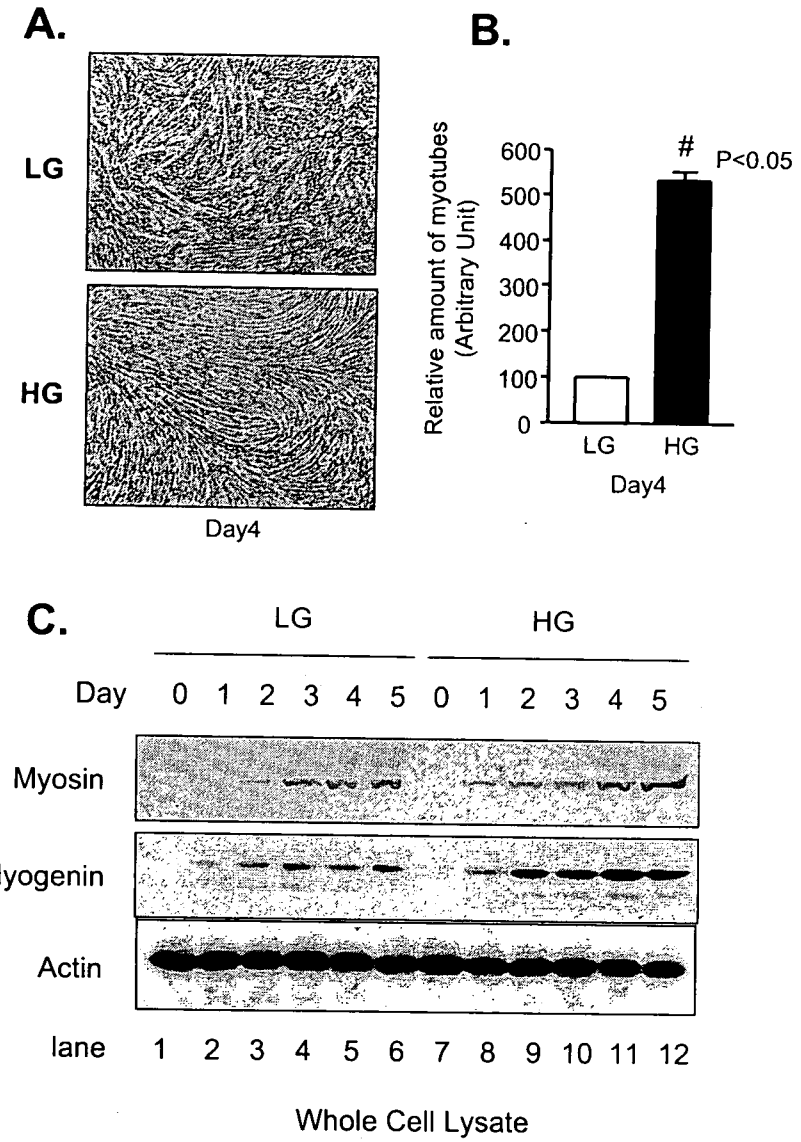
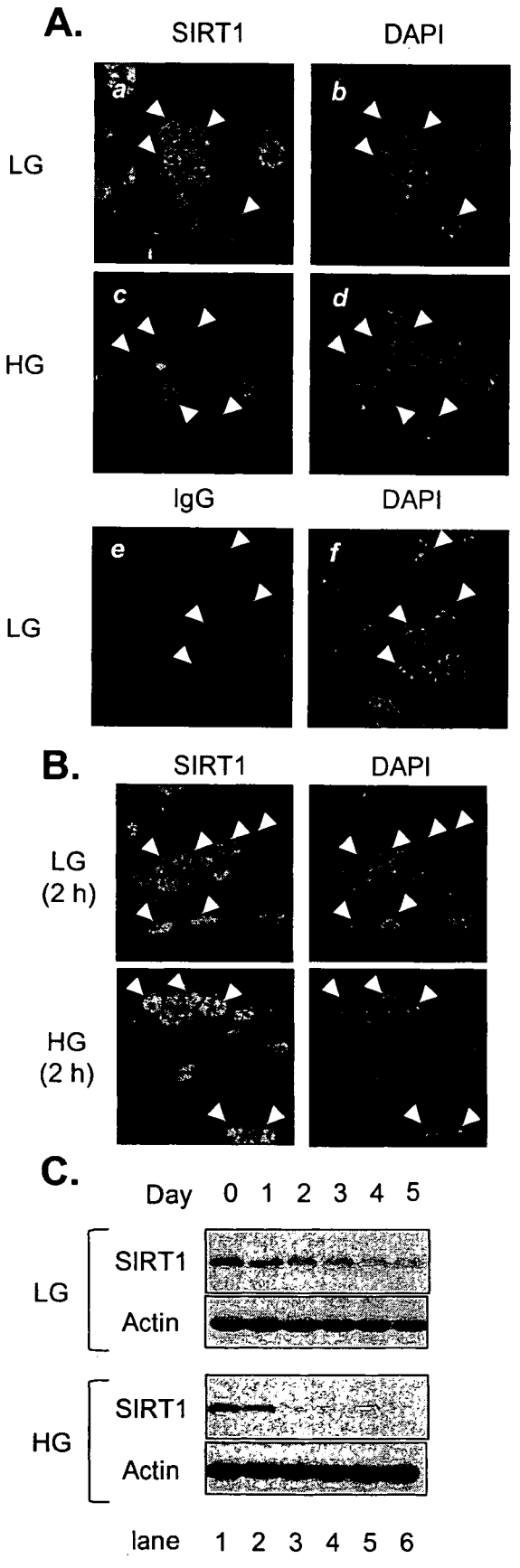
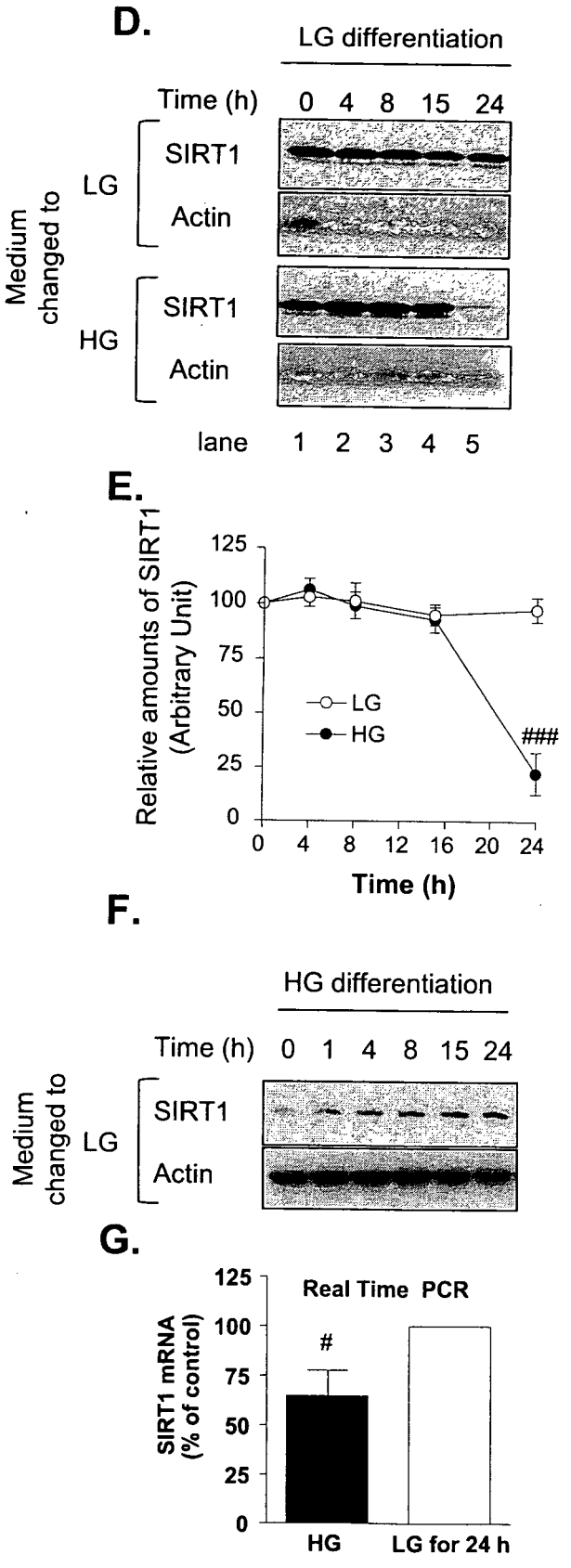


