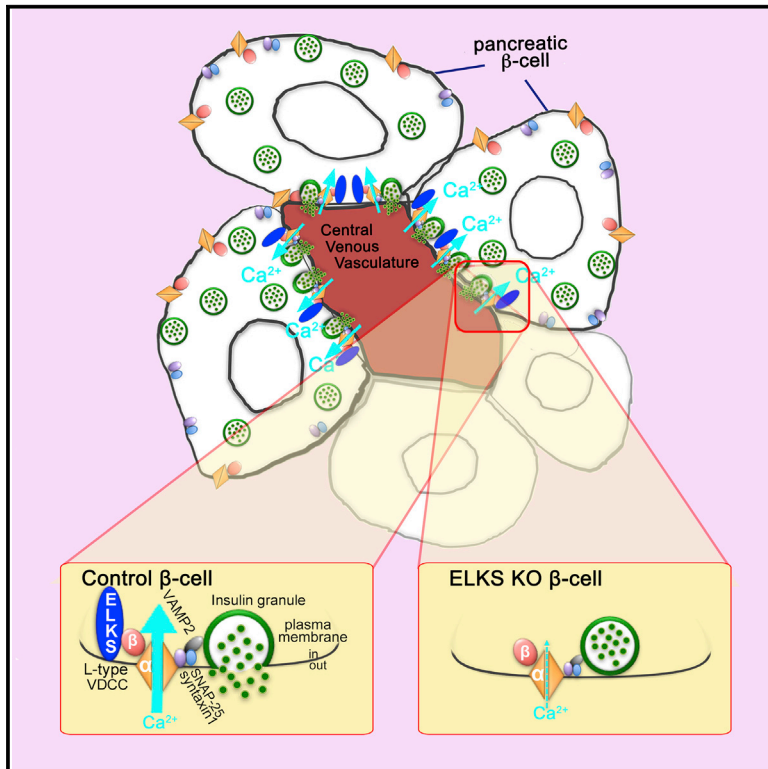


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ELKS/Voltage-Dependent Ca^{2+} Channel- β Subunit Module Regulates Polarized Ca^{2+} Influx in Pancreatic β Cells

Graphical Abstract



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In Brief

β cells secrete insulin by Ca^{2+} -triggered exocytosis; however, the mechanism underlying polarized exocytosis remains elusive. Ohara-Imaizumi et al. demonstrate that ELKS and voltage-dependent Ca^{2+} channels form a potent insulin secretion complex at the vascular side of the β cell plasma membrane for polarized Ca^{2+} influx and insulin secretion from islets.

Highlights

- ELKS regulates L-type VDCC opening and first-phase insulin secretion from β cells
- ELKS interacts with the VDCC- β subunits
- ELKS localizes at the vascular side of the β cell plasma membrane in islets
- ELKS/VDCC- β subunit module controls polarized Ca^{2+} influx in β cells



ELKS/Voltage-Dependent Ca^{2+} Channel- β Subunit Module Regulates Polarized Ca^{2+} Influx in Pancreatic β Cells

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SUMMARY

Pancreatic β cells secrete insulin by Ca^{2+} -triggered exocytosis. However, there is no apparent secretory site similar to the neuronal active zones, and the cellular and molecular localization mechanism underlying polarized exocytosis remains elusive. Here, we report that ELKS, a vertebrate active zone protein, is used in β cells to regulate Ca^{2+} influx for insulin secretion. β cell-specific *ELKS*-knockout (KO) mice showed impaired glucose-stimulated first-phase insulin secretion and reduced L-type voltage-dependent Ca^{2+} channel (VDCC) current density. *In situ* Ca^{2+} imaging of β cells within islets expressing a membrane-bound G-CaMP8b Ca^{2+} sensor demonstrated initial local Ca^{2+} signals at the ELKS-localized vascular side of the β cell plasma membrane, which were markedly decreased in *ELKS*-KO β cells. Mechanistically, ELKS directly interacted with the VDCC- β subunit via the GK domain. These findings suggest that ELKS and VDCCs form a potent insulin secretion complex at the vascular side of the β cell plasma membrane for polarized Ca^{2+} influx and first-phase insulin secretion from pancreatic islets.

INTRODUCTION

In nerve terminals, the polarized Ca^{2+} -dependent transmitter release is restricted to a specialized presynaptic membrane compartment called the active zone (AZ), characterized by its

high-electron density under electron microscopy (Landis et al., 1988). The AZ cytomatrix is thought to play an organizational role in defining the presynaptic sites of synaptic vesicle docking and fusion. So far, several AZ-specific proteins including ELKS (also known as ERC1), CAST (also known as ERC2), RIM1, Munc13-1, Piccolo (also known as Aczonin), and Bassoon have been characterized (Südhof, 2012). Among them, CAST and ELKS have a unique potency to directly interact with all the other AZ proteins, forming a core complex at the AZ (Hida and Ohtsuka, 2010). CAST regulates the size of specialized AZs, to enhance synaptic transmission at ribbon synapses of rod photoreceptors (tom Dieck et al., 2012). In conventional synapses, although there are still some discrepancies, CAST and ELKS regulate excitatory and inhibitory synaptic transmissions in the nervous system (Held et al., 2016; Liu et al., 2014; Takao-Rikitsu et al., 2004). Thus, it has been established that CAST and ELKS control the structure and functions of presynaptic AZs for tight regulation of polarized neurotransmitter release; however, outside the brain, little is known about the contribution of AZ proteins to cell functions such as exocytosis in endocrine cells.

Although pancreatic β cells lack ultrastructurally discernible AZs, polarized Ca^{2+} -dependent insulin secretion has been suggested. These cells play a central role in glucose homeostasis: glucose metabolism depolarizes β cells by inhibiting the K_{ATP} channels, causing Ca^{2+} to enter the cell through voltage-dependent Ca^{2+} channels (VDCCs) and trigger insulin granule exocytosis (Ashcroft and Rorsman, 2012). In rodents, β cells are typically organized in rosette-like structures around the central venous vasculature with arteries encircling the rosette (Bonner-Weir, 1988; Granot et al., 2009; Weir and Bonner-Weir, 1990). The insulin granules in the β cells are polarized toward the venous vasculature after cell stimulation (Bonner-Weir, 1988),



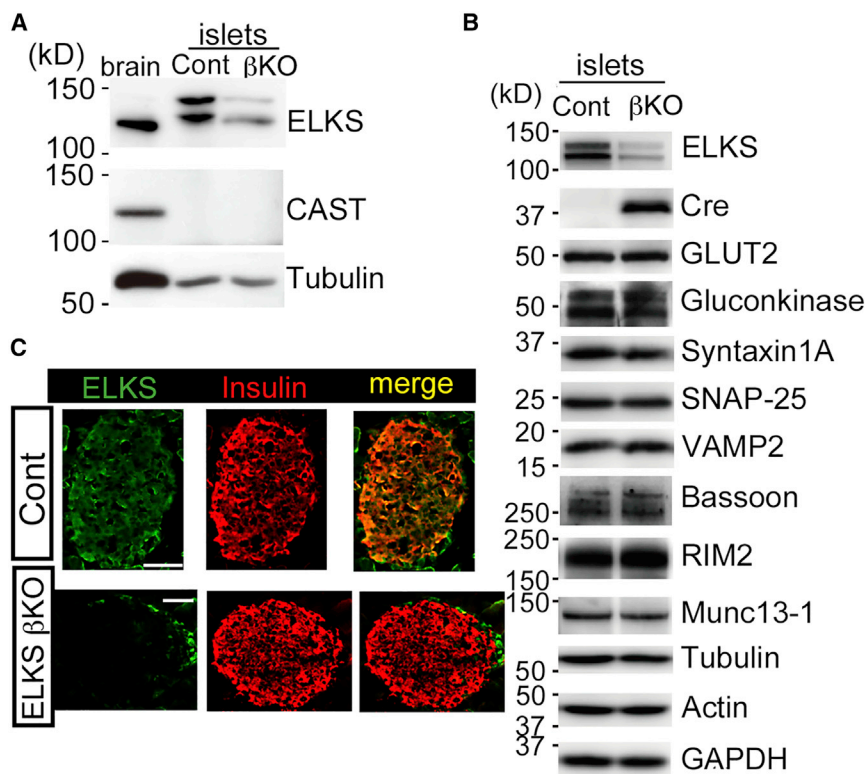


Figure 1. Pancreatic β Cell-Specific Disruption of ELKS

(A) Immunoblot analysis of pancreatic islet lysates from control (*ELKS^{flox/flox}*) and *ELKS* βKO (*ELKS^{flox/flox}; Cre*) mice using anti-ELKS and anti-CAST antibodies. A mouse brain homogenate was used as a positive control. Note that the control mouse islets expressed two major splice variants of ELKS (ELKS α and ϵ), but not CAST, whereas the mouse brain expressed both CAST and ELKS. (B) Expression of GLUT2, glucokinase, SNARE proteins, and other active zone (AZ) proteins in control and *ELKS* βKO islets observed by immunoblotting with specific antibodies, as indicated. (C) Pancreatic sections from control and *ELKS* βKO mice were double-immunostained for ELKS (green) and insulin (red). The intensity of ELKS staining in β cells was lower in *ELKS* βKO mice compared with the control. Bars, 50 μ m.

Here, we investigated whether ELKS controls polarized insulin secretion from β cells using β cell-specific *ELKS*-KO (*ELKS* βKO) mice and a newly established *in situ* Ca^{2+} -imaging system within islets expressing the membrane-bound Ca^{2+} sensor fluorescent protein, G-CaMP8b. We found that ELKS regulated L-type VDCC opening and first-phase insulin

secretion through direct binding to the VDCC- β subunit. Moreover, the glucose-evoked polarized $[\text{Ca}^{2+}]_i$ rise initially occurred at the vascular side of the β cell PM within islets, where ELKS is specifically localized. Thus, our functional analyses of ELKS strongly suggest that ELKS enhances Ca^{2+} influx through L-type VDCCs at the vascular side of the β cell PM and regulates the initial polarized Ca^{2+} influx and first-phase insulin secretion from pancreatic islets, providing insight into the AZ protein regulation of polarized Ca^{2+} influx and insulin secretion from β cells at the molecular level.

supporting the idea that insulin secretion into the blood stream occurs at the β cell plasma membrane (PM) facing the vasculature. Indeed, two-photon imaging has recently demonstrated that insulin granule secretion occurs mostly at the β cell membrane facing the vasculature (Low et al., 2014). Thus, polarized and targeted insulin secretion from β cells in islets into the vasculature has been proposed. Interestingly, several AZ-specific proteins including ELKS, RIM2, Munc13-1, Piccolo/Aczonin, and Bassoon are also expressed in pancreatic β cells (Fujimoto et al., 2002; Kang et al., 2006; Ohara-Imaizumi et al., 2005; Sheu et al., 2003; Yasuda et al., 2010). Except for Bassoon, these proteins have been reported to play a regulatory role in insulin secretion: (1) *ELKS* knockdown in MIN6 β cells reduced the glucose-induced insulin secretion (Ohara-Imaizumi et al., 2005), (2) treatment of islets with antisense oligodeoxynucleotides against *Piccolo* inhibited insulin secretion induced by glucose + the cAMP analog, 8-bromo-cyclic AMP (Fujimoto et al., 2002), (3) analysis of knockout (KO) mouse models revealed that RIM2 regulates the docking and priming states in insulin granule exocytosis (Yasuda et al., 2010), and (4) Munc13-1 is the priming factor in insulin exocytosis (Sheu et al., 2003). However, the functional relationship with the polarized and targeted insulin secretion is still obscure. Intriguingly, we have found that ELKS is enriched in the β cell PM facing the vasculature in rat islets, and that insulin granules' docking and fusion occurs selectively on ELKS clusters in the PM of MIN6 β cells (Ohara-Imaizumi et al., 2005). These findings suggest that ELKS may have a role in polarized Ca^{2+} -triggered insulin secretion into the vasculature from islets.

secretion through direct binding to the VDCC- β subunit. Moreover, the glucose-evoked polarized $[\text{Ca}^{2+}]_i$ rise initially occurred at the vascular side of the β cell PM within islets, where ELKS is specifically localized. Thus, our functional analyses of ELKS strongly suggest that ELKS enhances Ca^{2+} influx through L-type VDCCs at the vascular side of the β cell PM and regulates the initial polarized Ca^{2+} influx and first-phase insulin secretion from pancreatic islets, providing insight into the AZ protein regulation of polarized Ca^{2+} influx and insulin secretion from β cells at the molecular level.

