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Syntaxin 4 regulates the surface localization of a promyogenic receptor Cdo thereby promoting myogenic differentiation



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Abstract

Background: Syntaxins are a family of membrane proteins involved in vesicle trafficking, such as synaptic vesicle exocytosis. Syntaxin 4 (Stx4) is expressed highly in skeletal muscle and plays a critical role in insulin-stimulated glucose uptake by promoting translocation of glucose transporter 4 (GLUT4) to the cell surface. A cell surface receptor cell adhesion molecule-related, down-regulated by oncogenes (Cdo) is a component of cell adhesion complexes and promotes myoblast differentiation via activation of key signalings, including p38MAPK and AKT. In this study, we investigate the function of Stx4 in myoblast differentiation and the crosstalk between Stx4 and Cdo in myoblast differentiation.

Methods: The effects of overexpression or shRNA-based depletion of Stx4 and Cdo genes on C2C12 myoblast differentiation are assessed by Western blotting and immunofluorescence approaches. The interaction between Cdo and Stx4 and the responsible domain mapping are assessed by coimmunoprecipitation or pulldown assays. The effect of Stx4 depletion on cell surface localization of Cdo and GLUT4 in C2C12 myoblasts is assessed by surface biotinylation and Western blotting.

Results: Overexpression or knockdown of Stx4 enhances or inhibits myogenic differentiation, respectively. Stx4 binds to the cytoplasmic tail of Cdo, and this interaction seems to be critical for induction of p38MAPK activation and myotube formation. Stx4 depletion decreases specifically the cell surface localization of Cdo without changes in surface N-Cadherin levels. Interestingly, Cdo depletion reduces the level of GLUT4 and Stx4 at cell surface. Consistently, overexpression of Cdo in C2C12 myoblasts generally increases glucose uptake, while Cdo depletion reduces it.

Conclusions: Stx4 promotes myoblast differentiation through interaction with Cdo and stimulation of its surface translocation. Both Cdo and Stx4 are required for GLUT4 translocation to cell surface and glucose uptake in myoblast differentiation.

Keywords: Syntaxin 4, Cdo, Myogenic differentiation, p38MAPK, Cell surface localization

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Background

Skeletal myoblast differentiation is a well-coordinated multistep process that involves cell cycle withdrawal, expression of muscle-specific genes, and formation of multinucleated myofibers by cell fusion [1]. Two groups of transcription factors, the myogenic determination factors (Myf5, MyoD, Myogenin, and MRF4) and the myocyte enhancer factor 2 (MEF2), are central for the coordination of myogenesis [2–4]. The expression and activities of these transcription factors are tightly regulated to ensure efficient myogenic differentiation and to maintain the differentiated state of cells. Once activated, these transcription factors regulate numerous downstream target genes to initiate myogenic differentiation and reinforce each other's expression, resulting in a positive feedback network that amplifies and maintains the myogenic phenotype [5, 6].

Cell-cell adhesion between muscle precursors plays a crucial role in myoblast differentiation. A cell surface receptor cell adhesion molecule-related, down-regulated by oncogenes (Cdo) appears to be a critical component that integrates cell-contact-mediated signals from the cell surface into the myogenic regulatory network [7, 8]. Cdo forms a multiprotein complex with other cell adhesion molecules including N-Cadherin, Gas1, Boc, and neogenin/netrin-3, resulting in the promotion of myogenesis [9–12]. The depletion of Cdo in myoblasts shows impaired myogenic differentiation, and Cdo-deficient mice display delayed skeletal muscle development [7, 13]. In contrast, overexpression of Cdo in C2C12 cells enhances myoblast differentiation. The promyogenic function of Cdo involves a coordinated activation of p38 mitogen-activated protein kinase (p38MAPK) and AKT via association with scaffold proteins, JLP and Bnip2 for p38MAPK [13, 14] and APPL1 for AKT [15]. The level of Cdo protein, presumably at the cell membrane, appears to be critical for initiation of promyogenic signaling pathways; however, it is still unclear how the activity of the Cdo protein at the cell membrane is regulated.

Membrane fusion is an obligatory event in intracellular membrane trafficking and physically merges two lipid bilayers of separate compartments allowing content mixing [16]. Soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor proteins (SNAREs) play a key role in intracellular membrane fusion events and have been divided into vesicle-membrane SNAREs (v-SNAREs) and target-membrane SNAREs (t-SNAREs) based on their subcellular localization [17]. Syntaxin 4 (Stx4) is a member of t-SNAREs and expressed highly in various tissues, including the skeletal muscle, and plays a critical role in glucose uptake in response to insulin by delivery of glucose transporter 4 (GLUT4) to the cell membrane in skeletal muscle and adipose tissue [18, 19]. In addition, Stx4 has been shown to regulate glucose-stimulated insulin secretion in beta cells [20, 21]. The physiological importance of Stx4 in

the glucose uptake of skeletal muscle and whole body metabolism has been shown by studies with knockout and transgenic mice [19, 22]. Stx4 heterozygous mice display an insulin resistance with reduction of glucose uptake specifically in the skeletal muscle, without alterations in adipose tissue and liver [22]. Conversely, Stx4 transgenic mice exhibit enhanced glucose uptake and insulin-induced GLUT4 translocation to the cell membrane of the skeletal muscle [19]. The complete ablation of the Stx4 gene in mice causes early embryonic lethality before embryonic day 7.5 [23]. Thus, whether Stx4 plays any role in myogenesis is still unclear. The facts that GLUT4 expression and activity increase during myoblast differentiation [24] and the fusion of myoblasts into multinucleated myotubes is a critical step for efficient differentiation prompt us to examine the role of Stx4 in myoblast differentiation, especially in regulation of a promyogenic surface receptor Cdo.

We report here that Stx4 expression is upregulated upon induction of myoblast differentiation. Overexpression of Stx4 in C2C12 myoblasts increases myogenic differentiation via regulation of p38MAPK activity, whereas Stx4 depletion in C2C12 cells by small hairpin RNA (shRNA) decreases myogenesis. Stx4 and Cdo interact physically in differentiating myoblasts, and this interaction is mediated by the t-SNARE domain of Stx4, which is critical for the promyogenic function of Stx4. Stx4 depletion leads to declined levels of the cell surface resident Cdo without changes in the level of N-Cadherin, another Cdo-interacting protein. Interestingly, Cdo depletion affected the membrane translocation of GLUT4 and interaction of Stx4 with GLUT4, without altered total protein levels. Taken together, Stx4 promotes myogenic differentiation by binding to a promyogenic receptor Cdo and regulating its cell surface translocation thereby activating downstream p38MAPK pathway.

Methods

Cell culture and expression vectors

Myoblast C2C12 cells, primary myoblasts, embryonic fibroblast 10T1/2 cells, and embryonic kidney 293T cells were cultured as described previously [1]. To induce differentiation of C2C12 myoblasts, cells at near confluence were changed from Dulbecco modified Eagle's medium (DMEM) containing 15 % fetal bovine serum (FBS; growth medium, GM) to DMEM containing 2 % horse serum (HS; differentiation medium, DM), and myotube formation was observed at 2 or 3 days of differentiation. The efficiency of myotube formation was quantified by a transient differentiation assay as previously described [1]. To generate C2C12 cells that stably overexpress Cdo, Stx4, mutant forms of Stx4, or shRNAs against Stx4 or Cdo, cells were transfected with the indicated expression vectors and Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and cultures were selected in puromycin-

containing medium. Five different Stx4 shRNAs obtained from Sigma-Aldrich (St. Louis, MO) were screened for their effectiveness by transfection into 293T cells. From among them, the following sequences were chosen based on the strongest knockdown effect and reproducibility: shStx4#1, 5'-CCGGGAGAGACAGAGACCCAGCTTTC TCGAGAAAGCTGGGTCTCTGTCTCTCTTTTGG-3'; shStx4#2, 5'-CCGGGAGTCCGTGCCAGCAATTTGC TCGAGCAAATTGCTGGGACAGGACTCTTTTGG-3'. For the Stx4 deletion mutation study, the mouse Stx4 gene was amplified by Reverse Transcription Polymerase Chain Reaction (RT-PCR) of mRNAs purified from human embryonic kidney fibroblast cells. Full-length Stx4 (aa 1-299) and deletion forms of Stx4 (Stx4 Δ 1-153, Stx4 Δ 154-194, and Stx4 Δ 195-262) were inserted into mammalian expression vector pcDNA-myc and puroBABE-GFP-S, respectively. Hindlimb and satellite cells isolated from *Cdo*^{+/+} and *Cdo*^{-/-} mice were cultured as described previously [25]. Cells were grown in F10 medium containing 20 % FBS and basic fibroblast growth factor (bFGF; 100 ng/ml).

