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O-GlcNAcylation of eIF2α regulates the phospho-eIF2α-mediated ER stress response

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ABSTRACT

O-GlcNAcylation is highly involved in cellular stress responses including the endoplasmic reticulum (ER) stress response. For example, glucosamine-induced flux through the hexosamine biosynthetic pathway can promote ER stress and ER stress inducers can change the total cellular level of *O*-GlcNAcylation. However, it is largely unknown which component(s) of the unfolded protein response (UPR) is directly regulated by *O*-GlcNAcylation. In this study, eukaryotic translation initiation factor 2α (eIF2α), a major branch of the UPR, was *O*-GlcNAcylated at Ser 219, Thr 239, and Thr 241. Upon ER stress, eIF2α is phosphorylated at Ser 51 by phosphorylated PKR-like ER kinase and this inhibits global translation initiation, except for that of specific mRNAs, including activating transcription factor 4, that induce stress-responsive genes such as C/EBP homologous protein (CHOP). Hyper-*O*-GlcNAcylation induced by *O*-GlcNAcase inhibitor (thiamet-G) treatment or *O*-GlcNAc transferase (OGT) overexpression hindered phosphorylation of eIF2α at Ser 51. The level of *O*-GlcNAcylation of eIF2α was changed by dithiothreitol treatment dependent on its phosphorylation at Ser 51. Point mutation of the *O*-GlcNAcylation sites of eIF2α increased its phosphorylation at Ser 51 and CHOP expression and resulted in increased apoptosis upon ER stress. These results suggest that *O*-GlcNAcylation of eIF2α affects its phosphorylation at Ser 51 and influences CHOP-mediated cell death. This *O*-GlcNAcylation of eIF2α was reproduced in thiamet-G-injected mouse liver. In conclusion, proper regulation of *O*-GlcNAcylation and phosphorylation of eIF2α is important to maintain cellular homeostasis upon ER stress.

1. Introduction

Accumulation of misfolded proteins and disruption of calcium storage in the endoplasmic reticulum (ER) cause ER stress, which initiates the unfolded protein response (UPR). To alleviate ER stress and recover ER homeostasis, general translation is inhibited, production of ER chaperones is induced, and misfolded proteins are degraded. However, sustained ER stress leads to programmed cell death [1–3]. In response to ER stress, general translation is blocked through phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) at Ser 51 by phosphorylated PKR-like ER kinase (PERK) [4,5]. Aside from PERK, three other kinases, GCN2, PKR, and HRI, phosphorylate eIF2α upon diverse cellular stresses including amino acid deprivation [6], viral infection [7], and heme deficiency [8,9]. Phosphorylation of eIF2α at Ser 51 inhibits active 43S translation initiation complex formation and represses global protein synthesis [4], except for that of specific mRNAs, including activating transcription factor 4 (ATF4), which are involved in the induction of stress-responsive genes such as C/EBP homologous protein

Abbreviations: 1DLC–MS/MS, one-dimensional liquid chromatography/tandem mass spectrometry; ATF4, activating transcription factor 4; ATM, ataxia-telangiectasia mutated; CHOP, C/EBP homologous protein; CID, collision-induced dissociation; DTT, dithiothreitol; eIF2α, eukaryotic translation initiation factor 2α; ER, endoplasmic reticulum; ETD, electron transfer dissociation; GADD34, growth arrest and DNA damage-inducible protein; HBP, hexosamine biosynthetic pathway; HCD, high energy collision dissociation; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NCE, normalized collision energy; *O*-GlcNAc, *O*-linked β-*N*-acetylglucosamine; p-eIF2α, eukaryotic translation initiation factor 2α phosphorylated at Ser 51; PERK, PKR-like ER kinase; sWGA, succinylated wheat germ agglutinin; UPR, unfolded protein response

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(CHOP, also known as GADD153) [10]. CHOP is a transcription factor that regulates the expression of genes involved in apoptosis [11]. For instance, CHOP represses expression of the anti-apoptotic protein Bcl-2 and leads to cell death under ER stress [12].

Many nuclear, cytoplasmic, and mitochondrial proteins are dynamically modified with *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) [13–16]. In some proteins, *O*-GlcNAc modification reciprocally interplays with phosphorylation because these two modifications compete for the same serine/threonine residues (e.g., in c-myc) [17]. In addition, these two modifications can inhibit each other even when they do not occur at the same sites (e.g., in CK2 [18]) or can exist on the same protein simultaneously (e.g., in insulin receptor substrate-1 [19]). The *O*-GlcNAc modification is involved in various cellular activities including the cellular nutrient sensing pathway [20] and cellular stress responses [21]. *O*-GlcNAcylation is increased in response to diverse stresses such as heat stress, oxidative stress, and hypoxic conditions, and an increased level of *O*-GlcNAcylation acts as part of pro-survival signaling [21–23]. In recent decades, the number of reports suggesting that *O*-GlcNAcylation is highly involved in the ER stress response has increased. For example, increased flux through the hexosamine biosynthetic pathway (HBP) upon glucosamine treatment induces the ER stress marker GRP78 [24]. In addition, the total cellular level of *O*-GlcNAcylation changes in response to ER stress inducers and ER stress-induced cardiomyocyte death is attenuated by *O*-GlcNAcylation [21,23,25,26]. However, it is largely unknown which component(s) is directly regulated by *O*-GlcNAcylation under ER stress.

Here, we found that eIF2α, a major component of the UPR, is directly *O*-GlcNAcylated. This *O*-GlcNAcylation occurred at Ser 219, Thr 239, and Thr 241 of eIF2α and was tightly regulated under ER stress. *O*-GlcNAcylation of eIF2α affects its phosphorylation at Ser 51 and influences CHOP-induced apoptosis. In addition, eIF2α was *O*-GlcNAcylated in thiamet-G-injected mouse liver, concurrent with its reduced phosphorylation at Ser 51 and reduced CHOP expression. Taken together, our data suggest that *O*-GlcNAcylation of eIF2α hinders apoptotic cell death upon ER stress owing to its effects on phosphorylation of eIF2α at Ser 51 and CHOP expression.