RESULTS

Generation of β Cell-Specific *ELKS*-KO Mice

To explore the potential role of ELKS in pancreatic β cells, we generated *ELKS* βKO mice by crossing *ELKS* floxed (*ELKS^{flox/flox}*) mice (Dong et al., 2018), which served as controls, with RIP-Cre mice expressing Cre recombinase in pancreatic β cells (Aoyagi et al., 2012). Accordingly, control mouse islets expressed ELKS, but not its related protein, CAST, whereas the mouse brain expressed both ELKS and CAST (Figure 1A). The control islets expressed two major splice variants: ELKS α (brain isoform, ~120 kDa) and ELKS ϵ (ubiquitous isoform, ~140 kDa), which have distinct C termini, with the former containing the amino acid motif, IWA (Hida and Ohtsuka, 2010). By contrast, islets from *ELKS* βKO mice exhibited a marked reduction in the ELKS isoforms' expression (Figure 1A), with no changes in the expression levels of GLUT2, glucokinase and exocytotic SNARE proteins (syntaxin 1A, SNAP-25, and VAMP2), which

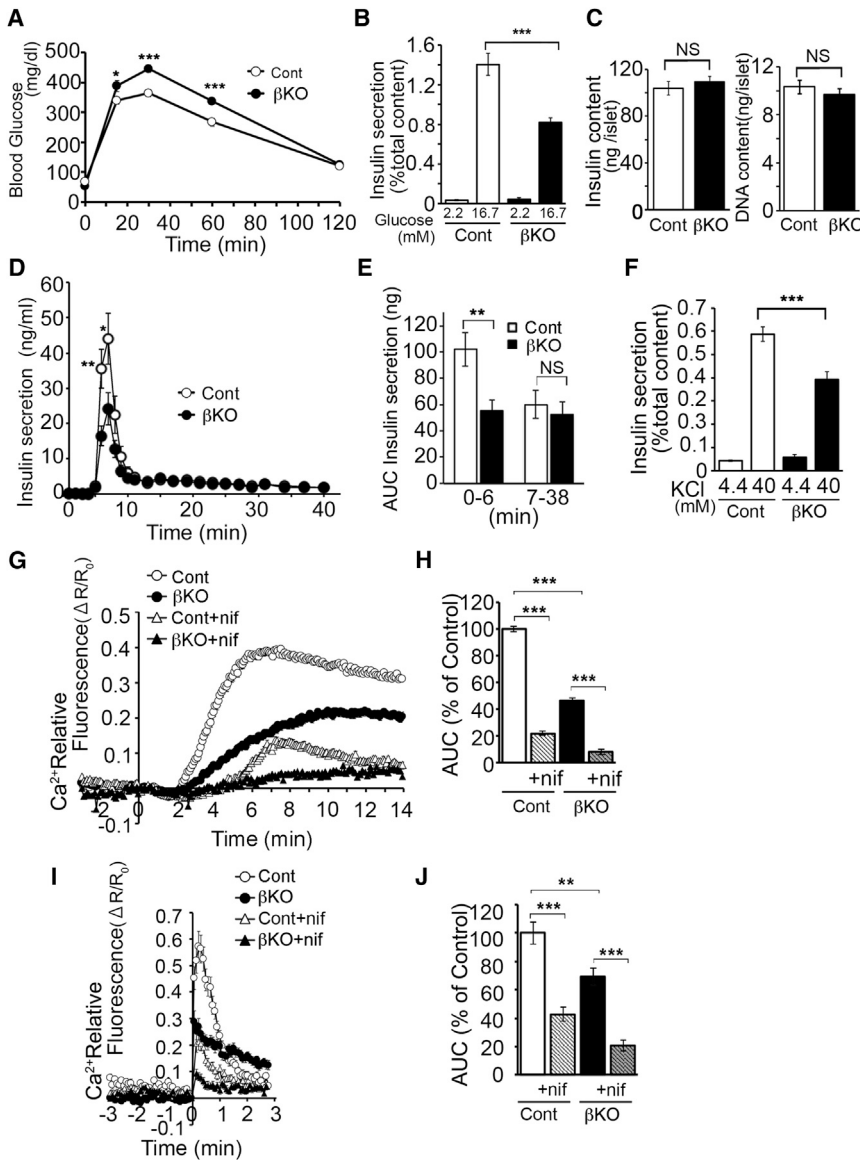


Figure 2. ELKS Deficiency Impairs the Glucose-Stimulated First-Phase Insulin Secretion and Cytosolic Ca²⁺ Increase

(A) Oral glucose tolerance was tested in control and *ELKS* βKO mice (*n* = 17 each). Blood glucose levels were measured at the indicated times after a glucose challenge of 2 g glucose/kg body weight. (B) Islets isolated from control and *ELKS* βKO mice were stimulated with 2.2 or 16.7 mM glucose for 30 min. The amount of secreted insulin was expressed as a percentage of the total cellular content (*n* = 14 separate challenges from six mice per group).

(C) Insulin content and DNA content per islet from control and *ELKS* βKO mice (*n* = 12 separate challenges from six mice per group).

(D) Insulin secretion from perfused control and *ELKS* βKO pancreata was measured as the glucose concentration was shifted from 2.8 to 16.7 mM.

(E) The area under the curve (AUC) for <6 min (first phase) and >7 min (second phase) in (D) (*n* = 13 per group).

(F) Islets isolated from control and *ELKS* βKO mice were stimulated with 4.4 or 40 mM KCl for 10 min (*n* = 18 separate challenges from six mice per group).

(G and I) Effects of *ELKS* βKO on changes in intracellular free calcium [Ca²⁺]_i. (G) Twenty-two millimolar glucose- and (I) 40 mM high K⁺-induced changes in [Ca²⁺]_i in β cells from control and *ELKS* βKO mice in the absence or presence of 1 μM nifedipine (nif). Changes in [Ca²⁺]_i were measured by Fura-2 acetoxymethyl (10 μM), and the ratio-metric 340/380 nm excitation signals were recorded. Time 0 indicates when the stimulants were added. Δ*R*/*R*₀ values were measured by quantifying the ratio between the change in the ratio-metric signal (Δ*R*) and the baseline value (*R*₀ at time 0; *n* > 50 cells from six mice per group).

(H and J) Quantitative analysis based on the AUC calculation is shown. (H) AUC 2–12 min in (G). (J) AUC 0–2 min in (I).

Results are means ± SEM. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; NS, not significant, two-tailed Student's *t* test in (A)–(F), one-way ANOVA with a Bonferroni post hoc test in (H)–(J).

are important for controlling glucose metabolism and insulin granule exocytosis (Figure 1B; Table S1). Moreover, the expression levels of the AZ proteins, Bassoon, RIM2, and Munc13-1, were unchanged in control and *ELKS* βKO islets. Immunostaining of pancreatic sections confirmed the efficient ablation of ELKS in insulin-positive β cells from *ELKS* βKO mice (Figure 1C).

First-Phase Insulin Secretion Is Impaired in Pancreatic Islets from *ELKS* βKO Mice

To examine pancreatic endocrine function in *ELKS* βKO mice, we performed the oral glucose tolerance test (OGTT). The fasting blood glucose levels (time 0 min) of *ELKS* βKO mice were not different from those of control mice. However, the OGTT did show impaired glucose tolerance in *ELKS* βKO mice at 15, 30, and 60 min after the challenge (Figure 2A). We next examined glucose-stimulated insulin secretion from isolated islets. As

shown in Figure 2B, glucose-stimulated insulin secretion (% of total insulin content) was markedly decreased in *ELKS* βKO mouse islets compared with control mouse islets, whereas the total insulin content and DNA content per islet were not altered (Figure 2C). Insulin secretion from pancreatic β cells shows a characteristic biphasic pattern, consisting of a transient first phase followed by a sustained second phase (Straub and Sharp, 2002). We investigated the effects of ELKS deletion on the biphasic insulin secretion in perfused pancreata. As shown in Figures 2D and 2E, the glucose-stimulated first-phase secretion (<6 min after onset of glucose stimulation) was selectively reduced in *ELKS* βKO mice (the area under the curve [AUC] during 0–6 min, but not during 7–38 min, was significantly lower in *ELKS* βKO mice than in control mice). In addition, high K⁺ depolarization (40 mM)-evoked insulin secretion, which elicits first-phase secretion (Ohara-Imaizumi et al., 2009), was markedly

reduced in KO mice compared with control mice (Figure 2F). These results strongly suggest that ELKS plays a major role in glucose-stimulated first-phase insulin secretion.

[Ca²⁺]_i Increase Is Impaired in *ELKS*-KO β Cells

Glucose metabolism depolarizes β cells by inhibiting K_{ATP} channels, resulting in Ca²⁺ influx by opening of primarily L-type VDCCs, and inducing first-phase insulin secretion (Rorsman et al., 2012). To determine whether the impairments in glucose-induced first-phase insulin secretion were associated with changes in [Ca²⁺]_i increase, we monitored the time course of the glucose-induced [Ca²⁺]_i increase using Fura-2 methods in primary cultured β cells. As shown in Figure 2G, the initial glucose-stimulated [Ca²⁺]_i increase was lower in *ELKS*-KO β cells than in control β cells, i.e., the AUC of the [Ca²⁺]_i increase was significantly lower in the *ELKS*-KO β cells than in the control β cells (Figure 2H). Furthermore, the high K⁺ depolarization-induced [Ca²⁺]_i rise was reduced in *ELKS*-KO β cells compared with the control β cells (Figures 2I and 2J). Both the glucose- and high K⁺-evoked [Ca²⁺]_i rise were remarkably decreased by the L-type VDCC blocker, nifedipine, both in the control and *ELKS*-KO β cells (Figures 2G–2J). These results suggest that ELKS deletion partially but substantially impairs the [Ca²⁺]_i rise via L-type VDCCs. Moreover, both *ELKS* α and *ELKS* ϵ gene transfer similarly restored the defect in glucose- and high K⁺-evoked [Ca²⁺]_i rise in *ELKS*-KO β cells (Figure S1), suggesting that both *ELKS* α and *ELKS* ϵ are responsible for the [Ca²⁺]_i rise in *ELKS*-KO β cells.

During the first phase of insulin secretion, exocytotic fusion events occur from the predocked granules, with some fusion events from newcomers (Ohara-Imaizumi et al., 2007), indicating that the number of morphologically docked insulin granules affects the first-phase secretion. However, the number of morphologically docked insulin granules observed by total internal reflection fluorescence (TIRF) microscopy (Figures S2A and S2B) and electron microscopy (Figures S2C and S2D) was not altered in *ELKS*-KO β cells, although ELKS-mediated vesicle targeting to the PM has been suggested in other cells (Grigoriev et al., 2007). In addition, the expression levels of the SNARE proteins, important mediators of insulin granule docking and fusion, were unchanged in *ELKS* β KO islets (Figure 1B), suggesting that insulin granule docking and fusion distal to the Ca²⁺ entry are not impaired in *ELKS*-KO β cells. Moreover, the results shown in Figure S3 suggest that ELKS does not affect the [Ca²⁺]_i oscillation and the endoplasmic reticulum (ER)-Ca²⁺ release. Thus, our data suggest that ELKS primarily regulates Ca²⁺ entry via L-type VDCCs during first-phase insulin secretion from pancreatic β cells.

ELKS Directly Interacts with VDCC- β Subunit

We next focused on the mechanism by which ELKS controls Ca²⁺ entry via L-type VDCCs. ELKS or the family member, CAST, interacts with VDCC- β subunits in the brain (Billings et al., 2012; Kiyonaka et al., 2012). Based on these results, we hypothesized that ELKS regulates VDCC function through binding to VDCC- β subunit in pancreatic β cells. In mouse islets, VDCC- β_2 and - β_3 are mainly expressed (Berggren et al., 2004; Lee et al., 2018) (Figure 3A; Table S1) with the L-type VDCC- α

subunit, Ca_v1.2, which is critical for insulin secretion (Rorsman et al., 2012). Therefore, we investigated the binding of ELKS to VDCC- $\beta_{2/3}$ subunits. First, Myc-ELKS was cotransfected with FLAG-VDCC- β_2 subunit into COS-7 cells, followed by immunoprecipitation with an anti-FLAG antibody. We found that ELKS coimmunoprecipitated with FLAG-VDCC- β_2 (Figure 3B, left). Moreover, we confirmed the binding of endogenous ELKS and VDCC- β_2 in MIN6 β cells (Figure 3B, right).

Next, to clarify which domain of ELKS binds to the VDCC- β_2 subunit, we performed a pull-down assay using various maltose-binding protein (MBP) fusion proteins with ELKS fragments (Figure 3C, left panel). FLAG-VDCC- β_2 subunit bound to the N-terminal region of ELKS (MBP-ELKS_{1–404}), but not to the other ELKS fragments (Figure 3C, right panel). Moreover, we examined which domain of the VDCC- β_2 subunit binds to ELKS. The VDCC- β_2 subunit contains two central conserved domains, src homology 3 (SH3) domain and guanylate kinase-like (GK) domain (Figure 3D, left panels) (Chien et al., 1998; Hanlon et al., 1999). A pull-down assay using GST fusion proteins of VDCC- β_2 -SH3 or -GK domain demonstrated that ELKS_{1–404} bound to GST-VDCC- β_2 -GK, but not to GST-VDCC- β_2 -SH3 (Figure 3D, right panel). Moreover, essentially the same results were obtained for the binding of ELKS and VDCC- β_3 subunit (Figure S4). Taken together, these results indicate that the binding of ELKS and VDCC- $\beta_{2/3}$ is directly mediated through the N-terminal region of ELKS and the GK domain of VDCC- $\beta_{2/3}$.

We further examined whether the binding of ELKS to VDCC- $\beta_{2/3}$ subunits is implicated in the [Ca²⁺]_i rise and insulin secretion from β cells. First, we performed a pull-down assay with Myc-ELKS and GST-VDCC- $\beta_{2/3}$ in the presence or absence of ELKS_{1–404}. ELKS_{1–404} inhibited the binding of ELKS to VDCC- $\beta_{2/3}$ in a dose-dependent manner (Figure S5A), indicating that ELKS_{1–404} inhibits the proper formation of the ELKS and VDCC- β complex. Next, ELKS_{1–404} was transfected into control mouse islets by electroporation (Aoyagi et al., 2012, 2016). Because of its low transfection efficacy, human growth hormone (hGH) was coexpressed as a reporter to evaluate its secretion from the transfected β cells. We have previously confirmed that a large part of hGH is stored in insulin granules and the amount of secreted hGH induced by glucose reflects the secretion ability of transfected β cells in islets (Aoyagi et al., 2012; 2016). As shown in Figures S5B and S5C, transient expression of ELKS_{1–404} significantly inhibited glucose- and high K⁺-stimulated hGH secretion from islets. Furthermore, the expression of ELKS_{1–404} markedly reduced the glucose- and high K⁺-induced Ca²⁺ response as assessed by Fura red imaging (Figures S5D and S5E). These findings suggest that the ELKS-VDCC- $\beta_{2/3}$ interaction contributes to the glucose-stimulated [Ca²⁺]_i rise and subsequent insulin secretion from pancreatic β cells.

