Glucose deprivation attenuates sortilin levels in skeletal muscle cells

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Abstract. In skeletal muscle, sortilin plays a predominant role in the sorting of glucose transporter 4 (Glut4), thereby controlling glucose uptake. Moreover, our previous study suggested that the sortilin expression levels are also implicated in myogenesis. Despite the importance of sortilin in skeletal muscle, however, the regulation of sortilin expression has not been completely understood. In the present study, we analyzed if the sortilin expression is regulated by glucose in C2C12 myocytes and rat skeletal muscles in vivo. Sortilin protein expression was elevated upon C2C12 cell differentiation and was further enhanced in the presence of a high concentration of glucose. The gene expression and protein degradation of sortilin were not affected by glucose. On the other hand, rapamycin partially reduced sortilin induction by a high concentration of glucose, which suggested that sortilin translation could be regulated by glucose, at least in part. We also examined if the sortilin regulation by glucose was also observed in skeletal muscles that were obtained from fed or fasted rats. Sortilin expression in both gastrocnemius and extensor digitorum longus (EDL) muscle was significantly decreased by 17-18h of starvation. On the other hand, pathological levels of high blood glucose did not alter the sortilin expression in rat skeletal muscle. Overall, the present study suggests that sortilin protein levels are reduced under hypoglycemic conditions by post-transcriptional control in skeletal muscles.

Key words: Sortilin, Glucose, AMPK, Skeletal muscle

SORTILIN is a vacuolar protein sorting (VPS) 10p domain containing receptor family member and is mainly expressed in skeletal muscle, adipose tissue, liver, and brain [1, 2]. An important function of sortilin is its role in sorting a variety of proteins from the trans-Golgi network to lysosome, endosome, or plasma membranes by forming membrane-derived vesicles [3, 4]. Especially, the role of sortilin in the formation of specialized storage vesicles containing Glut4 (Glut 4 storage vesicles, GSVs) has been well studied. GSVs consist of several proteins including Glut4, insulin-responsive aminopeptidase (IRAP), sortilin, insulin growth factor II/mannose 6-phosphate receptor (IGF-II/M6PR), ADP-ribosylation factor-binding protein GGA1 and GGA2, vesicle associated membrane protein (VAMP)-2 and/or VAMP3, receptor; VAMP, vesicle associated membrane protein; Glut1, glucose transporter 1; CS, calf serum; FBS, fetal bovine serum; BSA, bovine serum albumin; PBS, phosphate buffered saline; STZ, streptozotocin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BCA, bicinchoninic acid assay; TBS, Tris buffered saline; LG-DM, low glucose differentiation medium; HG-DM, high glucose differentiation medium; CHX, cycloheximide; mTORC1, mammalian target of rapamycin complex 1; mLST8/GbL, mammalian lethal with Sec13 protein 8; Raptor, regulatory-associated protein of mTOR; PRAS40, proline-rich Akt substrate 40kDa; DEPTOR, DEP-domain containing mTOR interacting protein; S6K1, p70 S6 kinase; elf4E, eukaryotic translation initiation factor 4E; AMPK, AMP-activated protein kinase.

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as well as several Rab proteins [5]. Sortilin is proposed to be essential and sufficient for the formation of GSVs [6, 7]. Under basal conditions, GSVs reside in the intracellular regions; and some stimuli such as insulin induce its translocation and fusion to the cell surface to initiate glucose uptake; however, the reduction of sortilin deprives this characteristic of GSVs and decreases insulin-dependent glucose uptake [6].

The other important role of sortilin is in controlling skeletal muscle cell differentiation. We previously studied the function of sortilin in the murine skeletal muscle cell line, C2C12 myocytes, and found that sortilin is directly involved in muscle differentiation, at least in part by potentiation of the p75NTR-proNGF autocrine loop [7]. Given that the interaction between p75NTR and proNGF can be augmented when sortilin associates with p75NTR [8], intriguingly, this interaction leads not only to skeletal muscle cell differentiation but also to various cellular responses including cell survival and apoptosis [8, 9]. Overall, sortilin seems to be a multifunctional protein that regulates glucose homeostasis, cell differentiation and cell death.

To maintain glucose homeostasis, blood glucose steady-state concentration is important. A condition when excessive amounts of glucose are present in blood, defined as hyperglycemia, promotes insulin resistance in skeletal muscle and adipose tissues and contributes to a diagnosis of Type 2 diabetes mellitus [10]. This impairment of insulin action by excess glucose can be explained primarily by a defect in insulin signal transduction. Evidence from both in vitro and in vivo models, strongly suggested that the prolonged exposure to high glucose attenuated insulin-dependent PKB/Akt activation, which has a prominent role in Glut4 translocation [11-14]. On the other hand, hypoglycemia, which is the opposite of hyperglycemia, is recognized as a major side effect of insulin therapy for diabetic patients [15], and also happens in non-diabetic conditions, such as starvation and overtraining [16]. It was widely accepted that glucose transport activity in many cell types is enhanced via up-regulating glucose transporter 1 (Glut1) under hypoglycemic conditions [17]. Together, the accumulated evidence clearly shows that the changes in surrounding glucose levels directly affect the glucose transport system. In addition to glucose homeostasis, the extracellular glucose levels also significantly alter the myogenic process. For example, we previously reported that C2C12 cell differentiation induced by low serum was significantly suppressed under glucose-restricted conditions [18].

Although sortilin appears to play crucial roles in both glucose incorporation and differentiation in skeletal muscle, the expression control of sortilin under hypoglycemic or hyperglycemic conditions remains to be further elucidated. In the present study, we analyzed whether and how the protein levels of sortilin in C2C12 myocytes are affected by changing environmental glucose levels in vitro and whether the expression of sortilin in rat skeletal muscles could also be controlled by changes in blood glucose levels in vivo.

