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The t-SNAREs syntaxin4 and SNAP23 but not v-SNARE VAMP2 are indispensable to tether GLUT4 vesicles at the plasma membrane in adipocyte

Takayuki Kawaguchi¹, Yoshikazu Tamori¹, Hajime Kanda¹, Mari Yoshikawa¹, Sanshiro Tateya¹, Naonobu Nishino¹, and Masato Kasuga²

¹Division of Diabetes, Metabolism, and Endocrinology, Department of Internal Medicine, Kobe University Graduate School of Medicine

²Research Institute, International Medical Center of Japan

Address all correspondence and requests for reprints to: Yoshikazu Tamori, M.D., Ph.D., Division of Diabetes, Metabolism, and Endocrinology, Department of Internal Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

Telephone: +81-78-382-5861

Fax: +81-78-382-2080

E-mail: tamori@med.kobe-u.ac.jp

Abbreviations

GLUT4, glucose transporter 4; t- and v-SNARE, target and vesicle soluble N-ethylmaleimide-sensitive factor-attachment protein (SNAP) receptor, respectively; VAMP2, vesicle-associated membrane protein 2; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PE, phycoerythrin; RNAi, RNA interference; siRNA, small interfering RNA.

Abstract

SNARE proteins (VAMP2, syntaxin4, and SNAP23) have been thought to play a key role in GLUT4 trafficking by mediating the tethering, docking and subsequent fusion of GLUT4-containing vesicles with the plasma membrane. The precise functions of these proteins have remained elusive, however. We have now shown that depletion of the vesicle SNARE (v-SNARE) VAMP2 by RNA interference in 3T3-L1 adipocytes inhibited the fusion of GLUT4 vesicles with the plasma membrane but did not affect tethering of the vesicles to the membrane. In contrast, depletion of the target SNAREs (t-SNAREs) syntaxin4 or SNAP23 resulted in impairment of GLUT4 vesicle tethering to the plasma membrane. Our results indicate that the t-SNAREs syntaxin4 and SNAP23 are indispensable for the tethering of GLUT4 vesicles to the plasma membrane, whereas the v-SNARE VAMP2 is not required for this step but is essential for the subsequent fusion event.

Keywords

GLUT4; VAMP2; syntaxin4; SNAP23; SNARE; adipocytes

Introduction

Glucose transporter 4 (GLUT4) is expressed specifically in insulin-sensitive tissues including adipose tissue, skeletal muscle, and the heart [1,2]. Regulation of glucose uptake by insulin in these tissues is mediated largely at the level of redistribution of GLUT4 from perinuclear depots to the plasma membrane [3,4] and is important for whole-body glucose homeostasis. Mice that lack GLUT4 specifically in adipose tissue develop insulin resistance [5], whereas those that lack the transporter specifically in muscle manifest both insulin resistance and glucose intolerance [6,7]. Conversely, overexpression of GLUT4 in adipose tissue enhances *in vivo* glucose tolerance [8]. These findings thus demonstrate the central role that glucose uptake by GLUT4 in adipose tissue and skeletal muscle plays in whole-body glucose metabolism, and they highlight the importance of characterization of the mechanism of GLUT4-mediated glucose uptake for clarification of the pathogenesis of type 2 diabetes.

GLUT4-mediated glucose uptake is thought to consist of several distinct steps [9,10]. The first step involves the movement of GLUT4-containing vesicles from a specialized intracellular compartment to the cell periphery. The second step includes two processes: tethering and docking. Tethering refers to a low-affinity interaction between GLUT4 vesicles and the plasma membrane, and docking is mediated by assembly of the soluble *N*-ethylmaleimide-sensitive factor-attachment protein (SNAP) receptor (SNARE) complex. The final step of GLUT4 trafficking to the cell surface is fusion, in which the lipid bilayers of each GLUT4 vesicle and the plasma membrane merge as a result of specific interactions between vesicle SNARE (v-SNARE) and cognate target SNARE (t-SNARE) proteins. A SNARE complex consisting of syntaxin4 and SNAP23 as t-SNAREs as well as vesicle-associated membrane protein 2 (VAMP2) as the v-SNARE has been shown to play an important role in insulin-stimulated externalization of GLUT4 in adipocytes and skeletal muscle [11–17], although a recent study showed that the disruption of VAMP2 did not inhibit insulin-stimulated GLUT4 insertion into the plasma membrane in adipocytes [18].

We have now examined the roles of the v-SNARE VAMP2, the t-SNAREs syntaxin4 and SNAP23 in each step of GLUT4 trafficking by forced expression of a GLUT4 reporter tagged with both the Myc epitope and green fluorescent protein (GFP) and by immunostaining of endogenous GLUT4 in cultured adipocytes. [19–21]. We found that the t-SNAREs, which have been recognized as important for fusion of GLUT4 vesicles with the plasma membrane, were also indispensable for the tethering of these vesicles to the plasma membrane. In contrast, the v-SNARE was indispensable for the fusion step but not for vesicle tethering.

Materials and Methods

Materials—Polyclonal antibodies to syntaxin4 were generated as described previously [15], whereas those to VAMP2 and to SNAP23 were obtained from Synaptic Systems. Rabbit monoclonal antibodies to GLUT4 were purchased from Abcam. Mouse monoclonal antibody to c-Myc was from Santa Cruz Biotechnology, and that to β -actin was from Sigma. Rabbit polyclonal antibodies to Akt and to Akt phosphorylated on Ser⁴⁷³ were obtained from Cell Signaling Technology.

