In pancreatic β-cells myosin 1b regulates glucose-stimulated insulin secretion by modulating an early step in insulin granule trafficking from the Golgi

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ABSTRACT Pancreatic β-cells secrete insulin, which controls blood glucose levels, and defects in insulin secretion are responsible for diabetes mellitus. The actin cytoskeleton and some myosins support insulin granule trafficking and release, although a role for the class I myosin Myo1b, an actin- and membrane-associated load-sensitive motor, in insulin biology is unknown. We found by immunohistochemistry that Myo1b is expressed in islet cells of the rat pancreas. In cultured rat insulinoma 832/13 cells, Myo1b localized near actin patches, the trans-Golgi network (TGN) marker TGN38, and insulin granules in the perinuclear region. Myo1b depletion by small interfering RNA in 832/13 cells reduced intracellular proinsulin content and glucose-stimulated insulin secretion (GSIS) and led to the accumulation of (pro)insulin secretory granules (SGs) at the TGN. Using an in situ fluorescent pulse-chase strategy to track nascent proinsulin, Myo1b depletion in insulinoma cells reduced the number of (pro)insulin-containing SGs budding from the TGN. The studies indicate for the first time that in pancreatic β-cells Myo1b controls GSIS at least in part by mediating an early stage in insulin granule trafficking from the TGN.

INTRODUCTION
Hormone biogenesis in endocrine cells involves multiple processes from protein translation and modification in the rough endoplasmic reticulum (ER) to budding of vesicles from the trans-Golgi network (TGN) and maturation into dense-core vesicles (Kim et al., 2006). Insulin is translated in the cytosol as preproinsulin and translocated to the lumen of the ER where the N-terminal signal peptide is subsequently removed, producing proinsulin (Davidson, 2004). After moving through the Golgi, proinsulin is packaged into immature insulin granules that exit the TGN and is then cleaved during granule maturation to form insulin and C-peptide. Mature secretory granules (SGs) containing insulin and C-peptide are secreted through the regulated secretory pathway in response to glucose stimulation. Defects in the biogenesis of insulin granules in pancreatic β-cells lead to a reduction in insulin secretion into the blood, a major cause of type II diabetes mellitus (DeFronzo et al., 2015).

Insulin trafficking is mediated by the actin cytoskeleton, and some members of the large myosin superfamily of actin-associated molecular motors have been shown to be involved in insulin trafficking (Ivarsson et al., 2005; Kasai et al., 2005; Varadi et al., 2005; Desnos et al., 2007; Arous et al., 2013; Arous and Halban, 2015). Glucose stimulation induces reorganization of the subcortical actin network and mobilization of insulin granules to the plasma membrane (Wang and Thurmond, 2009). Disruption of the actin cytoskeleton in cultured β-cells and pancreatic islets by treatment with the actin toxins latrunculin or cytochalasin enhances glucose-stimulated insulin secretion (GSIS) (Thurmond et al., 2003; Tomas et al., 2006; Jewell et al., 2008; Kalwat and Thurmond, 2013). Myosin-IIA coordinates actin reorganization and insulin SG shuttling to the plasma membrane (Arous et al., 2013); myosin-Va supports SG transport.
F-actin is evidence that Myo1b could mediate membrane tubule formation and membrane tubules along F-actin bundles in vitro, additional evidence that Myo1b could mediate membrane tubule formation and endosomes (Almeida et al., 2009). Recently, Myo1b was shown to enhance axon formation to be involved in both anchoring insulin granules to the actin cytoskeleton and assembling proteins necessary for exocytosis (Fan et al., 2017). Whether class I myosins, small, single-headed, nonfilamentous actin- and membrane-associated motor proteins (Coluccio, 2008, 2018) mediate insulin secretion is unknown. Whether Myo1b is a general modulator of regulated secretion is unknown. If so, then we predict that Myo1b participates in insulin secretion as 99% of proinsulin is sorted in pancreatic β-cells to the regulated pathway (Rhodes and Halban, 1987). The hypothesis that Myo1b modulates insulin secretion is addressed here.

