Munc18b is a major mediator of insulin exocytosis
in rat pancreatic β-cells

Patrick P.L. Lam1,2,7, Mitsuyo Ohno3,7, Subhankar Dolai1,7, Yu He1, Tairan Qin1, Tao Liang1, Dan Zhu1, Youhou Kang1, Yunfeng Liu1, Maria Kauppi4, Li Xie1, Wilson C.Y. Wan1, Na-Rhum Bin2,5, Shuzo Sugita2,5, Vesa M. Olkkonen6, Noriko Takahashi3, Haruo Kasai3,8, Herbert Y. Gaisano1,2,8

1Departments of Medicine and 2Physiology, University of Toronto, M5S 1A8, Toronto, Canada
3Laboratory of Structural Physiology, Center for Disease Biology and Integrative Medicine, The University of Tokyo, Hongo, Tokyo 113-0033, Japan
4National Institute for Health and Welfare, Biomedicum, FI-00290, Helsinki, Finland
5Division of Fundamental Neurobiology, University Health Network, 399 Bathurst Street, Toronto, Ontario, M5T 2S8, Canada
6Minerva Foundation Institute for Medical Research, Biomedicum 2U, FI-00290, Helsinki, Finland
7These three authors contributed equally to the work
8Equally contributing co-corresponding authors

Address correspondence to:
Herbert Y. Gaisano
Room 7368, Medical Sciences Building
1 King’s College Circle
Toronto, Ontario, M5S 1A8, Canada
Email: herbert.gaisano@utoronto.ca
Tel: (416) 978-1526

Haruo Kasai
Faculty of Medicine Bldg.1 #NC207
Laboratory of Structural Physiology
Center for Disease Biology and Integrative Medicine
The University of Tokyo
Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
Email: hkasai@m.u-tokyo.ac.jp
Tel: +81-(0)3-5841-1440

Running Title: Munc18b in primary exocytosis & sequential fusion
ABSTRACT
Sec1/Munc18 proteins facilitate the formation of trans-SNARE complexes that mediate fusion of secretory granule (SG) with plasma membrane (PM). The capacity of pancreatic β-cells to exocytose insulin becomes compromised in diabetes. β-cells express three Munc18 isoforms of which the role of Munc18b is unknown. We found that Munc18b depletion in rat islets disabled SNARE complex formation formed by Syntaxin-2 and Syntaxin-3. Two-photon imaging analysis revealed in Munc18b-depleted β-cells a 40% reduction in primary exocytosis (SG-PM fusion) and abrogation of almost all sequential SG-SG fusion, together accounting for a 50% reduction in glucose-stimulated insulin secretion (GSIS). In contrast, gain-of-function expression of Munc18b-wild type and more so, dominant-positive K314L/R315L mutant, promoted the assembly of cognate SNARE complexes, which caused potentiation of biphasic GSIS. We found that this was attributed to a >3-fold enhancement of both primary exocytosis and sequential SG-SG fusion, including long-chain fusion (6-8 SGs); not normally (2-3 SG fusion) observed. Thus, Munc18b-mediated exocytosis may be deployed to increase secretory efficiency of SGs in deeper cytosolic layers of β-cells as well as additional primary exocytosis, which may open new avenues of therapy development for diabetes.

INTRODUCTION
Glucose stimulation of islet β-cells triggers an initial robust 1st-phase GSIS, followed by a diminished but sustained 2nd-phase GSIS. In type-2 diabetes, islet insulin secretory capacity can’t meet the increasing insulin demand caused by insulin resistance; β-cells eventually decompensate with loss of 1st-phase GSIS and 2nd-phase GSIS becomes defective(1). Although previous studies have identified the molecular circuitry underlying β-cell stimulus-secretion coupling (reviewed in 2,3), the precise molecular determinants of the complex steps of insulin SG exocytosis with PM,
termed primary exocytosis, remain unclear. In mast cells and eosinophils(4,5), rapid and extensive sequential SG-SG fusions account for their high secretory efficiency. Such SG-SG fusions also occur in β-cells but at reduced frequency (1.9-2.6%) and extent (only 2-3 SGs)(6,7); and much less is known about the molecular machinery driving SG-SG fusion.

The membrane fusion machinery requires two key components: SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) and SM (Sec1/Munc18) proteins(8). The SNARE paradigm dictates that cognate vesicle-(v-) SNAREs (Vesicle Associated Membrane Proteins, VAMPs) and target membrane-(t-) SNAREs (syntaxins (Syn) and Synaptosome-Associated Protein of 25kDa, SNAP25) assemble into complexes that mediate different fusion events. Assembly of distinct SNARE complexes is regulated by cognate SM proteins to ensure subcellular compartmental specificity, and that fusion only occurs in response to cellular needs and demand(3,9). Of three SM proteins, Munc18a has been best-studied(9-11), including orchestrating SG docking and priming(12,13), chaperoning and inducing activated conformation of Syn-1A(14-16), and facilitating membrane fusion(17,18). In β-cells, much is known about Munc18a and Syn-1A in mediating insulin exocytosis(19,20). Munc18c and cognate Syn-4 mediate GLUT-4 translocation to PM in adipose tissue and muscle and also some aspects of GSIS(3). Relatively little is known about Munc18b, which preferentially binds Syn-2 and Syn-3(21).

Here, we examined the endogenous function of Munc18b by depleting Munc18b in rat β-cells, which showed that Munc18b accounted for a remarkable 50% of GSIS attributed to almost half of primary exocytosis and almost all of sequential SG-SG fusion. Gain-of-function expression of Munc18b-WT and Munc18b-K314L/R315L(KR) mutant, the latter facilitating SNARE complex assembly(21), greatly increased these two exocytic events. Mechanistically, Munc18b activated and induced the formation of distinct Munc18b/Syn-2 and Syn-3 SM/SNARE
complexes that mediated the primary exocytosis and SG-SG fusion.

**RESEARCH DESIGN AND METHODS**

**Islet isolation, cell culture, insulin secretion assays**

Islets from Sprague-Dawley male rats (275-300g) were isolated by collagenase digestion as described previously(22). Islets and INS-1 (832/13, C. Newgard, Duke University, NC) were cultured in RPMI 1640 medium. Batches of 30 rat islets (or INS-1) were transduced with Munc18b lenti-shRNA (siRNA for INS-1) or AdMunc18b mutants, loaded in perfusion chambers (~1.3mL capacity) and perifused at a flow rate of ~1mL/min (37°C). Stimulation was with glucose in presence or absence of 10nM glucagon-like peptide-1 (GLP-1) plus 150μM IBMX as indicated. Insulin secreted was measured by RIA (Linco Research, St. Louis, MO). The experiments were approved by the Animal Care Committees of the University of Toronto and University of Tokyo.

**Silencing Munc18b expression and adenovirus construction**

Silencing of endogenous Munc18b was by two strategies, siRNAs for INS-1 cells and lenti-shRNAs for islets. For siRNAs, two 64 base pair sequences of siRNA duplex targeted to Munc18b were created corresponding to rat Munc18b cDNA (GeneBank Access Number AF263346), with sense and antisense sequences, and control scrambled sequences shown in Supplementary Table 1. Transfection of INS-1 cells was with Lipofectamine2000.

For construction of lentiviruses, pLKO-Munc18b-eCFP plasmid was created by modifying the parental pLKO-Munc18b-puro plasmid we previously described(15) by replacing puromycin-resistance gene with eCFP. Knockdown plasmid was co-transfected with psPAX2 and
pMDG2 into HEK-293FT cells to generate recombinant lentiviruses, lenti-shRNA/Munc18b-eCFP and lenti-eCFP (Control).

Munc18b mutants tagged with GFP in a separate transcription cassette we previously reported(21) were subcloned into pAdTrack shuttle vector and inserted into pAdeasy-1 backbone. Positive clones screened were used to transfect HEK293; viruses released from single plaques then amplified to high titers.

**Two-Photon microscopy.**

Two-photon extracellular polar tracer (TEP) imaging of exocytosis in islets was performed with an inverted laser-scanning microscope (IX81; Olympus, Tokyo, Japan) equipped with water-immersion objective lens at 60x (NA=1.2) and femtosecond laser (MaiTai, Spectral Physics; Mountain View, CA) as previously described(23). Insulin exocytosis was visualized(6) with 0.3mM Alexa fluor® Hydrazide (polar fluorescent tracer, Molecular Probes) and normalized to the value per 800 µm² (per cell). Sequential exocytosis was detected as progression of exocytosis towards the cell interior. Sequential exocytosis occurred far more frequently than predicted from coincidental full fusion of a vesicle followed by another vesicle. Such coincidental events within 1min and within 0.5µm laterally and 1µm axially (T=2µm) from initial events can be estimated from frequency of exocytosis per membrane area using Poisson statistics as previously described(6). Given that frequencies of exocytosis of 7.7, 14.1, 26.4 and 5.4 events/cell/min in AdGFP, AdMunc18b-WT, AdMunc18b-KR and AdMunc18b-E59K-expressing islets, the proportions of random simultaneous events are estimated as 0.12-0.51%, 0.22-0.92%, 0.42-1.73% and 0.086-0.36%, respectively. We previously confirmed with anti-insulin immunostaining that glucose-induced exocytotic responses imaged by our 2-photon microscope are from β-cells(7).

