2DNet core in Cytoscape. T2DNet core was formed by restricting the coexpression analysis to at least four seeder genes of the 48 T2D with a cutoff value of $r > 0.8$ or < -0.8. We identified 14 genes whose expression was correlated to a cluster of 4–6 seeder T2D genes. Yellow nodes are the T2D genes and blue nodes are the identified 14 genes.

(F) Fold change in mean expression of the 14 genes (from T2DNet core) in 20 donors with hyperglycemia versus 30 donors with normoglycemia. A significantly reduced expression of *KLHDC5* ($p = 0.04$), *SNAP91* ($p = 0.01$), *MAFB* ($p = 0.01$), *PPM1K* ($p = 0.02$), *RGAG4* ($p = 0.03$), *SH3GL2* ($p = 0.01$), *FAM46C* ($p = 0.01$), *RPS6KA6* ($p = 0.04$), *MAPRE3* ($p = 0.03$), *CLCN4* ($p = 0.06$), *NMNAT2* ($p = 0.04$), *TMEM63C* ($p = 0.02$), *ELAVL4* ($p = 0.009$), and *NOL4* ($p = 0.03$) was observed. Bars represent mean ± SEM.

(G) Correlation of mean centroid expression of T2DNet core genes (23) with HbA_{1c} level and insulin secretion (ng/islet/hr). A negative correlation was observed to HbA_{1c} level and positive to insulin secretion.

(H) Low expression of T2DNet core mean centroid in diabetic compared to nondiabetic pancreatic islets ($p = 0.02$). Bars represent mean ± SEM.

adjusted the results for purity measured by dithizon and DNA content of the islets, but results remained virtually unchanged (data not shown). Those 18 genes included the receptors *IL1R2*, *GLP1R*, *GPR120*, *BDKRB1*, and the Wnt signaling regulator *SFRP4*. For *GPR120*, *CHL1*, *GLP1R*, and *LRNF2*, high expression in islets was associated with increased insulin

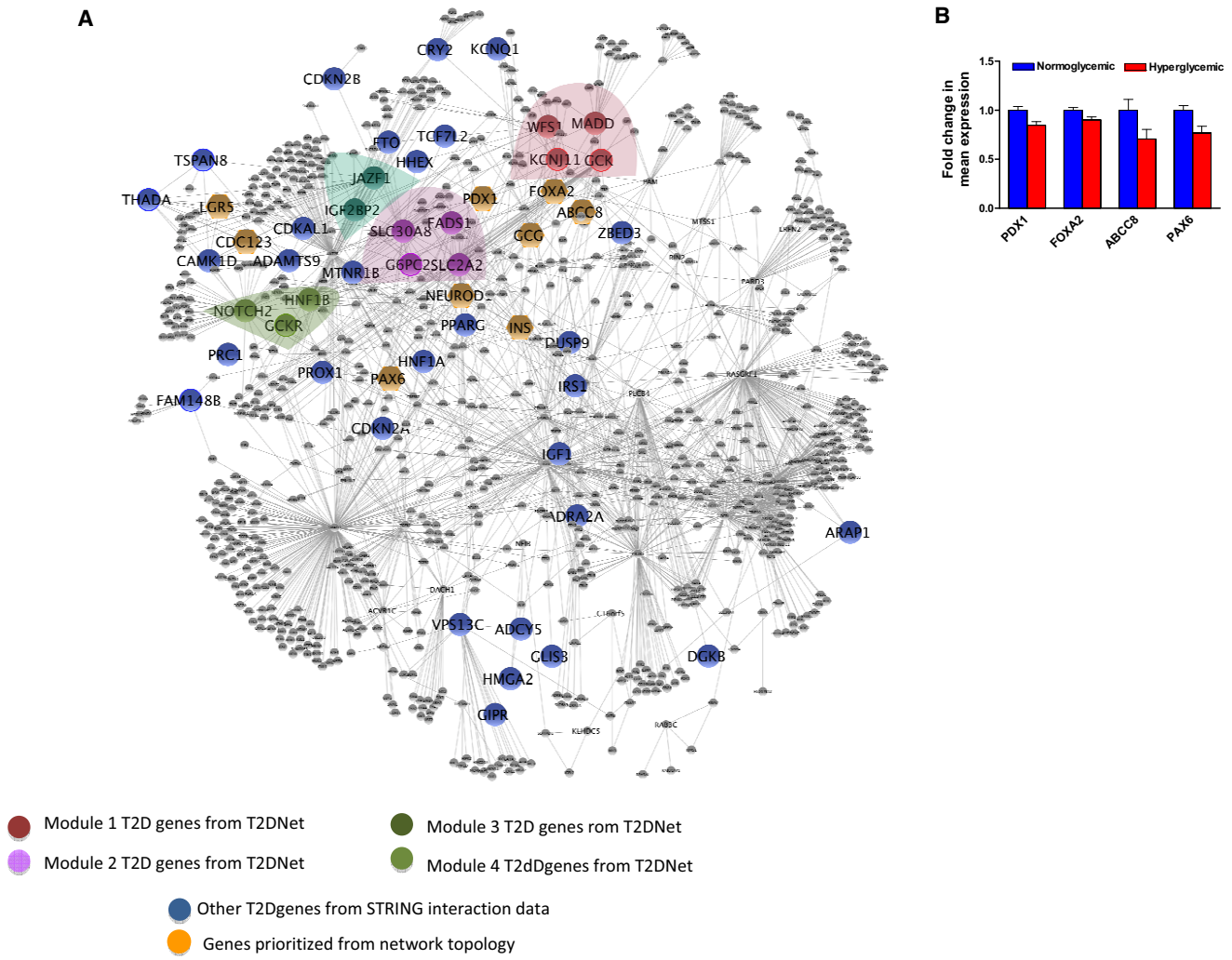

Figure 3. Molecular Interaction Networks of Known T2D Genes

(A) Protein-protein interaction network for the 248 genes in the T2DNet. Using EAGLE algorithm, four modules were identified. Module 1 included *WFS1*, *MADD*, *KCNJ11*, and *GCK*; module 2 *SLC30A8*, *SLC2A2*, *G6PC2*, and *FADS1*; module 3 *IGF2BP2* and *JAZF1*; and module 4 *NOTCH2*, *HNF1B*, and *GCKR*. Expansion of modules using STRING interaction data revealed 29-known T2D genes (nodes colored in blue). Genes prioritized from network topology are colored in yellow.

