Identification of a Novel Gene for Diabetic Traits in Rats, Mice, and Humans

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ABSTRACT The genetic basis of type 2 diabetes remains incompletely defined despite the use of multiple genetic strategies. Multiparental populations such as heterogeneous stocks (HS) facilitate gene discovery by allowing fine mapping to only a few megabases, significantly decreasing the number of potential candidate genes compared to traditional mapping strategies. In the present work, we employed expression and sequence analysis in HS rats (Rattus norvegicus) to identify Tpcn2 as a likely causal gene underlying a 3.1-Mb locus for glucose and insulin levels. Global gene expression analysis on liver identified Tpcn2 as the only gene in the region that is differentially expressed between HS rats with glucose intolerance and those with normal glucose regulation. Tpcn2 also maps as a cis-regulating expression QTL and is negatively correlated with fasting glucose levels. We used founder sequence to identify variants within this region and assessed association between 18 variants and diabetic traits by conducting a mixed-model analysis, accounting for the complex family structure of the HS. We found that two variants were significantly associated with fasting glucose levels, including a nonsynonymous coding variant within Tpcn2. Studies in Tpcn2 knockout mice demonstrated a significant decrease in fasting glucose levels and insulin response to a glucose challenge relative to those in wild-type mice. Finally, we identified variants within Tpcn2 that are associated with fasting insulin in humans. These studies indicate that Tpcn2 is a likely causal gene that may play a role in human diabetes and demonstrate the utility of multiparental populations for positionally cloning genes within complex loci.

To date, human genome-wide association studies (GWAS) have identified >60 genes involved in type 2 diabetes (T2D) (Zeggini et al. 2008; Voight et al. 2010; Morris et al. 2012; DIAbetes Genetics Replication and Meta-analysis (DIAGRAM) Consortium et al. 2014) and an additional 53 involved in related metabolic traits such as fasting glucose and insulin levels (Dupuis et al. 2010; Scott et al. 2012). Despite the relative success of human GWAS, when combined, these genes explain only a small percentage of the heritable variance (So et al. 2011; Morris et al. 2012), indicating many more genes have yet to be identified. Improving power with increased sample size, deep sequencing to identify rare variants, and identification of gene–gene and gene–environment interactions will help to identify at least some of this missing heritability in humans (Hu and Daly 2012). Animal models, however, offer an additional, complementary, method for identifying candidate genes and their related pathways. Advantages of animal models include the ability to control environment and genetics and assess gene expression levels in tissues that are not readily available from humans. Importantly, genes found for complex traits in animal models are frequently translated to humans (Swanberg et al. 2005; Altman et al. 2006; Samuelson et al. 2007; Behmoaras et al. 2012).
2008; Petretto et al. 2008), demonstrating the utility of animal models for uncovering at least some of the missing heritability in humans.

Particularly useful are multiparental or outbred populations such as heterogeneous stocks (HS). HS animals are derived from crossing eight inbred strains and outbreeding them for many generations (see Flint and Eskin 2012 or Solberg Woods 2014 for a review on HS populations). The genetic makeup of the resulting progeny represents a random mosaic of the founder animals, with each HS rat being phenotypically and genetically distinct. The distance between recombination events decreases with each generation of breeding, such that HS mice and rats have been used to map multiple complex traits to generally <5 Mb (Talbot et al. 1999, 2003; Demarest et al. 2001; Valdar et al. 2006; Solberg Woods et al. 2010, 2012; Johnsen et al. 2011; Baud et al. 2013). This fine-resolution mapping significantly decreases the number of potential candidate genes within each locus relative to those in conventional mapping methods (such as those using an F2 intercross or backcross). Once narrowed, HS founder sequence can be used to rapidly identify potentially causative genes within at least some of these loci (Keane et al. 2011; Baud et al. 2013), thus making these populations ideal for positional cloning of genes involved in complex traits.

We have used HS rats to fine-map traits involved in diabetes and related traits. Our work has shown that HS rats harbor alleles for glucose tolerance (Solberg Woods et al. 2010), as well as insulin resistance and β-cell dysfunction, the underlying causes of type 2 diabetes (Solberg Woods et al. 2012). We have identified a 3.1-Mb region on rat chromosome 1 that maps both fasting and postprandial glucose (Solberg Woods et al. 2010, 2012), and this region overlaps a larger 7-Mb region that maps fasting insulin and insulin sensitivity (Solberg Woods et al. 2012). Although a great improvement over previous mapping studies in F2 intercrosses (Galli et al. 1996; Gauguer et al. 1996; Chung et al. 1997; Kanemoto et al. 1998; Wei et al. 1999; Nobrega et al. 2009; Solberg Woods et al. 2009; Waliszewski et al. 2009), this region still contains a relatively large number of genes: 86 within the 3.1-Mb glucose region and >200 genes in the insulin region. Although a few plausible genes (including Kcnq1 and Igf2) stand out as likely candidates within the 7-Mb insulin region, none of the 86 genes within the 3.1-Mb region present themselves as obvious potential biological candidate genes.

The goal of the present study was to use expression analysis and next-generation sequencing to evaluate positional candidate genes within this 3.1-Mb glucose locus, located on rat chromosome 1: 204.38–207.48 Mb, a complex region that may harbor multiple causal genes (Granhall et al. 2006; Solberg Woods et al. 2010, 2012). Expression and sequence analysis in the HS rats identified two-pore segment channel 2 (Tpcn2) as a plausible candidate gene within this region. We confirm the role of Tpcn2 by demonstrating that Tpcn2 knockout mice exhibit altered fasting glucose and insulin in response to a glucose challenge. We also demonstrate significant association between SNPs in Tpcn2 and fasting insulin in humans. Together this work indicates that Tpcn2 is a strong candidate for being a causal gene that underlies the glucose and insulin phenotypes at this QTL.

