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Terminal differentiation program of skeletal myogenesis is negatively regulated by *O*-GlcNAc glycosylation

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ABSTRACT

Background: *O*-Linked β-*N*-acetylglucosaminylation (*O*-GlcNAcylation) on the Ser/Thr residue of nucleocytoplasmic proteins is a dynamic post-translational modification found in multicellular organisms. More than 500 proteins involved in a wide range of cellular functions, including cell cycle, transcription, epigenesis, and glucose sensing, are modified with *O*-GlcNAc. Although it has been suggested that *O*-GlcNAcylation is involved in the differentiation of cells in a lineage-specific manner, its role in skeletal myogenesis is unknown.
Methods and results: A myogenesis-dependent drastic decrease in the levels of *O*-GlcNAcylation was found in mouse C2C12 myoblasts. The global decrease in *O*-GlcNAcylation was observed at the earlier stage of myogenesis, prior to myoblast fusion. Genetic or pharmacological inactivation of *O*-GlcNAcase blocked both the myogenesis-dependent global decrease in *O*-GlcNAcylation and myoblast fusion. Although inactivation of *O*-GlcNAcase affected neither cell-cycle exit nor cell survival in response to myogenic stimulus, it perturbed the expression of myogenic regulatory factors. While the expression of *myod* and *myf5* in response to myogenic induction was not affected, that of *myogenin* and *mrf4* was severely inhibited by the inactivation of *O*-GlcNAcase.
Conclusion: These results indicate that the terminal differentiation program of skeletal myogenesis is negatively regulated by *O*-GlcNAcylation.
General significance: *O*-GlcNAcylation is involved in differentiation in a cell lineage-dependent manner, and a decrease in *O*-GlcNAcylation may have a common role in the differentiation of cells of muscle lineage.

1. Introduction

O-Linked β-*N*-acetylglucosaminylation (*O*-GlcNAcylation) on the serine or threonine residue of nucleocytoplasmic proteins is a ubiquitous post-translational modification found in the multicellular organisms studied to date [1]. Its addition and removal are dynamically regulated by *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase, respectively [1]. More than 500 proteins, including those involved in the regulation of the cell cycle [2,3], transcription [1,4], epigenesis [5,6], stress response [7,8], and glucose sensing [9,10], have now been identified as *O*-GlcNAcylated. Thus *O*-GlcNAcylation is thought to be an important modulator of various cellular functions throughout life. In addition, it has been reported that OGT is essential for mouse ES cell viability [11]. Tissue-specific mutations of *ogt* in mice results in apoptosis in T cells and neurons, and growth arrest in fibroblasts [12]. Hence, *O*-GlcNAcylation is thought to be not only essential for the development of mammals, but also to be involved in cells in a tissue-specific manner at later stages.

Recently, the involvement of *O*-GlcNAcylation for differentiation has been demonstrated in several cell lines. During ascorbic acid-induced osteoblastic differentiation, mouse MC3T3-E1 preosteoblasts show a global increase in *O*-GlcNAcylation, and the transcriptional activity of Runx2, an essential transcription factor for osteoblast differentiation, is positively regulated by *O*-GlcNAcylation [13]. In human HL60 promyelocytes, an increase in *O*-GlcNAcylation of a histone lysine methyltransferase, MLL5, facilitates retinoic acid-induced granulopoiesis through methylation of histone H3K4 [14]. The expression pattern of *O*-GlcNAcylated proteins in mouse embryonic neuroepithelial cells, which are rich in neural stem cells, is quite different from that in adult brain tissues [15]. During spontaneous cardiogenesis of mouse ES cells, a global decrease in *O*-GlcNAcylation is involved in cardiomyocyte development [16]. Although *O*-GlcNAcylation of C/EBPβ, a key transcription factor of

Abbreviations: FBS, fetal bovine serum; HRP, horseradish peroxidase; MHC, myosin heavy chain; MRF, myogenic regulatory factor; *O*-GlcNAcylation, *O*-linked β-*N*-acetylglucosaminylation; OGT, *O*-GlcNAc transferase; PVDF, polyvinylidene difluoride

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adipogenesis at the earlier stage, negatively regulates DNA binding activity of C/EBPβ [17], mouse 3T3-L1 preadipocytes show a drastic increase in global *O*-GlcNAcylation during adipogenesis at the later stage [18]. These lines of evidence suggest that *O*-GlcNAcylation is involved in differentiation in a cell lineage-dependent manner.

In this study, we demonstrate that mouse C2C12 myoblasts show a drastic decrease in the levels of *O*-GlcNAcylation during serum reduction-induced skeletal myogenesis. Inhibition of the myogenesis-dependent decrease in *O*-GlcNAcylation resulted in suppression of both the expression of muscle-specific genes such as myosin heavy chain (MHC), and myoblast fusion. Furthermore, we show that the myogenesis-dependent decrease in *O*-GlcNAcylation was required for expression of the myogenic regulatory factors (MRFs), such as *myogenin* and *mrf4*, but not for exit from the cell cycle. These results indicate that the skeletal myogenic program is negatively regulated by *O*-GlcNAcylation.

2. Materials and methods

2.1. Materials

Anti-*O*-GlcNAc (CTD110.6), anti-OGT (AL25), and anti-*O*-GlcNAcase (345) antibodies were kind gifts from Dr. Gerald W. Hart (Johns Hopkins University School of Medicine, Baltimore, MD). Anti-β-actin (I-19) and anti-myogenin (F5D) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MHC (MY-32) antibody was obtained from Sigma-Aldrich (St. Louis, MO), and anti-*O*-GlcNAc (RL2) antibody from Affinity Bioreagents (Golden, CO). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG and HRP-anti-rabbit IgG antibodies were obtained from GE Healthcare (Buckinghamshire, UK), and HRP-anti-goat IgG antibody from R & D systems (Minneapolis, MN). Thiamet G was obtained from Cayman Chemical Co. (Ann Arbor, MI).

2.2. Cell culture

C2C12 myoblasts (DS Pharma Biomedical, Osaka, Japan) were grown in DMEM supplemented with heat-inactivated 10% fetal bovine serum (FBS) (growth medium). To induce terminal differentiation, C2C12 myoblasts were plated at a density of 3×10^5 cells/9 cm^2 onto the dish coated with BD Matrigel (BD Biosciences, Bedford, MA) and cultured in growth medium. After 24 h, growth medium was replaced by DMEM supplemented with heat-inactivated 2% horse serum (differentiation medium), and the cells were re-fed differentiation medium every other day.

2.3. Protein extraction and Western blotting

Cells were resuspended in 100 μL of 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and protease inhibitor cocktail (Nacalai Tesque Inc., Kyoto, Japan), incubated for 15 min, and centrifuged at 13,200 rpm for 15 min. The clarified supernatants were used for Western blotting. Total protein concentration of the sample was measured by a BCA Protein Assay Kit (Pierce, Rockford, IL). For detection of the proteins in Western blots, 3–10 μg of each sample was run on SDS-polyacrylamide gel, electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA), and subjected to immunodetection using an appropriate primary antibody. Proteins were visualized by HRP-conjugated secondary antibody and enhanced chemiluminescence.