**Electron and confocal immunofluorescence microscopy.**
EM preparation as previously described(23) of 70nm-thick serial sectioning of AdMunc18b mutants-transduced islets was performed, followed by 3D reconstruction of the serial sections. 3-D modeling, texturing and rendering work were done with a trial version of Autodesk 3ds max9. As previously described(24), confocal microscopy was performed on an inverted confocal microscope system (DMIRE2, Leica Microsystems GmbH, Wetzlar, Germany), and deconvolution algorithm applied to images to remove out-of-focus background noise.

**Immunoprecipitation**

This was performed as we had described(24). 70-75% confluent INS-1 cells were transduced with a) Munc18b siRNA vs scrambled nonsense siRNA or b) mutant AdMunc18b mutants vs control AdGFP. INS-1 lysates were subjected to immunoprecipitation(IP) with specific 1.2-2.0µg syntaxin(-1A, -2 or -3) antibodies, and co-precipitated proteins identified with indicated antibodies by Western blotting. All were commercial antibodies except Munc18c (Y. Tamori, Kobe University, Japan), VAMP8 (W. Hong, Institute of Molecular and Cell Biology, Singapore), Syn-4 (J. Pessin, Albert Einstein College of Medicine, Bronx, NY) and Munc18b (generated by us)(21).

**Quantitative real-time PCR.** Total RNA was extracted from INS-1 (832/13) cells using TRIzol reagent and purified with RNeasy Kits according to manufacturer’s instructions. Purified RNA was converted to cDNA using SuperScript II RT; real-time PCR was performed using ABI PRISM 7900HT sequence detection system (PE Applied Biosystems, Foster City, CA) according to manufacturer’s protocol.

**Statistical analysis.** All data are presented as means±SEM. Statistical analysis was by Student’s t-test to compare two variables and ANOVA and Scheffe’ tests for multiple comparisons. Significance was $P<0.05$. 


RESULTS

Munc18b is a major regulator of biphasic GSIS in rat pancreatic islets

*Munc18b depletion in β-cells reduces biphasic GSIS.* Rat islets and INS-1 contain all three SM proteins, their four cognate syntaxins (Fig. 1A), SNAP25, VAMP-2 and -8. Syn-1A and Munc18a, known to drive primary exocytosis, are more abundant in PM (Fig. 1B). In contrast, Munc18b and cognate Syn-2 and Syn-3 are localized abundantly on insulin SGs, though not exclusively (Fig. 1B).

To examine the endogenous function of Munc18b, we employed lenti-shRNAs (co-expressing eCFP) to reduce Munc18b expression in rat islets (24hrs-32%, 48hrs-75%, 72hrs-97%, all \(P<0.05\), Fig. 1C). 48hr-transduced islets were used in the secretory studies as 72hr-treatment reduced secretory competence. CFP-expressing cells on outermost layers of the islet which were depleted of Munc18b were used for 2-photon imaging studies.

Physiologic 16.7mM GSIS was assessed by islet perifusion assay (Fig. 1D), which showed Munc18b depletion resulted in 44% reduction (AUC analysis: Munc18b shRNA:25.79±2.49; Control:45.54±2.37, \(P<0.001\)). Addition of cAMP-acting GLP-1 (10nM with 150µM IBMX) potentitated GSIS to ~3 times (Fig. 1D); Munc18b depletion reduced this by ~52% (Munc18b shRNA:68.63±19.3, Control:132.14±17.15, \(P<0.05\)). IBMX, a phosphodiesterase inhibitor which raises intracellular cAMP, was required to be added to GLP-1 to improve consistency. Consistently, Munc18b depletion in INS-1 averaging 86% reduction by either of two siRNAs (siMunc18b #1 and #2) caused a similar ~40% reduction in 10mM GSIS (data not shown).

*Gain-of-function Munc18b mutant potentiates biphasic GSIS.* As Munc18b preferentially binds and transduces its actions on Syn-2 and Syn-3, we had reported using *in vitro* binding
assays several Munc18b mutants which bind these syntaxins weaker than Munc18b-WT; Munc18b-K314L/R315L (abbreviated to KR) or not at all; Munc18b-E59K(21). We subcloned these constructs, each co-expressing EGFP to identify transfected cells (for 2-photon studies), into adenoviral vectors to transduce islets and INS-1. AdMunc18b mutant expression was ~4-5x that of endogenous levels (Fig. 2A); recombinant proteins were expressed in 60-70% of islet cells (GFP visualized by confocal microscopy). Syn-2 and Syn-3 localization to insulin SGs weren’t affected by any of the three Munc18b proteins (Fig. 2B; analysis by Pearson’s correlation, data not shown).

We examined the effects of AdMunc18b mutants on biphasic 16.7mM GSIS (Fig. 2C) and 10nM GLP-1-potentiated GSIS (Fig. 2D), the latter ~4-5-fold that stimulated by 16.7mM glucose alone. Munc18b mutant expression in islets didn’t affect the levels of syntaxins or cognate SNARE proteins (Fig. 2A). AdMunc18b-WT caused potentiation in 1\textsuperscript{st}-phase GSIS (encompassing 12-22mins in Fig. 2C, lower panel) by 63% (20.1±2.2; AdGFP:12.3±1.6, \( P=0.03 \)) and 2\textsuperscript{nd}-phase GSIS (encompassing 22-40mins) by 43% (36.0±1.6; AdGFP:25.2±1.8, \( P=0.02 \)). AdMunc18b-KR potentiated 1\textsuperscript{st}-phase GSIS by 95% (24.0±3.1, \( P=0.003 \)) and 2\textsuperscript{nd}-phase by 83% (46.2±4.2, \( P=0.003 \)), the latter effect higher than AdMunc18b-WT. AdMunc18b-E59K, intended to induce inhibition, however did not cause significant reduction (1\textsuperscript{st}-phase: 10.1±2.4, 2\textsuperscript{nd}-phase: 21.5±3.6). GLP-1-potentiated GSIS (Fig. 2D, lower panel) was remarkably similar in pattern to 16.7mM GSIS, with KR (1\textsuperscript{st}-phase:87.0±1.4, 2\textsuperscript{nd}-phase:257.4±5.5) > WT (1\textsuperscript{st}-phase:56.6±3.4, 2\textsuperscript{nd}-phase:198.1±20.9) > GFP (1\textsuperscript{st}-phase:46.4±3.4, 2\textsuperscript{nd}-phase:136.1±4.3). E59K (1\textsuperscript{st}-phase:40.7±3.9, 2\textsuperscript{nd}-phase:162±12.3) and GFP weren’t significantly different.

Like the islet study, overexpression of AdMunc18b constructs in INS-1 was ~4 times those of untransfected and AdGFP-transfected cells (Supplementary Fig. 1), but with 100%
transduction efficiency (all cells are green from co-expressed GFP) required for use in protein-binding studies. As with the islet study, expression of these Munc18b proteins didn’t affect the cellular levels of Syn-2 or Syn-3, which was confirmed at the mRNA level (Supplementary Fig. 2). There were also no effects on the levels of other syntaxins (Syn-1A, Syn-4), SNAP25 or SM proteins (Munc18a, Munc18c) (Supplementary Fig. 1). Effects of these Munc18b proteins on INS-1 GSIS (Fig. 2E) were similar to the islet results. Basal insulin release wasn’t affected by AdMunc18b mutants. GSIS (10mM; determined as stimulated minus basal values) was potentiated by AdMunc18b-KR expression by 98% (2.14±0.09%; AdGFP:1.08±0.80%, P<0.05) and 48% by AdMunc18b-WT expression (1.60±0.11%, P<0.05), which was lower than AdMunc18b-KR (P=0.007). AdMunc18b-E59K had no significant effect (0.86±0.05%). 10nM GLP-1-potentiated GSIS (Fig. 2E) to ~4x of 10mM GSIS alone, had similar a trend as GSIS alone, wherein AdMunc18b-KR (7.71±0.59%) and AdMunc18b-WT (5.99±0.19%) stimulated secretion by 68% and 30%, respectively, over AdGFP (4.60±0.16, P<0.05), with AdMunc18b-KR inducing higher secretion than AdMunc18b-WT (P=0.012); AdMunc18b-E59K (4.98±0.22%) didn’t cause significant change compared to AdGFP. Thus, Munc18b KD and mutants affected GSIS in INS-1 cells similarly as in β-cells.

**Munc18b controls the formation of distinct SM-activated SNARE complexes**

*Munc18b depletion reduces formation of distinct SM-activated SNARE complexes.*

INS-1 cell line was used as surrogate for β-cells to provide an abundance of protein required for protein-binding studies. We conducted co-IP experiments with antibodies to Syn-1A, Syn-2 and Syn-3 on scrambled (control) and Munc18b-siRNA-treated INS-1 cells, performed at non-stimulatory (0.8mM glucose) and maximal stimulatory conditions (preincubation with GLP-1+IBMX followed by 16.7mM glucose, Fig. 3: *left panels*-co-IPs, *right panels*-total lysate input.
controls; analysis in Supplementary Fig. 3).

In control scrambled siRNA-treated cells under non-stimulatory conditions, Syn-2 (Fig. 3B) and Syn-3 (Fig. 3C) bound Munc18b but very little VAMPs or SNAP25. Only upon stimulation which presumably ‘activates’ Munc18b, we observed formation of full and distinct SNARE complexes, with Syn-3 preferring to bind VAMP8, and Syn-2 binding both VAMPs (VAMP2>VAMP8). These results are consistent with our recent reports showing VAMP8 formed complexes with Syn-2 and Syn-3, but not Syn-1A, and which mediated additional primary exocytosis of newcomer insulin SGs(24), and SG-SG fusion in pancreatic acinar cells(25).