Western blot analysis and immunoprecipitation

Western blot analysis was performed as previously described [26]. Briefly, cells were lysed in cell extraction buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100) containing complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), and Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed. The primary antibodies used were anti-Stx4 (sc-101301), anti-MyoD (sc-32758), anti-Myogenin (sc-12732), anti-myc (sc-40), anti-GLUT4 (sc-53566, Santa Cruz Biotechnology, Santa Cruz, CA), anti-troponin T (SAB2102501), anti-pan-Cadherin (c3678, Sigma-Aldrich, St Louis, MO), anti-p-p38 (9211), anti-p38 (9212), anti-phospho-AKT (p-AKT; 9271), anti-AKT (9272, Cell Signaling Technology, Beverly, MA), anti-GFP (A11120, Invitrogen), anti-Cdo (AF2429, R&D Systems, Minneapolis, MN), and anti-myosin heavy chain (MHC) (MF20: Developmental Studies Hybridoma Bank, Iowa, IA). For immunoprecipitation assay, 293T cells were transfected with a combination of Cdo and either myc-tagged Stx4 or S-GFP-tagged Stx4. Thirty-six hours after transfection, whole cell extracts were incubated with anti-myc and protein G agarose beads (Roche Diagnostics) overnight at 4 °C. The beads were washed three times with extraction buffer and resuspended in extraction buffer, and samples were analyzed by western blotting. For pulldown experiments, 293T cells were transfected with Cdo and deletion mutant of Stx4-S. Cell extracts were incubated with anti-S beads (Novagen, Madison, WI), and the precipitates were assessed by immunoblotting.

Biotin labeling of cell surface protein

Cell surface biotinylation was performed essentially as described previously [27]. Briefly, C2C12 cells were induced to differentiate for indicated time points by switching to DM and incubating in phosphate-buffered saline (PBS) containing Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific, Rockford, IL) with the final concentration of 1 mg/ml for 30 min on ice. After quenching the biotinylation, cells were lysed in extraction buffer containing protease inhibitor. Biotinylated proteins were recovered on streptavidin-agarose beads (Thermo Fisher Scientific), followed by SDS-PAGE.

Immunocytochemistry and confocal microscopy

Immunostaining for MHC expression was performed as described previously [1]. Briefly, C2C12 cells were transfected with Cdo plus GFP vector or Stx4 and GFP vector, fixed with 4 % paraformaldehyde for 20 min, permeabilized with 1 % Triton X-100 in PBS for 10 min, blocked, and stained with anti-MHC, followed by an FITC-conjugated and Alexa Fluor 568-conjugated secondary antibody (Invitrogen). Images were captured and processed with a Nikon ECLIPSE TE-2000U microscope and NIS-Elements F software (Nikon). Quantitative differentiation assay was performed for at least three independent experiments.

For reactivation of p38 in Cdo-depleted cells by Stx4 overexpression experiment, C2C12 cells in 12-well plates were cotransfected with 100 ng of a GFP expression vector and 900 ng of the indicated DNA construct for 2 days and then fixed with 4 % paraformaldehyde for 20 min. Cultures were then permeabilized with 1 % Triton X-100 in PBS, blocked, and incubated with anti-p-p38 followed by incubation with an Alexa Fluor 568-conjugated secondary antibody. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). An image was obtained on a Zeiss LSM-510 Meta Confocal Microscope. Quantification of the fluorescent signal for p-p38 was performed with Image Gauge software (Fujifilm).

Luciferase assay

10T1/2 cells were seeded in 12-well plates at a density of 4×10^4 cells per well. Twenty-four hours after seeding, cells were transfected using Lipofectamine 2000 with 100 ng of the reporter plasmid of MyoD-luc and cotransfected with 50 ng MyoD. Twelve hours later, transfection cells were transferred into GM, harvested, and firefly luciferase activity was determined using a Luminometer with Luciferase Reporter Assay System (Promega, Fitchburg, WI). Experiments were performed in triplicates and repeated at least three times independently.

RNA extraction, RT-PCR, and quantitative RT-PCR

Total RNA was extracted using Easy-Blue reagent (iNTRON Biotechnology, Seongnam, Korea) according to

the manufacturer's instructions. Template cDNAs were reverse-transcribed from 2 µg of total RNA using oligo-dT primer and SuperScript II reverse transcriptase (Invitrogen). The PCR mixture contained the template DNA, primer, dNTPs, and DNA polymerase (Invitrogen). PCR reactions were performed in a Genepro-PCR model. Expression levels of glyceraldehyde 3-phosphate dehydrogenase (Gapdh) were used to normalize the expression levels of each sample. Primer sequences used for PCR were as follows: Stx4, 5'-GTCTGACGAGGAGCTGGAAC-3' and 5'-CCGAGCTCAGGATGTTCTTC-3'; Cdo intracellular region, 5'-ATAGGATCCTGGAAGAGTCGCCAACAG-3' and 5'-ATGGTACCTCAGGTCTCTTGGGCTTG-3'; Gapdh, 5'-ATGGGGAAGGTGAAGTTCG-3' and 5'-TTAC TCCTTGAGGCCATGT-3'. Each PCR reaction was analyzed on 1.2 % agarose gel containing ethidium bromide. Real-time PCR was performed using SYBR Green PCR master mix in an ABI cycler and quantified with ABI 7000 software (Applied Biosystems, Foster City, CA). Briefly, 1 µg of total RNAs was reverse-transcribed for 5 min at 72 °C and incubated for 5 min on ice followed by incubation for 60 min at 42 °C and 5 min at 95 °C. One hundred nanograms cDNA and 0.2 µl universal reverse (Invitrogen) and specific forward primer were used for the 20 µl PCR reaction. All PCR reactions were analyzed as triplicates.

Measurement of glucose uptake

Stable C2C12 cells transfected with control, Cdo, or shCdo expression vector were incubated in the serum-, glucose-free DMEM for 2 h at 37 °C. After the incubation, cells were treated with 10 µg/ml insulin in the serum-, glucose-free DMEM for 1 h, and 100 µM 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) was added, a fluorescent glucose analog (Invitrogen) for 1 h. Reactions were terminated by washing with a cold DPBS buffer, followed by measurement of the fluorescence intensity at an excitation of 485 nm and an emission of 535 nm using a Luminometer (Promega).

Statistics

The experiments were performed independently at least three times. The participants' *t*-test was used to access the significance of the difference between two mean values. **p* < 0.01 and ***p* < 0.05 were considered to be statistically significant.

Results

Stx4 is expressed in skeletal muscles and enhanced during myoblast differentiation

In the previous study, we performed a yeast two-hybrid screening to identify interacting proteins for Cdo, and JLP and Bnip2 are two such proteins implicated in Cdo-mediated myogenesis [13, 14]. In the same screen,

Syntaxin (Stx) 1 was identified as an interacting protein for Cdo. Stx1 and Stx4 share high homology and have similar domain structures consisting of Stx, t-SNARE, and transmembrane domain (TD) [28]. While Stx1 is expressed predominantly in neural cell types, Stx4 is the major form in skeletal muscles [29, 30]. Therefore, we examined whether Stx4 plays a role in myogenesis, especially in association with Cdo. First, we have assessed the expression pattern of Stx4 and Cdo in mouse hindlimb muscles from various developmental stages. The expression of Stx4 was detected throughout the examined stages; however, the level of Cdo, MyoD, and Myogenin decreased after the postnatal day 7 which may reflect the fast muscle growth during early postnatal life (Fig. 1a). Next, we have examined the expression pattern of Stx4 protein during myoblast differentiation. C2C12 cells were grown to near-confluency (D0) and induced to differentiate by switching to the differentiation medium for a total of 3 days (D3), followed by immunoblotting. As shown in Fig. 1b, the level of Stx4 is enhanced progressively during myoblast differentiation, while the Cdo protein is expressed throughout the differentiation time course, and the expression of Myogenin and myosin heavy chain (MHC) was dramatically enhanced at D2 or D3, respectively. These data suggest that Stx4 might be important for myoblast differentiation. Since Cdo and Stx4 were coexpressed in developing skeletal muscles, we examined the relationship between Cdo and Stx4 by using primary myoblasts isolated from *Cdo*^{+/+} or *Cdo*^{-/-} mice. Previously, we have shown that Cdo-deficient primary myoblasts display defects in myoblast differentiation and p38MAPK activation [26]. *Cdo*^{+/+} or *Cdo*^{-/-} myoblasts at high cell density (D0) were induced to differentiate by removal of basic fibroblast growth factor (bFGF) for 2 days. The expression of Stx4 in *Cdo*^{-/-} myoblasts was substantially increased at D2 compared to that of *Cdo*^{+/+} myoblasts, whereas there was only slight or no difference at D0 and D1 (Fig. 1c). In addition, the qRT-PCR analysis showed that Stx4 transcript levels were increased at D1 in Cdo-deficient myoblasts, but no difference in cells at D0 or D2 (Fig. 1d). These data suggest that the Stx4 expression level alone may not be sufficient to induce myoblast differentiation when Cdo is deficient.

