Islet proteomics reveals genetic variation in dopamine production resulting in altered insulin secretion

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The mouse is a critical model in diabetes research, but most research in mice has been limited to a small number of mouse strains and limited genetic variation. Using the eight founder strains and both sexes of the Collaborative Cross (C57BL/6J (B6), A/J, 129S1/SvImJ (129), NOD/ShiLtJ (NOD), NZO/HILtJ (NZO), PWK/PhJ (PWK), WSB/EiJ (WSB), and CAST/EiJ (CAST)), we investigated the genetic dependence of diabetes-related metabolic phenotypes and insulin secretion. We found that strain background is associated with an extraordinary range in body weight, plasma glucose, insulin, triglycerides, and insulin secretion. Our whole-islet proteomic analysis of the eight mouse strains demonstrates that genetic background exerts a strong influence on the islet proteome that can be linked to the differences in diabetes-related metabolic phenotypes and insulin secretion. We computed protein modules consisting of highly correlated proteins that enrich for biological pathways and provide a searchable database of the islet protein expression profiles. To validate the data resource, we identified tyrosine hydroxylase (Th), a key enzyme in catecholamine synthesis, as a protein that is highly expressed in β-cells of PWK and CAST islets. We show that CAST islets synthesize elevated levels of dopamine, which suppresses insulin secretion. Prior studies, using only the B6 strain, concluded that adult mouse islets do not synthesize L-3,4-dihydroxyphenylalanine (L-DOPA), the product of Th and precursor of dopamine. Thus, the choice of the CAST strain, guided by our islet proteomic survey, was crucial for these discoveries. In summary, we provide a valuable data resource to the research community, and show that proteomic analysis identified a strain-specific pathway by which dopamine synthesized in β-cells inhibits insulin secretion.

Approximately 50% of the variation in the risk of type 2 diabetes (T2D)4 in humans is due to genetic factors (1). Most of the candidate genes identified in genome-wide association studies for T2D affect pancreatic islet function either directly or indirectly from other tissues (2, 3). Humans have a wide range in insulin secretory capacity, and insulin secretion shows high heritability (4, 5). However, model organisms are necessary for detailed mechanistic studies to understand how specific genes affect insulin secretion.

The mouse has been indispensable in diabetes research (6). The phenotype spectrum present in the wide array of mouse strains offers the opportunity to discover gene action in relation to diabetes traits. However, most research in mice has been limited to a small number of mouse strains covering limited genetic variation. The majority of mouse gene knockout studies has been performed in C57BL/6J (B6), with most of the remaining studies done in FVB and 129/Sv. Often, a gene deletion results in “no phenotype,” but the absence of a discernible phenotype could be due to the strain background suppressing the phenotype of the gene deletion.

In 2002, the Collaborative Cross (CC) project was initiated to produce recombinant inbred strains from eight genetically diverse founder strains: five classical inbred mouse strains...
Genetic variation in dopamine regulates insulin secretion

We assessed the variability of diabetes-related metabolic phenotypes, conducted whole-islet proteomics, and measured isolated islet insulin secretory responses from the eight CC founder strains and both sexes. Our data show a wide range of diabetes-related metabolic phenotypes among the strains and indicate that genetic background exerts a strong influence on insulin secretion among the islet proteome, which can be causally linked to differences in insulin secretion among the strains. Furthermore, the data show that modules of highly correlated proteins are driven by specific strains and enrich for biological pathways.

We discovered that β-cells of PWK and CAST mice uniquely have elevated levels of tyrosine hydroxylase (Th), the first step in the catecholamine synthesis pathway. We show that increased Th in CAST islets leads to enhanced dopamine production, resulting in blunted insulin secretion. Our findings demonstrate the utility of exploiting the wide genetic diversity in the CC founder mouse strains available to the research community.

Results

Genetic diversity drives diabetes-related phenotypic variability

We assessed the variability of diabetes-related metabolic phenotypes of the following eight genetically diverse Collaborative Cross (CC) founder mouse strains: C57BL/6J (B6); A/J; 129S1/SvImJ (129); NOD/ShiLtJ (NOD); NZO/HILtJ (NZO); PWK/PhJ (PWK); WSB/EiJ (WSB); and CAST/EiJ (CAST), which were metabolically challenged with a Western-style diet high in fat and sucrose (HF/HS diet; 44.6% kcal from fat and 40.7% kcal from sucrose) for 16 weeks. This resulted in a large range in diet-induced weight gain and insulin resistance. All mice were obtained from The Jackson Laboratory, housed in the same vivarium, and maintained on the same diet through-out the study.

We observed an extraordinary range in diabetes-related metabolic phenotypes among the eight mouse strains and between the sexes, reflecting their genetic diversity. Body weight (Fig. 1, A and B), fasting plasma glucose (Fig. 1, C and D), insulin (Fig. 1, E and F), and triglyceride (Fig. S1, A and B) all showed strain- and sex-dependent differences over the course of the 16-week HF/HS dietary challenge. Body weight was lowest in the three wild-derived strains (CAST, PWK, and WSB) and was highest in NZO, which were sacrificed at 14 weeks of age due to severe hyperglycemia. Number of islets per pancreas (G) and insulin content per islet (H) were determined for all mice at 22 weeks of age, except for NZO male mice, which were sacrificed at 14 weeks of age due to severe hyperglycemia. Body weight, plasma glucose, and insulin levels were determined after a 3–4-h fast. Data are mean ± S.E., n = 3 mice/sex/strain.

Figure 1. Diabetes-related metabolic phenotypes vary with genetic background. Male and female mice of the eight CC founder strains (C57BL/6J (B6); A/J; 129S1/SvImJ (129); NOD/ShiLtJ (NOD); NZO/HILtJ (NZO); PWK/PhJ (PWK); WSB/EiJ (WSB); and CAST/EiJ (CAST)) were maintained on a HF/HS diet beginning at 4 weeks of age. Body weight (A and B), fasting plasma glucose (C and D), and insulin (E and F) were measured at multiple time points during the dietary challenge. Number of islets per pancreas (G) and insulin content per islet (H) were determined for all mice at 22 weeks of age, except for NZO male mice, which were sacrificed at 14 weeks of age due to severe hyperglycemia. Body weight, plasma glucose, and insulin levels were determined after a 3–4-h fast. Data are mean ± S.E., n = 3 mice/sex/strain.

The least amount of food but did not have the lowest body weight. Fasting glucose levels remained within a normal range in all mice (90–180 mg/dl for HF/HS-fed mice), except for NZO males. Fasting insulin levels, a marker of insulin resistance, however, showed dramatic strain- and sex-dependent variation; there was ~100-fold difference in fasting plasma insulin between the most insulin-resistant (NZO) and the most insulin-sensitive (CAST) strains, for the females at 20 weeks old and an ~10-fold difference in the males. Male NZO were the only mice to become overtly diabetic (fasting glucose >300 mg/dl) and did not survive the full 16-week dietary challenge. In contrast, female NZO mice were severely obese, yet maintained euglycemia by increasing insulin.

We previously evaluated the dynamic changes in plasma glucose and insulin in the eight male strains on regular chow or...
HF/HS diet during an oral glucose tolerance test (oGTT). We found remarkable strain-dependent variation in whole-body glucose homeostasis and circulating insulin (8). In particular, male CAST mice demonstrated a rapid and transient rise in plasma glucose and insulin during the oGTT. Furthermore, CAST was the only strain resistant to HF/HS diet-induced changes in all metabolic phenotypes. These results suggest that male CAST mice utilize unique physiological pathways to regulate glucose-stimulated insulin secretion and whole-body glucose homeostasis.