Nedachi et al. Fig. 1ABC



Nedachi et al.
Fig. 2ABC



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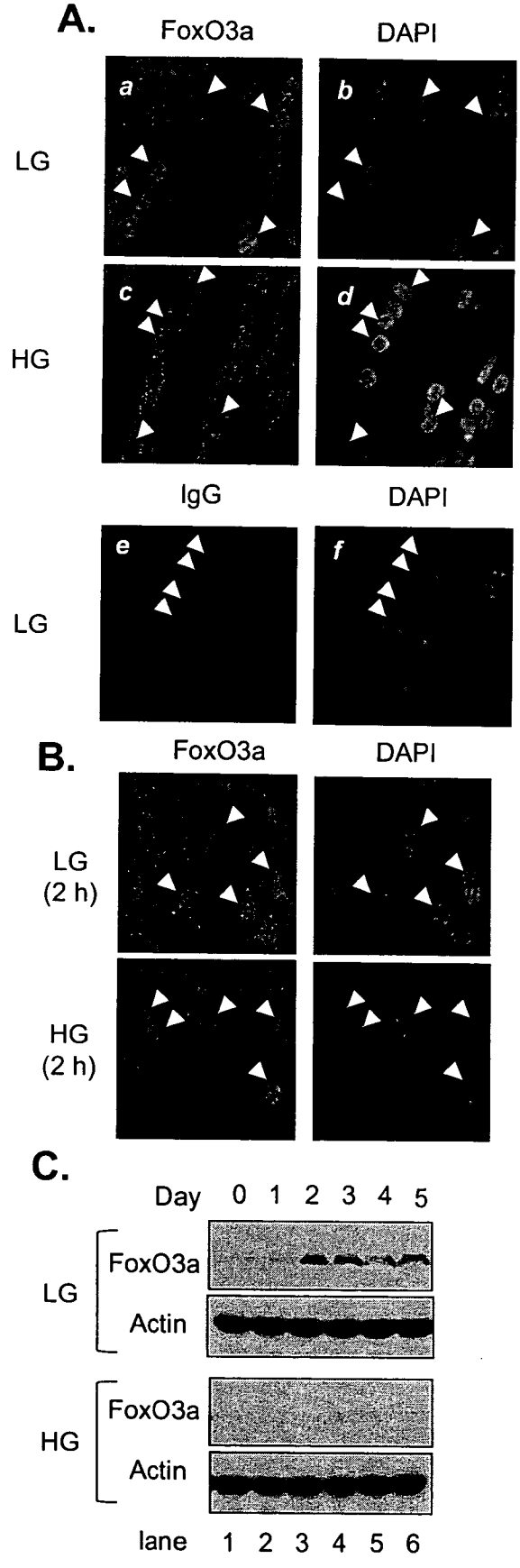

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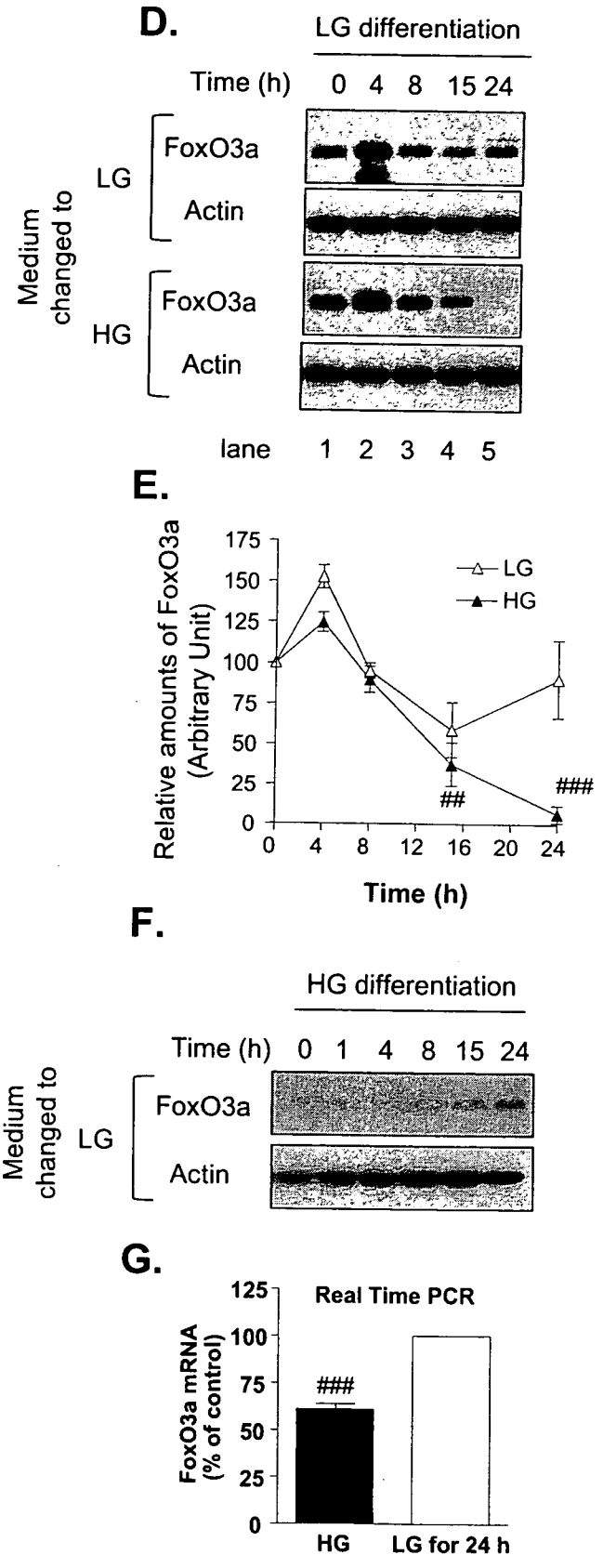

Nedachi et al.
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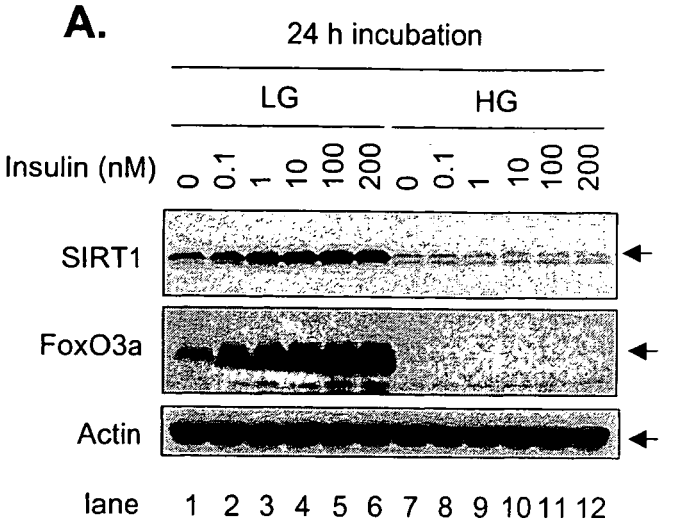