Overexpression of Stx4 enhances myogenic differentiation

To investigate the function of Stx4 in myogenesis, C2C12 cells were stably transfected with control or Stx4 expression vectors and induced to differentiate. Overexpression of Stx4 in C2C12 cells generally resulted in a twofold increase of Stx4 protein (Fig. 2a) and the expression of muscle-specific genes including MHC; Myogenin and Troponin T were significantly enhanced in Stx4-overexpressing C2C12 cells, compared to that of control cells, while MyoD levels were not altered (Fig. 2b). Next, we examined the effect of

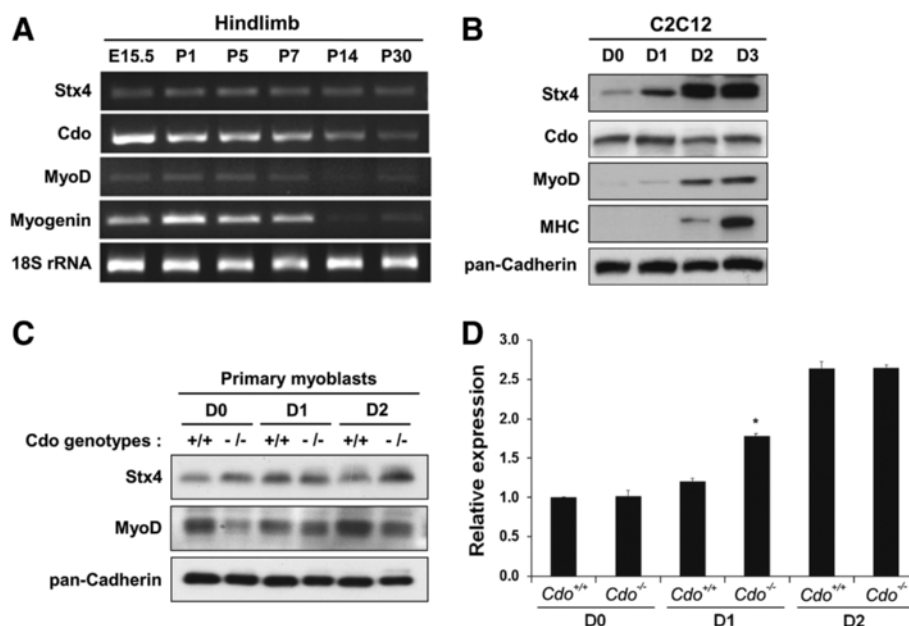


Fig. 1 Stx4 is expressed in skeletal muscles and induced in myoblast differentiation. **a** RT-PCR analysis of hindlimb muscles from E15.5 embryos and P1, P5, P7, P14, and P30 mice for the expression of Stx4, Cdo, MyoD, Myogenin, and 18S rRNA serves as a loading control. **b** Immunoblot analysis of C2C12 cells from various differentiation days (D) for the expression of Stx4, Cdo, MyoD, MHC, and pan-Cadherin serves as a loading control. **c** Immunoblot analysis for Stx4 protein expression in *Cdo*^{+/+} and *Cdo*^{-/-} primary myoblasts during differentiation, and pan-Cadherin serves as a loading control. **d** qRT-PCR analysis for Stx4 mRNA expression in *Cdo*^{+/+} and *Cdo*^{-/-} primary myoblasts during differentiation

Stx4 overexpression on myotube formation. Control (pcDNA) and Stx4-overexpressing C2C12 cells were induced to differentiate for 2 days, fixed, and immunostained with anti-MHC antibody followed by DAPI staining. Stx4-overexpressing C2C12 cells formed larger myotubes than the control (pcDNA) cells (Fig. 2c, d). MHC-positive cells were scored as mononucleate, containing two to five nuclei, containing six to nine nuclei, or containing ten or more nuclei. Stx4-overexpressing cells formed more larger myotubes containing six to nine nuclei (18 %) and ten or more nuclei (15 %), compared to control cells with 10 and 3 %, respectively. In contrast, the percentile of mononucleate cells decreased to 38 %, compared to 53 % of control cells (Fig. 2d). These data suggest that Stx4 promotes myoblast differentiation.

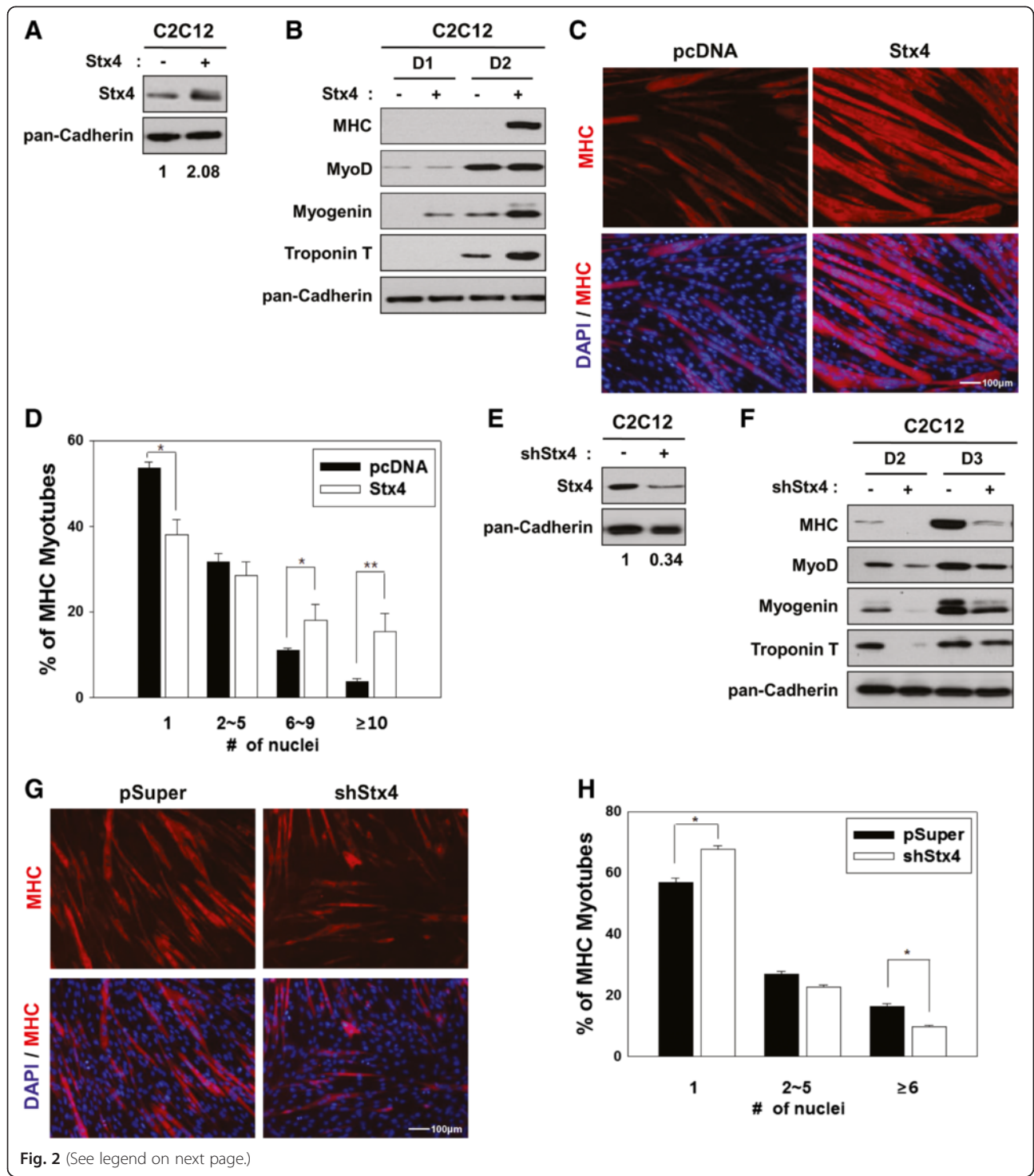
The depletion of Stx4 decreases myogenic differentiation

To examine whether Stx4 depletion inhibits muscle-specific gene expression and myotube formation, C2C12 cells were stably transfected with control pSuper or Stx4 shRNA (shStx4) expression vectors, induced to differentiate for 3 days and analyzed for their differentiation ability by Western blot analysis and immunostaining with anti-MHC antibody. We have tested five different Stx4 shRNA expression vectors, and among them, two shRNA constructs reproducibly resulted in a significant knockdown of Stx4. Among these, we used mostly shStx4#1 for this study (Additional file 1: Figure S1).