2.4. May–Grünwald Giemsa staining

Cells were fixed with cold methanol for 15 min and permeabilized with 1% Triton X-100 in PBS for 5 min. After two washes with phosphate-buffered saline (pH 7.2), cells were stained with May–Grünwald stain for 5 min, washed with phosphate-buffered saline (pH 6.4), and re-stained with Giemsa stain for 20 min. As a morphological parameter of myotube formation, the fusion index was calculated as the number of nuclei residing in cells containing three or more nuclei, divided by the total number of nuclei in May–Grünwald Giemsa stained cells.

2.5. Proliferation and cytotoxicity assays

To monitor proliferation or cytotoxicity, cells were seeded in triplicate into 96-well plates at a density of 2×10^4 cells/well and cultured in growth medium. After 24 h, growth medium was replaced by differentiation medium. The cells were re-fed differentiation medium every other day.

Viable cells were counted at the indicated time points using a XTT Cell Proliferation Kit II (Roche, Mannheim, Germany). Cytotoxicity was assessed at the indicated time points using LDH Cytotoxicity Detection Kit Plus (Roche). For both assays, plates were analyzed in a microtiter plate reader at 492 nm with a reference wavelength of 620 nm.

2.6. Quantitative RT-PCR analysis

RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcription was performed using ReverTra Ace (Toyobo, Osaka, Japan) and oligo $(dT)_{15}$ primer (Merck, Rahway, NJ). Real-time polymerase chain reaction (PCR) was performed using SYBR Green I Master (Roche), and real-time detection of PCR products was carried out using a Light Cycler 488 Real-Time PCR system (Roche). β-2 Microglobulin was used as the internal control to normalize the data. Samples were quantified relative to the CT (using the $2^{-\Delta\Delta CT}$ method [19], where CT is the threshold cycle value) of the internal control. All primers used are shown in Supplementary Table 1.

2.7. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.5% Triton X-100 in PBS for 15 min. After two washes with PBS, cells were incubated with PBS containing 4% FBS for blocking, and incubated overnight at 4 °C with an appropriate primary antibody diluted in PBS containing 4% FBS. Cells were washed once with PBS and incubated with Alexa fluor 488-conjugated secondary antibody (Invitrogen) diluted in PBS for 2 h. Nuclei were then stained with DAPI, and the samples were analyzed using an epifluorescence microscope. The index of myogenin- or MHC-positive cells was calculated as the number of nuclei residing in myogenin- or MHC-positive cells divided by the total number of DAPI-stained nuclei.

2.8. Small interfering RNA (siRNA)

Mouse *O*-GlcNAcase siRNA (siGENOME SMARTpool M-059543-01-0005) and non-targeting siRNA (siGENOME D-001206-14-05) were obtained from Thermo Fisher Scientific (Waltham, MA). siRNA was transfected into C2C12 cells using the N-TER Nanoparticle siRNA Transfection System (Sigma-Aldrich) according to the manufacturer's protocol. Briefly, C2C12 cells were transiently transfected with 50 nM of *O*-GlcNAcase siRNA (siOGA) or non-targeting siRNA (control) at four time points in the process of induction of differentiation. The time points of transient siRNA transfection were 24 h before induction of differentiation, on induction, and 48 h and 96 h after induction.

3. Results

3.1. Characterization of O-GlcNAcylation in skeletal myogenic differentiation

In response to myogenic stimulus, myoblasts exit from the cell cycle, activate the skeletal myogenic program, and begin to form multinucleated myotubes [20–22]. Upon induction of differentiation, C2C12 myoblasts arrested at G_0/G_1 phase and showed elongated cell morphology on day 1 (Fig. 1A and Supplementary Fig. 1). At the same time, the expression of *myogenin*, which is a critical MRF for terminal differentiation of myoblasts [21], was detected (Fig. 1B). Both the formation of multinucleated myotubes and the expression of *mhc*, a muscle-specific gene, were observed on and after day 3 (Fig. 1A and B). To characterize *O*-GlcNAcylation in myogenic differentiation, both the levels and patterns of *O*-GlcNAcylation were monitored at intervals of 24 h during myogenesis by Western blotting using