Materials and Methods

Materials

The Western blot detection kit (West super femto detection reagents) was from Pierce Biotechnology Inc. (Rockford, IL, USA). Dulbecco’s Modified Eagle Medium (DMEM), penicillin/streptomycin and Trypsin-EDTA were purchased from Sigma Chemicals (St. Louis, MO, USA). Cell culture equipment was from BD Biosciences (San Jose, CA, USA). Calf serum (CS) and fetal bovine serum (FBS) were obtained from Sigma-Aldrich or Nacalai Tesque. (Kyoto, Japan). Unless otherwise noted, all chemicals were of the purest grade available from Sigma-Aldrich or Nacalai Tesque.

Animal experiments

All animal care and experiments were approved by the Animal Care Committee at The University of Tokyo. Male Wistar rats (CLEA Japan, Inc., Tokyo, Japan, 5 weeks of age at the beginning of the experimental protocol) were individually housed in the temperature- and humidity-controlled room. The animals were fed rat chow (CLEA Japan, Inc.) and water ad libitum and were acclimatized to a 12-h light cycle (lights on between 0700 and 1900h) for a period of 1 week before experimental manipulation. For the dietary restriction, rats were divided into two groups; 1) fed: food was taken ad libitum, 2) fast: food was removed for overnight (17–18h) with n=8 per group. After rats from each group were euthanized by decapitation, blood glucose levels were measured using Antsense III (Fukuda Denshi, Tokyo, Japan). Gastrocnemius, soleus, and EDL skeletal muscles from each hindleg were then removed, washed in PBS(-), frozen in liquid
nitrogen and stored at -80°C. For the STZ experiments, rats were divided into two groups; 1) control and 2) streptozotocin-treated (STZ, Sigma-Aldrich) with n=8 per group. A single intra-peritoneal injection of STZ (70mg/kg in sterile sodium citrate buffer; 50mM sodium citrate, pH 4.5) was given to the STZ rats while control animals received a sodium citrate buffer injection under similar conditions. Body weight and food intake were measured daily and blood glucose concentration was measured as described above. Three or four days following the initial injection of saline or STZ, the rats were euthanized by decapitation. Gastrocnemius, soleus, and EDL skeletal muscles from each hindleg were removed, washed in PBS(-), frozen in liquid nitrogen and stored at -80°C.

To prepare the samples for western blotting, tissues in the NET buffer [50mM Tris-HCl, pH7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 100units/mL aprotinin, 1mM phenylmethylsulfonyl fluoride, 5μg/mL leupeptin, and 5μg/mL pepstatin A] were sonicated, centrifuged at 13,000×g for 10min at 4°C, and the supernatants were obtained as samples for western blotting.

Cell culture

Mouse skeletal muscle cell line, C2C12 myoblasts, were maintained in low-glucose (LG, 5mM) DMEM supplemented with 10% FBS, 30µg/mL penicillin, and 100µg/mL streptomycin (growth medium) at 37°C under a 5% CO2 atmosphere. For biochemical studies, cells were grown on 6 well plates (BD Biosciences) at a density of 3×10^4 cells/well in 3mL of growth medium. Three days after plating, cells had reached approximately 80–90% confluence (Day 0). Differentiation was then induced by switching the growth medium to DMEM supplemented with 2% CS, 30µg/mL penicillin, and 100μg/mL streptomycin (differentiation medium). The differentiation medium was changed every 24h.

Western blot analysis

The expression levels of each protein were analyzed by western blot analysis. Briefly, cells were lysed with NET buffer. The extracts were centrifuged at 13,000×g for 10min to remove insoluble materials and protein concentrations soluble fractions were determined using a bicinchoninic acid assay (BCA) (Pierce Biotech. Inc.). Equal amounts of total protein (22.5μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis (1: 30 bis: acrylamide). Proteins were transferred to a PVDF membrane (Immobilon-P, Millipore Corp., Bedford, MA, USA) or a nitrocellulose transfer membrane (Protran, Schleicher & Schuell BioScience, Heidelberg, Germany), and the membranes were then blocked for 1h at room temperature with 2–5% BSA in Tris buffered saline (TBS) containing 0.1% Tween 20. Immunostaining to detect each protein was achieved with an overnight incubation at 4°C with 3% BSA/TBS containing either anti-sortilin (1:1,000, BD Biosciences (San Jose, CA, USA) or Abcam plc (Cambridge, UK)), anti-UBC9 (1:500, BD Biosciences), anti-GGA1 (G-17, 1:200, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-GGA2 (AP2317, 1:500, Abgent, Inc., San Diego, CA, USA), anti-M6PR (1:1,000, Santa Cruz Biotechnology, Inc.), anti-actin (A-2066, 1:500, Sigma-Aldrich), anti-GAPDH (1:1,000, Cell Signaling Technology), anti-phosphorylated AMPK (Thr172) (1:1,000, Cell Signaling Technology, Danvers, MA, USA), anti-AMPK (1:1,000, Cell Signaling Technology), anti-phospho-p70 S6 Kinase (Ser371) (1:1,000, Cell Signaling Technology) or anti-Glut4 antibody (1:1,000, a generous gift from Dr. H. Shibata (Gunma University, Maebashi, Japan) [19]). Specific total proteins were visualized after subsequent incubation with a 1:5,000 dilution of anti-mouse, rabbit, or goat IgG conjugated to horseradish peroxidase and a SuperSignal Chemiluminescence detection procedure (Pierce Biotechnology Inc.). At least three independent experiments were performed for each condition.