Stx4 protein levels decreased to 34 % in C2C12/shStx4 cells, relative to that of C2C12/pSuper cells (Fig. 2e). Stx4-depleted cells exhibited a dramatic reduction in the expression of MHC, MyoD, Myogenin, and Troponin T, compared to C2C12/pSuper cells (Fig. 2f). Furthermore, C2C12/shStx4 cells formed smaller myotubes with fewer nuclei, relative to C2C12/pSuper cells (Fig. 2g). The quantification of MHC-positive cells showed that Stx depletion resulted in the formation of more mononucleated myocytes (~57 to ~67 %) and less myotubes with more than six nuclei (~17 to ~9 %), relative to C2C12/pSuper cells (Fig. 2h). These results indicate that Stx4 is required for efficient myoblast differentiation.

Stx4 and Cdo interact physically in differentiating myoblasts, and this interaction is mediated by the t-SNARE domain of Stx4

Next, we examined whether Stx4 and Cdo physically interacts in mammalian cells. To do so, 293T cells were transiently transfected with myc-tagged Stx4 and Cdo and then lysates were immunoprecipitated with the myc-tag antibody followed by immunoblotting with Cdo and myc antibodies. Consistent with the result obtained from a previous yeast two-hybrid screening [13], Stx4 and Cdo interacted in 293T cells when coexpressed (Fig. 3a). To assess whether Stx4 and Cdo interact endogenously in myoblasts, cell lysates of differentiating C2C12 myoblasts from a total of 3 days of differentiation



(See figure on previous page.)

Fig. 2 Overexpression or knockdown of Stx4 promotes or blocks myoblast differentiation, respectively. **a** Lysates of control or Stx4-overexpressing C2C12 cells were immunoblotted with antibodies against Stx4 and pan-Cadherin as a loading control. The relative signal intensity of Stx4 to pan-Cadherin was quantified and added under the blot. **b** Lysates of control or Stx4 expression vector transfected C2C12 cells from the differentiation day 1 (D1) and D2 were immunoblotted with antibodies to MHC, MyoD, Myogenin, Troponin T, and pan-Cadherin as a loading control. **c** Control or Stx4 expression vector transfected C2C12 cells were induced to differentiate for 3 days and immunostained with MHC antibody followed by DAPI staining to visualize nuclei. Size bar = 100 μ m. **d** The quantification of myotube formation shown in panel **c**. Values represent means of triplicate determinations \pm 1 SD. The experiment was repeated three times with similar results. Significant difference from control, * p < 0.01. **e** Control or shStx4 expression vector transfected C2C12 cells were analyzed by immunoblotting with antibodies to Stx4 and pan-Cadherin as a loading control. The relative knockdown levels of Stx4 to pan-Cadherin are quantified and added under the blot. **f** Control or shStx4 expression vector transfected C2C12 cells were induced to differentiate for 2 or 3 days and then lysates were subjected to immunoblotting with antibodies to MHC, MyoD, Myogenin, Troponin T, and pan-Cadherin as a loading control. **g** Control pSuper or shStx4 expression vector transfected C2C12 cells were induced to differentiate for 3 days and immunostained with an antibody to MHC followed by DAPI staining to visualize nuclei. Size bar = 100 μ m. **h** The quantification of myotube formation shown in panel **g**. Values represent means of triplicate determinations \pm 1 SD. The experiment was repeated three times with similar results. Significant difference from control, * p < 0.01, ** p < 0.005

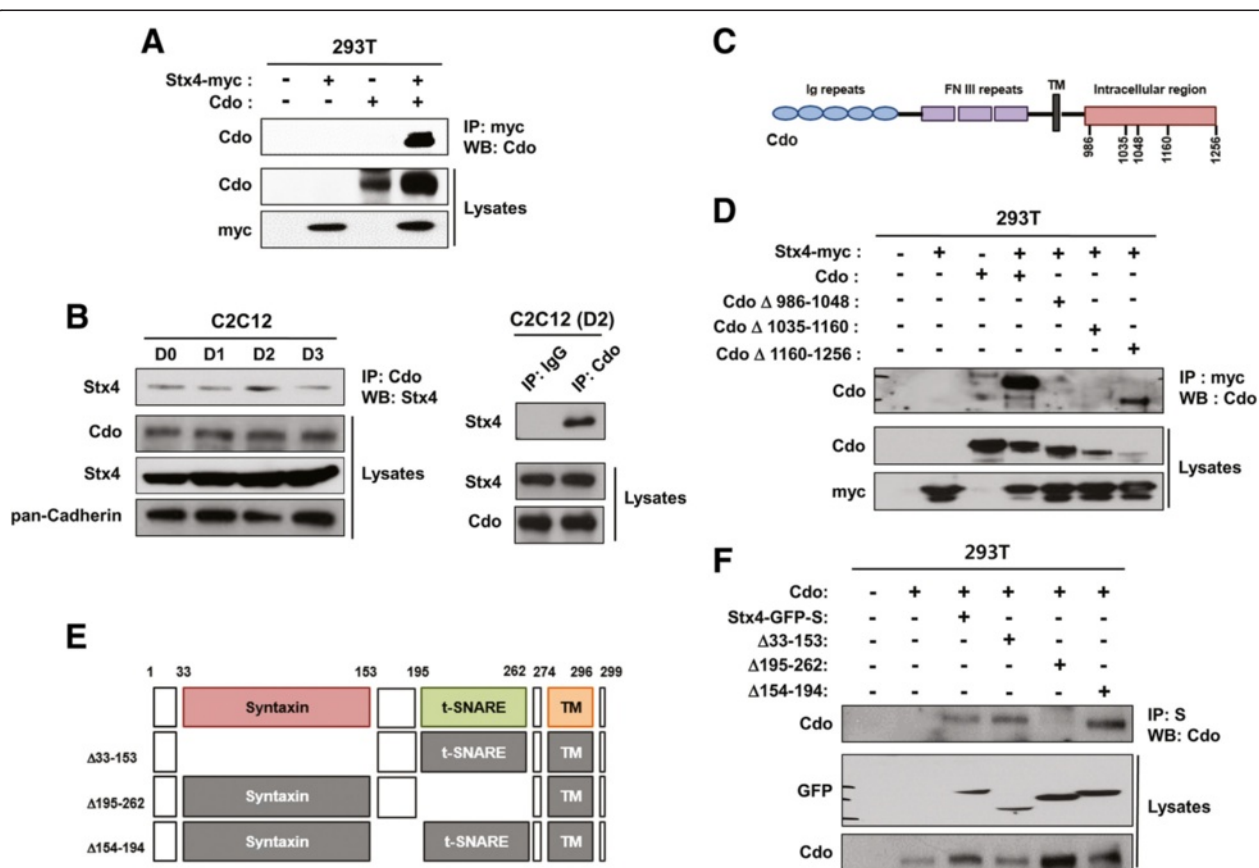


Fig. 3 Stx4 and Cdo interact physically in differentiating myoblasts, and the t-SNARE domain of Stx4 mediates the interaction with Cdo. **a** Lysates of 293T cells transfected with Stx4-myc, Cdo, or control vector were subjected to immunoprecipitation with myc and immunoblotting with Cdo or myc antibodies. **b** Lysates of C2C12 cells from various differentiation time courses were immunoprecipitated with control IgG or anti-Cdo antibody and immunoblotted with antibodies to Stx4, Cdo, and pan-Cadherin as a loading control. **c** The schematic representation of the domain structure of Cdo. Cdo consists of five immunoglobulin, three fibronectin type III, a single transmembrane domain, and a 270-amino-acid-long intracellular region. **d** 293T cells were transiently cotransfected with control or Stx4-myc along with either the full length or three deletion mutants of the Cdo's cytoplasmic region. Forty-eight hours later, cell lysates were subjected to immunoprecipitation with myc antibody followed by immunoblotting with Cdo antibody. Total lysates served as the expression controls. **e** The schematic representation depicts the domain structure of Stx4 and the deletion of the specific domain; Δ 33-153 (the Syntaxin domain deletion), Δ 195-262 (the t-SNARE region deletion), and Δ 154-194 (the linker region deletion). **f** 293T cells were transiently cotransfected with control or Cdo along with the full length or deletion mutants of Stx4, and the lysates were pulled down with S-agarose beads followed by immunoblotting with Cdo or GFP antibodies

time course were immunoprecipitated with control IgG or an anti-Cdo antibody and analyzed by Western blotting. Stx4 was precipitated with Cdo throughout differentiation time course, and the coprecipitation was highest at D2 (Fig. 3b) when myoblasts were differentiating (Fig. 1b). These results suggest that Stx4 and Cdo can physically interact in myoblasts during differentiation.