Materials and Methods

Animals

**Heterogeneous stock colony:** The NMcwi:HS colony, hereafter referred to as HS, was initiated by the National Institutes of Health (NIH) in 1984, using the following eight inbred founder strains: ACI/N, BN/SnHsd (BN), BUF/NHsd (BUF), F344/NHsd (F344), M520/N, MR/N, WKY/N, and WN/N (Hansen and Spuhler 1984). This colony has been maintained at the Medical College of Wisconsin (MCW) since 2006 and has been through >60 generations of breeding (see Solberg Woods et al. 2010 for history of the breeding colony).

**Founding inbred substrains:** Expression analysis was conducted in the following substrains (abbreviated names used throughout the article are in parentheses): ACI/Eur (ACI), BN/SnHsd (BN), BUF/NHsd (BUF), F344/NHsd (F344), M520/N, and WKY/NHsd (WKY). Other than M520/N, which is the original founder of the HS colony, substrains were chosen based on origin similarities to the original founders, as determined by data obtained from the Rat Genome Database: [http://rgd.mcw.edu/strains/](http://rgd.mcw.edu/strains/). The M520 and ACI rats are maintained at MCW. All other substrains were ordered from Harlan Sprague Dawley (Indianapolis). We were unable to identify substrains for two of the founders (MR/N and WN/N), so these strains were not tested.

Cluster analysis of glucose and insulin levels in HS rats

Rats for expression analysis were chosen from a group of 522 HS rats previously used to map glucose tolerance (Solberg Woods et al. 2010) (see Supporting Information, File S2). As previously described, HS rats underwent an intraperitoneal glucose tolerance test (IPGTT) test at 16 weeks of age in which blood glucose and plasma insulin were measured at fasting and at 15, 30, 60, and 90 min after a 1-g/kg glucose injection (Solberg Woods et al. 2010, 2012). Glucose area under the curve (glucose_AUC) and insulin_AUC were determined using a trapezoidal analysis. The quantitative insulin sensitivity check (QUICKI), a measure of insulin sensitivity that has been validated in both rats and humans (Katz et al. 2000; Cacho et al. 2008), was calculated as $1/\left[\log I_0 + \log G_0\right]$, where $I_0$ is fasting insulin and $G_0$ is fasting glucose (Solberg Woods et al. 2012). HS rats were killed at 17 weeks of age, 1 week after the glucose tolerance test. Fasting cholesterol, triglycerides, and c-peptide were determined from plasma at the time the animals were killed. Livers were immediately removed and frozen in liquid nitrogen for subsequent determination of transcript abundance. Rats were killed by decapitation to avoid gene expression changes that can occur when using inhalant drugs such as CO2 or isoflurane (Kadar et al. 2011; Taylor and Cummins 2012).
differences in expression levels based on sequence variation (Koressaar and Remm 2007; Untergasser with Primer3 (http://binoinfo.ut.ee/primer3-0.4.0/primer3/)) to determine signal log ratios (log2).

We initially conducted differential expression of genes of interest with quantitative RT-PCR (qRT-PCR), we did not correct for multiple testing. Because we planned to verify differential gene expression was determined through ANOVA, using Partek (St. Louis) Genomics Suite 6.5. Differential expression (DE) was defined as those genes with \( P \leq 0.05 \) and \( \log_{2} R \geq 0.5 \). Because we planned to verify differential expression of genes of interest with quantitative RT-PCR (qRT-PCR), we did not correct for multiple testing (Hessner et al. 2004).

**Real-time qRT-PCR**

We initially confirmed differential expression of Tpcn2 using qRT-PCR in 10 HS rats with glucose intolerance and 10 with normal glucose regulation. qRT-PCR was also conducted in the founder strains, using 2 animals from each inbred strain. Upon confirming Tpcn2 as a plausible candidate gene, we used qRT-PCR to assess gene expression levels in a total of 120 male HS rats. qRT-PCR was conducted on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Specific oligonucleotide primers were designed with Primer3 (http://binoinfo.ut.ee/primer3-0.4.0/primer3/) (Koressaar and Remm 2007; Untergasser et al. 2012). To avoid differences in expression levels based on sequence variation (Huang et al. 2009), we ensured no sequence variants were present in any of the HS founders at the location of the Tpcn2 primers. The primers for Tpcn2 were forward 5‘-TATGTGTTCGG CATGATTGG-3‘ and reverse 5‘-AACGACGAAAGTCGTAAGT-3‘ (Integrated DNA Technologies). We used primers for GAPDH, forward 5‘-CATGGAGAAGGCTGGGGCTC-3‘ and reverse 5‘-AAGCGATAACTGCGGTAG-3‘, and \( \beta \)-2 microglobulin (B2M), forward 5‘-CCGTGATCTTTCTGGTGCTT-3‘ and reverse 5‘-TTTGGGCTTCTTCTAGTG-3‘ (Integrated DNA Technologies), as internal controls. We used iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Synthesis of first-strand cDNA from 3 \( \mu \)g of RNA per animal was accomplished with random hexamers (Invitrogen, now Life Technologies) and Superscript III (Invitrogen, now Life Technologies), according to the manufacturer’s instructions. Triplicate Tpcn2 and GAPDH or B2M were performed for each sample in 20-\( \mu \)l reactions, which included 1 \( \mu \)l of cDNA and 10 \( \mu \)l of iQ SYBR Green Supermix (Bio-Rad) possessing 1.2 \( \mu \)l of Tpcn2 (10 \( \mu \)M), GAPDH, or B2M specific primers and 7.8 \( \mu \)l of deionized water. Reactions were cycled as follows: stage 1, 95°, 3 min; stage 2, 55 cycles of 95°.
using the cycle threshold (ct) for quantification. The data were analyzed with Sequence Detection System 2.3 software (Applied Biosystems), using the mean ct score of 120 animals as the reference. (2001), using the mean ct score of 120 animals as the reference.

30 sec, 55°, 30 sec, 72°, 30 sec; and stage 3, melt curve of 95°, 10 sec, 60°, 1 min, 95°, 1 sec, 40°, 10 sec. Standard curves were created using 1:10, 1:100, and 1:1000 concentrations of Tpcn2. Specificity for a qRT-PCR was verified by a melting curve analysis. Relative gene expression data (fold change) between samples was accomplished using the mathematical model described by Pfaffl (2001), using the mean ct score of 120 animals as the reference.

