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Exocytosis proteins as novel targets for diabetes prevention and/or remediation?

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Abstract

Diabetes remains one of the leading causes of morbidity and mortality worldwide, affecting an estimated 422 million adults. In the US, it is predicted that one in every three children born as of 2000 will suffer from diabetes in their lifetime. Type 2 diabetes results from combinatorial defects in pancreatic β-cell glucose-stimulated insulin secretion and in peripheral glucose uptake. Both processes, insulin secretion and glucose uptake, are mediated by exocytosis proteins, SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complexes, Sec1/Munc18 (SM), and double C2-domain protein B (DOC2B). Increasing evidence links deficiencies in these exocytosis proteins to diabetes in rodents and humans. Given this, emerging studies aimed at restoring and/or enhancing cellular levels of certain exocytosis proteins point to promising outcomes in maintaining functional β-cell mass and enhancing insulin sensitivity. In doing so, new evidence also shows that enhancing exocytosis protein levels may promote health span and longevity and may also harbor anti-cancer and anti-Alzheimer's disease capabilities. Herein, we present a comprehensive review of the described capabilities of certain exocytosis proteins and how these might be targeted for improving metabolic dysregulation.

Keywords: glucose homeostasis, diabetes, insulin resistance, SNARE proteins, glucose-stimulated insulin secretion, double C2-domain protein B, syntaxin 4

DIABETES MELLITUS is a complex disorder associated with increased risk of heart failure, stroke, blindness, neuropathy, and kidney disease, and it is reaching epidemic proportions. Out of the estimated 422 million people presently afflicted with diabetes worldwide, ~5% have type 1 diabetes (T1D), whereas the remaining ~95% of diabetics have type 2 diabetes (T2D). T2D results from defective insulin responsiveness/actions by peripheral tissues (skeletal muscle, adipose, liver), termed "insulin resistance" coupled with insufficient insulin production from the pancreatic islet β-cells. Peripheral insulin resistance is generally presumed to precede the demise of β-cells, although it has recently come to light that β-cell dysfunction occurs earlier in T2D etiology than once thought (17, 33, 66, 69). Because of the nature of T2D etiology, there exist various treatment approaches that aim to enhance insulin sensitivity and others that aim to improve insulin secretion.

Present T2D treatments range from diet and weight loss to various combinations of oral and injectable therapies (Table 1). If diet and exercise are not sufficient to meet the accepted hemoglobin A_{1c} (HBA1c) threshold (typically <7.5%), pharmacotherapy is initiated. The first line therapy, metformin, belongs to the family of biguanidine drugs and is thought to reduce blood glucose levels primarily by suppressing gluconeogenesis in the liver via activation of AMPK. However, the precise mechanism of action of metformin is poorly understood. Importantly, metformin is contraindicated in individuals with renal, liver, or cardiorespiratory failure and is known to cause gastrointestinal side effects, which significantly limits its use. For those patients who cannot take metformin, or have no significant improvement in HBA1c, the second line therapy recommended for T2D patients is a combination therapy of insulin and a sulfonylurea. Sulfonylureas work at the level of the β-cell to enhance insulin secretion via binding to the ATP-dependent potassium channel (K_{ATP}). Although sulfonylurea use is rapidly effective and has been shown to reduce microvascular complications (UKPDS), the risk of hypoglycemia and accompanying weight gain associated with sulfonylurea therapy are major treatment-limiting factors.

If sulfonylurea/insulin therapy is ineffective or causes side effects, the recommended Tier 2 therapy is the use of thiazolidinediones (TZDs). Tier 2 classification denotes therapies that are less validated than Tier 1 therapies. TZDs lower blood glucose levels by increasing the expression of genes that promote lipid storage and enhance hepatic insulin sensitivity (Table 1). However, the FDA has recently restricted TZDs due to affiliated severe risk of myocardial infarction, weight gain, and fractures. In lieu of prescribing TZDs, physicians may prescribe a glucagon-like receptor (GLP-1) agonist, a class of drugs that mimic the effects of the incretin GLP-1, which causes increased insulin secretion, decreased glucagon release, increased satiety, and reduced gastric motility. Although GLP-1 agonists have been shown to promote weight loss and have relatively lower risk of hypoglycemia, the frequent and often severe gastrointestinal side effects associated with GLP-1 agonists limit their use as well. Alarmingly, new evidence shows that long-term use of the GLP-1 agonist liraglutide compromises human β-cell function in vivo (1). Other therapies include dipeptydyl peptidase-4 (DPP-4) inhibitors, sodium-glucose cotransporter-2 inhibitors (SGLT2), α-glucosidase inhibitors, and meglitinides; these act by increasing endogenous GLP-1 levels, inhibiting glucose reabsorption from the proximal tubule of the kidney, delaying carbohydrate digestion, and increasing insulin secretion, respectively. In addition, DPP-4 inhibitors can increase heart failure risk, α-glucosidase inhibitors cause gastrointestinal side effects, and meglitinides carry the risk of hypoglycemic episodes.

Taken together, it is clear that presently available therapy options for T2D are limited in terms of long-term safety, achieving optimal glucose-dependent insulin secretion, or promoting/protecting functional β-cell mass. Furthermore, none of these therapies directly promotes skeletal muscle-mediated insulin sensitivity and action (i.e., glucose uptake), which accounts for ~80% of all excess glucose disposal by peripheral tissues. With 30–40% of the US population predicted to be at risk for diabetes (34), this creates an urgent need for novel therapies that can dually protect functional β-cell mass and peripheral insulin sensitivity.

A review of the literature spanning the past 25 years reveals dually active factors that are required in both β-cell function and peripheral insulin action: SNARE proteins. Indeed, in the late 1990s, Nagamatsu et al. (72) demonstrated that diabetic GK rat islets were deficient in two target membrane (t)-SNARE proteins, syntaxin 1A (STX1A) and SNAP25, and that replenishment of these factors resolved islet dysfunction. More recently, we demonstrated the capacity to restore normal function to dysfunctional T2D human islets by replenishing syntaxin 4 (STX4), which was previously considered to be an inactive and redundant t-SNARE isoform (78). Thus, this review aims to explore emerging evidence suggesting that there are deficiencies/defects in exocytosis proteins in diabetic rodents and humans and to explore the consequences of overexpressing certain exocytosis factors in the context of maintaining glucose homeostasis. In doing so, we will look for clues from other diseases, such as neurodegenerative disorders and cancer, for which there are accumulating data on the benefits of replenishing deficient exocytosis proteins. In summary, we

ultimately pose the idea that exocytosis proteins may carry unexplored therapeutic potential for diabetes prevention and/or remediation.

SNARE Exocytosis Machinery: The "Nuts and Bolts"

Exocytotic trafficking of proteins and lipids from the cytosol to the cell exterior constitutes one of the most important processes in the cell. Most eukaryotic cells achieve this via packaging of protein or lipid cargo in membrane-bound vesicles, which originate from the trans-Golgi network or recycling endosomes and are subsequently transported via cytoskeletal remodeling to the plasma membrane. Once at the membrane, vesicle docking, priming, and fusion depend on the high-affinity interaction of a complex of highly conserved proteins called SNAREs (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors). This fundamental mechanism of regulated SNARE complex assembly is conserved in many different cell types, including neuronal, exocrine, hematopoetic, and endocrine cells.

SNARE core complex.