Preparation of 3T3-L1 adipocytes expressing GLUT4myc7-GFP—3T3-L1 cells were obtained from American Type Culture Collection. Adipogenesis was induced in these cells as described previously [22]. Platinum-E (PLAT-E) ecotropic packaging cells were transfected with the retroviral vector pMX-GLUT4myc7-GFP (kindly provided by Harvey F. Lodish, Massachusetts Institute of Technology, Cambridge, MA) [19] with the use of the transfection reagent FuGENE 6 (Roche Diagnostics). Culture medium containing recombinant retroviruses was harvested 48 h after the onset of transfection. For infection of the target cells, 1 ml of the retrovirus-containing medium was added to 3T3-L1 fibroblasts (2×10^5 cells per 60-mm dish) cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, and the cells were incubated for 24 h. The efficiency of infection with the recombinant retroviruses was estimated at 60 to 80% on the basis of GFP fluorescence.

Confocal immunofluorescence microscopy—Adipocytes expressing GLUT4myc7-GFP were fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde, washed with PBS containing 100 mM glycine, and exposed to PBS containing 5% bovine serum albumin. Externalized GLUT4myc7-GFP was visualized without cell permeabilization by indirect immunofluorescence staining with antibodies to c-Myc and phycoerythrin (PE)–conjugated goat antibodies to mouse immunoglobulin G (Jackson ImmunoResearch Laboratories). The cells were examined for PE and GFP fluorescence with a confocal laser-scanning microscope (LSM5 PASCAL version 3, Carl Zeiss). The positive evaluation of cell surface externalization of GLUT4 or movement of GLUT4 to the cell periphery was determined by the fluorescence which reached more than half of cell surrounding.

Depletion of VAMP2, syntaxin4, or SNAP23 in 3T3-L1 adipocytes by RNA interference (RNAi)—Five days after the induction of adipocyte differentiation in 3T3-L1 fibroblasts, the cells were washed twice with PBS, detached from the culture dish by exposure to PBS containing 0.25% trypsin and collagenase (0.5 mg/ml), and resuspended in Hepes-buffered saline [10 mM Hepes-NaOH (pH 7.4), 150 mM NaCl, 5 mM CaCl₂]. The

adipocytes (3×10^6) were then mixed with small interfering RNA (siRNA) duplexes and subjected to electroporation with a Bio-Rad Gene Pulser II system at a setting of 0.18 kV and 975 μ F. The VAMP2, syntaxin4, and SNAP23 siRNAs were targeted to the cDNA sequences 5'-GGAAAAACCUCAAGAUGAUGAUCAU-3', 5'-GCAACTCAATGCAGTCCGATT-3', and 5'-GCAAGGGGAACAATAATCGCATA-3', respectively. The cells were mixed with fresh DMEM supplemented with 10% fetal bovine serum immediately after electroporation and were seeded into culture plates after incubation for 10 min. They were subjected to assays 2 days after electroporation.

Plasma membrane lawn assay—Translocation of GLUT4 to the plasma membrane was measured with a plasma membrane lawn assay as described previously [19]. In brief, 3T3-L1 adipocytes cultured on cover slips were washed with PBS, exposed to poly-L-lysine (0.5 mg/ml) in PBS, incubated in a hypotonic solution [KHMgE buffer: 30 mM Hepes-NaOH (pH 7.5), 70 mM KCl, 5 mM MgCl₂, and 3 mM EGTA], and disrupted by ultrasonic treatment in KHMgE buffer supplemented with 0.1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol. For detection of the plasma membrane of the disrupted cells, they were exposed to the lipophilic styryl dye FM1-43FX (Molecular Probes). The cells were then fixed in 4% paraformaldehyde, incubated with antibodies to GLUT4, washed three times with PBS, and incubated with fluorescein isothiocyanate-conjugated antibodies to rabbit immunoglobulin G (Bio-Rad). After a final wash with PBS, the cover slips were mounted in FluoroGuard Antifade Reagent (Bio-Rad) and examined with a confocal laser-scanning microscope (LSM5 PASCAL version 3, Carl Zeiss).

Assay of 2-deoxy-D-glucose transport—2-deoxy-D-glucose transport was measured in 3T3-L1 adipocytes as described previously [17].

Results

SNARE components are indispensable for insulin-stimulated glucose uptake in 3T3-L1 adipocytes—

The SNARE complex consisting of VAMP2, syntaxin4, and SNAP23 is essential for insulin-stimulated externalization of GLUT4 [11–17]. To precisely examine the roles of these molecules in GLUT4 trafficking process, we depleted 3T3-L1 adipocytes of VAMP2, syntaxin4, and SNAP23 by RNAi. Introduction of siRNAs specific for the mRNAs of these proteins into differentiated 3T3-L1 adipocytes resulted in substantial decrease in the abundance of each protein in these cells compared with that apparent in control cells (Fig. 1A, B). The morphology of these proteins-depleted cells did not differ substantially from that of the control cells,

however (data not shown). In addition, we confirmed that the extent of insulin-stimulated phosphorylation of Akt on Ser⁴⁷³ did not differ between these proteins-depleted and control adipocytes, suggesting that loss of SNAREs did not affect proximal insulin signaling (Fig. 1C). In contrast, insulin-stimulated 2-deoxy-D-glucose uptake was approximately half inhibited in the cells depleted of VAMP2, syntaxin4, or SNAP23 compared with that in control cells (Fig. 1D), indicating the importance of SNARE components for insulin-stimulated glucose uptake in adipocytes.