### Genome-wide genotyping

Tail samples from the original eight inbred founders were obtained from the NIH. We extracted DNA from tail tissue from HS and founder strains, using the QIAGEN (Valencia, CA) DNeasy kit. All eight original founder inbred strains and the 46 HS rats used for expression analysis were genotyped using the Affymetrix GeneChip Targeted Genotyping technology on a custom 10K SNP array panel as previously described (Saar et al. 2008). The samples were genotyped by the Vanderbilt Microarray Shared Resource center at Vanderbilt University in Nashville, Tennessee (currently VANTAGE: http://vantage.vanderbilt.edu). From the 10,846 SNPs that are on the array, 7634 were informative and produced reliable results in the HS rats. From these final informative markers, the average spacing was 353 kb apart, with an average heterozygosity of 28.8%. These data have been deposited in the Gene Expression Omnibus (GEO) database under accession no. GSE57118 (see: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=sjnjgkwulbebtmd&acc=GSE57118).

### Genetic mapping of expression QTL

Expression mapping was conducted for all genes on the expression array that were located within the QTL (57 of 86). Genome-wide fine mapping was conducted by linkage disequilibrium mapping, testing for the significance of association between expression levels and inferred haplotype descent at each locus, as previously described (Solberg Woods et al. 2010, 2012). Briefly, haplotype descent along each HS rat genome was inferred using the haplotype reconstruction method HAPPY (Mott et al. 2000), which applies a hidden Markov model simultaneously to the genotypes of the eight founder strains and the 46 HS rats. At each interval m = 1, ..., 7634 between adjacent pairs of markers, HAPPY produces for each rat i a vector $g_i(m)$ containing the probabilities of descent from each of the 8/(8 + 1)/2 = 36 unique haplotype pairs (diplotypes) (Valdar et al. 2009). Using Bagpipe software (Valdar et al. 2009), this is then used in the following mixed-model regression to predict rat i’s expression levels $y_i$,

$$f(y_i) = QTL_i(m) + \text{ship}_{k|i} + \text{residual},$$

where $f(y_i)$ is a transformed version of the expression levels, $QTL_i(m) = \beta^T g_i(m)$ models the putative effect of the QTL, $\text{ship}_{k|i}$ is the (random) effect of the shipk to which i belongs, and residual, accounts for individual variation (further parameters defined as in Solberg Woods et al. 2010). Models were fitted by restricted-estimate maximum likelihood, and the significance of association at each interval m was assessed by comparing the fit of Equation 1 with that of the null model, which is Equation 1 without the QTL term. Genome-wide significance thresholds were estimated by parametric bootstrap from the fitted null model (Valdar et al. 2009; Solberg Woods et al. 2010). Prior to genetic analysis, expression levels for each gene were normalized using a van der Waerden normal score.

### Sequencing and analysis

We sequenced 5 Mb within rat chromosome 1: 200–208 Mb in the eight inbred founder strains of the HS colony. We designed a custom 385K NimbleGen array that included all coding regions and all regions that are highly conserved. The array was composed of 385,000 long oligonucleotide probes and used a tiling design to target our unique genomic region. Five to eight μg of genomic DNA from each strain was nebulized and polished, and linkers were added according to the manufacturer’s standards (Roche NimbleGen). Samples were then hybridized to the custom NimbleGen 385K array. DNA not specific to these regions were washed off and targeted DNA sequences were eluted and amplified by emulsion PCR. To calculate whether targeted sequences were enriched, both pre- and postcapture DNAs were analyzed by qRT-PCR, using four real-time PCR assays.
developed and optimized by our laboratory. The region was then sequenced on the Roche GS-FLX 454 system (Roche 454, Branford, CT) according to the manufacturer’s protocol. Briefly, Roche “A” and “B” adapters were annealed onto captured fragments of template DNA. The library was single stranded and quantified on an Agilent 2100 Bioanalyzer. Library fragments were annealed to beads and emulsion PCR was performed. After clonal amplification by PCR, the emulsion was broken chemically and beads were collected and enriched. Enriched beads were then inserted into the Roche Picotiter Plate, using the appropriate gasket, and loaded onto the machine. Nucleotides flowed over the plate in a specific order and enzymatic cascade reaction caused incorporation of the specific nucleotides to illuminate. An image of every flow was taken with a CCD camera and images were strung together into a flowgram, which was transformed into usable sequence data.

Reads were separated by their multiplex identifiers, using the program sfftools (Roche). For each sample, demultiplexed reads were then trimmed and assembled against the rat chromosome 1 reference sequences (Rn4), using the GS Reference Mapper (http://454.com/products/analysis-software/index.asp). Variant detection was performed using the high-confidence differences strategies implemented in GS Reference Mapper software. The high-confidence differences (HCDiff) strategy requires the following criteria for a variant to be reported: (1) There must be at least three reads with the difference; (2) there must be both forward and reverse reads showing the difference, unless there are at least five reads with quality scores >20 (or 30 if the difference involves a 5-mer or higher); and (3) if the difference is a single-base overcall or undercall, then the reads with the difference must form the consensus of the sequenced reads.

Since the time of this work, all founder strains have been fully sequenced (Baud et al. 2013), and this information is currently available in the Rat Genome Database (http://rgd.mcw.edu). We have compared our sequence to what is available online. Some discrepancies were noted between the data sets. To determine the accurate sequence, these regions were sequenced using Sanger sequence technology.

**Taqman genotyping**

We genotyped 18 SNPs within the 3.1-Mb QTL in 508 male HS rats and all eight inbred founders. SNPs were chosen based on the founder strain distribution pattern (SDP) as a means of defining general haplotype blocks (Yalcin et al. 2004a). To ensure most haplotypes were represented, we used a measure of entropy as previously described (Yalcin et al. 2005). Briefly, a sliding window was used in which each SNP within the window is prioritized based on its ability to explain the diversity between the founder strains. SNPs were then given a score and those with the highest scores were chosen for genotyping. In addition to SNPs with high entropy scores, we also genotyped nonsynonymous coding variants in the F344 strain, the founder that contributes the susceptibility allele at the QTL (Solberg Woods et al. 2010, 2012). SNPs were spaced an average of 164.5 kb apart.