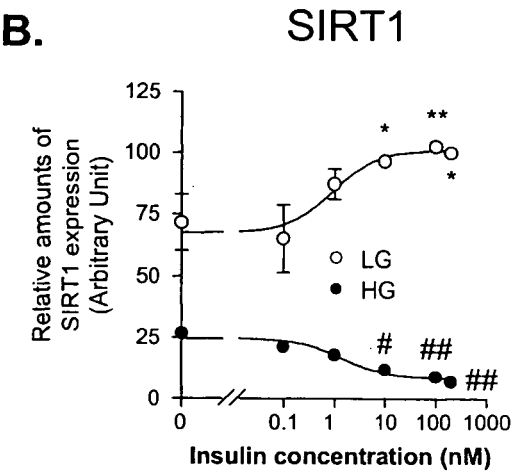

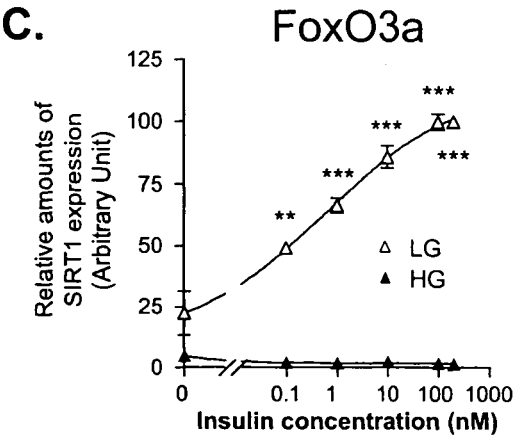

Nedachi et al.
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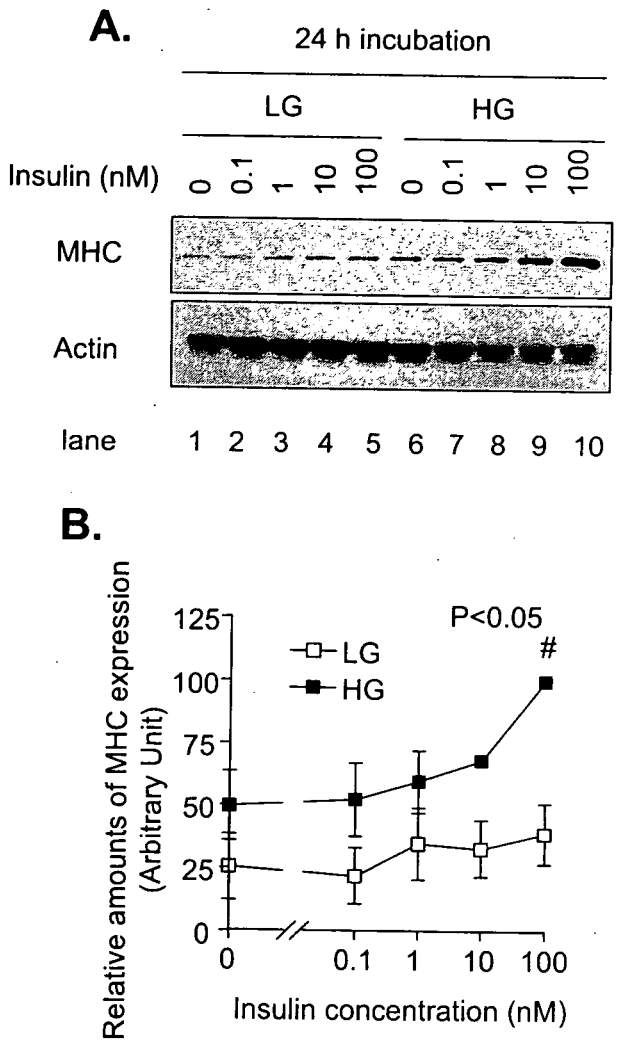

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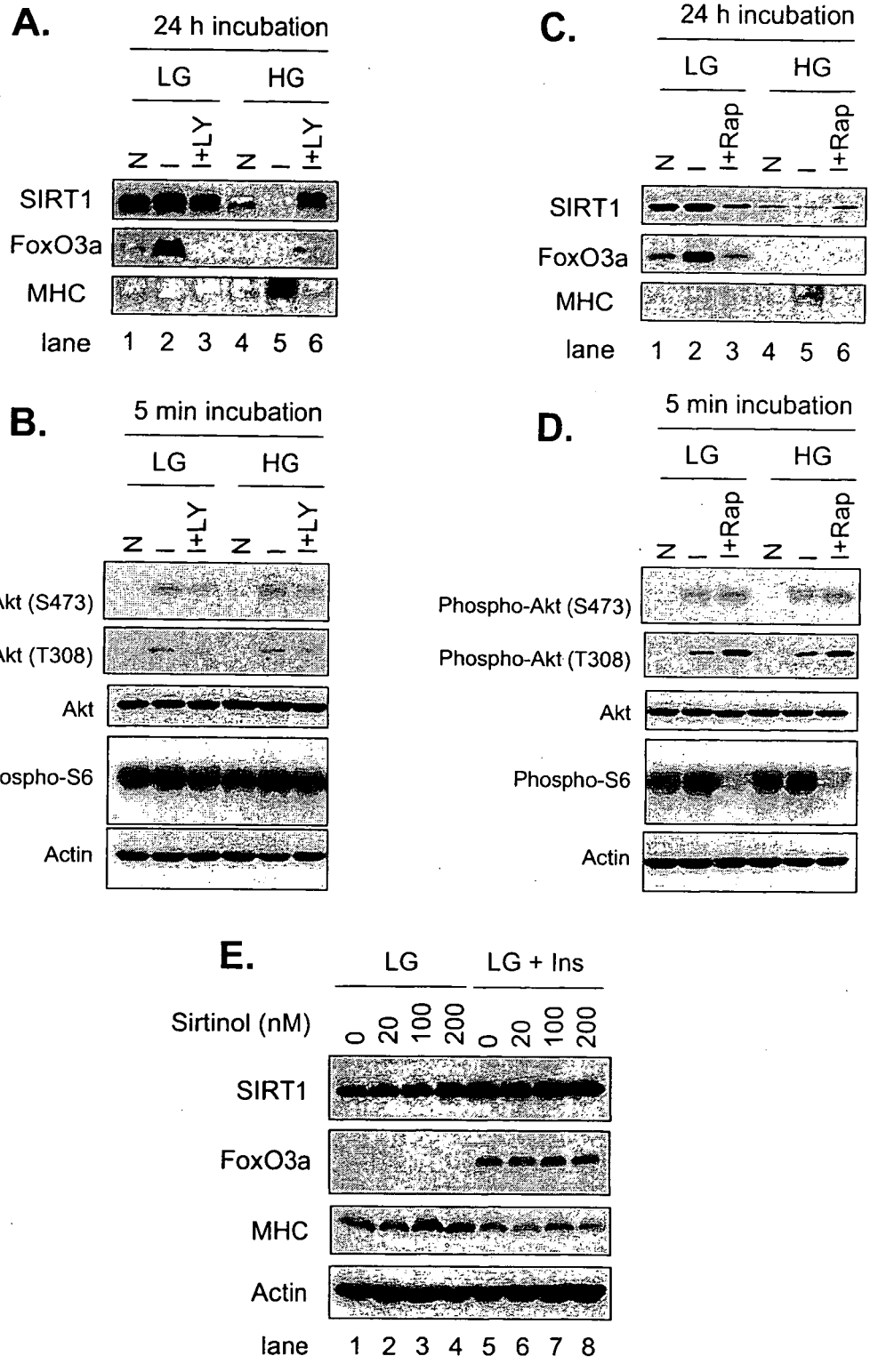

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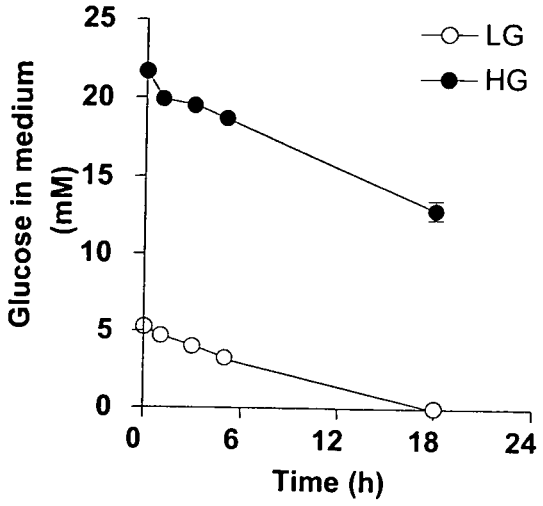

Nedachi et al.
Fig. 7

JBC Papers in Press. Published on February 7, 2008 as Manuscript M710604200
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M710604200

FUNCTIONAL ROLE OF SORTILIN IN MYOGENESIS AND DEVELOPMENT OF INSULIN-RESPONSIVE GLUCOSE TRANSPORT SYSTEM IN C2C12 MYOCYTES

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Running Title: Functional role of sortilin in skeletal muscle cells

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Sortilin has been implicated in the formation of insulin-responsive GLUT4 storage vesicles in adipocytes by regulating sorting events between the *trans*-Golgi-network and endosomes. We herein show that sortilin serves as a potent myogenic differentiation stimulator for C2C12 myocytes by cooperatively functioning with p75NTR, which subsequently further contributes to development of the insulin-responsive glucose transport system in C2C12 myotubes. Sortilin expression was up-regulated upon C2C12 differentiation, and over-expression of sortilin in C2C12 cells significantly stimulated myogenic differentiation, a response which was completely abolished by either anti-p75NTR- or anti-NGF-neutralizing antibodies. Importantly, siRNA-mediated suppression of endogenous sortilin significantly inhibited C2C12 differentiation, indicating the physiological significance of sortilin expression in the process of myogenesis. Although sortilin over-expression in C2C12 myotubes improved insulin-induced 2-deoxyglucose uptake, as previously reported, this effect apparently resulted from a decrease in the cellular content of GLUT1 and an increase in GLUT4 via differentiation-dependent alterations at both the gene transcriptional and the post-translational level. In addition, cellular contents of Ubc9 and SUMOylated proteins appeared to be increased by sortilin over-expression. Taken together, these data demonstrate that sortilin is involved not only in development of the insulin-responsive glucose transport system in myocytes, but is also directly involved in muscle differentiation via modulation of proNGF-p75NTR.

One of the major physiological roles of insulin is the control of postprandial blood glucose levels, and skeletal muscle is the primary tissue responsible for the bulk (70~80%) of insulin-stimulated postprandial glucose disposal (1,2). The effect of insulin on overall glucose disposal is primarily achieved via stimulation of glucose uptake into insulin-target tissues, and defects in this insulin action in skeletal muscle contribute to development of the insulin resistance which is characteristic of type 2 diabetes (3).