Cdo consists of an extracellular region that contains five immunoglobulin (Ig)-like repeats followed by three fibronectin type III (FNIII)-like repeats, a transmembrane segment, and a long cytoplasmic tail [31]. A schematic representation of the Cdo protein structure is shown in Fig. 3c. To determine the cytoplasmic region of Cdo which is responsible for interaction with Stx4, we have transiently transfected three Cdo mutants that harbor indicated deletions in the cytoplasmic tail and analyzed the ability of these mutants to coprecipitate with Stx4. All three Cdo mutants showed a reduction in Stx4 binding, relative to the full length. However, Cdo Δ 986-1048 and Cdo Δ 1035-1160 failed to coprecipitate Stx4 (Fig. 3d), suggesting the cytoplasmic region of Cdo is required for Stx4 interaction.

To identify the domain of Stx4 responsible for Cdo interaction, we have generated GFP-S-tagged Stx4 deletion mutants based on its domain structure (Fig. 3e). The full length or these deletion mutants of Stx4 were cotransfected into 293T cells with Cdo expression vector followed by a pulldown analysis with S-agarose beads and Western blot analysis. While Stx4 Δ 33-153 and Stx4 Δ 154-194 proteins pulled down Cdo similarly to the full-length Stx4, Stx4 Δ 195-262 failed to precipitate Cdo, suggesting that the t-SNARE domain (aa 195–262) of Stx4 is responsible for Cdo binding (Fig. 3f).

The deletion mutants for either the Syntaxin or the t-SNARE domain of Stx4 failed to enhance myoblast differentiation

The promyogenic function of Cdo involves the activation of MyoD via p38MAPK pathway [1]. Therefore, we assessed the effect of Stx4 or/and Cdo expression on MyoD activation by using a MyoD-responsive reporter. To do so, 10T1/2 fibroblasts were cotransfected with a MyoD-luciferase construct and a MyoD expression vector along with expression vectors for Stx4 and/or Cdo. Forty-eight hours later, lysates were subjected to a luciferase assay. The expression of Stx4 or Cdo singly with MyoD enhanced the luciferase activity approximately 2.5-fold and 2.7-fold, respectively, while coexpression of Stx4 and Cdo enhanced the MyoD-reporter activity to approximately 5.8-fold compared to control MyoD-expressing cells (Fig. 4a). These data suggest that Stx4 and Cdo can activate MyoD cooperatively.

To assess the functional significance of the Stx4 interaction with Cdo in myoblast differentiation, C2C12 cells stably transfected with the control, the full length, or

deletion mutants of Stx4 as indicated and induced to differentiate for 2 days followed by Western blotting for MHC expression. C2C12 cells expressing either the full-length Stx4 or Stx4 Δ 154-194 displayed enhanced MHC expression, compared to control-vector-expressing cells. In contrast, the expression of Stx4 Δ 33-153 or Stx4 Δ 195-262 resulted in starkly decreased MHC expression (Fig. 4b). To assess the effect of Stx4 deletion mutants on myotube formation, C2C12 cells were cotransfected with the control pcDNA, the full length, or deletion mutants of Stx4 and GFP to mark transfectants and induced to differentiate for 3 days followed by immunostaining for MHC expression. Consistent with the Western blot data, the expression of Stx4 Δ 154-194 enhanced myotube formation to a comparable level of the full-length Stx4, as seen by fewer GFP-positive MHC-negative cells and larger GFP-positive myotubes with more nuclei per myotube, relative to control cells (Fig. 4c, d). However, roughly 60 % of the control pcDNA, Stx4 Δ 33-153-, or Stx4 Δ 195-262-expressing cells were negative for MHC expression, and a large proportion of the GFP- and MHC-positive cells were mononucleated in these cultures. These results suggest that the Syntaxin and t-SNARE domains of Stx4 are required for the promyogenic function of Stx4.

Stx4 enhances p38MAPK phosphorylation, and Stx4 overexpression restores myoblast differentiation in Cdo-depleted cells

Previously, we have reported that Cdo promotes myoblast differentiation via activation of a key promyogenic kinase p38MAPK (p38) [13], and this is required for the efficient myoblast differentiation [13, 32]. Therefore, we examined the effect of Stx4 on p38 activation in C2C12 cells. Control or Stx4-overexpressing C2C12 cells were induced to differentiate for 2 days, and the status of p38 activation was analyzed by Western blot analysis with antibodies to an active phosphorylated form of p38 (p-p38) or total p38. Overexpression of Stx4 led to a substantial increase in p-p38 levels relative to that of control cells, while the level of total p38 was unchanged (Fig. 5a). In addition, C2C12/pSuper or C2C12/shStx4 cells were induced to differentiate for 3 days and analyzed for p38 activation. Stx4 knockdown in C2C12 cells caused a notable decrease in p-p38 levels relative to that of the control cells (Fig. 5b), suggesting that Stx4 is required for p38 activation during myoblast differentiation. We next asked whether the decreased p38 activation in Cdo-depleted C2C12 cells can be rescued by Stx4 expression. C2C12/pSuper and C2C12/shCdo cells were transiently transfected with pcDNA or Stx4 expression vector, plus GFP expression vector to label the transfectants. After 2 days of transfection, cells were immunostained with antibodies to p-p38 and GFP followed by DAPI staining to visualize nuclei. The representative pictures are shown in