(B) Out of the nine genes prioritized from the network topology, expression of *PDX1* ($p = 0.0001$), *FOXA2* ($p = 0.03$), *ABCC8* ($p = 0.006$), and *PAX6* ($p = 0.01$) was shown to have significantly lower expression in hyperglycemic compared to normoglycemic donors. Bars represent mean ± SEM.

secretion and lower HbA_{1c} (Figures 4B–4E and 4J). In contrast, the expression of *IL1R2*, *PDGFRA*, *SFRP4*, and *PTGES* was positively correlated with reduced insulin secretion and higher HbA_{1c} levels (Figures 4F–4I and 4K). We also tested whether 3,122 SNPs within a region of ± 50 kb of the 18 differentially expressed genes would show association with T2D in the DIAGRAM+ database at $p < 0.001$. As seen for the T2DNet, a SNP in the *LRFN2* gene (rs892367) showed nominal association with T2D ($p = 0.0001$).

Genes Associated with T2D Based upon Bioinformatic Ranking

Finally, we included the 14 genes from the T2DNet core, 8 genes from the PPI network (*Ins* gene was excluded from ranking as it was not annotated in our Affymetrix chip), 18 genes showing differential expression between hyperglycemic and normoglycemic patients and 10 genes with top-ranked SNPs in the DIAGRAM database together with the 48 T2D seeder genes to make a final rank list of 98 putative T2D genes. We used five different criteria to rank the genes: (1) correlation of gene expression in human islets with insulin secretion, (2) correlation of gene expression in human islets with HbA_{1c}, (3) SNPs within a region of ± 50 kb of the gene associated with T2D in the DIAGRAM+ database (with the lowest p value), (4) differential expression in intact pancreatic islets from diabetic and nondiabetic donors, and (5) bioinformatic analysis of a published data set on differential expression in pancreatic β cells from diabetic and nondiabetic individuals (Marselli et al., 2010). The genes

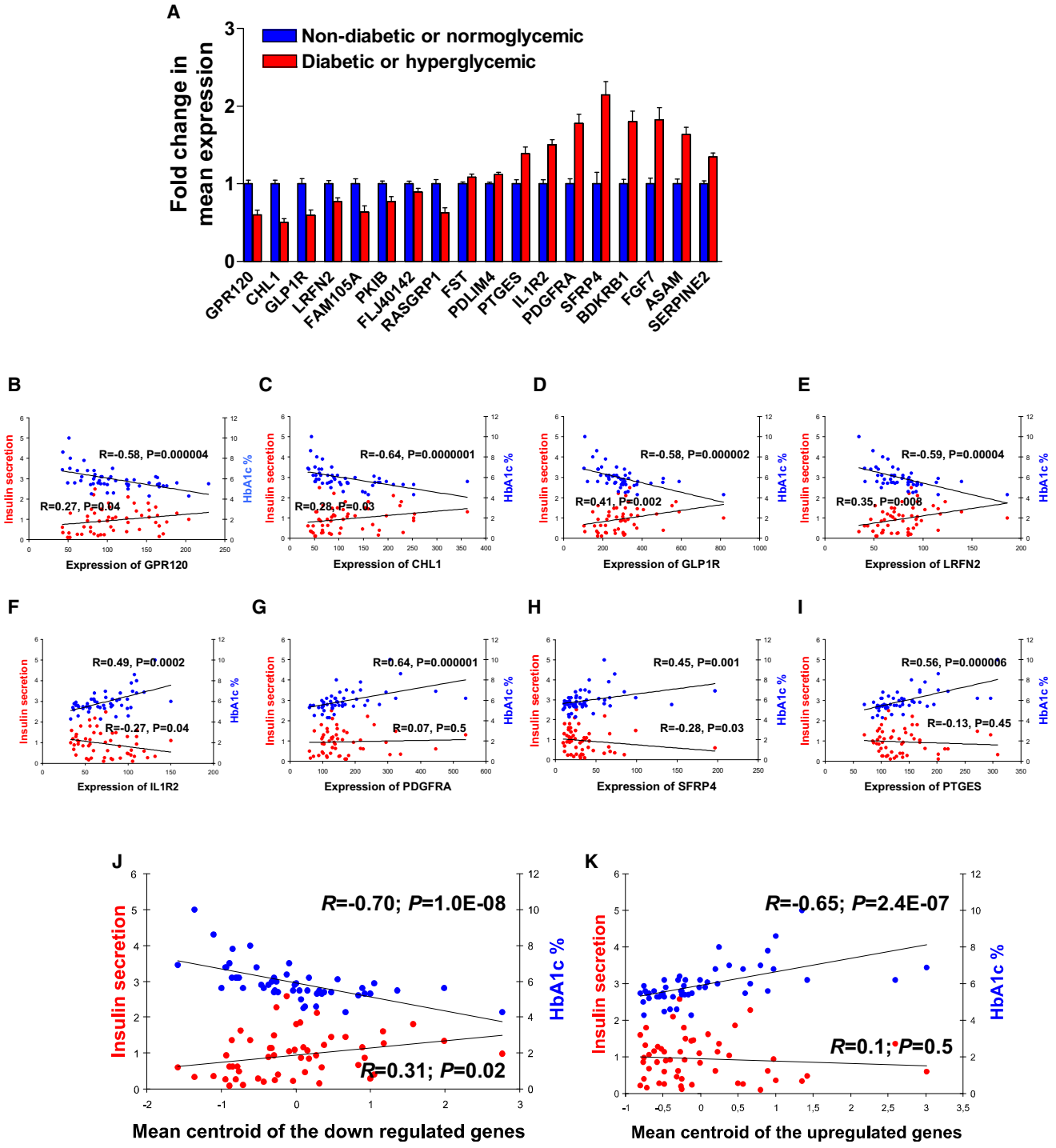

Figure 4. Genes Differentially Expressed in Human Islets

(A) Fold change in mean expression of the 18 overlapped differentially expressed genes in pancreatic islets between donors with hyperglycemia versus normoglycemia and donors with T2D versus nondiabetic donors. Bars represent mean ± SEM.