Remarkably, Munc18b depletion by Munc18b siRNA near-totally disabled Syn-2 (Fig. 3B) and Syn-3 (Fig. 3C) from forming complexes with both VAMPs and SNAP25. Disruption of SNARE complexes pulled down by Syn-3 antibody co-IP matched the ~80% reduction in Munc18b levels (Supplementary Fig. 3); disruption of SNARE complexes pulled down by Syn-2 antibody was slightly less (~70%). This is remarkable considering the total cellular levels of these SNARE proteins as shown in inputs controls were unaffected and were expected to be available to participate in SNARE complex formation. Munc18b depletion did not affect the amount of Munc18a co-IPed, indicating that formation of SNARE complexes by Syn-2 and Syn-3 is primarily through Munc18b. In contrast, Syn-1A co-IPed endogenous Munc18a and remained able to form complexes with SNAP25 and VAMP2 (Fig. 3A, Supplementary Fig. 3), inspite of Munc18b depletion; this indicates that Munc18a and not Munc18b is the primary SM protein to activate Syn-1A to form SNARE complexes.

**Gain-of-function Munc18b mutant promotes formation of distinct SM-activated SNARE complexes.** The ability of Ad-Munc18b-WT and KR mutant to potentiate GSIS (Fig. 2C-E) suggests that additional Munc18b-activated SNARE complexes must be formed to mediate the
additional exocytosis (Figs. 5-7). Association of Munc18b for native β-cell syntaxins likely within the context of assembled SNARE complexes, either at basal or stimulatory conditions, was unaffected by Munc18b-WT or Munc18b-KR (Fig. 4), but compromised by Munc18b-E59K. These results are different from our previous report(21) showing in vitro Munc18b-KR reduced association and Munc18b-E59K displayed almost no binding to uncomplexed recombinant syntaxins. This emphasizes the importance of conducting mutant analysis in native cells, which we here further examined such SM-activated SNARE complexes formed during basal and stimulated conditions. From AdMunc18b-KR and AdMunc18b-WT-transduced cells, abundant SM-activated SNARE complexes were indeed precipitated more than controls, with Munc18b-KR associated with more Syn-1A, Syn-2 and Syn-3 SNARE complexes than Munc18b-WT (analysis in Supplementary Fig. 4). Syn-3 (Fig. 4C), the putative syntaxin mediating SG-SG fusion(25), formed a complex with Munc18b to pull down exclusively VAMP8, along with SNAP25. A caveat in these co-IP studies is the possibility that syntaxin antibodies might be pulling down SM proteins independently from SNARE complexes, and hence may not be SM/SNARE quaternary protein complexes per se. Our Munc18b antibodies, although more appropriate, are not suitable for co-IP assays.

Since Munc18b also binds Syn-1A(26), Munc18b formed SNARE complexes with Syn-1A, SNAP25, and preferentially VAMP2 (Fig. 4A left panel). These actions of Munc18b mutants on Syn-1A mimicked those of Munc18a, thus likely serve similar functions as Munc18a in mediating primary exocytosis. The order for Munc18b mutant proteins inducing such SM-SNARE complexes is KR>WT>GFP>E59K (analyses in Supplementary Fig. 4) corresponded to their abilities to potentiate biphasic GSIS (Fig. 2C-D). What is less clear is the role of Syn-2, whose role is also unclear in the broader field of exocytosis. Syn-2’s ability to be induced by
Munc18b proteins to form SNARE complexes (KR>WT) with VAMP2 and VAMP8 (Fig. 4B, Supplementary Fig. 4), taken along with reduction of such complexes by Munc18b depletion (Fig. 3B), suggest that Syn-2 might share redundant functions with Syn-1A and Syn-3, respectively.

Munc18b-E59K bound the syntaxins under basal conditions, but upon stimulation wouldn’t induce syntaxins to bind SNAP25 or VAMPs, where levels of SM-activated SNARE complexes were similar or lower than AdGFP. Munc18b-E59K/Syn complexes were essentially functionally inert and didn’t significantly compete with endogenous Munc18b for any function essential for secretion. This explains why GSIS was similar between Munc18b-E59K and GFP control (Fig. 2C-E).

Taken together, the action of KR point mutant of Munc18b would mediate formation of activated states of SM-SNARE complexes (Fig. 4). Formation of SNARE complex by glucose stimulation, and its potentiation by Munc18b, is consistent with post-stimulus assembly of SNAREs(23,27) in insulin exocytosis.

**Munc18b gain-of-function promotes sequential SG-SG fusion and primary exocytosis**

We previously reported that sequential insulin SG-SG fusion visualized by 2-photon imaging occurs in β-cells(7), and mediated by diffusion of SNAP25 in PM into fused SGs following assembly of SNARE complex(6). We thus postulate that Munc18b-activated Syn-3/SNAP25/VAMP8 complexes might increase sequential SG-SG fusion, and examined whether overexpression of the Munc18b mutants could influence primary exocytosis and SG-SG fusions by 2-photon imaging analysis(6,7). Here, we also employed GLP-1+IBMX to maximally
potentiate 20mM glucose-stimulated insulin exocytosis, including maximal induction of sequential SG-SG exocytosis(22).

First we showed examples of single insulin SG fusion and sequential SG-SG exocytosis. Figure 5A shows a full fusion exocytosis of a single SG (Fig. 5Aii from inset in Fig. 5Ai) from AdGFP-transduced rat islet, with each vertical indicator temporally tracking the analysis of kinetics of these events in Figure 5Aiii, corresponding to images in Figure 5Aii. This represents fusion of an insulin SG at 1.2s (white arrow, Fig. 5Aii middle image indicates direction of fusion); upon discharge of contents at 14.6s (dotted circle, Fig. 5Aii right image), it collapses into the PM to create an omega figure. The most frequent mode of sequential exocytosis observed was that of two SGs; an example shown in Figure 5B in AdMunc18b-KR-transduced islet (Fig. 5Bii is inset in islet in Fig. 5Bi). Here, the first SG fuses with PM at 1.3s (arrow 1 indicates direction of fusion, Fig. 5Bii), then partially empties (dashed circle at 4.4s, Fig. 5Bii). Then, <2s later, it’s followed by fusion of a second SG at 5.6s coming from another direction (arrow 2) deeper into the cytosol relative to the first exocytosing SG. This resulted in a larger and brighter area of fluorescence intensity (temporal analysis in Fig. 5Biii) since it emanated from a 2-SG fusion, and is followed by a slower decline (than Fig. 5Aii) in fluorescence intensity reflecting insulin cargo emptying and eventual collapse of the compound granule onto the PM.

Employing this analysis, we counted the total number of exocytotic events as determined by number of omega figures (Fig. 5C, exocytotic events/cell/min) which showed AdMunc18b-KR induced the most exocytotic events (26.39±2.89, 58 cells/6 islets), followed by AdMunc18b-WT (14.06±1.39, 44 cells/7 islets), AdGFP (7.71±0.89, 68 cells/8 islets), and lastly AdMunc18b-E59K (5.4±0.53, 75 cells/5 islets). We then examined how many of these exocytotic events exhibited sequential exocytosis (Fig. 5D). AdMunc18b-KR (11.2±1.4%) and AdMunc18b-WT
(7.1±1.7%) induced ~3.2 and 2 times higher sequential exocytosis, respectively, than AdGFP-expressing cells (3.5±1.0%, \( P<0.05 \)). Sequential exocytosis constituted only 1.6±0.6% of total exocytotic events in AdMunc18b-E59K-expressing β-cells. We assessed the percentage of cells undergoing sequential exocytosis (vs no sequential exocytosis), which was higher in AdMunc18b-KR (74.1%) and AdMunc18b-WT (54.5%)-transduced cells, compared to AdGFP (30.9%) and AdMunc18b-E59K (13.3%) expression. These results indicate that Munc18b-KR and Munc18-WT expression recruited more β-cells to exhibit sequential exocytosis.

Considering that AdMunc18b-KR-infected β-cells exhibited the highest percentage (11.2%) of exocytotic events being sequential SG-SG fusion, majority of the increase in total exocytosis events in Figure 5C, ~89% is thus primary exocytosis. For AdMunc18b-WT expression, primary exocytosis accounted for 93% of total exocytotic events. It therefore appears that it’s the combination of a large increase in both primary exocytosis and SG-SG fusions that accounts for overall increase in biphasic GSIS caused by Munc18b-KR and -WT overexpression observed in Figure 2C-E. Munc18b-E59K expression apparently couldn’t induce a significant dominant-negative effect on exocytosis by competition with endogenous Munc18b, and thus didn’t cause detectable reduction in GSIS (Fig. 2C-E).

