Positional Cloning of a Type 2 Diabetes Quantitative Trait Locus; Tomosyn-2, a Negative Regulator of Insulin Secretion

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Abstract

We previously mapped a type 2 diabetes (T2D) locus on chromosome 16 (Chr 16) in an F2 intercross from the BTBR T (+) tf (BTBR) Lepob/ob and C57BL/6 (B6) Lepob/ob mouse strains. Intergression of BTBR Chr 16 into B6 mice resulted in a consomic mouse with reduced fasting plasma insulin and elevated glucose levels. We derived a panel of sub-congenic mice and narrowed the diabetes susceptibility locus to a 1.6 Mb region. Intergression of this 1.6 Mb fragment of the BTBR Chr 16 into lean B6 mice (B6.16BT36–38) replicated the phenotypes of the consomic mice. Pancreatic islets from the B6.16BT36–38 mice were defective in the second phase of the insulin secretion, suggesting that the 1.6 Mb region encodes a regulator of insulin secretion. Within this region, syntaxin-binding protein 5-like (Sxbp5l) or tomosyn-2 was the only gene with an expression difference and a non-synonymous coding single nucleotide polymorphism (SNP) between the B6 and BTBR alleles. Overexpression of the b-tomosyn-2 isoform in the pancreatic β-cell line, INS1 (832/13), resulted in an inhibition of insulin secretion in response to 3 mM 8-bromo cAMP at 7 mM glucose. In vitro binding experiments showed that tomosyn-2 binds recombinant syntaxin-1A and syntaxin-4, key proteins that are involved in insulin secretion via formation of the SNARE complex. The B6 form of tomosyn-2 is more susceptible to proteasomal degradation than the BTBR form, establishing a functional role for the coding SNP in tomosyn-2. We conclude that tomosyn-2 is the major gene responsible for the T2D Chr 16 quantitative trait locus (QTL). Our findings suggest that tomosyn-2 is a key negative regulator of insulin secretion.

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Introduction

Genetic factors are estimated to contribute approximately 50% towards the risk of developing type 2 diabetes (T2D) [1]. Recent genome-wide association studies have identified a number of “diabetes genes”, gene loci that act in an additive manner and conspire with obesity to augment the risk of T2D. Nearly all of these genes affect β-cell function and/or the maintenance of β-cell mass. Thus, it appears that diet and obesity place demands on β-cells for insulin by causing insulin resistance, but the genetic bottleneck that can lead to T2D involves genes that affect the capacity of pancreatic β-cells to meet the increased insulin demand.

Mouse intercrosses provide high power to detect linkage of gene loci to physiological traits related to obesity and diabetes. The mapping resolution of these intercrosses is typically not fine enough to identify individual genes. However, by generating panels of congenic strains carrying meiotic recombinations within disease loci, it is possible to map the genes underlying those loci with high resolution. We recently reported the positional cloning of a T2D gene, Sucs1, to sub-genetic resolution using this approach [2]. Several other genes have been identified using a similar approach; Zfp69, which encodes a transcription factor involved in regulating glucose levels, was identified as the gene underlying a diabetes susceptibility locus on mouse chromosome 4 [3]. Similarly, Lish-like was identified as the gene underlying a T2D locus on chromosome 1 and was shown to be involved in regulating β-cell mass and plasma glucose levels [4].

Borrowing from microbial genetics, mouse genetic studies employ a powerful tool for increasing the sensitivity to detect heritable phenotypes. This involves sensitized screens wherein a severe stressor provokes phenotypes that would otherwise be silent. The stressor need not be a normal feature in human disease pathogenesis to evoke phenotypes of great relevance to disease. For example, the apoE-deficient mouse is the most widely used animal model of atherosclerosis even though apoE deficiency is
Author Summary

Humans carry many genetic variants that confer small effects on metabolic traits relevant to type 2 diabetes. These effects are amplified by environmental stressors like obesity. We used morbid obesity as a sensitizer to identify genes that contribute to the diabetes susceptibility of the BTBR mouse strain. Using mapping and breeding strategies, we were able to narrow a genetic region to one containing just 13 genes. One of these genes, tomosyn-2, emerged as a prime candidate. Our functional studies showed that tomosyn-2 is an inhibitor of insulin secretion, and it binds to the proteins involved in the fusion of insulin containing granules with the plasma membrane. We found a coding mutation and demonstrated that this mutation affects the stability of the protein product. Our work with Tomosyn-2 provides new insights into the regulation of insulin secretion and emphasizes that negative regulation is critical for avoiding insulin-induced hypoglycemia.