In skeletal muscle, glucose transport is regulated by a facilitative glucose transport system involving at least two members of the glucose transporter family, GLUT1 and GLUT4 (4), and their expression levels are strictly regulated during myocyte differentiation (5,6). GLUT1 is targeted predominantly to the sarcolemma (muscle plasma membrane) and is therefore implicated in the regulation of basal glucose transport, although a marked reduction in GLUT1 expression occurs during muscle differentiation (5,6). In contrast, expression of the insulin-responsive glucose transporter GLUT4 is remarkably up-regulated upon muscle differentiation, and GLUT4 protein is predominantly localized in intracellular tubulo-

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vesicular elements under basal conditions (7,8), while insulin stimulates the translocation of GLUT4 from intracellular storage compartments to the cell surface, resulting in the insulin responsive augmentation of glucose uptake (9). Thus, differentiation-dependent changes in the amount and composition of these GLUT proteins, especially the increase in cellular GLUT4 content, contributes to development of the insulin responsive glucose transport system in myocytes and adipocytes (5,10). However, GLUT4 expression is not the only factor responsible for conferring the insulin-stimulated glucose uptake function in these insulin-responsive cells, because ectopic expression of GLUT4 in other cell types such as fibroblasts is insufficient to generate an insulin-stimulated glucose transport system (11,12).

Recent studies using adipocytes have revealed that one of the additional factors prerequisite for higher insulin responsiveness is a signaling cascade developed during the course of adipocyte differentiation that is absent in pre-adipocytes (13,14). In addition to differentiation-dependent establishment of this signaling cascade, Kandror and colleagues recently reported that sortilin, a type I transmembrane glycoprotein, which is implicated in the sorting of Vps10p-interacting proteins between the *trans*-Golgi network (TGN), plasma membrane and lysosomes (15,16), serves as another important factor which promotes development of the insulin-induced glucose transport system by producing insulin responsive GLUT4 storage vesicles. They found that fibroblasts ectopically expressing both sortilin and GLUT4 display significantly augmented glucose uptake in response to insulin stimulation (12). Although it has already been reported that sortilin is expressed in muscle (17,18), at present the functional role of sortilin in muscle is entirely unknown, though muscle is the tissue in which the insulin effect on postprandial glucose disposal is quantitatively most important.

Sortilin was originally purified from human brain extracts using receptor-associated protein (RAP) affinity chromatography (17) and was also identified as a major component of GLUT4-containing vesicles from rat adipocytes (18,19). Sortilin has a Vps10p domain in its luminal region at the amino terminus and therefore belongs to the mammalian VPS10p family of sorting receptors. The intra-luminal domain of sortilin was shown to interact with a wide array of proteins including an unprocessed form of nerve growth factor (proNGF), neurotensin, lipoprotein lipase, precursor of brain-derived neurotrophic factor (BDNF), and prosaposin, as well as RAP (16,17,20-23). Therefore, sortilin functions not only to direct the intracellular movements of such newly synthesized interacting proteins but also functions as a receptor for these proteins once sortilin is exposed to the cell surface plasma membrane. Nevertheless, the cytoplasmic tail of sortilin does not possess a domain enabling it to directly activate the intracellular signaling cascade (24). Recent studies have, however, revealed that sortilin forms a complex with proNGF and p75NTR, a low affinity NGF receptor, and triggers p75NTR signaling cascades leading to various cellular responses including differentiation, survival and apoptosis (22,25). Interestingly, genes encoding precursors of NGF and BDNF, and their common low-affinity receptor p75NTR, were shown to be expressed in C2C12 myoblasts (26). In addition, several lines of evidence indicate that NGF affects myogenic differentiation and muscle development in an autocrine fashion via p75NTR (26-28).

In the present study, we explored the functional roles of sortilin in muscle by using the skeletal muscle cell line C2C12. We herein report sortilin to be a potent differentiation stimulator functioning cooperatively with p75NTR, which subsequently further contributes to development of the insulin responsive glucose transport system in C2C12 cells. We present compelling evidence that sortilin is involved not only in generating insulin-responsive GLUT4 storage vesicles, but also in elaborating an entire glucose transport system exhibiting much higher insulin responsiveness via regulation of the processes of myogenesis, including expressions of GLUT proteins and also perhaps various other proteins involved in the development of insulin responsiveness. Thus, our results provide new insights into the functional roles of sortilin in myogenesis and in development of the insulin-responsive glucose transport system in myocytes.

EXPERIMENTAL PROCEDURES

Materials - We obtained 2-deoxy-[^{3}H]-glucose (37.2 Ci/mmol) from PerkinElmer Life and Analytical Science, Inc. (Boston, MA, USA). 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 BioWest (Nuaille, France). Immobilon-P was from Millipore Corp. (Bedford, MA, USA). Bovine serum albumin (BSA) was purchased from Wako (Osaka, Japan). Antibodies against sortilin, p115, syntaxin 6, Ubc9 and SUMOylated protein 1 (GMP1) were purchased from BD Biosciences. Anti-sortilin antibody obtained from Abcam (Cambridge, UK) was used for immunofluorescent analyses. Antibodies against cation-independent mannose 6-phosphate/insulin-like growth factor-2 receptor (CI-M6PR), insulin receptor β-subunit and c-myc (9E10) were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Anti-myosin heavy chain (MHC) (MF20), anti-myogenin (F5D), anti-troponin T (CT3) and anti-titin (9D10) antibodies were obtained from Iowa Hybridoma Bank (University of Iowa, Iowa City, IA, USA). Anti-sarcomeric α-actinin (EA-53) and anti-β-actin (A-2066) antibodies were purchased from Sigma Chemicals. Anti-GLUT1 antibody was purchased from Chemicon International Inc. (Temecula, CA, USA). Anti-p75NTR antibody (ME20.4) was obtained from Abcam. Anti-GLUT4 antibody was a generous gift from Dr. H. Shibata (Gunma University, Maebashi, Japan) (29). Fluorescent conjugated secondary antibodies were obtained from Invitrogen (Carlsbad, CA, USA). Unless otherwise noted, all chemicals were of the purest grade available from Sigma Chemicals.

Cell Culture - A mouse skeletal muscle cell line, C2C12 myoblasts (30), was maintained in DMEM supplemented with 10% FBS, 30 mg/ml penicillin, and 100 mg/ml streptomycin (growth medium) at 37° C under a 5% CO_2 atmosphere. For biochemical study, cells were grown on 6 well plates (BD Biosciences) at a density of 3 x 10^4 cells/well in 3 ml of growth medium. Three days after plating, the cells had reached approximately 80-90% confluence (*Day 0*). Differentiation was then induced by switching the growth medium to DMEM supplemented with 2% CS, 30 μg/ml penicillin, and 100 μg/ml streptomycin (differentiation medium). The differentiation medium was changed every 24 hours. For the immunofluorescent staining study, cells were grown on 22-mm glass coverslips (Matsunami C022221, Osaka, Japan) in 6-well plates.

Generation of C2C12 cells expressing sortilin - Human sortilin cDNA was inserted into a pMXs retroviral vector, which is generated by modifying the pBABE retroviral vector (31). The pMXs-sortilin or pMXs (control) was transfected into Plat E cells using Fugene 6 transfection reagents (Roche Applied Science, Indianapolis, IN, USA), and high-titer retroviral supernatants were obtained. C2C12 myoblasts were infected with the generated retrovirus in growth medium containing 10 μg/ml of Polybrene. Two days after infection, positive selection was performed in the presence of 5 μg/ml of puromycin, and 20 individual clones were isolated. The sortilin expression level in each clone was analyzed by western blotting as described below.

Adenoviral expression of myc-GLUT4-ECFP in C2C12 cells - An adenoviral technique was used to express rat GLUT4, possessing the c-myc epitope tag in the first extracellular loop and enhanced cyan fluorescent protein (ECFP) at the carboxyl terminus. Briefly, established exofacial-myc-GLUT4-ECFP cDNA (32) was inserted into adenoviral vector pHMCA5, generously provided by Dr. Mizuguchi (33,34). The high titer viruses were produced by transfection of pHMCA5-myc-GLUT4-ECFP or empty vector into 293 cells using Fugene 6 transfection reagents (Roche). Viral stocks of 5~50 x 10^7 plaque-forming units (PFU)/ml were prepared in serum-free DMEM and frozen at -80°C. The next day the medium was changed, and incubation was continued for another 24 hours. Then, 2-deoxy[^{3}H]glucose and myc-antibody uptake assays were performed as described below. Expression of myc-GLUT4-ECFP was confirmed by western blotting and immunofluorescent staining.