Samples were genotyped using 5’-exonuclease TaqMan technology (Applied Biosystems) with differently fluorescence-labeled probes. Custom TaqMan SNP Genotyping Assays (Applied Biosystems) were used. Ten nanograms of DNA was used in a total volume of 5 μl containing 2 μl of 5ng/μl DNA, 2 μl 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 0.125 μl probe, and 0.875 μl water for each sample. PCR was carried out on a GeneAmp PCR System 9700 (Applied Biosystems) and the post-PCR plate was read on the ViiA 7 real-time PCR system (Applied Biosystems), following the manufacturer’s instructions. ViiA 7 RUO version 1.2.1 software was used to assign genotypes, applying the allelic discrimination test. The overall call rate was 98.8%.

**Glucose tolerance test in Tpcn2 knockout mice**

Wild-type and knockout mice were created using embryonic stem cells from the 129P2 strain carrying a gene trap vector and injected into C57BL/6 blastocysts, as previously described (Calcraft et al. 2009). We confirmed that Tpcn2 is not transcribed in the knockout mice by running qPCR on multiple tissues in knockout and wild-type mice (see Figure S1). PCR was conducted as described above, using primers for GAPDH (forward 3’-CTGAAGGGCATCTTGCGTA-5’ and reverse 3’-GCCGTATTCAATGTCATACCA-5’ and Tpcn2 (forward 3’-CCAGGCTACCTGTCTCCTAAGG-5’ and reverse 3’-CAGGAAGCGAAAAACATCA-5’). Heterozygous mice were bred and set up as breeder pairs. Male Tpcn2 knockout, heterozygote, and wild-type mice from the heterozygote breeders were phenotyped at 9–13 weeks of age (n = 5–7
in each group), using the IPGTT, described above and in Solberg Woods et al. (2010, 2012). Blood was collected after an overnight fast and at 15, 30, 60, 90, and 120 min after a 1-g/kg glucose injection. We used the Ascensia Elite system for reading blood glucose values (Bayer, Elkhart, IN). We also collected blood at each time point for subsequent analysis of plasma insulin levels, which was assayed using an ultrasensitive ELISA kit from Alpco Diagnostics (Salem, NH).

**Statistical analysis**

A mixed model was used to determine association between 18 SNPs within the chromosome 1 QTl and diabetic phenotypes that map to this region (glucose_AUC, fasting glucose, fasting insulin, insulin_AUC, and QUICKI) and Tpcn2 expression levels (as determined by qPCR). The model included all appropriate covariates (location, injector, collector, and number of glucose injections) and accounted for the complex family relationships of the HS, as previously described (Solberg Woods et al. 2010, 2012). The significance threshold was determined using the Bonferroni method to account for multiple comparisons (18 SNPs by six traits). Correlations between Tpcn2 expression levels (as determined by qRT-PCR) and these traits were assessed using Pearson’s correlation coefficient. Pearson’s correlation coefficient was also used to determine correlations between all microarray probes within the chromosome 1 region and metabolic traits, using a Bonferroni correction to determine significance. A one-way ANOVA was used to determine whether there were statistical differences between wild-type, heterozygote, and Tpcn2 knockout mice. If a trend toward significance was noted, a t-test was used to assess significance between wild-type and knockout mice only.

**Tpcn2 lookup in human GWAS**

We used data from two large consortiums to determine whether Tpcn2 may play a role in human diabetes. Diabetes Genetics Replication and Meta-analysis (DIAGRAM) has collected 2.4 million SNPs in >47,000 cases and controls (Zeggini et al. 2008; Voight et al. 2010), with 34,840 cases and 114,981 controls recently genotyped on the custom Metabochip (Morris et al. 2012). The Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC) has collected phenotypic information for quantitative glycemic traits such as glucose and insulin in >40,000 individuals (Dupuis et al. 2010), with 133,010 individuals genotyped on the custom Metabochip (Scott et al. 2012). Both DIAGRAM and MAGIC cohorts consist of males and females mainly of European descent. Through collaboration with these investigators, we inquired whether a subset of 39 SNPs within Tpcn2 was associated with diabetic traits in humans. The 39 SNPs were chosen based on information from the HapMap project (www.hapmap.org), choosing SNPs with an average $r^2 > 0.8$. All but 2 SNPs had an average $r^2 > 0.9$. Fourteen of the 39 SNPs were upstream and 4 were downstream of the Tpcn2 gene (see Table S1). For those SNPs that reached an unadjusted level of significance at $P \leq 0.05$, we used the Benjamini and Hochberg (1995) false discovery rate to account for multiple comparisons.

**Results**

**Glucose and insulin phenotypes in HS rats separate into five main clusters**

Although we initially identified six clusters based on glucose and insulin values, one of the clusters (cluster 5) contained a single animal and was excluded from subsequent analysis. There are two dominant phenotypic factors that characterized the clusters. The first (which explained 65% of the variability between clusters) consists of the magnitude of the peak of the insulin curve and the glucose curve, the rate of increase of the glucose from baseline to peak, the rate of increase of insulin from baseline to peak, and glucose_AUC. The second factor (which explained 31% of the variability between the clusters) consists of the rate of decrease of the insulin curve after the peak and the rate of decrease of the glucose curve after the peak.