Islet insulin and glucagon secretory response is determined by genetic background

To evaluate the relationship between genetic diversity and islet function, we isolated islets from both sexes of each mouse strain that were maintained on the HF/HS diet for 16 weeks. The number of islets isolated per mouse (Fig. 1G), insulin content per islet (Fig. 1H), and glucagon content per islet (Fig. S3A) all varied greatly. In several strains (B6, A/J, WSB, CAST, NZO, and PWK), >400 islets were collected per mouse. 129 and NOD mice had fewer islets. NZO male mice yielded the fewest islets overall (~50 pooled from four mice), a consequence of their extreme diabetes. It is likely that other factors, such as effectiveness of pancreatic digestion by collagenase, affect the number of islets isolated per mouse. However, the small number of islets isolated from the severely diabetic animals suggests that the islet number measurement is also related to the physiological state of the mouse at the time the isolation was performed. Islets from A/J and NZO females had the highest insulin content, whereas islets from WSB, PWK, and NZO males had the lowest. Islets from female PWK mice had the highest glucagon content, whereas islets from male A/J mice had the lowest. There was a sex-effect on glucagon content, with islets from male mice generally having lower glucagon content than islets from female mice. Comparison of the patterns across the strains shows that glucagon content per islet was not strongly correlated with insulin content per islet.

To evaluate the relationship between genetic background and insulin secretion, we measured secretion in response to a variety of insulin secretagogues: glucose (3.3, 8.3, and 16.7 mM); the incretin hormone glucagon-like peptide-1 (GLP-1, 100 nM); the fatty acid palmitate (PA, 0.5 mM); amino acids (0.5 mM insulin secretion. Fatty acids, GLP-1 (via GLP-1 receptor), and containing granules with the plasma membrane, resulting in closure of ATP-sensitive potassium channels, followed by lytic pathway. This process induces a rise in the ATP/ADP ratio and secretion is usually represented by one of three metrics: total insulin secreted (total secretion), secretion as fold over basal (fold-change), and secretion as a percent of islet insulin content (% of content). The following conditions were used to stimulate insulin secretion from islets of the HF/HS diet-fed CC founder strains at 22 weeks of age: 3.3 mM glucose (G3.3, basal); 8.3 mM glucose (G8.3), 8.3 mM glucose + 100 nM GLP-1 (G8.3 + GLP-1), 8.3 mM glucose + 1.25 mM L-alanine, 2 mM L-glutamine, and 0.5 mM L-leucine (G8.3 + AA), 16.7 mM glucose (G16.7), 3.3 mM glucose + 40 mM KCl (G3.3 + KCl), and 16.7 mM glucose + 0.5 mM palmitic acid (G16.7 + PA). Values represent average secretory responses for ≥3 mice/sex/strain, except NZO male mice, where a pool of islets from four mice was used.

In all strains, 16.7 mM glucose plus palmitate (G16.7 + PA) elicited the largest insulin secretory response. With some exceptions, the remaining secretagogues had decreased potency in the following rank order: 3.3 mM glucose plus KCl (G3.3 + KCl), 16.7 mM glucose (G16.7), submaximal glucose with amino acids (G8.3 + AA), submaximal glucose with GLP-1 (G8.3 + GLP-1), submaximal glucose alone (G8.3), and low glucose (G3.3). Inter-strain variability was apparent, particularly in response to more moderate secretagogues (G8.3, G8.3 + GLP-1, G8.3 + AA, G16.7, and G3.3 + KCl). At the two extreme insulin secretory conditions (G3.3 and G16.7 + PA), we observed the most consistent secretion responses across all strains, suggesting that basal release and release in response to a strong stimulus can overcome genetic influences.

Islets from NZO mice secreted the greatest amount of total insulin in response to all secretagogues, including G3.3, the basal condition (Fig. 2, 1st panel). These results suggested that NZO islets secrete high levels of insulin under nonstimulatory conditions. When normalizing insulin secretion to insulin con-
tent, NZO islets appeared to demonstrate superb secretory capacity (Fig. 2, 3rd panel). This trend, however, is driven by the low insulin content in these mice (Fig. 1H). Indeed, NZO islets showed reduced responsiveness to several secretagogues, including G16.7 + PA, compared with the other strains, when represented as fold-change over basal (Fig. 2, 2nd panel), showing that the majority of insulin secreted from NZO islets is basal, unregulated secretion. In addition to strain, sex exerted a strong influence on insulin secretion in some (B6, CAST, 129, PWK, and NZO) but not all strains, suggesting strain–by–sex interactions. Strain–by–sex interactions became more apparent when insulin secretion was represented as fold-change over basal; fold-change in insulin secretion was much lower for females than males in several strains, including B6, 129, and CAST, and to a lesser degree in PWK and NZO. These data show that genetic background has a strong influence on insulin secretion in response to a variety of secretagogues, both metabolic and nonmetabolic.

In addition to insulin, we measured glucagon secretion from the isolated islets in response to KCl. Islets from PWK, NZO, and NOD mice secreted the highest amount of glucagon, and islets from B6, A/J, and 129 secreted the least amount (Fig. S3B). However, when glucagon secretion was expressed as a percent of content, these strain differences were reduced, demonstrating that glucagon content strongly influences the amount of glucagon secreted (Fig. S3C).

**Whole-islet proteomics reveals strain- and sex-dependent differences**

We measured the islet proteomes of the eight HF/HS-fed CC founder strains from both sexes, using high-resolution MS coupled with nano-flow LC (10–18). This did not include male NZO mice, as these animals yielded too few islets as a result of severe diabetes. Our analysis yielded an average detection of 23,148 unique peptides (Fig. S4A), corresponding to 4,705 quantified proteins per sample (Fig. S4B). We quantified 5,255 total proteins, and 4,775 across all eight strains (Fig. S4C and Table S1), yielding >90% overlap among the samples, which permitted across-strain comparisons. Quantitative reproducibility was good with a median coefficient of variation of 16.7% across all samples (Fig. S4D).

To identify strain-dependent patterns in the islet proteome, we computed the Z-score for all identified proteins, followed by unsupervised hierarchical clustering (Fig. 3). The Z-score indicates how many standard deviations a data point (in this case protein abundance) is from the mean (the mean abundance of that protein across all samples). All 5,255 proteins were used in the clustering, and those that were not detected in a sample are colored gray and denoted N/A. The clustering resulted in groups of proteins showing striking abundance differences among the strains. Six major clusters displayed both significant enrichment for Gene Ontology (GO) terms and marked differential expression among the mice.
mice clustered into two groups nearly according to sex. All but one NOD and one 129 mouse grouped by strain and sex. One male NOD and one female 129 grouped with the male WSB mice.

The protein clustering (horizontal axis) resulted in subsets of strain-specific up- and down-regulation of protein abundance that were significantly enriched for Gene Ontology (GO) terms (Fig. 3). The largest set contained 1,190 proteins and enriched for GO term “protein transport” ($p < 10^{-60}$). These proteins were down-regulated in CAST and male WSB and up-regulated in NZO female islets. Many of these proteins are involved in vesicle fusion (Vamps and Syntaxins). The up-regulation of these proteins in the NZO female islets suggests that an increase in vesicle transport and fusion could explain the high basal, unregulated insulin secretion from these islets (Fig. 2, 1st panel). There was a smaller set of 47 proteins associated with antigen processing and presentation ($p < 10^{-12}$) that was exclusively up-regulated in a subset of NOD mice, which included histocompatibility 2 class II antigen Aa (H2-Aa), Aβ1 (H2-Ab1), Eβ (H2-Eb1), and CD74 antigen (Cd74). These proteins have previously been shown to be enriched in intra-islet myeloid cells (19, 20). It is possible that one or more of these proteins is involved in the autoimmune-mediated death of β-cells in NOD, a model for type 1 diabetes. Two clusters of proteins showed differential regulation in NZO only, endoplasmic reticulum (ER) proteins (up-regulated; $p < 10^{-12}$) and mitochondrial proteins (down-regulated; $p < 10^{-40}$). Some of the proteins associated with mitochondrial function that are decreased in the NZO female islets may be involved in the relatively poor insulin secretory response that we observed in NZO islets (Fig. 2, 2nd panel). Our data show that genetic background exerts a strong influence on the islet proteome that can likely be causally linked to differences in insulin secretion.