The v-SNARE VAMP2 is indispensable for the insulin-induced fusion of GLUT4 vesicles with the plasma membrane but not for vesicle tethering in adipocytes—To determine the regulatory mechanism of SNARE complex more precisely, we first examined the function of VAMP2 in this process in adipocytes. We prepared 3T3-L1 adipocytes that express the GLUT4 reporter GLUT4^{myc7}-GFP, which contains seven copies of the Myc epitope in its first extracellular loop and is fused with GFP at its intracellular COOH-terminus [19]. These cells allowed us to monitor both the movement of GLUT4 from intracellular storage sites to the plasma membrane by detection of GFP fluorescence at the cell periphery as well as the subsequent externalization of GLUT4 by cell surface labeling with antibodies to the Myc tag [20]. Depletion of VAMP2 by RNAi did not affect the extent of insulin-stimulated GLUT4 movement to the cell periphery as estimated by the ratio of the number of cells positive for GFP fluorescence at the cell periphery to the total number of GFP-positive cells (Fig. 2A, B). In contrast, cell surface labeling of the GLUT4 reporter with antibodies to the Myc tag revealed that the insulin-induced appearance of GLUT4 at the cell surface was inhibited by ~41% in the VAMP2-depleted adipocytes (Fig. 2C, D). These results suggested that GLUT4 vesicles either are localized near the plasma membrane without association with it or are localized at the plasma membrane in the tethered or docked state without fusion in the VAMP2-depleted adipocytes stimulated with insulin.

Therefore, to further examine the insulin-stimulated localization of GLUT4 in adipocytes, we performed a plasma membrane lawn assay with VAMP2-depleted 3T3-L1 adipocytes and antibodies specific for the COOH-terminal domain of GLUT4. Because, GLUT4 can be stained on the plasma membrane lawns of 3T3-L1 adipocytes in this assay if GLUT4 vesicles are tethered or docked to plasma membrane. Insulin stimulation resulted in the almost same amount of GLUT4 staining on plasma membrane in both VAMP2-depleted adipocytes and control adipocytes (Fig. 3A, B). These results suggest that insulin induced similar extents of GLUT4 association with the plasma membrane lawns prepared from VAMP2-depleted adipocytes or control adipocytes irrespective of fusion event. Furthermore, given that the extent

of cell surface labeling of GLUT4 was decreased in VAMP2-depleted cells (Fig. 2C, D), these results altogether indicated that GLUT4 vesicles are localized at the plasma membrane in the tethered or docked state prior to fusion in VAMP2-depleted adipocytes stimulated with insulin. They thus also suggested that VAMP2 is indispensable for the fusion step, but not for the tethering step, of GLUT4 trafficking in adipocytes.

The t-SNAREs syntaxin4 and SNAP23 are important for the tethering of GLUT4 vesicles at the plasma membrane in adipocytes—We next examined insulin-stimulated GLUT4 translocation and externalization in the t-SNARE-depleted adipocytes with the use of the GLUT4 reporter GLUT4myc7-GFP. Whereas depletion of syntaxin4 or SNAP23 did not affect the insulin-induced movement of GLUT4 vesicles to the cell periphery (Fig. 4A, B), ablation of these proteins inhibited the appearance of GLUT4 at the cell surface by ~53 and ~57%, respectively (Fig. 4C, D). To determine whether the GLUT4 vesicles that moved to the periphery of the syntaxin4- or SNAP23-depleted cells in response to insulin were attached to the plasma membrane or not, we performed the plasma membrane lawn assay. In contrast to the lack of effect of VAMP2 depletion (Fig. 3A, B), depletion of syntaxin4 or SNAP3 resulted in inhibition of insulin-induced localization of GLUT4 to the plasma membrane by ~59 and ~64%, respectively (Fig. 4E, F). These results suggested that GLUT4 vesicles that moved to the cell periphery in response to insulin were not tethered to the plasma membrane in the t-SNARE-depleted cells, and that both syntaxin4 and SNAP23 are therefore important for insulin-stimulated tethering of GLUT4 vesicles to the plasma membrane.

Discussion

The t-SNAREs syntaxin4 and SNAP23 as well as the v-SNARE VAMP2 have been generally thought to be necessary for the fusion of GLUT4 vesicles with the plasma membrane [4,23]. However, we have now delineated more precisely the functions of these t-SNAREs in GLUT4 trafficking in 3T3-L1 adipocytes. We dissected the whole GLUT4 traffic to three steps using exogenously-expressed GLUT4 reporter and plasma membrane lawn assay: mobilization of GLUT4 vesicles from retention sites to the cell cortex, tethering of them to plasma membrane prior to fusion, and externalization to cell surface by fusion. Our results demonstrate that syntaxin4 and SNAP23 also play an important role in the tethering of GLUT4 vesicles at the plasma membrane before they undergo fusion. In contrast, the v-SNARE VAMP2 was found not to be required for this tethering step but to be essential for the subsequent fusion of GLUT4 vesicles with the plasma membrane. Our findings thus provide evidence for the new concept

that t-SNAREs (syntaxin4 and SNAP23), but not the v-SNARE (VAMP2), are indispensable for tethering of GLUT4 vesicles at the plasma membrane prior to their fusion in adipocytes.

The exocyst is implicated in capturing vesicles at relatively large distances from the plasma membrane and tethering them to exocytic sites at the membrane [24]. The exocyst complex in mammalian adipocytes is also thought to play an important role in GLUT4 trafficking, especially in the tethering-docking of GLUT4 vesicles at the plasma membrane [25]. Thus, whereas the SNARE complex was previously thought to be important for vesicle fusion and the exocyst for vesicle tethering, our results now suggest that both t-SNAREs and the exocyst are indispensable for the tethering of GLUT4 vesicles to the plasma membrane in adipocytes. Our results thus suggest the possibility that t-SNAREs, syntaxin4 and SNAP23 may function co-operatively with exocyst complex at the plasma membrane.

In summary, we have obtained direct evidence that the t-SNAREs syntaxin4 and SNAP23 are important for tethering of GLUT4 vesicles at the plasma membrane, whereas the v-SNARE VAMP2 is important for fusion of GLUT4 vesicles with the plasma membrane in adipocytes. These findings suggest that syntaxin4-SNAP23 and VAMP2 have distinct roles in GLUT4 trafficking in adipocytes.