The five clusters are shown in Figure 1 and demonstrate the importance of taking into account both glucose and insulin in the clustering. Note that animals in clusters 2 and 3 exhibit very similar plasma insulin levels, but differ significantly in blood glucose levels. Similarly, animals from clusters 3 and 4 exhibit similar blood glucose levels, but differ significantly in plasma insulin levels. For the expression analysis, glucose intolerant HS rats were chosen from cluster 1 (high glucose, moderate insulin) and HS rats with normal glucose regulation were chosen from clusters 2 and 4 (low glucose and insulin and moderate glucose and insulin, respectively). The glucose intolerant group is highly significantly
different from the normal glucose group (glucose_AUC = 13,458.9 vs. 9061, F_{1,44} = 398.7, P = 2e-16). Animals in the glucose intolerant group also exhibit differences in several other phenotypes, including significantly higher levels of body weight (349.1 vs. 314.4 g, F_{1,44} = 15.47, P = 0.000294), fasting glucose (90.3 vs. 77.5 mg/dl, F_{1,44} = 42.65, P = 5.59e-08), fasting insulin (1.8 vs. 1.1 ng/ml, F_{1,44} = 7.52, P = 0.009), insulin_AUC (311.3 vs. 204.3, F_{1,44} = 17.10, P = 0.000157), QUICKI (0.48 vs. 0.54, F_{1,44} = 7.96, P = 0.007) fasting c-peptide (1808.0 vs. 1505.8 pg/ml, F_{1,44} = 5.75, P = 0.021), and fasting triglycerides (86.2 vs. 72.3 mg/dl, F_{1,44} = 8.0, P = 0.007). No differences are seen between the groups for fasting cholesterol or fat pad weights (see Table S2).

**Tpcn2 is the only gene measured within the 3.1-Mb QTL that is differentially expressed between HS rats with different glucose profiles**

We identify 48 probes genome-wide that are differentially expressed between HS rats with glucose intolerance and those with normal glucose regulation, 3 of which had an unknown location (see Table S3). These data have been deposited in the GEO database under accession no. GSE57118 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=sjmjjgkwulbebtdl&acc=GSE57118). Of these 48 genes, only one falls within the 3.1-Mb glucose region on rat chromosome 1: Tpcn2. Statistics for the other probes within the 3.1-Mb region are listed in Table S4. Using qRT-PCR, we confirm differential expression of Tpcn2 against both GAPDH (F_{1,19} = 4.36, P = 0.05; see Figure 2A) and B2M (F_{1,18} = 7.03, P = 0.017; data not shown). HS founder strains also show different levels of Tpcn2 expression, with the F344 strain showing the least amount of Tpcn2 expression and the BN strain showing the greatest amount of Tpcn2 expression (see Figure 2B). All fold-change differences are expressed relative to the F344 founder strain.

**Tpcn2 maps as a cis-eQTL to the same region as the physiological QTL**

Using haplotype reconstruction and mixed-model linear regression, which takes into account sibship relationships, we identify a single sharp peak (marker interval gko.18d13.rp2.b1.157 and Cpn.1207450582, 205.22–207.45 Mb; log P = 12.09) for expression levels of Tpcn2 (see Table 1). Only one gene within this region, Acy3, maps as a cis-eQTL, but with a much lower log P (marker interval WKYOa01b03.r1.181 and rat106.025.f16.p1ca.195, 204.32–207.50 Mb; log P = 6.28). The genome-wide significance threshold is log P = 5.95 for both Tpcn2 and Acy3 expression levels, where −log P is defined as −log10(P-value).

**Nonsynonymous coding variants are identified within three genes, including Tpcn2**

As expected, we identify ~2000–3000 SNPs between each strain and the BN reference genome (Saar et al. 2008). Within the 3.1-Mb glucose locus, we identify three genes with a nonsynonymous amino acid change in the F344 founder strain: Tpcn2, RGD1311946, and Gpr152 (see Table 1). We focused on variants in the F344 strain because our previous work demonstrates that the F344 strain contributes to the susceptibility allele at the QTL for glucose tolerance (Solberg Woods et al. 2010) as well as for fasting glucose and insulin (Solberg Woods et al. 2012). It is important to note that loci for fasting glucose and insulin exhibit a complex genetic architecture with several other founder haplotype combinations leading to an increase in fasting glucose or insulin. Because of this, we were unable to run a simple haplotype analysis, as demonstrated by other groups (Svenson et al. 2012; Logan et al. 2013). The variants within Tpcn2 and RGD1311946 are not predicted to be damaging by PolyPhen or SIFT. The variant within Gpr152, however, results in a premature stop codon and could potentially lead to changes in protein function.

**Variants within and near Tpcn2 are significantly associated with fasting glucose and Tpcn2 expression levels**

Using a Bonferroni-adjusted region-wide threshold of 3.33, we found that 2 SNPs, located at 205,653,821 bp (~log P = 3.62) and 205,715,459 bp (~log P = 3.35), are significantly associated with fasting glucose (see Figure 4, Table 1, and Table S5). The SNP at 205,715,459 bp harbors the nonsynonymous
codeling variant within Tpcn2, while the SNP at 205,653,821 bp is located in the intron of the neighboring mammary cancer-associated protein Rmt1 gene. These SNPs and 3 additional SNPs are also associated with Tpcn2 expression levels: 205,653,821 bp (−log P = 6.58), 205,715,459 bp (−log P = 6.17), 205,898,738 bp (−log P = 4.90), 206,242,238 bp (−log P = 3.69), and 206,726,856 bp (−log P = 4.43). The additional SNPs include the nonsynonymous coding variants within RGD1311946 and Gpr152, as well as a coding variant within Cpt1a. No associations are found between the 18 SNPs and glucose_AUC, fasting insulin, insulin_AUC, or QUICKI. Genotypes for the 18 SNPs can be found in File S3.