2. Materials and methods

2.1. Cell culture, dithiothreitol (DTT), and inhibitor treatments

Human hepatocellular carcinoma HepG2 cells were grown in minimum essential medium (Gibco, Auckland, NZ). Human embryonic kidney Hek293 cells were grown in high-glucose (25 mM) Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA). All media were supplemented with 10% fetal bovine serum (HyClone, Logan, Utah) and 1% (v/v) penicillin/streptomycin (HyClone). Cells were cultured at 37 °C in 5% CO_2. Cells were treated with 2.5 mM DTT (GOLDBIO®COM), 1 μM thapsigargin (Sigma-Aldrich, St. Louis, MI), and 10 μM thiamet-G [27] for the indicated amount of time.

2.2. DNA plasmids and transfection

cDNA of human full-length eIF2α was generated by PCR and cloned into the p3XFlag-CMV™-7.1 expression vector (Sigma-Aldrich). Using wild-type Flag-eIF2α as a template, three point mutants (Flag-S219A eIF2α, Flag-T239V eIF2α, and Flag-T241V eIF2α) were generated by PCR and cloned into the p3XFlag-CMV™-7.1 expression vector. The sequences of all constructs were checked by DNA sequencing. Flag-eIF2α was subcloned into the pcDNA3.1/V5-HisA expression vector (Invitrogen). Untagged pCMV-OGT was used for transfection [28]. These DNA plasmids were transfected into cells using X-tremegene 9 DNA transfection reagent (Roche Applied Science, Basel, Switzerland) following the manufacturer's methods.

2.3. Western blotting

Thiamet-G (40 mg/kg/day) or saline solution was injected into the abdominal cavity of 8-week-old C57BL/6 mice every day for 7 days. Mouse liver was homogenized in lysis buffer containing 0.1% SDS, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM Tris–HCl, pH 7.4, and a protease inhibitor cocktail (Roche Applied Science) on ice for 1 h. HepG2 and Hek293 cells were solubilized in lysis buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, and a protease inhibitor cocktail on ice for 30 min and all lysates were centrifuged at 18,000 ×*g* for 20 min. The supernatant was collected and 4× SDS sample buffer was added. Samples were resolved on SDS-PAGE gels and transferred to nitrocellulose membrane. Immunoblotting was performed as described previously [29] using the following antibodies: anti-*O*-GlcNAc (CTD110.6; Covance Research Products, Denver, PA, USA, RL2; Thermo Scientific, USA), anti-eIF2α, anti-α-tubulin, anti-CHOP (Santa Cruz Biotechnology, CA, USA), anti-OGT, anti-Flag, anti-PARP (Sigma-Aldrich), anti-eIF2α phosphorylated at Ser 51 (p-eIF2α), anti-caspase 3 (Cell signaling), and anti-V5 (Invitrogen). All HRP-conjugated secondary antibodies were purchased from Santa Cruz Technology. Immunoreactive bands were detected using an ECL system (GE Healthcare, Bio-Science) and quantification was performed with Multi Gauge (Fuji Photo Film).

2.4. Succinylated wheat germ agglutinin (sWGA) precipitation and immunoprecipitation

For sWGA precipitation, cell lysates were incubated with agarose-sWGA (Vector Laboratories, Burlingame, CA, USA) at 4 °C overnight. For immunoprecipitation, cell lysates were incubated with Ezview™ Red anti-FLAG M2 affinity Gel (Sigma-Aldrich) or anti-V5 affinity Gel (Sigma-Aldrich) at 4 °C overnight. After rinsing four times with lysis buffer, precipitates were eluted in 2× SDS sample buffer.

2.5. Mass spectrometry (MS) to map the O-GlcNAcylation sites of eIF2α

Flag-eIF2α was overexpressed in Hek293 cells together with pCMV-OGT and immunoprecipitated with anti-FLAG M2 affinity Gel (Sigma-Aldrich). Immunopurified Flag-eIF2α was subjected to SDS-PAGE and in-gel digestion with 25 ng/μL trypsin (Promega, Madison, WI, USA) for 16 h at 37 °C. Peptides were analyzed using one-dimensional liquid chromatography/tandem mass spectrometry (1DLC–MS/MS) and identified using tandem mass spectrometry (MS/MS) with a nano-LC–MS system consisting of a nanoACQUITY UltraPerformance LC System (Waters, USA) and a LTQ Orbitrap Elite mass spectrometer (Thermo Scientific, USA) equipped with a nano-electrospray source. An autosampler was used to load 5-μL aliquots of the peptide solutions onto a C18 trap-column (i.d. 180 μm, length 20 mm, and particle size 5 μm; Waters). The peptides were desalted and concentrated on the column at a flow rate of 4 μL/min for 10 min. Thereafter, the trapped peptides were back-flushed and separated on a 150-mm home-made microcapillary column consisting of C18 (Aqua; particle size 3 μm) packed into 100-μm silica tubing with an orifice i.d. of 5 μm. The mobile phases A and B were composed of 0 and 80% acetonitrile, respectively, and each contained 0.02% formic acid and 0.5% acetic acid. The liquid chromatography (LC) gradient began with 5% B for 15 min and was sequentially ramped to 15% B over 5 min, to 50% B over 70 min, and to 95% B over 5 min, and remained at 95% B for 5 min and then at 5% B for another 5 min. The column was re-equilibrated with 5% B for 15 min before the next run. A voltage of 2.2 kV was applied to produce an electrospray. In each duty circle of mass analysis, one high-mass resolution (100,000) MS spectrum was acquired using the FT-ICR analyzer, followed by five data-dependent MS/MS scans using the linear ion trap analyzer. For MS/MS analysis, normalized collision energy (NCE, 35%) was used throughout the collision-induced dissociation (CID) phase. The mobile phases A and B were composed of 0% and 100%