Conclusions

The t-SNAREs syntaxin4 and SNAP23 are indispensable for the tethering of GLUT4 vesicles to the plasma membrane, whereas the v-SNARE VAMP2 is not required for this step but is essential for the subsequent fusion event.

Acknowledgements

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Figure legends

FIGURE 1. Depletion of each of VAMP2, syntaxin4, or SNAP23 inhibits insulin-stimulated glucose uptake in 3T3-L1 adipocytes. A, Immunoblot analysis (IB) of VAMP2, syntaxin4, SNAP23, and β -actin (loading control) in 3T3-L1 adipocytes 2 days after the introduction of siRNAs to VAMP2, syntaxin4, or SNAP23 as well as in control cells. B, Quantitation of abundance of VAMP2, syntaxin4, and SNAP23 by densitometry in experiments similar to that shown in A. Data are expressed relative to the value for control cells, and are means $\pm$ SEM of values from three separate experiments. * $P < 0.05$ versus control. C, VAMP2, syntaxin4, or

SNAP23-depleted or control 3T3-L1 adipocytes were stimulated with 100 nM insulin (or not) for 5 min, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to the Ser⁴⁷³-phosphorylated form of Akt (pAkt) or to total Akt. *D*, Basal and insulin-stimulated uptake of 2-deoxy-D-[1,2-³H]glucose was measured in VAMP2, syntaxin4, or SNAP23-depleted or control adipocytes. Data are means $\pm$ SEM of values from three separate experiments and are expressed as fold increase relative to the value for corresponding control cells not stimulated with insulin. **P* < 0.05 versus the value for control cells stimulated with insulin.

FIGURE 2. Depletion of VAMP2 inhibits insulin-stimulated GLUT4 externalization but not movement of GLUT4 vesicles to the plasma membrane. *A, C*, 3T3-L1 adipocytes expressing GLUT4^{myc7}-GFP were transfected with VAMP2 siRNA and 2 days later were stimulated (or not) with 100 nM insulin for 20 min, fixed, and subjected to indirect immunofluorescence staining with antibodies to the Myc tag and PE-conjugated secondary antibodies (red fluorescence) in order to detect externalized GLUT4 (*C*). Movement of GLUT4 vesicles to the cell periphery was detected on the basis of GFP fluorescence (green) on examination of the cells by confocal microscopy (*A*). Scale bar, 20 μ m. *B, D*, Movement of GLUT4 to the cell periphery in experiments similar to that shown in *A* was quantitated by determination of the percentage of GFP-positive cells that manifested GFP fluorescence at the cell periphery (*B*). GLUT4 externalization in experiments similar to that shown in *C* was quantitated by determination of the percentage of GFP-positive cells that exhibited PE fluorescence at the cell surface (*D*). Data are means $\pm$ SEM of values from three separate experiments, with 80 to 100 GFP-positive cells being examined in each experiment. **P* < 0.05 versus the corresponding value for control cells stimulated with insulin.

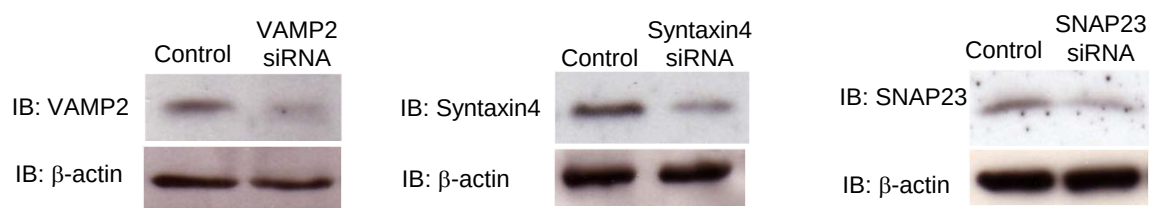
FIGURE 3. Depletion of VAMP2 does not affect insulin-stimulated tethering of GLUT4 vesicles to the plasma membrane. *A*, Two days after the introduction of VAMP2 siRNA, 3T3-L1 adipocytes were stimulated (or not) with 100 nM insulin for 20 min and then disrupted by ultrasonic treatment. The plasma membrane fragments were detected by staining with FM1-43FX (red fluorescence) and were subjected to immunofluorescence analysis with antibodies to GLUT4 (green fluorescence). Scale bar, 20 μ m. *B*, GLUT4 localization at the plasma membrane in experiments similar to that shown in *A* was quantitated by determination of the percentage of cells stained with FM1-43FX in which GLUT4 fluorescence was detected at the plasma membrane. Data are means $\pm$ SEM of values from three separate experiments,

with 80 to 100 FM1-43FX–stained cells being examined in each experiment.

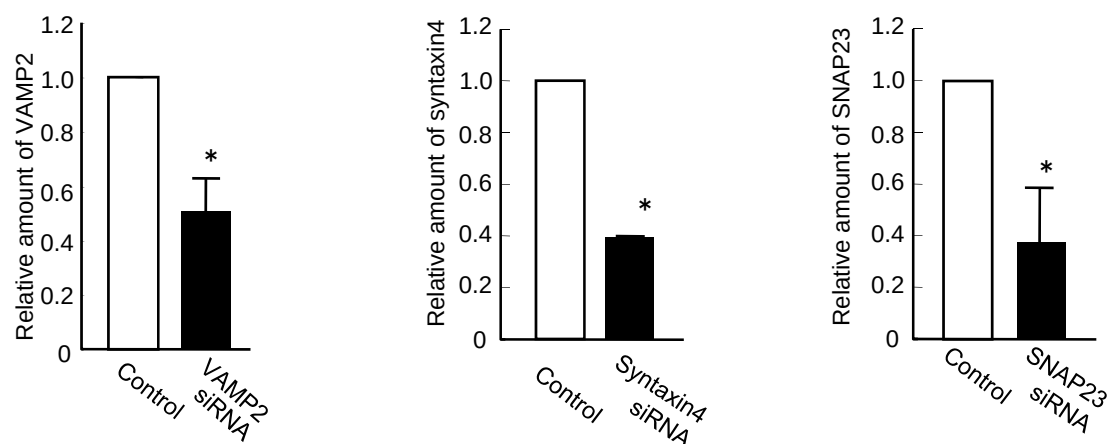
FIGURE 4. GLUT4 vesicles translocate to the cell periphery but do not associate with the plasma membrane in insulin-stimulated 3T3-L1 adipocytes depleted of syntaxin4 or SNAP23.