### Tpcn2 expression levels negatively correlate with fasting glucose

Using the qRT-PCR data in 120 HS rats, we demonstrate a significant negative correlation between Tpcn2 expression levels and fasting glucose and glucose_AUC (r = −0.302, P = 0.001 and r = −0.246, P = 0.007, respectively; see Figure 5). After removing 6 animals that have extremely high expression values (>2 SD from the mean), only fasting glucose remains significantly correlated with expression levels (r = −0.232, P = 0.013). Tpcn2 expression levels are not significantly correlated with fasting insulin, insulin_AUC, and QUICKI (r = −0.034, −0.063, and 0.078, respectively). qRT-PCR data can be found in File S4. To determine whether any other genes in the region are correlated with the diabetic traits, we determined correlation coefficients between expression levels of the microarray probes within the chromosome 1 region and six metabolic traits. We confirm the significant negative correlation between fasting glucose and Tpcn2 expression levels (r = −0.559, P = 0.0000549). Based on a Bonferroni-adjusted significance level of 0.00009, we also find a marginally significant correlation between RGD1307603, a gene with no known function, and QUICKI (r = 0.544, P = 0.0000925). Correlation coefficients and unadjusted P-values are reported in Table S6.

### Tpcn2 knockout mice exhibit decreased fasting glucose and insulin_AUC

We find a significant main effect of genotype on insulin_AUC (F2,15 = 4.41, P = 0.031), with knockout mice exhibiting significantly decreased insulin response relative to wild-type mice (KO = 126.2, Het = 147.2, WT = 165.4, P = 0.029, Tukey’s post-hoc test; see Figure 6 and Table S7). There is a trend toward significance for fasting glucose (KO = 96.2, Het = 116.3, WT = 111.0, F2,15 = 3.56, P = 0.056) and QUICKI (KO = 0.54, Het = 0.49, WT = 0.49, F2,15 = 3.32, P = 0.066). Upon running a t-test between knockout and wild-type mice only, we find that Tpcn2 knockout mice exhibit significantly decreased fasting glucose levels relative to wild-type mice (t(8.9) = 2.28, P = 0.048). Tpcn2 knockout mice do not differ significantly from wild-type mice in fasting insulin levels or glucose_AUC. Means for all traits are reported in Table S7. Raw data for the knockout, wild-type, and heterozygote mice can be accessed in File S5.

### Association between Tpcn2 and fasting insulin and HOMA_IR in humans

Although we looked up associations in 39 SNPs, MAGIC was able to provide statistics on only 14 SNPs and DIAGRAM provided statistics on 11 SNPs (see Table S1). Information
was not available for the other SNPs likely because they were either rare variants or failed imputation quality control for other reasons. Of the 14 SNPs from MAGIC, 4 SNPs reach unadjusted levels of significance for fasting insulin, 2 of which remain marginally significant after accounting for multiple comparisons (rs10736671, \(P = 0.051\), and rs11604251, \(P = 0.051\); see Table 2). Three of the 4 SNPs reach unadjusted levels of significance for HOMA IR, a measure of insulin sensitivity (Matthews et al. 1985), but do not remain significant after adjusting for multiple comparisons. Significance is not found between SNPs within \(Tpcn2\) and type 2 diabetes or fasting glucose (data not shown).

**Discussion**

In our previous work, we used HS rats to identify a 3.1-Mb region that contains a QTL for glucose tolerance and fasting glucose (Solberg Woods et al. 2010, 2012) and that overlaps larger QTL for fasting insulin and insulin sensitivity (Solberg Woods et al. 2012). In the present study, we use both analysis of gene expression and association analysis to identify \(Tpcn2\) as a likely causal gene within this locus. These findings are supported by altered glucose and insulin levels in a \(Tpcn2\) knockout mouse, as well as by an association between variants within \(Tpcn2\) and fasting insulin levels in humans.

The role of \(Tpcn2\) in regulating glucose and insulin levels is currently unknown. \(Tpcn2\) channels localize to the lysosome and are likely receptors for the calcium-mobilizing agent NAADP (Brailoiu et al. 2009; Calcraft et al. 2009; Schieder et al. 2010). Several studies indicate that NAADP may play a role in insulin signaling of the β-cells (Kim et al. 2008; Naylor et al. 2009; Shawl et al. 2009; Alejandro et al. 2010; Arredouani et al. 2010), and a recent study suggests NAADP is involved in glucose homeostasis (Park et al. 2013). It is also interesting to note that \(Tpcn2\) and other calcium-handling genes (\(Tpcn1\) and \(IP3R1\)) have recently been found to be differentially expressed in patients with heart failure (Garcia-Rua et al. 2012). Although previous studies have not looked at the role of \(Tpcn2\) in liver, alterations of calcium channels such as CaMKII have been shown to lead to impaired liver gluconeogenesis (Ozcan et al. 2012), while Cav2.3 calcium channel knockout mice exhibit altered glucose levels, likely as a result of reduced insulin sensitivity (Matsuda et al. 2001). Together these studies suggest a possible role for \(Tpcn2\) in both the β-cell and the liver. Future studies will test the hypothesis that \(Tpcn2\) is involved in glucose-stimulated insulin secretion of the β-cells as well as probe a potential role of this gene in regulating insulin sensitivity and gluconeogenesis.

Global gene expression analysis in HS rats was used to initially identify \(Tpcn2\) as a candidate gene within the glucose QTL. We show that \(Tpcn2\) is differentially expressed in HS rats with glucose intolerance relative to those with normal glucose regulation and demonstrate that this gene maps as a cis-eQTL to the same region as the physiological QTL. Several previous studies have found that genes that map as cis-eQTL within previously identified physiological QTL can be considered prime candidate genes (Hubner et al. 2005; Chen et al. 2008; Morrisey et al. 2011). Demonstrating a correlation between expression levels and fasting glucose levels further supports that this gene may be playing a causal role.
role at the QTL (Farber et al. 2009; Leduc et al. 2011; Morrissey et al. 2011).