Islet proteome co-expression modules enrich for physiological pathways

The results presented in Fig. 3 prompted us to use a weighted gene co-expression network analysis (WGCNA) approach (21, 21) to compute co-expression modules consisting of highly correlated protein subgroups (Table S2). Proteomics experiments can provide more information than a list of differentially expressed proteins. WGCNA can be used to analyze this higher-level information by considering relationships between measured proteins, which can be assessed by correlations between expression profiles. WGCNA starts with thousands of proteins, identifies co-expression modules, and uses correlation between an expression profile and a sample trait to identify important proteins for further validation (see “Experimental procedures” for computational details). When grouping proteins into co-expression modules, we did not utilize information about functional annotation. Among the 5,255 proteins identified from our whole-islet proteomics experiment, 83% were uniquely assigned to a co-expression protein module. The WGCNA approach computed 20 co-expression modules from the proteomics data, which are identified by a color name (Table S3). The modules contained varying numbers of proteins, ranging from 49 to 1,396. A cluster dendrogram shows the modules as downward branches (Fig. 4). The depth of the branches indicates the overall correlation between proteins in a module, with deeper branches having greater correlation. Table S2 lists all modules and their protein membership.

We and others have shown that highly correlated transcripts (in this case, proteins) are often associated with common physiological pathways (23–27). GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to determine whether the modules contained proteins that enriched for specific bio-
logical pathways. Remarkably, all modules were significantly enriched (Z-score >3) with one or more GO and/or KEGG terms. In the cluster dendrogram, module branches are labeled with a general description of the overall GO terms or KEGG pathways enriched in each module (Fig. 4). All significantly enriched categories for the modules are included in Table S4.

For each module, we computed a module eigengene (ME) (or first principal component (PC1)) to describe the pattern of protein abundance among the eight CC founder strains and both sexes. The ME can be considered a representative of the protein expression profiles in a module. MEs for all modules are shown in Fig. S5, A–D and illustrate the protein abundance pattern across the strain-sex combinations. The variance described by the MEs ranged from ~31% (blue module) to ~47% (lightcyan module) (Table S3). Proteins that were not identified within a co-expression module were put into the gray module (904 proteins), which had a variance described by the ME of ~6%. The variance described by the MEs is the percent variance among the proteins within a module that is explained by the PC1 or the ME. Typically, they can be ~30% or greater and much higher than the percent variance describing the ME for the gray module. This shows that proteins in the nongray modules have highly coordinated expression.

The top-enriched module was lightcyan, which enriched for the GO term “cytosolic ribosome” (Z = 36.3). The lightcyan module contained 83 proteins, including ribosomal protein S2 (Rps2), ribosomal protein L18 (Rpl18), and many other Rpl and Rps proteins. Proteins in this module were most highly up-regulated in islets from both sexes of CAST and female WSB, and most highly down-regulated in islets from female B6 and male 129 (Fig. S5A). The abundance of these ribosomal proteins may reflect the amount of protein turnover in these islets.

Other modules that were highly enriched for physiological pathways included tan, which enriched for the GO terms “response to interferon-γ” (Z = 15.6) and “immune response” (Z = 13.05) and the KEGG pathways “Staphylococcus aureus infection” (Z = 15.8) and “antigen processing and presentation” (Z = 10.0). This module contained histocompatibility 2 class II antigen Aα (H2-Aa), Aβ1 (H2-Ab1), Eβ (H2-Eb1), CD74 antigen (Cd74), and interferon-induced guanylate-binding protein 2 (Gbp2). This module describes the cluster of proteins highly abundant in NOD islets shown in Fig. 3.

The midnightblue module was highly enriched for the GO term “serine-type endopeptidase activity” (Z = 15.6), and the KEGG pathway “pancreatic secretion” (Z = 14.5). This module contained pancreatic lipase, pancreatic lipase-related protein 2 (Pnliprp2), and pancreatic colipase, which reflects the unavoidable contamination of acinar tissue in isolated islet preparations. The ME for midnightblue shows that proteins in this module were up-regulated in 129 and down-regulated in A/J islets, which may reflect the amount of contaminating acinar tissue in the islet preparations from these strains (Fig. S5B).

The turquoise module contained 1,396 proteins, which enriched for the GO term “Golgi vesicle transport” (Z = 8.6) and the KEGG pathway “SNARE interactions in vesicular transport” (Z = 7.0) and includes adaptor-related protein complex 1y1 subunit (Ap1g1), Rab8a, Sec22b, vesicle-associated membrane protein 7 (Vamp7), syntaxin 6 (Stx6), and many other Rabs, Secs, Vamps, and Stxs. Proteins in the turquoise module were up-regulated in female NZO and down-regulated in male WSB and female CAST islets and describe the “protein transport” cluster in Fig. 3. Islets from the NZO mice had high basal insulin secretion and poor insulin secretory response when secretion was presented as fold over basal (Fig. 2), suggesting that up-regulation of proteins involved in vesicle transport and SNARE interactions results in a high rate of unregulated basal insulin secretion.

The red module, enriched in the KEGG pathways “oxidative phosphorylation” (Z = 10.4) and “citrate cycle” (TCA cycle) (Z = 8.8), had an ME with the opposite pattern to that of turquoise. Proteins in the red module were down-regulated in female NZO and up-regulated in male WSB and female CAST islets. The red module included ATP synthase H⁺ transporting mitochondrial F1 (Atp5a1) and other mitochondrial ATP synthase subunits, cytochrome c oxidase subunit 5A (Cox5a), isocitrate dehydrogenase 3 (NAD⁺) (Idh3g), and pyruvate carboxylase (Pcx). This decrease in proteins in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OxPhos) pathway in the NZO islets could also explain their poor stimulated insulin secretion.

These results demonstrate that a network analysis yields robust protein sets (modules) that enrich for biological function, demonstrate striking strain- and sex-dependent patterns of protein abundance, and are describable by an ME that captures a large portion of the variance across the samples.

Islet proteome co-expression modules correlate with diabetes-related phenotypes

To determine the potential physiological significance of the islet modules, we asked whether the modules were correlated with the diabetes-related metabolic phenotypes we measured in the CC founder mice. Using MEs, we computed the correlation between the modules and several whole-body physiological traits (e.g. plasma insulin), and the insulin and glucagon secretory responses measured from the isolated islets (Fig. 5). Because islets isolated from the same mice were used for both the secretion studies and proteomics analysis, we were able to directly compare the protein and insulin secretion measurements.