Myo1b depletion by siRNA

To investigate Myo1b function in β-cells, we used small interfering RNA (siRNA) to reduce Myo1b expression in 832/13 cells. At 5 nM, siRNA1 and siRNA2 reduced Myo1b expression by 98% and 97%, respectively, as determined by immunoblotting with anti-Myo1b antibodies (Figure 3A) followed by densitometric analyses using ImageJ software (Figure 3B). The reduction in Myo1b expression was also confirmed by immunofluorescence microscopy where staining of Myo1b in cells treated with Myo1b-targeting siRNA (siRNA1, siRNA2) was significantly reduced versus cells treated with scrambled siRNA (Figure 3C).

Myo1b regulated GSIS and insulin/proinsulin content in 832/13 cells

To investigate the effect of Myo1b loss on insulin secretion, we measured GSIS in control and Myo1b-depleted 832/13 cells. Depletion of Myo1b expression resulted in a significant reduction in GSIS.
Myo1b depletion altered the distribution of insulin SGs

The distribution of (pro)insulin granules in glucose-stimulated 832/13 cells expressing reduced amounts of Myo1b differed from that of stimulated control cells expressing nontargeting siRNA. In merged images of cells stained with antibodies against TGN38, a marker for the TGN (red), and anti-insulin antibodies to localize (pro)insulin granules (green) following a 30-min incubation in high glucose, (pro)insulin granules appeared more dispersed throughout the cytoplasm in control versus Myo1b-depleted cells (Figure 5A). The distribution of (pro)insulin SGs in glucose-stimulated 832/13 control and Myo1b-depleted cells was quantitated (Figure 5B). The percentage of Myo1b-depleted cells with (pro)insulin

FIGURE 2: Myo1b expression in 832/13 cells. 832/13 cells were labeled with anti-Myo1b antibody (green), Alexa Fluor 594-phalloidin (red), and DAPI (blue). Myo1b localized at cell–cell contacts, in membrane projections, and in association with cytoplasmic puncta tentatively identified as organelles and vesicles. Actin filaments were observed at the cell cortex, in surface projections, and in puncta in the perinuclear region of 832/13 cells. In the merged confocal image (Merge), Myo1b and actin (yellow) were in close proximity at the plasma membrane. Scale bar, 10 µm.

FIGURE 3: Myo1b-specific siRNA reduced Myo1b expression in 832/13 cells. (A) Representative immunoblot probed for Myo1b and tubulin as an internal control of lysates from 832/13 cells treated with scrambled siRNA or Myo1b-specific siRNA1 or siRNA2. (B) Semiquantitative analysis of the amounts of Myo1b expressed in 832/13 cells treated with scrambled siRNA or Myo1b-specific siRNA1 or siRNA2 from four independent experiments as determined by immunoblotting and densitometry. Myo1b intensity was normalized to tubulin content. A significant reduction in Myo1b expression was obtained following treatment with either siRNA1 (98%) or siRNA2 (97%). *P < 0.01. (C) 832/13 cells were transfected with scrambled siRNA or Myo1b-specific siRNA and then stained with anti-Myo1b antibody (green), rhodamine-phalloidin (red), and DAPI (blue). In agreement with the immunoblotting results, the Myo1b signal was significantly reduced in cells treated with Myo1b-specific siRNA1 or siRNA2 vs. cells treated with scrambled siRNA. Scale bar, 20 µm.
SGs at the TGN was statistically higher in Myo1b-kd versus control cells, suggesting that Myo1b loss affects an early stage in the formation of nascent (pro)insulin granules.

**Myo1b was associated with the TGN region and insulin SGs**

To investigate the localization of Myo1b relative to the TGN region, 832/13 cells were stained with anti-Myo1b antibodies, anti-TGN38 antibodies, and 4′,6-diamidino-2-phenylindole (DAPI) to identify nuclei. By superresolution structured illumination microscopy (SR-SIM), Myo1b was observed in close association with the TGN region (Figure 6A). In addition, in SR-SIM images of cells immunostained for Myo1b and (pro)insulin, Myo1b was associated with insulin SGs in the perinuclear region (Figure 6B), evidence that Myo1b is within 100–200 nm, the resolution of superresolution microscopy (Wegel et al., 2016), of both the TGN region and (pro)insulin granules.