(B–I) Correlations between expression of eight of the differentially expressed genes and HbA_{1c} (n = 51) and insulin secretion (n = 53) (ng/islet/hr): *GPR120* (B), *CHL1* (C), *GLP1R* (D), *LRFN2* (E), *IL1R2* (F), *PDGFRA* (G), *SFRP4* (H), and *PTGES* (I). Genes in (B)–(E) negatively correlated to HbA_{1c} and positively to insulin secretion. Genes in (F)–(I) positively correlated to HbA_{1c}.

(J) Correlations of mean centroid of the downregulated genes (*GPR120*, *CHL1*, *GLP1R*, *LRFN2*, *FAM105A*, *PKIB*, *FLJ40142*, and *RASGRP1*) to HbA_{1c} level and insulin secretion (ng/islet/hr) in nondiabetic or normoglycemic donors.

(K) Correlations of mean centroid expression of the upregulated genes (*FST*, *PDLIM4*, *PTGES*, *IL1R2*, *PDGFRA*, *SFRP4*, *BDKRB1*, *FGF7*, *ASAM*, and *SERPINE2*) in nondiabetic or normoglycemic donors to HbA_{1c} level and insulin secretion (ng/islet/hr).

Table 1. Top 20 Ranked Genes

	SNP in DIAGRAM		T2D versus Healthy (β cell)		T2D versus Healthy (Islets)		Correlation with HbA1c		Correlation with Insulin		
Gene	P value	Rank	P value	Rank	P value	Rank	P value	Rank	P value	Rank	Sum of rank
JAZF1[a]	2.06E-08	8	0.03783304	11	0.0036	23	0.00106	24	0.0047	18	84
CHL1[b]	0.0015	52	0.00049799	3	0.0005	6	0.00000016	1	0.0338	32	94
LRNF2[b, d]	0.000106	35	0.36704424	41	0.0009	11	0.00000442	5	0.0077	22	114
RASGRP1[b]	0.00153	53	0.0032154	6	0.0001	1	0.000021	10	0.073	46	116
ABCC8[e]	1.60E-05	30	0.0005337	4	0.0067	27	0.017	49	0.00217	7	117
RASGRF1[d]	0.000216	38	0.00015519	2	0.0153	38	0.0054	39	0.0009	2	119
KLHDC5[d]	0.000003	20	0.14133106	24	0.0078	32	0.001406	28	0.02	29	133
ELAVL4[c]	0.003	58	0.28240375	33	0.0016	16	0.000885	22	0.0026	11	140
KCNJ11[a]	1.00E-06	14	0.62536277	64	0.0129	36	0.001016	23	0.0012	3	140
SLC2A2[a]	0.0287	74	4.46E-05	1	0.0085	34	0.0000024	4	0.034	33	146
FAM105A[b]	0.285	89	0.01057317	8	0.0038	24	0.00000968	9	0.0045	17	147
G6PC2[a]	0.0127	66	0.06640844	15	0.0330	41	0.001362	26	0.00142	4	152
CLCN4[c]	0.01956	71	0.08389617	17	0.0030	20	0.00152	29	0.0061	21	158
GLP1R[b]	0.00165	54	0.80635639	79	0.0011	13	0.00000829	8	0.00169	5	159
PLCB4[d]	0.000834	48	0.15950804	26	0.0518	45	0.00139	27	0.00353	13	159
GPR120[b]	0.00673	63	0.454	52	0.0004	2	0.00000823	7	0.0434	38	162
PDGFRA[b]	0.00565	62	0.06524248	14	0.0004	3	0.00000164	3	0.585	85	167
MAPRE3[c]	0.017	70	0.39069714	45	0.0011	12	0.004728	37	0.00213	6	170
PPM1K[c]	0.0105	65	0.1134122	21	0.0145	37	0.00371	35	0.0039	15	173
SLC30A8[a]	1.52E-08	7	0.20966361	30	0.2307	66	0.01847	52	0.0055	19	174

Genes were ranked based on their expression correlation with insulin secretion, HbA_{1c}, SNP associated with T2D in DIAGRAM+ database, and differential expression in donors with T2D versus healthy (in intact islets and pancreatic β cells). The genes were ranked for each parameter, and the sum score is denoted (low sum means highly ranked genes).

[a]Genes derived from the 48 T2D genes.

[b]Genes derived from the differential expression in islets from hyper/normoglycemic donors.

[c]Genes derived from the T2DNet.

[d]Genes derived from the top ten genes in the DIAGRAM data set.

[e]Genes derived from the PPI.

were given a rank from 1 to 98 according to the above-listed criteria (Table S7); genes with the lowest ranking sum will be the top-ranked genes. Out of the top 20 ranked genes, 7 genes were among the genes showing differential expression in islets from hyperglycemic and/normoglycemic donors (*RASGRP1*, *CHL1*, *PDGFRA*, *LRFN2*, *FAM105A*, *GLP1R*, and *GPR120*), 5 genes were derived from the T2DNet (*PPM1K*, *ELAVL4*, *KLHDC5*, *CLCN4*, and *MAPRE3*), 5 genes from the 48 T2D seeder genes (*JAZF1*, *SLC2A2*, *G6PC2*, *KCNJ11*, and *SLC30A8*), 3 genes from the top 10 in the DIAGRAM data set (*RASGRF1*, *LRFN2*, and *PLCB4*), and one gene from the PPI network (*ABCC8*) (Table 1).