Quantitative RT-PCR analysis

Total RNA from C2C12 cells was extracted with TRIzol reagent (Invitrogen) from three independently collected cell samples. First-strand cDNA was synthesized with ReverTra Ace qPCR Master Mix with gDNA remover (TOYOBO). Quantitative PCR was performed with THUNDERBIRD SYBR qPCR Mix (TOYOBO) on an ABI StepOnePlus Real Time PCR System (Applied Biosystems). To normalize the relative expression, a standard curve was prepared for each gene for relative quantification, and the expression level of each gene was normalized to the Rn18s gene. Specific primers for atrophy-related genes were used:

| Sort1 F | GCCTGTGGGTGTCCAAGAAAT |
| Sort1 R | GGCACCAAGATCAGCTTTGC |
| Rn18s F | AACAGGTCCTGTAGGCCTTAG |
| Rn18s R | ATCACGAATGGGGTCTACACG |
Subcellular fractionation

Sub-fractionation of plasma membrane (P), high-density microsome (H), low-density microsome (L), mitochondria/nuclei (M/N), and cytosol (C) of C2C12 myocytes were prepared by the differential centrifugation method described by McKeel and Jarret [20] with some modifications. C2C12 myocytes were washed twice in ice-cold phosphate-buffered saline and homogenized in HES buffer (20mM HEPES, pH 7.4, 1mM EDTA, 255mM sucrose, 100units/mL aproatin, supplemented with a mixture of protease inhibitors, Sigma) using a glass homogenizer with a Teflon pestle (Arthur H. Thomas Co., Philadelphia, PA). The homogenate was centrifuged at 4,000×g for 10min at 4°C to obtain supernatant (containing P, H, L, M/N and C). The pellets (cell debris) were discarded. The supernatant was layered on 1.12M sucrose cushion containing 20mM Hepes-Cl and 1mM EDTA, and centrifuged at 101,000×g for 1h at 4°C in a Beckman type SW28 rotor (Beckman Instruments Inc., Fullerton, CA). After the centrifugation, pellets (containing M/N) were suspended in a HES buffer. The band (containing P, H, L, and C) on the sucrose cushion was collected, re-suspended in a HES buffer and centrifuged at 48,000×g for 45min, and the pellet was collected as P fraction. The supernatant (containing H, L, and C) was re-centrifuged at 48,000×g to obtain H fraction in the pellets. Again, the obtained supernatant was re-centrifuged at 340,000×g for 100min, which allowed separation of pellets (containing L) from supernatant (containing C). The pellets of each fraction were re-suspended in HES buffer to a final concentration of 1–3mg protein/mL. All samples are stored at -80°C.

Statistical analysis

All statistical analyses were performed with Prism5 software (GraphPad Software Inc., La Jolla, CA, USA). Results are expressed as mean value ± SEM, and the data were analyzed by student t-test or the one-way analysis of variance (ANOVA) followed by Tukey’s posthoc test. Differences were considered to be significant at *p < 0.05, **p < 0.01, and ***p < 0.001.

Results

Changes in glucose levels reversibly control sortilin protein levels in C2C12 myotubes

Initially, we used murine C2C12 myocytes to analyze whether changing extracellular glucose levels altered the sortilin expression. C2C12 myoblasts were induced to differentiate in low glucose differentiation medium (LG-DM: DMEM containing 5mM glucose ± 2% CS) or high glucose differentiation medium (HG-DM: DMEM containing 22.5mM glucose ± 2% CS). In our experimental conditions, we had confirmed that the C2C12 myoblasts became hypoglycemic within 24h in LG-DM but not in HG-DM [18]. After inducing differentiation, sortilin expression was examined every 24h. As shown in Fig. 1A, sortilin protein levels were induced upon differentiation, and were further enhanced in HG-DM (Fig. 1A). It was demonstrated that the C2C12 cell differentiation was inhibited under LG-DM [18]. To exclude the possibility that the enhanced sortilin levels under HG-DM (compared to under LG-DM) may be dependent on the degree of differentiation, we next investigated whether the expression of sortilin is reversibly regulated by glucose in fully differentiated C2C12 myotubes. When C2C12 myoblasts were differentiated in LG-DM followed by switching medium to HG-DM, significant increase in sortilin protein can be seen at 3h after switching medium from LG-DM to HG-DM (Fig. 1B, lane 1-6, Fig. 1C). Conversely, when the cells were differentiated in HG-DM followed by switching medium to LG-DM, sortilin protein levels were also dramatically and significantly decreased (Fig. 1B, lane 7-12, Fig. 1C). In addition, our single experiment suggested that sortilin protein level was not affected by changing media from LG-DM to fresh LG-DM, or HG-DM to fresh HG-DM (data not shown).

We also analyzed the other components (UBC9, GGA1, GGA2, and M6PR) of GSVs and Glut1, and found that sortilin levels were most sensitively regulated by glucose (Fig. 1C). The expression of M6PR and GGA2 also tended to change chronologically after switching medium; however, our quantitative analysis revealed that their changes were very slight and did not show any statistical significance (Fig. 1D and 1E). Moreover, in contrast to the changes in sortilin, Glut1 tended to be decreased after increasing glucose concentration (Fig. 1B).

Moreover, the localization of sortilin was predominantly observed in high-density microsomes (H), plasma membrane (P), mitochondria and nucleus (M/N) fractions. Reduction of glucose (LG-DM) decreased sortilin levels in all fractions (Fig. 1F, lane 2, 4, 5, 7, 9, and 10 of sortilin). In addition, subcellular localization of Glut4 in C2C12 myotubes was
Sortilin regulation in skeletal muscles

Fig. 1  Sortilin protein levels are altered by glucose concentration in C2C12 myocytes.