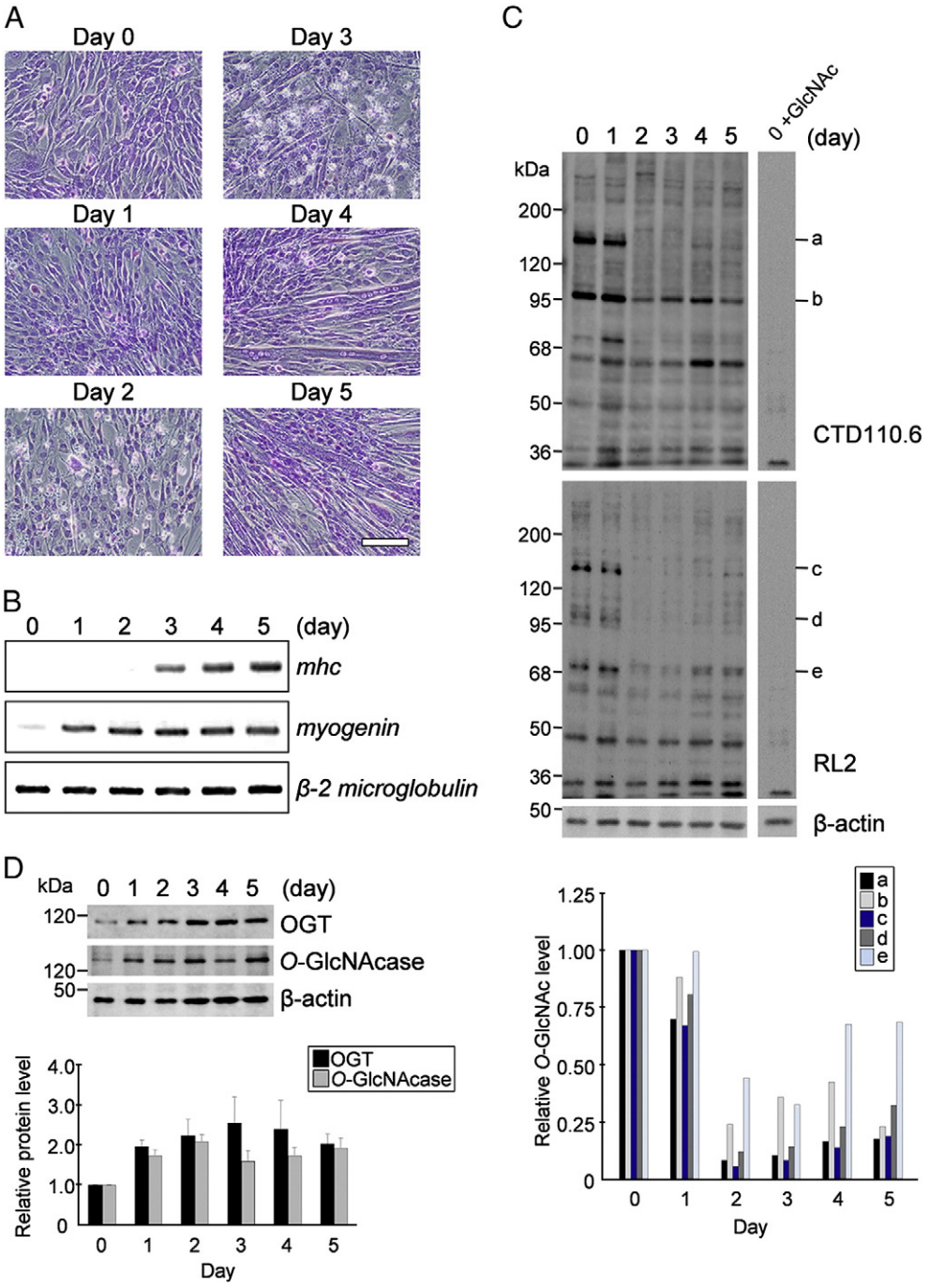

Fig. 1. Characterization of *O*-GlcNAcylation in skeletal myogenic differentiation. C2C12 myoblasts were induced to differentiate for 5 days. (A) Representative image of the differentiating cells at intervals of 24 h visualized with May–Grünwald Giemsa stain. Bar, 100 μm. (B) Representative RT-PCR analysis of the expression of *mhc* and *myogenin*. *β-2 Microglobulin* was used as loading control. (C and D) Representative Western blot of whole cell lysates probed with the indicated antibodies. β-Actin was used as loading control. In panel C, the specificity of the immunoreactivity of both CTD110.6 and RL2 was confirmed by hapten inhibition test using 0.5 M GlcNAc to day 0 sample (0 + GlcNAc). In panel C bottom, relative *O*-GlcNAc level of bands a to e obtained by densitometric analyses of Western blots was represented. In panel D bottom, relative expression levels of both OGT and *O*-GlcNAcase obtained by densitometric analyses of Western blots were represented as mean ± standard error (n = 3).

anti-*O*-GlcNAc antibodies, CTD110.6 and RL2 (Fig. 1C). It has been shown that both CTD110.6 and RL2 require *O*-GlcNAc as part of their epitopes, and that they show different specificities [23]. As shown in Fig. 1C, levels of *O*-GlcNAcylation in myoblasts were maintained until day 1 and then significantly decreased on and after day 2 in both CTD110.6 and RL2 blots, especially five protein bands which have molecular weights higher than 50 kDa (Fig. 1C, *bands a* to *e*). In addition to this decrease in levels, the cells had altered patterns of *O*-GlcNAcylated proteins on and after day 2. The immunoreactivity of both CTD110.6 and RL2 to day 0 sample was specifically competed away in the presence of 0.5 M GlcNAc (Fig. 1C, *lane 0 + GlcNAc*). This result indicates that *O*-GlcNAcylation may play a role in skeletal myogenesis before the formation of myotubes.

O-GlcNAc cycling (addition and removal) is regulated by OGT and *O*-GlcNAcase [1]. In order to understand the mechanism of the decrease in *O*-GlcNAcylation during myogenic differentiation of C2C12 cells, the expression levels of OGT and *O*-GlcNAcase were monitored at intervals of 24 h by both Western blotting and quantitative RT-PCR. As shown in Fig. 1D, expression levels of both OGT and *O*-GlcNAcase were increased on day 1 and remained constant thereafter