A, C, 3T3-L1 adipocytes expressing GLUT4^{myc7}-GFP were transfected with syntaxin4 or SNAP23 siRNAs and 2 days later were stimulated (or not) with 100 nM insulin for 20 min, fixed, and subjected to indirect immunofluorescence staining with antibodies to the Myc tag and PE-conjugated secondary antibodies (red fluorescence) in order to detect externalized GLUT4 (**C**). Movement of GLUT4 vesicles to the cell periphery was detected on the basis of GFP fluorescence (green) on examination of the cells by confocal microscopy (**A**). Scale bar, 20 μ m. **B, D,** Movement of GLUT4 to the cell periphery in experiments similar to that shown in **A** was quantitated by determination of the percentage of GFP-positive cells that manifested GFP fluorescence at the cell periphery (**B**). GLUT4 externalization in experiments similar to that shown in **C** was quantitated by determination of the percentage of GFP-positive cells that exhibited PE fluorescence at the cell surface (**D**). Data are means $\pm$ SEM of values from three separate experiments, with 80 to 100 GFP-positive cells being examined in each experiment. * $P < 0.05$ versus the corresponding value for control cells stimulated with insulin. **E,** Two days after the introduction of syntaxin4 or SNAP23 siRNAs, 3T3-L1 adipocytes were stimulated (or not) with 100 nM insulin for 20 min and then disrupted by ultrasonic treatment. The plasma membrane (PM) fragments were detected by staining with FM1-43FX (red fluorescence) and were subjected to immunofluorescence analysis with antibodies to GLUT4 (green fluorescence). Scale bar, 20 μ m. **F,** GLUT4 localization at the plasma membrane in experiments similar to that shown in **E** was quantitated by determination of the percentage of cells stained with FM1-43FX in which GLUT4 fluorescence was detected at the plasma membrane. Data are means $\pm$ SEM of values from three separate experiments, with 80 to 100 FM1-43FX–stained cells being examined in each experiment. * $P < 0.05$ versus the corresponding value for control cells stimulated with insulin.

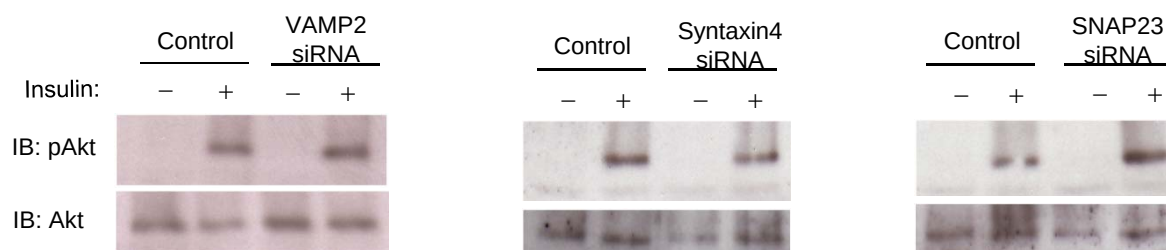
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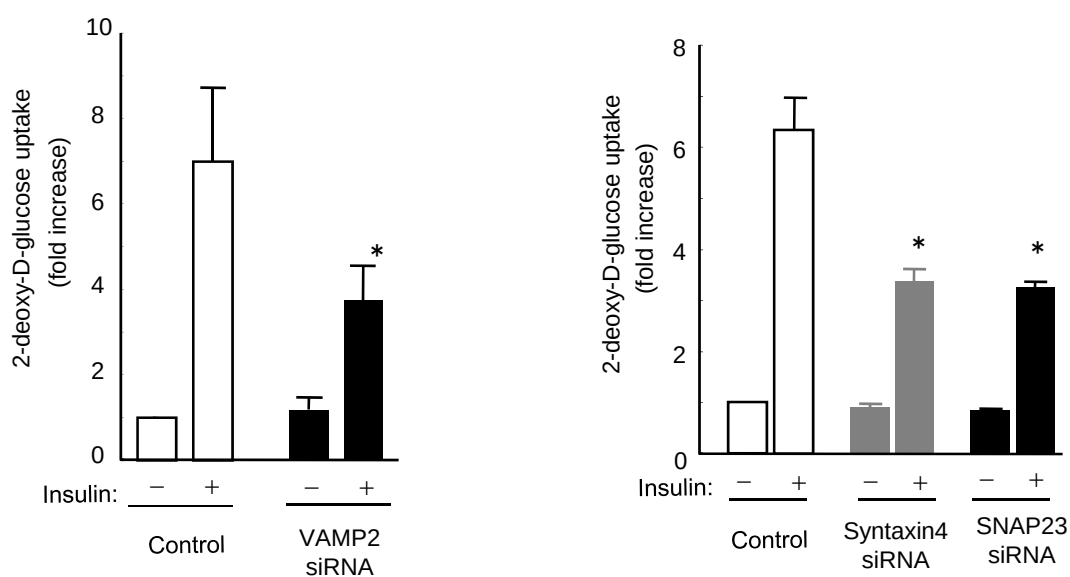
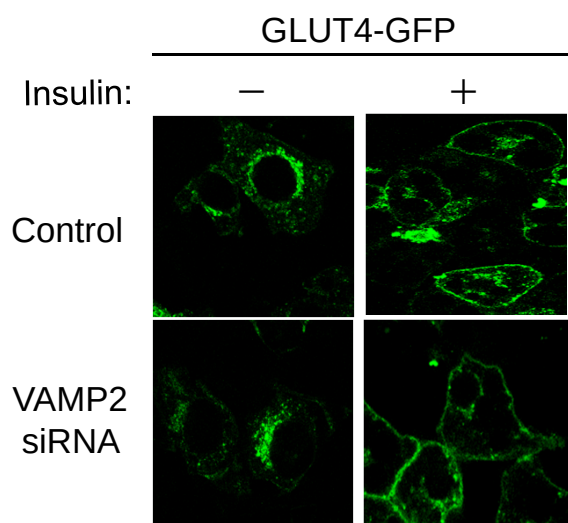
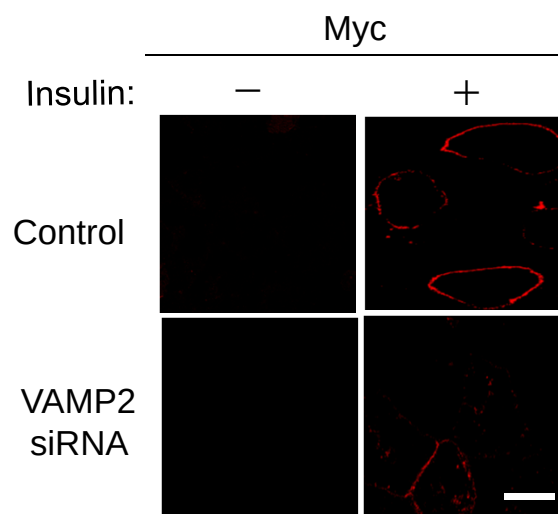