The SNARE complex consists of two target membrane (t)-SNARE proteins, syntaxin (STX) and SNAP23 (or SNAP25), and one vesicle-associated (v-SNARE) protein, VAMP2 (8, 32, 53, 60, 104, 105). STX proteins are ~35 kDa, containing a carboxy-terminal transmembrane domain spanning the plasma membrane and an amino terminus oriented toward the cytoplasm (87). The other t-SNARE type, SNAP-23/25 (23 or 25 kDa in size), is associated with the plasma membrane via palmitoylation of four cysteine residues in the central region of the protein (38). The v-SNARE VAMP2 is an 18-kDa protein with a vesicle membrane-spanning carboxyl terminus and an amino terminus oriented away from the vesicle toward the cytoplasm (8). Ultrastructural evidence of the SNARE complex shows that one v-SNARE binds with two cognate t-SNARE proteins in a heterotrimeric 1:1:1 ratio (53, 104). This SNARE core complex is extremely stable and is sufficient to withstand the energy barrier required to fuse the vesicle to the membrane (120). Once fused, the SNARE complex is described as being in "*cis*" configuration, as opposed to when the vesicle has been docked previously or tethered and in "*trans*" configuration (when the lipid bilayer of the vesicle is distinct from the plasma membrane, not yet having merged). The SNARE core complex is notoriously SDS resistant, requiring boiling in SDS-containing buffer to dissociate into free monomers. After vesicle fusion, the *cis*-complex SNARE proteins are bound by α-SNAP and *N*-ethylmaleimide sensitive factor (NSF) proteins to catalyze SNARE complex dissociation, allowing endocytosis of the v-SNARE and recycling of the individual t-SNAREs back to their respective plasma membrane compartments (104).

Regulation of the core complex: SNARE accessory factors.

SNARE-mediated vesicle fusion is tightly regulated by accessory binding proteins such as "SM" (Sec1/Munc18), Munc13, and DOC2 (double C2-domain containing proteins). Munc18 proteins, also called "syntaxin binding proteins (STXBP)," are ~66–68 kDa in size, are soluble, and do not contain a transmembrane domain (36). These proteins are localized to the cytosol and to the plasma membrane through direct interaction with their cognate syntaxin partners (88, 112, 113). It is proposed that, upon stimulation, Munc18 proteins assist the conformational change of syntaxin to its accessible "open" or "active" conformation for its subsequent engagement as part of the SNARE core complex (i.e., VAMP, SNAP23/25, STX) and docking and fusion of vesicles (15, 67).

In addition to SM proteins, the calcium- and phosphoinositide-binding protein isoform DOC2B has been shown to be essential for SNARE core complex assembly to occur in islet β-cells and muscle/fat cells (90). DOC2B is a ubiquitously expressed 46- to 50-kDa protein. In islet β-cells, DOC2B binds to the SNARE regulatory proteins Munc18-1 and Munc18c via its C2A and C2B domains, respectively (52, 89, 117). Other calcium-sensing C2-domain-containing proteins, including Munc13 and synaptotagmin, are

implicated in accelerating SNARE complex formation and insulin release from β-cells via fostering STX1-based actions (31, 57, 100, 125). In skeletal muscle cells, DOC2B facilitates STX4-based actions. Since many of these regulatory factors are implicated in T2D, we discuss in detail their tissue-specific and conserved mechanisms required for insulin secretion and peripheral insulin action in the following section.

Tissue-Specific and Conserved SNARE Proteins in Diabetes-Related Tissues

Among the diabetes-related tissues (pancreatic β-cells, skeletal muscle cells, fat cells), there are exocytosis factors that are cell-type specific and other factors that are conserved but exhibit distinct mechanisms suited to the function of that tissue in maintaining glucose homeostasis. As depicted in Fig. 1, the set of SNARE core complex proteins required by β-cells, fat cells, and muscle cells consists of STX4, SNAP23, and VAMP2. Islet β-cells, being neuroendocrine derived, also express and utilize neuronal-specific isoforms of SNARE proteins, namely STX1A, SNAP25, DOC2A, and Munc18-1. Hence, β-cells have some overlapping and functional redundancy in SNARE isoform usages. By contrast, skeletal muscle and fat cells have little to no redundancy, with only one t-SNARE of each type expressed (STX4 and SNAP23). Additional t- and v-SNAREs shown in Fig. 1 are reportedly expressed and used in some cases but are not necessarily required for normal function (e.g., VAMP3). The following sections will explore the detailed mechanisms underlying β-cell insulin secretion and skeletal muscle glucose uptake. Of note, although the focus of this review is to highlight prediabetes and T2D through the lens of SNAREs levels, SNARE defects are one of a number of other defects (e.g., signaling) that contribute to T2D.

Exocytosis in β-Cell: Biphasic Glucose-Stimulated Insulin Secretion

Interestingly, although the majority of the SNARE isoforms were identified in the early 1990s (41), only the neuronal cluster of STX1A, SNAP25, and VAMP2 was initially studied for functionality in insulin release mechanisms. SNAP23 was found capable of substituting for SNAP25 in insulin secretion (96) shortly thereafter, but it was another decade before the functional requirements for other syntaxin and SM isoforms, such as syntaxin 4 (STX4), Munc18b, and Munc18c, were examined in the process of insulin release (59, 77, 108). The β-cell is now known to express and use all four plasma membrane-localized STX isoforms, STX1–4. At the plasma membrane, isoforms STX1–3 can bind to Munc18-1 (also called Munc18a) and Munc18-2 (Munc18b) (36, 93, 94), whereas only STX4 can bind to Munc18c (111, 113). The following section discusses the ornate complexity of how these SNARE proteins are utilized, which contributes to the biphasic pattern of insulin release.

Biphasic secretion and affiliated SNARE complexes in the β-cell.

Insulin granule exocytosis in the pancreatic β-cell is elicited in two discrete phases in response to a nutrient stimulus, commonly referred to as glucose-stimulated insulin secretion (GSIS). GSIS is evoked by sensing of the β-cell to a stimulatory level of extracellular glucose (16.7 mM is most commonly used experimentally). This glucose enters the β-cell by passing through the constitutively plasma membrane-localized glucose transporter GLUT2. Once inside the β-cell, glucose is metabolized to yield an increased ratio of cellular ATP to ADP. Increased ATP induces closure of the K_{ATP} channels, causing membrane depolarization and opening of voltage-dependent calcium channels, increasing intracellular calcium ($[Ca^{2+}]_i$) levels. Elevated $[Ca^{2+}]_i$ triggers the SNARE and regulatory proteins to facilitate priming and fusion of insulin-filled granules with the plasma membrane to prompt release of the insulin cargo from the cell. This chain of events constitutes the first phase of GSIS (Fig. 2), occurring within 5–10 min of glucose stimulation, and uses STX1A- or STX4-based SNARE core complexes involving SNAP25 or SNAP23 and VAMP2 (80, 96, 97, 108, 121). Second-phase insulin release, which is characterized by the mobilization of insulin granules from intracellular storage pools out to the plasma membrane, occurs beyond 10 min of glucose stimulation. Second-phase GSIS utilizes STX4, SNAP25 or SNAP23, and VAMP2 (37, 80, 108) (

Fig. 2). Other syntaxin isoforms, (e.g., STX2 and STX3) have also been shown to participate in β-cell exocytosis events, although they are affiliated with more rare forms of exocytosis (48, 124, 131). Another v-SNARE protein, VAMP8, also functions in insulin secretion but is required selectively for glucagon-like peptide (GLP-1)-enhanced insulin release (132).

Regulation of insulin secretion: SNARE accessory proteins.