The gene encoding *JAZF1* (zinc finger 1) was top ranked, followed by *CHL1* (cell adhesion molecule with homology to *L1CAM*), *LRFN2* (leucine-rich repeat and fibronectin type III domain containing 2) and *RASGRP1* (RAS guanyl releasing protein 1). Interestingly, the top 20 genes explained 7% and 24% of the variance in insulin secretion and HbA_{1c}. Moreover, the mean centroid of these 20 genes differed strongly between hyperglycemic and normoglycemic donors ($p = 0.005$) and was positively correlated with insulin secretion ($R = 0.45$; $p = 0.001$) and negatively with HbA_{1c} levels ($R = -0.50$; $p = 0.0001$) (Figures 5A and 5B).

GPR120 Knockdown Increased Apoptosis in Human Islets

GPR120 is an omega-3 fatty acid receptor and has been shown to mediate GLP-1 secretion (Hirasawa et al., 2005). Culturing of human islets with eicosapentaenoic acid (EPA), a *GPR120* activating agent, prevented palmitate and linoleic acid-induced apoptosis and increased cell viability, but did not appreciably influence glucose-induced apoptosis. Knockdown of *GPR120* in human islets diminished the ability of EPA to prevent apoptosis induced by palmitate or linoleic acid by 50% (Figures 5C and 5D). However, we did not observe a significant effect of EPA on insulin secretion during the 1 hr incubation period.

Functional Studies in Clonal Rat β Cells

To obtain some insight into whether any of the other top-ranked genes would influence glucose-stimulated insulin secretion, we used RNA interference to silence expression of *JAZF1*, *CHL1*, *LRNF2*, *RASGRP1*, *RASGRF1*, *KLHDC5*, *PLCB4*, *ELAVL4*, *IL1R2*, *PDGFRA*, and the *PPM1K* genes in INS-1 cells (831/13). Silencing of *CHL1*, *LRFN2*, and *PPM1K* resulted in reduced glucose-stimulated insulin secretion, whereas *RASGRP1* silencing resulted in increased insulin secretion (Figures 6 and S6). No

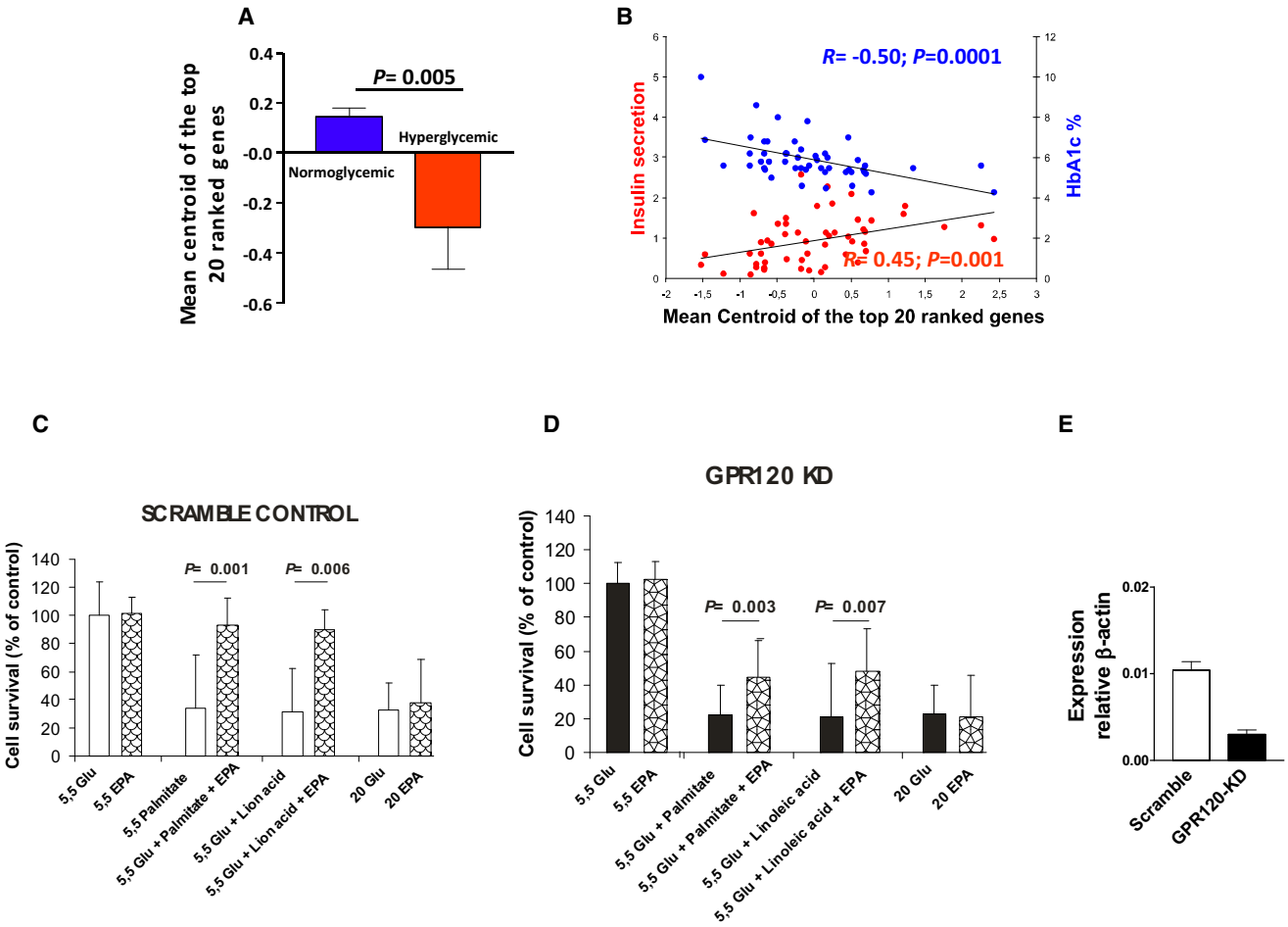

Figure 5. *GPR120* Knockdown Enhanced Apoptosis in Human Islets

(A) Low expression of the mean centroid of the top 20 ranked hyperglycemic donors compared to normoglycemic donors (p = 0.005). Bars represent mean ± SEM.