Several modules were significantly correlated with more than one phenotype (Fig. 5). For example, the lightgreen module, enriched for the GO terms “cell-substrate adherens junction” (Z = 8.8) and “focal adhesion” (Z = 8.8), showed the strongest positive correlation with insulin secretion in response to all secretagogue classes, as well as positive correlation with triglyceride (TG) and to a lesser extent plasma insulin. Proteins in the lightgreen module included integrin-linked kinase (Ilk), melanoma cell-adhesion molecule (Mcam), Thy-1 cell-surface antigen (Thy1), and lectin galactoside-binding soluble 1 (Lgals1) and were up-regulated in islets from NOD and NZO and down-regulated in islets from B6 (Fig. S5A). This suggests that the high total insulin secretion from the NZO and NOD islets and low insulin secretion from the B6 islets shown in Fig. 2 could be due to an increase or decrease, respectively, in proteins involved in cell-substrate junctions.
The cyan and black modules, enriched in endoplasmic reticulum proteins, also showed a similar correlation pattern to these phenotypes. The cyan module enriched for the KEGG pathway “protein processing in endoplasmic reticulum” (Z/H11005 3.3) and included DnaJ heat-shock protein family (Hsp40) member C1 (DnaJc1), protein-disulfide isomerase family A member 15 (Txndc5), signal sequence receptor subunit 1 (Ssr1), and SEC13 homolog nuclear pore and COPII coat complex component (Sec13). The ME for cyan showed that proteins in this module are highly up-regulated in islets from female NZO mice (Fig. S5C). The black module enriched for the GO terms “endoplasmic reticulum chaperone complex” (Z/H11005 13.7) and “response to endoplasmic reticulum stress” (Z/H11005 6.8) and included protein-disulfide isomerase family A member 4 (Pdia4), heat-shock protein 90 β family member 1 (Hsp90b1), calreticulin (Calr), and endoplasmic reticulum lectin 1 (Erelc1), and these proteins are also up-regulated in islets from female NZO mice and down-regulated in female B6 and PWK islets. This suggests that the high insulin secretion from the NZO islets and low insulin secretion from the B6 and PWK islets shown in Fig. 2 could be due to an increase or decrease, respectively, in proteins involved in the ER stress response. Modules enriched for the GO terms “aerobic respiration” (Z/H11005 10.7) (red), “mRNA processing” (Z/H11005 9.8) (blue), and “lipid metabolic process” (Z/H11005 3.1) (gray60) showed the strongest negative correlation to insulin secretion in response to all secretagogue classes, as well as a negative correlation to body weight and plasma insulin.

The magenta module was the most highly negatively correlated with glucagon secretion in response to KCl and enriched for the GO term “glycosphingolipid metabolic process” (Z/H11005 8.8) and the KEGG pathway “lysosome” (Z/H11005 6.6). Proteins in the magenta module included galactosylceramidase (Galc), GM2 ganglioside activator (Gm2a), hexosaminidase subunits a and b (Hexa and Hexb), and prosaposin (Psap). The ME for magenta showed that proteins in this module are generally up-regulated in B6, A/J, and 129 islets and down-regulated in female NZO, PWK, and CAST islets (Fig. S5B).

Tyrosine hydroxylase is highly abundant in β-cells of PWK and CAST islets

Previously, we have shown that CAST mice were resistant to HF/HS diet and demonstrated remarkably rapid insulin and glucose responses during an oGTT (8). Furthermore, a preliminary survey of the islet phosphoproteome in islets from CAST mice showed that serine 31 on Th was phosphorylated in response to glucose. Th activity is regulated by phosphorylation at specific residues (28). ERK1/2-mediated phosphorylation at serine 31 stabilizes the enzyme and stimulates catalytic activity (29, 30). Th is the first step in the catecholamine synthesis pathway, converting L-tyrosine to L-3,4-dihydroxyphenylalanine (l-DOPA). Catecholamines are potent inhibitors of insulin secretion (31–33).

Our survey of the islet proteome in the CC founder mice shows that Th is ~70-fold higher in islets from CAST and PWK mice compared with the other strains (Fig. 6A). Th is present in
the blue module. The ME for blue shows that blue module proteins are generally up-regulated in PWK and CAST islets and down-regulated in female NZO and male B6 islets (Fig. 6B). GO terms that describe Th in this module include “cell body” (Z = 3.4), “axon” (Z = 2.4), and “catecholamine metabolic process” (Z = 2.1) (Table S4). These GO terms for the blue module also include neural cell adhesion molecule 1 (Ncam1) and neuropeptide Y (Npy), among others. Ncam1 has been shown to be required for cell-type segregation and normal ultrastructure in pancreatic islets (34). Npy is a secreted neuropeptide that influences many physiological processes, including cortical excitability, stress response, and food intake (35), and it also inhibits insulin secretion (36). These other proteins in the blue module should also be elevated in islets from PWK and CAST mice like Th. Indeed, Npy is 4-fold higher in PWK islets and 12-fold higher in CAST islets over B6 islets. These proteins in the blue module are associated with inhibition of insulin secretion, consistent with the negative correlation between the ME of the blue module and insulin secretion in Fig. 5.

In pancreatic sections from B6, PWK, and CAST mice, we determined the proportion of β-cells (insulin-positive), α-cells (glucagon-positive), and unidentified cells (neither insulin nor glucagon-positive) that were Th-positive using immunohistochemistry (Fig. 6, C and D). PWK and CAST islets had ~35-fold more Th-positive β-cells per islet area, compared with B6 islets. There was no statistically significant difference in the number of Th-positive α-cells or Th-positive unidentified cells per islet area across the strains. In summary, our data show that β-cells from CAST and PWK mice have greatly elevated Th protein, suggesting that islets from these mice utilize catecholamines as an additional regulatory mechanism for insulin secretion that is absent in strains that have low Th levels.

**Increased dopamine production in CAST islets is associated with decreased insulin secretion**

Catecholamine synthesis begins with Th converting l-tyrosine to l-DOPA, which in turn becomes dopamine via DOPA decarboxylase (Ddc). Dopamine can then become norepinephrine via dopamine β-hydroxylase (Dbh) and norepinephrine can become epinephrine via phenylethanolamine N-methyltransferase (Pnmt). Excess dopamine is metabolized by two enzymes, Comt, producing 3-methoxytyramine (3-MT), and Mao, producing 3,4-dihydroxyphenylacetic acid (DOPAC). These two metabolites are further metabolized to homovanillic acid (HVA) by Mao or Comt, respectively. Interestingly, our proteomic data revealed that Ddc is highly expressed among all eight mouse strains and sexes; Comt is more abundant in CAST islets than other strains, and Mao is equally abundant among
To bypass the strain difference in Th activity, we incubated B6 islets with L-DOPA, the product of Th. L-DOPA is transported into cells via the cell-surface large amino acid transporter (Laat) (37, 38), which is equally abundant in all eight strains (Table S1). Pre-incubating B6 islets with L-DOPA led to a dramatic increase in islet levels of dopamine, as well as its metabolites, mimicking what we observed with CAST islets (Fig. 7, A–F). These results strongly suggest that the elevated levels of dopamine in CAST islets are due to the increased abundance and activity of Th.

We hypothesized that the increased dopamine levels in CAST islets or that achieved in B6 islets by preincubation with L-DOPA would result in reduced glucose-stimulated insulin secretion (GSIS). We measured GSIS in islets isolated from B6 and CAST. To enhance the suppressive autocrine effect of secreted dopamine on insulin secretion, we incubated 15 islets in 125 μl of secretion media for these experiments. In response to high glucose (16.7 mM), insulin secretion from CAST islets was reduced by ~60% compared with B6 islets (Fig. 7G). Pre-incubating B6 islets with L-DOPA (50 μM, 45 min) mimicked the response observed in CAST islets; insulin secretion from B6 islets was reduced by ~40% in response to L-DOPA preincubation. The addition of 1 μM dopamine suppressed insulin secretion from B6 islets by ~50%, confirming the autocrine negative feedback previously reported (31, 39, 40). Interestingly, addition of exogenous dopamine (1 μM) did not cause an additional suppression of secretion from CAST islets, suggesting that endogenously produced dopamine was sufficient to suppress the insulin secretory response.

In summary, β-cells of CAST islets express high levels of Th, the first step in catecholamine synthesis, resulting in elevated levels of dopamine. In response to glucose, dopamine is co-secreted with insulin, establishing a negative autocrine feedback that blunts the secretory response.

**Discussion**

In this study, we used the eight genetically diverse CC founder mouse strains fed a HF/HS diet to assess the contribution of genetic variation to diabetes-related phenotypes. We found that genetic diversity strongly influenced a host of metabolic phenotypes, including body weight, fasting plasma glucose and insulin, and insulin secretion from isolated islets in response to a range of metabolic stimuli. The eight strains displayed a wide range in insulin resistance, as judged by the level of fasting plasma insulin.