Results

Chr 16 consomic mice have increased fasting plasma glucose and reduced insulin levels

We previously identified a fasting plasma glucose locus on Chr 16 from a Lep<sup>ob/ob</sup> F2 intercross derived from the B6 and BTBR mouse strains [9]. This locus acts in a fully dominant fashion on plasma glucose and a semi-dominant fashion on fasting plasma insulin [9]. The LOD peak on Chr 16 of the fasting glucose locus from the F2 intercross is located at approximately 36–38 Mb (Figure 1A). To determine if the Chr 16 locus could act autonomously to affect T2D susceptibility, we derived a chromosome substitution (i.e. consomic) mouse strain by introgression of either a B6 or BTBR mouse chromosome 16 in the B6 Lep<sup>ob/ob</sup> mouse. The approximate QTL position was derived using a previously described method [9]. Fasting plasma glucose (B) and fasting plasma insulin (C) were measured after a 4 h fast in male B6.16<sup>B6</sup>B6 and B6.16<sup>BT</sup> Lep<sup>ob/ob</sup> mice. Values are means ± S.E. of N=6. * p<0.05 for B6.16<sup>BT</sup> Lep<sup>ob/ob</sup> mice vs. control B6.16<sup>B6</sup> mice at each time point. doi:10.1371/journal.pgen.1002323.g001

Congenic mice with a 1.6 Mb fragment of the BTBR Chr 16 have an insulin secretion defect

To assess whether the B6.16<sup>BT</sup> Lep<sup>ob/ob</sup> mice have a defect in insulin secretion, we isolated pancreatic islets from 10-week old B6.16<sup>BT</sup> Lep<sup>ob/ob</sup> mice and measured fractional insulin secretion in response to high glucose (16.7 mM). We observed a ~50% reduction in fractional insulin secretion in the B6.16<sup>BT</sup> Lep<sup>ob/ob</sup> islets relative to control mice (B6.16<sup>B6</sup> Lep<sup>ob/ob</sup> (Figure 2, left graph). To avoid the metabolic complexities that are attributed to the leptin mutation in the Lep<sup>ob/ob</sup> mice [10], we performed experiments in lean mice. Islets isolated from the congenic
B6.16BT and B6.16B6 lean mice were treated with 8-bromo cAMP (3 mM) at sub-maximal glucose (11.1 mM); this combination of secretagogues was used for phenotyping lean congenic mice because it evoked more insulin secretion from lean islets than glucose alone. We observed ~40% reduction in fractional insulin secretion in islets isolated from the lean B6.16BT mice relative to the lean control B6.16B6 mice (Figure 2, right graph). The data show that the insulin secretion defect, although initially mapped in a screen of F2 mice sensitized by the Lepob mutation, manifests itself independent of leptin deficiency.

To investigate the region of the BTBR Chr 16 that confers the insulin secretion defect, a panel of lean congenic mouse strains was generated from the B6.16BT mice, each containing a small introgressed region from the BTBR Chr 16 in the B6 background (Figure 3, left panel). The B6/BTBR boundaries for each congenic strain were determined via microsatellite marker, single nucleotide polymorphism (SNP) sequencing or deletion/insertion polymorphism (DIF) sequencing (Dataset S1). By phenotyping each strain, we were able to fine-map the location of the gene responsible for the insulin secretion defect.

Islets were isolated from each lean congenic mouse strain and fractional insulin secretion was determined in the presence of 3 mM 8-bromo cAMP at sub-maximal 11 mM glucose. The islets isolated from the lean congenic mice that carry the 2 Mb Chr 16 derived from the BTBR strain (B6.16BT36–38) were defective in insulin secretion when compared to islets isolated from congenic mice B6.16B6, B6.16BT37–55, or B6.16BT24–37 (Figure 3, right panel). The overlapping region of BTBR Chr 16 represented by congenic strains, B6.16 BT24–37 and B6.16 BT37–55 enabled us to further narrow the region to 1.6 Mb. Islets isolated from congenic mice, B6.16BT24–38, B6.16BT24–47, or B6.16BT24–37, which contained the 1.6 Mb region derived from the BTBR strain, also displayed a reduced level of insulin secretion, indicating that this 1.6 Mb region contains a gene responsible for regulating insulin secretion.

Reduced insulin secretion in islets from lean B6.16BT36–38 mice

Insulin secretion from pancreatic β-cells is biphasic. The first phase represents a brief but rapid secretion from pre-docked insulin granules in response to an initial depolarization of the plasma membrane. Non-nutrient secretagogues like KCl predominantly invoke the first phase of insulin secretion. The second phase of insulin secretion is associated with metabolic signals derived from the metabolism of fuel-based insulin secretagogues like glucose. Glucose affects both the first and second phase of insulin secretion. Briefly, glucose oxidation increases the ATP/ADP ratio, resulting in the closure of ATP-sensitive K+ channels. This causes depolarization of the plasma membrane and influx of Ca2+ via L-type voltage-dependent calcium channels. Glucose promotes the second phase of insulin secretion without causing a further increase in intracellular Ca2+ levels. Diazoxide

Figure 3. Effect on insulin secretion of introgressing 1.6 Mb of BTBR Chr 16 into B6 mice. Left panel: Congenic mice were generated by introgression of varying fragments of the BTBR chromosome 16 into the B6 mice. The B6/BTBR boundaries for each congenic strain were determined via microsatellite marker, SNP sequencing or DIF sequencing and are listed in Datasets S1 and S3. Overlying the congenic diagram is the position of the fasting glucose QTL derived from a (B6 x BTBR) F2 intercross. Right panel: Fractional insulin secretion (% of islet insulin content) from the islets of the 10-week lean congenic mice. Isolated islets were incubated for 45 min in KRB-based buffer containing low glucose (1.7 mM). After 45 min, the islets were exposed to 8-bromo cAMP (3 mM) at sub-maximal glucose (11.1 mM) for another 45 min. Values are mean ± S.E. of N=3. *p<0.05 for fractional insulin secretion from islets of congenic mice that contains the 1.6 Mb fragment of the BTBR chromosome 16 mice vs. congenic mice carrying this 1.6 Mb fragment derived from the B6 strain.