(B) Correlations of mean centroid expression of the top 20 ranked genes with HbA_{1c} level and insulin secretion (ng/islet/hr). A negative correlation was observed to HbA_{1c} level and positive to insulin secretion.

(C–E) Human islets transfected with scrambled (Scr) or shRNA for *GPR120* (KD) were treated with 5.5 mM glucose, 100 μM eicosapentaenoic acid (EPA), 250 μM palmitate, or 250 μM linoleic acid for 1 hr prior to measurement of cell apoptosis by MTS. Treatment of human islets with EPA *GPR120* prevented palmitate and linoleic acid-induced apoptosis and increased cell viability but had only a modest effect on insulin secretion (C). Knockdown of *GPR120* in human islets resulted in diminished ability of EPA to prevent apoptosis induced by palmitate or linoleic acid by 50%. No effect was observed on insulin secretion (D). The knockdown of *GPR120* was estimated to be 65% as measured by RT-PCR (E). Bars represent mean ± standard deviation (SD).

effect on secretion was seen after siRNA silencing of *JAZF1*, *RASGRF1*, *PLCB4*, *KLHDC5*, *ELAVL4*, *IL1R2*, and *PDGFRA*. In an independent study, we observed that overexpression of *SFRP4* (Figure 4A) was associated with increased expression of inflammatory markers and impaired insulin secretion.

DISCUSSION

The current paper presents a comprehensive map linking genetic variation with gene expression and function in a large number of well-characterized human islets. The analyses provide a list of potential T2D genes based upon coexpression with known T2D genes, differential expression in islets from T2D and nondiabetic islet donors, and correlation with metabolic phenotypes.

The PPI network extended the analysis of transcripts to predicted proteins and protein pathways. Most of the proteins identified as having strong connection to known T2D-associated genes encode key genes in pancreatic development (*PDX1*, *NEUROD1*, *FOXA2*, and *PAX6*) in addition to insulin and glucagon. The genes involved in pancreatic development have also been suggested to cause monogenic forms of diabetes (MODY) (Malecki et al., 1999; Stoffers et al., 1997).

To summarize the findings from the different subanalyses, we ranked the identified genes based upon five different criteria. The ten top-ranked genes explained 22% of the variance in HbA_{1c}, which is higher than 2.4%, explained by ten SNPs in a GWAS of HbA_{1c} concentrations in > 46,000 Europeans (Soranzo et al., 2010). HbA_{1c} provides a robust estimate of glycemic excursions during the preceding weeks and has been suggested