Figure. 1 Kawaguchi *et al.*

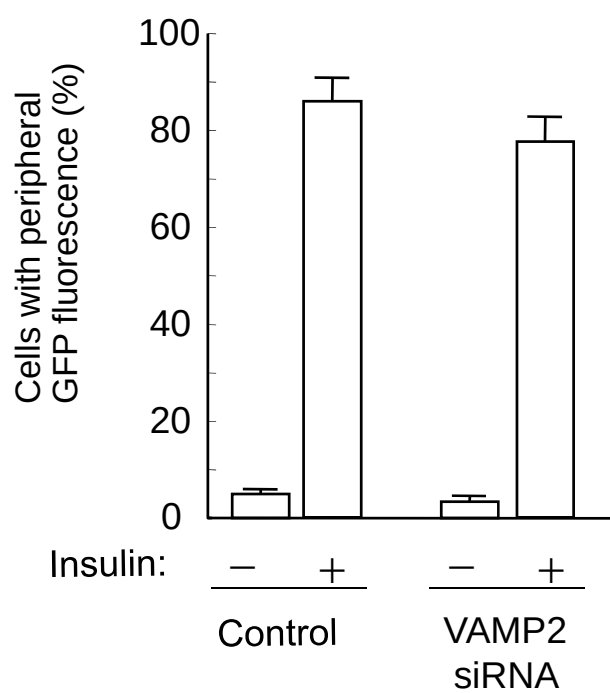
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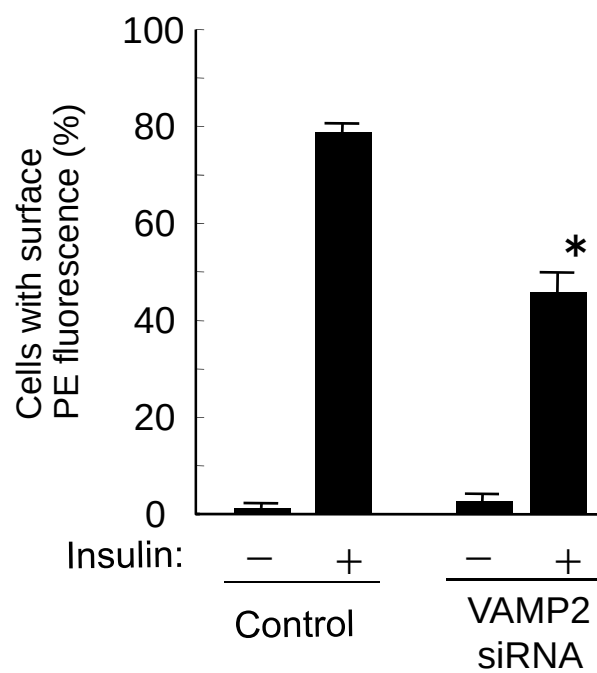