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Figure 4. Plasma insulin and glucose levels in the B6.16BT36–38 male mice. Random-fed plasma insulin (A and B) and glucose (C and D) were measured in 10-week Lepobob/ob and lean B6.16B6 and B6.16BT36–38 male mice. Values are means ± S.E. of N=10. *p<0.05 for plasma glucose and insulin levels in B6.16BT36–38 mice vs. control B6.16B6 mice.

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The 1.6 Mb region of the BTBR Chr 16 is sufficient to increase plasma glucose levels

We next determined the effect of introgression of the BT36–38 region of Chr 16 into B6 mice on susceptibility to obesity-induced diabetes. Plasma insulin and glucose levels were determined in random-fed 10-week B6.16BT36–38 and control B6.16B6 Lepobob/ob congenic mice. We observed a ~40% reduction in plasma insulin levels in the B6.16BT36–38 Lepobob/ob compared to B6.16B6 Lepobob/ob mice (Figure 4A). The reduction in plasma insulin was accompanied by an increase in plasma glucose by ~100 mg/dl (Figure 4C).

Although not nearly as dramatic as in the Lepobob/ob mice, lean congenic mice with the 36–38 Mb BTBR insert had a significant reduction in plasma insulin and increase in plasma glucose (Figure 4B and 4D, respectively). Detection of this very small rise in plasma glucose required a very large sample size (n = 80) to achieve statistical significance. Clearly, we would not have found this modest phenotype in a screen of lean mice, showing that severe stressors like the Lepob mutation are required to identify subtle allelic variation in QTLs that contribute to T2D risk.
inhibits closure of the K⁺ channels, therefore adding this drug along with 16.7 mM glucose will result only in the glucose-induced second phase of insulin secretion and eliminates any glucose induction of the first phase of insulin secretion [11–13].

To determine which phase of insulin secretion is defective in our lean congenic B6.16BT36–38 mice, we carried out perfusion studies of isolated islets. Islets were perfused in Krebs-Henseleit Ringer bicarbonate (KRB) buffer at the rate of 1 ml/min. The perfusate was sampled every 30 sec, and the secreted insulin was measured by ELISA. After an initial 60 min equilibration period in KRB containing 1.7 mM glucose, islets were perfused for 10 min in KRB containing 40 mM KCl and 250 μM diazoxide to elicit first phase of insulin secretion. After 10 min, the islets were perfused for an additional 30 min in KRB containing 16.7 mM glucose with 40 mM KCl and 250 μM diazoxide to evoke the second phase of insulin secretion. The peak of the first phase of insulin secretion from B6.16B6 islets was observed within 1–2 min of KCl treatment. Following the first peak, the more sustained second phase of insulin secretion was observed for an additional 30 min, mimicking the well-studied biphasic kinetics of insulin secretion [14].

Islets from B6.16BT36–38 lean mice secreted ~40% less insulin during the second phase than islets from the B6.16 B6 mice, as determined by calculating the area under the curve (AUC) (Figure 5A, 5B). There was also a small, but statistically significant reduction of first-phase insulin secretion (p = 0.044) (Figure 5A, 5B).

To complement the perfusion experiments, static insulin secretion experiments were performed in islets isolated from the B6.16BT36–38 and control B6.16B6 lean mice. Isolated islets were incubated for 45 min in KRB containing 1.7 mM glucose. Following a 45-min incubation, the islets were treated with 40 mM KCl in KRB containing 1.7 mM glucose. No difference in fractional insulin secretion was observed in response to KCl (Figure 6C, left panel). However, we observed a significant decrease in fractional insulin secretion between islets from the B6.16BT36–38 lean mice and those from the control B6.16B6 lean mice in response to 15 mM arginine, 3 mM 8-bromo cAMP in KRB containing 11 mM glucose, and 16.7 mM glucose alone (Figure 5C, middle and right panels).

**Sequencing and alignment of overlapping congenic mouse strains narrowed the Chr 16 locus to 0.94 Mb**

To further narrow the 1.6 Mb region of BTBR Chr 16 responsible for the phenotype, we used Agilent’s SureSelect Target Enrichment to capture DNA from 35.55 Mb to 38.65 Mb on mouse Chr 16. 55,336 RNA baits were designed using the Agilent eArray and were used to enrich for our region from tail DNA of the B6, BTBR, B6.16BT36–38, B6.16BT24–37 and B6.16BT24–38 mice. DNA was sequenced by Next Generation Sequencing using an Illumina GA IIx sequencer at the UW-Madison Biotechnology Center. Using CLC Genomics 4.0.3 Software, we were able to identify 470 SNPs; 3 non-synonymous coding SNPs and 46 DIPs between the B6 and BTBR DNA (Figure 6A). 83 SNPs and 8 DIPs were further confirmed by manual base reading to confirm the accuracy of the software (listed in Dataset S2). Using this sequence and known overlapping regions derived from the BTBR strain in the sub-congenic strains exhibiting normal insulin secretion (B6.16BT24–37, B6.16BT36–38, B6.16BT24–38), we were able to narrow the region responsible for the insulin secretion defect to 0.94 Mb containing 13 genes (Figure 6B).