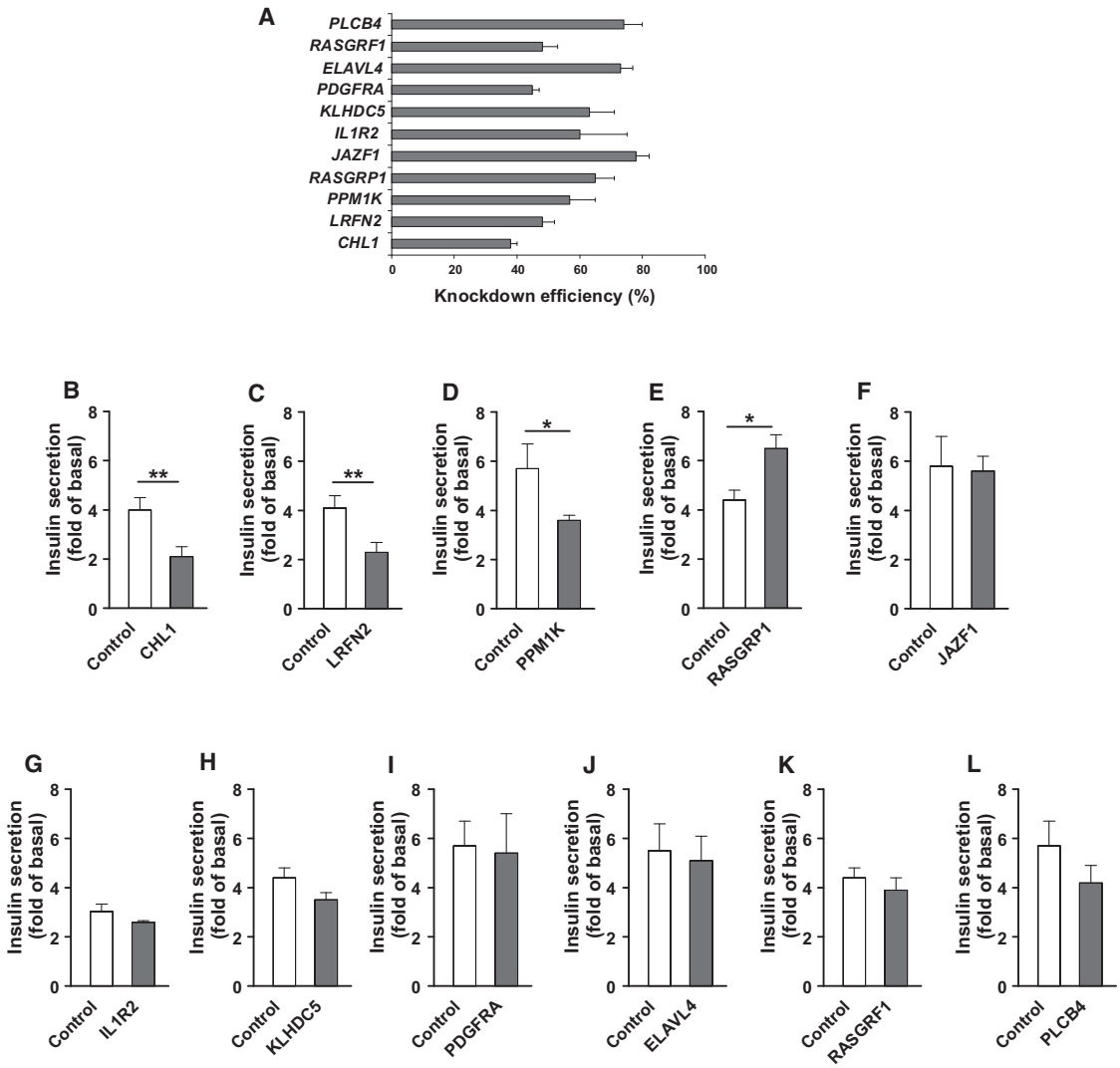

Figure 6. Glucose-Stimulated Insulin Secretion in Transfected Clonal β Cells

(A) Knockdown efficiency of siRNA of *CHL1*, *LRNF2*, *PPM1K*, *RASGRP1*, *JAZF1*, *IL1R2*, *KLHDC5*, *PDGRFA*, *ELAVL4*, *RASGRF1*, and *PLCB4* in INS-1 cells. (B–L) individual siRNA experiments for the above-listed genes show insulin secretion in response to 2.8 mM and 16.7 mM glucose 72 hr after siRNA transfection as measured during 1 hr static incubation. Secretion was expressed as fold increase (insulin secreted at 16.7 mM/insulin secreted at 2.8 mM glucose) and normalized for protein content (ng/mg/hr). Data are shown from three independent experiments for each siRNA. Bars represent mean ± SEM. * < 0.05, ** < 0.01.

as a diagnostic test for diabetes (http://www.who.int/diabetes/publications/report-hba1c_2011). HbA_{1c} also has the advantage that it is unlikely to be influenced by the ultimate treatment the donors have received.

We acknowledge that the use of HbA_{1c} to differentiate between expressions in islets from hyperglycemic and normoglycemic individuals may infer a circular element to the ranking. However, excluding the 18 genes derived from this analysis for the ranking did not alter the results markedly; the top 20 genes still explained 22% of the variance in HbA_{1c}. The cutoff for ranking is of course arbitrary, but increasing the number of top-ranked genes from ten to 20 or 40 did not significantly change the proportion of the variance of HbA_{1c} explained. It remains to be shown whether this is an indication that the first ten top-ranked genes already picked key pathways contributing to abnormal glucose tolerance and that most additional genes from the ranking list came from the same pathways, thereby adding little to the explanation of the variance in HbA_{1c}.

Importantly, the correlation between gene expression in human islets and HbA_{1c} and insulin secretion cannot be interpreted as evidence of causality, i.e., the changes in gene expression precede the changes observed in insulin secretion and HbA_{1c}. It could also be the other way around, that elevated glucose induces changes in gene expression. There is some further information to clarify these relationships. Of the 50 top-ranked genes, 84% (6% after Bonferroni correction) and 34.1% of the 48 T2D seeder genes (2%) correlated with HbA_{1c} compared with 12% (0.7%) of all 20,000 genes on the array. The corresponding figures for genes correlating with both

HbA_{1c} and insulin secretion were 54% for the top-ranked genes, 12% for the T2D seeder genes, and 3.4% for all genes on the array. From these data we can conclude that more of the 48 seeder genes and particularly the top-ranked 50 genes are associated with measures of glucose homeostasis, the direction of which cannot be inferred from the data.

To gain insight into the direction of the effect, we silenced expression of 12 genes, *JAZF1*, *CHL1*, *LRNF2*, *RASGRP1*, *RASGRF1*, *KLHDC5*, *PLCB4*, *ELAVL4*, *IL1R2*, *PDGFRA*, and *PPM1K*, using siRNA in INS-1 cells, as well as of *GPR120* using shRNA in human islets. These results obtained in a clonal rat insulinoma cell line suggest that disruption of *CHL1*, *LRFN2*, *RASGRP1*, and *PPM1K* results in altered insulin secretion. The *CHL1* gene encodes for a neural adhesion molecule, which has been ascribed a role in regulation of GABA and activation of the MAP/ERK pathway (Huang et al., 2011). The gene has been shown to be differentially expressed in pancreas from patients with pancreatic cancer (Senchenko et al., 2011). Expression of *CHL1* in human islets was strongly related to HbA_{1c} and insulin secretion (Figure 4C) and decreased in islets and β cells from patients with T2D (Marselli et al., 2010) (Figure 4A). Additionally, expression of *CHL1* was significantly correlated with the insulin content ($R = 0.30$; $p = 0.02$). It has been suggested that adhesion molecules might be involved in the development of islet structure and determine distribution of β, α, and δ cells in pancreatic islets (Cirulli et al., 1994).

LRFN2 (leucine-rich repeat and fibronectin type III) encodes a protein known to promote growth of hippocampal neurons and regulate cell surface expression of NMDA receptor subunits. High expression of *LRFN2* in islets was associated with better insulin secretion and lower HbA_{1c} (Figure 4E).

The *PPM1K* gene encodes for the mitochondrial branched-chain α-ketoacid dehydrogenase phosphatase, which catalyzes oxidative decarboxylation of branched-chain α-ketoacids from leucine, isoleucine, and valine (Wynn et al., 2012). Given the central role ascribed to branched-chain amino acids in the pathogenesis of T2D and insulin resistance (Newgard et al., 2009; Wang et al., 2011) and a recent finding demonstrating that SNPs in the *PPM1K* gene showed strong association with the Fisher ratio (ratio of branched-chain amino acids to aromatic amino acids) (Kettunen et al., 2012), it is interesting to speculate that similar mechanisms as described in muscle might also be operative in islets.