Western blot analysis - The expression levels of each protein were analyzed by western blotting. In

brief, cells were lysed with NET buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 100 units/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml pepstatin A). The extracts were centrifuged at 13,000 x *g* for 10 min at 4°C to remove insoluble materials. The protein concentrations of supernatants were determined, and 3 x Laemmli's sample buffer was added to achieve a 1 x final concentration. From 50-100 μg of total protein were subjected to 7.5 ~ 10% SDS-polyacrylamide gel electrophoresis (1: 30 bis: acrylamide). Proteins were transferred to a PVDF membrane (Immobilon-P: Millipore Corp.), and the membranes were then blocked for 2 hours at room temperature with 5% BSA in TRIS buffered saline (TBS) containing 0.1% Tween 20. Immunoblotting to detect each protein was achieved with an overnight incubation at 4° C with 3% BSA/TBS containing either anti-sortilin antibody (1:1000), anti-myosin heavy chain (MHC) antibody (1:1000), anti-myogenin antibody (1:1000), anti-sarcomeric α-actinin antibody (1:1000), or anti-troponin T antibody (1:1000). Anti-β-actin (1:500) or anti-insulin receptor β-subunit (1:1000) antibodies were used as a loading control. Specific total proteins were visualized after subsequent incubation with a 1:5000 dilution of anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase and a SuperSignal Chemiluminescence detection procedure (Pierce). Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce). Three independent experiments were performed for each condition.

2-deoxy-glucose uptake assay - A 2-deoxy[^{3}H]glucose uptake assay was performed as previously described (35). Briefly, cells were starved in serum-free DMEM for 4 hours, and then washed with Krebs-Ringer phosphate HEPES buffer (KRPH buffer; 10 mM phosphate buffer, pH 7.4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 136 mM NaCl, 4.7 mM KCl, 10 mM HEPES (pH 7.6)). The serum-starved cells were incubated without or with 100 nM insulin for 60 min. in KRPH buffer and then chilled on ice. After washing with ice-cold KRPH buffer, glucose transport was determined by addition of 2-deoxy[^{3}H]glucose (PerkinElmer Life and Analytical Science, Inc.; Boston, MA, USA; 0.1 mM, 0.5 μCi/ml). After four minutes of incubation with the KRPH buffer containing 2-deoxy[^{3}H]glucose, the reaction was stopped by adding phosphate buffered saline (PBS) containing 10 μM cytochalasin B (Sigma), and the cells were washed three times with ice-cold PBS. The cells were then lysed in 0.2 N NaOH solution, and 2-deoxy[^{3}H]glucose uptake was assessed by scintillation counting. Twenty uM of cytochalasin B were added to the assay buffer for the measurement of nonspecific background. Specific uptake, i.e. the background subtracted from the total uptake, was obtained. The protein content was determined in each experiment with a BCA protein assay kit. Data are presented as picomoles of 2-deoxy[^{3}H]glucose per milligram protein per minute. For each experiment, at least three assays were performed under each condition, and each experiment was repeated at least three times.

Anti-myc antibody uptake assay - For the measurement of insulin-induced GLUT4 translocation, the C2C12 cells expressing myc-GLUT4-ECFP were starved in serum-free DMEM for 4 hours, washed three times with KRPH buffer, and then placed in a CO_2 incubator with 2 ml of KRPH buffer. Ten minutes after incubation, the cells were treated with or without 100 nM insulin in the presence of 4 μg/ml of the anti-myc antibody for 60 min. Next, the cells were washed three times with ice-cold PBS, harvested using 1 X Laemmli's sample buffer without bromo-phenol blue (BPB) and 2-mercaptoethanol (2-ME). After the protein concentration had been determined, BPB and 2-ME were added, denatured by incubation for 5min at 95°C, and subjected to western blotting using anti-mouse IgG antibody, anti-myc antibody, or anti-Sortilin antibody.

Immunofluorescence and image analysis - After experimental treatments, the cells were washed in PBS and fixed for 20 min in 2% paraformaldehyde (PFA)/PBS containing 0.1 % Triton X-100. The cells were washed, and then blocked in PBS containing 5% calf serum plus 1% BSA for 1 h at room temperature. Primary and secondary antibodies were used at 1:100 and 1:1000 dilutions, respectively (unless otherwise indicated), in 1% BSA/PBS, and samples were mounted on glass slides with Vectashield (Vector Laboratories, Burlingame, CA, USA). Cells were

imaged using a confocal fluorescence microscope (Olympus Fluoview FV-1000) with associated application program ASW Ver. 1.3 (Olympus, Tokyo, Japan). Images were then imported into Adobe Photoshop (Adobe Systems, Inc) for processing. Myogenin-positive cells were counted within a 500 μm x 500 μm area. At least five fields were counted for each sample under 20X microscopic magnification. For all microscopic analyses, at least three independent experiments were performed for each condition.

Subcellular fractionation of C2C12 cells - To detect endogenous GLUT1 and GLUT4, fractionation was performed according to the method of Tortorella and Pilch (36). Briefly, cells (three 150 mm plates for each sample) were washed with PBS three times, harvested with a cell scraper, suspended in 0.25 M sucrose, 20 mM Hepes–HCl (pH 7.4), 1 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride, then homogenized with a Teflon-glass homogenizer. The homogenate was centrifuged at 600 x *g* for 10 min at 4°C. The supernatant was centrifuged at 100,000 x *g* for 1 hour to collect the pellet for the total membrane fraction. For detection of GLUT1, the total membrane fraction was lysed in 100 μl of 1 X Laemmli's sample buffer, and subjected to western blotting using anti-GLUT1 antibody (1: 5840). For detection of GLUT4, the total membrane fraction was lysed in 100 μl of 8 M urea, 5% SDS, and 50mM Tris-HCl (pH 6.8). After the protein concentration had been determined as described above, dithiothreitol (DTT) and BPB were added to each sample (final concentrations; 343.6 μM and 0.005%, respectively). After incubation for 30 min at 37°C, samples were subjected to SDS-PAGE followed by immunoblotting using anti-GLUT4 antibody (1:500) (37).

Real-Time RT PCR - Total RNA was prepared using TRIzol reagent according to the manufacturer's instructions, and was quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription was carried out using the First Strand cDNA Synthesis kit for PCR from Roche. Real-time PCR reactions were performed using the Roche Light Cycler, utilizing Roche SYBR Green reagents according to the manufacturer's instructions. Amplification of PCR products was quantified during PCR by measuring fluorescence associated with binding of double-stranded DNA to the SYBR Green dye incorporated into the reaction mixture. The sequences of the oligonucleotides used to PCR-amplify the cDNAs of interest were: GLUT4 upper primer: 5'-TGCTCTCCTGCAGCTGATT-3', lower primer: 5'–TTCAGCTCAGCTAGTGCGTC-3'; GLUT1 upper primer: 5'-CTTCCTGCTCATCAATCGT-3', lower primer: 5'–AGCTCCAAGATGGTGACCTT-3', sortilin upper primer: 5'–AATGGTCGAGACTATGTTGTG-3', lower primer: 5'-CCGGTA CCCATTTGTTGT-3', Ubc9 upper primer: 5'-GGCACAATGAACCTGATGAAC-3', lower primer: 5'-TTGGTGGTGAGGACGGATAGT-3'. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified as a housekeeping gene by using: upper primer 5'-GGAGAAACCTGCCAAGTATGA-3', and lower primer 5'–GCATCGAAGGTGGAAGAGT-3'. Following an initial denaturation step of 95°C for one min, 30-45 cycles of 95°C for 10 seconds, 60°C for 1 second, and 72°C for 10 seconds were used for GLUT4, GLUT1, sortilin and Ubc9. For GAPDH, an initial denaturation step of 95°C for 10 minutes, followed by 45 cycles of 95°C for 10 seconds, 57.5°C for 15 seconds, and 72°C for 15 seconds was used.

siRNA-mediated reduction of sortilin in C2C12 cells - The siRNA species purchased from Nippon EGT Co. Ltd. (Toyama, Japan) were designed to target the following cDNA sequences: scrambled, 5'-AGGGUGGGUUUGGCCAAAATT-3'; and sortilin siRNA-1, 5'-GGUGGUGUUAACAGCAGAGTT-3'; sortilin siRNA-2, 5'- CCAUUGGUGUGAAAAUCUA -3', and sortilin siRNA-3, 5'-GGACCACAUUACUAUACCA-3'. Scramble and Sortilin siRNA-1 were modified from the reference (38), and the sequences of Sortilin siRNA-2 and -3 were provided by NIPPON EGT Co. Ltd. (Toyama, Japan). Two hundred nmol of sortilin siRNA, or scrambled siRNA species, were introduced into C2C12 myoblasts using Oligofectamine (Invitrogen). Ninety-six hours after transfection (day 3 of differentiation), cells

were harvested with NET buffer followed by SDS-PAGE and immunoblotting using anti-sortilin antibody, anti-myogenin antibody or anti-β-actin antibody (as a control).