(A) C2C12 myoblasts were cultured in low glucose (LG)-DMEM (5mM glucose) + 10% FBS and then switched to differentiation medium [DM: DMEM + 2% calf serum (CS)] containing 5mM (LG) or 22.5mM high glucose (HG). The DM was changed every 24h. Whole cell lysates were obtained daily until day 5 of differentiation. Total protein extracts (22.5μg/lane) were subjected to SDS-PAGE followed by western blot analysis using anti-sortilin antibodies (upper panel). (B) C2C12 myoblasts were differentiated into myotubes under LG-DM for 6 days. Then the media were switched to HG-DM, and myotubes were then further incubated for the indicated time (LG→HG). Conversely, they were differentiated into myotubes under HG-DM for 6 days, then media were switched to LG-DM (HG→LG). Cell lysates were subjected to Western blotting using indicated antibodies. (C, D, E) Densitometric analysis of the bands of sortilin (C), GGA2 (D), and M6PR (E) were performed from the results obtained in Fig. 2B. The amounts of sortilin protein are expressed relative to the densitometry unit of sortilin protein at the 0h in HG-DM. Significantly different from 0h in LG-DM or 0h in HG-DM (** p<0.01, *** p<0.001 or ### p<0.001, n=3, unpaired t-test). (F) C2C12 myoblasts were differentiated under LG-DM or HG-DM and cell lysates were fractionated into cytosol (C), high-density microsome (H), low-density microsome (L), plasma membrane (P), and nuclei/mitochondria (N/M) as described in Materials and Methods. Sortilin, Glut4, or Glut1 expression in each fraction was monitored by western blotting analysis. All experiments were repeated at least three times, and representative data are shown.
predominantly observed in H and low-density microsome (L) fractions and was increased by elevating glucose concentration (Fig. 1F, lane 2, 3, 7, and 8 of Glut1). In contrast, Glut1, which was observed in different molecular sizes on SDS-PAGE perhaps because of its glycosylation [21], was dramatically decreased in H and P fractions in the HG-DM condition (Fig. 1F, lane 2, 4, 7, and 9 of Glut1).

The series of experiments suggested that the sortilin induction was not only the resultant of differentiation, but also observed in differentiated myotubes where sortilin induction can be reversibly controlled by either increasing or decreasing glucose.

**Post-transcriptional regulation is important for glucose-dependent sortilin induction in C2C12 myotubes**

We next studied the molecular mechanisms controlling sortilin protein expression in C2C12 myotubes in response to extracellular glucose levels. Three possible mechanisms could be considered; (a) changes in mRNA level, (b) post-transcriptional regulation, and/or (c) changes in protein stability.

First, we measured sortilin mRNA levels during C2C12 cell differentiation (Fig. 2A). Sortilin mRNA gradually increased after inducing differentiation under both LG-DM and HG-DM conditions. On days 1 and 3 after inducing differentiation, sortilin gene expression was higher in LG-DM compared to in HG-DM (Fig. 2A, n=3, p=0.002, p=0.043, respectively); however, at day 5 after inducing differentiation, the difference had disappeared. Moreover, we found that changes in glucose concentration in differentiation medium did not alter sortilin gene expression, at least for 24 hours, in differentiated C2C12 myotubes (Fig. 2B). These results suggested that other system(s) were involved in sortilin protein induction by high glucose, rather than the changes in its gene expression.

Next, we measured the stability of sortilin protein by using the protein synthesis inhibitor, cycloheximide (CHX). C2C12 myoblasts were differentiated in HG-DM. At day 6 of differentiation, medium was changed from HG-DM to LG-DM with CHX, or from HG-DM to HG-DM with CHX and incubated for the indicated time (Fig. 2C). After addition of CHX, the half-life of sortilin protein was approximately 15h in both conditions. In addition, we also measured the stability of sortilin protein in the C2C12 myotubes differentiated in LG-DM followed by switching medium to LG-DM or HG-DM in the presence of CHX. The duration of degradation showed no difference under these conditions (Supplemental Fig. 1). From these results, the rate of degradation of sortilin protein was not affected by extracellular glucose level.

Thus, these results suggested that the sortilin protein level can be regulated at the post-transcriptional step. Indeed, the increased sortilin protein level after switching media, from LG-DM to HG-DM, was markedly reduced by the addition of mammalian target of rapamycin complex 1 (mTORC1) inhibitor, rapamycin (Fig. 2D). Although HG-DM-induced sortilin expression was not completely abolished by rapamycin, these results indicated that extracellular glucose regulates the sortilin protein levels through rapamycin-sensitive post-transcriptional regulation, at least in a part.

In skeletal muscle, AMP-activated protein kinase 1 (AMPK) is known as a major regulator of mTORC1 [22]. The glucose-restricted condition, such as glucose deprivation, can be sensed by AMPK [23], and the activation of AMPK down-regulates anabolic pathways by phosphorylating several target molecules including mTORC1 [24]. Indeed, we also confirmed whether the AMPK activity, assessed by its phosphorylation, was regulated by glucose in C2C12 myotubes. The phosphorylation of AMPK was greater under LG-DM compared to HG-DM, whereas the phosphorylation of S6K1, which reflects the activity of mTORC1, was reduced by LG-DM (Fig. 2E). We next analyzed if the amount of sortilin was changed by treating with an activator of AMPK, AICAR, in C2C12 myotubes. The expression of sortilin was decreased by administration of 0.5mM AICAR under HG-DM (Fig. 2F).