**Munc18b can mediate long-chain sequential SG fusion.** Long-chain sequential exocytosis is the mode of exocytosis in highly efficient secretory cells like mast cells(28), eosinophils(5) and pancreatic acinar cells, but not in less efficient β-cells. The molecular mechanism underlying long-chain sequential exocytosis is unknown. We postulated that up-regulating Munc18b expression or action could induce β-cells to increase the number of SGs participating in long-chain sequential SG-SG fusion. We therefore assessed the proportions of the different extent of sequential SG fusions as determined by number of omega figures (Fig. 6A). Both AdMunc18b-
KR and –WT transduced cells had higher order SG-SG fusions of up to five long-chain sequential SG-SG fusions. AdGFP cells only had up to 2-SG sequential fusions as is the case with AdMunc18b-E59K, the latter at much reduced frequency. The distinct increases in fluorescence predict the sizes of SGs of about 0.3µm as shown in Supplementary Figure 5. This value is consistent with reported EM analysis of insulin SG size (as in Fig. 6C), and strongly supports our interpretation that increases in fluorescence in our 2-photon studies genuinely reflected exocytosis of insulin SGs.

We occasionally found very long-chain sequential SG fusions in AdMunc18b-KR expressing β-cells (Fig. 6B, corresponding Supplementary Movie 1), which shows sequential fusion of seven insulin SGs. The time course in this exocytosis event shows an initial delay in fusion of the second SG (Fig. 6Bii) followed by rapid successive fusions up to fifth SG, whose positions are indicated by dashed circles (Fig. 6Bi); temporal analysis tracking kinetics of fusion events correspondingly numbered in Figure 6Bii, (Supplementary Movie 1). The diameters of puncta are noted to actually increase with each successive fusion. Supplementary Movie 1 better shows events 2, 3 and 4 to be associated with abrupt changes in shapes of the compound SGs in addition to increases in fluorescence. In Figure 6Bi, arrows shown indicate direction of exocytosis of individual SGs, which are distinct from event to event. The focal plane wasn’t deviated during acquisition of images (see Supplementary Fig. 6) where low magnification images demonstrated that landmarks weren’t changed at all. This rapid successive fusion might be due to ‘primed’ insulin SGs (3rd-5th SGs, and resulting sustained plateau of fluorescence intensity (Fig. 6Bii) might reflect slower flattening of compound SGs. Note that positions of compound SGs (point of highest fluorescent intensity) resulting from each sequential SG-SG fusion tend to shift from previous positions (dashed circles, Fig. 6Bi). This suggests that fusion pores of primary SGs were stably maintained even though its position was altered by subsequent
sequential exocytosis. These events are also better observed in **Supplementary Movie 1**.

We next visualized these very-long chains of sequentially-fused SGs induced by $Ad$Munc18b-KR expression by EM (**Fig. 6C**). Since a single EM slice (70nm-thick) won’t capture such a large number of sequential-fused SGs (encompassing 2-3 SG diameters, ~300nm diameter per SG), we sectioned numerous consecutive 70nm-thick slices. **Fig. 6C** shows EM images from four-consecutive slices (of 8 slices) exhibiting distinct SG-SG fusions (green-numbered asterisks) visualized in different slices. Here, exocytotic fusion of SG1 (G1) formed a large fusion pore with PM sufficient to empty the insulin cargo (slice 4). Note that some SGs appeared deceivingly empty on one slice (G2, slices 3 and 4), yet actually have complete core cargo in another slice (G2, slice 1). We created cartoons of these four slices (left cartoons) to reconstruct a 3D image (middle and right cartoons) that more clearly shows the relationships of these six sequentially-fused SGs (fusion pores indicated by numbered asterisks in EM images and blow-up cartoon images (middle cartoons). **Supplementary Figure 7** shows another example of a long-chain (seven SGs) sequential fusion, with G2 and G7 undergoing fusion with PM. In both (and other) examples, we consistently saw sequentially-fused SGs arranged in parallel and close to the PM, as was the case with our 2-photon study, where compound SGs never reached further than 2µm from the PM. From these EM images, we quantified the number of SGs within 120nm from the PM that would participate in these exocytotic events (**Fig. 6D**), which was increased by ~2.1 times in $Ad$Munc18b-KR (36.42±0.5 SGs, p<0.01) compared to $Ad$GFP cells (17.5±0.6 SGs), and ~1.5 times in $Ad$Munc18b-WT cells (27.11±0.4 SGs, p <0.05); and mild reduction of ~27% in $Ad$Munc18b-E59K-infected cells (11.52±0.7 SGs, p< 0.05).

**Depletion of endogenous Munc18b reduces primary exocytosis by half and completely abrogates sequential SG-SG fusion**
Finally, we examined how primary and sequential exocytoses would be diminished by lenti-Munc18b-shRNA depletion of endogenous Munc18b in β-cells. We compared these exocytotic events in eCFP-expressing (therefore Munc18b-depleted) β-cells of lenti-Munc18b-shRNA-eCFP-treated (48hrs) islets versus Control lenti-eCFP-expressing β-cells. Total exocytotic events (Fig. 7A) in Munc18b-depleted (67 cells/8 islets) vs control β-cells (48 cells/8 islets) over a 10min period of recording were 9.47±0.65 vs 6.1±0.50, respectively, which is a ~40% reduction (P<0.001). The distribution of these exocytotic events over time (exocytotic events/cell, Fig. 7B) showed a 35% reduction (Control:49.4±7.9; Munc18b-shRNA:31.9±4.2, P<0.05) in the first 5min, and 41% reduction (Control 44.2±8.8; Munc18b shRNA:26±5.1, P<0.05) in the second 5min (Fig. 7B right panel). This extent and pattern of sustained reduction of exocytosis over time remarkably mimicked the sustained suppression of GSIS observed in the islet perifusion results (Fig. 1E). We examined how many of these exocytotic events exhibited sequential exocytosis (Fig. 7C). Of the total number of exocytotic events, sequential exocytotic events in control β-cells constituted only 3.57±0.59%, consistent with our previous report(7). Remarkably, Munc18b depletion reduced this to 0.6±0.22%, which is an 83% reduction (P<0.001), indicating almost total abrogation.

**DISCUSSION**

This work revealed that Munc18b-mediated pathways in β-cells account for half of the physiologic biphasic GSIS, and that Munc18b mediated glucose-induced assembly of SNAREs to effect primary exocytosis and sequential SG-SG fusion. We discuss several points below that led us to these conclusions.

First, Munc18b deletion abolished half of primary exocytosis and almost all sequential
SG-SG fusion. Conversely, overexpression of Munc18b increased insulin exocytosis as reported(29), and overexpression of dominant-positive Munc18b-KR mutant greatly up-regulated recruitment of insulin SGs to PM to undergo increased primary exocytosis and sequential SG-SG fusions, leading to ~50% (Munc18b-WT) and ~100% increase (Munc18b-KR) in biphasic GSIS, respectively. Munc18b-E59K, caused some disruption of SNARE complexes but had only minor insignificant reduction on exocytosis events likely because of promiscuous binding of endogenous Munc18b to several SNARE complexes conferring redundant actions on exocytosis. Although SG-SG fusion contributed to a small degree (3-12%) of total insulin exocytosis, it may play a more important role in other experimental conditions, such as cholinergic stimulation shown to induce compound exocytosis(30).

Second, only upon stimulation would Munc18b become activated to effect formation of distinct SNARE complexes required for SG-SG fusion and primary exocytosis. Munc18b on SGs could bind Syn-1A on PM perhaps during SG approach to the PM; this SM-activated SNARE complex may also participate in primary exocytosis. These roles of Munc18b are similar to Munc18a(18) and Munc18c(31) in inducing formation of their respective SNARE complexes. These actions of Munc18b are in line with reports showing glucose stimulation induces SNARE assembly(23) and vesicle docking(32,33) leading to insulin exocytosis. Activation of Munc18b is likely by PKC phosphorylation, reminiscent of Munc18a(34), likely at Ser313 that’s conserved in both.

Third, Munc18b-KR-activated SNARE complexes represent superior ‘activated’ conformations than WT-Munc18b-activated complexes, similar to the Munc18a-SNARE complex(14,15). Munc18b-KR-activated SNARE complexes thus enabled enhanced efficiency of SG-SG and SG-PM fusions resulting in larger increases in GSIS and GLP-1-potentiated GSIS than Munc18b-WT-activated SNARE complexes. We estimate in Munc18b-KR-expressing islets
that sequential insulin exocytosis *per se* stimulated by maximal cAMP enhancement was augmented three times (11%), with infrequent artificially-enhanced very long-chain sequential SG fusion occurring more in Munc18-KR-transduced β-cells, along with recruitment of more β-cells to exhibit sequential exocytosis. This is a large increase considering that sequential exocytosis (normally only 2-3 SGs) occurs normally in only 2-3% of β-cells(6,7).

Finally, what are the v-SNAREs and t-SNAREs activated by Munc18b that specifically mediate SG-SG fusion and primary exocytosis. We recently reported that primary exocytosis of newcomer insulin SGs is mediated by VAMP8(24) and Syn-3(35), and that insulin SG-SG fusion is mediated Syn-3(35). Indeed, both these SNARE proteins were co-IPed by Munc18b in this study. Nonetheless, some of the increase in primary exocytosis is likely mediated by Syn-1A(19), which could also be activated by Munc18b to form SM-SNARE complexes with VAMP2. It is conceivable that enhancing these actions (or expression) of Munc18b-activated SNARE complexes in mobilizing more insulin SGs (‘newcomer’ SGs) to PM to undergo sequential SG-SG fusion and primary exocytosis, which are responsive to GLP-1 stimulation, could compensate and be deployed to treat the exocytotic defects of diabetic β-cells to attain normoglycemic control(36).