Myo1b depletion did not alter actin patches at the perinuclear region of 832/13 cells

In SR-SIM images, actin patches and Myo1b were found in close proximity in the perinuclear region (Figure 7A). To investigate the effect of reduced Myo1b expression on the actin cytoskeleton at the perinuclear region, cells were stained with fluorescent phallolidin and anti-TGN38 antibody. No obvious changes in the actin cytoskeleton at the TGN region were observed in localization studies of Myo1b-depleted (siRNA1, siRNA2) versus control cells (Figure 7B). Analysis showed that the percentage of cells with actin patches at the TGN did not change with Myo1b depletion (Figure 7C).

**Myo1b is necessary for efficient budding of nascent insulin granules from the TGN region**

The increased (pro)insulin localization at the TGN in glucose-stimulated 832/13 cells (Figure 5) suggested a role for Myo1b in (pro)insulin granule trafficking at the TGN. For insight into Myo1b function at the TGN, an in situ pulse-chase fluorescence-labeling strategy developed by Bearrows et al. (2019) was used to examine the effect of Myo1b loss on nascent granule trafficking from the Golgi region. The assay utilizes a strategy in which the modified DNA repair enzyme SNAP-tag self-labels by transfer of a fluorescent probe from a benzylguanidine-conjugated substrate (Ivanova et al., 2013). SNAP-tag was inserted within the C-peptide region of human preproinsulin, which yields proCpepSNAP (proinsulin) and the mature processed fragments insulin and CepSNAP (C-peptide); proCpepSNAP is processed, trafficked, and released in a manner consistent with the kinetics of native insulin and C-peptide. Importantly, SNAP-tag pulse-chase labeling allows the visualization of granule loading and the initial trafficking of (pro)insulin-rich granules from the TGN region. To this end, insulinoma 832/3 cells stably expressing proCpepSNAP were transfected with Myo1b-targeting siRNA or nontargeting control siRNA, prelabeled with a nonfluorescent SNAP-tag probe to mask the existing pool of (pro)CpepSNAP and then after a 2-h recovery period pulse labeled with SNAP-Cell TMR-STAR, a cell-permeable, red fluorescence-conjugated substrate for labeling SNAP-tag fusions, and chased for 2 h before fixation and immunofluorescence microscopy. Confocal images showed that initial tetrachromatidine (TMR) labeling of proCpepSNAP occurred in the perinuclear region near the TGN marker TGN38 both in control cells treated with nontargeting siRNA and cells treated with Myo1b-targeting siRNA. After a 2-h chase, TMR-labeled (pro)CpepSNAP was distributed throughout the cell body of cells treated with control siRNA, with a large fraction ≥2 μm away from the TGN, consistent with the trafficking of nascent insulin granules and their subsequent maturation on their way to the plasma membrane (Figure 8A). In contrast, more TMR-labeled (pro)CpepSNAP granules remained near the TGN region in Myo1b-kd cells with far fewer granules ≥2 μm away from the TGN region, suggesting that Myo1b loss delayed the early trafficking of nascent granules from the TGN region (Figure 8C). Image analysis of only TMR-labeled granules ≤1 μm from the TGN showed that after a 2-h chase,
granules from the TGN. The reduced number of nascent granules in Myo1b-depleted 832/3 cells may indicate that the budding of immature insulin granules is impeded in the absence of Myo1b.

Reexpression of Myo1b rescues Myo1b siRNA-mediated defects in proCpepSNAP Golgi exit

To determine whether the observed delay in early trafficking of nascent granules was a consequence of Myo1b depletion, we determined whether expression of RNAi-resistant Myo1b would rescue the kd phenotype. Insulinoma 832/3 cells stably expressing proCpepSNAP were transfected with control siRNA, Myo1b-targeting siRNA1, or Myo1b-targeting siRNA1 and RNA interference (RNAi)-resistant Myo1b (Figure 9). Myo1b overexpression and kd were confirmed by immunostaining. In the SNAP-tag pulse-chase assay, Myo1b depletion in proCpepSNAP-expressing 832/3 cells resulted at t = 2 h in an increase in the frequency of TMR-labeled granules ≤2 µm from the TGN region as compared with cells treated with control siRNA (Figure 9A); this is consistent with the results described above. Importantly, no difference in the distribution of TMR-labeled granules ≤2 µm from the TGN region was observed between cells treated with Myo1b-targeting siRNA followed by RNAi-resistant Myo1b and cells treated with control siRNA. Moreover, introduction of RNAi-resistant Myo1b into cells expressing Myo1b-targeting siRNA restored the frequency of TMR-labeled granules >2 µm from the TGN to that observed in cells treated with control siRNA. These data indicate that Myo1b depletion is responsible for the defects in intracellular trafficking of (pro)insulin granules observed in Myo1b-kd cells.