**Starvation reduced sortilin protein levels in rat gastrocnemius and EDL muscles**

In order to evaluate if glucose starvation alters sortilin protein amounts in rat skeletal muscle, rats were fed *ad libitum* (feeding group) or were starved for 17–18h (fasting group) and sortilin expression was measured in gastrocnemius, soleus and EDL muscle. Before executing the experiments, we monitored blood glucose levels in each group. The blood glucose levels in fasting group were significantly lower compared to those in the feeding group (Fig. 3A, ***p<0.001, n=8, unpaired t-test). These results suggested fasting rats for 17–18h was sufficient to induce the hypoglycemic condition as described previously [25]. After 17–18h fasting, the expression of sortilin was significantly decreased compared to that in the feeding group (Fig. 3A, ***p<0.001, n=8, unpaired t-test). These results suggested fasting rats for 17–18h was sufficient to induce a decreased sortilin expression.
Fig. 2 Changes in glucose availability modify post-transcriptional regulation of sortilin in C2C12 myocytes.

(A) C2C12 myoblasts were differentiated under LG-DM or HG-DM for 1, 3, and 5 days, and sortilin mRNA expression was evaluated with quantitative RT-PCR analysis. On the same day of starting differentiation, cell lysates were obtained as “Day 0”. The data are presented as means ± SEM for three samples per group normalized to Rn18s mRNA. Significant differences in gene expression between sortilin and Rn18s. * p<0.05, ** p<0.01, n=3, unpaired t-test, N.S.: no significant differences.

(B) C2C12 myoblasts were differentiated into myotubes under LG-DM or HG-DM for 6 days. Then the media were switched as indicated below the graph (from LG to LG, from LG to HG, from HG to LG, from HG to HG), and the cells were further incubated for 24h and sortilin gene expression was evaluated as described in Materials and methods.

(C) C2C12 myoblasts were differentiated into myotubes under HG-DM condition for 6 days. Culture medium was then switched to HG-DM or LG-DM with 30µM cycloheximide (CHX) for the indicated time. Cell lysates were subjected to Western blotting analysis using anti-sortilin antibody. The graph is representative of a total of three independent experiments and relevant readings at each individual time point were normalized to the densitometry units of actin (lower plot).

(D) C2C12 myoblasts were differentiated into myotubes under LG-DM or HG-DM condition for 6 days. Cultured media were switched to HG-DM or LG-DM in the presence of the indicated concentrations of rapamycin. Phosphorylated and total AMPK, phosphorylated S6K1, and mTOR were analyzed by western blotting analysis.

(E) C2C12 myoblasts were differentiated under LG-DM or HG-DM. Phosphorylated and total AMPK, phosphorylated S6K1, and mTOR were analyzed by western blotting analysis.

(F) C2C12 myoblasts were differentiated under HG-DM, followed by stimulating with 0.5mM AICAR for indicated times. Sortilin expression was measured by western blotting analysis. All experiments were repeated for at least three times, and similar results were obtained.
reduced in both gastrocnemius and EDL muscles [Fig. 3B, *p<0.05 (Gastrocnemius) and **p<0.01 (EDL), n=5, unpaired t-test]. In contrast, sortilin protein levels in soleus muscles were not affected by 17–18h of starvation (Fig. 3B).

We also tested if streptozotocin (STZ)-induced hyperglycemia also affects sortilin expression in skeletal muscles (Fig. 4). STZ-treated rats indeed lost weight and the blood glucose levels in these rats were significantly increased (Fig. 4A and 4B, *p<0.05, **p<0.01, ***p<0.001, n=8, one-way ANOVA for analyzing body weights, unpaired t-test for analyzing blood glucose levels). These results suggested STZ-treatment indeed produced hyperglycemic conditions in rats as reported previously [23]. However, sortilin protein levels in gastrocnemius, soleus and EDL muscles were not significantly changed by STZ-treatment (Fig. 4C, n=5, unpaired t-test, N.S.: no significant differences).

These results suggested that the hypoglycemic conditions rather than hyperglycemic conditions control sortilin levels.
The present study demonstrated that the environmental glucose levels directly control skeletal muscle sortilin levels both in vitro and in vivo. Particularly, the glucose-restricted condition appears to be a crucial determinant for sortilin levels in skeletal muscle.

As described, one of the physiological functions of sortilin is to control the incorporation of the extracellular glucose into skeletal muscle cells [6, 7]. We previously found that sortilin overexpression markedly decreased basal glucose uptake in C2C12 myocytes, which was associated with reduction in Glut1 protein levels [7]. As shown in Fig. 1F, most of the Glut1 protein localized in the plasma membrane and its expression was dramatically reduced by high glucose (Fig. 1B). Together, these data suggest the possibility that Glut1 down-regulation by high glucose is mediated by sortilin expression.

Decreased Glut1 expression was also crucial for acquiring the insulin-responsive Glut4 translocation system in C2C12 myocytes [7]. Hence, the reduction in sortilin under hypoglycemic conditions may have at least two distinct roles in controlling the glucose uptake.

**Discussion**

**A**, **B** Saline (control) or 70mg/kg STZ was administrated to male Wister rats as described in Materials and Methods and the body weight (everyday) and blood glucose levels (6 days after the administration) in each rat were monitored (*p<0.01, **p<0.001, ***p<0.0001, n=8, one-way ANOVA for analyzing body weights, unpaired t-test for analyzing blood glucose levels). **C** Total cell lysates were obtained from gastrocnemius, soleus or EDL muscles, and equal amounts of protein were subjected to SDS-PAGE followed by western blotting analysis using anti-sortilin antibody (N.S.: no significant differences, n=3).

**Fig. 4** Streptozotocin (STZ)-induced hyperglycemia did not alter sortilin protein levels in rat skeletal muscles.
transporter systems, increasing Glut1 protein to enhance basal glucose uptake levels and decreasing insulin-sensitivity by regulating GSV formations. Intriguingly, in contrast to skeletal muscle cells, the overexpression of sortilin in 3T3-L1 adipocytes did not alter the expression of glucose transporters, whereas, sortilin overexpression enhanced insulin-dependent Glut4 translocation [6]. In other words, the sortilin-dependent GSV regulation is a common mechanism in both skeletal muscle and adipocytes, whereas the sortilin-dependent expression control of Glut1 exists only in skeletal muscles, which may be an integrated system for giving preference to skeletal muscle cells over adipocytes for glucose usage during starvation.