ACKNOWLEDGEMENTS

This work was supported by grants to HYG (Canadian Institute of Health Research (CIHR), MOP89889 and MOP 86544), to HK (Grants-in-Aids for Specially Promoted Area No. 2000009; Global COE Program (Integrative Life Science Based on the Study of Biosignaling Mechanisms) from Ministry of Education, Culture, Sports, Science, and Technology, Japan, CREST of JST, a Research Grant from Human Frontier Science Program), and to VMO (Academy of Finland, grants 50641 and 121457; Sigrid Juselius Foundation). P.P.L.L. was funded by doctoral
studentships from Canadian Digestive Health Foundation and CIHR, S.D. is funded by a postdoctoral fellowship from Banting and Best Diabetes Center, University of Toronto.

**Author’s contribution.** H.Y.G., H.K. and P.P.L.L. formulated the original hypothesis. H.Y.G and P.P.L.L. designed the molecular and biochemical experiments, which were performed by P.P.L.L., Y.H., S.D., Y.K., and W.C.Y.W. M.O., N.T., and H.K. performed the 2-photon imaging experiments and analysis. V.M.O. designed the Munc18b mutant constructs and M.K. subcloned these mutants into adenoviruses. S.S. and N.R.B. designed and generated the Munc18b lentishRNA. S.D. performed the immunoprecipitation studies. Y.H., P.P.L.L., L.X. and D.Z. performed the E.M. experiments. T.Q. and Y.L. performed islet perfusion experiments. T.L. performed the confocal quantification analysis. H.Y.G., H.K., V.M.O, P.P.L.L. and M.O. wrote the manuscript. All authors discussed the results and commented on the manuscript. H.Y.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors declare no conflict of interest.

**REFERENCES:**

1. Hosker JP, Rudenski AS, Burnett MA, Matthews DR, Turner RC: Similar reduction of first- and second-phase B-cell responses at three different glucose levels in type II diabetes and the effect of gliclazide therapy. Metabolism 1989;38:767-772
2. Kwan EP, Gaisano HY: Rescuing the subprime meltdown in insulin exocytosis in diabetes. Ann N Y Acad Sci 2009;1152:154-164
3. Jewell JL, Oh E, Thurmond DC: Exocytosis mechanisms underlying insulin release and glucose uptake: conserved roles for Munc18c and syntaxin 4. Am J Physiol Regul Integr Comp Physiol 2010;298:R517-531
4. Fernandez JM, Neher E, Gomperts BD: Capacitance measurements reveal stepwise fusion events in degranulating mast cells. Nature 1984;312:453-455
5. Hafez I, Stolpe A, Lindau M: Compound exocytosis and cumulative fusion in eosinophils. J Biol Chem 2003;278:44921-44928
6. Takahashi N, Hatakeyama H, Okado H, Miwa A, Kishimoto T, Kojima T, Abe T, Kasai H: Sequential exocytosis of insulin granules is associated with redistribution of SNAP25. J Cell Biol 2004;165:255-262
7. Takahashi N, Kishimoto T, Nemoto T, Kadowaki T, Kasai H: Fusion pore dynamics and insulin granule exocytosis in the pancreatic islet. Science 2002;297:1349-1352
8. Kasai H, Takahashi, N., and Tokumaru, H.: Distinct initial SNARE configurations underlying the diversity of exocytosis. Physiol Rev 2012;92:In press
9. Sudhof TC, Rothman JE: Membrane fusion: grappling with SNARE and SM proteins. Science 2009;323:474-477
10. Burgoyne RD, Barclay JW, Ciufo LF, Graham ME, Handley MT, Morgan A: The functions of Munc18-1 in regulated exocytosis. Ann N Y Acad Sci 2009;1152:76-86
11. Rizo J, Rosenmund C: Synaptic vesicle fusion. Nat Struct Mol Biol 2008;15:665-674
12. Deak F, Xu Y, Chang WP, Dulubova I, Khvotchev M, Liu X, Sudhof TC, Rizo J: Munc18-1 binding to the neuronal SNARE complex controls synaptic vesicle priming. J Cell Biol 2009;184:751-764
13. Voets T, Toonen RF, Brian EC, de Wit H, Moser T, Rettig J, Sudhof TC, Neher E, Verhage M: Munc18-1 promotes large dense-core vesicle docking. Neuron 2001;31:581-591
14. Gerber SH, Rah JC, Min SW, Liu X, de Wit H, Dulubova I, Meyer AC, Rizo J, Arancillo M, Hammer RE, Verhage M, Rosenmund C, Sudhof TC: Conformational switch of syntaxin-1 controls synaptic vesicle fusion. Science 2008;321:1507-1510
15. Han L, Jiang T, Han GA, Malintan NT, Xie L, Wang L, Tse FW, Gaisano HY, Collins BM, Meunier FA, Sugita S: Rescue of Munc18-1 and -2 double knockdown reveals the essential functions of interaction between Munc18 and closed syntaxin in PC12 cells. Mol Biol Cell 2009;20:4962-4975
16. Arunachalam L, Han L, Tassew NG, He Y, Wang L, Xie L, Fujita Y, Kwan E, Davletov B, Monnier PP, Gaisano HY, Sugita S: Munc18-1 is critical for plasma membrane localization of syntaxin1 but not of SNAP-25 in PC12 cells. Mol Biol Cell 2008;19:722-734
17. Rodkey TL, Liu S, Barry M, McNew JA: Munc18a scaffolds SNARE assembly to promote membrane fusion. Mol Biol Cell 2008;19:5422-5434
18. Shen J, Tareste DC, Paumet F, Rothman JE, Melia TJ: Selective activation of cognate SNAREpins by Sec1/Munc18 proteins. Cell 2007;128:183-195
19. Ohara-Imaizumi M, Fujiwara T, Nakamichi Y, Okamura T, Akimoto Y, Kawai J, Matsushima S, Kawakami H, Watanabe T, Akagawa K, Nagamatsu S: Imaging analysis reveals mechanistic differences between first- and second-phase insulin exocytosis. J Cell Biol 2007;177:695-705
20. Oh E, Kalwat MA, Kim MJ, Verhage M, Thurmond DC: Munc18-1 Regulates First-phase Insulin Release by Promoting Granule Docking to Multiple Syntaxin Isoforms. J Biol Chem 2012;287:25821-25833
21. Kauppi M, Wohlfahrt G, Olkkonen VM: Analysis of the Munc18b-syntaxin binding interface. Use of a mutant Munc18b to dissect the functions of syntaxins 2 and 3. J Biol Chem 2002;277:43973-43979
22. Kwan EP, Gaisano HY: Glucagon-like peptide 1 regulates sequential and compound exocytosis in pancreatic islet beta-cells. Diabetes 2005;54:2734-2743
23. Takahashi N, Hatakeyama H, Okado H, Noguchi J, Ohno M, Kasai H: SNARE conformational changes that prepare vesicles for exocytosis. Cell Metab 2010;12:19-29
24. Zhu D, Zhang Y, Lam PP, Dolai S, Liu Y, Cai EP, Choi D, Schroer SA, Kang Y, Allister EM, Qin T, Wheeler MB, Wang CC, Hong WJ, Woo M, Gaisano HY: Dual Role of VAMP8 in Regulating Insulin Exocytosis and Islet beta Cell Growth. Cell Metab 2012;16:238-249
25. Cosen-Binker LI, Binker MG, Wang CC, Hong W, Gaisano HY: VAMP8 is the v-SNARE that mediates basolateral exocytosis in a mouse model of alcoholic pancreatitis. J Clin Invest 2008;118:2535-2551
26. Hata Y, Slaughter CA, Sudhof TC: Synaptic vesicle fusion complex contains unc-18 homologue bound to syntaxin. Nature 1993;366:347-351
27. Song WJ, Seshadri M, Ashraf U, Mdluli T, Mondal P, Keil M, Azevedo M, Kirschner LS, Stratakis CA, Hussain MA: Snapin mediates incretin action and augments glucose-dependent insulin secretion. Cell Metab 2011;13:308-319
28. Alvarez de Toledo G, Fernandez JM: Compound versus multigranular exocytosis in peritoneal mast cells. J Gen Physiol 1990;95:397-409
29. Mandic SA, Skelin M, Johansson JU, Rupnik MS, Berggren PO, Bark C: Munc18-1 and Munc18-2 proteins modulate beta-cell Ca2+ sensitivity and kinetics of insulin exocytosis differently. J Biol Chem 2011;286:28026-28040
30. Hoppa MB, Jones E, Karanauksaite J, Ramracheya R, Braun M, Collins SC, Zhang Q, Clark A, Eliasson L, Genoud C, Macdonald PE, Monteith AG, Barg S, Galvanovskis J, Rorsman P: Multivesicular exocytosis in rat pancreatic beta cells. Diabetologia 2012;55:1001-1012
31. Latham CF, Lopez JA, Hu SH, Gee CL, Westbury E, Blair DH, Armishaw CJ, Alewood PF, Bryant NJ, James DE, Martin JL: Molecular dissection of the Munc18c/syntaxin4 interaction: implications for regulation of membrane trafficking. Traffic 2006;7:1408-1419
32. Shibasaki T, Takahashi H, Miki T, Sunaga Y, Matsumura K, Yamanaka M, Zhang C, Tamamoto A, Satoh T, Miyazaki J, Seino S: Essential role of Epac2/Rap1 signaling in regulation of insulin granule dynamics by cAMP. Proc Natl Acad Sci U S A 2007;104:19333-19338
33. Kasai K, Fujita T, Gomi H, Izumi T: Docking is not a prerequisite but a temporal constraint for fusion of secretory granules. Traffic 2008;9:1191-1203
34. Wierda KD, Toonen RF, de Wit H, Brussaard AB, Verhage M: Interdependence of PKC-dependent and PKC-independent pathways for presynaptic plasticity. Neuron 2007;54:275-290
35. Zhu D, Koo E, Kwan E, Kang Y, Park S, Xie H, Sugita S, Gaisano HY: Syntaxin-3 regulates newcomer insulin granule exocytosis and compound fusion in pancreatic beta cells. Diabetologia 2013;56:359-369
36. Gaisano HY: Deploying insulin granule-granule fusion to rescue deficient insulin secretion in diabetes. Diabetologia 2012;55:877-880