Although it is generally agreed that the Munc18 proteins facilitate the SNARE complex assembly process, the detailed protein-protein interaction changes in response to glucose stimulation are not agreed upon. For example, both Munc18-1 and Munc18c have been demonstrated to undergo phosphorylation and subsequent dissociation from their cognate STX partner (43, 74, 79). However, other reports suggest that the Munc18 proteins remain bound or reposition to become part of the final SNARE complex (16, 40). In general, studies favoring the dissociation model stem from cell or tissue studies, whereas the other model stems from in vitro assays. Interestingly, Munc18c is required only for second-phase insulin secretion, whereas STX4 and DOC2B are required for both phases (77, 91, 108) (Fig. 2). Munc18-1 regulates first-phase secretion, and although initially this was presumed to occur via STX1A binding changes, it was later discovered that Munc18-1 overexpression enhances STX4 activation (75); this has been since proposed as an explanation for why STX4 functions in first-phase insulin release.

Akin to the controversy described above regarding the molecular binding mechanisms of Munc18 proteins, the details regarding DOC2B's mechanism(s) of action are controversial. For example, Fig. 3*A* depicts DOC2B binding to Munc18-1 and Munc18c directly via DOC2B's C2A and C2B domains, respectively, scaffolding both Munc18 isoforms concurrently in a heterotrimeric complex (89). Alternatively, it has been suggested that DOC2B binds to STX4 rather than Munc18 proteins, which involves a calcium-dependent mechanism (Fig. 3*B*) (70). The key difference between the proposed mechanisms is the direct or indirect nature of DOC2B binding to STX4 to evoke its activation. Although the details of the direct binding model (Fig. 3*B*) are still evolving, the Munc18-binding model (Fig. 3*A*) purports that glucose stimulation triggers rapid Munc18c phosphorylation, switching its affinity for binding to DOC2B and away from STX4, which permits STX4 opening for its engagement in SNARE core complexes (43). As described below, some of these mechanistic concepts are also proposed in GLUT4-dependent glucose uptake in peripheral tissues.

Exocytosis of GLUT4 Vesicles and Glucose Uptake in Skeletal Muscle and Fat Cells

After a meal, glucose uptake into peripheral skeletal muscle accounts for ~80% of whole body glucose clearance, whereas fat cells account for the remaining 20% via intracellular processes that promote trafficking of the vesicle containing the insulin-responsive glucose transporter GLUT4 (26, 55, 62, 92, 109). This process begins with insulin binding to the insulin receptor (IR) to induce its tyrosine autophosphorylation, which in turn activates the canonical phosphatidylinositol 3-kinase (PI3K)→Akt signaling pathway, triggering the intracellularly localized GLUT4-containing vesicles to translocate to the sarcolemmal and T-tubule membranes, where the vesicles are docked and fused via SNARE proteins.

In the late 1990s, numerous groups independently deduced that the t-SNARE isoforms STX4 and SNAP23 and the v-SNARE VAMP2 comprised the machinery necessary and sufficient for GLUT4 vesicle docking/fusion (51, 81, 118, 119, 127). This mechanism was later expanded to include a pivotal regulatory step by which the SNARE complex was assembled; the insulin-activated IR directly phosphorylates Munc18c to trigger the activation of STX4, fostering SNARE complex formation (5, 44). Because Munc18c phosphorylation occurs independently of PI3K, these results gave rise to the present model, wherein insulin elicits a coordinated response through activation of IR to evoke t-SNARE assembly in sync with PI3K-mediated vesicle mobilization to the plasma membrane, culminating in coordinate SNARE core complex formation and vesicle fusion. Given the ubiquitous expression of this grouping of SNARE and SNARE accessory factors, this also provides a testable model for other exocytosis events initiating from

pivotal extracellular signals.

Regulation of GLUT4 Translocation/Glucose Uptake: Controversy Surrounding SNARE Accessory Proteins

Although all groups agree fully that DOC2B plays a positive role in regulating insulin-stimulated GLUT4 vesicle exocytosis and SNARE complex formation, DOC2B's mechanism(s) of action in peripheral tissues, like those described in the β-cell, remain controversial, depending upon the experimental system used to derive the mechanism. For example, studies of primary mouse skeletal muscle or GLUT4myc-L6 myoblasts show that insulin stimulation increased DOC2B binding to phosphorylated Munc18c in coordination with increased STX4 activation and SNARE complex formation (90, 91). Consistent with this, DOC2B-knockout (−/−) mouse skeletal muscle harbors abundant Munc18c-STX4 complexes and reduced SNARE complex formation (43, 89, 91). In contrast, studies using 3T3L1 adipocytes or in vitro mixing assays using recombinant DOC2B report a direct STX4-DOC2B binding interaction (27, 128); this interaction is not observed in primary tissues or L6 muscle cells (91). Although this has yet to be experimentally reconciled, differences that might underlie these distinct mechanisms could be that the in vitro and 3T3L1 adipocyte studies assessed DOC2B association with STX4 using coimmunopreciptations from cell lysates, a method that cannot distinguish direct from indirect binding interactions (27, 101). In vitro studies also exclude additional DOC2B binding factors (such as microtubule and/or actin cytoskeletal factors) that could impact how DOC2B associates with STX4. Indeed, both STX4 and DOC2B have each been shown to bind to microtubule-associated Tctex-1 type proteins (46, 73, 101), and hence, microtubule factors might bridge the DOC2B-STX4 interaction (as modeled in Fig. 3*C*). DOC2B also harbors an amino terminal Munc13-interacting domain (referred to as the MID domain), yet no Munc13 partners for DOC2B have yet been identified in fat or skeletal muscle cells. Bridging factors remain relatively unexplored as potential targets for improving peripheral insulin sensitivity.

Deficient, Defective, and Mislocalized Exocytosis Factors in T2D Tissues

After the discovery of important SNARE isoforms involved in insulin secretion and glucose uptake, several reports published data correlating deficiencies in SNARE and regulatory proteins to T2D and obesity in human subjects (as referenced in Table 2). Several recent reports link polymorphisms in STX1A to impaired glucose metabolism in obese human subjects and to the age of onset and insulin requirement in T2D individuals (95, 115). Another report links decreased STX4 gene expression with T2D and psoriasis (2) and suggests that STX4 be considered a biomarker for T2D development in psoriasis cases. STX4, among other SNARE proteins such as STX1A, SNAP25, and VAMP2, have all been shown to be deficient in T2D human islets (Table 2). Notably, the regulatory factors of these SNARE proteins, such as multiple Munc18 isoforms, DOC2B, Munc13-1, multiple synaptotagmin isoforms, and synaptophysin, are also deficient (3, 6, 9, 30, 72, 78, 83, 100). Modeling this in spontaneous rodent models of prediabetes and T2D, such as the obese and diabetic Zucker rats and the nonobese diabetic GK rat, revealed that many of these same exocytosis factor deficits are conserved (Table 2). Although recent studies point to significant differences between human and mouse islets in terms of islet architecture and islet cell function, deficiencies in SNARE proteins under conditions of obesity and T2D are uniformly similar. Many SNARE deficiencies have been studied using gene-targeted ablation knockout mouse models, resulting in dysregulated metabolic phenotypes. This raises the question as to whether deficiencies of SNARE/regulatory proteins are a cause or a consequence of diabetes. The prevailing hypothesis is that deficiencies of SNAREs are likely a consequence of diabetes. However, STX1 gene mutations and polymorphisms have been linked with T2D, and SNAREs are noted as being targets of miRNA (61, 65) and lncRNAs (19, 20), suggesting that genetic and/or epigenetic links will need to be explored to fully address this question.

Can Deficient/Defective Exocytosis Factors be Replenished to Remediate Disease? Clues from Neurodegenerative Disorders

One of the earliest reports linking defects in exocytosis proteins to human disease involved senile plaques of Alzheimer subjects, which were found to be deficient in exocytosis proteins, namely STX1A, synapsin, snaptophysin, and synaptotagmin (22). Akin to findings in islets described above, deficiencies in SNAP25 and STX1A were found in subjects with Creutzfeldt-Jakob disease (Fig. 4) (23). Decreased abundances or functions in exocytosis proteins were also reported in subjects with Huntington's disease, schizophrenia, and attention-deficit/hyperactivity disorder (18, 21, 29, 71, 103). In each disease, treatments involved restoration of SNARE proteins (Fig. 4). Related to this, STX4 replenishment to human T2D islet cells restored glucose-stimulated insulin secretory function equivalent to that of nondiabetic age-matched human islets (78), providing proof of concept for this as a restorative approach.