As described, the expression of glucose transporters under hypoglycemic conditions is regulated, at least in part by sortilin. Similarly, suppression of myogenesis under hypoglycemic conditions appears to be mediated by sortilin. In addition, it has been shown that the glucose-restricted condition inhibits skeletal myoblast differentiation [18], and the overexpression of sortilin in C2C12 cells significantly stimulated myogenic differentiation [7]. These results together with the present findings suggest that sortilin is an important intermediate molecule for glucose-dependent regulation of myogenic differentiation.

Sortilin is also known to have a role of mediating lysosomal trafficking of prosaposin and acid sphingomyelinase [26]. Intriguingly, Tsuchiya et al. recently reported that palmitate induced sortilin down-regulation as well as impaired lysosomal motility in C2C12 myotubes [27]. Since nutrient starvation, which suppresses mTORC1 activity, also restricts lysosome motility mainly by inducing FLCN and Rab34 dependent perinuclear lysosome clustering [28], it could be speculated that the glucose-dependent sortilin regulation also plays a role on controlling lysosomal functions.

Our results suggested that the sortilin induction by high glucose was partially inhibited by rapamycin, which suggested mTORC1 controlled sortilin expression. mTORC1 consists of mTOR, mammalian lethal with Sec13 protein 8 (mLST8/GbL), regulatory-associated protein of mTOR (Raptor), proline-rich Akt substrate 40kDa (PRAS40), and DEP-domain containing mTOR interacting protein (DEPTOR) [29]. Two major downstream targets for mTORC1 are also well defined, p70 S6 kinase (S6K1) and 4E-BP1. Phosphorylation of S6K1 at Thr389 by mTORC1 is a trigger for its activation [29], and the activated S6K1 phosphorylate S6 ribosomal protein, subsequently enhances the translation of mRNAs that have 5′ terminal oligopyrimidine [30]. On the other hand, 4E-BP1 normally binds to eukaryotic translation initiation factor 4E (eIF4E) and inhibits eIF4E-dependent protein translation. mTORC1 activation by administering nutritional factors such as glucose or amino acids induces hyperphosphorylation of 4E-BP1, and the resultant free eIF4E initiates cap-dependent protein translation [31]. In addition to the glucose-dependent regulation, mTORC1 can be activated by amino acids [32]. Consistently, our preliminary data suggested that the amino acid administration into C2C12 myotubes also enhanced sortilin protein expression (data not shown).

Therefore, AMPK-mTORC1 axis appears to be an important system to control glucose-dependent sortilin regulation. Given that sortilin expression regulated skeletal muscle cell differentiation, it is consistent that the impairment of differentiation of myoblasts under hypoglycemic conditions is controlled by AMPK [33].

Our animal experiments clearly demonstrated that the expression control of sortilin under glucose-restricted conditions is indeed physiological. Interestingly, hypoglycemic conditions reduced sortilin levels in gastrocnemius and EDL muscles, whereas apparent effects of starvation were not observed in soleus muscle (Fig. 3B). Although all three muscles obtained from rats displayed insulin-dependent glucose uptake [34], a series of experiments suggested that the glucose-dependent regulation of sortilin appeared to differ among skeletal muscle types. EDL muscle has a fast-twitch characteristic and higher potential for producing ATPs anaerobically, presumably because of higher activities of glycolytic enzymes [35]. On the other hand, soleus muscle is predominantly composed of slow-twitch oxidative muscle fiber and has higher aerobic potential [35]. Gastrocnemius muscle is mixed fast and slow-twitch fiber type. Hence, the down-regulation of sortilin under hypoglycemic conditions appeared mainly in fast-twitch muscle fiber.

The progressive loss of muscle mass is often associated with aging, and defined as one of the symptoms of sarcopenia. In this age-associated sarcopenia, it has been demonstrated that mTORC1 signaling is negatively controlled in those atrophied skeletal muscle cells [36, 37]. As described, the reduction of glucose or amino acids can inactivate mTORC1 followed by decreased protein translation in skeletal muscle [30,
Because the small interference RNA-mediated suppression of endogenous sortilin inhibited C2C12 differentiation [7], it is indeed possible to speculate that the mTORC1-dependent sortilin regulation may contribute to the loss of skeletal muscle mass. Intriguingly, it has been reported that the age-dependent individual fiber atrophy is specifically observed in the fast-twitch fibers but not in the slow-twitch ones [38]. Although the impact of reduced sortilin protein levels in terminaly differentiated skeletal muscle atrophy is currently not yet clear; however, coincidently, our present study suggests the sortilin reduction under hypoglycemic conditions was apparently observed in gastrocnemius and EDL muscles that contain fast-twitch fibers. It should also be noted that differentiated C2C12 myotubes, which we utilized in the present study, predominantly express fast type myosin heavy chains [39]. To test the possibility whether sortilin regulation by glucose in vivo skeletal muscle was controlled by same mechanism, which we observed in C2C12 cells, is a future project in our laboratory. We observed that AMPK activity in EDL and gastrocnemius muscles tended to slightly increase by fasting (Supplemental Fig. 2); however, the variation in each group was quite large. We speculate this is because of the complexity of AMPK regulation in skeletal muscle, since it has been reported that AMPK activity was regulated not only by glucose but also by exercise or amino acids [40-44]. Indeed, our preliminary result suggested that sortilin protein expression was also induced by administering non-essential amino acids (data not shown). Together with the fact that serum amino acid concentration is affected by fasting in rats [45], we plan to analyze different animal models (i.e. tissue-specific knock out animals) to investigate if AMPK and/or mTORC1 activation directly engages sortilin gene and protein expression in skeletal muscle in future.