**DISCUSSION**

In regulated secretory cells, newly synthesized proteins are sorted at the TGN to one of three routes: the constitutive pathway, the endosomal/lysosomal pathway, and the regulated secretory pathway (Arvan and Castle, 1998). Secretory proteins like insulin aggregate in the lumen of the TGN due to Ca\(^{2+}\) and low pH and thus segregate from constitutively secreted proteins. The aggregated proteins directly or indirectly attach to lipid rafts at the TGN to induce the formation of immature SGs through budding (Kim et al., 2006). The actin cytoskeleton and its associated motors are implicated in post-Golgi trafficking, but whether the same or specific myosin motors mediate biogenesis of carriers destined for all or specific post-Golgi pathways is unknown.

In superresolution images, Myo1b puncta were observed in 832/13 cells at the TGN region near F-actin patches, short rhodamine-phalloidin–stained actin filaments. Previously, localization of Myo1b in the perinuclear region of HeLa cells, in close proximity to the TGN, was reported (Almeida et al., 2011). Although not enriched at the TGN region in 832/13 cells, Myo1b need not be to perform an important function there; other proteins determined to affect Golgi structure/function have little or no enrichment at the Golgi (Ramabhadran et al., 2011; Zilberman et al., 2011).

The reduction in insulin secretion and accumulation of insulin SGs at the TGN region in response to glucose stimulation in Myo1b-depleted 832/13 cells are consistent with Myo1b loss causing a defect in insulin granule biogenesis. Using a pulse-chase assay designed to track nascent insulin granules (Bearrows et al., 2019), more TMR-labeled granules remained at the TGN region (0 µm) in Myo1b-kd versus control cells (Figure 8B). These data suggest that Myo1b depletion reduced the early trafficking of nascent insulin granules from the TGN. The reduced number of nascent granules in Myo1b-depleted 832/3 cells may indicate that the budding of immature insulin granules is impeded in the absence of Myo1b.

**FIGURE 5:** Myo1b knockdown increased insulin localization at the TGN in glucose-stimulated 832/13 cells. (A) Representative confocal images of 832/13 cells treated with scrambled siRNA or Myo1b-specific siRNA1 or siRNA2 following incubation in 16.7 mM glucose for 30 min and staining for insulin (green), TGN38 (red), actin (cyan), and nuclei (blue). Scale bar, 20 µm. For each condition, the rightmost panel (Magnified) is a 2.5x image of the box in the panel labeled Merge. Note that insulin (green) is more highly dispersed throughout the cells treated with scrambled siRNA but not Myo1b-targeting siRNA. (B) Percentage of insulin fluorescence at the TGN/total fluorescence in glucose-stimulated control (scrambled siRNA; 20.87% ± 0.57 SEM) and Myo1b-kd (siRNA1, 28.95 ± 0.66 SEM; siRNA2, 27.75 ± 0.77 SEM) cells as determined with ImageJ. Data were collected from five different cells in five different areas for each of three conditions in three independent experiments. *P<0.01 for both control vs. siRNA1 and control vs. siRNA2.