Measurement of GLUT stability – Adenoviral infected WT- and sort10-C2C12 cells expressing myc-GLUT4-ECFP were treated with 10 μg/ml of cycloheximide and the cells were then harvested with NET buffer at the indicated time intervals. Cell lysates were subjected to SDS-PAGE, followed by western blotting analysis using anti-GLUT1 or anti-myc antibody.

Statistical analysis - Results are expressed as means ± S.E.M., and the data were analyzed by ANOVA followed by Student's *t*-test. Differences were considered to be significant at * $P < 0.05$., ** $P < 0.01$., and *** $P < 0.001$.

RESULTS

Sortilin expression during differentiation of C2C12 cells - To investigate the physiological role of sortilin in C2C12 myocytes, we first determined changes in expression levels of sortilin during myogenesis of C2C12 cells by Western blotting (Fig. 1A, *upper panel*). Sortilin was undetectable in undifferentiated C2C12 myoblasts (*Day 0*), but was observed as a ~100 kDa protein at *Day 2* of differentiation when these myoblasts were differentiated in a differentiation medium containing 5 mM glucose. The amount of sortilin then gradually increased until *Day 5*, and remained unchanged up to *Day 8*. As a control for myogenic differentiation, expression levels of myogenin (*middle panel*) and MHC (*lower panel*) were also determined. Immunofluorescent staining demonstrated sortilin containing endosomes to be located throughout the cytoplasm. Thus, co-localizations with GLUT4 (*panels a* and *b, red*), CI-M6PR (*panels c* and *d, red*) and Syntaxin 6 (*panels e* and *f, red*) were obvious, though the sortilin-positive endosomes were apparently devoid of p115 (*panels g* and *h, red*), a *cis*-Golgi marker in differentiated C2C12 myotubes.

Expression of sortilin induces spontaneous myogenic differentiation of C2C12 cells - In order to explore the functional roles of sortilin in C2C12 myocytes, we established C2C12 cells in which sortilin is stably expressed using a retroviral technique. In the process of isolating positive clones, we unexpectedly found that over-expression of sortilin in C2C12 myoblasts often resulted in spontaneous myotubular formation even in growth medium containing 10% FBS (data not shown). Twenty clones of drug-resistant C2C12 cells were isolated, nine of which stably expressed sortilin as assessed by western blotting analysis. All 9 clones expressing detectable levels of exogenous sortilin displayed the myotubular phenotype once they reached confluence. Three independent clones expressing sortilin, two control clones expressing empty vector and parental wild type cells were further analyzed. As shown in Fig. 2, in growth medium, neither wild type C2C12 (WT-C2C12) nor the control clone expressing empty vector expressed sortilin (Fig.2A, *right panel, lanes 1* and *2*). Furthermore, neither displayed a myotubular phenotype even after reaching confluence (Fig. 2A, *panels a* and *b*). In sharp contrast, C2C12 clones expressing exogenous sortilin (Sort10- and Sort15-C2C12) (Fig.2, *right panel, lanes 3* and *4*) underwent spontaneous differentiation as evidenced by myotube formation (Fig. 2A, *left panels, panels c* and *d*). Immunofluorescent analysis revealed that even before differentiation began Sort10-C2C12 cells already expressed various muscle differentiation markers including myogenin (Fig. 2B, *panel d*), MHC (Fig.2, *panel e*), and titin (Fig.2, *panel f*), observations not made in either WT-C2C12 cells (Fig 2B, *panels a~c*) or empty vector-expressing C2C12 cells (Fig.2B, *panels d-f*). Essentially the same results were obtained in the other clones over-expressing sortilin, but not in other control C2C12 clone obtained by infection with empty retrovirus lacking an insert, and levels of sortilin protein in the control cells were not significantly different from those observed in the non-infected C2C12 myocytes (data not shown).

This stimulatory effect of sortilin over-expression on myogenesis was further confirmed by Western blot analysis using antibodies against various muscle differentiation marker proteins including MHC, sarcomeric α-actinin, troponin T and myogenin (Fig. 2C). Even at *Day 0*, Sort10-C2C12 cells already showed considerable elevations of all muscle differentiation marker proteins tested (*lane 2*), observations not made in WT-C2C12 cells (*lane 1*). At *Day 4* of

differentiation, all of these differentiation marker proteins were also detected in WT-C2C12 cells, although their expression levels were obviously lower in these (*lane 3*) than in Sort10-C2C12 cells (*lane 4*). At *Day 7* of differentiation, most of these expressions reached comparable levels in WT-C2C12 (*lane 5*) and Sort10-C2C12 (*lane 6*) cells. Together, these data clearly demonstrate that sortilin over-expression in C2C12 myoblasts results in spontaneous myogenic differentiation once confluence is reached even under growth stimulating conditions.

Involvement of the proNGF-p75NTR autocrine loop in the spontaneous differentiation of sortilin-over-expressing C2C12 cells - Since recent studies have revealed direct interactions among sortilin, p75NTR and proNGF exerting biological effects in neuronal cells (22,25), we examined the possible involvement of proNGF and p75NTR, the existence of which in C2C12 cells was previously reported (26,27), in the process of spontaneous differentiation induced by sortilin over-expression. To address this question, Sort10-C2C12 cells were cultured in the presence or absence of a p75NTR-neutralizing antibody that blocks p75NTR-mediated biological responses (39). The p75NTR-neutralizing antibody significantly reduced the number of Sort10-C2C12 myocytes expressing myogenin in the growth medium, whereas control mouse IgG had no such effect (Fig. 3A, *left panel*). In addition, essentially the same result was obtained when an NGF-neutralizing antibody that blocks both NGF and proNGF action was added to the culture instead of the p75NTR-neutralizing antibody (Fig. 3A, *right panel*). We also found the anti-p75NTR antibody to be very efficiently endocytosed only in C2C12 cells expressing sortilin (Fig.3B, *panel d, sortilin; green, mouse IgG; red*), not in control cells expressing empty vector (Fig.3B, *panel b*). No control mouse IgG was incorporated into C2C12 cells, regardless of sortilin expression. during the incubation (Fig.3B, *panels a* and *c*). This observation suggests that sortilin overexpression may facilitate complex formation with p75NTR, leading to efficient incorporation of the anti-p75NTR antibody. These data indicate that the stimulatory effect of sortilin over-expression on myogenesis is dependent on the existence of functional p75NTR that has an ability to form the high-affinity proNGF receptor with sortilin.

Physiological significance of endogenous sortilin expression in myogenic differentiation of C2C12 cells - Since sortilin expression was markedly up-regulated during C2C12 differentiation (Fig. 1), we examined the physiological significance of sortilin expression in the process of myogenic differentiation using the siRNA-mediated gene silencing technique to decrease the expression of endogenous sortilin (Fig. 3C). Three of the sortilin sequence-specific siRNA oligos (Oligo #1, #2 and #3) significantly reduced endogenous sortilin expression at *Day 2* of C2C12 differentiation (Fig. 3C, *upper panel, lanes 1, 2* and *3*), resulting in a concomitant suppression of myogenin expression (*middle panel, lanes 1, 2* and *3*), whereas a control scramble oligo had no effect on the expression of either sortilin or myogenin (*lane 4*). β-actin, used as a loading control, was unaffected by siRNA introduction (*lower panel*). In addition, consistent with the sortilin over-expression experiments (Fig. 3A), either p75NTR- or NGF-neutralizing antibody also significantly inhibited the conventional differentiation process of wild type C2C12 cells in a low-serum differentiation medium (Fig. 3D). These data indicate that endogenous sortilin up-regulated during myogenesis is directly involved in the myogenic differentiation of C2C12 cells, a process possibly mediated through the proNGF-p75NTR autocrine loop.

Involvement of ROCK in the spontaneous differentiation of sortilin-overexpressing C2C12 cells – Since the p75NTR has been shown to activate the small G-protein Rho and its downstream effector ROCK in various cell types (40,41), we examined whether this signaling cascade is involved in the spontaneous differentiation of sortilin-overexpressing C2C12 cells (Fig. 3E). The myogenin expression observed in sortilin-overexpressing C2C12 cells at *Day 0* was diminished when the cells were cultured for 24 h in the presence of an inhibitor of ROCK, Y27632 (Fig. 3E, *lower panel*).