Are Exocytosis Proteins Implicated in Cell Survival and Proliferation? Clues from Cancer

STX4 and DOC2B as targets to combat cancer?

Recent investigations have suggested that STX4 and DOC2B may act as tumor suppressors. For example, downregulation of STX4 was recently shown to be associated with cancer cell proliferation, as the STX4 is required for acid sphingomylinase translocation, a vesicle-trafficking event needed for normal apoptotic mechanisms (86). Similarly, DOC2B transcription was found to be downregulated in several human cancer cell lines due to hypermethylation of the DOC2B promoter (45). DOC2B is required to regulate proapoptotic mechanisms in human cervical cancer cells, combating their proliferation. DOC2B replenishment in cervical cancer cells led to increased actin cytoskeleton remodeling and inhibition of Akt and ERK hyperphosphorylations, yielding attenuation of cell migration to decrease cancer cell growth (45). Importantly, no increases in common mitogenic pathway proteins such as MAPK, ERK, or echanistic target of rapamycin were reported with overexpression of STX4 or DOC2B.

STX4 and DOC2B: potential roles in proliferation.

Despite studies suggesting that STX4 and DOC2B have tumor suppressor capabilities, other studies have implicated STX4 and DOC2B in playing roles in proliferating cells during early embryogenesis. For example, STX4 homozygous (−/−) null mice died during early embryogenesis (127), and this was later linked to STX4's vital role in facilitating GLUT8 exocytosis to support glucose influx for the growing blastocysts (123). In neurons, DOC2B expression occurs long before neurotransmitter release is functional, and this early expression pattern of DOC2B correlates with that of genes involved in neuronal proliferation and differentiation, such as the neuroepithelial stem cell marker nestin (56). Furthermore, studies have shown that DOC2B has a critical role in synaptic vesicle trafficking as early as *embryonic day 18*, as neurons from DOC2B-deficient (−/−) knockout mice of this age exhibited impaired spontaneous release frequency (35). This points to a putative role for DOC2B in the delivery of membrane proteins to the surface of proliferating neurons and/or to the tip of outgrowing axons. Whether overexpression of STX4 or DOC2B could be harnessed as a means to protect/promote β-cell proliferation will be an important area of future investigation.

Exocytosis Protein Replenishment and Glucose Homeostasis: Isoform Specificity Matters

The first demonstration that restoration of t-SNAREs to normal levels could recover insulin secretion in diabetic GK rat islets provided proof of concept for SNARE replenishment as an approach for restoring islet function (72). The increasing evidence of exocytosis protein deficiencies in islets of obese and diabetic humans and rodents spurred the generation of several transgenic rodent models designed to test the effect of increased abundance of specific SNARE isoforms on the regulation of islet function and glucose

homeostasis in vivo (Table 3).

STX1A and Munc18c: unexpectedly ineffective.

Despite a deficiency of STX1A and Munc18c in diabetic human and rodent islets (Table 2), overexpression of either STX1A or Munc18c in transgenic mice did not improve glucose tolerance as expected; instead, both models exhibited profound glucose intolerance (58, 107). β-Cell-specific STX1A-overexpressing mice harbored β-cells that were functionally defective, with reduced depolarization-evoked membrane capacitance and reduced currents through the Ca^{2+} channels (58). Years later, STX1A was shown to bind to the SUR1 regulatory subunit of islet β-cell K_{ATP} channels when overexpressed, inhibiting the activity of the channels and causing secretion defects (12). Munc18c overexpression revealed a similar limitation; Munc18c transgenic mice, overexpressing Munc18c by as little as two- to threefold in pancreas, skeletal muscle, and adipose tissues, harbored dysfunctional islets as well as insulin-resistant skeletal muscle (107). In both cell types, the additional Munc18c bound and sequestered endogenous STX4, reducing the formation of STX4-based SNARE complexes. These inhibitory actions leave STX1A and Munc18c with too narrow a window of efficacy for remediating islet function or skeletal muscle functions.

STX4: an expandable hub for excitosomes?

In contrast to mice overexpressing STX1A, mice overexpressing STX4 (simultaneously in pancreatic islets, skeletal muscle, and adipocytes) exhibited enhanced glucose homeostasis, resulting from increased skeletal muscle insulin sensitivity and islet function (Table 3) (106). This is an important distinction between the two STX isoforms and points to important structure-function differences between these t-SNARE proteins. Amino acid alignment of STX1A and STX4 shows only 45% sequence similarity, supporting the concept that STX4 partners with significantly different factors. One such factor is filamentous actin (F-actin). Through its unique amino terminal α-spectrin like domain, STX4 is the only SNARE protein capable of direct F-actin binding (7, 42, 46, 122). Moreover, in β-cells, STX4 can associate with the actin binding and severing protein gelsolin and in 3T3-L1 adipocytes with the F-actin cross-linking protein α-fodrin (46, 63). Both STX4-cytoskeletal protein associations are affiliated with positive effects upon vesicle exocytosis in β-cells and fat cells. Although insulin granule exocytosis from β-cells requires actin depolymerization (39, 47, 82), whereas GLUT4 vesicle exocytosis requires F-actin polymerization (49, 54, 116), both are considered F-actin remodeling events that may coordinate the timing of STX4 activation with the arrival of incoming granules/vesicles. If true, the interaction of STX4 with the actin cytoskeleton may constitute an "excitosome," a localized site at the plasma membrane with concentrated SNARE complex proteins and accessory proteins that promote granule docking to the membrane (110, 129). Overexpression of STX4 may provide the basis for more excitosomes, i.e., more docking sites for exocytosis in skeletal muscle and β-cells.

The translational implication of targeting exocytosis proteins for diabetes remediation has been demonstrated effectively with STX4 in human pancreatic islets. Islets from human T2D cadaveric donors transduced to replenish STX4 levels to that of nondiabetic islets showed fully restored biphasic insulin secretion (78). Furthermore, consistent with STX4 being limiting for function, biphasic insulin release was enhanced to supranormal levels in nondiabetic human islets transduced to overexpress STX4, and transplantation of a very minimal number of these islets (200/graft) was capable of attenuating STZ-induced diabetes (78). These intriguing data support the concept of STX4 enrichment as a potential novel therapeutic target for enhancing β-cell function in humans.

STX4 promotes health span and longevity.

Recent evidence has shown that beyond enhancing insulin secretion and glucose uptake, STX4 overexpression also promotes health span and longevity (76). Transgenic mice overexpressing STX4 in

pancreas, skeletal muscle, and adipocytes lived ~33% longer than wild-type control littermates (76). Furthermore, when challenged with high-fat diet-induced obesity, STX4 transgenic mice showed preserved islet insulin secretion and skeletal muscle GLUT4 translocation compared with high-fat-fed wild-type mice (76). Microarray analysis of muscle from the STX4 transgenic mice showed changes in the pathways of leptin and AMPK signaling as well as reduced Fox01 protein levels in pancreata. These intriguing results suggest that preservation of insulin sensitivity into old age via retaining a rapid rate of glucose uptake/clearance of excess circulating glucose may protect against hyperglycemia-related damage to cells and diabetes and may improve overall health span and lifespan (Fig. 5).

DOC2B as a restorative?