ELKS Regulates L-type VDCC Opening

ELKS interacting with the VDCC- β GK domain regulates channel gating by binding to pore-forming VDCC- α (Buraei and Yang, 2013). To investigate whether ELKS regulates VDCC activity in β cells, we used whole-cell voltage-clamp experiments (Figure 4). The Ca²⁺ current amplitudes were markedly reduced at membrane potentials between –20 mV and +30 mV, reducing

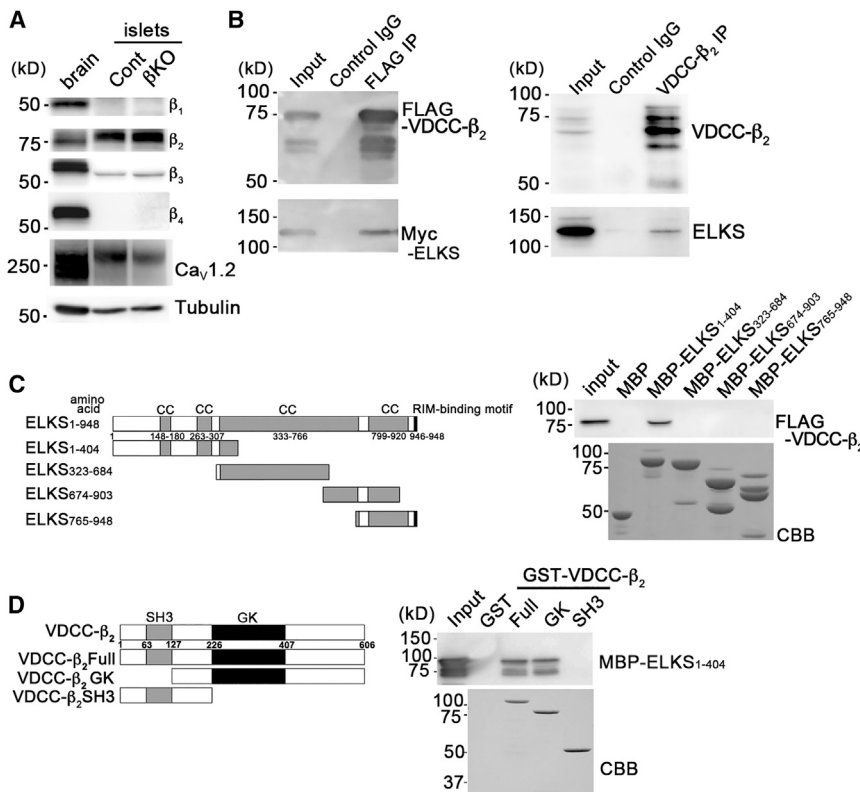


Figure 3. Interaction of ELKS with VDCC-β₂ and -β₃

(A) Expression of the VDCC subunits (Ca_v1.2, β₁₋₄) in control and *ELKS* βKO mouse islets assessed by immunoblotting. Note that in these islets, mainly VDCC-β₂ and -β₃ were expressed.

(B) Coimmunoprecipitation of ELKS with VDCC-β₂ in COS-7 cells and MIN6 β cells. For FLAG immunoprecipitation, Myc-ELKS and FLAG-VDCC-β₂ expression plasmids were transfected into COS-7 cells. The cell extracts were subjected to immunoprecipitation with the anti-FLAG antibody. The immunoprecipitates were analyzed by immunoblotting using the anti-FLAG or anti-Myc antibodies. For endogenous VDCC-β₂ immunoprecipitation, the cell extracts were subjected to immunoprecipitation with the anti-VDCC-β₂ antibody. The immunoprecipitates were analyzed by immunoblotting using the anti-ELKS or anti-VDCC-β₂ antibodies. Input contained 0.05% and 0.01% of the extract used in the anti-VDCC-β₂ and anti-ELKS immunoblot assay, respectively.

(C) The VDCC-β binding domain of ELKS. Pull-down assay of MBP fusion proteins containing various fragments of ELKS with the VDCC-β₂ subunit. The protein extract of COS-7 cells expressing FLAG-VDCC-β₂ was incubated with amylose beads containing various MBP-ELKS constructs (MBP-ELKS₁₋₄₀₄, -ELKS₃₂₃₋₆₈₄, -ELKS₆₇₄₋₉₀₃, -ELKS₇₆₅₋₉₄₈) as indicated. Domain structures were analyzed with the SMART program (Schultz et al., 1998). Proteins that bound to the

beads were analyzed by immunoblotting using the anti-FLAG antibody. Equal amounts of the MBP fusion proteins were loaded and assessed by protein staining with Coomassie brilliant blue. Input contained 1.5% of the extract used in the assay.

(D) The ELKS-binding domain of VDCC-β₂. Pull-down assay of GST fusion proteins containing the SH3 or GK domain of VDCC-β₂ (as indicated) with MBP-ELKS₁₋₄₀₄. The GST fusion proteins containing these VDCC-β₂ subunit regions as well as GST alone were immobilized by glutathione-Sepharose beads and then analyzed by immunoblotting using the anti-MBP antibody. Domain structures were analyzed with the SMART program. Input contained 1.5% of the extract used in the assay. All the ELKS fragments used in Figure 3 are derived from ELKS_α.

See Figure S4.

by 30% at +10 mV (-14.0 ± 1.0 pA/pF, $p < 0.01$, $n = 39$) in *ELKS*-KO β cells compared with control cells (-20.1 ± 1.6 pA/pF, $n = 22$; Figures 4A and 4D). In Figure 4B, averaged currents were plotted by the same voltage protocol as in A for control and *ELKS*-KO β cells in the presence of the L-type VDCC blocker, nifedipine (1 μM); the currents were identical in both cell types. To examine whether the current component inhibited in *ELKS*-KO β cells is an L-type VDCC current, the β cells were exposed to nifedipine to isolate nifedipine-sensitive currents, which were calculated by subtracting the current of the control in B from that in A and compared with that in *ELKS*-KO β cells in Figure 4C. The major component of the VDCC current reduced in the *ELKS*-KO β cells was the L-type Ca²⁺ channel (Figure 4D). In the current-voltage (I-V) relationship in control and *ELKS*-KO β cells in the presence or absence of nifedipine, the current decreased in *ELKS*-KO β cells only in the absence of nifedipine without shifting the I-V relationship along the voltage axis. We also found that ELKS deletion had no effect on the steady-state voltage-dependent activation (VDA) and inactivation (VDI) (Figures S6A–S6F). These results suggest that ELKS regulates the Ca²⁺ current through L-type VDCCs without affecting the voltage-dependent gating process.

Furthermore, the functional effect of ELKS on L-type VDCCs was confirmed in the exogenous expression system of human embryonic kidney (HEK293) cells (Figure S7). We measured the currents from L-type VDCCs (Ca_v1.2) with or without overexpression of ELKS with Ba²⁺ as the charge carrier under whole-cell recording. The peak current density at +10 mV in the cells coexpressing VDCC (Ca_v1.2/β_{2a}/α_{2δ}) and ELKS was -34.1 ± 3.8 pA/pF (ELKS), whereas it was -12.7 ± 3.4 pA/pF in the control cells, which is a 2.8 ± 0.2 -fold increase (Figure S7D). However, the I-V relationship was not shifted along the voltage axis, and the steady-state VDI was almost identical between these two cellular settings (Figures S7C and S7E). Therefore, the results from the exogenous system strengthen the conclusion from the *ELKS*-KO β cells (Figures 4D, S6E, and S6F) that ELKS positively regulates L-type VDCCs without affecting the voltage-dependent gating process.

Although the L-type current is the major VDCC current in mouse β cells, the P/Q- and R-type VDCC currents contribute to the L-type VDCC blocker-resistant current (Schulla et al., 2003; Yang and Berggren, 2005). To determine whether ELKS deletion also reduces non-L-type VDCC currents, we examined the effect of non-L-type VDCC blockers on the L-type VDCC blocker (nifedipine)-resistant current. As shown in Figure S6G,

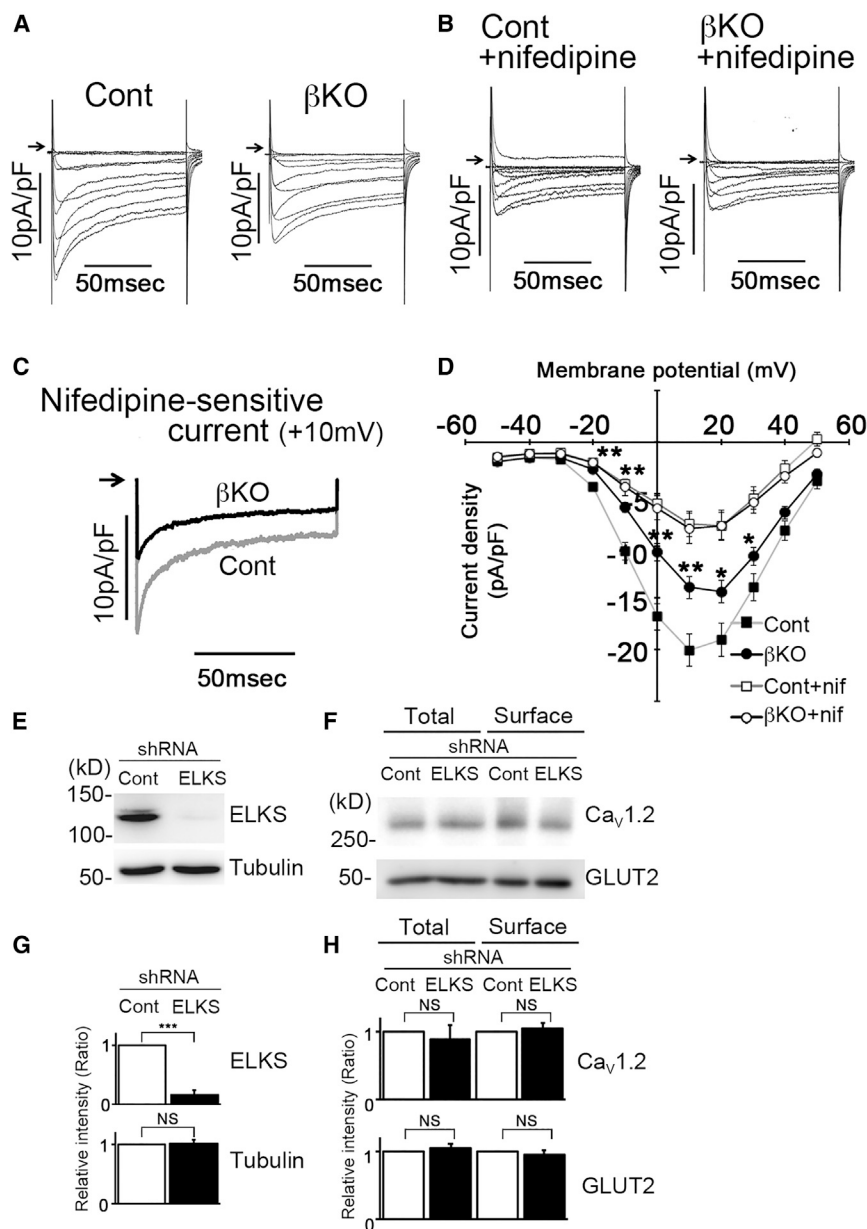


Figure 4. ELKS Deficiency Reduces the L-type Voltage-Dependent Ca^{2+} Current

(A and B) Current data of VDCCs elicited by test pulses converted to current density with cell capacitance were averaged ($n = 22$ challenges for cont. and $n = 39$ challenges for βKO in A from four mice per group) and the mean current density at each test potential was plotted for control (left panel) and *ELKS*-KO β cells in (A). The mean current density was similarly obtained in the presence of $1 \mu\text{M}$ nifedipine and is shown in (B) ($n = 8$ challenges for cont. and $n = 10$ challenges for βKO from four mice per group).

(C) Nifedipine-sensitive inward currents as mean current density in control and *ELKS*-KO β cells in response to $+10$ mV were obtained by subtracting the corresponding current data of (B) from those of (A) in control and *ELKS*-KO β cells.

(D) The I-V relationship of the peak Ca^{2+} current amplitude of the control and *ELKS*-KO β cells in the absence or presence of $1 \mu\text{M}$ nifedipine (nif). Cont, control; βKO , *ELKS* βKO . The arrows indicate zero current. The glucose concentration in the bath solution was 5.6 mM.

(E and G) shRNA against *ELKS* suppressed the expression of *ELKS* in MIN6 cells. MIN6 cells were infected with control shRNA or *ELKS* shRNA and were subjected to immunoblotting using antibodies against *ELKS* and tubulin. Representative immunoblots (E) and a graph (G) showing the intensity of protein levels in *ELKS* shRNA-treated cells relative to that in control shRNA-treated cells are presented. *ELKS* shRNA significantly down-regulated *ELKS* expression levels (18% of control), whereas tubulin expression was unchanged. The data represent five separate experiments.

(F and H) *ELKS* KD did not affect the amount of $\text{Ca}_v1.2$ in the PM. MIN6 cells were infected with control shRNA or *ELKS* shRNA, and then proteins in the PM were extracted as described in the STAR Methods. The purified cell-surface proteins and total cell lysates were subjected to immunoblotting using antibodies against $\text{Ca}_v1.2$ and GLUT2. Representative immunoblots (F) and a graph (H) showing the intensity of protein levels in *ELKS* shRNA-treated cells relative to that in control shRNA-treated cells are presented. shRNA KD of *ELKS* had no effect on the amount of $\text{Ca}_v1.2$ in the PM, similar to the situation of GLUT2, which was used as a positive control.