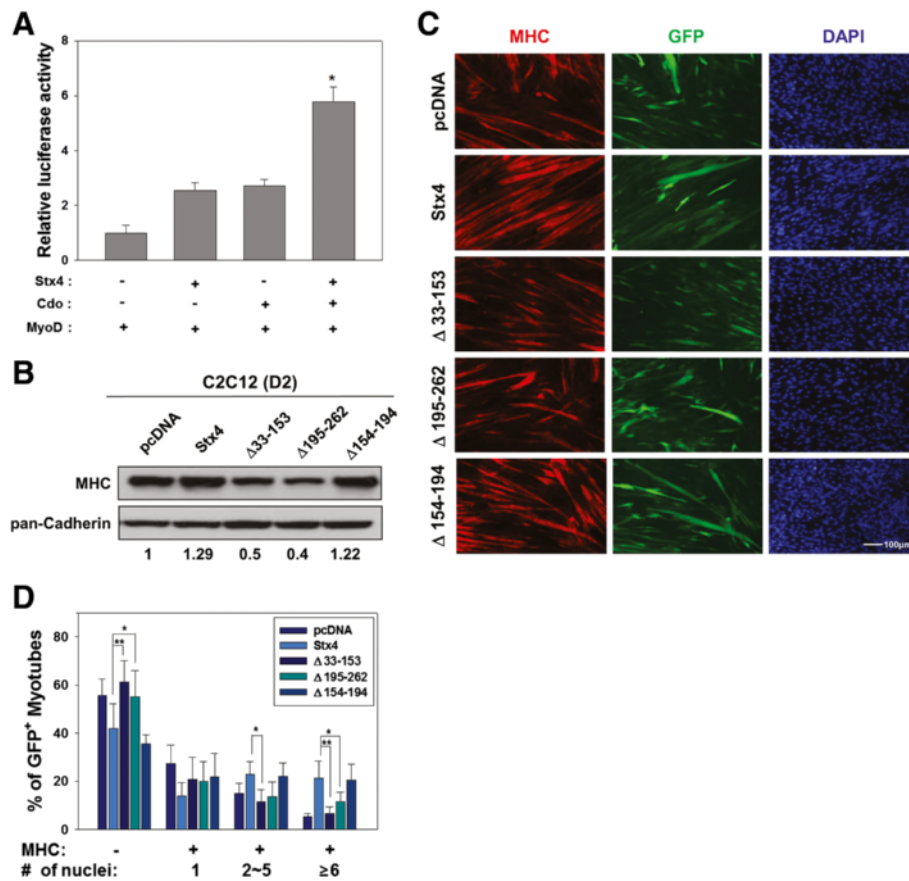


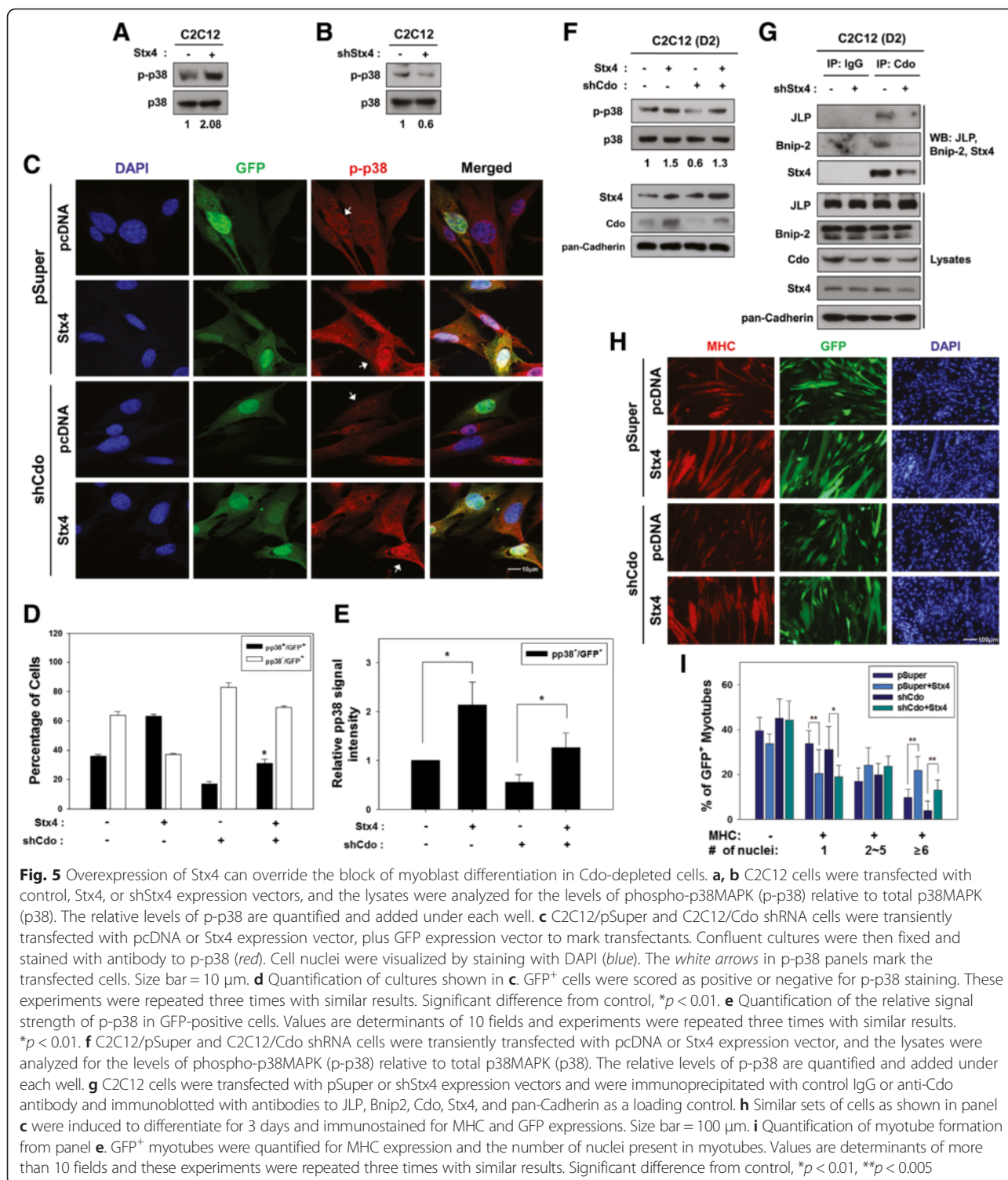
Fig. 4 Stx4 and Cdo induce MyoD activities synergistically, and the Cdo-binding deficient Stx4 mutant failed to enhance myotube formation. **a** 10T1/2 cells were cotransfected with a MyoD-luciferase reporter and the expression vectors for MyoD and β-galactosidase as an internal control. In addition, control, Stx4, and/or Cdo expression vectors were cotransfected as indicated. Forty-eight hours later, the reporter activities were measured and normalized relative to the internal control. The experiment was performed as triplicates and repeated three times with similar results. **p* < 0.01. **b** Lysates of C2C12 cells stably transfected with indicated Stx4 vectors were immunoblotted with antibodies to MHC and pan-Cadherin as a loading control. The relative signal intensities of MHC to pan-Cadherin were quantified and added under the blot. **c** C2C12 cells were transiently cotransfected with control (pcDNA), Stx4, or Stx4 mutants along with a GFP expression vector to mark the transfectant. Then, cells were induced to differentiate for 2 days, followed by immunostaining with an antibody to MHC and DAPI stain. Size bar = 100 μm. **d** Quantification of myotube formation of cell lines shown in panel **c**. Values represent means of triplicate determinations ± 1 SD. The experiment was repeated three times with similar results. Significant difference from control, **p* < 0.01, ***p* < 0.005

Fig. 5c. Roughly 36 % of control-transfected C2C12/pSuper cells were positive for the nuclear p-p38 accumulation (marked with a white arrow), whereas 63 % of Stx4-transfected C2C12/pSuper cells were positive for p-p38. On the other hand, only 17 % of control-transfected C2C12/shCdo cells were weakly positive for p-p38, whereas Stx4-expressing C2C12/shCdo cells displayed restored p-p38 levels with 31 % which is similar to control-transfected C2C12/pSuper cells (Fig. 5d). The quantification of relative p-p38 signal intensities in GFP-positive cells revealed the increased signal intensity in Stx4-overexpressing control cells. Furthermore, the overexpression of Stx4 restored p38 activation in C2C12/shCdo cells (Fig. 5e).

To further confirm these results, C2C12/pSuper and C2C12/shCdo cells were transfected with control or Stx4 expression vectors and induced to differentiate for

2 days, followed by Western blot analysis. As expected, C2C12/pSuper cells overexpressing Stx4 displayed elevated p-p38 levels while control transfected Cdo-depleted cells showed a reduction in p-p38 levels. In consistent with the aforementioned data, Stx4 expression in C2C12/shCdo cells restored p38 activation (Fig. 5f). Since Cdo can activate AKT via interaction with APPL1 in promotion of myoblast differentiation [15], we next examined the effect of Stx4 on AKT activation in C2C12 cells. However, the active phosphorylated AKT levels were not altered by depletion or overexpression of Stx4 (Additional file 2: Figure S2). These data suggest that Stx4 overexpression can override the block of p38MAPK activation caused by Cdo depletion in C2C12 myoblasts.

To further examine the role of Stx4 in Cdo-mediated p38 activation, we have assessed the effect of Stx4



depletion on the complex formation of Cdo with Bnip2 and JLP which has been shown to be critical for p38 activation and myogenic differentiation [13, 14]. C2C12 cells were transfected with control or shStx4 expression vector and induced to differentiate for 2 days. Cell lysates were

subjected to immunoprecipitation with control IgG or anti-Cdo antibody followed by immunoblotting. The interaction of Cdo with JLP, Bnip2, and Stx4 was abrogated in Stx4-depleted cells compared to control cells (Fig. 5g). Interestingly, Cdo levels in total lysates were slightly

decreased in Stx4-depleted C2C12 cells, while the levels of JLP and Bnip2 were not altered. These data suggest that Stx4 is required for Cdo/Bnip2/JLP complex formation.