Mice overexpressing DOC2B simultaneously in β-cells, skeletal muscle, and adipocytes show a significantly increased capacity for glucose-stimulated insulin granule exocytosis and insulin-stimulated GLUT4 vesicle translocation (90). While overexpressing DOC2B by only approximately threefold compared with endogenous levels, these transgenic mice were exquisitely insulin sensitive, showing supranormal levels of glucose tolerance (Table 3). Importantly, despite the capacity of the islets to secrete 30–50% more insulin in response to a glucose bolus, the mice never exhibited hypoglycemia. This may be due to the rapid response of the skeletal muscle to the insulin release during the first phase, triggering rapid glucose clearance and rapid return to normoglycemia, with this return being the natural cue for the pancreas to decrease release of insulin. With this exquisite coordination and because of its ability to promote STX4 activation, DOC2B overexpression presents an attractive target for restoring glucose homeostasis. The advantage to DOC2B is that it can enhance both phases of insulin release, and if delivered systemically, it might enhance skeletal muscle insulin sensitivity concurrently. Whether DOC2B overexpression carries the capacity to protect against diabetogenic stimuli remains to be tested.

Stoichiometry.

Since overexpression of either STX4 or DOC2B potentiates selective exocytosis events in vivo and in clonal cells, STX4 and DOC2B are considered to be present in β-cells and muscle cells at limiting levels (52, 70, 90, 98, 106). This concept is supported by quantification of stoichiometric ratios of STX4 and SNAP23 in skeletal muscle from C57BL6 mice, where SNAP23 was found present in a threefold molar excess over that of STX4 (106), providing sufficient SNAP23 for appropriate stoichiometric ratios of t-SNARE binary complexes in the muscle of the STX4-overexpressing mice. DOC2B is not a SNARE protein per se, but its overexpression in skeletal muscle increased the abundance of STX4-based SNARE complexes in coordination with an increase in DOC2B-Munc18c complexes and increased STX4 activation (90). This trio of effects is consistent with earlier cell culture findings pointing to DOC2B “freeing” STX4 from Munc18c (43). The only stoichiometric data reported on DOC2B comes from β-cells, wherein DOC2B was recently shown to be capable of binding in 1:1:1 ratio with Munc18-1 and Munc18c (83). Stoichiometry of SNARE proteins and SNARE accessory factors still needs to be carefully quantified in primary tissue, although to date there is full consensus regarding beneficial effects of STX4 and DOC2B overexpression among primary islets and multiple different β-cell lines. Finally, studies to investigate alterations in STX4 or DOC2B and their stoichiometry with other factors involved in proliferative and anti-tumorigenic mechanisms will require further investigation. Clearly, therapies designed to enhance STX4 and DOC2B could be clinically useful for a variety of metabolic aberrations (Fig. 5).

Perspectives: Targeting Exocytosis Proteins for Ideal Diabetes Treatment Outcomes

T2D treatments ideally aim to enhance both functional β-cell mass and peripheral insulin sensitivity. It is thus plausible that proteins that can dually enhance functional β-cell mass, and glucose uptake should be investigated for potential clinical relevance. Beyond functional enrichment, increasing evidence shows that

exocytosis proteins may be involved in cell survival processes. For example, in neurons, it has been shown that STX1A and SNAP25 promote neuron survival via membrane-recycling processes (85). In lymphocytes, SNAP23 is limiting and necessary for B and T cell development and fibroblast survival (50). SNAP25 has also been shown to play a role in neuronal spine morphogenesis and plasticity (4). With this in mind, investigations into SNARE/accessory proteins potentially protecting β-cell mass and promoting β-cell survival are necessary and would certainly be ideal outcomes for T2D therapeutics.

What are the potential perils of overexpressing SNARE/accessory proteins? With increasing evidence that exocytosis proteins may be involved in cell survival processes, there is controversy regarding exocytosis protein involvement in tumorigenesis. Some studies have shown that certain SNAREs may facilitate tumor cell migration, mediate inflammation involved in cancer development, and be under regulation by oncogenes (68). Despite this, both STX4 and DOC2B have been shown to possess proapoptotic/anticancer properties (45, 86), indicating that isoform-type and context-type specificities are crucial to determine when considering replenishment or overexpression strategies for treatment/prevention of diabetes and/or cancer.

How to target overexpression of exocytosis factors.

Potential delivery options for increasing cellular levels of exocytosis proteins are likely to vary in an isoform-type and context-specific manner as well. Based on present therapeutic options being explored in neurons, a recent study has utilized a truncated form of SNAP25 conjugated to human Hph-1, a protein transduction domain that allows penetration of various macromolecules into the cytoplasm and nucleus both in vitro and in vivo through local or systemic administrations (84). This approach may be useful for treating neurodegenerative disorders, as Hph-1-conjugated proteins can cross the blood-brain barrier. Additional delivery options involve gene therapy approaches using adeno-associated virus vectors (AAV), a delivery system presently being investigated in clinical trials for several diseases (14, 25, 102). AAV vectors have recently been implicated in treating neurodegenerative disorders, as characterization of several novel AAV serotypes has shown that a single intravenous injection in adult mice leads to transduction of neural cells throughout the entire central nervous system (126). Recent characterization of several novel AAV serotypes has revealed ideal serotypes for targeting skeletal muscle and pancreas (11, 13, 24, 25) and could potentially be utilized for gene therapy to target exocytosis proteins in treating prediabetes and T2D. Other options include enhancing human pancreatic islets with STX4 or DOC2B slated for subsequent clinical transplantation, as clinical trials using transplantation procedures in T2D are presently being pursued (99, 130). Notably appealing for clinically effective druggable targets is that achieving high levels of overexpression of STX4 and DOC2B would not be necessary. In view of their potential to correct and protect healthy metabolic parameters coordinately in the tissues overseeing glucose homeostasis, these unexpected exocytosis proteins may be ideal candidate targets for remediation and/or prevention of type 2 diabetes.

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DISCLOSURES

Neither A. Aslamy nor D. C. Thurmond has conflicts of interest with this work.

AUTHOR CONTRIBUTIONS

A.A. and D.C.T. conceived and designed research; A.A. prepared figures; A.A. drafted manuscript; A.A. and D.C.T. edited and revised manuscript; A.A. and D.C.T. approved final version of manuscript; D.C.T. interpreted results of experiments.