The data represent five separate experiments. Results are means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, not significant, two-tailed Student's t test in (D), (G), and (H).

nifedipine reduced the β cell Ca^{2+} currents (elicited by depolarizations to $+10$ mV) to nearly the same values in control and *ELKS* βKO cells. Furthermore, the P/Q- and R-type channel blockers significantly inhibited the L-type VDCC blocker-resistant current to similar values in control and *ELKS* βKO cells (Figures S6H and S6I). These results suggest that *ELKS* deletion reduced the Ca^{2+} currents conducted by L-type VDCCs, but not by non-L-type VDCC.

The expression of the L-type VDCC subunits, $\text{Ca}_v1.2$, β_2 , and β_3 , was normal in *ELKS* βKO islets (Figure 3A). However, if *ELKS*

controls VDCC expression at the PM of β cells, it may affect the Ca^{2+} current in *ELKS*-KO β cells. To evaluate the changes in VDCC expression at the PM due to *ELKS* deletion, we biochemically examined the cell-surface levels of $\text{Ca}_v1.2$ using surface biotinylation and *ELKS*-knockdown (KD) MIN6 β cells. Short hairpin RNA (shRNA) adenoviruses against *ELKS* effectively reduced the amount of endogenous *ELKS* (Figures 4E and 4G) and inhibited evoked insulin secretion and $[\text{Ca}^{2+}]_i$ increase in MIN6 cells (Figures S8A–S8C), similarly to *ELKS*-KO β cells. After infection of MIN6 cells with control or *ELKS* shRNA

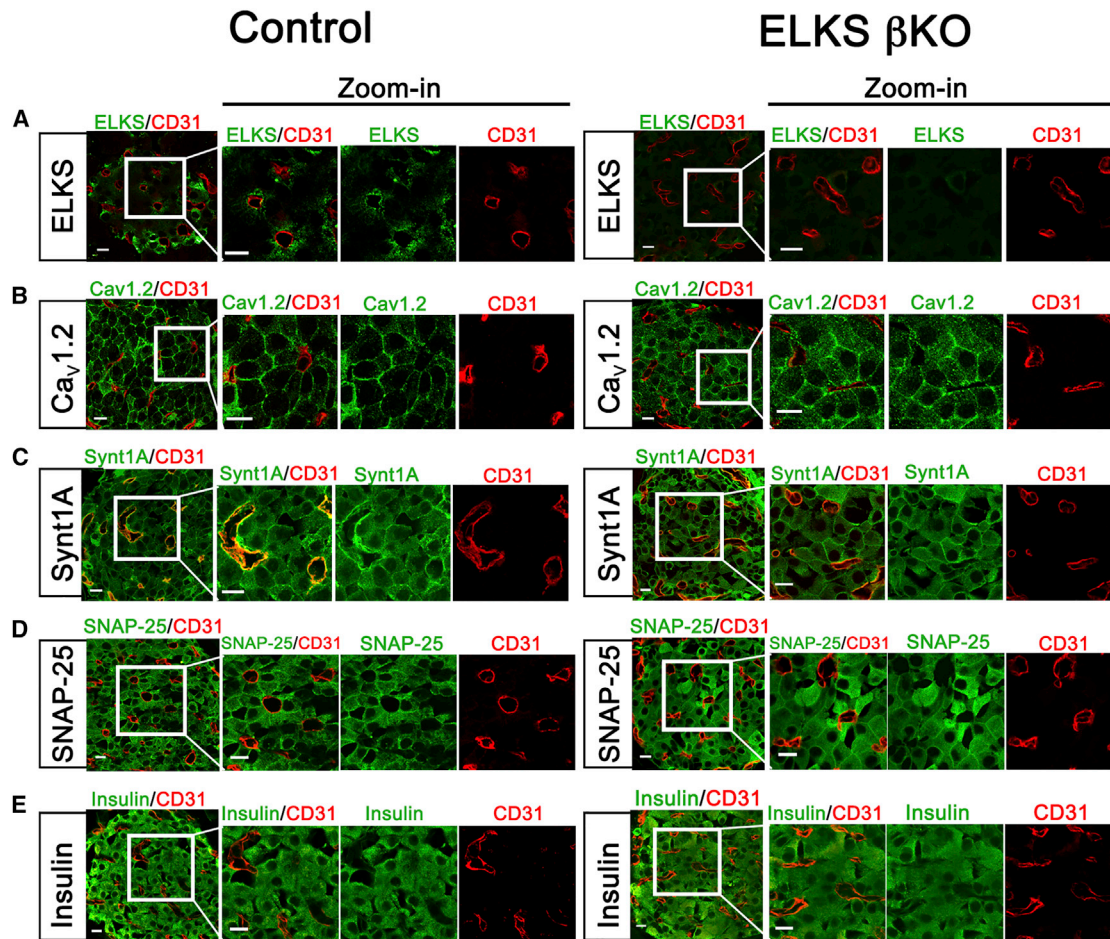


Figure 5. Expression Pattern of Insulin, ELKS, $\text{Ca}_v1.2$, Syntaxin1A, and SNAP-25 in the Core of Islets of Control and *ELKS* β KO Mice

(A–E) Representative immunohistochemistry images of coimmunostaining of CD31, a marker of blood capillary endothelial cells, and ELKS (A), $\text{Ca}_v1.2$ (B), Syntaxin1A (Synt1A) (C), SNAP-25 (D), or insulin (E) in control and *ELKS* β KO mouse islets. Zoomed-in images are of the area within the square in the first panel from the left.

Note that the immunofluorescence images show that insulin is positive in the core of both control and *ELKS* β KO islets, indicating that β cells compose the core of the islets in (E). Bars, 10 μm .

adenoviruses, membrane proteins exposed to the extracellular surface were labeled with a membrane-impermeable biotinylation reagent, and biotinylated proteins were recovered by NeutrAvidin beads. As shown in Figures 4F and 4H, *ELKS* KD did not affect the amount of $\text{Ca}_v1.2$ on the PM, similar to the situation of GLUT2, which was used as a positive control, indicating that ELKS regulates the Ca^{2+} current by a mechanism independent of regulating the VDCC expression levels at the PM. Taken together, our data suggest that ELKS controls the opening of L-type VDCCs, which may lead to Ca^{2+} influx and first-phase insulin secretion from pancreatic β cells.

ELKS Is Enriched at the β Cell PM Facing the Islet's Vasculature

We next examined the localization of ELKS in β cells within the mouse islet (Figure 5). Confocal imaging revealed that the islet core region is composed of insulin-stained β cells (Figure 5E),

similar to the case in rodent islets (Steiner et al., 2010). Insulin seemed to be evenly distributed in the cytosol both in control and *ELKS* β KO islet β cells (Figure 5E). In fact, electron microscopy studies also showed that the insulin granules were evenly distributed in control β cells, which was not affected by ELKS deletion (data not shown). Note that in control mouse islets, ELKS was preferentially enriched at the β cells' PM facing the vasculature labeled with CD31, a marker of endothelial cells (Figure 5A), as we described previously in rat islets (Ohara-Imaizumi et al., 2005), whereas the intensity of ELKS was significantly reduced in *ELKS* β KO islets. In contrast, the immunostaining pattern of $\text{Ca}_v1.2$ and exocytotic t-SNARE proteins (syntaxin 1A and SNAP-25) was seen on the entire β cell PM both in control and *ELKS* β KO islets (Figures 5B–5D). These results prompted us to hypothesize that ELKS locally regulates L-type VDCCs at the β cell PM facing the vasculature, followed by Ca^{2+} influx and insulin granule exocytosis.

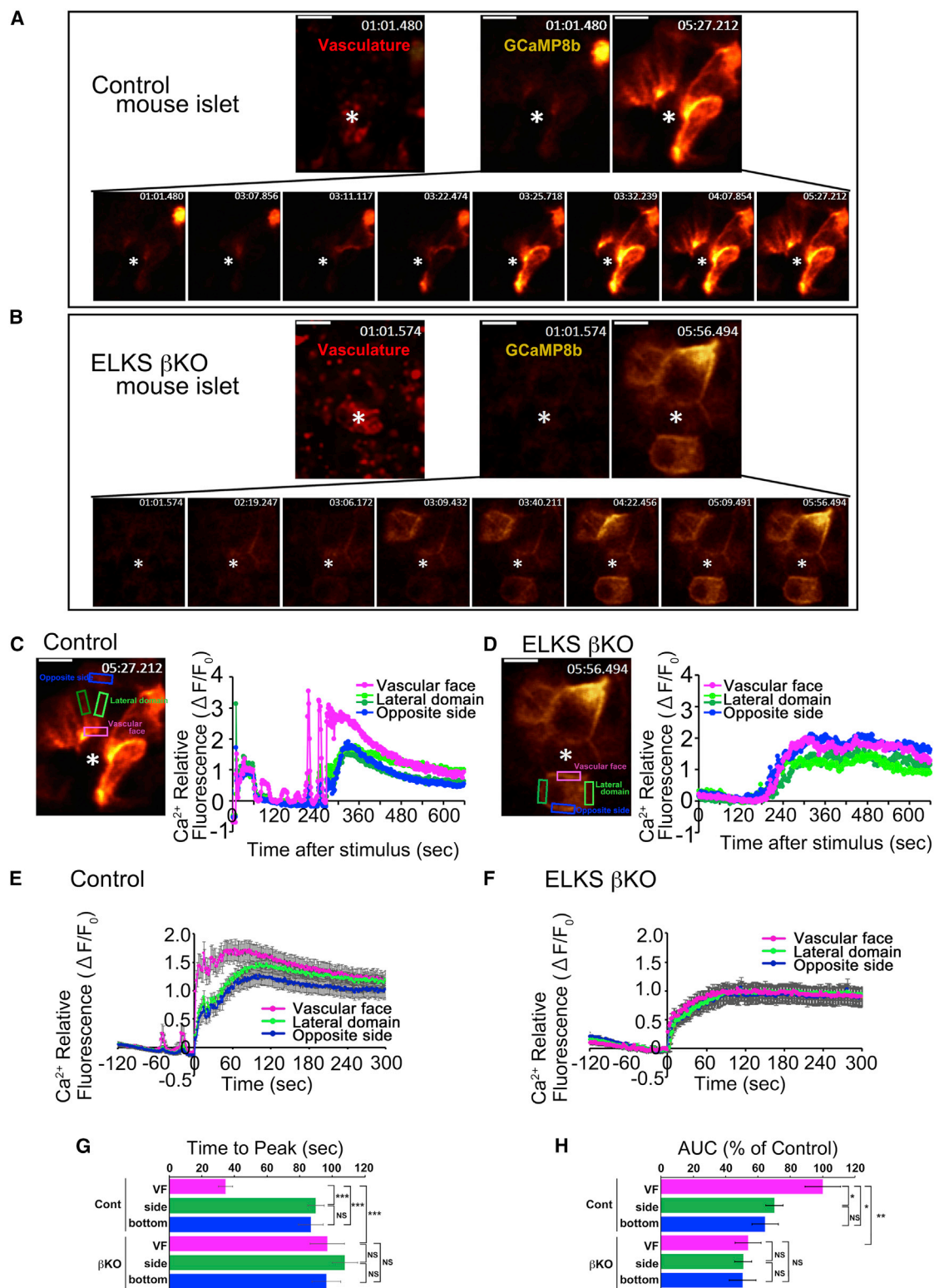


Figure 6. ELKS Facilitates the Ca²⁺ Rise at the PM Facing the Vasculature in Intact Islets

(A) Membrane-bound G-CaMP8b Ca²⁺ fluorescence images of the β cell PM in close vicinity to tomato lectin-labeled blood vessels during a 22-mM glucose stimulation in control islets using confocal microscopy. Islets were treated with adenoviruses carrying the membrane-bound Ca²⁺ indicator, G-CaMP8b, and DyLight 594-conjugated tomato lectin using the whole-pancreas perfusion-based transduction procedure, and cultured for 48 h. Top: the middle and right panels

(legend continued on next page)

ELKS Facilitates Ca^{2+} Influx at the PM Facing the Vasculature

If ELKS controls L-type VDCCs at the vascular side of the β cell PM in islets, glucose-evoked local Ca^{2+} signals induced by VDCCs would initially appear at the PM facing the vasculature during the first phase of insulin secretion. Therefore, to examine the subplasmalemmal Ca^{2+} signals in β cells and the vasculature within intact cultured islets, islets were infected with adenoviruses encoding membrane-bound G-CaMP8b, which was newly synthesized as a high-affinity Ca^{2+} probe (Ohkura et al., 2012; Figure S9A), and DyLight 594-labeled tomato lectin, which binds the luminal surface of endothelial cells in blood vessels (Brissova et al., 2004). For highly efficient transduction of intact islets, we used a whole-pancreas perfusion-based transduction procedure. Using confocal microscopy, we observed the subplasmalemmal Ca^{2+} increase in islet β cells as the appearance of G-CaMP8b fluorescence in close vicinity to blood vessels labeled with DyLight 594 fluorescence. As shown in Figure 6A and Video S1, when control mouse islets were stimulated with glucose, the local Ca^{2+} fluorescence initially appeared at the β cell PM facing the vasculature, but then the Ca^{2+} fluorescence spread over the entire PM. To better understand the dynamics of the Ca^{2+} fluorescence, the mean intensities of three PM regions, the vascular face, the lateral domain, and the opposite side of the vasculature, were plotted against time (Figures 6C and 6E). Note that the Ca^{2+} signal initially increased in the vascular face region, while the Ca^{2+} fluorescence intensity in the lateral domain and the opposite side increased slowly: in the vascular region, the time to peak (Figure 6G) was significantly reduced and the AUC of the Ca^{2+} rise (Figure 6H) was significantly increased compared to in the lateral domain and the opposite side of control β cells.