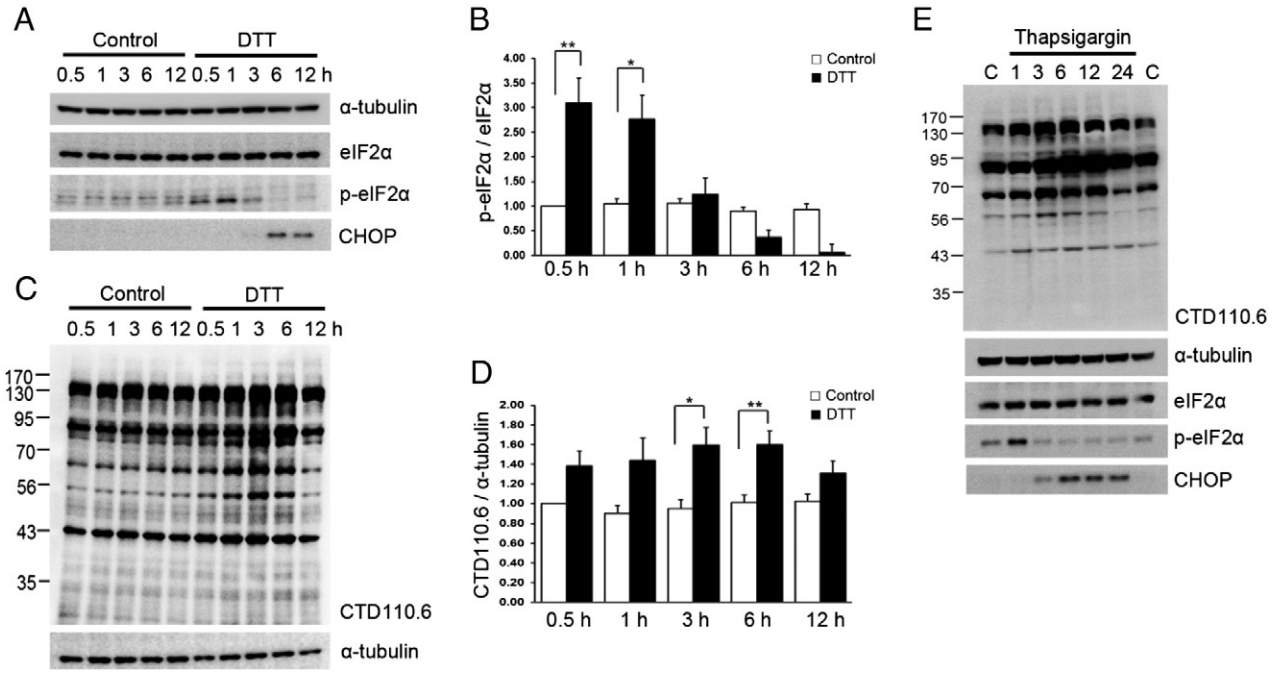


Fig. 1. Total cellular *O*-GlcNAcylation was dynamically changed in response to endoplasmic reticulum stress in HepG2 cells. (A–D) HepG2 cells were incubated in 2.5 mM dithiothreitol (DTT)-containing media for the indicated amount of time (0.5–12 h). A. Eukaryotic translation initiation factor 2α (eIF2α) phosphorylated at Ser 51 (p-eIF2α), total eIF2α, C/EBP homologous protein (CHOP), and α-tubulin were immunoblotted. B. Quantification of the level of p-eIF2α induced by DTT treatment. The band density of p-eIF2α was normalized against that of total eIF2α. C. *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) was detected using the monoclonal CTD110.6 antibody. D. Quantification of the amount of total cellular *O*-GlcNAc upon DTT treatment. The density of total bands stained by CTD110.6 antibody was normalized to the amount of α-tubulin. E. HepG2 cells were treated with 1 μM thapsigargin for the indicated amount of time (1–24 h). Lysates of control cells (C) were prepared at 1 and 24 h. *O*-GlcNAc, p-eIF2α, total eIF2α, CHOP, and α-tubulin were immunoblotted. Statistical significance was determined by the two-tailed Student's t test ($^{*}p < 0.05$, $^{**}p < 0.01$). All results are given as the mean ± standard error based on at least three independent experiments.

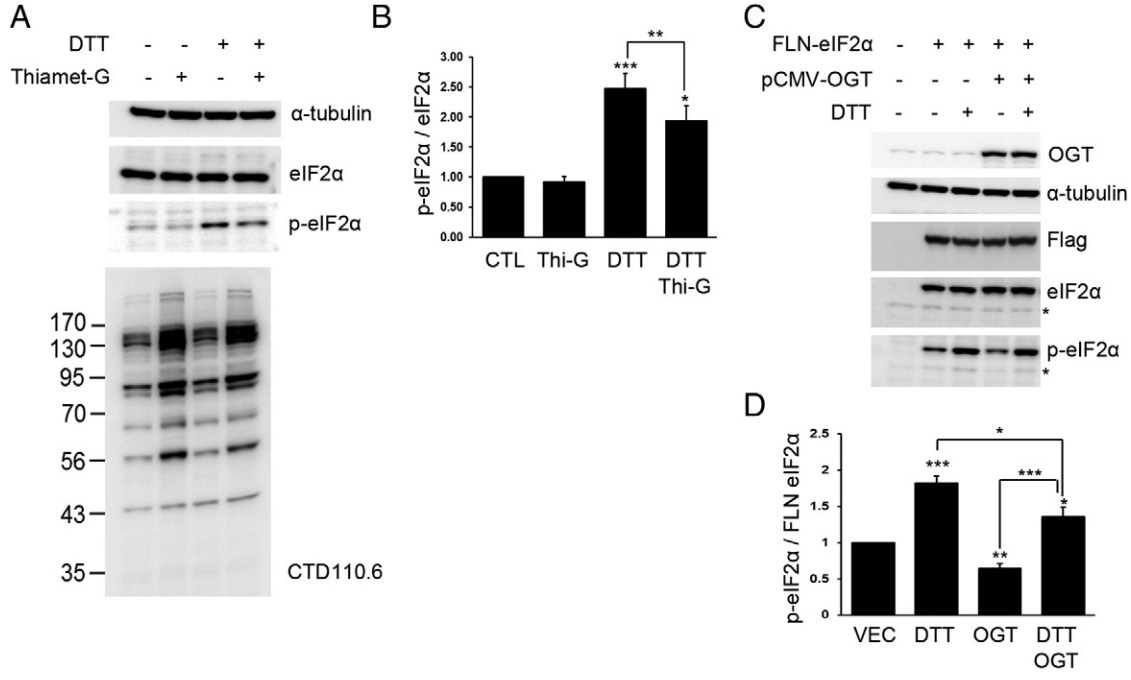


Fig. 2. *O*-GlcNAcase inhibitor (thiamet-G) treatment or *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) transferase (OGT) overexpression reduced phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) at Ser 51. A. HepG2 cells were treated with 10 μM thiamet-G with or without 2.5 mM dithiothreitol (DTT) and then endogenous eIF2α phosphorylated at Ser 51 (p-eIF2α), total eIF2α, *O*-GlcNAc, and α-tubulin were immunoblotted. Cells were pretreated with thiamet-G for 8 h and harvested after DTT treatment for 1 h. B. Quantification of the amount of p-eIF2α normalized to the amount of total eIF2α. C. Flag-tagged eIF2α (FLN-eIF2α) was overexpressed in Hek293 cells together with pCMV-OGT. Thereafter, cells were treated with 2.5 mM DTT for 1 h. Total eIF2α, p-eIF2α, Flag, OGT, and α-tubulin were immunoblotted. Endogenous eIF2α (denoted by asterisks) was detected using anti-eIF2α and anti-p-eIF2α antibodies. D. Quantification of the amount of Flag-p-eIF2α normalized to the amount of Flag. Statistical significance was determined by the two-tailed Student's t test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$). All results are given as the mean ± standard error based on at least three independent experiments.