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Figures and Tables

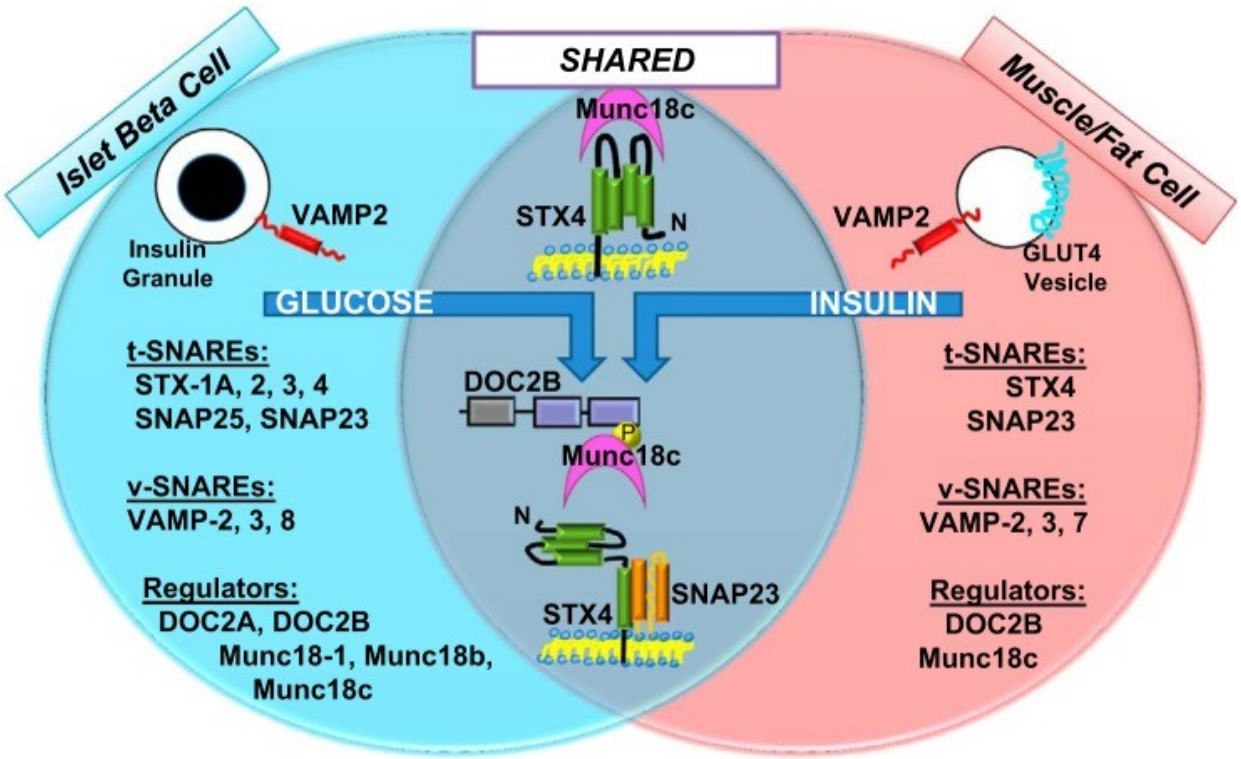
Table 1.

Present T2D treatments

Treatment	Target	Mechanism	Advantages	Disadvantages
		Tier 1: first/second line therapies		
Biguanide (metformin)	Liver	↑Hepatic insulin sensitivity; ↓gluconeogenesis and lipogenesis via activating hepatic AMPK	↓Macrovascular events and mortality (UKPDS); weight neutral; no hypoglycemia	GI side effects; contraindicated in patients with kidney, liver, cardiorespiratory insufficiency, alcoholism, or older age
Insulin	Skeletal muscle, adipose; liver	↑Glucose uptake in muscle, fat; ↓hepatic glucose output	Rapidly effective; improved lipid profile	Hypoglycemia; daily injections; constant monitoring
Sulfonylurea (chlorpropamide, glipizide, etc.)	Pancreatic β-cell	↑Insulin secretion via binding to ATP-dependent K+ channel	↓Microvascular complications (UKPDS); rapidly effective	Severe hypoglycemia; GI issues, weight gain; CV safety issues; hastening of β-cell death
		Tier 2: less validated therapies		
TZDs (pioglitazone, rosiglitazone)	Liver adipose	↑Expression of genes that promote lipid storage and enhance hepatic insulin sensitivity	↓Loss of β-cell function; ↑insulin sensitivity	↑MI risk; weight gain; fluid retention; ↑LDL, cholesterol; ↑bone fracture risk in women
GLP-1 agonist (liraglutide, exenatide)	Pancreatic β-cell	↑Insulin secretion via binding to GLP-1 receptor	Weight loss; ↓hypoglycemic episodes	↑GI side effects; requires injection; fail if patients are insulinopenic
		Other therapies		
DPP-4 inhibitor (sitagliptin, linagliptin)	Pancreatic β-cell	Inhibit cleavage of endogenous GLP-1	Weight neutral	Risk of heart failure; long-term safety?
SGLT2 inhibitor (empagliflozin, canagliflozin)	Renal proximal tubule	Inhibits sodium glucose cotransporters,	Weight loss; ↓systolic blood pressure	Urinary tract infections; long-term safety?
α-Glucosidase inhibitor (acarbose, miglitol)	Small intestine	Delays carbohydrate digestion	Weight neutral; no hypoglycemia if taken alone	↑GI side effects

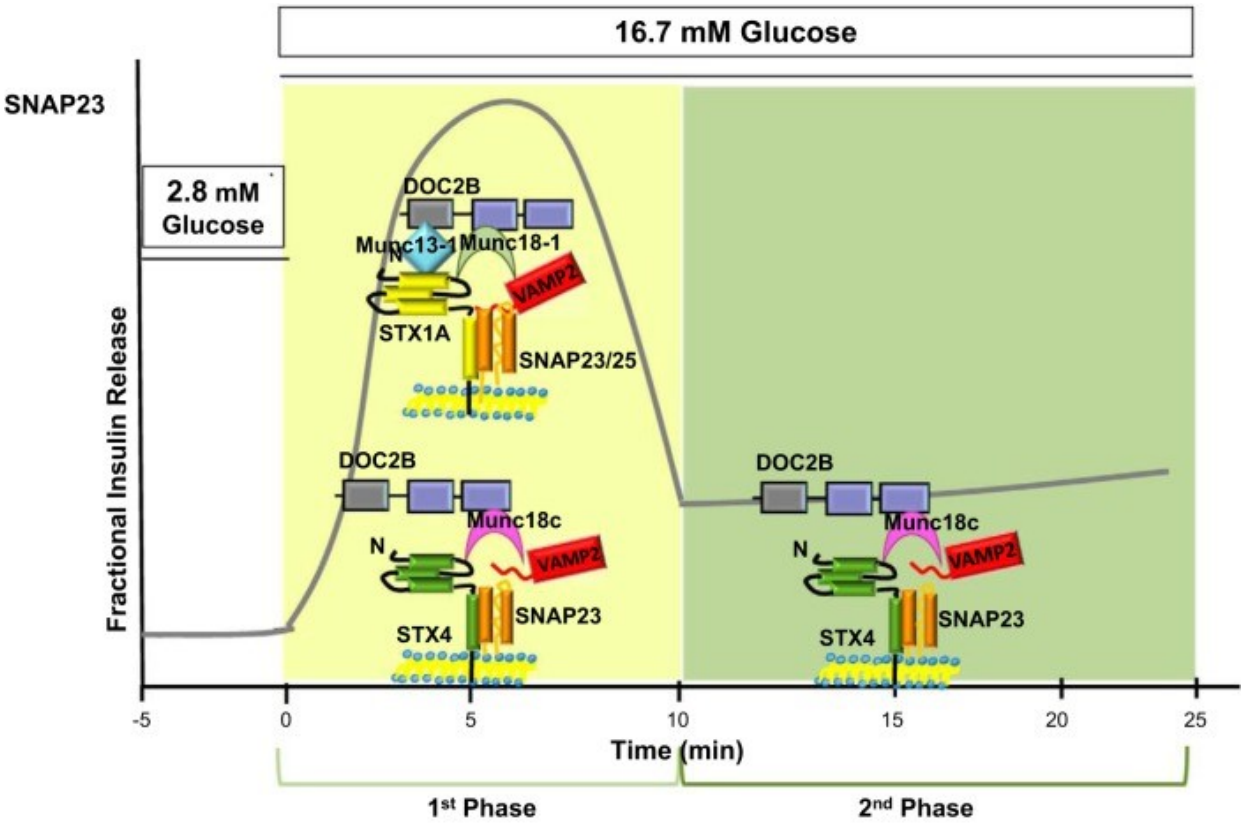
T2D, type 2 diabetes; GI, gastrointestinal; TZDs, thiazolidinediones; CV, cardiovascular; GLP-1, glucagon-like peptide-1; DPP-4, dipeptydyl peptidase-4; SGLT2, sodium-glucose cotransporter-2. ↑Increased aspects; ↓decreased aspects.

Fig. 1.