FIGURE LEGENDS

FIG. 1. Munc18b is a major mediator of GSIS in pancreatic islet β-cells

(A) Pancreatic β-cells (rat islets, INS-1) express SM proteins and Syntaxins. Rat pancreatic acini and brain were used as positive and negative controls.
**B** Immunofluorescence images showing cognate Munc18b and Syn-2 and Syn-3 are abundant in insulin SGs in rat β-cells. Cognate Munc18a and Syn-1A are abundant in the PM. These images are representative of 4 independent experiments. *Scale bars*, 10µm.

**C** Munc18b depletion in rat islets by lenti-Munc18b shRNA/eCFP vs eCFP control. *Top*: representative blots, *bottom*: analysis of 3 experiments, shown as means ± SEMs. *P<0.05, **P<0.01.

**D** Islet perifusion assays of 48hrs lenti-shRNA/Munc18b-eCFP (vs lenti-eCFP, Control) depletion of endogenous Munc18b in rat islets showing reduction of GSIS and GLP-1 (10nM) + IBMX (150µM) potentiated GSIS, shown as means ± SEMs of 3 independent experiments, and their area under the curve (AUC) analysis; *P<0.05, ***P<0.001.

**FIG. 2. Munc18b gain-of-function mutant potentiates GSIS**

**A** AdMunc18b mutants (-WT, -KR, -E59K) transduction of rat islets did not influence the expression of SNARE or other SM proteins. Shown are representative of 3 independent experiments; analysis of Munc18b proteins expression shown in *bottom*.

**B** Confocal imaging showing AdMunc18b mutants did not influence Syn-2 or Syn-3 targeting to insulin SGs in rat β-cells. Shown are representative of 4 independent experiments. *Scale bars*, 10µm.

**C, D** Islet perifusion assays showing AdMunc18b mutants-transduced rat islets influencing GSIS (in C) and 10nM GLP-1 +150µM IBMX-potentiated GSIS (in D). Data shown are means ± SEMs from 3-4 sets of experiments, each experiment performed simultaneously on all the 4 conditions. *Bottom panels* show quantification of AUC analysis of 1st Phase (encompassing 11-22min) and 2nd Phase (encompassing 22-40min) GSIS. *P<0.05, **P<0.01, ***P<0.001 by one-way ANOVA.
(E) Effects of AdMunc18b mutants on GSIS and 10nM GLP-1 +150µM IBMX-potentiated GSIS in INS-1 cells. Results shown are means ± SEMs from 3 independent experiments performed in duplicates or triplicates (n=6-8). *P<0.05 by ANOVA and Scheffe tests.

FIG. 3. Munc18b depletion in INS-1 cells disrupts SM-activated SNARE complex formation.

INS-1 cells were transduced with Munc18b siRNA or control scrambled RNA, then kept at basal condition (0.8 mM glucose) or simulated with 16.7 mM glucose+10 nM GLP-1 with IBMX (150 µM), then subjected to co-ip (left panels) with antibodies against (A) Syn-1A, (B) Syn-2 or (C) Syn-3. Corresponding right panels show ‘Input’ controls (25µg protein, total INS-1 lysates), confirmed the reduction in Munc18b levels and similar levels of indicated SNARE proteins. Results shown are representative of 3 independent experiments, with densitometry analyses in Supplementary Figure 3.

FIG. 4. Munc18b gain-of-function mutant increases SM-activated SNARE complex formation

INS-1 cells were transduced with AdMunc18 mutants, kept in non-stimulated (lanes 1-4) or simulated (lanes 5-8) condition as in Fig 3, and subjected to co-ip (left panels) with antibodies against (A) Syn-1A, (B) Syn-2 or (C) Syn-3. Exogenous Munc18b (tagged with Myc) expression was detected by Myc antibody. Corresponding right panels are ‘Input’ controls (25µg protein, total INS-1 lysates) showing similar levels of indicated SNARE proteins. Results shown are representative of 3 independent experiments, with densitometry analyses shown in Supplementary Figure 4.
FIG. 5. Munc18b mediates primary exocytosis and sequential SG-SG fusion in rat β-cells

(A) Example of TEP imaging of single insulin SG exocytosis in an AdGFP-transduced rat islet stimulated with 20 mM glucose plus 10 nM GLP-1 and 150 µm IBMX in a solution containing 0.3 mM Alexa fluor® 594 hydrazide. An omega construction appeared when the SG(s) coalesced with the PM.

(B) Example of TEP imaging of sequential exocytosis involving two SGs in an AdMunc18b-KR-transduced rat islet stimulated as in (A).

(A, B) (i) Scale bar represents 10µm. The Alexa fluorescence distinguishes several adjacent β-cells in the islet, along with small blood vessels (white arrowheads) and major blood vessels (white arrows). White square shows the position of (ii). An example of a single SG exocytosis shown in (A) with imaging interval of 1.2sec; in (B), an example of 2-SG sequential events with imaging interval of 0.6sec. The numbers below each panel represent time after onset of exocytosis. Dashed circles show position of an omega construction in a former panel. Arrows point to the direction of SG fusion towards the cell interior. (iii) Time courses of fluorescence at the area containing the exocytotic events. Vertical bars indicate the times when images in (ii) were acquired. The fluorescence value before exocytosis was set to zero.

(C) Total number of exocytotic events calculated as number of exocytotic events per cell per min in β-cells from islets treated with AdGFP (8 islets, 68 cells), AdMunc18b-WT (7 islets, 44 cells), AdMunc18b-KR (6 islets, 58 cells), and AdMunc18b-E59K (5 islets, 75 cells) during 10min period of stimulation as in (A).

(D) Fractions of total exocytotic events which are sequential exocytosis. The number of secondary exocytotic events considered to be sequential exocytosis is expressed as a percentage of total number of exocytotic events assessed in (C). Data in (C) and (D) are shown as means ±
SEMs, and analyzed by Steel-Dwass’s test for multiple comparisons using AdGFP as control, *$P<0.05$, **$P<0.01$, ***$P<0.001$.

**FIG. 6. Munc18b mediates long-chain sequential SG fusion.**

(A) The ordinate represents the ratios for increasing chain-length of sequential SG fusion events relative to total numbers of exocytotic events from >20 β-cells (>2 rat islets) transfected with AdGFP, AdMunc18b-WT, AdMunc18b-KR and AdMunc18b-E59K. Here, we also assessed the distinct increases in fluorescence that predicts the sizes of SGs, which was ~0.3µm, shown in Supplementary Figure 5.

(B) TEP imaging of long-chain sequential exocytosis of insulin SGs in a rat β-cell treated with AdMunc18b-KR. (i) Sequential images for Supplementary Movie 1 of an islet stimulated with 20mM glucose plus 10nM GLP-1 and 150µM IBMX. Dashed circles show positions of an omega construction in a former panel. Arrows indicate the direction of oncoming SG undergoing sequential fusion. (ii) Time-course of fluorescent intensity within the region that contained all exocytotic events shown in (i) Asterisk indicates an independent single exocytotic event, occasionally seen close to compound SGs. Scale bars, 1µm.

(C) EM demonstrating long-chain sequential exocytosis of insulin SGs in an islet treated with AdMunc18b-KR. An example of AdMunc18b-KR-transduced islet stimulated with 20mM glucose plus 10nM GLP-1 and 150µM IBMX that illustrates long-chain sequential fusion of SG-SG events up to six SGs in length. Left panels, 70nm thick sections cut serially, showing only four of eight consecutive slices containing the SG-SG fusions. G plus number (in red) indicates the numbered SGs involved in long-chain exocytosis; M (in red) indicates PM; numbered asterisks (in green) indicate SG-SG fusion sites. Scale bar, 500nm. Cartoon panels. SG-SG fusion events constructed from the EM images in left panels and converted into cartoons (first
column) followed by reconstruction into a 3D image (third column). The second column shows blowups of this 3D image that can be rotated to selectively visualize fusion pores between indicated SGs, which correspond to raw EM images in the left panels. Blue arrows indicate locations of fusion pore openings. A second example of a seven-SG fusion is shown in Supplementary Figure 7.

(D) Insulin SGs within 120nm from PM calculated as SG per µm2 area, expressed as means ± SEMs of 3- 4 independent experiments. AdGFP: 17 sections AdMunc18b-KR: 16 sections; AdMunc18b-WT: 17 sections; AdMunc18b-E59K: 21 sections; * p < 0.05 and ** p < 0.01.