In conclusion, we demonstrate that the sortilin expression in skeletal muscle is tightly regulated by changes in extracellular glucose concentration through the mTORC1 pathway. Moreover, animal experiments suggest the expressionional reduction of sortilin mainly occurred under hypoglycemic conditions and also seems to be mainly observed in fast-twitch muscle fiber. In the case of hypoglycemia, the reduced sortilin protein levels can regulate glucose transport systems as well as suppression of skeletal muscle cell differentiation for coordinating glucose homeostasis in the whole body.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

Supplemental Fig. 1 Sortilin protein degradation rate was not affected by extracellular glucose level. C2C12 myoblasts were differentiated into myotubes in LG-DM for 6 days. Culture medium was then switched to LG-DM or HG-DM in the presence of 30µM cycloheximide (CHX) for the indicated time. Cell lysates were subjected to Western blotting analysis using anti-sortilin or anti-GAPDH. Densitometric analysis were performed for measuring sortilin amounts that were normalized by GAPDH amounts.
Supplemental Fig. 2 AMPK activity in EDL and gastrocnemius muscles were tended to slightly increase by fasting. (A, B) Skeletal muscles (gastrocnemius, soleus, and EDL muscles) were obtained from the rats divided into two groups; fed and fasted for overnight (A), and the rats treated with saline (control) or 70mg/kg STZ for 6 days (B). Homogenates from skeletal muscles were subjected to SDS-PAGE, followed by western blot analysis using antibodies against phosphorylated (Thr172) or total AMPK. Data are means ± SEM (*p<0.05, n=5/group, unpaired t-test).

References

1. Lefrancois S, Canuel M, Zeng J, Morales CR (2005) Inactivation of sortilin (a novel lysosomal sorting receptor) by dominant negative competition and RNA interference. Biol Proced Online 7: 17-25.
2. Nielsen MS, Madsen P, Christensen EI, Nykjaer A, Gliemann J, et al. (2001) The sortilin cytoplasmic tail conveys Golgi-endosome transport and binds the VHS domain of the GGA2 sorting protein. EMBO J 20: 2180-2190.
3. Shi J, Kandror KV (2007) The luminal Vps10p domain
of sortilin plays the predominant role in targeting to insulin-responsive Glut4-containing vesicles. *J Biol Chem* **282**: 9008-9016.

4. Huang G, Buckler-Pena D, Nauta T, Singh M, Asmar A, et al. (2013) Insulin responsiveness of glucose transporter 4 in 3T3-L1 cells depends on the presence of sortilin. *Mol Biol Cell* **19**: 3115-3122.

5. Shi J, Huang G, Kandror KV (2008) Self-assembly of Glut4 Storage Vesicles during Differentiation of 3T3-L1 Adipocytes. *J Biol Chem* **283**: 30311-30321.

6. Shi J, Kandror KV (2005) Sortilin is essential and sufficient for the formation of Glut4 storage vesicles in 3T3-L1 adipocytes. *Dev Cell* **9**: 99-108.

7. Ariga M, Nedachi T, Katagiri H, Kanzaki M (2008) Functional role of sortilin in myogenesis and development of insulin-responsive glucose transport system in C2C12 myocytes. *J Biol Chem* **283**: 10208-10220.

8. Nykjaer A, Lee R, Teng KK, Jansen P, Madsen P, et al. (2004) Sortilin is essential for proNGF-induced neuronal cell death. *Nature* **427**: 843-848.

9. Bronfman FC, Fainzilber M (2004) Multi-tasking by the p75 neurotrophin receptor: sortilin things out? *EMBO Rep* **5**: 867-871.

10. Pandey A, Chawla S, Guchhait P (2015) Type-2 diabetes: Current understanding and future perspectives. *IUBMB Life* **67**: 506-513.

11. Kurowski TG, Lin Y, Luo Z, Tsichlis PN, Buse MG, et al. (1999) Hyperglycemia inhibits insulin activation of Akt/protein kinase B but not phosphatidylinositol 3-kinase in rat skeletal muscle. *Diabetes* **48**: 658-663.

12. Nedachi T, Kanzaki M (2006) Regulation of glucose transporters by insulin and extracellular glucose in C2C12 myotubes. *Am J Physiol Endocrinol Metab* **291**: E816-824.

13. Krook A, Kawano Y, Song XM, Efendić S, Roth RA, et al. (1997) Improved glucose tolerance restores insulin-stimulated Akt kinase activity and glucose transport in skeletal muscle from diabetic Goto-Kakizaki rats. *Diabetes* **46**: 2110-2114.

14. Oku A, Nawano M, Ueta K, Fujita T, Umebayashi I, et al. (2001) Inhibitory effect of hyperglycemia on insulin-induced Akt/protein kinase B activation in skeletal muscle. *Am J Physiol Endocrinol Metab* **280**: E816-824.

15. McCall AL (2012) Insulin signaling and hypoglycemia. *Endocrinol Metab Clin North Am* **41**: 57-87.

16. Brun JF, Dumortier M, Fedou C, Mercier J (2001) Exercise hypoglycemia in non diabetic subjects. *Diabetes Metab* **27**: 92-106.

17. von der Cronen S, Deppe C, Barthel A, Sasson S, Joost HG, et al. (2000) Glucose deprivation induces Akt-dependent synthesis and incorporation of GLUT1, but not of GLUT4, into the plasma membrane of 3T3-L1 adipocytes. *Eur J Cell Biol* **79**: 943-949.

18. Nedachi T, Kadotani A, Ariga M, Katagiri H, Kanzaki M (2008) Ambient glucose levels qualify the potency of insulin myogenic actions by regulating SIRT1 and FoxO3a in C2C12 myocytes. *Am J Physiol Endocrinol Metab* **294**: E668-678.