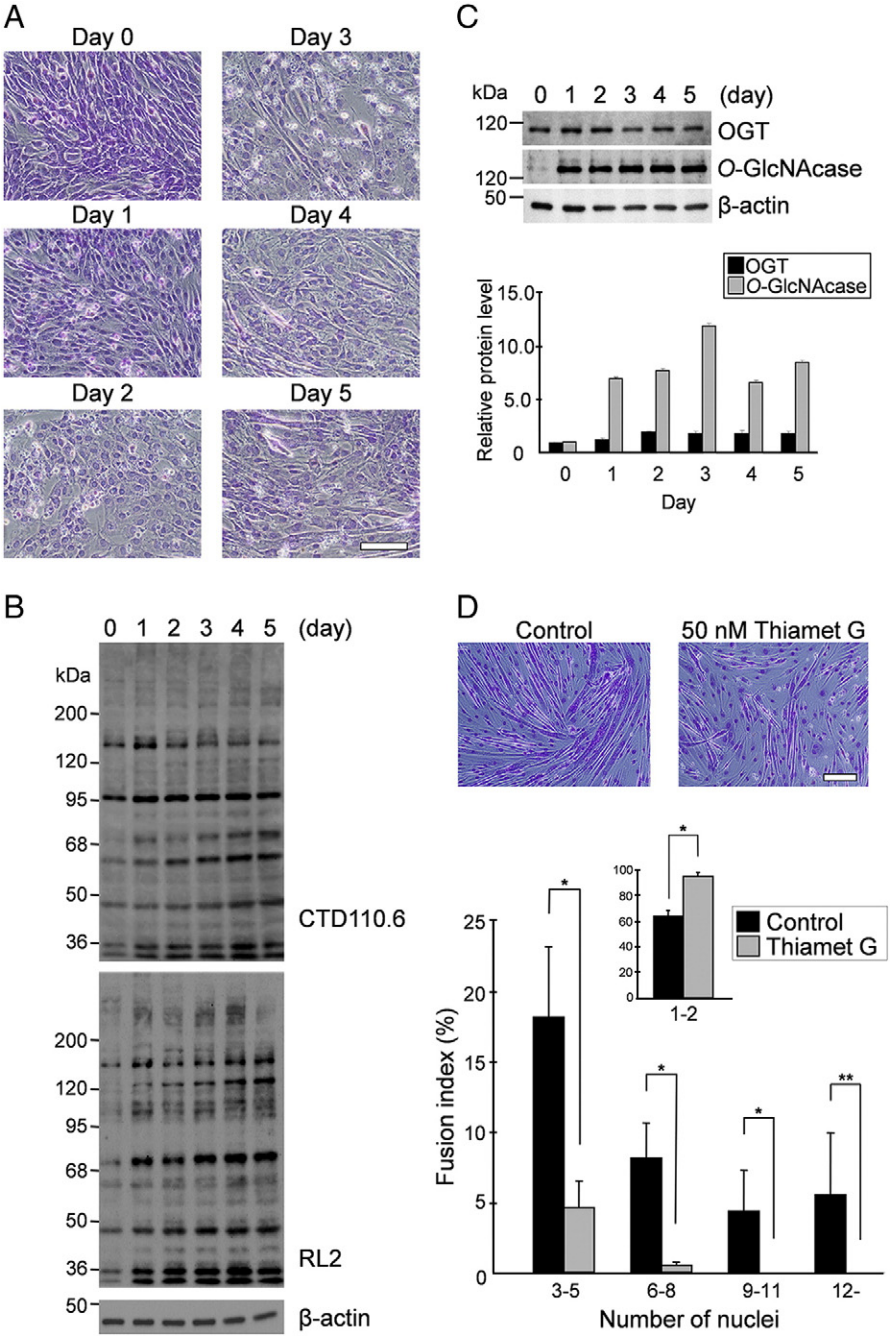

Fig. 2. Pharmacological inhibition of *O*-GlcNAcase blocks myogenic differentiation. C2C12 myoblasts were induced to differentiate in the presence of 50 nM of thiamet G for 5 days. (A) Representative image of the May–Grünwald Giemsa stained cells. Bar, 100 μm. (B and C) Representative Western blot of whole cell lysates probed with the indicated antibodies. β-Actin was used as loading control. In panel C bottom, relative expression levels of both OGT and *O*-GlcNAcase obtained by densitometric analyses of Western blots were represented as mean ± standard error (n = 3). (D) Top panels, the cells were induced to differentiate for 5 days in the absence (control) or the presence of 50 nM of thiamet G, and visualized with May–Grünwald Giemsa stain. Bar, 200 μm. Bottom panel, the fusion index was calculated as the number of nuclei residing in cells containing three or more nuclei, divided by the total number of nuclei in May–Grünwald Giemsa stained cells. Data represents mean ± standard error (n = 5, $^{*}p<0.01$, $^{**}p<0.05$).

(approximately 2-fold for OGT and 1.7-fold for *O*-GlcNAcase compared with day 0). Similarly, mRNA expression levels of both *ogt* and *o-glcnacase* were increased by several-fold on and after day 1 (Supplementary Fig. 2). This result indicates that the expression of both OGT and *O*-GlcNAcase is stimulated by the induction of differentiation, and the increase of *O*-GlcNAcase expression may participate at least in part in the decrease in *O*-GlcNAcylation during myogenesis of C2C12 cells.

3.2. Inhibition of O-GlcNAcase blocked myogenic differentiation

To examine whether the decrease in *O*-GlcNAcylation is required for skeletal myogenesis, C2C12 myoblasts were induced to differentiate in the presence of thiamet G, a very potent and highly specific inhibitor for *O*-GlcNAcase [24]. Interestingly, thiamet G-treated C2C12 myoblasts showed elongated cell morphology on day 1, but most of the cells remained mononucleated until at least day 5 (Fig. 2A). Thiamet G blocked the myogenesis-dependent global decrease in *O*-GlcNAcylation, increased the level of *O*-GlcNAcylation on day 1, and remained constant thereafter during myogenesis (Fig. 2B). Although the expression level of both OGT and its mRNA remained constant, that of both *O*-GlcNAcase and its mRNA increased significantly on and after day 1 by thiamet G treatment (Fig. 2C and Supplementary Fig. 2). The increase in *O*-GlcNAcase expression by thiamet G treatment would be a compensatory effect of cells in response to the change in *O*-GlcNAc level as reported [2, 25]. C2C12 myoblasts were induced to differentiate in the presence or the absence of thiamet G until day 5, and cells were visualized with May–Grünwald Giemsa stain (Fig. 2D). As a morphological parameter of myotube formation, the fusion index was calculated as described in the Materials and methods. As shown in Fig. 2D, the fusion index of the cells containing three or more nuclei was 5.04 ± 2.84% in the cells that differentiated in the presence of thiamet G, which is significantly lower than that of the cells that differentiated in the absence of thiamet G (control, 36.09 ± 5.14%). In particular, multinucleated myotubes containing six or more nuclei were hardly observed in the thiamet G-treated cells. Similar results were obtained by another *O*-GlcNAcase inhibitor, PUGNAc (Supplementary Fig. 3).

To confirm that the effect of *O*-GlcNAcase inhibitor on the suppression of the formation of myotubes depends on its inhibitory activity toward *O*-GlcNAcase, we performed an RNA interference of *O*-GlcNAcase and monitored its effect on the suppression of myotube formation. The dose-dependent efficiency of the transient transfection of siOGA in C2C12 myoblasts was confirmed (Supplementary Fig. 4). As shown in Fig. 3A, the expression of *O*-GlcNAcase was clearly suppressed on and after day 1 by the transient transfection of siOGA. The myogenesis-dependent global decrease in *O*-GlcNAcylation (Fig. 1C) was almost blocked by the knockdown of *O*-GlcNAcase (Fig. 3B). Similar to the *O*-GlcNAcase inhibitor-treated C2C12 cells, most of the *O*-GlcNAcase-knockdown cells remained mononucleated