Although we did not observe any effect of silencing of *PDGFRA* (platelet-derived growth factor receptor A) on insulin secretion, there is prior evidence that disruption of *PDGFRA* impairs β cell regeneration in streptozotocin-treated mice and that *PDGFR* levels decline with age in parallel with a decline in β cell replication (Chen et al., 2011). This could suggest that a decrease in expression of *PDGFRA* influences insulin secretion only under diabetic conditions.

In contrast to expectations from findings in diabetic islets showing decreased expression of *RASGRP1*, silencing of *RASGRP1* resulted in markedly increased insulin secretion in INS-1 cells. In keeping with the findings in islets, *RASGRP1* (RAS guanyl releasing protein 1) showed marked downregulation in β cells from patients with T2D (Marselli et al., 2010). The encoded protein activates the MAP/ERK pathway and enhances endocytosis of the sodium-chloride cotransporter (Ko et al., 2010). Variation in the gene has been associated with T1D (Qu et al., 2009). These data thereby highlight the problem of generalizing data from short-term perturbations to the situation of chronic hyperglycemia characteristic of T2D.

We did not observe any effect of silencing of *JAZF1*, *RASGRF1*, *KLHDC5*, *ELAVL4*, *PLCB4*, and *IL1R2* on glucose-stimulated insulin secretion. It therefore still remains to be shown whether this reflects a failure of the experimental situation, that the observed differences in expression of these genes could be a consequence of chronic hyperglycemia, or that effect on insulin secretion also requires perturbation of coexpressed networks. Of these genes, *ELAVL4* is particularly interesting, not only because it is a putative target of *miRNA-375*, a key miRNA involved in regulation of β cell mass and function (Poy et al., 2004), but also since *ELAVL4* was shown to reside in a region of open chromatin as assessed by FAIRE enrichment (Gaulton et al., 2010) or H3K4me3 peaks (Stitzel et al., 2010) in human islets.

SFRP4 (secreted frizzled-related protein 4) contains cysteine-rich domains homologous to the Wnt binding sites of Frizzled proteins. High expression in islets was associated with impaired insulin secretion and high HbA_{1c} levels (Figure 4A). This was further supported by independent results from our laboratory showing that recombinant *SFRP4* inhibited in vitro insulin secretion by 30% and β cell exocytosis by 50% in human islets.

GPR120 encodes for a G protein-coupled receptor known to stimulate secretion of *GLP-1*. It is stimulated by unsaturated fatty acids, particularly omega-3 fatty acids (Hirasawa et al., 2005). Here we noted that its expression in human islets was positively correlated with insulin secretion and insulin content ($R = 0.25$; $p = 0.04$) and with lower HbA_{1c} (Figure 4B). Second, activation of *GPR120* with EPA prevented lipid-induced apoptosis and increased cell viability. However, this effect was reduced by 50% in human islets after disruption of *GPR120*. Although we could not observe a significant effect on insulin secretion during the short incubation period, the data suggest that *GPR120* can protect pancreatic islets from lipotoxicity (Figures 5C and 5D).

On the other hand, several genes from loci associated with T2D gathered at the bottom of the list, including *KCNQ1*, *CDKAL1*, *ADRA2A*, *CAMK1D*, and *GIPR*, etc. This may suggest that the effect of these variants on risk of T2D affects a specific splice isoform pattern rather than total gene expression. Another explanation could be that expression of most of these genes in islets was relatively low. This type of reasoning is relevant to *TCF7L2*, for which there is accumulating evidence that the risk genotype in *TCF7L2* influences splicing pattern rather than absolute levels of expression (Le Bacquer et al., 2011; Osmark et al., 2009).

There are some limitations with the study we need to take into account. One caveat when generalizing from SNPs to genes is that SNPs usually are associated with a chromosomal locus rather than a gene. Although we here focused on the nearest gene to the SNP, we cannot exclude the possibility that some of the other nearby genes are the true targets of the SNP. We therefore hoped that the analysis of *cis* eQTLs would help in identifying causal genes, but only five *cis* eQTLs were observed using these stringent criteria, and these SNPs were from genes/loci (*CAMK1D*, *HHEX*, *MADD*, *KCNQ1*, and *GCKR*) with fairly

well-described functions in metabolism. None of the *cis* eQTLs showed differential expression in islets from normoglycemic and hyperglycemic donors.

Another caveat could be purity of human cadaver islets and differences in contribution of exocrine and endocrine tissue or different contribution of α and β cells between normoglycemic and hyperglycemic donors. Although there was a small decrease in purity as measured by dithizone staining in islets from T2D versus nondiabetic islets (10%, p = 0.15), adjusting gene expression for differences in purity did not significantly change the results, nor were they influenced by adjustment for DNA content. Also, the contribution of exocrine and endocrine tissue did not significantly differ between diabetic and nondiabetic islets (see Experimental Procedures). In analogy with the issue of whether changes in gene expression are the cause or consequence of increased HbA_{1c}, we cannot from the current cross-sectional data conclude whether changes in gene expression are the cause or a consequence of a reduction in β cell mass. A recent paper on patients undergoing pancreatic surgery showed that 65% of β cell mass was lost at onset of diabetes, but the paper did not provide any information on gene expression (Meier et al., 2012). Notably, we did not attempt to include two groups of human islets, i.e., islets from T2D and nondiabetic donors; rather, the HbA_{1c} values indicate that the donors represent the whole spectrum from normal to impaired and diabetic glucose tolerance. However, only large enough numbers can outweigh the problems of heterogeneity and purity.

Taken together, these results combining SNP information and islet gene expression with relevant in vitro and in vivo measurements in humans present a map of potential genes involved in T2D pathogenesis. Thereby, systems genetics may help to link the plethora of T2D-associated SNPs and loci to pathways of relevance for islet function and pathogenesis of T2D. Functional studies will be required to pinpoint the mechanisms by which they impair islet function and increase risk of T2D.