19. Shibata H, Suzuki Y, Omata W, Tanaka S, Kojima I (1995) Dissection of GLUT4 recycling pathway into exocytosis and endocytosis in rat adipocytes. Evidence that GTP-binding proteins are involved in both processes. *J Biol Chem* **270**: 11489-11495.

20. McKeel DW, Jarett L (1970) Preparation and characterization of a plasma membrane fraction from isolated fat cells. *J Cell Biol* **44**: 417-432.

21. Mitsumoto Y, Klip A (1992) Development regulation of the subcellular distribution and glycosylation of GLUT1 and GLUT4 glucose transporters during myogenesis of L6 muscle cells. *J Biol Chem* **267**: 4957-4962.

22. Kahn BB, Alquier T, Carling D, Hardie DG (2005) AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab* **1**: 15-25.

23. Hardie DG (2011) Energy sensing by the AMP-activated protein kinase and its effects on muscle metabolism. *Proc Nutr Soc* **70**: 92-99.

24. Hardie DG, Scott JW, Pan DA, Hudson ER (2003) Management of cellular energy by the AMP-activated protein kinase system. *FEBS Lett* **546**: 113-120.

25. Wapnir RA, Lifshitz F (1977) Fasting-induced hypoglycemia in experimentally malnourished rats. *J Nutr* **107**: 383-390.

26. Braulke T, Bonifacino JS (2009) Sorting of lysosomal proteins. *Biochim Biophys Acta* **1793**: 605-614.

27. Tsuchiya Y, Hatakeyama H, Emoto N, Wagatsuma F, Matsushita S, et al. (2010) Palmitate-induced down-regulation of sortilin and impaired GLUT4 trafficking in C2C12 myotubes. *J Biol Chem* **285**: 34371-34381.

28. Starling GP1, Yip YY, Sanger A, Morton PE, Eden ER, et al. (2016) Folliculin directs the formation of a Rab34-RILP complex to control the nutrient-dependent dynamic distribution of lysosomes. *EMBO Rep* **17**: 823-841.

29. Thoreen CC, Chantranupong L, Keys HR, Wang T, Gray NS, et al. (2012) A unifying model for mTORC1-mediated regulation of mRNA translation. *Nature* **485**: 109-113.

30. Moschetta M, Reale A, Marasco C, Vacca A, Carratù MR (2014) Therapeutic targeting of the mTOR-signalling pathway in cancer: benefits and limitations. *Br J Pharmacol* **171**: 3801-3813.

31. Pause A, Belsham GJ, Ginqaras AC, Donzé O, Lin TA, et al. (1994) Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5′-cap function. *Nature* **371**: 762-767.

32. Dann SG, Thomas G (2006) The amino acid sensitive TOR pathway from yeast to mammals. *FEBS Lett* **580**: 2821-2829.
33. Palm W, Park Y, Wright K, Pavlova NN, Tuveson DA, et al. (2015) The Utilization of Extracellular Proteins as Nutrients Is Suppressed by mTORC1. Cell 162: 259-270.
34. Gaudreault N, Santuré M, Pitre M, Nadeau A, Marette A, et al. (2001) Effects of insulin on regional blood flow and glucose uptake in Wistar and Sprague-Dawley rats. Metabolism 50: 65-73.
35. Takekura H, Yoshioka T (1987) Determination of metabolic profiles on single muscle fibres of different types. J Muscle Res Cell Motil 8: 342-348.
36. Sandri M, Barberi L, Bijlsma AY, Blaauw B, Dyar KA, et al. (2013) Signalling pathways regulating muscle mass in ageing skeletal muscle: the role of the IGF1-Akt-mTOR-FoxO pathway. Biogerontology 14: 303-323.
37. Fry CS, Drummond MJ, Glynn EL, Dickinson JM, Gundermann DM, et al. (2011) Aging impairs contraction-induced human skeletal muscle mTORC1 signaling and protein synthesis. Skelet Muscle 1: 11.
38. Welle S (2002) Cellular and molecular basis of age-related sarcopenia. Can J Appl Physiol 27: 19-41.
39. Meissner JD, Umeda PK, Chang KC, Gros G, Scheibe RJ (2007) Activation of the beta myosin heavy chain promoter by MEF-2D, MyoD, p300, and the calcineurin/NFATc1 pathway. J Cell Physiol 211: 138-148.
40. Ai H, Ihlemann J, Hellsten Y, Lauritzen HP, Hardie DG, et al. (2002) Effect of fiber type and nutritional state on AICAR- and contraction-stimulated glucose transport in rat muscle. Am J Physiol Endocrinol Metab 282: E1291-1300.
41. Cantó C, Jiang LQ, Deshmukh AS, Mataki C, Coste A, et al. (2010) Interdependence of AMPK and SIRT1 for Metabolic Adaptation to Fasting and Exercise in Skeletal Muscle. Cell Metab 11: 213-219.
42. Frier BC, Jacobs RL, Wright DC (2011) Interactions between the consumption of a high-fat diet and fasting in the regulation of fatty acid oxidation enzyme gene expression: an evaluation of potential mechanisms. Am J Physiol Regul Integr Comp Physiol 300: R212-221.
43. Liu X, Yuan H, Niu Y, Niu W, Fu L (2012) The role of AMPK/mTOR/S6K1 signaling axis in mediating the physiological process of exercise-induced insulin sensitization in skeletal muscle of C57BL/6 mice. Biochim Biophys Acta 1822: 1716-1726.
44. Holecová M, Sisera L (2016) Effects of Arginine Supplementation on Amino Acid Profiles in Blood and Tissues in Fed and Overnight-Fasted Rats. Nutrients 8: 206-216.