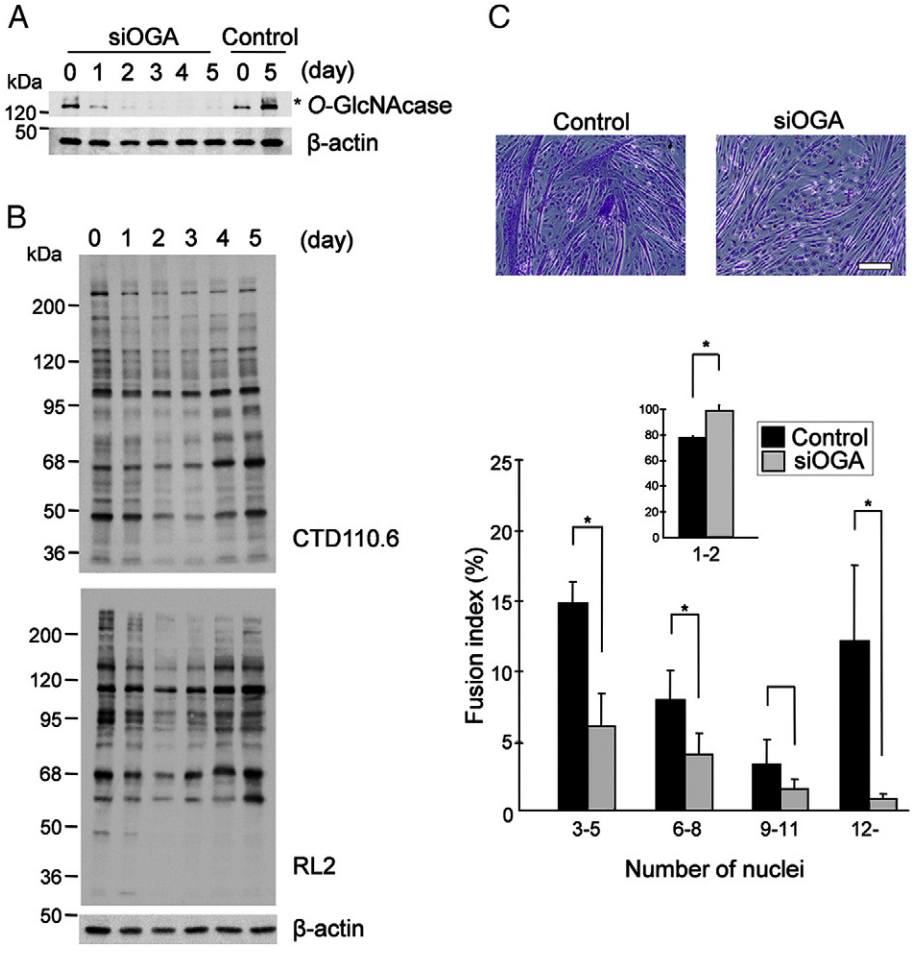

Fig. 3. Myogenic differentiation is suppressed by knockdown of *O*-GlcNAcase. *O*-GlcNAcase was knocked down during myogenic induction of C2C12 cells for 5 days. (A and B) Representative Western blot of whole cell lysates probed with the indicated antibodies. β-Actin was used as loading control. An asterisk indicates non-specific signal. (C) Top panels, *O*-GlcNAcase siRNA (siOGA) or non-targeting siRNA (control) was treated during myogenic induction, and the cells were visualized with May–Grünwald Giemsa stain. Bar, 200 μm. Bottom panel, the fusion index was calculated as mentioned in Fig. 2. Data represents mean ± standard error (n = 5, $^{*}p < 0.01$).

until at least day 5 (Fig. 3C). As shown in Fig. 3C, the fusion index of the cells containing three or more nuclei was 9.47 ± 3.85% in the *O*-GlcNAcase-knockdown cells, which is clearly lower than that of the control siRNA-treated cells (29.21 ± 4.06%). In addition, the fusion index of the multinucleated myotubes containing six or more nuclei in the *O*-GlcNAcase-knockdown cells was significantly smaller than that of the control siRNA-treated cells.

These results indicate that the myogenesis-dependent decrease in *O*-GlcNAcylation is involved in the process before the formation of myotubes.

3.3. Inhibition of O-GlcNAcase does not affect cell survival during myogenic differentiation

To address the effect of *O*-GlcNAcase inhibition on the earlier step of myogenic differentiation before the formation of myotubes, we analyzed the effect of thiamet G on the early events upon induction of myogenic differentiation. Flow cytometric analysis showed that thiamet G did not affect cell cycle arrest of differentiation-induced C2C12 cells at G_0/G_1 phase on day 1 (Supplementary Fig. 1). As shown in Fig. 4A and B, thiamet G-treated cells showed a similar change in the quantities of both living and dead cells compared with control cells until day 3, indicating that inhibition of *O*-GlcNAcase did not affect cell survival during myogenic differentiation of C2C12 cells.

During myogenic differentiation, migration and alignment of the cells precedes cell fusion to form multinucleated myotubes [22]. To assess the effect of thiamet G on cell motility, C2C12 myoblasts were cultured in growth medium in the absence or presence of thiamet G, and a Boyden chamber assay was performed. Thiamet G treatment did not affect the number of cells that traversed the membrane (Supplementary Fig. 5), indicating that inhibition of *O*-GlcNAcase may have no influence on the motility of C2C12 cells during myogenic differentiation.

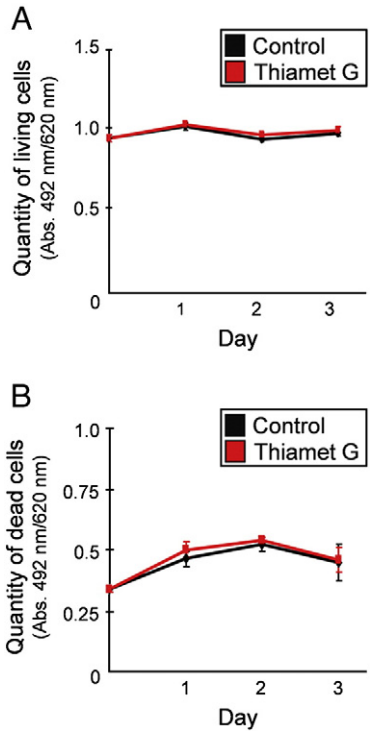

Fig. 4. Pharmacological inhibition of *O*-GlcNAcase does not affect cell survival during myogenic differentiation. C2C12 myoblasts were induced to differentiate in the absence (control) or the presence of 50 nM of thiamet G for 3 days, the quantity of living cells (A) or dead cells (B) was assessed at the indicated time points. Data represents mean ± standard error (n = 3).

3.4. Inhibition of O-GlcNAcase decreases in the appearance of both myogenin- and MHC-positive cells

Next we analyzed the effect of thiamet G on the expression of myogenin and MHC by immunostaining. As shown in Fig. 5A, the appearance of myogenin-positive cells induced to differentiate until day 5 clearly decreased in the thiamet G-treated cells. In the thiamet G-treated cells, 23.3 ± 6.9% of cells were myogenin-positive in sharp contrast with 78.2 ± 6.9% of those in the control cells. Similarly, the appearance of MHC-positive cells decreased in the thiamet G-treated cells (control cells vs thiamet G-treated ones: 43.3 ± 5.6% vs 20.4 ± 2.4%; Fig. 5B). The appearance of MHC-positive cells also decreased in the *O*-GlcNAcase-knockdown cells (control cells vs *O*-GlcNAcase-knockdown cells: 34.4 ± 5.8% vs 17.3 ± 1.3%; Supplementary Fig. 6). This result indicates that the myogenesis-dependent decrease in *O*-GlcNAcylation may be involved in the activation of the skeletal myogenic program.

3.5. Inactivation of O-GlcNAcase inhibits the expression of both myogenin and mrf4

Since the appearance of the myogenin-positive cells decreased by inhibition of *O*-GlcNAcase, we monitored the effect of thiamet G on the expression of MRFs in response to myogenic induction by quantitative RT-PCR. C2C12 myoblasts were induced to differentiate in the absence or presence of thiamet G, and the expression patterns and levels of MRFs were analyzed until day 5 (Fig. 6). The expression patterns of both *myod* and *myf5* were not affected by thiamet G treatment, although the expression levels of both genes in the thiamet G-treated cells were slightly lower than those in the control cells at every time point. In contrast, the expression levels of *myogenin* were significantly reduced compared to those in the control cells at every time point (control versus thiamet G: day 1, 6.85 ± 0.76% versus 4.22 ± 0.61%, $p = 0.009$; day 2, 20.18 ± 1.02% versus 11.42 ± 0.91%, $p = 0.0003$; day 3, 34.11 ± 3.71% versus 22.29 ± 2.36%, $p = 0.01$; day 4, 73.31 ± 7.97% versus 36.40 ± 4.10%, $p = 0.002$; day 5, 125.87 ± 14.02% versus 52.84 ± 4.48%, $p = 0.001$). The expression of *mrf4* and muscle-specific genes, such as *mhc*, *troponin t1*, *troponin t2*, and *caveolin 3*, was also inhibited by thiamet G treatment (Fig. 6 and Supplementary Fig. 7). These results indicate that the myogenesis-dependent decrease in *O*-GlcNAcylation may have a critical role in the activation of the terminal differentiation program of myoblasts in the process of *myogenin* and *mrf4* expression.