To identify the gene(s) responsible for the insulin secretion defect, each candidate gene in the 0.94 Mb region was scored for the difference in mRNA abundance between the islets B6.16B6 and B6.16BT36–38 islets, the presence of non-synonymous coding SNPs, and similarity to a protein that have a functional role in exocytosis. Tomosyn-2 or Stxbp5l (syntaxin binding protein 5-like) quickly emerged as the top candidate gene. The mRNA abundance of tomosyn-2 was elevated 2.6 fold in the B6.16BT36–38 lean mice compared to control B6.16B6 mice (Figure 7A). Tomosyn-2 has a coding SNP (Ser-912→Leu). Eight other SNPs were also identified in the introns and additional SNPs were identified in the intergenic regions 5’ and 3’ of the gene (Dataset S1, Table S1). The tomosyn-2 protein shares 95% identity in the C-terminal
soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor (SNARE) domain with several syntaxin-binding proteins. Finally, a related protein, tomosyn-1, has been shown to inhibit insulin secretion [15].

Increased expression of tomosyn-2 in islets of the B6.16BT36–38 lean mice

To understand the role of tomosyn-2 in the regulation of insulin secretion, the expression of tomosyn-2 was determined in key metabolic tissues; islet, liver, brain, cerebellum, kidney, adrenal, adipose (perigonadal), heart, skeletal muscle (gastrocnemius, soleus, and quadriceps) of the lean B6.16BT36–38 and B6.16B6 mice. We found that the b-tomosyn-2 isoform is the most abundant isoform in mouse islets. This was confirmed by RT-PCR with a primer pair that simultaneously amplified all of the tomosyn-2 isoforms (data not shown). The relative expression of b-tomosyn-2 and s-tomosyn-2 mRNA was ~6-fold higher in islets of the lean B6.16BT36–38 mice than in lean B6.16B6 mice (Figure 7B). We observed no significant difference between the two-congenic mouse strains in the expression of xb- and m-tomosyn-2 isoforms.

Together, the data indicate that increased expression of tomosyn-2 may be responsible for the insulin secretion defect observed in the lean B6.16BT36–38 mice.
Tomosyn-2 inhibits insulin secretion in pancreatic β-cells

To investigate the role of tomosyn-2 in insulin secretion, we investigated the effect of overexpressing β-tomosyn-2 in the pancreatic β-cell line, INS1 (832/13). The cells were transfected with GFP or β-tomosyn-2 expression plasmids. After 36 h, the cells were incubated in KRB containing 1.5 mM glucose for 2 h. Following the low glucose incubation, the cells were incubated for additional 10 min or 2 h in 3 mM 8-bromo cAMP at 7 mM glucose. Overexpressing β-tomosyn-2 decreased insulin secretion by ~40% vs. GFP expressing cells at both 10 min and 2 h (Figure 8A). No inhibition in fractional insulin secretion was observed at low glucose (1.5 mM) (data not shown).

To determine if β-tomosyn-2 binds to syntaxin-1A and syntaxin-4, key t-SNARE proteins involved in the fusion of insulin granules to the plasma membrane, in vitro binding experiments were performed using GST fused syntaxin-1A and syntaxin-4 recombinant proteins (soluble, lacking transmembrane domains) by pull-down assays using glutathione beads. All isoforms of tomosyn-2 bound to GST-syntaxin-1A and GST-syntaxin-4 (Figure 8B). The quantification for the amount of bound tomosyn-2 isoforms as a fraction of total is shown in Figure 8C. Tomosyn-1 was used as a positive control for binding. The GST tag did not pull down tomosyn-1 or tomosyn-2, confirming that the interaction between tomosyn-2 and syntaxin-1A and -4 is specific. Together, these data suggest that the mechanism by which β-tomosyn-2 inhibits insulin secretion involves binding to the syntaxin proteins. This suggests the possibility that tomosyn-2, like tomosyn-1, inhibits insulin secretion by preventing the binding of VAMP2 to syntaxin-1A and syntaxin-4.

The B6 allelic product of β-tomosyn-2 is susceptible to proteasomal degradation

We have shown that tomosyn-2 is a negative regulator of insulin secretion and also binds to syntaxin-1A and syntaxin-4. To investigate the possibility that the serine-912leucine SNP in tomosyn-2 affects its stability, HEK293T cells were transfected with empty vector (mock), β-tomosyn-2 (Serine-912), or β-tomosyn-2 (Leucine-912). After 16 h, the cells were treated with or without the proteasomal inhibitor, MG132 (100 μM) for 6 h. The MG132 treatment rescued the B6 allelic form of the protein, β-tomosyn-2 (serine-912), from proteasomal degradation by ~50% (Figure 9A and 9B). However, the BTBR allelic form of the protein, β-tomosyn-2 (leucine-912) was not resistant to MG132 treatment, suggesting that an increased stability of the tomosyn-2 protein might be responsible for the attenuation in insulin secretion from islets of the BTBR mice.