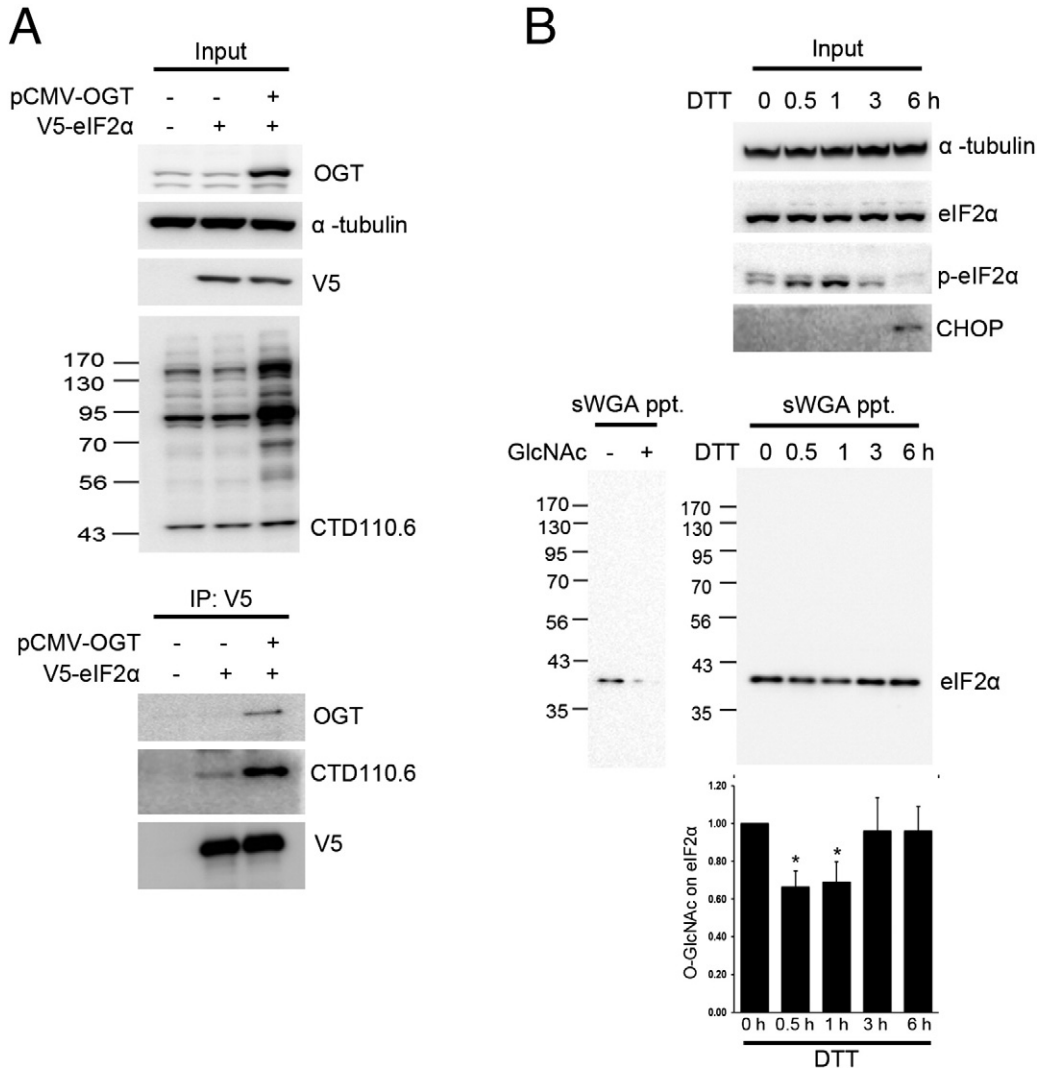


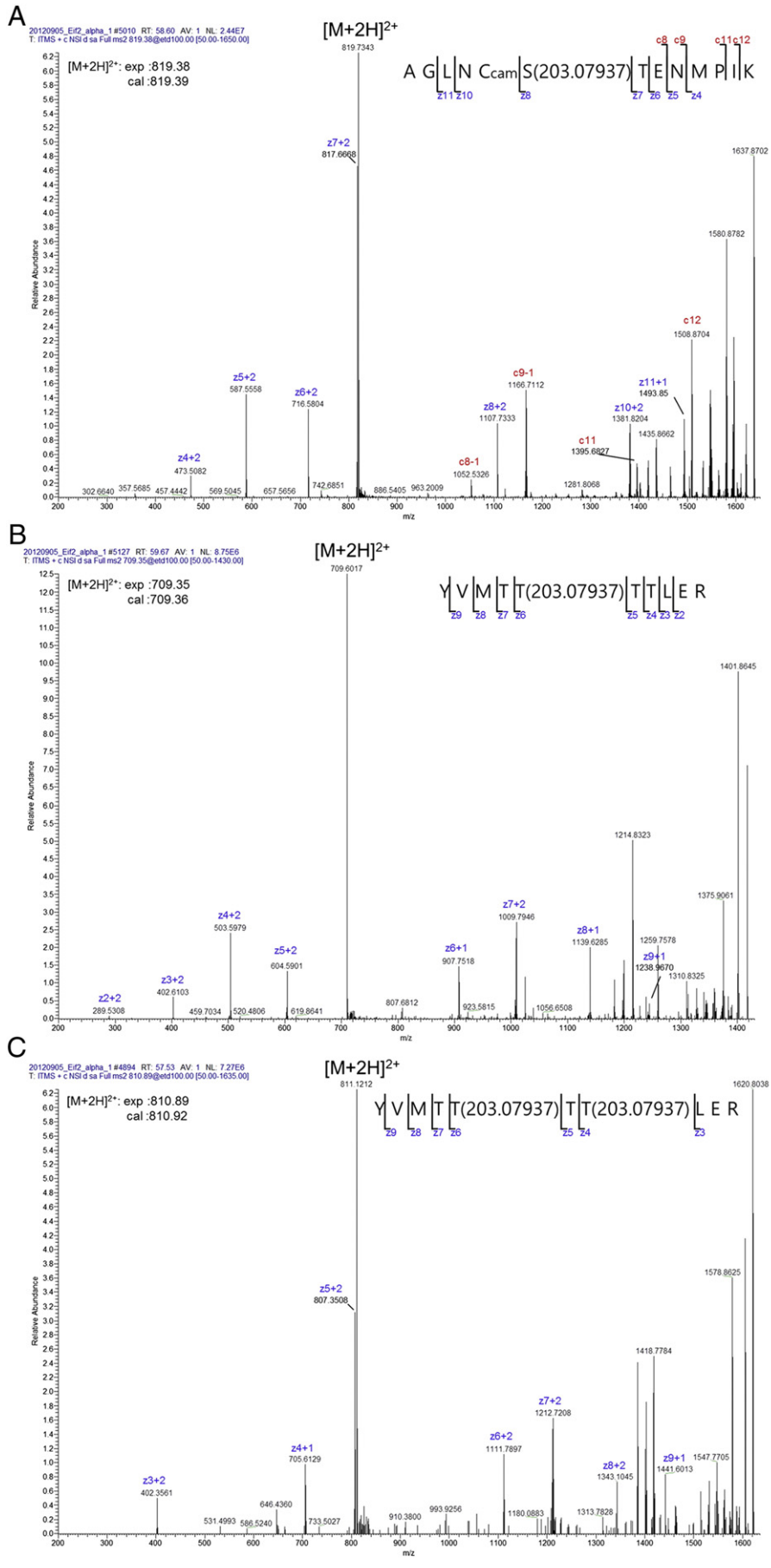
Fig. 3. Eukaryotic translation initiation factor 2α (eIF2α) was directly *O*-GlcNAcylated and this was dynamically regulated by dithiothreitol (DTT) treatment. A. V5-tagged eIF2α (V5-eIF2α) was overexpressed in Hek293 cells together with pCMV-OGT. After immunoprecipitation with V5 affinity Gel, *O*-GlcNAcylated V5-eIF2α was immunoblotted. B. HepG2 cells were cultured in 2.5 mM DTT-containing media for the indicated amount of time (0–6 h) and endogenous eIF2α was precipitated with succinylated wheat germ agglutinin (sWGA). eIF2α phosphorylated at Ser 51 (p-eIF2α), C/EBP homologous protein (CHOP), total eIF2α, α-tubulin, and eIF2α precipitated with sWGA were immunoblotted. For competition, 25 mM N-acetylglucosamine (GlcNAc) was added to control lysates during incubation with sWGA agarose. The amount of eIF2α precipitated with sWGA was quantified and normalized to the amount of total eIF2α. Statistical significance was determined by two-tailed Student's t test (*$p < 0.05$). All results are given as the mean ± standard error based on at least three independent experiments.

acetonitrile, respectively, and each contained 0.1% formic acid. The LC gradient began with 5% B for 1 min and was sequentially ramped to 20% B over 5 min, to 50% B over 80 min, and to 95% B over 5 min, and remained at 95% B for 9 min and then at 5% B for another 5 min. The column was re-equilibrated with 5% B for 15 min before the next run. A voltage of 2.0 kV was applied to produce an electrospray. During chromatographic separation, the LTQ Orbitrap Elite was operated in a data-dependent mode. The MS data were acquired using the following parameters: three data-dependent triple MS/MS scans (CID–high energy collision dissociation (HCD)–electron transfer dissociation (ETD)) per full scan. CID and ETD scans were acquired in linear trap quadrupole with two-microscan averaging. Full scans and HCD scans were acquired in Orbitrap at a resolution of 1,200,000 and 15,000, respectively, with two-microscan averaging. A NCE of 35% was used in all MS/MS scans and an activation time of 30, 20, and 100 ms was used in CID, HCD, and ETD, respectively, ±1 Da isolation window. Previously fragmented ions were excluded for 60 s. In CID–HCD–ETD triple scans, each selected parent ion was first fragmented by CID, by HCD, and then by ETD.