4. Discussion

The status of *O*-GlcNAcylation during terminal differentiation of two cell lines of mesenchymal origin has recently been characterized [13,18,26]. In mouse MC3T3-E1 preosteoblasts, the levels of *O*-GlcNAcylation increase during osteoblastic differentiation [13]. Similarly, the levels of *O*-GlcNAcylation increase during adipogenesis in mouse 3T3-L1 preadipocytes [18,26]. In contrast, the present study demonstrated that the levels of *O*-GlcNAcylation decrease during skeletal myogenesis in mouse C2C12 myoblasts, suggesting that the status of *O*-GlcNAcylation in terminal differentiation differs among the respective cell lines, even in those of the same origin. Interestingly, Kim et al. have reported that the levels of *O*-GlcNAcylation decreases during spontaneous cardiac development of mouse ES cells, and excessive *O*-GlcNAcylation impairs spontaneous cardiogenesis in ES cells [16]. A transition of a decrease in *O*-GlcNAcylation may have a common role in the differentiation of cells of muscle lineage.

Our data showed that the levels of *O*-GlcNAcylation begin to fall at the stage of myogenesis before the formation of myotubes. Inactivation of *O*-GlcNAcase causes suppression of MHC expression and myotube formation, suggesting that a decrease in *O*-GlcNAcylation is required for the terminal differentiation of myoblasts. Myoblasts exit from the

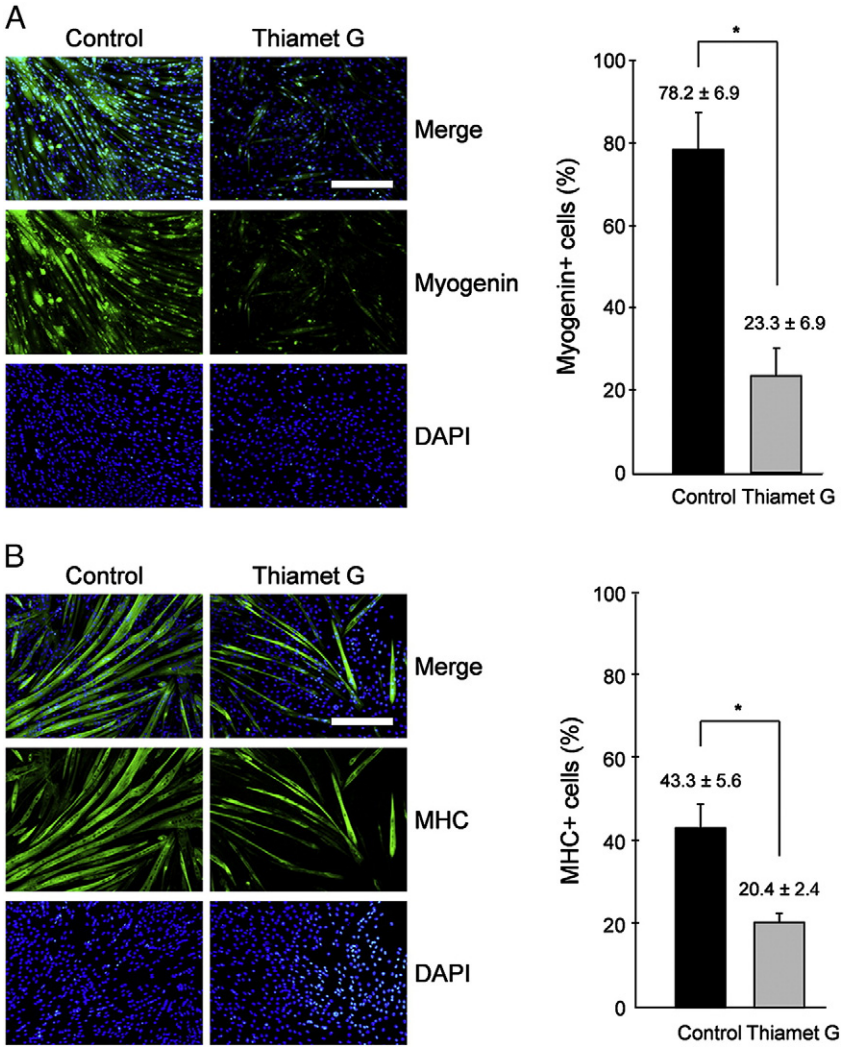

Fig. 5. Pharmacological inhibition of *O*-GlcNAcase decreases in the appearance of both myogenin- and MHC-positive cells. (A and B) Left panels, C2C12 myoblasts were induced to differentiate in the absence (control) or the presence of 50 nM of thiamet G until day 5, and stained with anti-myogenin antibody or anti-MHC antibody (green) and DAPI (blue). Bar, 200 μm. Right panel, the index of myogenin- or MHC-positive cells was calculated as the number of nuclei residing in myogenin- or MHC-positive cells divided by the total number of DAPI-stained nuclei. Data represents mean ± standard error (n = 5, $^{*}p<0.01$).

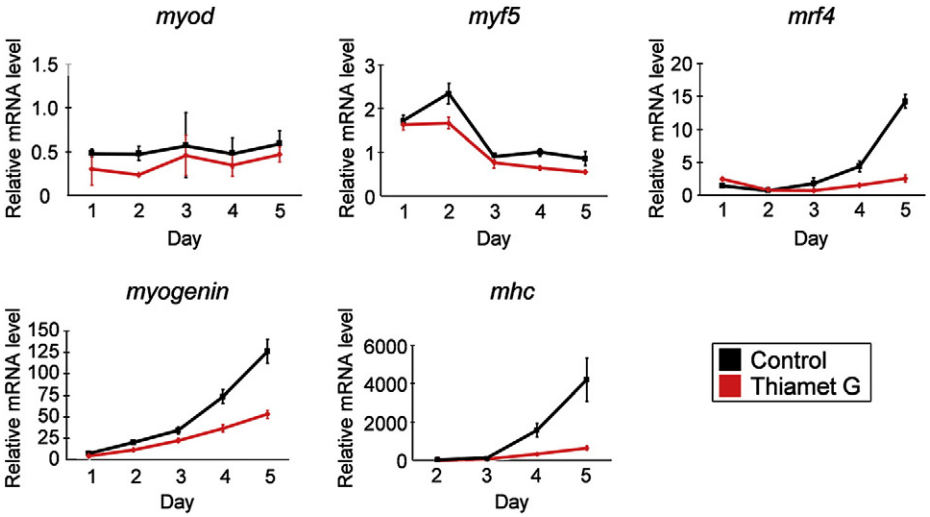

Fig. 6. Pharmacological inhibition of *O*-GlcNAcase inhibits the activation of the terminal differentiation program of myoblasts. C2C12 myoblasts were induced to differentiate for 5 days in the absence (control) or the presence of 50 nM of thiamet G, and the expression profiles of *myod*, *myf5*, *mrf4*, *myogenin*, and *mhc* were analyzed by quantitative RT-PCR. Data represents mean ± standard error (n = 3).