Discussion

Our pursuit of genes conferring susceptibility to obesity-induced T2D focuses on two mouse strains that differ in diabetes susceptibility. BTBR mice, when made obese with the Leptin<sup>−/−</sup> mutation, are susceptible to T2D, whereas B6 mice with the same mutation are relatively diabetes resistant [16,17]. The diabetes susceptibility of the obese BTBR mice has multiple causes, including an insulin secretion defect and a failure to increase β-cell mass. As early as 4 weeks of age, islets from the obese B6, but not BTBR mice have an increase in expression of a module of cell cycle genes whereas the obese BTBR mice fail to induce the expression of this module [16].

Through genetic mapping in an F2 intercross, we identified a strong QTL on Chr 16 wherein the BTBR allele is linked to increased glucose levels. In complex trait genetics, it is often the case that gene loci do not act autonomously, but must act along with specific alleles at other loci to exert their phenotypic effects [18–20]. To determine if the Chr 16 locus acts autonomously, we derived a chromosome substitution strain. Substitution of Chr 16 in the B6 strain with Chr 16 from the BTBR strain led to a ~100 mg/dl increase in glucose and a ~50% decrease in plasma insulin. This established that a locus on Chr 16 acts autonomously and is sufficient to account for a major part of the diabetes phenotype of the BTBR mouse strain.

QTL mapping in an F2 intercross does not provide the resolution required to identify individual genes. To narrow the interval, we derived a panel of interval-specific congenic strains. The strains were phenotyped on the basis of insulin secretion from isolated islets, a far more robust phenotype than fasting glucose or insulin levels. This enabled us to narrow the position of the QTL to <1 Mb, containing just thirteen genes. Of these thirteen genes, tomosyn-2 was the only gene that had both altered mRNA abundance and a coding SNP (S912L).

Recent studies by Williams et al. show that tomosyn-2 inhibits exocytosis in PC12 cells [21]. Our experiments establish a role for tomosyn-2 in insulin secretion. When we overexpressed tomosyn-2 in the pancreatic β-cell line INS1 (832/13), insulin secretion in response to 8-bromo cAMP at sub-maximal glucose was attenuated. In vitro GST-pull-down experiments showed that tomosyn-2 has the ability to bind t-SNARE proteins, syntaxin-1A and syntaxin-4. Syntaxin-1A is involved in the first phase and syntaxin-4 is involved in regulating both the first and second phase of insulin secretion [22]. These results establish that tomosyn-2,
like its homologue, tomosyn-1, inhibits insulin secretion [23]. The fact that allelic variation in tomosyn-2 is sufficient to produce this phenotype suggests that tomosyn-1 cannot compensate for this deficiency, implying that their functions may not completely overlap.

The mRNA abundance of tomosyn-2 was increased in the congenic mouse strains expressing the BTBR allele. It is difficult to determine if this difference in expression level is sufficient to produce the difference in insulin secretion in islets of the two mouse strains because it would require accurately titrating the gene dosage (and the amount of protein product) in a null background.

Sequence analysis revealed a SNP in tomosyn-2 (S912L). We utilized a recent study showing that the proteasome inhibitor MG132 increases the abundance of tomosyn-2 [21]. We found that the S912L SNP abolishes the ability of MG132 to rescue tomosyn-2 from proteasomal degradation, thus establishing a functional role for the S912L SNP. Therefore, the decreased insulin secretion associated with the BTBR allele might be the result of increased stability of the tomosyn-2 protein as a consequence of the SNP at amino acid 912. We also identified SNPs in the introns and the intergenic regions 5' and 3' of the tomosyn-2 gene (Figure 7). The intronic SNPs may regulate the stability of the tomosyn-2 mRNA and the intergenic SNPs might affect the level of transcription of the gene.

To investigate the role of the S912L SNP in pancreatic islets, we conducted perifusion experiments. Our perifusion experiments demonstrated that islets from the B6.16BT36-38 congenic mice were defective in the 2nd phase of insulin secretion. Our results suggest that tomosyn-2 is likely to be responsive to metabolic signals. With static incubation experiments, we tested various insulin secreting agents. Islets with the BTBR allele of tomosyn-2 were clearly less responsive to cAMP or arginine at sub-maximal glucose. These secretagogues are involved in both phases of insulin secretion, but the exact mechanisms by which they stimulate the 2nd phase of insulin secretion are not fully understood. The involvement of tomosyn-2 provides a plausible new target for the actions of these secretagogues.

We show that tomosyn-2, similar to tomosyn-1, binds to syntaxin-1A and -4 [24–27]. Recent studies suggest that the binding to syntaxin is necessary but not sufficient for tomosyn-2’s inhibition of insulin secretion [21].

Our studies suggest that tomosyn-2 imposes a critical brake on insulin secretion. This is particularly important during fasting when inappropriate insulin secretion could cause life-threatening hypoglycemia. We hypothesize that under fasting conditions when
glucose levels are low, tomosyn-2 blocks exocytosis and prevents hypoglycemia.