2.6. Protein identification

MS/MS spectra were analyzed using various software analysis protocols with the eIF2α database (UniProt P05198). ProLuCID was used to identify peptides, a precursor mass error of 25 ppm, and a fragment ion mass error of 600 ppm. Trypsin was selected as the enzyme, with

Fig. 4. Eukaryotic translation initiation factor 2α (eIF2α) was *O*-GlcNAcylated at Ser 219, Thr 239, and Thr 241. (A–C) Flag-eIF2α was overexpressed in Hek293 cells together with pCMV-OGT. Purified Flag-eIF2α was subjected to SDS-PAGE and in-gel digestion with trypsin for 16 h at 37 °C. Peptides were analyzed using one-dimensional liquid chromatography/tandem mass spectrometry and identified using tandem mass spectrometry with a nano-LC–MS system consisting of a nanoACQUITY UltraPerformance LC System and a LTQ Orbitrap Elite mass spectrometer equipped with a nano-electrospray source. Ser 219, Thr 239, and Thr 241 were identified as *O*-linked β-*N*-acetylglucosamine-modified sites. D. Empty vector (VEC), wild-type Flag-eIF2α (WT), Flag-S219A eIF2α (S219A), Flag-T239V eIF2α (T239V), and Flag-T241V eIF2α (T241V) were transfected into Hek293 cells together with pCMV-OGT. Each construct was immunoprecipitated with anti-FLAG M2 affinity Gel, and *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) was immunoblotted using two anti-*O*-GlcNAc antibodies: CTD110.6 and RL2. E. Empty vector (VEC) and Flag-S51A eIF2α (S51A) were transfected into Hek293 cells together with pCMV-OGT and immunoprecipitation was performed as described above.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cal :819.39
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cal :709.36
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C
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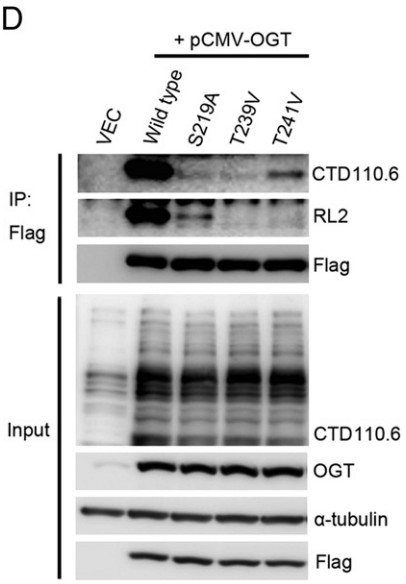
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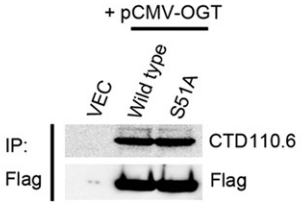
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one potential missed cleavage. *N*-acetylhexosamine modification (+203.07937) at serine and threonine residues by *O*-glycosylation and carbamidomethylation at cysteine residues were chosen as static modifications. Oxidation at methionine residues was chosen as the variable modification. Only peptides with high confidence were included. All MS/MS spectra identified as modified peptides were manually confirmed.