**FIGURE 6:** Myo1b was associated with the TGN and insulin granules at the perinuclear region in 832/13 cells. (A) 832/13 cells were stimulated with 16.7 mM glucose for 1 h and then fixed, stained for Myo1b (green), TGN38 (red), and nuclei (blue), and viewed with SR-SIM. Myo1b puncta were observed at the TGN (arrows). Scale bar, 1.2 µm. (B) 832/13 cells were stimulated for 1 h with 16.7 mM glucose and then fixed, stained for Myo1b (green), insulin (red), and nuclei (blue), and viewed by SR-SIM. Insulin staining was evident near Myo1b staining (arrows). Scale bar, 0.9 µm.
Myo1b mediates insulin secretion

The restoration in the frequency of insulin granules >2 μm from the TGN in Myo1b-kd cells to control levels by expression of exogenous Myo1b (Figure 9) indicated that Myo1b depletion is responsible for the kδ phenotype. The results are consistent with the idea that Myo1b loss inhibits insulin granule biogenesis. Insulin stalls at the TGN in β-cells expressing a dominant-negative form of the receptor SORCS1 (Kebede et al., 2014) and near the TGN network in β-cells deficient in the prohormone VGF (Stephens et al., 2017) and chromogranin B (Bearrows et al., 2019), factors determined to mediate SG biogenesis. The studies are also consistent with previous results showing that Myo1b localizes to the TGN in HeLa cells and that its depletion impacts exit of the lysosomal marker MPR from the Golgi region. Evidently, Myo1b promotes the formation of MPR-positive membrane tubules at the TGN (Almeida et al., 2011). Indeed, small numbers of Myo1b molecules are able to support membrane tubulation in vitro (Yamada et al., 2014). Thus, Myo1b likely participates in both the endosomal/lysosomal pathway and, based on our studies, regulated secretory pathways.

β-cells maintain an optimal intracellular store of insulin granules by balancing insulin production with insulin secretion and degradation (Boland et al., 2017). For example, to compensate for the reduced insulin secretion in transgenic mice lacking the GTPase Rab3, which is necessary for granule transport to the cell surface, β-granule degradation is increased (Marsh et al., 2007). Thus, the reduction in proinsulin and insulin content observed in Myo1b-kd cells may be a consequence of reduced formation of nascent insulin granules in the absence of Myo1b. Alternatively, the reduction in proinsulin in Myo1b-kd cells may indicate that Myo1b loss affects proinsulin to proinsulin processing; however, given what is known about Myo1b function and the conversion of proinsulin to proinsulin, it is hard to envision how.

Previous studies showed that Myo1b depletion affects regulated chromogranin A (CgA) secretion in PC12 cells and a model system consisting of COS7 cells expressing exogenous CgA (COS7-CgA cells) (Delestre-Delacour et al., 2017). Importantly, although our studies show that reduced Myo1b expression also affects regulated insulin secretion, there are significant differences between the two studies. In PC12 and COS7-CgA cells, no decrease in stimulated chromogranin B (Bearrows et al., 2019), factors determined to mediate SG biogenesis. The studies are also consistent with previous results showing that Myo1b localizes to the TGN in HeLa cells and that its depletion impacts exit of the lysosomal marker MPR from the Golgi region. Evidently, Myo1b promotes the formation of MPR-positive membrane tubules at the TGN (Almeida et al., 2011). Indeed, small numbers of Myo1b molecules are able to support membrane tubulation in vitro (Yamada et al., 2014). Thus, Myo1b likely participates in both the endosomal/lysosomal pathway and, based on our studies, regulated secretory pathways.

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Myo1b depletion also reportedly leads to fewer CgA granules in COS7-CgA cells, suggesting that Myo1b controls the biogenesis of SGs, although the immunoblots show that the total amount of CgA did not change with Myo1b depletion, data inconsistent with this conclusion (Delestre-Delacour et al., 2017). Although we found it difficult to count the insulin granules in control and Myo1b-kd cells, our impression from the pulse-chase experiments is that Myo1b-kd cells contained fewer TMR-labeled granules than control cells (Figure 8C). Thus, although Myo1b may participate in the regulated secretion of both CgA and insulin, Myo1b may play different roles with different cargoes and in different cell types. The idea that Myo1b functions differently in different cell types is supported by examination of the actin cytoskeleton. The distribution of F-actin at the Golgi requires Myo1b expression and Arp2/3 complex activation in COS7-CgA cells, and Myo1b-depletion reduces the amount of actin at the Golgi region in these cells (Delestre-Delacour et al., 2017). In contrast, no obvious reduction in the actin cytoskeleton at
Here. These data suggest that other class I myosins may mediate aspects of insulin secretion.