In contrast, in *ELKS*-KO β cells, the initial Ca^{2+} fluorescence in the vascular face region was decreased (Figure 6B; Video S2). Furthermore, the Ca^{2+} fluorescence increased similarly in these three subplasmalemmal regions (Figures 6D and 6F): the time to peak (Figure 6G) and the AUC of the Ca^{2+} rise (Figure 6H) were not significantly different among the three regions of the KO β cells. Furthermore, these Ca^{2+} signals were reduced by nifedipine in the control and β KO islets (Figures S9B and S9C). These data suggest that ELKS and L-type VDCCs form an insulin secretion complex at the ELKS-localized vascular side of the β cell PM, and that this complex regulates the initial polarized Ca^{2+} influx and first-phase insulin secretion from pancreatic islets.

DISCUSSION

In this study, we showed that ELKS regulates the L-type VDCC opening and first-phase insulin secretion using *ELKS* β KO mice. Moreover, ELKS is localized at the β cell PM facing the vasculature within islets, where the polarized $[\text{Ca}^{2+}]_i$ rise initially occurs after glucose stimulation as assessed by the newly established Ca^{2+} sensor, G-CaMP8b. Thus, our data strongly suggest that ELKS plays a pivotal role in L-type VDCC activation at the vascular side of the β cells' PM, and in the polarized $[\text{Ca}^{2+}]_i$ rise and first-phase insulin secretion probably into the vasculature.

ELKS Controls L-type VDCC Opening and First-Phase Insulin Secretion

The first-phase insulin secretion from β cells is significantly larger than the second-phase secretion, and it greatly affects the subsequent glucose tolerance (Gerich, 1998). We found that ELKS

show representative G-CaMP8b fluorescence images before and after the Ca^{2+} rise evoked by high glucose in control islet β cells. The asterisk represents the location of the DyLight 594-tomato lectin-labeled vasculature (left). High glucose was added at time 0. Bars, 10 μm . A time stamp (min:s.ms) is overlaid on the images. Bottom: representative sequential fluorescence images of G-CaMP8b during high-glucose stimulation of control islet β cells. Note that in control islets, initially the local Ca^{2+} fluorescence appeared at the β cell PM facing the vasculature, and then it was spread through the entire PM during high-glucose stimulation (see Video S1).

(B) Membrane-bound G-CaMP8b Ca^{2+} fluorescence images of β cell PM in close vicinity to tomato lectin-labeled blood vessels during a 22-mM glucose stimulation in *ELKS* β KO islets using confocal microscopy. Top: the middle and right panels show representative G-CaMP8b fluorescence images before and after the Ca^{2+} rise evoked by high glucose in *ELKS* β KO islet β cells. The asterisk represents the location of the DyLight 594-tomato lectin-labeled vasculature (left). High glucose was added at time 0. Bars, 10 μm . (Bottom: representative sequential fluorescence images of G-CaMP8b during high-glucose stimulation of *ELKS* β KO islet β cells. Note that the slow increase in Ca^{2+} fluorescence was uniformly observed in the entire β cell PM during high-glucose stimulation (see Video S2).

(C) Representative time course of the changes in G-CaMP8b fluorescence was plotted for three β cell PM regions in the control islets (A): the vascular face (magenta), the lateral domain (light green and deep green), and the opposite side of the vasculature (blue). Changes in the average fluorescence intensities of the β cell PM regions in the control islets were plotted against time. Note that the Ca^{2+} signal rose up at first in the vascular face, whereas the Ca^{2+} fluorescence intensity slowly increased in the lateral domain region and in the opposite side of the vasculature. The fluorescence intensities are expressed relative to the baseline fluorescence intensity at time 120 s. Stimulation started at 0 s.

(D) Representative time course of the changes in G-CaMP8b fluorescence was plotted for three β cell PM regions in *ELKS* β KO islets (B): the vascular face (magenta), the lateral domain (light green and deep green), and the opposite side of the vasculature (blue). Changes in the average fluorescence intensities of the β cell PM regions in *ELKS* β KO islets were plotted against time. Note that the Ca^{2+} fluorescence intensity uniformly increased in all the regions. The fluorescence intensities are expressed relative to the baseline fluorescence intensity at time 120 s. Stimulation started at 0 s.

(E) The time course of the mean changes in G-CaMP8b fluorescence was plotted for three β cell PM regions in control islets: the vascular face (magenta), the lateral domain (green), and the opposite side of the vasculature (blue); the onset time of the Ca^{2+} rise was standardized as time 0 s. $\Delta F/F_0$ values were calculated as described in (C). Results are means $\pm$ SEM ($n = 45$ cells; 16 islets; nine mice).

(F) The time course of the mean changes in G-CaMP8b fluorescence was plotted for three β cell PM regions in *ELKS* β KO islets: the vascular face (magenta), the lateral domain (green), and the opposite side of the vasculature (blue); the onset time of the Ca^{2+} rise was standardized as time 0 s. $\Delta F/F_0$ values were calculated as described in (D). Results are means $\pm$ SEM ($n = 43$ cells; 18 islets; 10 mice).

Quantitative analysis based on time to peak (G) and on AUC (0–150 s) (H) of the Ca^{2+} rise for (E) and (F) is shown. Results are means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, not significant, one-way ANOVA with a Bonferroni post hoc test.

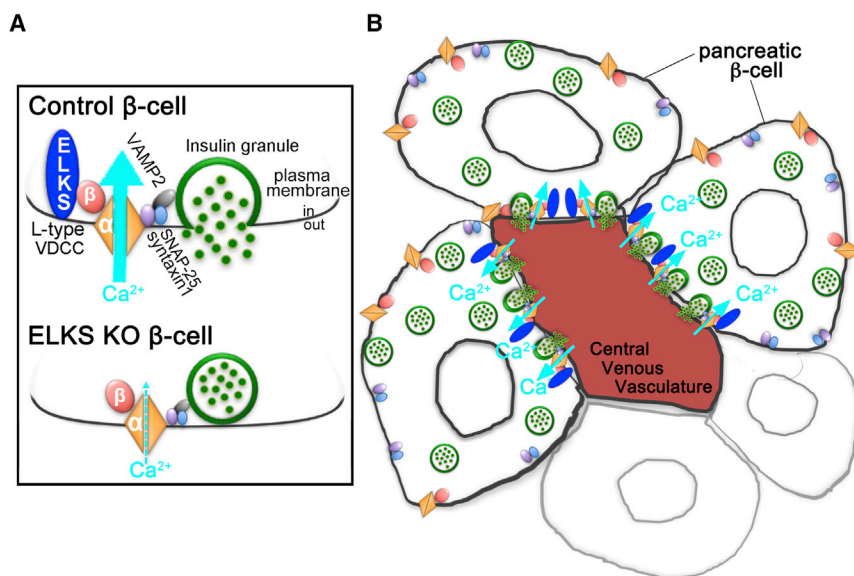


Figure 7. A Model of the Roles of ELKS in the First-Phase Insulin Secretion from β Cells within Islets

(A) ELKS directly binds to VDCC- $\beta_{2/3}$ and supports the L-type VDCC opening by glucose-induced β cell membrane depolarization. This causes a boosted Ca^{2+} influx via VDCC α ($\text{Ca}_v1.2$) and exocytosis of insulin granules, which are located near the L-type VDCCs by tethering the assembly of SNAREs (Syntaxin1, SNAP-25, VAMP2).

(B) In islets, ELKS localizes at the vascular side of the β cell PM and regulates the initial Ca^{2+} influx through binding to L-type VDCCs, followed by first-phase insulin secretion probably into the vasculature. Thus, ELKS is required for the initial polarized Ca^{2+} influx in β cells within islets.

deletion impaired the first-phase secretion in perfused pancreata experiments and in high K^+ depolarization (known to elicit first-phase secretion)-evoked insulin secretion from islets (Figure 2). The first-phase secretion requires a rapid and marked elevation in Ca^{2+} influx via opening of L-type VDCCs (Rorsman et al., 2012; Schulla et al., 2003). Indeed, ELKS deletion impaired the nifedipine-sensitive $[\text{Ca}^{2+}]_i$ rise evoked by glucose and high K^+ stimulation (Figure 2), suggesting that ELKS primarily regulates Ca^{2+} entry via L-type VDCCs. Moreover, the N-terminal region of ELKS directly interacted with the GK domain of VDCC- $\beta_{2/3}$ subunits (Figures 3 and S4), which interact with pore-forming VDCC α (Buraei and Yang, 2013). Inhibition of the interaction between ELKS and the β subunits reduced the Ca^{2+} response in control β cells (Figure S5). Consistently, an electrophysiological study revealed that ELKS-KO β cells had decreased L-type VDCC current density (Figure 4). The exogenous system strengthens the view that ELKS positively regulates L-type VDCCs (Figure S7). Previously, we have found that ELKS clusters were on the docking sites of insulin granules that were colocalized with clusters of the exocytotic t-SNARE, syntaxin 1A, in the PM of MIN6 β cells (Ohara-Imaizumi et al., 2005). The insulin granules are localized near L-type VDCCs by tethering the assembly of SNAREs (syntaxin1, SNAP-25, VAMP2) (Barg et al., 2002). Thus, ELKS is important for L-type VDCC opening through binding to the VDCC- $\beta_{2/3}$ GK domain, which leads to enhanced Ca^{2+} influx and insulin exocytosis during first-phase insulin secretion from pancreatic β cells (Figure 7A).

ELKS controls the Ca^{2+} influx through L-type Ca^{2+} channels via an interaction with VDCC β subunit. The β subunit associates the VDCC as an auxiliary subunit of the pore-forming VDCC α subunit and contributes to trafficking to the PM as well as the voltage-dependent gating of VDCC (Buraei and Yang, 2013). The RGK (Rem/Gem/Kir) family, which belongs to the Ras-related GTPase subfamily, interacts with the β subunit. Similar to ELKS, RGK also interacts with the GK domain of the β subunit; however, it potently inhibits the membrane

trafficking of Ca_v1 or Ca_v2 channels (Yang and Colecraft, 2013), which is a different functionality to ELKS that did not affect the $\text{Ca}_v1.2$ expression in the PM (Figures 4F and 4H). RIM, a synaptic

protein that is required for vesicle docking and priming (Kaesler et al., 2012), interacts with the β subunit as well as the C-terminal domain of VDCC α (Kaesler et al., 2011; Hirano et al., 2017; Kiyonaka et al., 2007). This synaptic protein interaction regulates the VDCC functions of both trafficking, to enhance the current density, and the voltage-dependent gating process, to delay inactivation (Hirano et al., 2017; Kiyonaka et al., 2007). So far, we have not found a clear effect of ELKS on the voltage-dependent gating process (Figures S6 and S7); thus, the mechanism of ELKS via the β subunit interaction may be different from that of these well-known β subunit-interacting molecules. Hence, ELKS may enhance L-type VDCC activity via β subunit interaction by an as-yet-unknown mechanism. Considering ELKS had no effect on the voltage-dependent gating process, it may be speculated that ELKS influences the number of functional VDCCs or the single Ca^{2+} -channel conductance. Nevertheless, further studies are necessary to reveal the molecular mechanism for controlling ELKS-mediated VDCC facilitation.

In β cells, the AZ proteins, RIM2 and its binding partner, Piccolo, have been reported to interact with $\text{Ca}_v1.2$ in pancreatic β cells (Shibasaki et al., 2004). RIM2 and Piccolo interact with ELKS through binding to the C-terminal IWA region and the central region (445–606 aa) of ELKS, respectively. However, ELKS directly binds to the β subunit via the N-terminal region (Figures 3 and S4); therefore, the VDCC regulation by ELKS is independent of RIM2 and Piccolo. Indeed, (1) ELKS $^{\text{sc}}$, which lacks the RIM2-interacting IWA region, restored the defect in the $[\text{Ca}^{2+}]_i$ rise in ELKS-KO β cells, as did ELKS α overexpression, which possesses the IWA region (Figure S1), and (2) RIM regulates VDCC functions in an electrophysiologically different way than ELKS as described above, suggesting that ELKS regulation of VDCC is not mediated by RIM2. It would be interesting to explore the relationship among the AZ proteins in the VDCC control and the Ca^{2+} -dependent insulin secretion from β cells.

ELKS Roles in Ca^{2+} Influx and Insulin Secretion at the β Cell PM Facing the Islet's Vasculature

In rodents, β cells are organized in the form of a rosette around the central venous vasculature (Bonner-Weir, 1988). Growing evidence suggests that there are functional domains in the β cell PM, which implies β cell polarity, e.g., the glucose transporter, GLUT2, and primary cilia located on the lateral membrane between the cells, and the nucleus is located close to the PM in the opposite side of the vasculature (away from the center of the rosette) (Granot et al., 2009; Orci et al., 1989; Gan et al., 2017). Furthermore, interestingly, the insulin granules are enriched along the vascular face after cell stimulation (Bonner-Weir, 1988), supporting the concept that Ca^{2+} -dependent insulin secretion into the bloodstream occurs at the PM facing the vasculature. Although the site of insulin granule exocytosis has been debated (Takahashi et al., 2002), recently, insulin exocytosis was observed mostly at the β cell PM facing the vasculature using two-photon imaging with 3D measurements (Low et al., 2014). However, the molecular mechanism underlying the polarized Ca^{2+} influx and insulin secretion from islets is still unclear.

Here, we found in membrane-bound G-CaMP8b-expressing β cells within islets that the local Ca^{2+} fluorescence initially appeared at the vascular side of the β cell PM, while such initial Ca^{2+} fluorescence was decreased in ELKS-KO β cells (Figure 6), which showed impaired first-phase insulin secretion. Thus, the β cell PM is functionally compartmentalized for Ca^{2+} rise. Importantly, the Ca^{2+} signal at the vascular side of the PM is crucial for the large first-phase elevation in insulin. Despite the fact that Cav1.2 is distributed over the entire β cell PM, ELKS is preferentially enriched at the β cells PM facing the vasculature (Figure 5), thereby glucose-induced membrane depolarization may initially activate ELKS-interacting L-type VDCCs at the vascular side of the β cell PM during first-phase insulin secretion. Taken together, ELKS controls the initial polarized Ca^{2+} influx at the vascular face and the first-phase insulin secretion probably into the islets' vasculature (Figure 7B).