FIG. 7. Munc18b depletion reduces primary and sequential exocytosis in rat β-cells
TEP images of single SG exocytosis and SG-SG fusions were analysed from islets treated with lenti-shRNA/Munc18b-eCFP (67 cells, 8 islets) vs lenti-eCFP (Control, 48 cells, 8 islets) during 10min period of stimulation with 20 mM glucose plus 10 nM GLP-1 and 150 µM IBMX, and the following were calculated. (A) Total number of exocytotic events. (B) Number of exocytotic events at each indicated time interval in the 10min recording. Right panel, summation of the first 5 min and second 5 min of recording. (C) Secondary exocytotic events considered to be sequential exocytosis expressed as a percentage of total number of exocytotic events assessed in (A) Data shown as means ± SEMs, *P<0.05, ***P<0.001.
FIG. 1. Munc18b is a major mediator of GSIS in pancreatic islet β-cells

(A) Pancreatic β-cells (rat islets, INS-1) express SM proteins and Syntaxins. Rat pancreatic acini and brain were used as positive and negative controls.

(B) Immunofluorescence images showing cognate Munc18b and Syn-2 and Syn-3 are abundant in insulin SGs in rat β-cells. Cognate Munc18a and Syn-1A are abundant in the PM. These images are representative of 4 independent experiments. Scale bars, 10µm.

(C) Munc18b depletion in rat islets by lenti-Munc18b shRNA/eCFP vs eCFP control. Top: representative blots, bottom: analysis of 3 experiments, shown as means ± SEMs. *P<0.05, **P<0.01.

(D) Islet perifusion assays of 48hrs lenti-shRNA/Munc18b-eCFP (vs lenti-eCFP, Control) depletion of endogenous Munc18b in rat islets showing reduction of GSIS and GLP-1 (10nM) + IBMX (150µM) potentiated GSIS, shown as means ± SEMs of 3 independent experiments, and their area under the curve (AUC) analysis; *P<0.05, **P<0.001.

180x189mm (300 x 300 DPI)
FIG. 2. Munc18b gain-of-function mutant potentiates GSIS

(A) AdMunc18b mutants (-WT, -KR, -E59K) transduction of rat islets did not influence the expression of SNARE or other SM proteins. Shown are representative of 3 independent experiments; analysis of Munc18b proteins expression shown in bottom.

(B) Confocal imaging showing AdMunc18b mutants did not influence Syn-2 or Syn-3 targeting to insulin SGs in rat β-cells. Shown are representative of 4 independent experiments. Scale bars, 10µm.

(C, D) Islet perifusion assays showing AdMunc18b mutants-transduced rat islets influencing GSIS (in C) and 10nM GLP-1 +150µM IBMX-potentiated GSIS (in D). Data shown are means ± SEMs from 3-4 sets of experiments, each experiment performed simultaneously on all the 4 conditions. Bottom panels show quantification of AUC analysis of 1st Phase (encompassing 11-22min) and 2nd Phase (encompassing 22-40min) GSIS. *P<0.05, **P<0.01, ***P<0.001 by one-way ANOVA.

(E) Effects of AdMunc18b mutants on GSIS and 10mM GLP-1 +150µM IBMX-potentiated GSIS in INS-1 cells. Results shown are means ± SEMs from 3 independent experiments performed in duplicates or triplicates.
(n=6-8). *P<0.05 by ANOVA and Scheffe tests.
FIG. 3. Munc18b depletion in INSL1 cells disrupts SM/SNARE complex formation. INSL1 cells were transduced with Munc18b siRNA or control scrambled RNA, then kept at basal condition (0.8 mM glucose) or simulated with 16.7 mM glucose+10 nM GLP-1 with IBMX (150 µM), then subjected to co-ip (left panels) with antibodies against (A) Syn-1A, (B) Syn-2 or (C) Syn-3. Corresponding right panels show 'Input' controls (25µg protein, total INSL1 lysates), confirmed the reduction in Munc18b levels and similar levels of indicated SNARE proteins. Results shown are representative of 3 independent experiments, with densitometry analyses in Supplementary Figure 3.
FIG. 4. Munc18b gain-of-function mutant increases SM/SNARE complex formation
INS-1 cells were transduced with AdMunc18 mutants, kept in non-stimulated (lanes 1-4) or simulated (lanes
5-8) condition as in Fig 3, and subjected to co-ip (left panels) with antibodies against (A) Syn-1A, (B) Syn-2
or (C) Syn-3. Exogenous Munc18b (tagged with Myc) expression was detected by Myc antibody.
Corresponding right panels are 'Input' controls (25µg protein, total INS-1 lysates) showing similar levels of
indicated SNARE proteins. Results shown are representative of 3 independent experiments, with
densitometry analyses shown in Supplementary Figure 4.
FIG. 5. Munc18b mediates primary exocytosis and sequential SG-SG fusion in rat β-cells

(A) Example of TEP imaging of single insulin SG exocytosis in an AdGFP-transduced rat islet stimulated with 20 mM glucose plus 10 nM GLP-1 and 150 µm IBMX in a solution containing 0.3 mM Alexa fluor® 594 hydrazide. An omega construction appeared when the SG(s) coalesced with the PM.

(B) Example of TEP imaging of sequential exocytosis involving two SGs in an AdMunc18b-KR-transduced rat islet stimulated as in (A).

(A, B) (i) Scale bar represents 10µm. The Alexa fluorescence distinguishes several adjacent β-cells in the islet, along with small blood vessels (white arrowheads) and major blood vessels (white arrows). White square shows the position of (ii). An example of a single SG exocytosis shown in (A) with imaging interval of 1.2sec; in (B), an example of 2-SG sequential events with imaging interval of 0.6sec. The numbers below each panel represent time after onset of exocytosis. Dashed circles show position of an omega construction in a former panel. Arrows point to the direction of SG fusion towards the cell interior. (iii) Time courses of fluorescence at the area containing the exocytotic events. Vertical bars indicate the times when images in (ii) were acquired. The fluorescence value before exocytosis was set to zero.

(C) Total number of exocytotic events calculated as number of exocytotic events per cell per min in β-cells from islets treated with AdGFP (8 islets, 68 cells), AdMunc18b-WT (7 islets, 44 cells), AdMunc18b-KR (6 islets, 58 cells), and AdMunc18b-E59K (5 islets, 75 cells) during 10min period of stimulation as in (A).

(D) Fractions of total exocytotic events which are sequential exocytosis. The number of secondary exocytotic events considered to be sequential exocytosis is expressed as a percentage of total number of exocytotic events assessed in (C). Data in (C) and (D) are shown as means ± SEMs, and analyzed by Steel-Dwass’s
test for multiple comparisons using AdGFP as control, *P<0.05, **P<0.01, ***P<0.001.
FIG. 6. Munc18b mediates long-chain sequential SG fusion.

(A) The ordinate represents the ratios for increasing chain-length of sequential SG fusion events relative to total numbers of exocytotic events from >20 β-cells (>2 rat islets) transfected with AdGFP, AdMunc18b-WT, AdMunc18b-KR and AdMunc18b-E59K. Here, we also assessed the distinct increases in fluorescence that predicts the sizes of SGs, which was ~0.3µm, shown in Supplementary Figure 5.

(B) TEP imaging of long-chain sequential exocytosis of insulin SGs in a rat β-cell treated with AdMunc18b-KR. (i) Sequential images for Supplementary Movie 1 of an islet stimulated with 20mM glucose plus 10nM GLP-1 and 150µM IBMX. Dashed circles show positions of an omega construction in a former panel. Arrows indicate the direction of oncoming SG undergoing sequential fusion. (ii) Time-course of fluorescent intensity within the region that contained all exocytotic events shown in (i) Asterisk indicates an independent single exocytotic event, occasionally seen close to compound SGs. Scale bars, 1µm.

(C) EM demonstrating long-chain sequential exocytosis of insulin SGs in an islet treated with AdMunc18b-KR. An example of AdMunc18b-KR-transduced islet stimulated with 20mM glucose plus 10nM GLP-1 and
150µM IBMX that illustrates long-chain sequential fusion of SG-SG events up to six SGs in length. Left panels, 70nm thick sections cut serially, showing only four of eight consecutive slices containing the SG-SG fusions. G plus number (in red) indicates the numbered SGs involved in long-chain exocytosis; M (in red) indicates PM; numbered asterisks (in green) indicate SG-SG fusion sites. Scale bar, 500nm. Cartoon panels.

SG-SG fusion events constructed from the EM images in left panels and converted into cartoons (first column) followed by reconstruction into a 3D image (third column). The second column shows blowups of this 3D image that can be rotated to selectively visualize fusion pores between indicated SGs, which correspond to raw EM images in the left panels. Blue arrows indicate locations of fusion pore openings. A second example of a seven-SG fusion is shown in Supplementary Figure 7.