Over-expression of sortilin contributes to development of an insulin-induced glucose

transport system in C2C12 cells - To assess whether an insulin responsive glucose transport system develops in skeletal muscle C2C12 cells under the same conditions as previously reported (12), WT- and Sort10-C2C12 myoblasts expressing myc-GLUT4-ECFP were obtained using an adenoviral vector, and 2-deoxy[^{3}H]glucose (Fig. 4) and Myc Ab (Fig. 5) uptake assays (35) were then performed. Since Sort10-C2C12 cells tend to undergo spontaneous myotubular formation, we carried out the assay before they reached confluence (*Day -1*). Nevertheless, some differentiation markers were already being expressed at this time point (data not shown). Under the conventional 2-deoxy[^{3}H]glucose uptake assay protocol (42), insulin-induced augmentation of 2-deoxy[^{3}H]glucose uptake was not observed in either WT-C2C12 myoblasts (Fig. 4A, WT) or WT-C2C12 myoblasts expressing exogenous myc-GLUT4-ECFP (Fig. 4A, WT+G4). Intriguingly, in Sort10-C2C12 myoblasts, basal 2-deoxy[^{3}H]glucose uptake was remarkably decreased, by approximately ~25 % (2.89 +/- 0.2 pmol/min./mg protein) as compared with that of WT-C2C12 myoblasts, while Sort10-C2C12 myoblasts failed to display any insulin-responsiveness probably due to negligible expression of GLUT4 (Fig. 4A, Sort). However, consistent with a previous study using 3T3 fibroblasts (12), a slight but significant insulin-stimulated 2-deoxy[^{3}H]glucose uptake (approximately 1.5 –fold) was observed in Sort10-C2C12 myoblasts expressing myc-GLUT4-ECFP (Fig. 4A, Sort+G4). This occurred concurrently with a small increase in basal 2-deoxy[^{3}H]glucose uptake.

Effects of sortilin over-expression on insulin-induced glucose uptake were also examined in differentiated C2C12 myotubes (*Day 5~6*) (Fig. 4B). Since differentiated C2C12 myotubes contained massive amounts of various muscle proteins such as skeletal muscle type myosins and α-actin, a normalization by total protein contents for comparing glucose uptake between myoblasts and myotubes may not directly reflect the actual capacity for glucose transport into individual cells. However, it should be noted that the net amount of 2-deoxy[^{3}H]glucose uptake was remarkably low (1.26 +/- 0.1 pmol/min./mg protein) in differentiated C2C12 myotubes, being nearly ten fold lower than that in undifferentiated C2C12 myoblasts (Fig. 4AB). Consistent with our previous report (35), insulin induced 2-deoxy[^{3}H]glucose uptake was marginal even in differentiated C2C12 myotubes (Fig. 4B, WT) under the conventional uptake assay protocol. The adenovirus-mediated expression of myc-GLUT4-ECFP raised 2-deoxy[^{3}H]glucose uptake under both basal and insulin-stimulated conditions, and insulin-responsiveness was slightly improved in differentiated WT-C2C12 myotubes (Fig.4B, WT+G4). Consistent with the result obtained in undifferentiated myoblasts expressing sortilin (Fig. 4A, Sort), differentiated Sort10-C2C12 myotubes showed a further reduction in net 2-deoxy[^{3}H]glucose uptake (Fig. 4B, Sort). Because of this suppression of basal glucose uptake, a slight but significant augmentation of insulin-induced 2-dexoy[^{3}H]glucose uptake was subsequently seen even without exogenous expression of myc-GLUT4-ECFP (Fig. 4B, Sort), presumably reflecting glucose uptake mediated through endogenous GLUT4 translocation in differentiated Sort10-C2C12 myotubes. The adenovirus-mediated expression of myc-GLUT4-ECFP in Sort10-C2C12 myotubes further enhanced insulin-responsiveness (2.1 fold increase in response to insulin), and this was concurrent with a slight increase in basal 2-deoxy[^{3}H]glucose uptake (Fig. 4B, Sort+G4).

Effect of sortilin over-expression on insulin-induced GLUT4 translocation in C2C12 cells - To confirm that insulin-induced 2-deoxy[^{3}H]glucose uptake was in fact achieved through the translocation of GLUT4 to the plasma membrane, the amount of myc-GLUT4-ECFP exposed to the cell surface during insulin stimulation was assessed using an anti-myc antibody (Myc Ab) uptake assay (Fig. 5) (35). The serum-starved WT- or Sort10-C2C12 myotubes expressing myc-GLUT4-ECFP (*Days 5~6*) were incubated for 1 hour in KRPH buffer containing 4 μg/ml of Myc Ab in the absence (Fig. 5, *lanes 1* and *3*) or presence (*lanes 2* and *4*) of insulin (100 nM) and whole cell lysates were then subjected to SDS-PAGE followed by Western blotting using antibodies against anti-mouse IgG for detecting Myc Ab uptake and anti-BD-living colors for detecting the total amount of myc-GLUT4-ECFP expressed. Consistent with our

previous report (35), a small but significant increase in the uptake of Myc Ab was observed with insulin stimulation in WT-C2C12 myotubes expressing myc-GLUT4-ECFP (Fig. 5, *upper panel, lanes 1* and *2*). Importantly, insulin-responsive GLUT4 translocation as assessed by the Myc Ab uptake assay appears to be significantly improved in Sort10-C2C12 myotubes (Fig. 5, *lane 4*), despite marked suppression of net 2-deoxy[^{3}H]glucose uptake in comparison with WT-C2C12 myotubes (Fig. 4B). Together, these data clearly demonstrate that sortilin over-expression improves insulin-responsive glucose transport by increasing insulin-responsive GLUT4 translocation in C2C12 myotubes. Our data also indicate the importance of decreased basal glucose uptake in the emergence of markedly increased insulin responsive glucose uptake.

Effects of sortilin over-expression on expressions of GLUT1 and GLUT4 in C2C12 cells - Since sortilin over-expression markedly decreased basal glucose uptake (Fig. 5) and also appeared to stimulate C2C12 differentiation (Fig. 1 and 2), we examined protein and mRNA expressions of endogenous GLUT1 and GLUT4 in WT- and Sort10-C2C12 cells during myogenesis (Fig. 6). Western blot analysis demonstrated that GLUT1 protein decreased remarkably during differentiation of C2C12 cells (Fig. 6A, *left panel, lanes 1, 3 and 5*). Consistent with our finding that sortilin over-expression suppresses basal glucose uptake (Fig. 4), an obvious reduction of GLUT1 protein was observed in Sort10-C2C12 myoblasts (Fig. 6A, *left panel, lane 2*) as compared with that in WT-C2C12 myoblasts (*lane 1*). Upon differentiation, Sort10-C2C12 myotubes displayed marginal levels of GLUT1 protein (Fig. 6A, *left panel, lanes 6 and 7*), resulting in lower basal 2-deoxy[^{3}H]glucose uptake than that observed in WT-C2C12 myotubes (Fig. 4B). Real time PCR analysis using a Lightcycler (Roche) revealed that GLUT1 mRNA expression was significantly decreased in Sort10-C2C12 cells as compared with WT-C2C12 cells even at *Day 0* (Fig. 6B). Upon differentiation, expression of GLUT1 mRNA reached minimum levels, and was present in comparable amounts in WT- and Sort10-C2C12 myotubes on *Day 4* and *Day 7* (Fig. 6B). We have also confirmed that sortilin over-expression resulted in increased expression of GLUT4 as assessed by both western blotting (Fig. 6A, *right panel*) and real-time PCR analysis (Fig. 6B, *right graph*). Since decreased GLUT1 and increased GLUT4 expression are important criteria for myogenesis (43,44), these data further confirm that sortilin functions as a potent differentiation stimulator in C2C12 cells. It is also noteworthy that despite the similar expression levels of GLUT1 mRNA in differentiated WT- and Sort10-C2C12 myotubes (Fig. 6B, *Day 4* and *Day 7*), cellular contents of GLUT1 protein were significantly reduced in Sort10-C2C12 (Fig. 6A, *lanes 4* and *6*) in comparison with WT-C2C12 (Fig. 6A, *lanes 3* and *5*) myotubes.

To assess whether sortilin overexpression alters the stability of GLUT proteins, we next examined time-dependent changes in cellular contents of GLUT1 and GLUT4 in the presence of cycloheximide (Fig. 6C). Consistent with a previous report (12), GLUT4 protein displayed greater stability in sortilin-overexpressing C2C12 myoblasts (*right panels*). On the other hand, we found that GLUT1 tended to be less stable in sortilin-overexpressing cells (*left panel*). Taken together, these data indicate ~~suggest~~ that, in addition to the translational regulation, post-transcriptional regulation of GLUT1 protein is also altered by sortilin over-expression.