Upon assessing association between 18 SNPs within the QTL and traits that map to this region (including five metabolic traits and Tpcn2 expression levels), we find that the nonsynonymous coding variant within Tpcn2, as well as an intronic variant in the neighboring Rmt1 gene, exhibits the highest level of association with fasting glucose levels and Tpcn2 expression levels, further supporting a potential causal role for this gene. Despite having a slightly higher level of association, we do not believe that Rmt1 plays a causal role in diabetes because it is neither correlated with the diabetic traits nor exhibits expression differences. It is interesting to note that three additional SNPs in the region exhibit significant association with Tpcn2 expression levels, indicating that Tpcn2 expression may be regulated by other genes in the region. Three of these SNPs, including the Tpcn2 SNP, are from the F344 founder, the strain that exhibits very low Tpcn2 expression levels (Figure 2) and that contributes the susceptibility allele at this locus (Solberg Woods et al. 2010, 2012). It is unlikely that any of the identified variants are causal, however, because the significance levels of these SNPs do not surpass that of the haplotype mapping for either fasting glucose (Solberg Woods et al. 2012) or Tpcn2 expression levels (Figure 3). A merge analysis, in which genotypes for all SNPs in the region are imputed, may be useful to identify the causative variant (Yalcin et al. 2005; Keane et al. 2011; Baud et al. 2013). However, because this method is most successful when a single causative variant underlies the locus (Baud et al. 2013), it may prove less useful for the current region in which multiple genes/variants may be playing a role.

Upon investigating glucose and insulin levels in Tpcn2 knockout mice, we find that knockout mice exhibit significantly decreased fasting glucose and decreased insulin response to a glucose challenge relative to wild-type mice. These findings contrast with the results in the HS rats, in which decreased Tpcn2 expression levels are associated with increased, as opposed to decreased, fasting glucose and insulin levels. There are several possible reasons for this discrepancy. Because the mice were created on a mixed C57/129 background, we cannot rule out the possibility that alleles from the 129 strain, as opposed to the gene knockout, are causing the differences in phenotype. To minimize this effect, however, only siblings from heterozygous breeder pairs were tested. Another possibility is related to the fact that a full gene knockout can have a very different effect on a phenotype than natural allelic variation (Flint and Eskin 2012). Furthermore, several studies have shown that different variants within the same gene, or within the same GWAS location, can have opposite effects on a single phenotype (Cohen et al. 2005; Flister et al. 2013). To test the role specifically of the F344 variant on glucose and insulin regulation, quantitative complementation can be used (Yalcin et al. 2004b, 2010). Alternatively, if we are able to identify the causal variant(s) within this region, we can use genetic techniques to investigate the specific role of this (or these) variant(s) (Katter et al. 2013).

To determine whether Tpcn2 may be playing a role in human diabetes, we collaborated with investigators from the DIAGRAM and MAGIC consortiums. Tpcn2 has not previously reached significance levels in any of the published genome-wide scans (Morris et al. 2012; Scott et al. 2012). However, genome-wide thresholds are conservative because of the need to account for the multiple testing across the genome. In the present study, we were able to assess 14 of 39 SNPs within the Tpcn2 gene and find that two are marginally significantly associated with fasting insulin levels in humans. Although no significant associations were found between these SNPs and diabetes or fasting glucose levels, the majority of SNPs that we investigated were not represented within the MAGIC or DIAGRAM meta-analyses. A potential reason for this is that some of these SNPs may be rare variants and thus could not be properly imputed. It is therefore possible that rare variants within Tpcn2 may be associated with diabetes and related traits (see Steinthorsdottir et al. 2014; Zuk et al. 2014). The fact that variants within all three species alter diabetic phenotypes, however, is strong evidence that this gene plays a role in metabolic regulation.

This work demonstrates the power of highly recombinant animal models such as the HS for relative rapid identification of a candidate gene within a QTL. Despite relatively complex genetic architecture at the QTL, we were able to identify a highly plausible causal gene within only a few years. It is important to recognize, however, that we are not able to rule out any other genes within this QTL at this time, particularly the 29 genes that were not assessed on the

---

**Table 2**: Association between Tpcn2 SNPs and diabetic traits in humans

<table>
<thead>
<tr>
<th>SNP</th>
<th>Location</th>
<th>Fasting insulin</th>
<th>HOMA_IR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unadjusted P-value</td>
<td>BH adjusted P-value</td>
</tr>
<tr>
<td>rs11604251</td>
<td>chr11: 68,820,429</td>
<td>0.0073 (+)</td>
<td>0.0511</td>
</tr>
<tr>
<td>rs7127082</td>
<td>chr11: 68,822,023</td>
<td>0.02475 (-)</td>
<td>0.0937</td>
</tr>
<tr>
<td>rs753559</td>
<td>chr11: 68,851,605</td>
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<td>0.0937</td>
</tr>
<tr>
<td>rs10736671</td>
<td>chr11: 68,858,907</td>
<td>0.00398 (+)</td>
<td>0.0511</td>
</tr>
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</table>

Only the 4 most significant SNPs (of 14) are reported. Statistics on the additional SNPs are reported in Table S1. SNPs that remain marginally significant after adjusting for multiple comparisons using the Benjamini and Hochberg FDR method (BH) are in boldface type.
expression array. In fact, our mapping studies indicate that more than one gene likely underlies the insulin locus (Solberg Woods et al. 2012), and it is reasonable to expect that Tpcn2 may interact with one or more genes within this region. Of particular interest is Gpr152, a G-protein coupled receptor in which the F344 variant encodes a premature stop codon, thus likely affecting protein function. The variant within Gpr152 was also significantly associated with Tpcn2 expression levels, suggesting a potential interaction between these genes. Future studies will investigate a potential role for these other genes, as well as potential interactions between genes within this region.

In sum, we have identified Tpcn2 as a highly plausible causal gene within a QTL for both glucose and insulin traits. We have used expression and sequence analysis in a highly recombinant population to identify this gene in only a few years. A causal role for this gene is supported by alterations in both glucose and insulin in a Tpcn2 knockout mouse as well as data from clinical cohorts. Future work will identify the causative variant within this gene and begin to unravel the underlying mechanisms involved, as well as investigate a potential role for other genes in the region. The gene expression data, association analysis, and confirmation in both mice and humans demonstrate Tpcn2 as a new gene contributing to glucose and insulin regulation.