In this study, we succeeded in observing the changes in the subplasmalemmal Ca^{2+} rise in islet β cells using the membrane-bound G-CaMP8b, which we newly synthesized as a higher-affinity Ca^{2+} probe (K_d : ~44 nM, see STAR Methods) than Fluo-3 (K_d : ~325 nM) and Fura-2 (K_d : ~230 nM) (Paredes et al., 2008). A recent study on mouse islets using another Ca^{2+} probe, R-CaMP1h, has reported that a Ca^{2+} -sensing microdomain was seen both in the PM facing the vasculature and in the lateral cell edges between β cells (Geron et al., 2015); however, it is unclear which Ca^{2+} microdomain appears first. The difference from our observations may be due to the use of the cytosol expressed R-CaMP1h with a lower K_d (~1.3 μM) (Akerboom et al., 2013). In the future, it will be important to perform simultaneous imaging of the Ca^{2+} rise and insulin exocytosis in β cells within islets.

Because ELKS deletion significantly impaired the glucose tolerance in ELKS βKO mice, ELKS-induced potentiation of the $[\text{Ca}^{2+}]_i$ rise and the first-phase insulin secretion are essential for normal blood glucose homeostasis. Thus, does ELKS have any implications in diabetes? Interestingly, ELKS expression was decreased in the islets of a type 2 diabetes mouse model (*db/db* mice) (Figure S10A), which showed elevated fasting

blood glucose, and impaired glucose-induced insulin secretion and $[\text{Ca}^{2+}]_i$ rise (Figures S10B–S10E). These findings imply that downregulated ELKS expression may have implications in diabetes. Indeed, *in situ* Ca^{2+} imaging of β cells within islets (Figure S11) demonstrated that although the Ca^{2+} signal initially increased in the vascular face region in nondiabetic *db/+* mouse islet β cells as well as in control mouse islet β cells (Figure 6), in *db/db* mouse islet β cells the initial Ca^{2+} rise at the vascular face was impaired, and Ca^{2+} increased uniformly over the PM regions in ELKS-KO mouse islet β cells. Thus, it is tempting to speculate that the downregulated ELKS expression is involved in the impaired insulin secretion in type 2 diabetes through reduced polarized Ca^{2+} rise. However, it is still unclear whether overexpression of ELKS in *db/db* islet β cells restores the polarized Ca^{2+} rise and the first-phase insulin secretion. Therefore, further work is needed to clarify the close relationship between downregulated ELKS expression and diabetes.

Conclusion and Outlook

Our data suggest that ELKS enhances Ca^{2+} influx through L-type VDCCs at the vascular side of the β cell PM in islets, and that it controls the initial polarized Ca^{2+} influx and first-phase insulin secretion from pancreatic islets. Our results also raise new questions: how is ELKS delivered to the vascular side of the β cell PM and how does ELKS form secretory hotspots? ELKS localized at the vascular side, suggesting it may receive polarity cues; however, the underlying mechanisms are largely unknown. Furthermore, a recent study on mouse islets showed that the ELKS-binding AZ proteins, RIM2, Piccolo, and Liprin, were enriched at the vascular face in β cells (Low et al., 2014). In addition, it has been reported that local integrin activation at the vascular face targets insulin secretion (Gan et al., 2018). Thus, it is important to elucidate the mechanisms of ELKS trafficking and the formation of the secretory hotspots together with other AZ proteins, L-type VDCCs, and cell adhesion molecules at the β cell PM facing the vasculature.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes eleven figures, one table, and two videos and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.12.106>.

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AUTHOR CONTRIBUTIONS

M.O.-I. and T.O. designed the research; M.O.-I., K.A., H.Y., M.Y., M.X.M., Y.H., H.N.T., M.O., M.A., Y.A., Y.N., and C.N. performed the experiments; M.O.-I., T.O., H.K., K.H., K.S., S.N., Y.M., J.N. and M.K. analyzed the data; M.O.-I. and T.O. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
rabbit anti-ELKS	Deguchi-Tawarada et al., 2004	N/A
rabbit anti-CAST	Takao-Rikitsu et al., 2004	N/A
rabbit anti-ELKS ϵ	This paper	N/A
mouse anti-Cre	Millipore	Cat #MAB3120; RRID:AB_2085748
rabbit anti- β_1	Alomone Lab	Cat #ABR-011; RRID:AB_2039752
rabbit anti- β_2	Alomone Lab	Cat #ACC-105; RRID:AB_2340905
rabbit anti- β_3	Alomone Lab	Cat #ACC-008; RRID:AB_2039787
rabbit anti- β_4	Kiyonaka et al., 2007	N/A
rabbit anti-Cav1.2	Alomone Lab	Cat #ACC-003; RRID:AB_2039771
rabbit anti-GLUT2	Millipore	Cat #07-1402; RRID:AB_1587076
sheep anti-Glucokinase	a gift from Mark Magnuson, Vanderbilt University, Nashville, TN	N/A
mouse anti-Bassoon	Enzo Life Sciences	Cat #ADI-VAM-PS003; RRID:AB_10618753
rabbit anti-RIM2	Synaptic Systems	Cat #140-103; RRID:AB_88776
mouse anti-Munc13-1	Sigma-Aldrich	Cat #M6194; RRID:AB_477220
mouse anti-Syntaxin 1A	Sigma-Aldrich	Cat #S0664; RRID:AB_477483
mouse anti-SNAP-25	Wako	Cat #014-15771(BR05); RRID:AB_2315334
rabbit anti-VAMP2	Enzo Life Sciences	Cat #ADI-VAS-SV006; RRID:AB_10534308
mouse anti-Tubulin	Millipore	Cat #CP06; RRID: AB_310035
mouse anti-Actin	Millipore	Cat #MAB1501; AB_2223041
rabbit anti-GAPDH	Cell Signaling	Cat # 3683; RRID:AB_1642205
mouse anti-Myc	WAKO	Cat #011-21874; RRID:AB_390912
rabbit anti-Myc	Cell Signaling	Cat #2278; RRID:AB_490778
mouse anti-FLAG	MBL	Cat #M185-3S; AB_10971972
rabbit anti-FLAG	MBL	Cat # PM020; AB_591224
mouse anti-MBP	New England BioLabs	Cat #E8032S; RRID:AB_1559732
mouse anti-GST	Cell Signaling	Cat #2624; RRID:AB_2189875
mouse anti-insulin	Sigma-Aldrich	Cat #I2018; RRID:AB_260137
rat anti-CD31	BD PharMingen	Cat #557355; RRID:AB_396660
horseradish peroxidase-labeled anti-(rabbit IgG)	GE healthcare	Cat #NA934; RRID:AB_772206
horseradish peroxidase-labeled anti-(mouse IgG)	GE healthcare	Cat #NA931; RRID:AB_772210
Polyclonal Rabbit Anti-Sheep Immunoglobulins/HRP	Dako	Cat #P0163
Goat anti-mouse IgG conjugated to Alexa Fluor-488	Thermo Fisher Scientific	Cat #A-11001; RRID:AB_2534069
Goat anti-rabbit IgG conjugated to Alexa Fluor-488	Thermo Fisher Scientific	Cat #A-11008; RRID:AB_143165
Goat anti-rat IgG conjugated to Alexa Fluor-546	Thermo Fisher Scientific	Cat #A-11081; RRID:AB_141738
Chemicals, Peptides, and Recombinant Proteins		
nifedipine	SIGMA-Aldrich	N7634
Liberase RI	Roche	11-815-032-001
fibronectin	SIGMA-Aldrich	F4759-5MG
RPMI1640 medium	Thermo Fisher Scientific	22400
polyethyleneimine	SIGMA-Aldrich	P3143
fetal bovine serum	Thermo Fisher Scientific	26140-079
bovine serum albumin	SIGMA-Aldrich	A-8022

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
EGTA	Nacalai tesque	15214-92
DMEM	SIGMA-Aldrich	D5796-500ML
Hank's balanced salt solution	Thermo Fisher Scientific	24020-117
2-mercaptoethanol	Thermo Fisher Scientific	21985-023
Trypsin-EDTA	Thermo Fisher Scientific	25300-062
dextran	SIGMA-Aldrich	31390-100G
Prolong Gold antifade reagent	Thermo Fisher Scientific	P36934
4% paraformaldehyde phosphate buffer solution	Wako	163-20145
Tissue-Tek OCT compound	Sakura Finetek	4583
Superfect	QIAGEN	301305
Fura-2 acetoxymethyl	Thermo Fisher Scientific	F1221
Fluo-3 acetoxymethyl	Thermo Fisher Scientific	F1242
Fura red acetoxymethyl ester	Thermo Fisher Scientific	F3021
DyLight 594-labeled Lycopersicon Esculentum (Tomato) lectin	VECTOR Laboratories	DL-1177
Critical Commercial Assays		
Insulin ELISA	Morinaga	M1103
hGH ELISA kit	Roche Applied Science	1115-858-780-01
PicoGreen dsDNA assay kit	Thermo Fisher Scientific	P7589
Pierce Cell Surface Protein Isolation Kit	Thermo Fisher Scientific	89881
Experimental Models: Cell Lines		
MIN6 cell line	a gift from Dr. Jun-ichi Miyazaki, Osaka University, Osaka	N/A
COS-7 cell line	RIKEN BRC	RCB0539 RRID:CVCL_0224
HEK293 cell Line	ATCC	CRL-1573
Experimental Models: Organisms/Strains		
<i>ELKS^{fllox/fllox}</i> mice	Dong et al., 2018	N/A
RIP-Cre mice	Aoyagi et al., 2012	N/A
db/db mice	CLEA Japan	BKS.Cg-+ Lepr ^{db} /+ Lepr ^{db} /Jcl
db/m+ (mysty)	CLEA Japan	BKS.Cg-m+/+ Lepr ^{db} /Jcl
m+/m+ (mysty / mysty) mice	CLEA Japan	BKS.Cg-m+/m+/Jcl
Oligonucleotides		
shRNA targeting sequence: mouse <i>ELKS</i> (5'-GTGGGAAAACCCCTTTCAAT-3')	This paper	N/A
control shRNA sequence: 5'-ACTACCGTTGTTATAGGTG-3'	This paper	N/A
Recombinant DNA		
Plasmid:Myc-rat <i>ELKS</i> α	Deguchi-Tawarada et al., 2004	N/A
Plasmid:MBP-rat <i>ELKS</i> α_{1-404}	This paper	N/A
Plasmid:MBP-rat <i>ELKS</i> $\alpha_{323-684}$	This paper	N/A
Plasmid:MBP-rat <i>ELKS</i> $\alpha_{674-903}$	This paper	N/A
Plasmid:MBP-rat <i>ELKS</i> $\alpha_{765-948}$	This paper	N/A
Plasmid:FLAG-rabbit VDCC- β_2	This paper	N/A
Plasmid:GST-rabbit VDCC- β_2 -Full ₁₋₆₀₆	This paper	N/A
Plasmid:GST-rabbit VDCC- β_2 -GK ₁₂₈₋₆₀₆	This paper	N/A
Plasmid:GST-rabbit VDCC- β_2 -SH3 ₁₋₂₂₅	This paper	N/A
Plasmid:FLAG-rabbit VDCC- β_3	This paper	N/A
Plasmid:GST-rabbit VDCC- β_3 -Full ₁₋₄₇₇	This paper	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Plasmid:GST-rabbit VDCC- β_3 -GK ₁₂₇₋₄₇₇	This paper	N/A
Plasmid:GST-rabbit VDCC- β_3 -SH3 ₁₋₁₇₆	This paper	N/A
Plasmid:Mouse ELKS α	This paper	N/A
Plasmid:Mouse ELKS ϵ	This paper	N/A
Plasmid:EGFP-rat ELKS α_{1-404}	This paper	N/A
Plasmid:human growth hormone	a gift from Dr. Masami Takahashi, Kitasato University School of Medicine	N/A
Plasmid:membrane-bound-G-CaMP8b	This paper	N/A
Plasmid:pCI-neo	Inoue et al., 2006	N/A
Plasmid:Rabbit Cav1.2	Mori et al., 2004	N/A
Plasmid:rat β_{2a}	Mori et al., 2004	N/A
Plasmid:rat $\alpha_{2\delta}$	Mori et al., 2004	N/A
Software and Algorithms		
GraphPad Prism	GraphPad Software	https://www.graphpad.com/
Metamorph 7.8	Molecular Devices	https://www.moleculardevices.com/
pCLAMP10.2 software	Molecular Devices	https://www.moleculardevices.com/
MATLAB	MathWorks	https://www.mathworks.com/

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to and will be fulfilled by Lead Contact, Mica Ohara-Imaizumi (mimaizu@ks.kyorin-u.ac.jp).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse Studies

To generate β cell-specific *ELKS*-KO (*ELKS* β KO) mice, the *ELKS*^{flox/flox} mice (Dong et al., 2018), which served as controls, were bred with RIP (rat insulin 2 gene promoter)-Cre transgenic mice (Aoyagi et al., 2012). By mating *ELKS*^{flox/flox} mice carrying the RIP-Cre transgenic allele with *ELKS*^{flox/flox} mice, we generated *ELKS* β KO mice and the control Flox mice in the same litter. All experiments in this study were performed on 10–14-wk-old male mice. Male *db/db*, *db/m+* (misty) and *m+/m+* (misty/misty) mice age 11–12 wk were obtained from CREA Japan. This research was approved by the Animal Care and Experimentation Committee of Kyorin University, Yamanashi University, Jichi Medical University and Niigata University, and all experiments were performed according to the guidelines of the Committees. All mice were housed in a 12-h light/dark cycle in climate-controlled facilities and fed standard rodent food (EC-2; Clea Japan, Inc., Tokyo, Japan). In this study, we used the minimum number of mice required to draw conclusions and tried to minimize their suffering as much as possible.