Increased Ubc9 expression in Sort10-C2C12 cells - Cellular contents of GLUT1 and GLUT4 are reportedly regulated post-transcriptionally by exogenous Ubc9 expression in the L6 skeletal muscle cell line (45). We therefore examined expression levels of Ubc9 by western blotting (Fig. 7A) and real-time PCR analysis (Fig. 7B) in WT- and Sort10-C2C12 cells during myogenesis. We found that the amount of Ubc9 protein was significantly increased in Sort10-C2C12 cells (Fig. 7A, *lanes 2, 4* and *6*) as compared with WT-C2C12 cells (*lanes 1, 3* and *5*) at all differentiation time points examined. Consistent with this, a slight increase in Ubc9 mRNA expression was also observed in Sort10-C2C12 cells (Fig. 7B, *closed bars*) as compared with WT-C2C12 cells (*open bars*), while expression levels of Ubc9 mRNA decreased upon differentiation in both WT- and Sort10-C2C12 cells. As a result of the increased Ubc9 in Sort10-

C2C12 cells, SUMOylated proteins were also increased (Fig. 7A, *right panel, lanes 2, 4* and *6*).

DISCUSSION

While substantial progress has been made in our understanding of the insulin-induced GLUT4 translocation process in adipocytes, achieved mainly using the excellent 3T3L1 adipogenic cell culture model (46), much less information is available about the mechanistic details involved in this event in skeletal muscle, the tissue in which the effect of insulin on glucose disposal is quantitatively most important. Recently, we have reported that, much like the L6 skeletal muscle cell line, a C2C12 myogenic cell line derived from mouse skeletal muscle possesses the basic machinery required for GLUT4 translocation in response to insulin stimulation (43,47). However, the insulin-induced glucose uptake achieved by this GLUT4 translocation is masked by relatively high basal glucose transport activity, presumably mediated through GLUT1 (35). Since it would be highly desirable to establish cell culture models of skeletal muscle that clearly and accurately reflect muscle glucose disposal *in vivo*, we have attempted to further characterize the molecular details underlying development of the insulin-induced glucose transport system in these C2C12 cells. We therefore explored the functional roles of sortilin, a sorting receptor implicated in the formation of insulin-responsive GLUT4 vesicles in adipocytes (12,48), by using C2C12 myocytes.

A key finding of the present studies is that sortilin functions as a potent differentiation regulator for C2C12 skeletal muscle cells, at least in part by modulating the p75NTR-proNGF autocrine loop (Fig. 2 and 3). As previously demonstrated in adipocytes (12), sortilin over-expression apparently improves the insulin-induced glucose uptake in C2C12 myocytes (Fig. 4 and 5), a novel observation which, however, reveals that this effect of sortilin on the development of insulin responsiveness in C2C12 cells reflects a combination of consequences of its myogenic stimulatory action. Indeed, sortilin over-expression significantly stimulates C2C12 differentiation (Fig. 2) and thereby raises the cellular content of GLUT4 and decreases that of GLUT1 by altering both transcriptional and post-transcriptional levels (Fig. 6). Importantly, the differentiation-dependent expression of endogenous sortilin (Fig. 1) appears to be directly involved in the process of myogenesis, because siRNA-mediated suppression of sortilin significantly inhibited C2C12 differentiation (Fig. 3C), which is at least in part mediated through the p75NTR-NGF autocrine loop (Fig. 3D). In addition, we found that cellular contents of Ubc9 and SUMOylated proteins are remarkably increased by sortilin-over-expression (Fig. 7). Although it is not clear from the present study whether the up-regulated Ubc9 is directly responsible for post-translational regulation of GLUT proteins as previously reported (45), we observed that GLUT4 became more stable, while GLUT1 became less stable, in sortilin-overexpressing C2C12 cells (Fig. 6C).

Sortilin serves as a stimulator of C2C12 myogenic differentiation via activation of the p75NTR-proNGF autocrine loop -

In cultured myoblasts including mouse C2C12 cells and rat L6 cells, serum withdrawal initiates the myogenic differentiation program, and it is generally accepted that autocrine/paracrine actions of insulin-like growth factors (IGFs) in response to serum withdrawal play an important role in the process of muscle differentiation (49,50). In addition to the IGF-mediated autocrine system, several lines of evidence have demonstrated that NGF, and presumably its unprocessed form termed proNGF, is also involved in the initiation of muscle differentiation in an autocrine fashion (26,27,51). Furthermore, over-expression of p75NTR, a low affinity receptor for various neurotrophins including proNGF, proBDNF, NGF and BDNF, has been shown to induce spontaneous myogenic differentiation in a growth medium (26) which is similar to what we have observed in C2C12 myoblasts over-expressing sortilin (Fig. 2). Since p75NTR appeared to form a complex with sortilin creating high affinity receptors for proNGF and thereby evoking an intracellular signaling cascade (22), we hypothesized that sortilin over-expression allows endogenous p75NTR to respond to limiting concentrations of neurotrophins being secreted by C2C12 cells in an autocrine manner and/or fed with medium containing FBS. Consistent with this idea, neutralizing antibodies against either p75NTR or

NGF significantly inhibited the spontaneous differentiation of Sort10-C2C12 cells over-expressing sortilin (Fig. 3A). In addition, sortilin overexpression resulted in an efficient endocytosis of the anti-p75NTR antibody from culture media (Fig. 3B), suggesting that exogenously expressed sortilin facilitates formation of the functional tripartite complex comprised of proNGF, p75NTR and sortilin, as previously reported (22). More importantly, either siRNA-mediated suppression of endogenous sortilin expression or specific antibody-mediated neutralization of endogenous p75NTR/proNGF significantly inhibits low-serum-induced C2C12 differentiation (Fig. 3CD). Together, these data demonstrate that sortilin serves as a stimulator of C2C12 myogenic differentiation mediated through potentiation of the p75NTR-proNGF autocrine loop, and that differentiation-dependent increases in endogenous sortilin participate directly in the promotion of myogenesis. It remains to be clarified whether sortilin has a similar capability in the process of adipocyte differentiation, which is currently under investigation.

It has been reported that sortilin binds mature NGF with low affinity ($Kd=10^{-8}$ M) and proNGF with high affinity ($Kd=10^{-9}$ M), and that the latter affinity is further increased ($Kd=10^{-10}$ M) when sortilin associates with p75NTR (22). Therefore, our data raise the novel possibility that levels of sortilin expression regulate myogenic differentiation by modulating the biological actions mediated through the p75NTR-proNGF autocrine loop. Since sortilin expression is up-regulated (Fig. 1) while p75NTR and NGF expressions are down-regulated (26), upon differentiation, fine tuning of the expression levels of these proteins would be required for proper development and maintenance of healthy muscle tissues (28). Although the signaling cascades involved in its myogenic actions have yet to be clarified, several lines of evidence indicate a signaling relationship between p75NTR and the Rho family of small GTP-binding proteins in the process of cellular differentiation (40,41). In this regard, regulation of Rho family members has been directly implicated in myogenesis (52,53), and the inhibition of ROCK by either Y29632 or siRNA-mediated gene silencing reportedly potentiated myogenesis of C2C12 cells (54-56). In the present study, however, we found that Y29632 significantly inhibited the myogenin expression observed in sortilin-overexpressing C2C12 cells early in differentiation (*Day 0*) (Fig. 3E). Since fine tuning of both Rho family members and ROCK has been shown to be important for myogenesis (54-56), the precise molecular mechanisms underlying the phenomena mentioned above remain to be clarified. However, our results strongly suggest an involvement of ROCK in the potentiation of myogenesis induced by sortilin-overexpression.

Effects of sortilin expression on the insulin-responsive glucose transport system in C2C12

Sortilin is expressed in a number of tissues, including the brain, testis and fat, as well as in skeletal muscle and the heart (17,18,57). Sortilin has been shown to play an important role in TGN-to-endosome and TGN-to-lysosome trafficking events (15). Like CI-M6PR, a well known sorting receptor that traffics among the TGN, lysosomes and the plasma membrane, sortilin possesses a short cytoplasmic tail at the carboxyl-terminal containing an acidic cluster-dileucine motif that is specifically recognized by the recently identified adaptor proteins termed GGAs (15,58). In the context of GLUT4 regulation in adipocytes, sortilin expression is up-regulated during 3T3L1 adipocyte differentiation (18,19), and recent studies by Kandror and colleagues demonstrated a crucial role of sortilin in the formation of insulin-sensitive GLUT4 storage vesicles responsible for the insulin-stimulated glucose transport in 3T3L1 adipocytes (12,48). While the precise molecular mechanism involved in the formation of insulin-responsive GLUT4 storage compartments remains largely unknown, the available evidence indicates that sortilin induces the biogenesis of vesicles containing GLUT4 in concert with GGA adaptor proteins in cultured adipocytes (59,60). More recently, the importance of luminal interactions between sortilin and GLUT4 in endosomes for producing insulin-responsive GLUT4 storage vesicles has been demonstrated (48).

Similar to observations in the 3T3L1 adipogenic cell line (18,19), our results demonstrate that sortilin is up-regulated upon C2C12 differentiation but is only partially co-localized to GLUT4-containing endosomes (Fig.