This led us to investigate whether overexpression of Stx4 can restore the differentiation ability of Cdo-depleted myoblasts. C2C12/pSuper and C2C12/shCdo cells were transiently transfected with pcDNA or Stx4 plus GFP expression vectors to label the transfectants and induced to differentiate for 3 days, followed by immunostaining with a MHC antibody and DAPI staining. Consistently, Stx4 overexpression in C2C12/pSuper cells enhanced myotube formation as seen by the increased proportion of larger myotubes containing more than six nuclei compared with the control transfected cells (Fig. 5h, i). Similarly to the previous reports [26], C2C12/shCdo cells transfected with the control pcDNA exhibited impaired myotube formation. Overexpression of Stx4 in these cells restored myotube formation to similar levels of control cells (Fig. 5h, i). These results demonstrate that overexpression of Stx4 can restore the differentiation ability of Cdo-depleted C2C12 myoblasts.

Depletion of Stx4 causes a reduction in Cdo protein levels at the cell surface

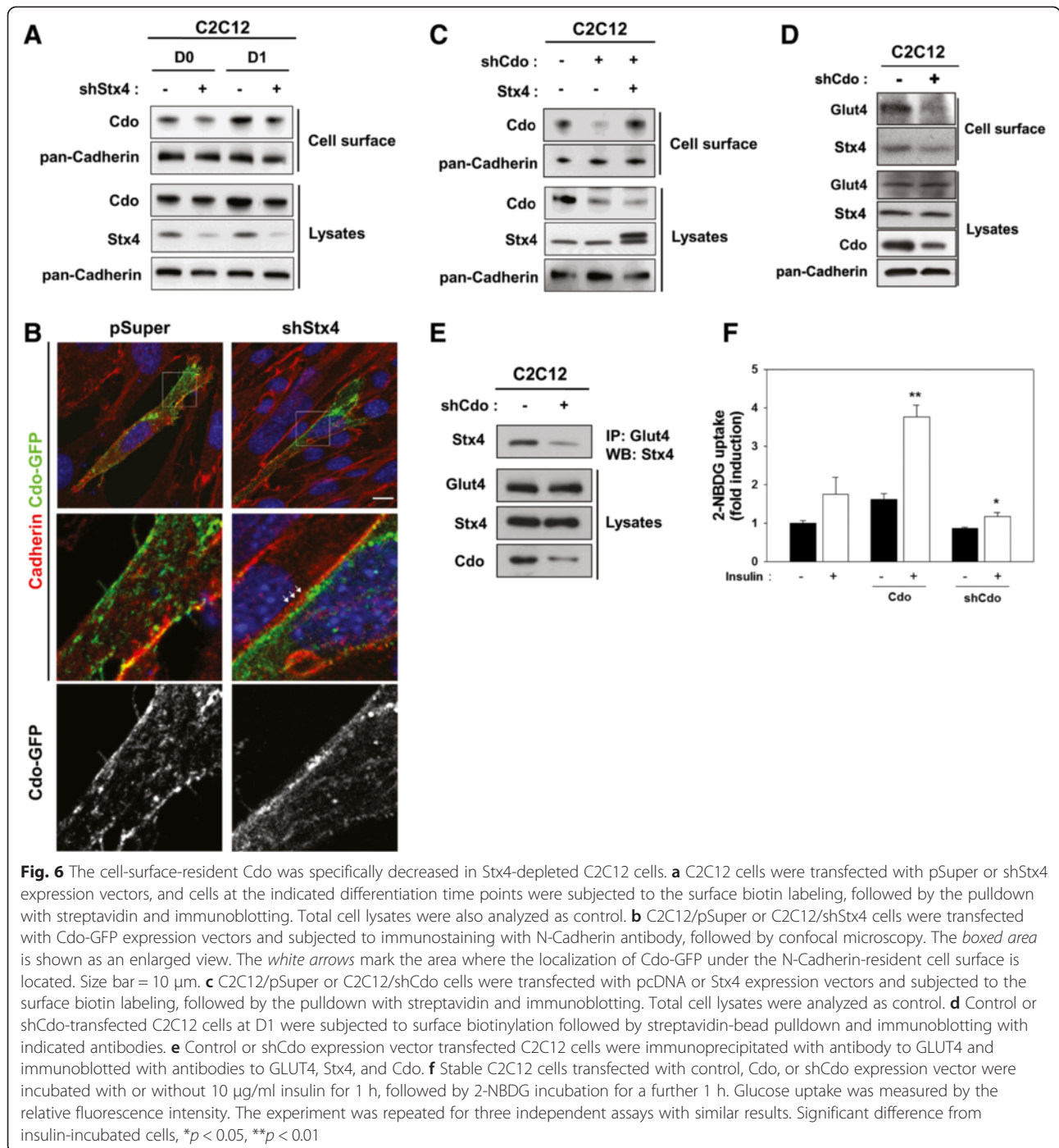
Next, we examined whether Stx4 regulated Cdo translocation to cell surface. To do so, we have assessed whether Stx4 depletion altered the level of Cdo at the cell surface by surface biotinylation. Stx4 knockdown C2C12 cells displayed decreased Cdo protein levels at the cell surface as well as total Cdo proteins in lysates (Fig. 6a). Furthermore, this effect on Cdo levels appears to be specific since N-Cadherin levels did not alter in these cells. To further examine, C2C12/pSuper or C2C12/shStx4 cells were transfected with a Cdo-GFP vector and subjected to immunostaining with a Cadherin antibody to label the membrane and confocal microscopy. Cdo-GFP proteins were found at the cell membrane and intracellular compartments in both cell types. However, the signals of Cdo-GFP and Cadherin were partially superimposed at the membrane in control cells, whereas Cdo-GFP and Cadherin signals did not largely overlap at the membrane in Stx4-depleted cells (Fig. 6a). Next, we assessed whether the amount of Cdo at the cell surface is rescued by Stx4 in Cdo-depleted C2C12 cells. C2C12/shCdo cells were transfected with the Stx4 expression vector, and 24 h later, cells were analyzed by surface biotinylation. At this condition, Cdo was decreased in both pcDNA and Stx4-transfected C2C12/shCdo cells, compared to the control C2C12/pSuper cells (Fig. 6c). Consistently, the biotinylated Cdo levels were decreased in control C2C12/shCdo cells while Stx4 overexpression restored the biotinylated Cdo levels in C2C12/shCdo cells to the control C2C12/pSuper cells.

These data suggest that Stx4 enhances Cdo translocation to the cell surface thereby stimulating Cdo-mediated p38 activation and myoblast differentiation.

Next, we have assessed whether Cdo is involved in GLUT4 trafficking to the cell surface mediated by Stx4. To do so, C2C12/pSuper or C2C12/shCdo cells were induced to differentiate for 1 day and analyzed for the surface biotinylation of GLUT4. The biotinylated GLUT4 levels were decreased in Cdo-depleted cells, while total GLUT4 levels did not alter (Fig. 6d). Interestingly, GLUT4 interaction with Stx4 was decreased in Cdo-depleted C2C12 cells, without affecting the total expression levels of these proteins (Fig. 6e). To assess the effect of Cdo on glucose uptake in C2C12 myoblasts, cells were stably transfected with control, Cdo, or shCdo expression vectors and treated with insulin and a fluorescent glucose analog 2-NBDG. Overexpression of Cdo in C2C12 cells generally resulted in a twofold increase of 2-NBDG uptake, while Cdo depletion reduced the level of 2-NBDG uptake to about 71 % in C2C12/shCdo cells, relative to that of control cells (Fig. 6f). Taken together, these data suggest that Stx4 regulates Cdo protein levels at the cell surface thereby enhancing the promyogenic signal triggered by Cdo, such as p38MAPK. In turn, this signaling appears to be critical for GLUT4 translocation to the cell membrane mediated by Stx4.