cell cycle prior to commitment to skeletal muscle differentiation [20]. It has been demonstrated that *O*-GlcNAc is an important regulator of the cell cycle [27], especially in mitotic progression and cytokinesis, in growing cells [2,3]. We observed that the inhibition of *O*-GlcNAcase in myoblasts does not affect exit from the cell cycle in response to myogenic stimulus. Hence *O*-GlcNAc appears not to have a critical role in the exit from the cell cycle in this case, and the decrease in *O*-GlcNAcylation is involved in myogenesis progression at the stage posterior to the exit from the cell cycle.

It has been reported that the induction of myogenic differentiation and apoptosis may use overlapping cellular mechanisms and activated caspase 3 is required for skeletal myogenesis [28]. Since *O*-GlcNAcase is a known substrate for caspase 3 [29,30], caspase 3 may cleave *O*-GlcNAcase for the induction of myogenesis. We did not, however, observe a clear caspase 3-cleaved fragment of *O*-GlcNAcase (data not shown). In addition, our data showed that the inhibition of *O*-GlcNAcase does not affect cell survival during myogenic differentiation. Therefore, the decrease in *O*-GlcNAcylation does not participate in either the regulation of caspase 3-mediated signal cascade in the promotion of myogenesis, or in cell survival during myogenesis.

The most striking finding in this study is that inactivation of *O*-GlcNAcase inhibits the expression of two MRFs, *myogenin* and *mrf4*. The process of myogenic specification and terminal differentiation are coordinated by MRFs (Myf5, MyoD, myogenin, and MRF4) [20–22]. In particular, both myogenin and MRF4 play a critical role in the terminal differentiation of myoblasts [21]. Consistent with this, the expression of muscle-specific genes such as *mhc*, *troponin t1*, *troponin t2*, and *caveolin 3* was also inhibited by the inactivation of *O*-GlcNAcase. We therefore propose for the first time that the myogenesis-dependent decrease in *O*-GlcNAcylation is required for the activation of the terminal differentiation program of myoblasts in the process of *myogenin* and *mrf4* expression. It is well-known that *myogenin* is one of the target genes of MyoD [21]. The *myogenin* promoter is also occupied by different myogenic factors, including myogenin, at different times of myogenesis [31]. It is likely that MyoD and myogenin regulate the expression of *mrf4* on terminal differentiation [32]. The transcriptional activity of MyoD has been shown to be regulated by a complex mechanism including regulation of the half-life and the subcellular localization [33]. Interestingly, the half-life of MyoD is regulated by post-translational modifications such as phosphorylation [34,35]. The transcriptional activity of MyoD may be regulated in part by *O*-GlcNAcylation, and the functional characterization of *O*-GlcNAcylation of MyoD in skeletal myogenesis is an attractive issue to be unveiled. In addition, it will be important to survey *O*-GlcNAcylation of the other regulators involved in the *myogenin* expression, including myogenin, in deciphering the complex mechanism of skeletal myogenesis.