(D) Insulin SGs within 120nm from PM calculated as SG per µm² area, expressed as means ± SEMs of 3-4 independent experiments. AdGFP: 17 sections AdMunc18b-KR: 16 sections; AdMunc18b-WT: 17 sections; AdMunc18b-E59K: 21 sections; * p < 0.05 and ** p < 0.01.
FIG. 7. Munc18b depletion reduces primary and sequential exocytosis in rat β-cells

TEP images of single SG exocytosis and SG-SG fusions were analysed from islets treated with lenti-shRNA/Munc18b-eCFP (67 cells, 8 islets) vs lenti-eCFP (Control, 48 cells, 8 islets) during 10 min period of stimulation with 20 mM glucose plus 10 nM GLP-1 and 150 µM IBMX, and the following were calculated. (A) Total number of exocytotic events. (B) Number of exocytotic events at each indicated time interval in the 10 min recording. Right panel, summation of the first 5 min and second 5 min of recording. (C) Secondary exocytotic events considered to be sequential exocytosis expressed as a percentage of total number of exocytotic events assessed in (A) Data shown as means ± SEMs, *P<0.05, ***P<0.001.

73x59mm (600 x 600 DPI)
SUPPLEMENTARY DATA

Supplementary Table 1

Two 64 base pair (bp) sequences of siRNA duplex targeted to Munc18b were created corresponding to rat Munc18b cDNA (GeneBank Access Number AF263346), with sense and antisense sequences, and control scrambled sequences as shown below.

| siRNA oligo                  | siRNA oligo sequence                      |
|------------------------------|-------------------------------------------|
| Munc18b siRNA                |                                           |
| stealth_271 sense            | 5’-GCC CUG AUU GCG GAC UUC CAG GGA A-3’   |
| stealth_271 antisense        | 5’-UUC CCU GGA AGU CCG CAA UCA GGG C-3’   |
| stealth_1214 sense           | 5’-CCU ACG ACA AGA UCC GGG UUC UGU U-3’   |
| stealth_1214 antisense       | 5’-AAC AGA ACC CGG AUC UUG UCG UAG G-3’   |
| Scrambled siRNA              |                                           |
| stealth_376 sense            | 5’-CGG UUU GGG UGU GCAG UAC AAA CAG-3’    |
| stealth_376 antisense        | 5’-CUG UUU GUA CUG CAC ACC CAA ACC G-3’   |
Supplementary Figure 1. Munc18b mutants do not influence expression of SNARE or other SM proteins in INS-1. INS-1 (832/13) cells were transduced with AdMunc18b-WT, AdMunc18b-K314L/R315L (KR), AdMunc18b-E59K or AdeGFP (control). The indicated exocytotic proteins were identified by Western blotting from whole cell lysates of adenovirus transduced cells; Na⁺/K⁺ ATPase and β-actin used as protein loading controls. Shown is a representative of five independent experiments, each performed in duplicates.
Supplementary Figure 2. Munc18b mutants have no effect on Syn-2 and Syn-3 transcription levels. INS-1 (832/13) β-cells were treated with the indicated adenoviruses vs. untreated sample used as the blank control. Total RNA was then extracted, processed to convert to cDNA and real-time PCR performed. Primers were designed to amplify a 50bp fragment from the cDNA corresponding with Syn-2 and Syn-3. Data was normalized to the expression level of internal control GAPDH from which comparative quantification by ΔΔCT method (ΔΔCT: CT represents threshold cycle) and fold changes of gene of interest were then calculated. Each reaction condition was conducted in triplicate and the mean ± S.E.M. shown were collected from three independent runs.

| Sample               | Syntaxin-2A average CT | GAPDH average CT | ΔCT syntaxin-2A- GAPDH | ΔΔCT treated -ΔCT untreated | Fold difference in syntaxin-2A relative to untreated |
|----------------------|------------------------|------------------|------------------------|----------------------------|---------------------------------------------------|
| Untreated INS-1 832/13 | 23.32±0.08            | 17.74±0.14       | 5.59±0.04              | 0.00±0.04                  | 1                                                 |
| AdGFP Control        | 22.27±0.11             | 17.85±0.11       | 4.41±0.01              | -1.18±0.01                 | 2.2                                               |
| AdMunc18b-WT         | 22.13±0.09             | 17.58±0.04       | 4.55±0.03              | -1.04±0.03                 | 2.1                                               |
| AdMunc18b-KR         | 22.82±0.13             | 17.74±0.05       | 5.09±0.06              | -0.51±0.06                 | 1.4                                               |
| AdMunc18b-E59K       | 23.80±0.06             | 18.20±0.05       | 5.59±0.01              | 0.00±0.01                  | 1                                                 |

| Sample               | Syntaxin-3A average CT | GAPDH average CT | ΔCT syntaxin3A- GAPDH | ΔΔCT treated -ΔCT untreated | Fold difference in syntaxin-3A relative to untreated |
|----------------------|------------------------|------------------|------------------------|----------------------------|---------------------------------------------------|
| Untreated INS-1 832/13 | 23.60±0.03            | 17.74±0.14       | 5.87±0.08              | 0.00±0.08                  | 1                                                 |
| AdGFP Control        | 22.53±0.06             | 17.85±0.11       | 4.68±0.04              | -1.19±0.04                 | 2.2                                               |
| Ad-Munc18b-WT        | 22.70±0.13             | 17.58±0.04       | 5.12±0.06              | -0.75±0.06                 | 1.7                                               |
| Ad-Munc18b-KR        | 23.07±0.20             | 17.74±0.05       | 5.33±0.11              | -0.54±0.11                 | 1.4                                               |
| Ad-Munc18b-E59K      | 23.85±0.06             | 18.20±0.05       | 5.65±0.01              | -0.22±0.01                 | 1.1                                               |
Supplementary Figure 3. Summary of NIH-Image densitometry analysis of Figure 3.

Quantitative analysis of the levels of immunoprecipitated (IPed) Syn-1A (top), Syn-2 (middle) and Syn-3 (bottom) proteins, and SNARE and SM proteins co-precipitated (co-IPed) with these syntaxins were analysed (left panels). Their corresponding input controls are shown on the right panels. Quantification of the IPed syntaxins and co-IPed proteins was performed by normalizing the IPed protein band intensity to the corresponding input protein band intensity and then comparing that value with total protein (500 µg) used for each immunoprecipitation assay. Input controls were analyzed taking the most intense band as 100 in the blot. The results are expressed as means ± SEM of 3 experiments; and * indicates $P<0.05$ as per Student’s t test.
Supplementary Figure 4. Summary of NIH-Image densitometry analysis of Figure 4.

After IP with Syn-1A (top), Syn-2 (middle) and Syn-3 (bottom) antibodies, proteins co-precipitated with these syntaxins were analysed (left panels). Their corresponding input controls are shown on the right panels. Quantification of the IPed syntaxins and co-IPed proteins was performed as described in Supplementary Figure 3. * indicates $P<0.05$ in comparison to the variable indicated by the arrows as per Student’s t-test, $n=3$. 
Supplementary Figure 5. Frequency distribution of SG diameters estimated by \( \Delta V \)-TEP-IQ (image-based quantification) analysis of the TEP (Two-photon extracellular polar tracer) imaging.

By the \( \Delta V \)-TEPIQ method, the fluorescence intensity of the vesicle \( (F_V) \) was divided by \( F_E \), representing the fluorescence intensity per unit area in an xy-image of a solution with an infinite depth, as \( \Delta V = F_V/F_E/P_{xy}(0) \), where \( P_{xy}(0) \) is the efficiency of focal illumination, and was 0.56 in our setup. Using this analysis we found no significant difference in the diameters between single, first and second SGs in each adenovirus infected group (significance of differences was evaluated by Scheffe method for multiple comparison, data not shown). The black smooth lines represent the Gaussian distribution of the means and standard deviation of the single granule fusion.
**Supplementary Figure 6.** Long chain sequentially-fused SGs in AdMunc18b-KR transduced rat β-cell. These low magnification images correspond to those in Figure 6B and Supplementary Movie 1. The large oblique areas (red space surrounded with white dotted line) showed high fluorescence intensity that did not change. These represent intercellular spaces, which serve as landmarks to indicate that the focal plane was not deviated during the acquisition of these successive images. An example of exocytosis is shown in the upper middle area of each panel, which demonstrated changes in fluorescence intensity and pattern reflecting sequential SG-SG fusion, indicated by the numbered arrows, and these images correspond to the high magnification images in Figure 6B and Supplementary Movie 1. Note the other scattered independent exocytosing vesicles appearing at the right lower regions between the two oblique fluorescent areas in the later panels.
Supplementary Figure 7. 3D E.M. imaging of long chain sequentially-fused SGs in islet expressing Munc18b-KR.

This is an additional example of long-chain sequential SG fusion (seven SGs in length) from AdMunc18b-KR-transduced islets. Corresponding cartoons of the E.M. slices were performed and the 3D-reconstructed model shown. Note both G2 and G7 are undergoing exocytosis. Also note homotypic fusion occurring between G8 and G9, and between G10 and G11 located further into the cell interior. Scale bar, 500nm.
Supplementary Movie 1. TEP imaging of long chain sequentially-fused SGs in an islet expressing Munc18b-KR.

This is an example of a movie of long-chain sequential SG fusion from AdMunc18b-KR-transduced islets, which detected SG-SG fusion events of up to seven SGs in length. The successive numbers correspond to the SG number (first to seventh SG) undergoing fusion with the compound SG, which corresponds to the representative images from this movie shown in Figure 6B(i), and temporally corresponds to Figure 6B(ii).

NOTE: During submission of this ms, the Journal indicated that this movie file can/will not be made available to Reviewers. We hope to be permitted to upload the movie if/when the manuscript is accepted. We request the reviewers to accept our apology for this inconvenience of not providing this data during the review process.