Pancreatic Islets, β Cells and MIN6 Cells

Pancreatic islets were isolated from mice by collagenase (Liberase RI) digestion, as previously described (Ohara-Imaizumi et al., 2004, 2013). The isolated islets were cultured on fibronectin-coated normal coverslips (Matsunami, Ohsaka, Japan) in RPMI-1640 medium (11 mM glucose) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 200 units/ml penicillin and 200 μ g/ml streptomycin at 37°C in an atmosphere of 95% air/5% CO₂. For intracellular Ca²⁺ imaging and TIRF analysis, the isolated islets were dissociated into single cells by incubation in calcium-free Krebs-Ringer buffer (KRB) [110 mM NaCl, 4.4 mM KCl, 1.45 mM KH₂PO₄, 1.2 mM MgSO₄, 4.8 mM NaHCO₃, 2.2 mM glucose, 10 mM HEPES, 0.3% bovine serum albumin (BSA); pH 7.4] containing 1 mM EGTA and cultured on fibronectin-coated normal coverslips and high refractive index cover glass (Olympus Corporation, Tokyo, Japan), respectively, in RPMI-1640 medium at 37°C in an atmosphere of 5% CO₂. MIN6 cells, a gift from Dr. J. Miyazaki (Osaka University, Osaka, Japan), at passage 20–30 were cultured as described previously (Ohara-Imaizumi et al., 2005).

METHOD DETAILS

Plasmids

The rat *ELKS* constructs contained the following amino acid residues: *ELKS*_{1–404} (amino acid 1–404), *ELKS*_{323–684} (amino acid 323–684), *ELKS*_{674–903} (amino acid 674–903) and *ELKS*_{765–948} (amino acid 765–948). The rabbit VDCC- β_2 constructs contained the following amino acid residues: Full (amino acid 1–606), GK (amino acid 128–606) and SH3 (amino acid 1–225). The rabbit

VDCC- β_3 constructs contained the following amino acid residues: Full (amino acid 1–477), GK (amino acid 127–477) and SH3 (amino acid 1–176). The construction of the rat Myc-ELKS expression vector has been described previously (Deguchi-Tawarada et al., 2004). The expression vectors for MBP-ELKS_{1–404}, -ELKS_{323–684}, -ELKS_{674–903} and -ELKS_{765–948}, GST-VDCC- $\beta_{2/3}$ -Full, -GK and -SH3, mouse ELKS α and ELKS ϵ , FLAG-VDCC- $\beta_{2/3}$ and EGFP-ELKS_{1–404} were constructed in pMAL-c2 (New England BioLabs, #E8200, Ipswich, MA, USA), pGEX-4T1 (GE Healthcare, #28954549, Chicago, IL, USA), pCAII, pCAII-FLAG and pCAII-EGFP, respectively, by standard molecular biology methods. pCAII-ELKS α and -ELKS ϵ were subcloned into pCAG from C. Cepko (Addgene plasmid No. 11160) to construct the mammalian expression vectors, pCAG-ELKS α and pCAG-ELKS ϵ , respectively. DNA fragments corresponding to the red fluorescent protein mCherry and the human growth hormone (hGH) were also subcloned into pcDNA3.2 (Thermo Fisher Scientific, #12489019, Carlsbad, CA, USA).

Glucose Tolerance Testing

For oral glucose tolerance test, control and ELKS β KO mice were fasted for 16 h before the test. Glucose was administered orally at 2 g glucose/kg body weight (Ohara-Imaizumi et al., 2007). Blood samples were collected from the tail vein at 0, 15, 30, 60 and 120 min after loading. Blood glucose levels were measured by Glutest R (Sanwa Kagaku Kenkyusho, Aichi, Japan).

Insulin Secretion Experiments

Islets were handpicked and preincubated for 30 min in KRB containing 110 mM NaCl, 4.4 mM KCl, 1.45 mM KH₂PO₄, 1.2 mM MgSO₄, 2.3 mM calcium gluconate, 4.8 mM NaHCO₃, 2.2 mM glucose, 10 mM HEPES, pH 7.4, and 0.3% BSA, and then challenged with 2.2 mM or 16.7 mM glucose for 30 min, or with 4.4 mM or 40 mM KCl for 10 min. At the end of the stimulation period, the islets were disrupted by 1% Triton X-100 and sonication. Aliquots of media and cell extracts were analyzed by insulin ELISA. The secreted insulin was expressed as a percentage of total cellular content. The amount of total cellular DNA was measured by a PicoGreen dsDNA assay kit.

Pancreas Perfusion Experiments

Mouse pancreata were isolated with segments of the duodenum and spleen. An arterial cannula was introduced into the celiac artery, and a venous cannula was inserted into the portal vein. The perfusate consisted of KRB containing 2.8 or 16.7 mM glucose supplemented with 3% dextran and 0.2% BSA, gassed with 95% O₂/5% CO₂. The perfusate was introduced into the celiac artery at 1 ml/min, and the effluent was collected at 1-min intervals from the portal vein after a single passage through the pancreas. The insulin secretion in the perfusate was measured by insulin ELISA.

Patch-Clamp Experiments

Conventional whole-cell currents of VDCCs were recorded from single mouse β cells, and data were stored online in a computer using an amplifier (Axopatch, 200B, Foster, CA, USA) with pCLAMP10.2 software (Gong et al., 2000; Yoshida et al., 2009). The electrode pipettes were made from glass tubing with a 1.5-mm diameter (Narishige, Tokyo, Japan) using a patch pipette puller. The resistances of patch pipettes ranged from 3 to 5 M Ω when the following pipette solution was filled. To isolate the VDCC current from the whole-cell current, we used a pipette solution containing (in mM): 90 CsOH, 5 MgCl₂, 10 EGTA, 20 TEA-Cl, 5 ATP-Mg and 10 HEPES at pH 7.2 with CsOH. The modified HKRB solution contained (in mM): 133.7 NaCl, 5 NaHCO₃, 10 CaCl₂, 1.2 MgCl₂ and 10 HEPES at pH 7.4 with NaOH. Mouse β cells were voltage-clamped at a holding potential of –40 mV and the membrane potential was stepped to test potentials of 100 ms duration from –50 mV to +50 mV with 10 mV increments every 10 s. The VDCC current amplitudes were divided by cell capacitance to obtain the current density at each test potential in control and ELKS-KO β cells. The temperature throughout the experiments was maintained at 30°C.

Transfection of Human Embryonic Kidney 293 Cells and Electrophysiological Recording

Human embryonic kidney (HEK293) cells were transiently transfected (Superfect). For expression of recombinant calcium channels, we used 2 μ g of cDNA encoding rabbit Ca_v1.2 (α_1 subunit) along with 2 μ g each of rat β_{2a} and rat $\alpha_{2\delta}$ subunits (Mori et al., 2004), and pCI-neo vector (control) or ELKS in pCI-neo vector (ELKS). Whole-cell current recordings were performed 1–3 d after transfection, using glass pipettes with resistances of 1.5–3 M Ω prior to series resistance compensation. The internal solution contained 125 mM Cs-aspartate, 20 mM CsCl, 5 mM EGTA, 2 mM MgCl₂, 4 mM Mg-ATP and 10 mM HEPES (pH 7.2); 290 mOsm. The bath solution contained 142 mM TEA-Cl, 10 mM HEPES (pH 7.4) and 5 mM BaCl₂; 300 mOsm. Standard patch-clamp techniques were used with an Axopatch 200B amplifier (Axon Instruments/Molecular Devices, Carlsbad, CA, USA). Currents were filtered at 2 kHz and sampled at 10 kHz; series resistance was typically 1–2 M Ω after > 70% compensation; and leaks and capacitive transients were subtracted by a P/8 protocol. Test pulse depolarizations were delivered every 25 s. Holding potential was –90 mV. Data analysis was done by custom-written software using MATLAB software (MathWorks).

Antibody

A polyclonal antibody against mouse ELKS ϵ was produced by immunization of rabbits with a GST-ELKS ϵ (amino acids 1066–1120) fusion protein, followed by affinity-purification as reported previously (Takao-Rikitsu et al., 2004).

Immunoblotting

The amount of protein in mouse islets, MIN6 cells and C57BL/6 mouse whole brain was analyzed by immunoblotting. SDS-PAGE was performed on 8% polyacrylamide gels equipped with a 5% stacking gel. After separation, the proteins were transferred to PVDF membranes following standard procedures with a semi-dry transblotting apparatus. The membranes were blocked with 5% (w/v) non-fat milk powder in TBST (Tris-buffered saline with Tween 20; 25 mM Tris/HCl, pH 7.5, 150 mM NaCl and 0.05% Tween 20) for 30 min at room temperature (22 °C), followed by an overnight incubation with rabbit anti-ELKS (1:500), rabbit anti-CAST (1:500), mouse anti-Cre (1:200), rabbit anti-VDCC- β_1 (1:500), rabbit anti-VDCC- β_2 (1:500), rabbit anti-VDCC- β_3 (1:200), rabbit anti-VDCC- β_4 (1:500), rabbit anti-Cav1.2 (1:500), rabbit anti-GLUT2 (1:500), sheep anti-Glucokinase (1:200), mouse anti-Bassoon (1:500), rabbit anti-RIM2 (1:500), mouse anti-Munc13-1 (1:200), mouse anti-Syntaxin 1A (1:1000), mouse anti-SNAP-25 (1:500), rabbit anti-VAMP2 (1:2000), mouse anti-Tubulin (1:1000), mouse anti-Actin (1:1000), rabbit anti-GAPDH (1:1000), mouse anti-Myc (1:1000), rabbit anti-Myc (1:1000), mouse anti-FLAG (1:1000), rabbit anti-FLAG (1:1000), mouse anti-MBP (1:1000) or mouse anti-GST (1:1000) antibodies diluted in 5% (w/v) non-fat milk powder in TBST at 4 °C. After washing with TBST, membranes were incubated for 1 h at room temperature with horseradish peroxidase-labeled anti-rabbit, anti-mouse or anti-sheep IgG antibodies (1:5000) in TBST containing 5% (w/v) non-fat milk powder. After washing, membranes were treated with ECL solution [100 mM Tris-Cl (pH 8.5), 1.25 mM Luminol, 0.01% (v/v) H₂O₂] and the immunoreactive bands were visualized by a luminescence image analyzer with an electronically cooled CCD camera system (LAS-3000; Fuji Photo Film, Tokyo, Japan).

Immunoprecipitation

For *in vitro* binding studies of complex formations of ELKS with VDCC- β_2 or - β_3 , immunoprecipitation was performed as described previously (Ohtsuka et al., 2002). For anti-FLAG immunoprecipitation, Myc-ELKS, FLAG-VDCC- β_2 or - β_3 expression plasmids were transfected into COS-7 cells (RIKEN BRC, Tsukuba, Japan), and cell lysates were extracted. Immunoprecipitation was performed using anti-FLAG antibodies. For endogenous studies, anti-VDCC- β_2 or - β_3 immunoprecipitation was performed as described previously (Ohtsuka et al., 2002) with minor modifications. In brief, MIN6 cells in 16 10-cm dishes were lysed in 10 mL of lysis buffer and subjected to immunoprecipitation. Protein A beads were extensively washed with a wash buffer containing 20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.5 mM EDTA, 1.0% (w/v) NP-40, 1 mM DTT and complete protease inhibitor cocktail. All samples were then analyzed by immunoblotting.

Pull-down assay

The pull-down assay was performed as described previously (Ohtsuka et al., 2002). In brief, COS-7 cells expressing Myc-ELKS, FLAG-VDCC- β_2 or - β_3 in 10-cm dishes were lysed in 0.5 mL of lysis buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% (w/v) NP-40 (FLAG-VDCC- $\beta_2/3$) or 1% Triton X-100 (Myc-ELKS) and complete protease inhibitor cocktail] at 4°C for 1 h. The sample was centrifuged at 100,000 $\times g$ at 4°C for 30 min to collect the supernatant, which was subjected to the pull-down assay. GST and MBP fusion proteins were produced and purified according to the manufacturer's instructions. For Figure 3C and S4B, the lysates of COS-7 cells expressing FLAG-VDCC- β_2 or - β_3 were incubated with 20 μ L of amylose beads containing MBP-ELKS₁₋₄₀₄, -ELKS₃₂₃₋₆₈₄, -ELKS₆₇₄₋₉₀₃, -ELKS₇₆₅₋₉₄₈ or MBP alone at 4°C for 1 h. For Figures 3D and S4C, the MBP-ELKS₁₋₄₀₄ protein was incubated with 20 μ L of glutathione-Sepharose beads containing GST- $\beta_{2/3}$ -Full, -GK, -SH3 or GST alone. For Figure S5A, the Myc-ELKS protein was incubated with 20 μ L of glutathione-Sepharose beads containing GST- $\beta_{2/3}$ in the presence of MBP-ELKS₁₋₄₀₄ at the indicated concentration. After the beads were extensively washed with lysis buffer, the bound proteins were eluted by boiling the beads in SDS sample buffer for 5 min. The samples were then analyzed by immunoblotting.

Immunofluorescence Staining

Immunofluorescence staining of mouse pancreas was performed as described previously (Ohara-Imaizumi et al., 2005). Mouse pancreas was fixed with 4% paraformaldehyde phosphate buffer solution, cryoprotected with graded concentrations of sucrose in phosphate-buffered saline (PBS), embedded in OCT compound, and then frozen by immersion in liquid nitrogen. Semithin frozen sections were cut with a Miles cryostat (Elkhart, Indiana, USA), transferred to MAS-coated glass slides (Matsunami Glass, Osaka, Japan) and immunostained. Sections were incubated with primary antibodies [rabbit anti-ELKS_e (1:100), mouse anti-insulin (1:100), rabbit anti-Cav1.2 (1:100), mouse anti-Syntaxin 1A (1:200), mouse anti-SNAP-25 (1:200) and rat anti-CD31 (1:200)] in PBS containing 4% FBS, 0.1% sodium azide, and 0.25% Triton X-100, followed by secondary antibodies (1:400) [goat anti-mouse IgG conjugated to Alexa Fluor-488, goat anti-rabbit IgG conjugated to Alexa Fluor-488, goat anti-rat IgG conjugated to Alexa Fluor-546]. Slides were mounted using Prolong Gold, and then examined using laser-scanning confocal microscopy (LSM510, Carl Zeiss, Germany).