2.7. Statistical analysis

Statistical significance was determined by two-tailed Student's t test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$). All results are given as the mean ± standard error based on at least three independent experiments.

3. Results

3.1. Total cellular O-GlcNAcylation and phosphorylation of eIF2α in HepG2 cells reciprocally change upon DTT treatment

A change in total cellular *O*-GlcNAcylation is a pro-survival mechanism to protect cells in response to various cellular stresses, and treatment with an ER stress inducer, such as tunicamycin, increases the level of total *O*-GlcNAcylation [21–23]. To determine whether ER stress induced a change in *O*-GlcNAcylation in our system, HepG2 cells were incubated in 2.5 mM DTT-containing media for 0.5–12 h and phosphorylation of eIF2α at Ser 51, a well-known ER stress marker, was examined using a specific antibody. p-eIF2α appeared at 0.5 h and its level started to decline at 3 h (Fig. 1A and B). The level of CHOP was also increased by DTT after 6 h (Fig. 1A). The level of total cellular *O*-GlcNAcylation, as determined using the monoclonal CTD110.6 antibody, was increased by DTT treatment (Fig. 1C and D). Interestingly, the level of p-eIF2α decreased as the level of total cellular *O*-GlcNAcylation increased. These reciprocal changes between the levels of p-eIF2α and total cellular *O*-GlcNAcylation were also observed when HepG2 cells were treated with another ER stress inducer, thapsigargin (Fig. 1E). *O*-GlcNAcylation reportedly interplays with phosphorylation on some proteins [17–19]. Thus, we considered the possibility that *O*-GlcNAcylation may regulate phosphorylation of eIF2α.

3.2. Hyper-O-GlcNAcylation reduces phosphorylation of eIF2α at Ser 51

To determine if the level of total cellular *O*-GlcNAcylation affects phosphorylation of eIF2α at Ser 51, HepG2 cells were treated with the potent *O*-GlcNAcase inhibitor thiamet-G [27] with or without DTT. Induction of eIF2α phosphorylation at Ser 51 upon DTT treatment was decreased when cells were pretreated with thiamet-G (Fig. 2A and B). OGT overexpression is another powerful method to enhance total cellular *O*-GlcNAcylation. Flag-tagged eIF2α was transfected into Hek293 cells with or without pCMV-OGT. Flag-eIF2α was phosphorylated at Ser 51 upon DTT treatment; however, this was reduced by OGT overexpression (Fig. 2C and D). When OGT was overexpressed, the phosphorylation on Flag-eIF2α was slightly reduced. Possibly, overexpression of OGT modified *O*-GlcNAc on Flag-eIF2α more, which might affect basal level of phosphorylation of Flag-eIF2α at Ser 51 without DTT treatment (Fig. 2C and D). These results indicate that phosphorylation of eIF2α at Ser 51 is directly or indirectly affected by the *O*-GlcNAcylation status of the cell.

3.3. eIF2α is O-GlcNAcylated and this is tightly regulated during the ER stress response

To determine whether eIF2α is directly *O*-GlcNAcylated, we transfected V5-tagged eIF2α into Hek293 cells together with pCMV-OGT and performed immunoprecipitation using anti-V5 affinity Gel. Immunoprecipitated V5-eIF2α was modified with *O*-GlcNAc and *O*-GlcNAcylation of V5-eIF2α was increased by OGT overexpression. OGT co-immunoprecipitated with V5-eIF2α (Fig. 3A). As shown in Fig. 2, hyper-*O*-GlcNAcylation reduced phosphorylation of eIF2α at Ser 51. This implies that *O*-GlcNAcylation of eIF2α affects its phosphorylation at Ser 51. To confirm the *O*-GlcNAcylation of endogenous eIF2α and to determine whether this modification is tightly regulated under ER stress, similar to phosphorylation of eIF2α at Ser 51 [4,5], we precipitated sWGA from HepG2 cells following DTT treatment for 0.5–6 h. Endogenous eIF2α was detected in sWGA precipitates, and this was abolished following treatment with 25 mM GlcNAc (Fig. 3B). The amount of eIF2α in sWGA precipitates was reduced following treatment with DTT for a short amount of time (0.5 or 1 h), when eIF2α was highly phosphorylated at Ser 51, and this was recovered after treatment with DTT for 3 h, when the level of eIF2α phosphorylation at Ser 51 began to decrease (Fig. 3B). These results show that eIF2α is directly *O*-GlcNAcylated. In addition, well controlled *O*-GlcNAcylation and phosphorylation of eIF2α are important for the proper ER stress response.