We previously found that of the eight strains, CAST is the only strain completely resistant to HF/HS diet-induced changes in glucose homeostasis during an oGTT, consistent with the high insulin sensitivity of the CAST mice (8). At the other extreme, the NZO mice are the most insulin-resistant and essentially HF/HS diet-intolerant. NZO male mice become severely hyperglycemic, resulting in death by 14 weeks of age. Thus, genetic variation in the CC founder strains results in a range of phenotypes from complete resistance to lethality in response to the Western-style dietary challenge.

We evaluated the relationship between genetic diversity and islet function. The number of islets isolated per mouse, the insulin and glucagon content per islet, and the islet insulin and
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glucagon secretory response varied widely among the mice. Ranking the mice based on their insulin secretory response to different classes of secretagogues illustrates how genetic background drives the insulin secretory response. When focusing on the fold-change in insulin secretion in response to the different secretagogues, islets from NOD males had the highest insulin secretion in response to G8.3 + GLP-1, whereas islets from B6 females had the lowest response. In contrast, in response to G16.7 + PA, islets from PWK males had the highest insulin secretory response, and islets from NZO males had the lowest response. In response to the nonmetabolic secretagogue KCl, islets from PWK females secreted the most insulin, whereas islets from B6 females secreted the least.

Characterization of islet protein composition is key to unlocking the molecular details of diabetes pathophysiology. One drawback is that islet scarcity has confounded extensive analyses and has required pooling islets from multiple animals. However, recent developments in mass spectrometric technologies have improved sensitivity and permit deep profiling of single animals without the need for pooling, pre-fractionation, or heavy-isotope quantitative tagging (17, 41). The CC founder strains have been extensively studied individually, and Chick or heavy-isotope quantitative tagging (17, 41). The CC founder single animals without the need for pooling, pre-fractionation, whereas islets from B6 females secreted the most insulin, whereas islets from B6 females secreted the least.

Unsupervised clustering of the islet proteome revealed that the mice clustered based on strain and sex. This shows that genetic background has a strong influence on the islet proteome that can be linked to differences in insulin secretion. We used the WGCNA approach to compute co-expression protein modules consisting of highly correlated proteins. We found that these protein groups enriched for biological pathways and correlated with the diabetes-related phenotypic measures.

Correlations can lead to hypotheses that can be tested for causality. For example, both the black and cyan modules enriched for protein critical for ER homeostasis and were positively correlated with insulin secretion, plasma insulin, and body weight. Because the ER is involved in protein folding, modification, and trafficking to the Golgi, ER homeostasis is critical in β-cells (44–47). Proteins in the black module include hypoaxia up-regulated 1 (Hyou1), protein-disulfide isomerase-associated 4 (Pdia4), and heat-shock protein 90 β (Grp94) member 1 (Hsp90b1), all reported to be up-regulated in islets under conditions that elicit ER stress (48–50). Other potentially novel proteins in the black module may be important for ER stress-induced changes in islet function and/or health.

The lightgreen module was enriched for proteins involved in cell–substrate junctions and positively correlated with insulin secretion. It is known that adherens junctions between β-cells is required for proper insulin secretion (51–56). Proteins in the lightgreen module include annexin A1 (Anxa1) and paxillin (Pxn), both reported to be important in insulin secretion (57, 58). There may be novel proteins in the lightgreen module important for cell–cell communication through adherens junctions and focal adhesions.

The gray60 module enriches for the GO terms “phosphoric ester hydrolase activity” and “lipid metabolic process” and negatively correlates with insulin secretion. Proteins in the gray60 module include carnitine palmitoyltransferase 2 (Cpt2), TAM41 mitochondrial translocator assembly and maintenance homolog (Tamm41), and acyl-CoA synthetase short-chain family member 2 (Acss2). Testable hypotheses can be generated about the function of these proteins in negatively regulating insulin secretion.

Interestingly, 5 of the 20 modules (salmon, brown, gray60, yellow, and red) were most highly enriched in distinct mitochondrial-associated pathways, enriching for mitochondrial dicarboxylic acid metabolism (salmon), transport across the mitochondrial membrane (brown), mitochondrial lipid metabolism (gray60), mitochondrial purine nucleoside, branched-chain amino acid, carbohydrate metabolism (yellow), and TCA cycle/OxPhos (red). Each of these modules consists of different proteins that have distinct expression patterns across the strains and sexes. The presence of these mitochondrial-enriched modules is consistent with the importance of mitochondrial function in islets. Mitochondrial proteins appear to be down-regulated in the islets of NZO mice, which show the greatest total insulin secretion of all the strains in Fig. 2, 1st panel. However, when represented as fold-change over basal secretion (Fig. 2, 2nd panel), islets from the NZO mice show a clear deficit in regulated insulin secretion. This shows that the NZO mice have a high nonstimulatory basal insulin secretion and a poor stimulated insulin secretion, for which mitochondrial oxidative metabolism is important (59–61).

A caveat to performing omics studies on whole islets is that changes in islet omics may reflect differences in compositions of islet cell types. Mouse islets are composed of 60–80% β-cells producing insulin and amylin in a central core and a layer of other endocrine cells surrounding the core, which is composed of 15–20% α-cells producing glucagon, <10% δ-cells producing somatostatin, <5% PP-cells producing pancreatic polypeptide, and <1% ε-cells producing ghrelin (62–65). Also, axon endings can remain within islets after isolation, and fragments of acinar and ductal cells can remain attached to the islets, and their abundance could plausibly be strain-specific. Not only can these compositions be altered by genetic background, but different regions of the pancreas within the same mouse can have islets with different endocrine cell contents (62, 65). Furthermore, recent papers employing single-cell RNAseq and mass spectrometric studies on islet cells have found heterogeneity within islet cell types (66–71). There are strain- and sex-specific differences in the abundances of the major islet hormones. These differences could be plausibly due to differences in islet cell type composition and/or hormone content per cell; fluorescence-activated cell sorting (FACS) purification of the different
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cell types in each of the strains would need to be conducted to investigate this.

A recent report by Cruciani-Guglielmacci et al. (72) compared the variation in body weight, glucose homeostasis, insulin secretion, and islet gene expression across six different mouse strains (C57BL/6J, DBA/2J, A/J, AKR/J, 129S2/SvPas, and BALB/cJ), all maintained on either regular chow or HF/HS diet. Three of these strains (C57BL/6J, A/J, and 129J) were included in our current and previous studies (8). Like our study, striking strain-specific differences in diabetes-related phenotypes were observed in response to the HF/HS diet. The HF/HS diet resulted in obesity, glucose intolerance, and insulin resistance in DBA/2J and AKR/J mice, whereas these same phenotypes were separable in BALB/cJ, which only showed evidence of glucose intolerance. A major difference between the report by Cruciani-Guglielmacci et al. (72) and our studies is the inclusion of CAST mice, which were completely resistant to HF/HS dietary challenge; they showed no change in body weight, glucose homeostasis, or insulin dynamics. Thus, the wild-derived strains, which contain greater genetic diversity than the classical inbred strains, yielded a higher level of phenotypic diversity.

In addition to surveying diabetes-related physiological phenotypes, Cruciani-Guglielmacci et al. (72) performed islet transcriptomics on mice maintained on either regular chow or HF/HS diet, enabling them to identify transcripts that were diet-responsive in each of the six strains studied. In contrast, our study surveyed whole-islet proteomics in eight mouse strains, all maintained on the HF/HS diet. Interestingly, Cruciani-Guglielmacci et al. (72) showed that the islet transcriptional profile was more closely related to genetic background than dietary conditions, length of time on a particular diet, or diet composition.