Finally, our results indicating that Myo1b modulates an early event in the trafficking of (pro)insulin granules from the TGN do not rule out the possibility that Myo1b may also play other roles in β-cells. As in other cell types, in β-cells Myo1b is at the plasma membrane, which is critical for insulin granule docking, fusion, and secretion. Whether Myo1b plays a role in insulin secretion at the plasma membrane of β-cells awaits further investigation.

**MATERIALS AND METHODS**

**Reagents**  
Rabbit monoclonal anti-Myo1b antibody (ab194356) was obtained from Abcam (Cambridge, MA). The specificity of the Myo1b antibody was confirmed by immunoblotting, where it recognized a 130-kDa polypeptide, the same molecular weight as Myo1b, and by both immunoblotting and immunocytochemistry of insulinoma 832/13 cells treated with Myo1b-specific siRNA, which resulted in reduced expression of the 130-kDa band and reduced staining of the cell membrane and cytoplasm versus control cells. Mouse monoclonal anti-β-tubulin antibody (T5168; clone B-5-1-2), which recognized a single band at 55 kDa, the size of tubulin, was obtained from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal anti-insulin antibody (SAB42006791; clone K36AC10), which recognized intracellular organelles in insulinoma 832/13 cells, was also obtained from Sigma-Aldrich. Rabbit monoclonal anti-insulin antibody (3014S; clone C27C9) was obtained from Cell Signaling Technology (Danvers, MA). On immunoblots, this antibody recognized a band of 3.5 kDa, which is attributed to the B chain of insulin. Mouse anti-TGN38 antibody (610898; clone 2/TGN38) was obtained from BD Biosciences (Billerica, MA). Its specificity for the TGN was confirmed by immunocytochemistry of insulinoma 832/13 cells. A widely used guinea pig polyclonal anti-insulin antibody obtained from Abcam (ab7842) was used in immunoblotting to identify proinsulin, which migrated at 30 kDa. Cell Signaling Technologies anti-insulin antibody (813S) was used in immunoblotting to identify proinsulin (L6B10) antibody (813S) was used in immunoblotting to identify proinsulin. Alexa Fluor 488- (A-11034), 594- (A-11037), and 647-conjugated goat anti-mouse, Alexa Fluor 488- (A-11029), and Alexa Fluor 488- (A-11034), 594- (A-11037), and 647-conjugated goat anti-rabbit, mouse, and Alexa Fluor 488–conjugated goat anti-mouse, and Alexa Fluor 488–conjugated goat anti-guinea pig (A-11073) secondary antibodies were obtained from Molecular Probes (Eugene, OR). Control tissue samples stained with secondary antibody only were routinely used to rule out nonspecific staining. Rhodamine-phalloidin (R415) and Alexa Fluor 594–phalloidin (A12381) were also obtained from Molecular Probes. Paraffin sections of rat pancreas were purchased from Abcam and Zyagen (San Diego, CA).
SNAP-Cell TMR-Star (S9105S) and SNAP-Cell Block (S9106S) were obtained from New England Biolabs (Ipswich, MA). Restriction enzymes PmeI (R0560S), Paci (R0547S), BglII (R0144S), and XhoI (R0146S) were also obtained from New England Biolabs. Lipofectamine and Lipofectamine MAX were obtained from Invitrogen (Thermo Fisher Scientific, Waltham, MA).

Cell culture

The study used 832/13 and 832/3 cell lines, which are clones derived from the stable transfection of the parental rat INS-1 insulinoma cell line with a plasmid containing the human proinsulin gene driven by the cytomegalovirus promoter (Hohmeier et al., 2000); 832/3 cells stably expressing proCpepSNAP (Bearrows et al., 2019) were the kind gift of Samuel B. Stephens (University of Iowa). 832/13 and 832/3 cells have features expected of pancreatic β-cells as they contain insulin granules as observed by immunostaining with anti-insulin antibody, which recognizes both rat and human insulin and proinsulin, and they secrete insulin in response to glucose stimulation as determined by insulin assays. Insulinoma cells were cultured in RPMI 1640 medium (+ l-glutamine, – d-glucose (Life Technologies 11879-020; Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) 10 mM HEPES (pH 7.4), 1 mM sodium pyruvate, 50 µM β-mercaptoethanol, and 10 mM glucose.