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References

[1] Q. Zeidan, G.W. Hart, The intersections between *O*-GlcNAcylation and phosphorylation: implications for multiple signaling pathways, J. Cell Sci. 123 (2010) 13–22.
[2] C. Slawson, N.E. Zachara, K. Vosseller, W.D. Cheung, M.D. Lane, G.W. Hart, Perturbations in *O*-linked β-*N*-acetylglucosamine protein modification cause severe defects in mitotic progression and cytokinesis, J. Biol. Chem. 280 (2005) 32944–32956.
[3] K. Sakabe, G.W. Hart, *O*-GlcNAc transferase regulates mitotic chromatin dynamics, J. Biol. Chem. 285 (2010) 34460–34468.
[4] C. Butkinaree, K. Park, G.W. Hart, *O*-Linked β-*N*-acetylglucosamine (*O*-GlcNAc): extensive crosstalk with phosphorylation to regulate signaling and transcription in response to nutrients and stress, Biochim. Biophys. Acta 1800 (2010) 96–106.
[5] K. Sakabe, Z. Wang, G.W. Hart, -*N*-Acetylglucosamine (*O*-GlcNAc) is part of the histone code, Proc. Natl. Acad. Sci. USA 107 (2010) 19915–19920.
[6] D.C. Love, M.W. Krause, J.A. Hanover, *O*-GlcNAc cycling: epigenetic roles in development and epigenetics, Semin. Cell Dev. Biol. 21 (2010) 646–654.
[7] N.E. Zachara, N. O'Donnell, W.D. Cheung, J.J. Mercer, J.D. Marth, G.W. Hart, Dynamic *O*-GlcNAc modification of nucleocytoplasmic proteins in response to stress: a survival response of mammalian cells, J. Biol. Chem. 279 (2004) 30133–30142.
[8] C. Guinez, A.-M. Mir, V. Dehennaut, R. Cacan, A. Harduin-Lepers, J.-C. Michalski, T. Lefebvre, Protein ubiquitination is modulated by *O*-GlcNAc glycosylation, FASEB J. 22 (2008) 2901–2911.
[9] X. Yang, P.P. Ongusaha, P.D. Miles, J.C. Havstad, F. Zhang, W.V. So, J.E. Kudlow, R.H. Michell, J.M. Olefsky, S.J. Field, R.M. Evans, Phosphoinositide signaling links *O*-GlcNAc transferase to insulin resistance, Nature 451 (2008) 964–970.
[10] S.A. Whelan, W.B. Dias, T. Lakshmanan, M.D. Lane, G.W. Hart, Regulation of insulin receptor 1 (IRS-1)/Akt kinase mediated insulin signaling by *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) in 3T3-L1 adipocytes, J. Biol. Chem. 285 (2009) 5204–5211.
[11] R. Shafi, S.P.N. Iyer, L.G. Ellies, N. O'Donnell, K.W. Marek, D. Chui, G.W. Hart, J.D. Marth, The *O*-GlcNAc transferase gene resides on the X chromosome and is essential for embryonic stem cell viability and mouse ontogeny, Proc. Natl. Acad. Sci. USA 97 (2000) 5735–5739.
[12] N. O'Donnell, N.E. Zachara, G.W. Hart, J.D. Marth, *Ogt*-dependent X-chromosome-linked protein glycosylation is a requisite modification in somatic cell function and embryo viability, Mol. Cell. Biol. 24 (2004) 1680–1690.
[13] S.-H. Kim, Y.-H. Kim, M. Song, S.H. An, H.-Y. Byun, K. Heo, S. Lim, Y.-S. Oh, S.H. Ryu, P.-G. Suh, *O*-GlcNAc modification modulates the expression of osteocalcin via OSE2 and Runx2, Biochem. Biophys. Res. Commun. 362 (2007) 325–329.
[14] R. Fujiki, T. Chikanishi, W. Hashiba, H. Ito, I. Takada, R.G. Roeder, H. Kitagawa, S. Kato, GlcNAcylation of a histone methyltransferase in retinoic-acid-induced granulopoiesis, Nature 459 (2009) 455–461.
[15] M. Yanagisawa, R.K. Yu, *O*-Linked β-*N*-acetylglucosaminylation in mouse embryonic precursor cells, J. Neurosci. Res. 87 (2009) 3535–3545.
[16] H.-S. Kim, S.Y. Park, Y.R. Choi, J.G. Kang, H.J. Joo, W.K. Moon, J.W. Cho, Excessive *O*-GlcNAcylation of proteins suppresses spontaneous cardiogenesis in ES cells, FEBS Lett. 583 (2009) 2474–2478.
[17] X. Li, H. Molina, H. Huang, Y.-Y. Zhang, M. Liu, S.-W. Qian, C. Slawson, W.B. Dias, A. Pandey, G.W. Hart, M.D. Lane, Q.-Q. Tang, *O*-Linked *N*-acetylglucosamine modification on CCAAT enhancer-binding protein β: role during adipocyte differentiation, J. Biol. Chem. 284 (2009) 19248–19254.
[18] K. Ishihara, I. Takahashi, Y. Tsuchiya, M. Hasegawa, K. Kamemura, Characteristic increase in nucleocytoplasmic protein glycosylation by *O*-GlcNAc in 3T3-L1 adipocyte differentiation, Biochem. Biophys Res. Commun. 398 (2010) 489–494.
[19] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (2001) 402–408.
[20] M. Kitzmann, A. Fernandez, Crosstalk between cell cycle regulators and the myogenic factor MyoD in skeletal myoblasts, Cell. Mol. Life Sci. 58 (2001) 571–579.
[21] C.A. Berkes, S.J. Tapscott, MyoD and the transcriptional control of myogenesis, Semin. Cell Dev. Biol. 16 (2005) 585–595.
[22] R.S. Krauss, F. Cole, U. Gaio, G. Takaesu, W. Zhang, J.-S. Kang, Close encounters: regulation of vertebrate skeletal myogenesis by cell–cell contact, J. Cell Sci. 118 (2005) 2355–2362.
[23] F.I. Comer, K. Vosseller, L. Wells, M.A. Accavitti, G.W. Hart, Characterization of a mouse monoclonal antibody specific for *O*-linked *N*-acetylglucosamine, Anal. Biochem. 293 (2001) 169–177.
[24] S.A. Yuzwa, M.S. Macauley, J.E. Heinonen, X. Shan, R.J. Dennis, Y. He, G.E. Whitworth, K.A. Stubbs, E.J. McEachern, G.J. Davies, D.J. Vocadlo, A potent mechanism-inspired *O*-GlcNAcase inhibitor that blocks phosphorylation of tau in vivo, Nat. Chem. Biol. 4 (2008) 483–490.
[25] T.M. Gloster, W.F. Zandberg, J.E. Heinonen, D.L. Shen, L. Deng, D.J. Vocadlo, Hijacking a biosynthetic pathway yields a glycosyltransferase inhibitor within cells, Nat. Chem. Biol. 7 (2011) 174–181.
[26] T.J. Hsieh, T. Lin, P.C. Hsieh, M.C. Liao, S.J. Shin, Suppression of glutamine:fructose-6-phosphate amidotransferase-1 inhibits adipogenesis in 3T3-L1 adipocytes, J. Cell. Physiol. (2011) DOI: 10.1002/jcp.22707.
[27] V. Dehennaut, T. Lefebvre, C. Sellier, Y. Leroy, B. Gross, S. Walker, R. Cacan, J.-C. Michalski, J.-P. Vilain, J.-F. Bodart, *O*-Linked *N*-acetylglucosaminyltransferase inhibition prevents G_2/M transition in *Xenopus laevis* oocytes, J. Biol. Chem. 282 (2007) 12527–12536.
[28] P. Fernando, J.F. Kelly, K. Balazsi, R.S. Slack, L.A. Megeney, Caspase 3 activity is required for skeletal muscle differentiation, Proc. Natl. Acad. Sci. USA 99 (2002) 11025–11030.
[29] L. Wells, Y. Gao, J.A. Mahoney, K. Vosseller, C. Chen, A. Rosen, G.W. Hart, Dynamic *O*-glycosylation of nuclear and cytosolic proteins: further characterization of the nucleocytoplasmic β-*N*-acetylglucosaminidase, J. Biol. Chem. 277 (2002) 1755–1761.
[30] C. Butkinaree, W.D. Cheung, S. Park, K. Park, M. Barber, G.W. Hart, Characterization of β-*N*-acetylglucosaminidase cleavage by caspase-3 during apoptosis, J. Biol. Chem. 283 (2008) 23557–23566.

[31] Y. Ohkawa, S. Yoshimura, C. Higashi, C.G.A. Marfella, C.S. Dacwag, T. Tachibana, A.N. Imbalzano, Myogenin and the SWI/SNF ATPase Brg1 maintain myogenic gene expression at different stages of skeletal myogenesis, J. Biol. Chem. 282 (2007) 6564–6570.
[32] S.J. Tapscott, The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription, Development 132 (2005) 2685–2695.
[33] P. Ferri, E. Barbieri, S. Burattini, M. Guescini, A. D'Emilio, L. Biagiotti, P.D. Grande, A.D. Luca, V. Stocchi, E. Falcieri, Expression and subcellular localization of myogenic regulatory factors during the differentiation of skeletal muscle C2C12 myoblasts, J. Cell. Biochem. 108 (2009) 1302–1317.
[34] A. Song, Q. Wang, M.G. Goebl, M.A. Harrington, Phosphorylation of nuclear MyoD is required for its rapid degradation, Mol. Cell. Biol. 18 (1998) 4994–4999.
[35] M. Kitzmann, M. Vandromme, V. Schaeffer, G. Carnac, J.-C. Labbé, N. Lamb, A. Fernandez, Cdk1- and cdk2-mediated phosphorylation of MyoD Ser200 in growing C2 myoblasts: role in modulating MyoD half-life and myogenic activity, Mol. Cell. Biol. 19 (1999) 3167–3176.