In mice, the two tomosyn genes, tomosyn-1 and tomosyn-2, encode seven alternatively spliced variants [23]. Tomosyn-1 contains three distinct isoforms (s, m, and b), whereas tomosyn-2 has four different spliced variants (s, m, b, and x). The spliced exons encode the hypervariable region (HVR), which in tomosyn-1 has been shown to be subject to SUMOylation and PKA-mediated phosphorylation [21,28]. The amino acid sequences of tomosyn-1 and tomosyn-2 are quite similar in the N-terminal WD40 repeats and C-terminal VAMP-like domain (VLD) [23]. Tomosyn-1 was identified in neurons as a syntaxin-1-binding protein that sequesters t-SNAREs on the plasma membrane by forming a “dead end”, nonfusogenic SNARE complex, resulting in inhibition of the formation of the SNARE complex [24,26]. Deletion of the tomosyn-1 gene in C. elegans or in mice resulted in enhanced asynchronous neurotransmitter release [29,30]. Gain of function studies demonstrated that tomosyn-1 is responsible for inhibiting exocytosis of dense core granules in primary adrenal chromaffin cells [27], PC12 cells [23], and pancreatic β-cells [15]. Moreover, in vitro biochemical evidence further supports the conclusion that tomosyn-1 inhibits the formation of the SNARE complex [31,32].

The Ca^2+ -independent inhibitory effects of the tomosyn-1 have been attributed to the VLD. More recently, Yamamoto et al demonstrated that in the presence of Ca^{2+}, tomosyn-1, via the N-terminal WD40 domain, binds to synaptotagmin and inhibits SNARE complex-mediated neurotransmitter release [26,30,33,34]. Together, the evidence is accumulating for tomosyn-1 as a negative regulator of exocytosis in both the stimulated and unstimulated states.

Insulin resistant animals compensate for their insulin resistance and maintain normal glucose levels by increasing insulin secretion. Our studies show that mutations in tomosyn-2 that increase its inhibitory activity can create a bottleneck and in the presence of obesity-induced insulin resistance, tip the balance towards T2D. However, it is also possible that tomosyn-2 plays an important role in regulating insulin secretion during daily starvation cycles by preventing inappropriate insulin secretion during fasting.

Tomosyn-2 may regulate exocytosis by modulating the formation of the SNARE complex in tissues other than islets. We observed significant tomosyn-2 expression in brain, cerebellum, islets, kidney, liver, and gastrocnemius (Figure 7). Therefore it is possible that tomosyn-2, like tomosyn-1, may have an important regulatory role in tissues where regulation of the SNARE complex can be limiting for an important transport process; e.g. insulin-mediated GLUT4 translocation in adipocytes [35] and transport of LDL-derived cholesterol from the trans-Golgi network to the endoplasmic reticulum in hepatocytes [36]. Thus, this tomosyn-2 could be playing a critical role in regulating vesicle trafficking in other tissues.

In summary, we have identified tomosyn-2 as a gene underlying a T2D susceptibility QTL on Chr 16. We show that tomosyn-2 is a negative regulator of insulin secretion. We identified a SNP in tomosyn-2 that affects the stability of the protein and thus suggest a molecular mechanism by which allelic variation in this gene increases diabetes susceptibility. Future studies will focus on the pathways that link nutrient sensing with the role of tomosyn-2 in the regulation of insulin secretion.

Materials and Methods

Materials

The enzymatic glucose reagent was purchased from Thermo Scientific. Insulin in lean mice was measured using a radio-immunooassay kit from Linco Research (St. Charles, MO). In Left^+/ob mice, insulin was measured with an in-house ELISA using an anti-insulin antibody from Fitzgerald Industries (Acton, MA). The mouse anti-myc antibody and Z-Leu-Leu-Leu-al (MG132) were purchased from Sigma-Aldrich, USA. The mouse secondary antibodies were purchased from Cell Signaling Technology (Boston, MA). Glutathione 4B Sepharose beads were purchased from GE Healthcare, USA.

Animals and breeding strategy

The C57BL/6 (B6) and BTBR T^+ tf (BTBR) mice were intercrossed and were crossed to generate F1 mice. The B6.16^B6 and B6.16^BT mice were created by backcrossing the F1 mice to B6 mice using microsatellite markers to select for BTBR (or B6 in the case of B6.16^B6) homozygosity on mouse chromosome 16 in an otherwise B6 background. The B6.16^BT mice were further backcrossed to B6 with marker assisted selection to create congenic strains. Further identification of the B6/BTBR genetic boundaries were determined by SNP sequencing for some of the congenic strains (listed in Dataset S2). The Left^+ mutation was introgressed into all strains using Left^+/+ mice as breeders [37]. All mice were maintained at the Department of Biochemistry, University of Wisconsin-Madison animal care facility on a 12 h dark-light cycle (6 PM to 6 AM). The mice were fed Purina Formulab Chow 5008 and water ad libitum.
The mice were kept in accordance with the University of Wisconsin-Madison Research Animal Resource Center and the NIH guidelines for care and use of laboratory animals.

Plasma measurements

For plasma glucose and insulin measurements, blood was taken from the retro orbital sinus from random fed mice at 8 AM or from fasting mice at 12 PM (fasted at 8 AM). For both Lep<sup>ob/ob</sup> and lean mice, glucose was measured via glucose oxidase method (Thermo Scientific). For Lep<sup>ob/ob</sup> mice, insulin was measured via ELISA using a matched rat insulin antibody pair ( Fitzgerald Industries International Inc.). For lean mice, insulin was measured by Linco Sensitive rat insulin radioimmunoassay.