Figure. 2 Kawaguchi *et al.*

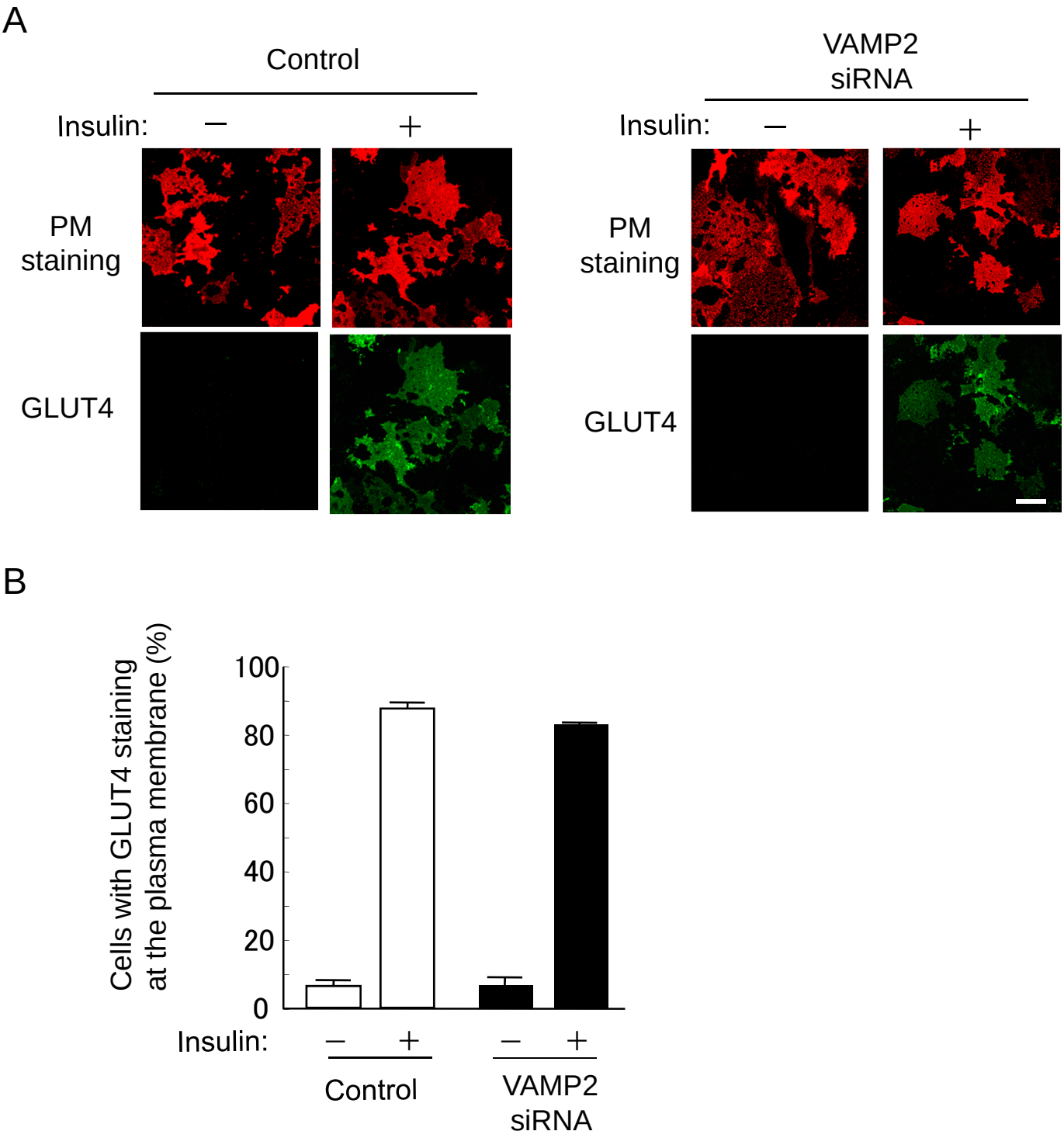


Figure. 3 Kawaguchi *et al.*

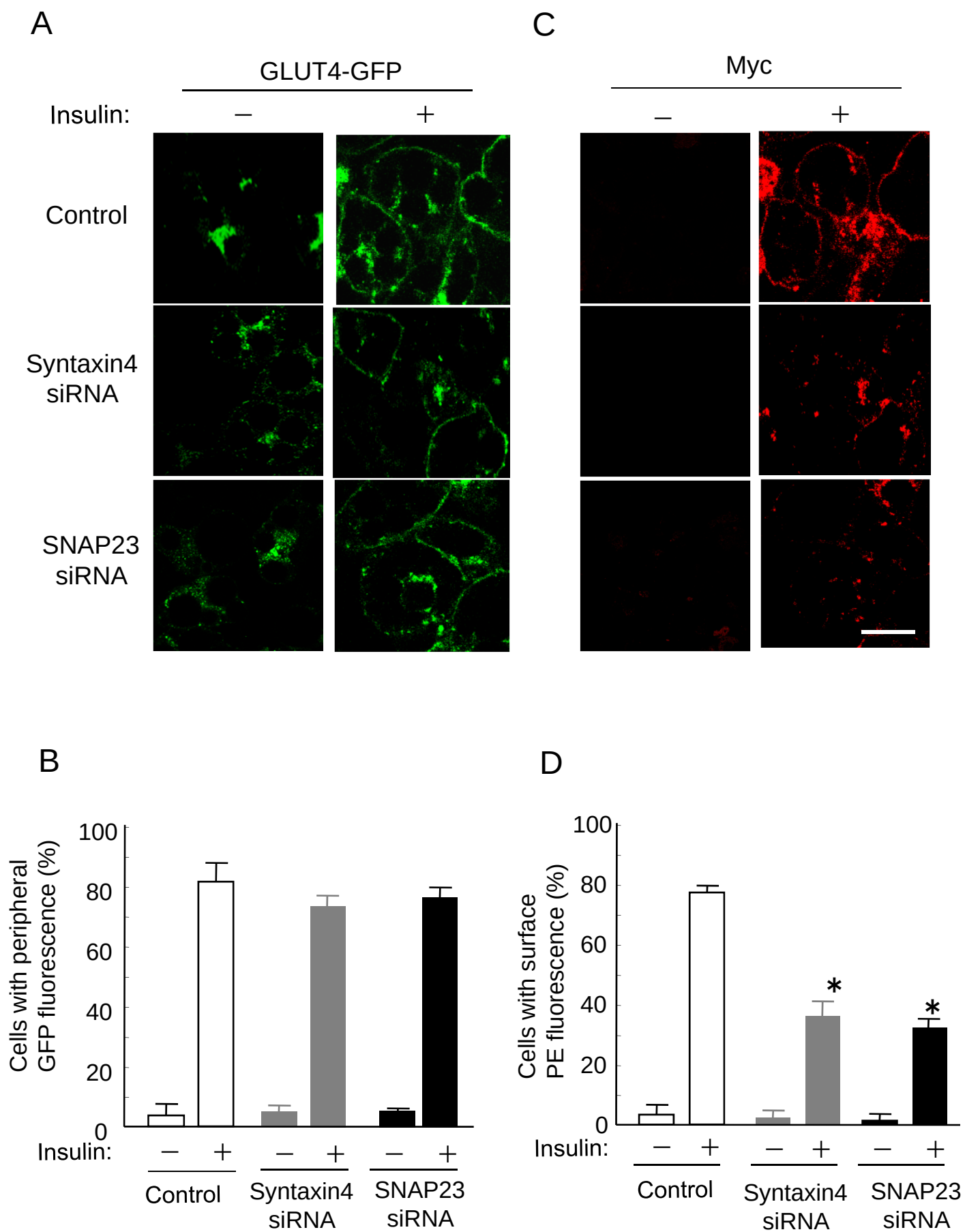