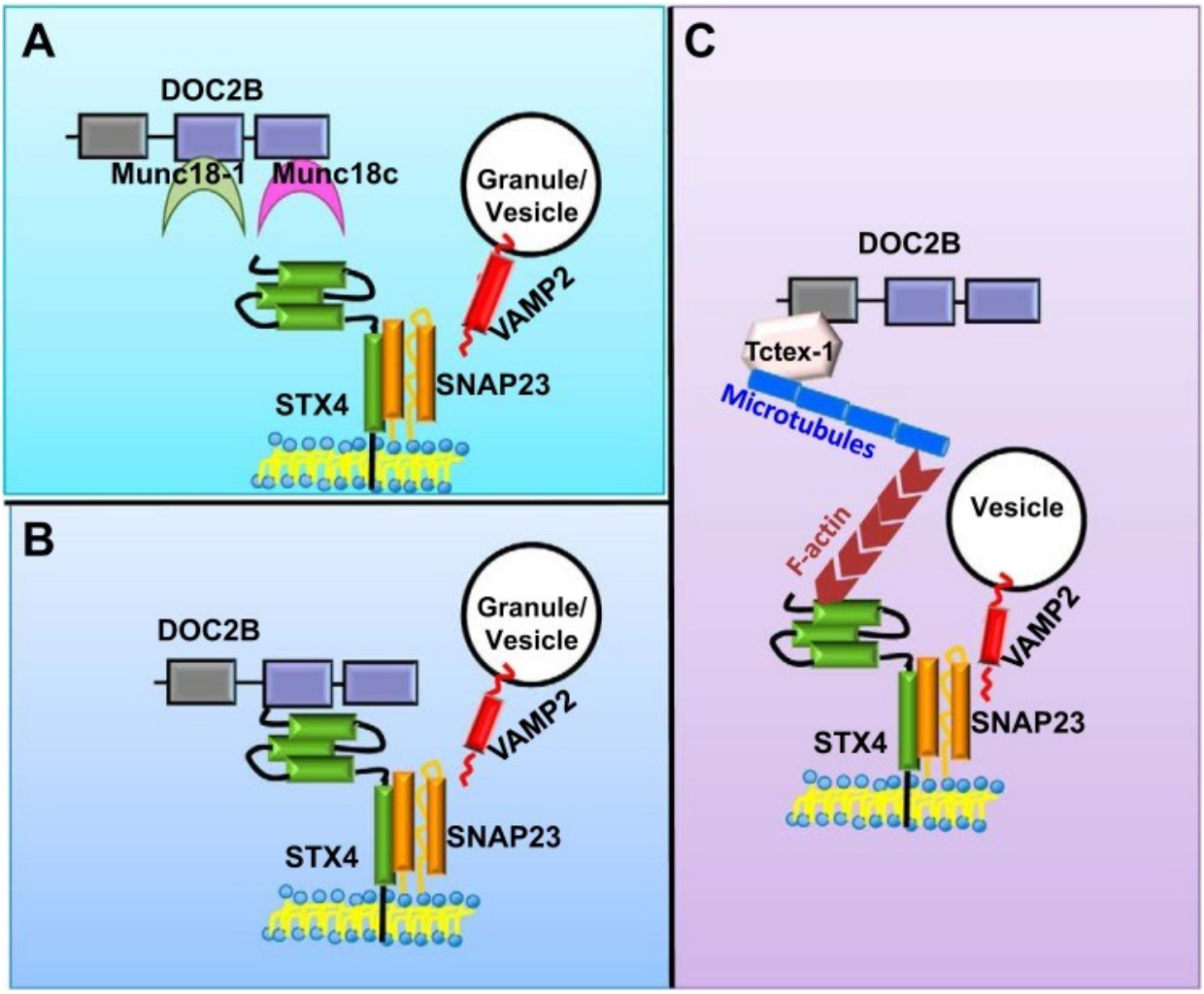
Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) and SNARE accessory protein isoforms utilized by islet β-cells and skeletal muscle cells for vesicle exocytosis: a common subset of factors. Factors listed in the β-cell (blue circle) are known to facilitate glucose-stimulated insulin secretion, and those listed in the skeletal muscle cell (red circle) are required for insulin-stimulated glucose uptake. Those isoforms utilized by both β-cells and muscle cells are shown in the center overlap of the blue and red circle. VAMP2, -3, -7, and -8, vesicle-associated membrane proteins 2, 3, 7, and 8, respectively; STX1A, -2, -3, and -4, syntaxin 1A, 2, 3, and 4, respectively; t-SNARE, target membrane SNARE; v-SNARE, vesicle-associated SNARE; DOC2B, double C2-domain protein B.

Fig. 2.



Differential requirement of SNARE and SNARE accessory protein isoforms during each phase of glucose-stimulated insulin secretion from islet β-cells. Whereas the first phase of insulin release uses both STX1- and STX4-based complexes, the second phase of insulin release relies upon STX4-based SNARE complexes. Accessory factors shown to be affiliated with these complexes are also grouped with the corresponding t- and v-SNARE core complexes. Note: SNAP25/23 denotes that SNAP25 and SNAP23 both suffice in the affiliated complex.

Fig. 3.



Proposed mechanisms underlying the benefits of DOC2B overexpression. Overexpression of DOC2B has shown positive effects on whole body glucose homeostasis. The mechanism by which DOC2B overexpression exerts its positive effects in β-cells/muscle cells is still yet to be elucidated. *A*: DOC2B proposed to function as a scaffold for Munc18-1 and Munc18c binding and subsequent activation of STX4. *B*: DOC2B proposed to bind directly to STX4, allowing for STX4 activation and promotion of SNARE formation. *C*: DOC2B proposed to bind the light chain (Tctex-1) of the motor protein dynein, which functions along microtubules. DOC2B may act direct/indirectly on actin remodeling, subsequently allowing for translocation of vesicles to the plasma membrane.

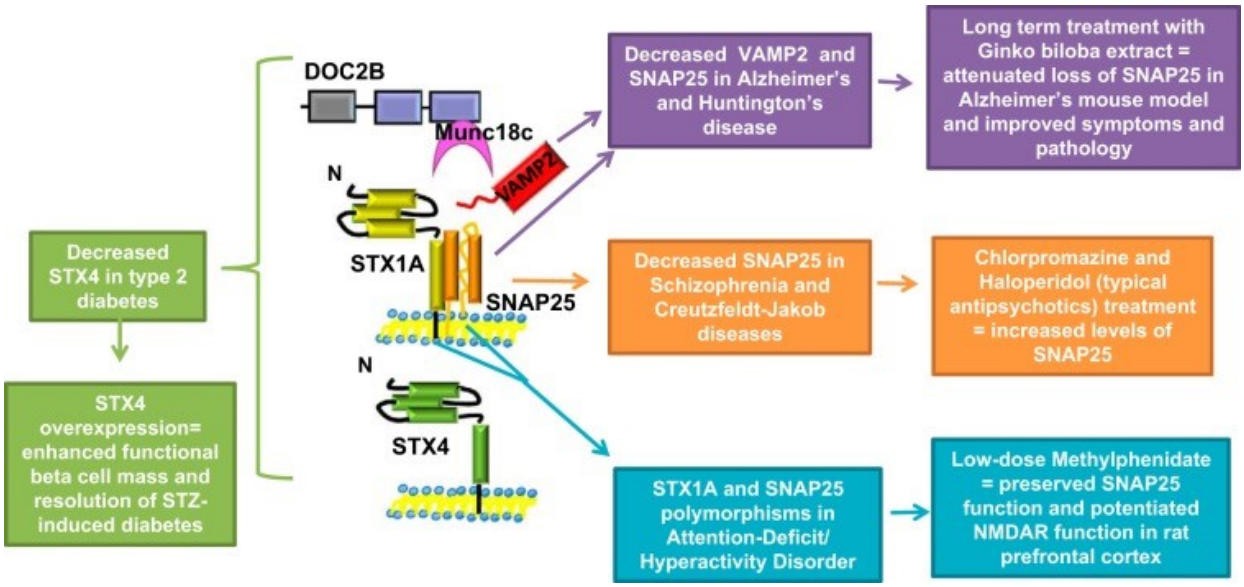
Table 2.