Acknowledgments

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Communicating editor: S. F. Chenoweth
Identification of a Novel Gene for Diabetic Traits in Rats, Mice, and Humans

Shirng-Wern Tsaih, Katie Holl, Shuang Jia, Mary Kaldunski, Michael Tschannen, Hong He, Jaime Wendt Andrae, Shun-Hua Li, Alex Stoddard, Andrew Wiederhold, John Parrington, Margarida Ruas da Silva, Antony Galione, James Meigs, Meta-Analyses of Glucose and Insulin-Related Traits Consortium (MAGIC) Investigators, Raymond G. Hoffmann, Pippa Simpson, Howard Jacob, Martin Hessner, and Leah C. Solberg Woods
Figure S1  PCR on RNA from multiple tissues in wild-type and knock-out Tpcn2 mice. Results confirm that the knock-out mice do not transcribe Tpcn2.
File S1

** List of MAGIC (Meta-Analysis of Glucose and Insulin-Related traits) Consortium investigators


Files S2-S5


File S2  Phenotype data used for the cluster analysis

File S3  HS genotype data for 18 SNPs within the 3.1 Mb QTL on rat chromosome 1

File S4  Quantitative rt-PCR data for Tpcn2 and GAPDH in 120 HS rats

File S5  Glucose and insulin levels during the IPGTT in Tpcn2 knock-out, heterozygous and wild-type mice
<table>
<thead>
<tr>
<th>SNP</th>
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<th>Functional location</th>
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39 human SNPs within or near Tpcn2 were used to determine association with diabetes and related traits. DIAGRAM assessed association between 11 SNPs (in italics) and diabetes while MAGIC assessed association between 14 SNPs (in bold) and fasting glucose, fasting insulin and the homeostatic model assessment of insulin resistance (HOMA_IR). Associations between 14 SNPs and fasting insulin and HOMA_IR, as assessed by MAGIC, are listed above. Multiple comparisons were adjusted for using the Benjamini and Hochberg FDR method (BH). No significant associations were found between the SNPs and diabetes or fasting glucose. Associations could not be determined for SNPs that are not in bold because of insufficient data. *p = 0.051 after adjusting for multiple corrections.
## Table S2  Phenotypic differences between glucose intolerant and normal glucose HS rats

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<thead>
<tr>
<th>Trait</th>
<th>Normal Glucose HS (mean ± SD)</th>
<th>Glucose Intolerant HS (mean ± SD)</th>
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<tr>
<td>Glucose_AUC</td>
<td>9061.0 ± 209.1</td>
<td>13,458.9 ± 1,035.5***</td>
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<tr>
<td>Insulin_AUC</td>
<td>204.3 ± 63.3</td>
<td>311.3 ± 106.7****</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>77.5 ± 6.6</td>
<td>90.3 ± 6.7***</td>
</tr>
<tr>
<td>Fasting insulin (ng/ml)</td>
<td>1.1 ± 0.5</td>
<td>1.8 ± 1.2**</td>
</tr>
<tr>
<td>Fasting c-peptide (pg/ml)</td>
<td>1505.8 ± 479.8</td>
<td>1808.0 ± 367.8*</td>
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<tr>
<td>Fasting cholesterol (mg/dL)</td>
<td>100.1 ± 46.8</td>
<td>125.5 ± 56.0</td>
</tr>
<tr>
<td>Fasting triglycerides (mg/dL)</td>
<td>72.3 ± 20.1</td>
<td>86.2 ± 12.2**</td>
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<tr>
<td>QUICKI (1/(log I0 + log G0))</td>
<td>0.54 ± 0.06</td>
<td>0.48 ± 0.08**</td>
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<tr>
<td>Body Weight (g)</td>
<td>314.4 ± 25.8</td>
<td>349.1 ± 33.5***</td>
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<tr>
<td>Retroperitoneal fat pad weight (g)</td>
<td>0.014 ± 0.004</td>
<td>0.014 ± 0.005</td>
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<tr>
<td>Epidydimal fat pad weight (g)</td>
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<td>0.016 ± 0.005</td>
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</table>

***p < 0.0001, **p < 0.01, *p < 0.05, see manuscript for full statistics
Tables S3-S6

Table S3  Differentially Expressed Genes between Glucose Intolerant and Normal Glucose HS rats (Genome-wide)

Table S4  Mean expression levels for all probes within the 3.1 Mb chromosome 1 region

Table S5  Significance of association (-\log_{10}P) between 18 SNPs within the 3.1 Mb QTL on rat chromosome 1 and all traits that map to this locus

Table S6  Correlations between probes within the chromosome 1 region and six diabetic traits
Table S7  Phenotypic differences in Tpcn2 knock-out, heterozygote and wild-type mice

<table>
<thead>
<tr>
<th>Trait</th>
<th>Wild-type (mean ± SD)</th>
<th>Heterozygotes (mean ± SD)</th>
<th>Knock-out (mean ± SD)</th>
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<tbody>
<tr>
<td>Glucose_AUC</td>
<td>22,917.5 ± 4,384.2</td>
<td>21,811.2 ± 1,609.2</td>
<td>20,700.0 ± 3,660.1</td>
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<tr>
<td>Insulin_AUC</td>
<td>165.4 ± 13.4</td>
<td>147.2 ± 35.8</td>
<td>126.2 ± 6.4**</td>
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<td>Fasting glucose (mg/dL)</td>
<td>111.0 ± 12.0</td>
<td>116.3 ± 15.5</td>
<td>96.2 ± 9.6*</td>
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<td>Fasting insulin (ng/ml)</td>
<td>1.04 ± 0.22</td>
<td>1.03 ± 0.51</td>
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<tr>
<td>QUICKI (1/(logI0 + logG0))</td>
<td>0.49 ± 0.02</td>
<td>0.49 ± 0.04</td>
<td>0.54 ± 0.04</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>25.3 ± 2.1</td>
<td>24.3 ± 2.4</td>
<td>27.0 ± 3.6</td>
</tr>
</tbody>
</table>

**p = 0.031 (wild-type mice differ from knock-out mice based on a Tukey-post-hoc based after an ANOVA between all three genotypes), *p = 0.048 (t-test between wild-type and knock-out mice only)