Discussion

In the skeletal muscle, the role of Stx4 in glucose uptake through stimulation of GLUT4 translocation to the cell membrane in response to insulin has been well documented [19]. The fact that GLUT4 expression and translocation, likely via Stx4, exclusively induced in differentiating muscle cells [33] suggests a potential link between promyogenic signaling pathways and Stx4/GLUT4 activation to promote myoblast differentiation. Our current study suggests that Stx4 plays a critical role for myoblast differentiation. Overexpression or knockdown of Stx4 enhances or inhibits myogenic differentiation via regulation of promyogenic signaling molecules Cdo and p38. Stx4 and Cdo interact physically in differentiating myoblasts, and this interaction is mediated by the t-SNARE domain of Stx4, which is critical for the promyogenic function of Stx4. Through this interaction, Stx4 appears to regulate translocation of Cdo to the plasma membrane. It is noteworthy that Stx4 depletion results in a specific decrease in Cdo protein at the cell surface without altering N-Cadherin levels which can also interact with Cdo and promote myoblast differentiation. Vesicle transport is regulated via multiple steps including formation of vesicles or tubular intermediates, movement of vesicles towards the target compartments, tethering/docking with the acceptor membrane, and fusion of the lipid bilayers [34]. For the membrane trafficking, the specific interaction of



membrane tethering and fusion is critical. Small GTPases of the Rab family are important in the stage of vesicle tethering, and SNAREs might mediate membrane fusion [34]. The mechanism how some SNAREs function in several trafficking steps and substitute for other SNAREs [35] is still unclear. In this study, we show that Cdo is a new SNARE-binding protein and directly interacts with Stx4 that regulates its cell surface localization. Considering the relatively short half-life of Cdo protein (~2 h) [36], the

increased levels of Stx4 in differentiating myoblasts might ensure fast and continuous membrane trafficking of Cdo to promote myogenic differentiation. The interaction of Stx4 and Cdo can be detected already at D0 before the differentiation initiation. Considering cell adhesion signaling is an important regulator for myogenic differentiation and both Cdo and Stx4 are induced by the high cell density at D0, Cdo and Stx4 might be required for the initial stage of myogenic differentiation. In consistent with this notion,

overexpression of Cdo [1] or Stx4 accelerates differentiation while depletion of these genes delays it. Thus, it is conceivable that Cdo-mediated signaling might be regulated via two sequential steps. At D0 with cell adhesion signaling, Cdo is induced and translocated to the cell surface. Subsequently, the removal of serum might relieve Cdo-mediated signaling from inhibition by growth factor signaling resulting in activation of Cdo-mediated signaling and induction of myoblast differentiation. Currently, it is unclear whether growth factor signaling directly affects Cdo-mediated signaling.

In muscle and fat cells, insulin stimulates the delivery of GLUT4 from an intracellular location to the cell surface, where it facilitates glucose uptake thereby controlling the plasma glucose levels [37]. Insulin-stimulated GLUT4 translocation seems to be regulated mainly via activation of phosphatidylinositol 3-kinase (PI3-kinase) and the AKT pathway [38]. Muscle contraction and hypoxia associated with exercise have been shown to regulate glucose uptake mainly via AMP-activated protein kinase and the CAMKII pathway [39]. In addition, p38 has been shown to regulate GLUT4 activity and glucose uptake during L6 myoblast differentiation, and inhibition of p38 reduces GLUT4 translocation and glucose uptake [33]. Previously, we have shown that the Cdo's promyogenic function is mainly mediated by p38 which in turn activates post-translationally myogenic bHLH transcription factors, such as MyoD [26]. Stx4 overexpression and knockdown increased and reduced p38 activities, respectively (Fig. 5a, b). However, it appears that Stx4-mediated p38 activation requires Cdo, since Cdo-deficient myoblasts exhibit a decrease in the level of p-p38 and the membrane-resident GLUT4 proteins (Fig. 5c, d and Fig. 6d). Other components of Cdo-multiprotein complexes, including Boc, neogenin, and Cadherins, might be also regulated through membrane trafficking by SNARE proteins, since their expression is upregulated during myogenic differentiation [8], though Stx4 appears not to be regulating translocation of Cadherins. Interestingly, Stx4 overexpression in C2C12/shCdo cells restores the level of Cdo at the cell surface to the similar levels of the control C2C12/pSuper cells. Thus, it is likely that Stx4 might restore differentiation of Cdo-knockdown C2C12 cells via enhancing translocation of the residual Cdo. In addition, Stx4 might also regulate the translocation of other components of the Cdo-multiprotein complex such as Boc or neogenin which can stimulate Cdo-mediated myogenic differentiation. However, we cannot exclude the possibility that Stx4 may stimulate translocation of other cell membrane protein which can also activate p38 and induce myogenic differentiation. Further study will be required for elucidation of detailed mechanisms.

Cdo knockdown resulted in decreased interaction between Stx4 and GLUT4, and overexpression or knockdown

of Cdo increases or decreases glucose uptake, respectively. These data suggest that Cdo-mediated p38MAPK activation may trigger Stx4 and Glut4 interaction which may trigger GLUT4 translocation to plasma membrane and glucose uptake in myoblast differentiation (Fig. 6e, f). Thus, it is conceivable that Stx4 induces translocation of Cdo first which in turn activates p38MAPK leading to the activation of Glut4 and glucose uptake during myoblast differentiation [33]. The expression of deletion mutants for the Syntaxin or t-SNARE domain responsible for Cdo binding had a dominant negative effect on MHC expression further supporting for the importance of interaction between Stx4 and Cdo and Stx4 function for its promyogenic function (Fig. 4b). It appears that the Stx4-mediated Cdo activation induces specifically p38MAPK activation since overexpression or knockdown of Stx4 did not affect the activity of another promyogenic kinase AKT which can be also upregulated by Cdo/APPL1 complexes [15]. This result suggests that the surface translocation of Cdo may not be required for AKT activation and further studies are needed to understand the detailed mechanism.

Microtubule dynamics have been shown to play an essential role in trafficking of vesicles and protein transports mediated by Stxs [40]. We have previously reported that Cdo interacts with Stim1 to regulate calcium-mediated signaling which is critical for induction of myoblast differentiation [41]. Interestingly, Stim1 can also interact with the microtubule plus end tracking protein EB1 that stabilizes the microtubule structure thereby regulating the interaction between endoplasmic reticulum and cell membrane [42]. Thus, it is plausible that Cdo localization may be regulated by a transport network involving Stim1/EB1/microtubule and Stx4 at the cell membrane. Further studies will determine whether components of vesicle trafficking and microtubule organization are involved in regulation of Cdo proteins and myogenesis. In the original yeast two-hybrid screening, Stx1B was identified as an interacting protein for Cdo [13]. Stx1 is a central component of the neuronal SNARE complex and believed to play an essential role in neurotransmitter exocytosis [17]. In addition, Stx1B has been implicated in neuronal survival [43, 44]. In our previous studies, Cdo has been shown to regulate neuronal differentiation via p38MAPK activation [45]; a similar mechanism may be applied to regulation of neuronal differentiation. The defined underlying mechanism by which Cdo and Stx1B may regulate neuronal differentiation will be addressed in the future.

Conclusions

In conclusion, we have examined the roles of Stx4 in myoblast differentiation. Overexpression or knockdown of Stx4 enhances or inhibits myogenic differentiation, respectively, via regulation of promyogenic signaling molecules Cdo and p38MAPK. Stx4 and Cdo interact physically in

differentiating myoblasts, and this interaction is mediated by the t-SNARE domain of Stx4, which is critical for the promyogenic function of Stx4. Stx4 depletion decreases specifically the cell surface resident Cdo, and the level of cell surface resident GLUT4 and interaction between GLUT4 and Stx4 are declined in Cdo-depleted cells. Therefore, interaction with Cdo and regulation of its cell surface localization by Stx4 are necessary for myogenic differentiation. In turn, this signaling appears to be critical for GLUT4 translocation to the cell membrane by Stx4 and glucose uptake.

Additional files

Additional file 1: Figure S1. Knockdown of Stx4. Control or shStx4 expression vector transfected C2C12 cells were analyzed by immunoblotting with antibodies to Stx4 and pan-Cadherin as a loading control. The relative knockdown levels of Stx4 to pan-Cadherin is quantified and added under the blot.

Additional file 2: Figure S2. Phosphorylation of AKT in Stx4-depleted or Stx4-overexpressed C2C12 cells. C2C12 cells were transfected with control, Stx4, or shStx4 expression vectors, and the lysates were analyzed for the levels of phospho-AKT (p-AKT) relative to total AKT.

Abbreviations

Cdo: cell adhesion molecule-related, down-regulated by oncogenes; DM: differentiation medium; GLUT4: glucose transporter type 4; GM: growth medium; MHC: myosin heavy chain; p38MAPK: p38 mitogen-activated protein kinase; SNAREs: soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor proteins.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MY participated in the design of the study; acquisition, assembly, analysis, and interpretation of data; and drafting the initial manuscript. BGK participated in the collection and assembly of data. SJL has been involved in drafting the manuscript and analyzed the data. HJJ participated in the collection and assembly of data and revised critically for important intellectual content. JP, DWS, YK, HL, and JWH provided guidance and helped edit the manuscript. JSK and GUB conceived the study, participated in its coordination, provided guidance with data interpretation, and revised critically for important intellectual content and final approval of the manuscript. All authors read and approved the final manuscript.

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