RNAi experiments

siRNA targeting rat Myo1b (siRNA1: CCAAGAUAUUCAUCGGAAA and siRNA2: UAAAAUCGAAUCAACGAAA) and nontargeting siRNA as a negative control were designed by and obtained from Dharmacon (Lafayette, CO). After being cultured for 1 d, insulinoma cells were transfected with either 5 nM Myo1b-targeting siRNA or scrambled siRNA using Lipofectamine RNAiMax reagent according to the manufacturer’s suggestions (Invitrogen, Thermo Fisher Scientific, Waltham, MA). Transfected cells were cultured for 3 d and analyzed for Myo1b expression by immunoblotting and/or immunocytochemistry with anti-Myo1b antibody.

Immunoblotting

Proteins separated by SDS–PAGE were transferred to polyvinylidene difluoride membrane, blocked in 5% nonfat milk in phosphate-buffered saline (PBS) for 1 h and then incubated with a 1/100,000 dilution of anti-Myo1b antibody or 1/1,000,000 dilution of anti-insulin antibody and 1/200,000 dilution of anti-tubulin antibody in PBS with nonfat milk followed by washing in PBS and incubation in 1/20,000 horseradish-conjugated secondary antibody. To probe for insulin and proinsulin, polycyclamide gels and transfers were performed according to a previously described method (Okita et al., 2017). Color development was achieved with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Marlborough, MA). The blot was then exposed to x-ray film, and the film was developed in an X-omat film developer. Densitometry was performed using ImageJ software (Schindelin et al., 2012).

Immunocytochemistry

832/13 or 832/3 cells were fixed with 4% formaldehyde in PBS for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min.
Cells were blocked in 5% goat serum and 2.5% bovine serum albumin (BSA) in PBS for 1 h and then incubated with the appropriate primary antibodies (1:500 anti-insulin; 1:10,000 anti-Myo1b; and 1:500 anti-TGN38), fluorescently labeled secondary antibodies (1:500), and/or rhodamine- or Alexa Fluor 594–conjugated phalloidin (1:500) to identify actin filaments. For staining of sections from rat pancreas, after deparaffinization and rehydration, the sections were subjected to antigen retrieval by being boiled in 10 mM sodium citrate, pH 6.0, for 5 min. Sections were stained as described above. Sections and cells grown on #1.0 coverslips to be viewed with a Leica TCS SP5 Broadband confocal microscope (Leica, Söhms, Germany) were fixed and mounted on glass slides in Vectashield Hardset antifade mounting medium with DAPI (H-1500; Vector Laboratories, Burlingame, CA). Alternatively, cells grown on #1.5 coverslips were fixed and mounted in ProLong Gold mounting reagent (P10144; Molecular Probes, ThermoFisher Scientific) and viewed with a Nikon N-SIM superresolution system (Minato, Tokyo, Japan) using a 40× or 100× objective.

Insulin secretion assays
832/13 cells were grown in 24-well plates for 3 d and then cultured overnight in RPMI medium supplemented with 2.5% FBS and 2 mM glucose. The culture medium was replaced with RPMI medium containing 2 mM glucose without FBS, and cells were incubated for 2 h. Cells were preincubated in modified Krebs-Ringer bicarbonate (KR) buffer consisting of 119 mM NaCl, 4.6 mM KCl, 2 mM CaCl2, 1 mM MgSO4, 0.15 mM Na2HPO4, 0.4 mM KH2PO4, 20 mM HEPES, and 5 mM NaHCO3 with 0.2% BSA and 2 mM glucose, pH 7.4, for 30 min. Insulin secretion assays were performed in KR buffer in the presence of 2 or 16.7 mM glucose for 30 min. Insulin secretion was measured and reported as a percentage of total cellular insulin fluorescence (mean insulin fluorescence × cellular area). At least five cells in five different areas for each condition (control, siRNA1, and siRNA2) were examined. The results are drawn from three independent experiments.