EXPERIMENTAL PROCEDURES

Human Pancreatic Islets

Islets from cadaver donors (54 nondiabetic and 9 diabetic) were provided by the Nordic Islet Transplantation Programme (http://www.nordicislets.org), coordinated by Olle Korsgren, Uppsala University. Islets were obtained from 54 nondiabetic donors (25 females, 29 males, age 59 ± 9, BMI 25.9 ± 3.5, HbA_{1c} 5.5 ± 1.1) and 9 T2D donors (4 females, 5 males, age 57 ± 4, BMI 28.5 ± 4.5, HbA_{1c} 7.2 ± 1.1). Purity of islets was assessed by dithizone staining and was 57% ± 19% in the T2D and 67% ± 17% in the nondiabetic islets (p = 0.15). We also measured the DNA content in islets from diabetic and nondiabetic donors; no difference was observed between the groups (2.5 ng/islet versus 2.2 ng/islet; p = 0.6). In addition, we also tried to obtain an estimate of the contribution of exocrine and endocrine tissue by measuring expression of pancreatic lipase, alpha 2 amylase and chymotrypsin 2 as markers of exocrine and somatostatin and glucagon as markers of endocrine tissue (probes for insulin were not on the Affy chip). Using this approach, the contribution of endocrine tissue did not differ between nondiabetic and T2D donors (72% versus 68%, p = 0.29). A modest, insignificant decrease in insulin content in islets from hyperglycemic versus normoglycemic donors was observed (8.8 ± 3.2 ng/islet versus 10.6 ± 3.2 ng/islet; p = 0.4). All procedures were approved by the ethics committees at Uppsala and Lund Universities.

Microarray Gene Expression in Human Pancreatic Islets

The microarrays were performed using GeneChip Human Gene 1.0 ST whole transcript according to Affymetrix standard protocol. The microarrays were performed using GeneChip Human Gene 1.0 ST whole transcript according to Affymetrix standard protocol. All data are MIAME compliant, and the raw data have been deposited in a MIAME database (GEO, accession number: GSE38642). Microarray procedure is described in detail in Supplemental Experimental Procedures.

Glucose-Stimulated Insulin Secretion

Insulin secretion analysis is described in detail in the Supplemental Experimental Procedures.

Genotyping

GWAS was performed using Genome-Wide Human SNP array (SNP 6.0), and the 47 T2D-associated SNPs were genotyped by allelic discrimination with TaqMan assay on the ABI 7900 platform. More details are given in Supplemental Experimental Procedures.

Coexpression Analysis

Genes showing correlation of Spearman rho (r^s) > 0.8 or < −0.8 where considered coexpressed. All coexpression data passed Bonferroni correction ($p = 4.73 \times 10^{-8}$). For more details see Supplemental Experimental Procedures.

Molecular Interaction Networks

Genes in the connection groups were analyzed in the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database, version 8, for protein-protein interactions (Szklarczyk et al., 2011). The EAGLE clustering algorithm was used for detecting the subnetworks from the protein-protein (PPI) interaction network. The EAGLE algorithm was used to identify the network modules with CliqueSize threshold = 3 and OutputThreshold = 2. Cytoscape software (version 2.6) was used to visualize and analyze molecular and interaction networks (http://www.code.google.com/p/clusterviz-cytoscape/). Topological network properties were calculated using Network Analyzer in Cytoscape. Nodes in the network were classified according to the degree of connectivity based on the Lu et al. scheme (Lu et al., 2007). Superhubs were defined as nodes having connectivity greater 100, hubs as nodes with > 20 and < 100, peripheral-A as nodes with connectivity > 2 and < 20, and peripheral-B as those with only one interacting partner. Gene ontology (GO) enrichment of the network was carried out using the Cytoscape-BINGO program to detect significantly overrepresented GO biological processes (Maere et al., 2005). BINGO applies Benjamini and Hochberg multiple-test corrections at a significance level of p < 0.05.

cis and *trans* eQTL Detection

To identify *cis* and *trans* eQTLs, we assessed significance of expression changes in the data set of 20,000 genes by applying a linear model with the assumption of additive effect (corrected for age and sex). *cis* eQTLs were defined as significant associations between the 47 T2D-associated SNPs and expression of genes within 1 Mb distance, whereas *trans* were considered associations with expression of any other gene outside this interval. A permutation significance threshold (p < 0.001) for *cis* eQTLs was defined based on 1,000 permutations of the sample expression vectors. The p value threshold (p < 0.00019) for the *trans* eQTLs was defined by looking at the distribution of best p values from 1,500 random SNP-gene test pairs, then selecting the top 5% most significant p value (p < 0.00019).

Differences in Expression of Genes between Patients with and without T2D

Omics Explorer, Version 2.0 Beta (Qlucore AB, Lund, Sweden, http://www.qlucore.se) was used to identify any gene on the chip showing differential expression between patients with and without T2D and donors with HbA_{1c} < 6% and > 6%). A nominal p value of < 0.001 was used to identify differentially expressed genes. The mean centroid represents the normalized gene expression levels of all genes from all individuals in the analysis to a mean of 0 and a variance of 1 (Mootha et al., 2003).

DIAGRAM Database

DIAGRAM database is described in details in Supplemental Experimental Procedures.

Statistical Analysis

Differences in expression of 48 putative known T2D genes in diabetic versus nondiabetic or normoglycemic versus hyperglycemic donors were tested using nonparametric Mann-Whitney test (two-tailed). Nonparametric Spearman's test was used for testing correlation between gene expression, HbA_{1c}, and/or insulin secretion. Data are presented as means ± SEM (standard error of the mean) or ± SD (standard deviation). Expression variation of the top 20 genes in HbA_{1c} and insulin secretion were analyzed using linear regression test. All statistical tests were performed using statistical package for the social sciences (SPSS) version 19.0 software (SPSS, Chicago, IL, USA) and R package.

ACCESSION NUMBERS

The raw data have been deposited in a MIAME database under accession number GSE38642

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, seven tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2012.06.006.

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