Exocytosis protein expression in type 2 diabetic humans and rodents

Protein	Tissue	Protein Levels in T2D Humans	Protein Levels in T2D Rodents	Ref. No(s).
DOC2B	Islets	Decreased	ND	6
Munc13-1	Islets	Decreased	Decreased in diabetic GK and obese Zucker *fa/fa* rats	83, 100
Munc18-1	Islets	Decreased	ND	78, 83
Munc18c	Islets; skeletal muscle; adipose	All decreased	Decreased in diabetic GK rats	9, 28, 30, 72, 83
SNAP25	Islets	Decreased	Decreased in diabetic GK rats	28, 72, 83
STX1A	Islets	Decreased SNP (D68D, T to C) correlates to age at onset and insulin requirement in T2D	Decreased in hyperglycemic GK rats	3, 72, 78, 83, 115
STX4	Islets, Skeletal muscle	Decreased in both tissues	ND	9, 78
Synaptophysin	Islets	Decreased	ND	83
Synaptotagmin 4, 7, and 11	Islets	Decreased	ND	3
VAMP2	Islets	Decreased	Decreased in diabetic GK rats	28, 83

DOC2B, double C2-domain protein B; ND, not determined; PM, plasma membrane; SNP, single nucleotide polymorphism; GK, Goto-Kakizaki; VAMP2, vesicle-associated membrane protein 2. Note: presented in alphabetical order by protein name.

Fig. 4.



Exocytosis proteins implicated in human neurological and metabolic diseases. SNARE protein abundances (STX4, STX1A, SNAP25, and VAMP2) are decreased and genetic polymorphisms implicated in a variety of neurodegenerative, autoimmune, and metabolic diseases. Therapies are proposed based on present studies that aim to replenish/preserve abundance of exocytosis proteins. STZ, streptozotocin.

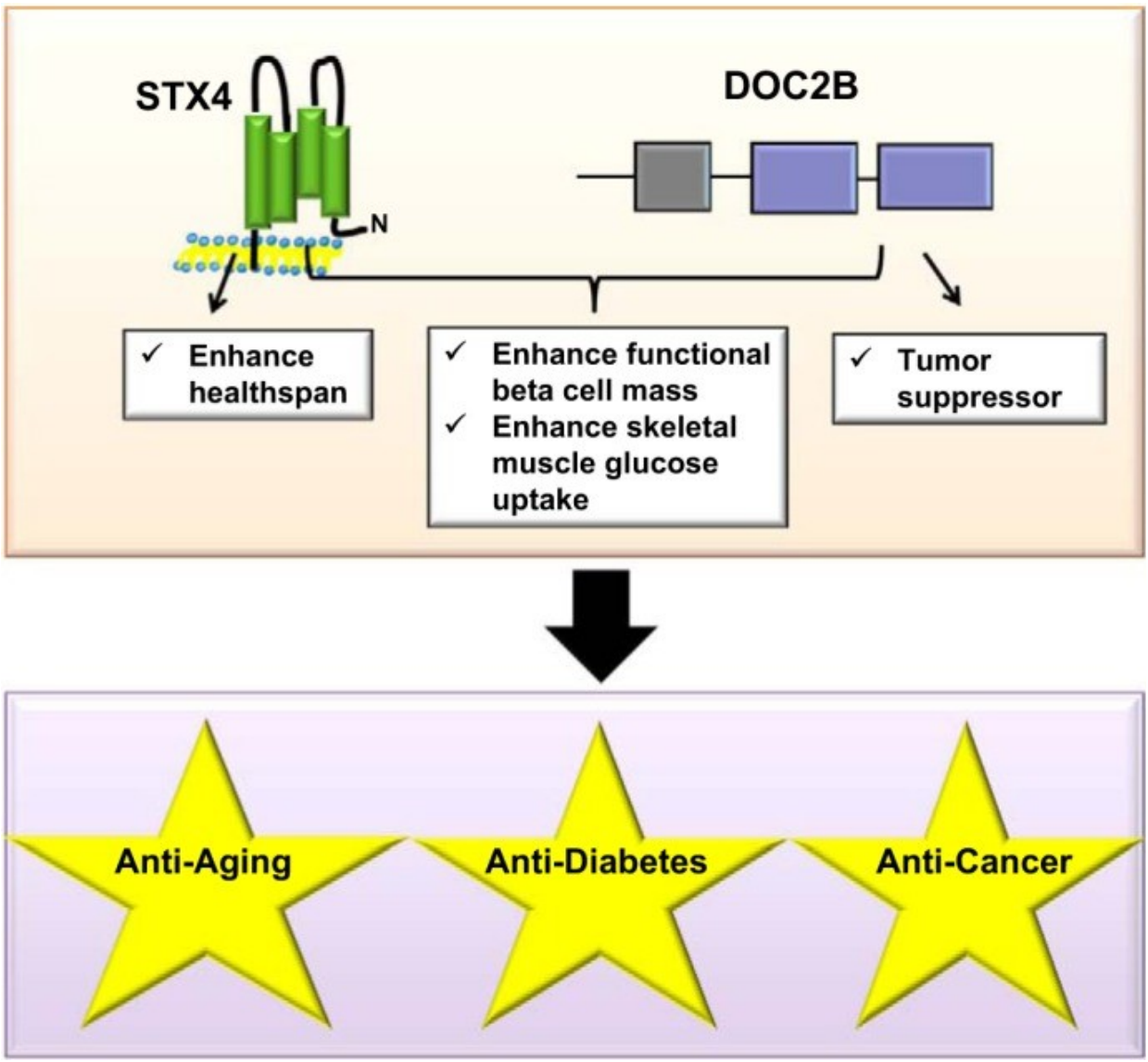
Table 3.

Transgenic mouse models of exocytosis protein overexpression

Genotype	Alteration	Metabolic Phenotype	Ref. No(s).
STX1A	Increased STX1A in pancreatic β cells	Fasting hyperglycemia, reduced insulin secretion, insulin intolerance in male mice	58
STX4	Increased STX4 in skeletal muscle, fat, and pancreas	Enhanced insulin sensitivity, GLUT4 translocation and skeletal muscle glucose uptake; enhanced insulin secretion from islets; increased healthspan, lifespan; protected from age- and HFD-induced metabolic dysfunction	76, 78, 106, 108
VAMP2	Increased VAMP2 in neurons	ND	64
Munc18-1	Increased Munc18-1 in neurons	ND	114
Munc18c	Increased Munc18c in fat, skeletal muscle and pancreas	Insulin resistant, glucose intolerant with impaired skeletal muscle glucose uptake; impaired insulin secretion from islets	107
DOC2B	Increased DOC2B in skeletal muscle, fat, and pancreas	Enhanced insulin sensitivity, GLUT4 translocation and skeletal muscle glucose uptake; enhanced insulin secretion from islets	90

STX1A and -4, syntaxin 1A and 4, respectively; GLUT4, glucose transporter 4; ND, not determined; HFD, high-fat diet.

Fig. 5.



Translational implications for SNARE/accessory proteins? Based on preliminary animal studies and in vitro analysis, targeting exocytosis proteins such as STX4 and DOC2B for enrichment appears to be beneficial in treatment strategies to deter aging/diminished healthspan, diabetes, and cancer.