In both studies, WGCNA-based clustering was used to compute islet gene modules (transcriptomics or proteomics), and gene set analysis was conducted on the modules to identify enriched biological pathways. Pathways that were enriched within modules from both studies included cell–substrate junction, immune response, lipid metabolism, actin cytoskeleton, ribosome (biosynthesis), tricarboxylic acid cycle, oxidative phosphorylation, carbohydrate metabolism, and antigen processing and presentation. Some pathways were enriched in only one study (e.g. DNA repair and replication, vesicular transport) and may reflect post-transcriptional regulatory mechanisms, including protein turnover. Unfortunately, Elov2, a gene validated to play a role in the regulation of insulin secretion by Cruciani-Guglielmacci et al. (72) was not included in the 5,255 proteins that were identified in our study. Future studies would be required to directly assess the genetic dependence of Elov2 protein abundance differences in the eight Collaborative Cross-founder strains and to what extent these differences play a role in differential insulin secretion among these strains.

Driven by our preliminary finding that glucose promotes phosphorylation of serine 31 on Th in CAST islets, we asked whether Th was differentially abundant across the strains. Our proteomic survey showed that Th was expressed far more highly in PWK and CAST islets. It has been known for over 40 years that mouse islets can synthesize and secrete dopamine, but seemingly only after supplementing them with its precursor L-DOPA (73, 74). Mouse β-cells contain all of the components necessary to synthesize dopamine from L-DOPA. The large aromatic amino acid transporter on the surface of the β-cell rapidly transports L-DOPA into the cell. L-DOPA is decarboxylated into dopamine by Ddc (75). Dopamine is packaged into insulin granules via the vesicular monoamine transporter 2 (Vmat2) (76, 77), resulting in co-secretion of dopamine with insulin in response to a stimulus. Dopamine acts in an autocrine fashion to inhibit insulin secretion by binding to dopamine receptors on the surface of the β-cells (31, 39, 40). Furthermore, β-cells express Mao and Comt, which degrade excess cytoplasmic dopamine (78).

Th, the enzyme that converts L-tyrosine to L-DOPA, is the only dopamine biosynthetic enzyme thought to be essentially absent in mouse β-cells. However, these conclusions were drawn from studies that utilized B6 mice (31, 39, 40, 73–78). Therefore, the consensus has been that for mouse β-cells to synthesize and secrete dopamine, they must first import L-DOPA.

In the central nervous system, dopamine is secreted from neurons and functions as a neurotransmitter, although it is not released into the bloodstream. Peripheral, non-neuronal production of L-DOPA results in nanomolar levels of circulating L-DOPA (79). One source of this circulating L-DOPA is thought to be intestinal cells, which express high levels of Th (79). Although some speculate that β-cells import L-DOPA from the circulation (40), evidence that circulating L-DOPA is taken up by the β-cells is elusive.

Here, with a survey that included several strains, we show that β-cells from CAST mice express high levels of Th, leading to the synthesis of dopamine. This de novo synthesis of dopamine is associated with reduced insulin secretion, which can be mimicked in islets from B6 mice by preincubating the islets in L-DOPA (79). One source of this circulating L-DOPA is thought to be intestinal cells, which express high levels of Th (79). Although some speculate that β-cells import L-DOPA from the circulation (40), evidence that circulating L-DOPA is taken up by the β-cells is elusive.

Why would CAST β-cells synthesize a molecule that potently inhibits insulin secretion? One explanation is that they require an additional mechanism to control insulin secretion because of their high level of insulin sensitivity. We show that CAST mice are extremely insulin-sensitive, requiring the lowest plasma insulin of all the strains to maintain euglycemia and being resistant to HF/HS diet. Islets in CAST mice may employ an autocrine dopamine-mediated break on insulin secretion to ensure a brief rise in insulin, followed by a suppression of secretion (as occurs during an oGTT), to avoid hypoglycemia.

The B6 mouse strain has become the most widely used mouse model for studying human physiology, as well as the most common strain used in gene editing. However, when a gene alteration fails to produce a phenotype, it is possible that the B6 strain was not the best choice to study the gene’s function (81), rather than concluding that mice are not appropriate models to study human pathophysiology (82). The “absence of a phenotype” in a single mouse strain can be the result of strain-to-strain variation.
The phenotype variation between mouse strains motivates the search for comparable variation across the human population. In this study, we saw dramatic strain variation in Th expression in mouse islets, which determines the ability to produce dopamine de novo. Based on these results, we predict that there may be genetic variation in humans in the contribution of β-cell-derived dopamine to the regulation of insulin secretion.

Data resource for the research community

Our work provides a resource to identify the presence or absence of specific biological pathways and proteins in the islets of the eight genetically and phenotypically diverse CC founder mouse strains. Both collaborating labs have made available searchable databases of our islet proteomics data from the eight strains of mice (http://diabetes.wisc.edu/cc_founder.php) and (http://coonlabdata.com/founder_mice). http://diabetes.wisc.edu/cc_founder.php under the “Whole-islet proteomics” link is a user-friendly web interface that allows the user to enter a gene symbol. If the query was one of the 5,255 proteins identified by our whole-islet proteomic survey, the average abundance of that protein will be displayed across the 15 experimental groups (eight strains of each sex, except NZO males). In addition, we have incorporated a protein-to-protein correlation tool that can be used to identify groups of proteins with highly correlated expression profiles. Lists of correlated proteins can be directly uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/), an NIH-funded bioinformatics resource that provides functional annotation to large gene lists.

As an example of the database utility, searching for glucagon (Gcg) and then clicking the “Plot” bar graph icon under “Actions” reveals a striking strain- and sex-dependent pattern of protein abundance (Fig. S6A). A correlation analysis can then be performed by clicking the “Correlation” icon under “Actions” to determine whether other proteins show a similar pattern as Gcg. After setting the desired options and submitting, clicking “Show” for a correlated protein will produce a graph of the correlation between that protein and Gcg across the samples (Fig. S6B). Clicking “View Details,” selecting all of the proteins in the list, and selecting “Heat Map” generates a heat map of the Z-scores of the proteins across the samples, where proteins, mice, or both can be hierarchically clustered (Fig. S6C). Returning to the protein list and clicking “DAVID” automatically uploads the correlated list to the DAVID functional annotation tool website. Selecting “Functional Annotation Clustering” reveals that proteins that correlated with Gcg across the strains enrich for chaperone (p = 2.7 × 10⁻⁷) and RNA binding (p = 1.7 × 10⁻⁸). Proteins within each of these annotation clusters can be identified by clicking on the blue bar for each enrichment term.

The http://www.coonlabdata.com/founder_mice/main.php allows for proteins of interest to be queried to generate simple abundance column plots across strains. Individual strains can be compared with one another to determine significant changes across the dataset, and outlier analysis can be performed to identify significant changers specific to individual strains.

Our aim is to provide a valuable tool to the scientific community to support further biological inquiry and guide future studies. Our study provides an extremely valuable tool to help determine the appropriate strain and sex in which to study a specific biological pathway or to knock out a gene.

Experimental procedures

Animals

Animal care and study protocols were approved by the University of Wisconsin-Madison Animal Care and Use Committee. Mice were housed within the Biochemistry Department vivarium and maintained on a 12-h light/dark cycle (6 a.m. to 6 p.m.). The eight Collaborative Cross founder strains (C57BL/6J (B6); A/J; 129S1/SvImJ (129); NOD/ShiLtJ (NOD); NZO/HILtJ (NZO); PWK/PhJ (PWK); WSB/EiJ (WSB); and CAST/EiJ (CAST)) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were bred at the University of Wisconsin-Madison Biochemistry Department, except for CAST and NZO. Mice were group-housed by strain and sex (2–5 mice/cage) except for CAST that required individual housing. Mice were housed under temperature- and humidity-controlled conditions and received ad libitum access to water and food. Beginning at 4 weeks of age, mice were maintained on HF/HS diet (TD.08811, Envigo Teklad Custom Diet, 44.6% kcal from fat, 14.7% kcal from protein, 40.7% kcal from carbohydrate). Mice were sacrificed at 22 weeks of age, except for NZO males that were sacrificed at 14 weeks, due to high mortality attributable to severe diabetes.