TIRF microscopy

The Olympus total internal reflection system was used with a high-aperture objective lens (Apo 100 $\times$ OHR; NA 1.65, Olympus, Tokyo, Japan) as previously described (Ohara-Imaizumi et al., 2007). Isolated islets were dispersed and cultured on fibronectin-coated high refractive index (ND = 1.788) coverslips (Olympus). To evaluate the number of docked insulin granules by TIRF microscopy, control and ELKS-KO β cells were fixed, permeabilized with 4% paraformaldehyde/0.1% Triton X-100, and processed for immunohistochemistry. Cells were labeled with anti-insulin antibodies and processed with goat anti-mouse IgG conjugated to Alexa Fluor-488. Immunofluorescence was detected by TIRF microscopy.

Electron microscopy

Tissues were fixed with phosphate-buffered 2.5% glutaraldehyde (pH 7.4), postosmicated, dehydrated with an alcohol gradient and embedded in Epon 812. After staining with uranyl acetate and lead citrate, ultrathin sections were examined under a transmission electron microscope (TEM-1010C, JEOL, Tokyo, Japan). In the electron microscopy, granules at their shortest distance of < 10 nm from the plasma membrane were defined as morphologically docked granules.

Transfection and hGH secretion assay

Islets were transfected with plasmids encoding ELKS_{1–404} together with hGH. Freshly isolated islets were suspended in 0.5% BSA-containing Hank's balanced salt solution with 1.0 or 1.5 mg/ml DNA, and placed in an electrode chamber. Six rectangular electric pulses (200 V, 1-ms duration, 50-ms interval, decay constant: 10%), followed by five pulses (50 V, 50-ms duration, 50-ms interval, decay constant: 40%) and another five pulses in the opposite direction of the electric field were delivered using a pulse generator (Nepa21, Nepa gene, Chiba, Japan) (Aoyagi et al., 2012). The electroporated islets were washed twice with culture medium and plated on 35-mm or 60-mm culture dishes. Two days after transfection, 15 size-matched islets were subjected to the hGH secretion assay. Islets were preincubated for 30 min in KRB containing 2.2 mM glucose, and then challenged with 2.2 mM or 16.7 mM glucose for 30 min, or with 4.4 mM or 40 mM KCl for 10 min. At the end of the stimulation period, the islets were disrupted by 1% NP-40 and sonication. The secreted hGH and total cellular content of hGH were measured by an hGH ELISA kit. For β cell transfection by electroporation, isolated islets were dispersed and suspended in 0.5% BSA-containing Hank's balanced salt solution with 1.0 mg/ml DNA and placed in an electrode chamber. Six rectangular electric pulses (120 V, 1-ms duration, 50-ms interval, decay constant: 10%), followed by five pulses (20 V, 50-ms duration, 50-ms interval, decay constant: 40%) and another five pulses in the opposite direction of the electric field were delivered using a pulse generator (Nepa21, Nepa gene). The electroporated β cells were seeded and cultured on fibronectin-coated normal glass coverslips (Matsunami) in RPMI-1640 medium supplemented with 10% FBS, 200 units/ml penicillin and 200 μ g/ml streptomycin at 37°C in an atmosphere of 5% CO₂.

Insulin secretion assay in MIN6 cells

MIN6 cells were cultured on polyethyleneimine-coated 35-mm dishes and were infected with control or ELKS shRNA adenoviruses for 3 days. MIN6 cells were washed three times with KRB containing 2.2 mM glucose. After washing, cells were incubated with KRB containing 2.2 mM glucose for 15 min, and then stimulated with KRB containing 2.2 or 22 mM glucose for 30 min. At the end of the experiments, 1% Triton X-100 was added, and then the samples were sonicated (30 s; UR-20P, Tomy Seiko, Tokyo, Japan) on ice. Secreted insulin and the total cellular content of insulin were measured with an Insulin ELISA kit.

Measurements of Intracellular Free Ca²⁺ ([Ca²⁺]_i)

β cells and MIN6 cells were loaded with 10 μ M Fura-2 acetoxymethyl ester for 30 min at 37°C in KRB (2.2 mM glucose), and then washed and incubated for an additional 15 min with KRB. Coverslips were mounted on an ARGUS/HiSCA system (Hamamatsu Photonics, Hamamatsu, Japan) and cells were stimulated with 22 mM glucose or 40 mM high K⁺. Fura-2 fluorescence was detected by a cooled CCD camera after excitation at 340 nm (F340) and 380 nm (F380), and the ratio image (F340/F380) was calculated with the ARGUS/HiSCA system. For Fluo-3 fluorimetry, 10 μ M Fluo-3 acetoxymethyl ester was loaded into cultured β cells for 40 min at 37°C. After rinsing, cells on cover glass in the chamber were placed on the stage of a confocal microscope (FV1000, Olympus) and stimulated with 22 mM glucose or 40 mM high K⁺. Excitation was at 488 nm (Argon laser), and emissions were collected using a 505–525 nm band-pass emission filter. Time-lapse images (3 s/frame) were captured and analyzed with Metamorph software. For Fluo-3 Ca²⁺ imaging of cells expressing mock (empty vector) or ELKS α/ϵ and mCherry (Figure S1) by electroporation, the FV1000 microscope was powered by Argon and HeNe lasers for the detection of Fluo-3 (excitation at 488 nm, emission at 505–525 nm) and mCherry (excitation at 543 nm, emission at 560 nm long pass) fluorescence. Cells with the mCherry signal were selected, then Fluo-3 signals were monitored as described above. For Fura Red fluorimetry, 10 μ M Fura Red acetoxymethyl ester was loaded into cultured β cells for 30 min at 37°C. After rinsing, cells on cover glass in the chamber were placed on the stage of a confocal microscope (FV1000, Olympus) and stimulated with 22 mM glucose or 40 mM high K⁺ as previously described (Ohara-Imaizumi et al., 2009). Excitation was at 488 nm (Argon laser), and emissions were collected using a 650 nm long pass emission filter. Time-lapse images (3 s/frame) were captured and analyzed with Metamorph software (Molecular Devices). The fluorescence of the Fura Red excited at 488 nm decreases once the indicator binds Ca²⁺. The Fura Red trace was inverted to show the [Ca²⁺]_i increases as upward deflections. For Fura Red Ca²⁺ imaging of cells expressing mock-EGFP or ELKS_{1–404}-EGFP (Figure S5), we used the 488 nm laser line for excitation, and emissions were collected using a 505–525 nm band pass filter for mock-EGFP or ELKS_{1–404}-EGFP, and 650 nm long pass filter for Fura Red. Cells with the EGFP signal were selected, then Fura Red signals were monitored as described above.

Calcium Indicator G-CaMP8b

We developed a novel fluorescent calcium indicator G-CaMP8b (Figure S9A). Briefly, the complementary DNA (cDNA) encoding G-CaMP8a was constructed by replacing the 0.31-kb PstI/NotI fragment of *G-CaMP8* cDNA (Ohkura et al., 2012) with the corresponding 0.31-kb fragment of *G-CaMP8a* cDNA (Muto et al., 2013). G-CaMP8a differs from G-CaMP8 by the N97D and N137D mutations in the calmodulin (CaM) domain. Next, the cDNA encoding G-CaMP8b was constructed by replacing the 0.25-kb PstI/Clal fragment of *G-CaMP8a* cDNA with the corresponding 0.25-kb fragment of a chemically synthesized CaM cDNA carrying the

T79R and S81T mutations from G-CaMP6s (Chen et al., 2013) and an E87V mutation. G-CaMP8b differs from G-CaMP8 by the T79R, S81T, E87V and N137D mutations in the CaM domain (Figure S9A). To target G-CaMP8b to the PM, a G-CaMP8b(12aa)CT indicator (membrane-bound-G-CaMP8b) was generated by fusing a cDNA sequence encoding the C-terminal peptide of the small G-protein, H-Ras (KLNPPDESGPGCMSCCKVLS in one-letter code) (Hancock, 2003), to the 3' end of a cDNA encoding G-CaMP8b via a peptide linker (GGGTGGSEFTSG in one-letter code). The membrane-bound-G-CaMP8b cDNA was subcloned into the pAd/CMV/V5-DEST vector (Thermo Fisher Scientific) by site-specific recombination to generate adenovirus vectors. Recombinant adenoviruses were generated according to the manufacturer's instructions. All constructs were sequenced to confirm their identities with the designed sequences.

Measurements of Subplasmalemmal Ca^{2+} Rise

To image the subplasmalemmal Ca^{2+} signals in individual β cells and the vasculature within intact cultured islets, islets were infected with adenoviruses encoding membrane-bound-G-CaMP8b and DyLight 594-labeled *Lycopersicon Esculentum* (Tomato) lectin, which was used as a capillary endothelial cell marker. For highly efficient transduction of the intact islets, we used the pancreas perfusion experiments as described above with minor modifications. The control, *ELKS* β KO, *db/m+* and *db/db* mice pancreata were perfused for 60 min at a flow rate of 0.1 ml/min, with the perfusate consisting of KRB (2.8 mM glucose) with the membrane-bound-G-CaMP8b adenoviruses, DyLight 594-labeled tomato lectin (10 $\mu\text{g/ml}$), 3% dextran and 0.2% BSA. Islets were then isolated from the pancreas by collagenase digestion (as described above), and then cultured on fibronectin-coated cover glass (Matsunami) in RPMI-1640 medium supplemented with 10% FBS, 200 units/ml penicillin and 200 $\mu\text{g/ml}$ streptomycin at 37°C in an atmosphere of 5% CO_2 . After 2 days the cultured islets were mounted on an open chamber and incubated for 30 min with KRB. Islets were placed on the stage of a confocal microscope (FV1000, Olympus) using 40 $\times$ magnification and stimulated with 22 mM glucose. An FV1000 inverted microscope was powered by Argon and HeNe lasers for the detection of G-CaMP8b (excitation at 488 nm, emission at 505–525 nm) and tomato lectin (excitation at 543 nm, emission at 630 nm long pass) fluorescence. Time-lapse images (1.5 s/frame) were captured and analyzed with Metamorph software.

ELKS RNA Interference

To generate the expression vectors for the shRNA against mouse *ELKS*, the *U6* promoter and shRNA sequence were subcloned into pENTR1A vector with mCherry, followed by the SV40 polyA sequence. The target sequence was 5'-GTGGGAAAACCCTTTCAAT-3'. To generate adenovirus vectors, these sequences were introduced into the pAd/CMV/V5-DEST vector by site-specific recombination. Recombinant adenoviruses were generated according to the manufacturer's instructions. All constructs were sequenced to confirm their identity with the designed sequences. Adenoviruses expressing mCherry and scrambled shRNA (5'-ACTACCGTTGT TATAGGTG-3') were used as a nonspecific control. MIN6 cells were infected with control or *ELKS* shRNA-containing adenoviruses for 3 days, and used for the experiments.

Purification of Cell-Surface Proteins by Biotinylation and Immunoblotting

For the isolation and collection of surface proteins, we used the Pierce Cell Surface Protein Isolation Kit (Thermo Fisher Scientific). Control shRNA or *ELKS* shRNA-treated MIN6 cells grown on 60-mm dishes were washed twice with KRB containing 4 mM glucose, and incubated in KRB for 20 min at 37°C (Aoyagi et al., 2010). After the incubation, proteins expressed in the PM were labeled with sulfo-NHS (N-hydroxysuccinimido)-biotin and isolated by NeutrAvidin beads according to the instructions provided. Following binding, beads were washed and bound proteins were eluted with sample buffer plus DTT, and then SDS-PAGE and immunoblotting were performed.

$[\text{Ca}^{2+}]_i$ Oscillations and ACh-evoked Cytosolic Ca^{2+} Rise

To investigate the effects of *ELKS* KO on Ca^{2+} oscillation frequency, we examined the cytosolic Ca^{2+} dynamics in control and *ELKS* KO β cells using Fura-2 methods (described above). Clusters of islet cells attached to the coverslips were loaded with Fura-2, and the coverslips were transferred into a perfusion chamber (A8819, Hamamatsu Photonics) on an ARGUS/HiSCA system (Hamamatsu Photonics). Clusters of islet cells were perfused with KRB containing 2.2 or 16.7 mM glucose at 1 ml/min at 37°C. To investigate whether *ELKS* KO affects endoplasmic reticulum (ER)- Ca^{2+} release, we examined the acetylcholine (ACh)-evoked cytosolic Ca^{2+} increase in control and *ELKS* KO β cells using Fura-2 methods. The release of Ca^{2+} from the ER is triggered by phospholipase C (PLC)-inositol 1,4,5 trisphosphate (IP_3) pathway-activating agonists, such as ACh in pancreatic β cells (Gilon et al., 2014). However, dual action of ACh has been reported to release ER- Ca^{2+} and cause membrane depolarization (Gilon et al., 1995; Rolland et al., 2002), so we imaged the Ca^{2+} rise in the presence of nifedipine, which prevents the depolarization-induced Ca^{2+} rise via L-type VDCCs.

STATISTICAL ANALYSIS

Data are presented as mean $\pm$ SEM. Statistical significance was performed using Microsoft Excel and GraphPad Prism. Details of statistical tests used can be found in the figure and table legends. Significance is indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; N.S., not significant. $p < 0.05$ was considered statistically significant throughout the study.