Islet isolation and insulin secretion assays

Intact pancreatic islets were isolated from mice using a collagenase digestion procedure [38]. Static insulin secretion assays were performed on preparations consisting of three islets incubated with various secretagogues [38]. For perfusion insulin secretion assays, approximately 100 medium size islets were washed three times, placed in a sterile Petri dish, and incubated overnight in culture media (RPMI 1640, with 11.1mM glucose, antibiotics and 10% heat inactivated fetal bovine serum). The following day, 50 islets were washed and transferred in 100 µl of Krebs Ringer Buffer (KRB) to the Swinnex filter holder (Millipore). The islets were sandwiched between two layers of Bio-Gel P-2 bead (Bio-Rad) solution (200 mg beads/ml in KRB; Millipore). The islets were perifused at the holder was attached in-line with a Minipuls 3 pump (Gilson) and a FC 204 Fraction Collector (Gilson). Islets were perfused at the rate of 1ml/min and samples were collected at 30 sec intervals. Ilet insulin content and secretion were determined by ELISA.

Next generation sequencing

Tail DNA was extracted from B6, BTBR, B6.16<sup>BTm3J-TcR2R<BR></sup> and B6.16<sup>Bt124<BR></sup> mice using the QIAGEN Puregene Core Kit. RNA bait was designed using Agilent eArray and used for Agilent SureSelect Target Enrichment to capture sequence from a 10-50 Mb region on mouse chromosome 16. Target enrichment was followed by DNA amplification and confirmation of enrichment using SNP sequencing inside and outside of the target region. DNA was sequenced by Next Generation Sequencing using an Illumina GA IIx sequencer at the University of Wisconsin-Madison Biotechnology Center. CLC Genomics 4.0.3 Software was used to identify SNPs and DIPs between B6 and BTBR sequence. For some SNPs and DIPs an accuracy of the software (listed in Dataset S1).

Isolation and quantitation of total RNA

RNA from islets, kidney, and liver was extracted using the QIAGEN RNasy Plus Kit. RNA from epididymal fat pads, brain, cerebellum, and adrenal glands was extracted using QIAGEN RNasy Lipid Kit. RNA from heart, soleus, gastrocnemius and quadriceps was extracted using QIAGEN RNasy Fibrous Tissue Kit. Following extraction, RNA was used for cDNA synthesis (Applied Biosystems). The mRNA abundance was determined by quantitative PCR using FastStart SYBR Green (Roche) and gene expression was represented by comparative CT method.

Plasmids

MMLV-based retroviral vector (RVV, 3051) [gift from Dr. Bill Sugden, University of Wisconsin, Madison] containing a MCS-ires GFP was used to generate b-tomosyn-2-RVV construct for expression studies. The pcDNA3-m-tomosyn-1, pCR-Script-xb, -b, -m, and s-tomosyn-2 constructs were generously provided by Dr. Alexander Groffen, Virje Universiteir, Netherlands. We corrected a mutation (AG) at nucleotide 3245 of the b-Tomosyn-2 cDNA. The tomosyn-1 or tomosyn-2 cDNA from these vectors were used for subsequent subcloning. The b-tomosyn-2-RVV construct was generated by subcloning the b-tomosyn-2 cDNA with 5'-BspDI and 3'-NotI overhangs into the compatible 5'-BstBI and 3'-NotI ends of the RVV vector. For binding studies, the tomosyn-2-pcDNA/TO/myc-His was generated by subcloning a PCR-amplified tomosyn-2 cDNA in to 5'-BamHI and 3'-Xhol sites of the pcDNA4/TO/myc-His C vector (Invitrogen). The primers that were used to amplify tomosyn-2 cDNA with the restriction sites for cloning, a partial KOZAK, and a 3'-precision protease cleavage site are: forward (5'-TTAAGGATCCGCCAC- CACGAAAGAAGTTTAATTTCCG) and reverse (5'-ATATCCTG- GAGGCCCCCTTCTACCCGGACTTGGTAC-CACCTTTTATCCT) (Invitrogen). Similar subcloning strategy was used for generating m-tomosyn-1-pcDNA/TO/myc-His construct. The primers were used as follows: forward (5'-CGAGAGCCGATCCGCCCAC- CACCATGAGAAGTTTAATTTCCG) and reverse (5'-ATATCCTGAGGCCCCCTTCTACCCGGACTTGGTAC-CACCTTTTATCCTTTG) primes. The pGEX-4T1-syntxin-4 construct encoding soluble GST-syntxin-4 (1-273) fusion protein was generated as previously described [39]. The pGEX-2T1-syntxin construct (1-265) was a generous gift from Dr. Tom Martin, University of Wisconsin, Madison. All constructs were verified by sequencing.