Reagents

Collagenase type XI (C7657), BSA (A4503), Ficoll type 400-DL (F9378), FBS (12306C), dopamine (H8502), L-DOPA (D9628), ascorbic acid (A5960), and all general chemicals were purchased from Sigma. Dextrose (D16) was purchased from Thermo Fisher Scientific. Hanks’ balanced salt solution (14065056) and RPMI 1640 medium (11879-020) were from Thermo Fisher Scientific.

In vivo measurements

Body weight was measured weekly beginning at 4 weeks of age. Blood was collected by retro-orbital bleed following a 4-h fast (8 a.m. to noon) at 6, 10, and 14 weeks of age and a 3-h fast (5 a.m. to 8 a.m.) at sacrifice (22 weeks of age) and used to measure plasma glucose, insulin, and triglyceride (TG). Glucose was measured by the glucose oxidase method using a commercially available kit (TR15221, Thermo Fisher Scientific). Insulin was measured by radioimmunoassay (RIA; SRL-13K, Millipore). TG was measured using a commercially available kit (TR22421, Thermo Fisher Scientific). If plasma insulin was off the low end of the standard curve for the assay (some CAST male mice), the value of the lowest standard on the assay was reported (0.1 ng/ml). Beginning at 4 weeks of age, food intake was calculated by weighing the food remaining after 1 week, subtracting it from the amount fed, then dividing by number of mice per cage, and days to get g/mouse/day.

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Islet isolation

For all experiments that include islet isolation, intact pancreatic islets were isolated from mice using a collagenase digestion procedure as described previously (83). Islets were hand-picked and counted under a stereomicroscope to minimize contaminating acinar tissue.

Insulin and glucagon secretion measurements

After isolation, islets were placed in recovery media (RPMI 1640, 11.1 mM glucose, anti/anti antibodies, 10% FBS) for 2 h at 37 °C and 5% CO₂. All insulin secretion media (3.3 mM glucose (G3.3), 8.3 mM glucose (G8.3), 8.3 mM glucose plus 100 mM GLP-1 (G8.3 + GLP-1), 8.3 mM glucose plus 1.25 mM L-alanine, 2 mM L-glutamine, 0.5 mM L-leucine (G8.3 + AA), 16.7 mM glucose (G16.7), 3.3 mM glucose plus 40 mM KCl (G3.3 + KCl), and 16.7 mM glucose plus 0.5 mM palmitate (G16.7 + PA), was made in Krebs Ringer Buffer (KRB: 118.41 mM NaCl, 4.69 mM KCl, 1.18 mM MgSO₄, 1.18 mM KH₂PO₄, 2 mM NaHCO₃, 5 mM HEPES, 2.52 mM CaCl₂ (pH 7.4) containing 0.5% BSA, G16.7 + PA), which contained 0.67% BSA from the PA that was conjugated to BSA. For each mouse, 50 average sized islets were transferred from the recovery media to a 35-mm Petri dish containing 3 ml of preincubation media (KRB + 0.5% BSA + 3.3 mM glucose). The rest of the islets from each mouse were washed twice with PBS, snap-frozen in liquid nitrogen, stored at −80 °C, and then used for the whole-islet proteomics (see under “Whole-islet proteomics on islets from the eight CC founder strains”). For NZO male mice, islets from four mice were pooled to have enough for the secretion measurements. There were not enough islets to conduct proteomics on the NZO males. Islets were returned to the 37 °C incubator for a 45-min preincubation period. 100 µl of each secretagogue incubation media was placed in six wells of a 96-well plate. At the end of the preincubation period, individual islets were transferred to individual wells containing the incubation media, alternating the transfer between all seven incubation conditions, to ensure similar sized islets were distributed between all seven secretion conditions. Half-way through the islet transfers, three islets were placed in 1 ml of acid EtOH for measuring insulin and glucagon content. At the end of the 45-min incubation period, the media were transferred to a 96-well polypropylene storage plate. Media were frozen at −20 °C until analyzed.

Whole-islet proteomics on islets from the eight CC founder strains

Proteomic sample preparation—After islets from each mouse were isolated and allowed to recover for 2 h in recovery media (see “Insulin and glucagon secretion measurements”), 50 islets from each mouse were used for the secretion measurements, and the rest of the islets were washed twice with PBS, snap-frozen in liquid nitrogen, stored at −80 °C, and then used for the whole-islet proteomics. Islets from each mouse were lysed by boiling in 6 M guanidine, and protein concentration was determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific). 50 µg of protein was aliquoted from each sample, precipitated with 90% methanol, mixed, and centrifuged at 12,000 × g for 5 min. The supernatants were discarded, and the protein pellets were resuspended in 8 M urea, 10 mM tris(2-carboxyethyl)phosphine, 40 mM chloroacetamide, and 100 mM Tris (pH 8). Lysates were diluted to 1.6 M urea with 50 mM Tris (pH 8) and digested overnight at room temperature with trypsin (Promega) at a ratio of 1:50 enzyme to protein. Samples were desalted using Strata X columns (Phenomenex Strata-X Polymeric Reversed Phase, 10 mg/ml). Desalting columns were equilibrated with 1 ml of 100% acetonitrile (ACN) followed by 1 ml of 0.2% formic acid. Samples were acidified with TFA and loaded onto the equilibrated Strata X columns, which were then washed with 1 ml of 0.2% formic acid. Peptides were eluted into clean tubes with 1 ml of 80% ACN, dried, and reconstituted in 0.2% formic acid. Peptide concentration was measured prior to MS analysis using the Pierce Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific).

LC-MS/MS analysis—2 µg of islet peptides were loaded onto a reversed phase nano-LC column for chromatographic separation prior to MS analysis. Columns were prepared in-house from 35 cm of 75-µm inner diameter, 360-µm outer diameter fused-silica capillary tubing with polyimide coating with a laser-pulled electrospray tip. They were packed with 1.7 µm diameter, 130 Å pore size, bridged ethylene hybrid C18 particles (Waters). Columns were fitted onto an Ultimate3000 UHPLC system (Thermo Fisher Scientific) and heated to 55 °C using a home-built column heater. Mobile phase buffer A was composed of 0.2% formic acid. Mobile phase B was composed of 70% ACN, 0.2% formic acid. Samples were separated over a 120-min gradient, including time for column re-equilibration. Flow rates were set at 325 nL/min.

Peptide cations were converted to gas-phase ions by electrospray ionization and analyzed on an Orbitrap Fusion Lumos (Q-OT-qIT, Thermo Fisher Scientific). Precursor scans were collected from 300 to 1,350 m/z at 60,000 resolution (at 400 m/z) using a 1e6 AGC target. Precursors selected for MS/MS
analysis were isolated at 0.7 Th with the quadrupole mass filter and fragmented by HCD with a collision energy of 25. The maximum injection time for MS/MS analysis was 15 ms with an AGC target of 3e4. Only precursors from charge state 2–8 were selected. Dynamic exclusion time was set to 5 s, with a mass tolerance of 25 ppm. Analyses were performed in top-speed mode with a cycle time of 2 s.

**Database searching**—The raw data were processed using MaxQuant (Version 1.5.5.1) (84, 85). Searches were performed against a target-decoy database of mouse proteins, including isoforms (Uniprot, downloaded November 6, 2014), using the Andromeda search algorithm. The precursor search tolerance was set to 4.5 ppm, and the product mass tolerance was set to 0.5 Da. Search parameters included fixed modification for carbamidomethylation of cysteine residues, variable modification for oxidation of methionine, N-terminal acetylation, and a maximum of two missed cleavages. Peptide spectral match false discovery rate (FDR), and protein FDR were both set to 1%. Proteins were quantified using MaxLFQ, a label-free, intensity-based method that obviates the need for additional chemical or metabolic labeling, with an LFQ minimum ratio count of 1. LFQ intensities were calculated using the match between runs feature, and MS/MS spectra were not required for LFQ comparisons.