3.4. eIF2α is O-GlcNAcylated at Ser 219, Thr 239, and Thr 241

To identify the *O*-GlcNAcylation sites of eIF2α, Flag-eIF2α was overexpressed in Hek293 cells together with pCMV-OGT and immunoprecipitated with anti-FLAG M2 affinity Gel. Immunopurified Flag-eIF2α was subjected to SDS-PAGE and in-gel digestion with trypsin for 16 h at 37 °C. Peptides were analyzed using 1DLC–MS/MS. As a result, we found three *O*-GlcNAcylated sites (Ser 219, Thr 239, and Thr 241) in eIF2α (Fig. 4A–C). To verify that all three residues were *O*-GlcNAcylated, we generated three point mutants (Flag-S219A eIF2α,

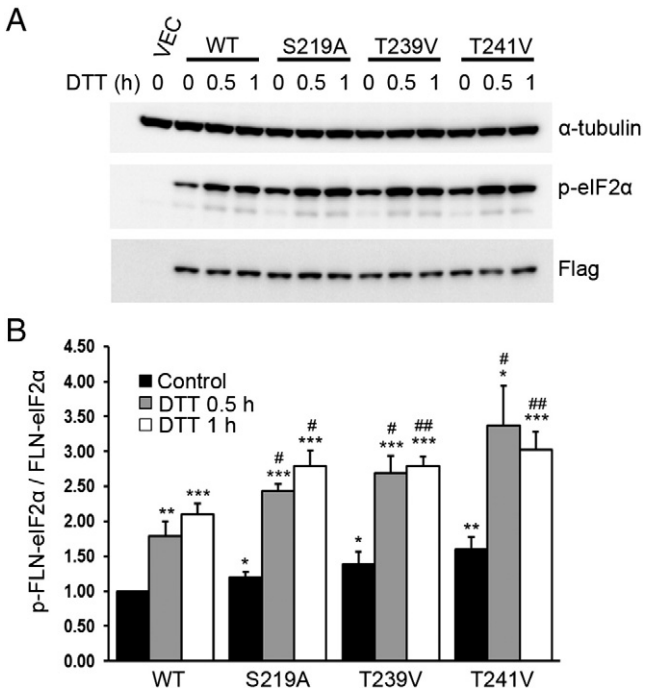

Fig. 5. Phosphorylation of Ser 51 was higher in eukaryotic translation initiation factor 2α (eIF2α) *O*-linked β-*N*-acetylglucosamine site point mutants than in wild-type eIF2α following dithiothreitol (DTT) treatment. A. Empty vector (VEC), wild-type Flag-eIF2α (WT), Flag-S219A eIF2α (S219A), Flag-T239V eIF2α (T239V), and Flag-T241V eIF2α (T241V) were overexpressed in Hek293 cells. Thereafter, cells were treated with 2.5 mM DTT for 0.5 or 1 h. eIF2α phosphorylated at Ser 51 (p-eIF2α) was detected with a specific antibody. Flag, p-eIF2α, and α-tubulin were immunoblotted. B. Quantification of the amount of p-eIF2α. Each value was normalized to the amount of Flag. Statistical significance was determined by two-tailed Student's t test (*, $^{\#}p < 0.05$, **, $^{\#\#}p < 0.01$, $^{***}p < 0.005$). *Indicates a significant difference compared to control wild-type Flag-eIF2α. $^{\#}$Indicates a significant difference between wild-type Flag-eIF2α and mutants at each time point. All results are given as the mean ± standard error based on at least three independent experiments.

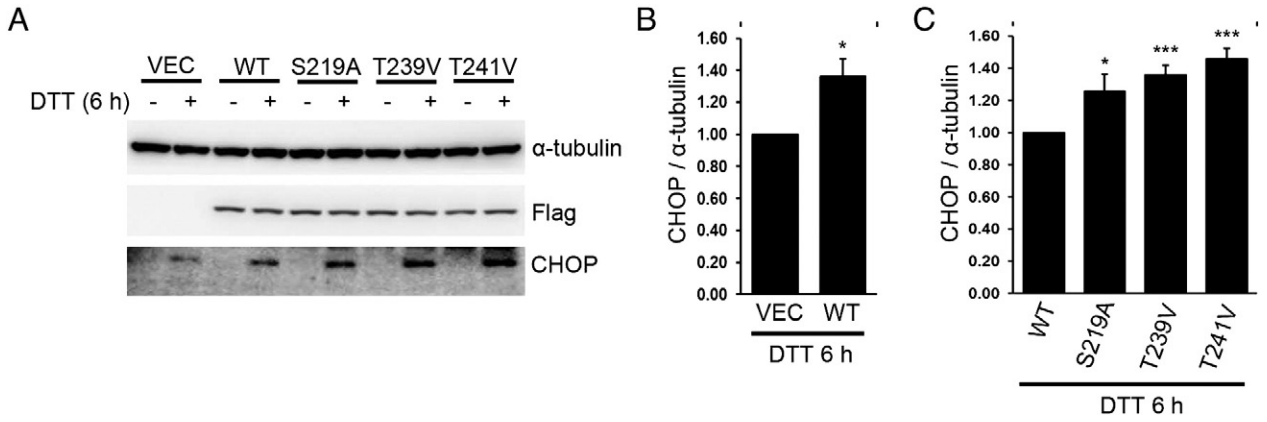

Fig. 6. C/EBP homologous protein (CHOP) expression was higher in Hek293 cells expressing eukaryotic translation initiation factor 2α (eIF2α) *O*-linked β-*N*-acetylglucosamine site point mutants than in cells expressing wild-type Flag-eIF2α. A. Empty vector (VEC), wild-type Flag-eIF2α (WT), Flag-S219A eIF2α (S219A), Flag-T239V eIF2α (T239V), and Flag-T241V eIF2α (T241V) were overexpressed in Hek293 cells. Thereafter, cells were treated with 2.5 mM dithiothreitol (DTT) for 6 h. Flag, CHOP, and α-tubulin were immunoblotted. B. Quantification of the amount of CHOP in empty vector-transfected cells (VEC) and wild-type Flag-eIF2α-transfected cells (WT) normalized to the amount of total α-tubulin. C. Quantification of CHOP in cells overexpressing wild-type Flag-eIF2α (WT), Flag-S219A eIF2α (S219A), Flag-T239V eIF2α (T239V), and Flag-T241V eIF2α (T241V). Values were normalized to the amount of Flag. Statistical significance was determined by two-tailed Student's t test ($^{*}p < 0.05$, $^{***}p < 0.005$). All results are given as the mean ± standard error based on at least three independent experiments.