Cell culture, transient transfection, and insulin secretion measurements

The glucose responsive rat b-cell line, INS1 (832/13, a gift from Dr. Chris Newgard, Duke University) was cultured in RPMI 1640 medium containing 11 mM glucose supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 100 Units/ml of antibiotic-antimycotic, and 50 µM β-mercaptoethanol. Approximately 100,000 cells/well were plated in a 96-well plate. The following day, INS1 (832/13) cells at 80-90% confluency were transfected with 0.4 µg of plasmid DNA using Lipofectamine 2000 (Invitrogen). After 36 h of incubation, cells were washed once with 200 µl and incubated for 2 h in 100 µl of modified Krebs-Henseleit Ringer bicarbonate buffer (KRB: 118.41 mM NaCl, 4.69 mM KCl, 1.18 mM NaHCO<sub>3</sub>, 1.85 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 20 mM HEPES, 2.52 mM CaCl<sub>2</sub>, pH 7.4, and 0.2% BSA) containing 1.5 mM glucose. After 2 h, cells were stimulated for 2 h in 100 µl of KRB buffer containing 7 mM glucose + 3 mM 8-bromo-cAMP. The incubation buffer was collected to determine the amount of insulin secreted under varying conditions. The cells were lysed (lysis buffer: 1 M Tris-HCl, pH 8.0, 1 M NaCl, 0.5 M NaF, 200 mM Na<sub>2</sub>VO<sub>4</sub> 2% NP-40, and protease inhibitor cocktail tablet (Roche)) to determine insulin content. The percent fractional insulin secretion was calculated as the amount of insulin secreted divided by total insulin content. Insulin was determined using ELISA.

The human embryonic kidney 293T cells (HEK293T) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM glucose were supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acid, 6 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml of penicillin, 100 units/ml of streptomycin, and 500 µg/ml of geneticin. HEK293T cells at 70-80% in 100 mm tissue culture dishes were transfect with plasmid DNA constructs using 40 µl of 1 µg/ml
polypehyleneimine. Following day, cells were lysed (lysis buffer: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 mM PMSF, and protease inhibitor cocktail tablet (Roche)) and total protein lysates were prepared via sonication. Western blotting was performed as described [40]. For protein stability, 16 h post transfection, cells were treated with or without 100 μM MG132 for 6 h. After 6 h, cells were lysed and whole cell lysates were prepared.

Recombinant proteins, in vitro binding assays, and immunoblotting
Recombinant proteins encoding GST or GST-fusion proteins with the cytoplasmic domain of syntaxin-1A and syntaxin-4 were expressed in E.coli strain BL21 (DE3) and were purified using glutathion-affinity chromatography [41]. The concentration and the purity of the fusion proteins were assessed by SDS-PAGE followed by Coomassie-blue staining against BSA standards. The binding studies were performed by incubating 10 μg of recombinant proteins with 25 μl of 100% Glutathione-Sepharose 4B beads (Amersham Biosciences) with 1 mg of HEK293T lystate over-expressing tomosyn-1 or isoforms of tomosyn-2 (xb, b, m, and s) in 1% Triton-lysix buffer for 2 h at 4°C. After 2 h, the complexes were washed three times with Triton lysis buffer and was eluted in Western loading buffer. The denatured samples were subjected to 10% SDS-PAGE gels followed by transfer to PVDF membrane for immunoblotting. The immunoblotting was performed using a standard protocol [40].

Statistical analysis
Data was expressed as means ± standard error of means. The statistical comparisons were made using Student’s t test at p<0.05.

Supporting Information

**Dataset S1** SNPs and DIPs in QTL Region. Single nucleotide polymorphisms and deletion/insertion polymorphisms (DIPs) in the 36–38.5 Mb region underlying the chromosome 16 QTL were determined by Next Generation sequencing. Using the CLC Genomics 4.0.3 software, for each strain (B6, BTBR, B6.16BT24–36, B6.16BT24–37, and B6.16BT24–38), an algorithm generated a list of SNPs/DIPs with our criteria. Listed are the base calls for the SNPs/DIPs for both B6 and BTBR. This will be an overestimate of actual SNPs/DIPs with our criteria. Listed are the bp boundaries of the missing B6 and BTBR sequence.

**Dataset S2** Tomosyn-2 SNPs. Listed are those SNPs/DIPs that were located upstream and downstream of the tomosyn-2 gene. We identified 1 non-synonymous coding SNP and 8 intronic SNPs in tomosyn-2.

**Dataset S3** SNP map for chromosome 16. Listed are the microsatellite markers, SNPs and DIPs used to identify the chromosome 16 B6/BTBR boundaries of our congenic mouse strains. Only those with a B6 or BT call were tested at each marker, SNP or DIP. The rest of the region was inferred from flanking markers.

**Dataset S4** Gap data. Within our 36–38.5 Mb region on chromosome 16 there were 30531 bp of gap sequence (representing ~ 1.2% of the region) where there was not sufficient sequence for both B6 and BTBR. This will be an overestimate of actual gaps, due to the fact that the sequence from other strains compared to either B6 or BTBR could still be used to determine SNPs/DIPs with our criteria. Listed are the bp boundaries of the missing B6 and BTBR sequence.

**Table S1** Strain distribution of S912L SNP. Non-synonymous coding SNPs identified within Stxbp5l from mouse strains sequenced at the Sanger Institute. Reference sequence corresponds to C57BL/6NJ. - indicates no change. Data obtained from http://www.sanger.ac.uk/cgi-bin/modelorgs/mousegenomes/snps.pl (DOCX)

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**Author Contributions**
Conceived and designed the experiments: SB ATO MER ADA BSY SPS. Performed the experiments: SB ATO MER DSS KLS NAT. Analyzed the data: SB ATO MER ADA BSY SPS. Contributed reagents/materials/analysis tools: DCT SB ATO MER DSS SLW. Wrote the paper: SB ATO ADA.
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