**Hierarchical clustering**—MaxLFQ protein groups output was further processed using the Perseus software platform (Version 1.5.6.0) (86). LFQ values were log2-transformed. Z-scores were calculated for each protein across all samples \((x - \mu)/\sigma\), and the data were hierarchically clustered in an unsupervised manner using Pearson’s correlations as the distance metric. In the resulting dendrogram, clusters were defined using a distance threshold of 0.82. Uniprot accession numbers from each cluster were used for gene ontology enrichment analysis via the DAVID tool; reported \(p\) values have been corrected for multiple tests using the Benjamini-Hochberg method.

**Generating co-expression modules**—We used a previously developed method to identify protein co-expression modules (WGCNA) (21, 22). An extensive overview of WGCNA, including numerous tutorials, can be found at www.genetics.ucla.edu/labs/horvath/CoexpressionNetwork/.5 Because proteomics were conducted on three or more mice from each strain/sex combination, any protein that was detected in two or fewer mice/sample was considered not included in the module calculation. This resulted in the exclusion of 27 proteins out of the 5,255 identified. For those proteins that were detected in three or more samples (5,228 proteins), any samples where it was not detected we entered a zero value, followed by rank transformation of all values. An adjacency matrix was constructed for these proteins. Each entry in the matrix was the absolute Pearson’s correlation, adjusted so that the overall network is approximately scale-free. Connection strength between two proteins \((x_i, x_j)\) in the network was determined according to the adjacency function, \(a_{ij} = 0.5 + 0.5 \times \text{cor}(x_i, x_j)^\beta\), using the estimated power parameter \(\beta\) of 12, resulting in a weighted network (21, 87). This yields a “signed” co-expression network that preserves the directionality of the correlation between the protein pairs, yielding values that range from 0 to 1. We note that this allows for all correlations to be used, unlike approaches that invoke arbitrary thresholds. For a discussion of the advantage of weighted versus unweighted networks, see Ref. 21 and references therein. For the WGCNA, suggestions in the following tutorial were followed, with a power parameter of 12, Pearson correlation, and signed modules: https://labs.genetics.ucla.edu/horvath/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/FemaleLiver-02-networkConstr-blockwise.pdf.5 The minimum number of proteins to make up a module was set at 30.

**Calculating correlations between MEs and physiological traits and GO/KEGG enrichment**—The proteins were clustered into modules by color, and the ME was calculated as the first principal component for the proteins in the module (21). The first principal component estimate for each module was then used along with Pearson’s correlation to correlate the modules with the clinical traits. Normalized ranks of the clinical trait values were used when calculating the correlation. We used a previously developed method for GO/KEGG enrichment of co-expression modules (88).

**Immunohistochemistry**—Immunohistochemistry was performed on 40 islets in pancreas sections from each of three B6, CAST, and PWK male mice, which were 20 weeks of age and maintained on the HF/HS diet. Mice were euthanized by CO2 asphyxiation, perfused with 4% paraformaldehyde through the heart, and the pancreas was removed, embedded in paraffin, and sectioned. Briefly, paraffin-embedded pancreas sections were de-waxed in xylene, rehydrated in decreasing percentages of ethanol, and boiled in antigen retrieval solution (Vector Labs, H3300). After the sections were cooled and washed with PBS, sections were blocked with 10% normal donkey serum in PBS for 1 h at room temperature. Primary antibody solution in 1% normal donkey serum in PBS was incubated overnight at 4 °C. After a PBS wash, secondary antibody solution in 1% normal donkey serum in PBS was incubated 1 h at room temperature. After a PBS wash, slides were allow to dry and mounted in mounting media (Vector Labs, H-1000). Primary antibodies used were as follows: polyclonal guinea pig anti-insulin (Agilent, A056401-2); monoclonal mouse anti-glucagon antibody (Sigma, G2654); and polyclonal rabbit anti-tyrosine hydroxylase (Millipore, AB152). Secondary antibodies used were as follows: goat anti-rabbit AlexaFluor 488 (Thermo Fisher Scientific, A-11008), chicken anti-mouse AlexaFluor 647 (Thermo Fisher Scientific, A-21463), and donkey anti-guinea pig Cy3 (Jackson ImmunoResearch, 706-165-148). All primary and secondary antibodies were used at a dilution of 1:500. DAPI was added to the secondary antibody solution at a concentration of 1.3 μg/ml to view nuclei. Images were acquired on a Nikon A1R+ point scanning confocal system that uses photomultiplying tubes at room temperature with a Nikon ×40 Pan Apo oil immersion lens with a numerical aperture of 1.3. Acquisition software is NIS-Elements Ar. Post-processed using ImageJ software by adjusting the brightness and contrast. The area of each islet was measured, and the number of Th+ /insulin+ /glucagon− (Th+ β-cells), Th+/insulin− /glucagon+ (Th+ α-cells), and Th− /insulin− /glucagon− (Th− unidentified cells) cells were counted. Statistics were performed using unpaired, parametric, and two-tailed \(t\) tests in GraphPad Prism 7.

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**Genetic variation in dopamine regulates insulin secretion**
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Measuring dopamine-related metabolites in B6 and CAST islets—100 islets each from four B6 and four CAST male mice (20 weeks of age on HF/HS diet) were recovered in culture media (RPMI 1640, 1.7 mM glucose, 10% FBS) for 2 h at 37 °C and 5% CO₂. Islets were then washed twice with PBS and frozen as a pellet in liquid nitrogen for storage at −80 °C.

Metabolites were extracted from the frozen islets by the addition of 50 μl of 80% (v/v) ice-cold acetonitrile, followed by sonication. The mixture was centrifuged for 5 min at 12,100 × g. The supernatant was derivatized with benzoyl chloride as described previously (89). Briefly, the supernatant was derivatized by sequential addition of 10 μl of 100 mM sodium carbonate, 10 μl of 2% (v/v) benzoyl chloride in acetonitrile, and 10 μl of the internal standard solution. The resulting solution was diluted with 50 μl of water. The internal standard solution consisted of metabolites derivatized with 13C₆-benzoyl chloride in 20% (v/v) acetonitrile with 1% (v/v) sulfuric acid. Protein content was determined using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Walther, MA), and metabolite concentrations were normalized to protein content. Calibration standards were prepared in water, diluted in acetonitrile to match the sample composition, and derivatized.

Samples were analyzed using a Waters nanoAcquity UPLC coupled to an Agilent 6410B triple quadrupole mass spectrometer. An Acquity HSS T3 C18 (100 mm, 1.8 μm, 100-Å pore size) column was used, and the injection volume was 5 μl. Mobile phase A was 10 mM ammonium formate with 0.15% formic acid. Mobile phase B was acetonitrile. The flow rate was 100 μl/min, and the gradient used was as follows: initial, 0% B; 0.1 min, 17% B; 0.5 min, 17% B; 3 min, 25% B; 3.3 min, 56% B; 4.9 min, 70% B; 5 min, 100% B; 6 min, 100% B; 6.1 min, 0% B; 8 min, 0% B.

Electrospray ionization was used in positive mode, and the capillary was at 4 kV. The nebulizer pressure was 15 p.s.i.; the drying gas was at 11 liters/min, and the gas temperature was 350 °C. Detection was performed in dynamic multiple reaction monitoring mode (MRM), and the MRM conditions are listed in Table S5. Automated peak integration was performed with an in-house developed insulin ELISA (27). Statistics were performed using unpaired, parametric, two-tailed t tests in GraphPad Prism 7.

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