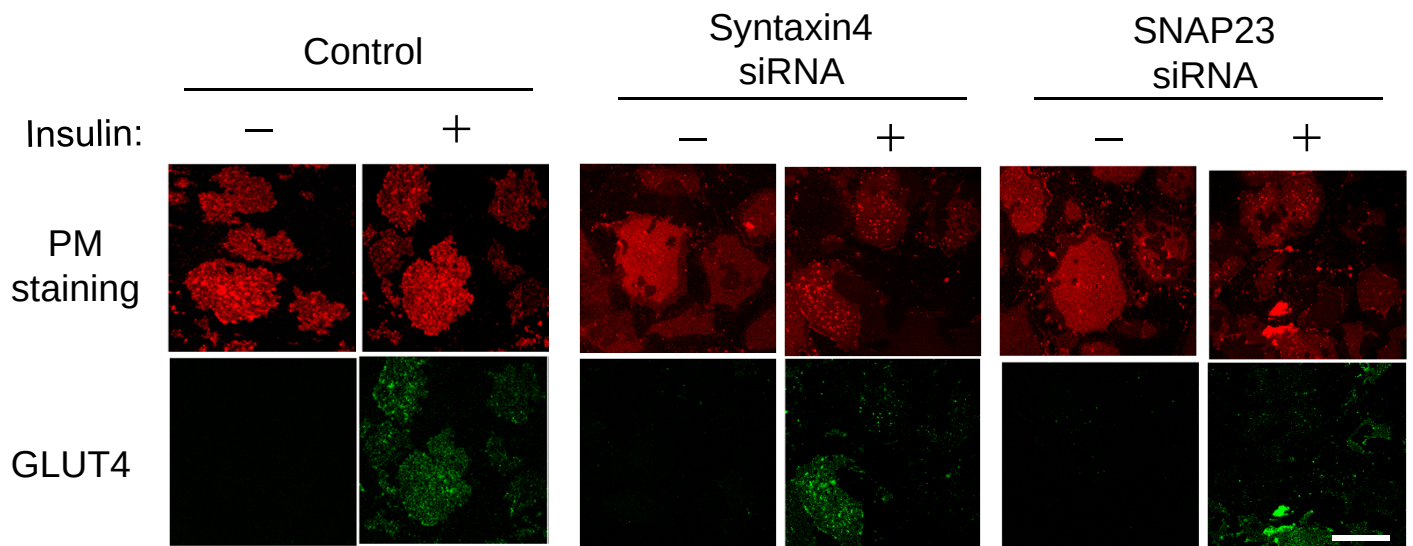
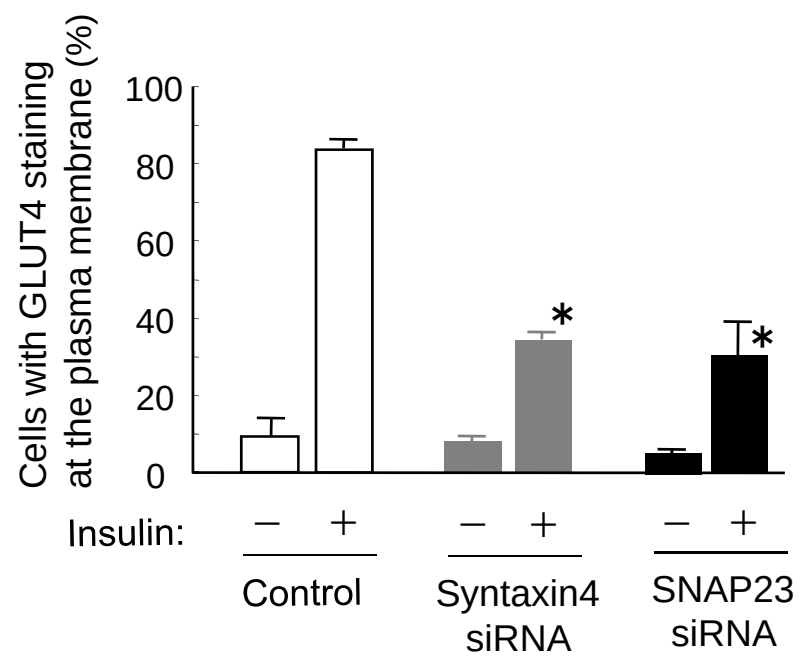


Figure. 4 Kawaguchi *et al.*

E



F

Figure. 4-continued Kawaguchi *et al.*