Analysis of insulin at the TGN region
Following glucose stimulation for 30 min, 832/13 control and Myo1b-kd cells were stained for actin, insulin, TGN38, and nuclei. Cell images were captured by immunofluorescence microscopy and analyzed with ImageJ software in the cases of Figures 4, 5, and 7. The Freehand function was used to outline cells based on actin staining in order to determine cell area. The area occupied by the TGN was determined similarly based on TGN38 staining. The measurement function was then used to determine the mean insulin fluorescence in the cellular region and in the TGN region. The insulin fluorescence in the TGN region (mean insulin fluorescence at the TGN area of the TGN) was measured and reported as a percentage of total cellular fluorescence (mean cellular insulin fluorescence × cellular area). At least five cells in five different areas for each condition (control, siRNA1, and siRNA2) were examined. The results are drawn from three independent experiments.

Analysis of actin foci at the TGN region
The number of 832/13 cells with actin patches within 2 µm of the TGN area was determined with ImageJ of confocal images of multiple (n > 69 for each condition in three separate experiments) cells stained for actin with Alexa Fluor 594–phalloidin and TGN38 with anti-TGN38 antibodies.

Pulse-chase labeling of nascent proinsulin/insulin-rich SGs
An in situ pulse-chase fluorescence-labeling strategy (Bearrows et al., 2019) was used to examine the effect of Myo1b loss on nascent insulin granule trafficking from the Golgi region. This assay uses 832/3 cells expressing a construct in which the modified DNA repair enzyme SNAP-tag, which self-labels by transfer of a fluorescent probe from a benzylguanidine-conjugated substrate, is inserted within the C-peptide region of human preproinsulin; preproinsulin is processed to proCpepSNAP and the mature forms insulin and CpepSNAP (C-peptide) (Bearrows et al., 2019). 832/3 cells expressing proCpepSNAP were grown on HTB9-coated coverslips (Hayes et al., 2017; Stephens et al., 2017; Bearrows et al., 2019) and then transfected with either 5 µM control siRNA or Myo1b-targeting siRNA1 overnight. Two days later, the cells were pulse-labeled with 1 µM SNAP-Cell TMR-STAR substrate (S91955; New England Biolabs) for 20 min and chased for 2 h before fixation, staining with anti-TGN38 antibody to identify the TGN region, counterstaining with DAPI, and evaluation by immunofluorescence microscopy. The ImageJ plug-in DiAna (Gilles et al., 2017) was used to analyze the shortest distance from the surface of TMR-labeled granules to the nearest surface of TGN, and the results are reported as frequency of TMR-labeled granules as a function of distance from the TGN.

Rescue experiments
To confer siRNA resistance to the exogenous Myo1b, the target sequence of siRNA1, CCAAGATATTCCATCCCGA, was mutated to CGAAAATCTTATACGTAA in Myo1b–green fluorescent protein (GFP) plasmid (a kind gift of Martin Bähler, Westfälische Wilhelms-Universität, Münster, Germany). On day 1, 832/3 cells stably expressing proCpepSNAP were transfected with Myo1b-targeting siRNA1 or a nontargeting control siRNA. On day 2, these cells were subsequently transfected ± RNAi-resistant Myo1b in suspension by electroporation with a Neon transfection system (Invitrogen, Thermofisher Scientific) according to the manufacturer’s instructions and cultured on HTB9-coated coverslips. On day 4, cells were immunostained with anti-Myo1b antibody to confirm Myo1b kd and pulse-labeled with SNAP-Cell TMR-STAR (red) for 20 min and chased for 2 h. Rescued cells were confirmed as GFP-positive cells. The frequency distribution of granules as a function of distance from the TGN was determined using the ImageJ plug-in DiAna (Gilles et al., 2017).

Statistics
Data from multiple independent experiments are expressed as means ± SD and compared using analysis of variance (ANOVA) followed by Tukey’s post-hoc test. The data of TMR-proCpepSNAP granule frequency are presented as the mean ± SEM and analyzed by two-way ANOVA with Sidak posttest analysis (GraphPad Prism). The horizontal lines over the bar graphs indicate which two conditions were compared. The asterisks above the horizontal lines refer to the P values recorded in the legends.

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