Flag-T239V eIF2α, and Flag-T241V eIF2α) by single amino acid substitution from serine to alanine or from threonine to valine and confirmed whether these three point mutants were *O*-GlcNAcylated when overexpressed with pCMV-OGT. As expected, all three immunopurified point mutants exhibited a lower level of *O*-GlcNAcylation than wild-type Flag-eIF2α (Fig. 4D). As mentioned above, phosphorylation

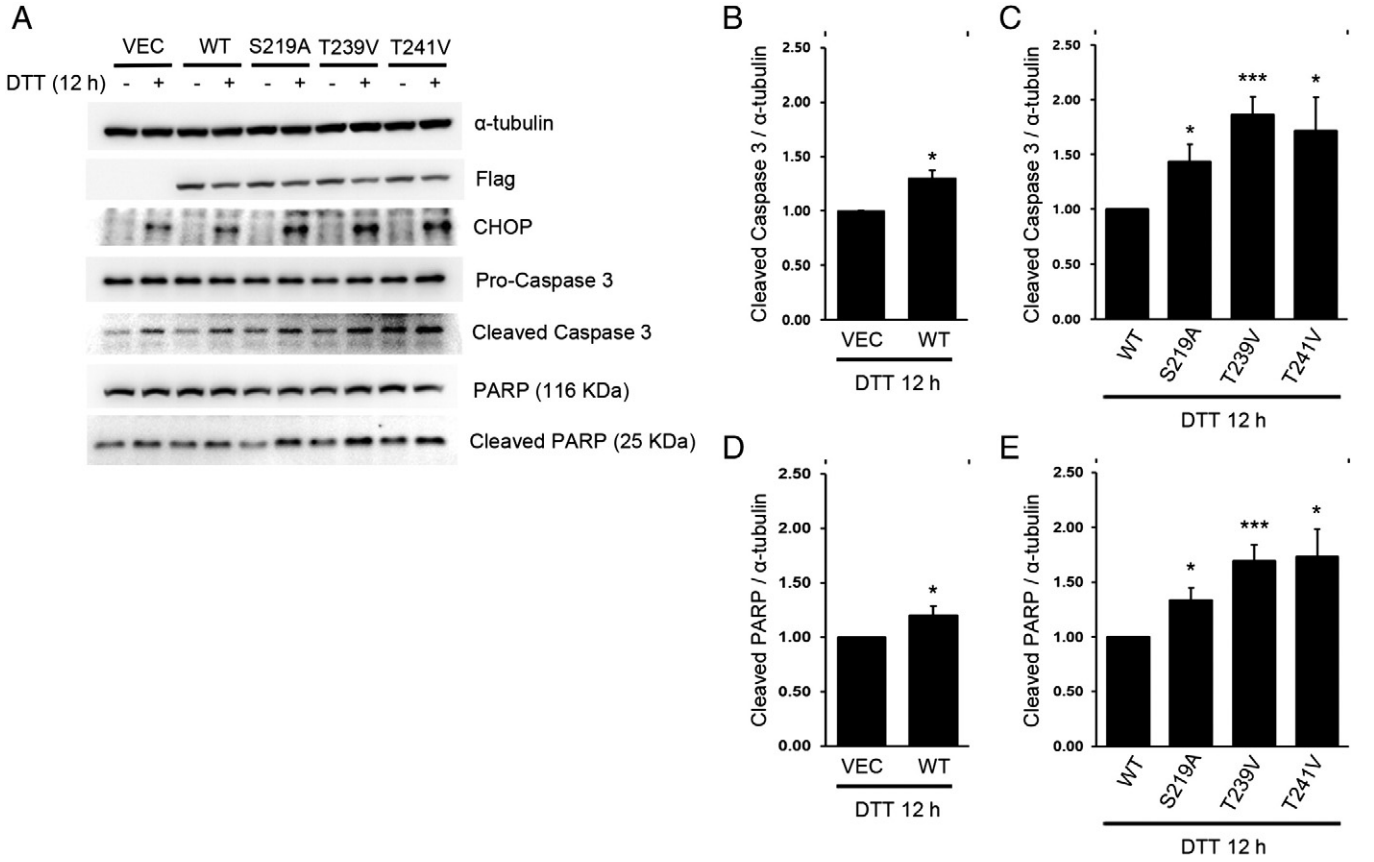

Fig. 7. The level of apoptosis was higher in Hek293 cells expressing eukaryotic translation initiation factor 2α (eIF2α) *O*-linked β-*N*-acetylglucosamine site point mutants than in cells expressing wild-type Flag-eIF2α. A. Empty vector (VEC), wild-type Flag-eIF2α (WT), Flag-S219A eIF2α (S219A), Flag-T239V eIF2α (T239V), and Flag-T241V eIF2α (T241V) were overexpressed in Hek293 cells. Thereafter, cells were treated with 2.5 mM dithiothreitol (DTT) for 12 h. Flag, C/EBP homologous protein (CHOP), pro-caspase 3, cleaved caspase 3, PARP, cleaved PARP, and α-tubulin were immunoblotted. B. Quantification of the amount of cleaved caspase 3 in empty vector-transfected cells (VEC) and wild-type Flag-eIF2α-transfected cells (WT) normalized to the amount of total α-tubulin. C. Quantification of the amount of cleaved caspase 3 in cells overexpressing wild-type Flag-eIF2α (WT), Flag-S219A eIF2α (S219A), Flag-T239V eIF2α (T239V), and Flag-T241V eIF2α (T241V). Values were normalized to the amount of Flag. D. Quantification of the amount of cleaved PARP in empty vector-transfected cells (VEC) and wild-type Flag-eIF2α-transfected cells (WT) normalized to the amount of total α-tubulin. E. Quantification of the amount of cleaved PARP in cells overexpressing wild-type Flag-eIF2α (WT), Flag-S219A eIF2α (S219A), Flag-T239V eIF2α (T239V), and Flag-T241V eIF2α (T241V). Values were normalized to the amount of Flag. Statistical significance was determined by two-tailed Student's t test ($^{*}p < 0.05$, $^{***}p < 0.005$). All results are given as the mean ± standard error based on at least three independent experiments.

of eIF2α at Ser 51 was decreased under hyper-*O*-GlcNAcylation (Fig. 2). For this reason, we checked whether the Ser 51 residue was also *O*-GlcNAcylated. We generated Flag-S51A eIF2α, a point mutant in which Ser 51 was substituted to alanine. Flag-S51A eIF2α was overexpressed in Hek293 cells together with pCMV-OGT and *O*-GlcNAcylation was investigated. Unlike the aforementioned three point mutants, Flag-S51A eIF2α exhibited a similar level of *O*-GlcNAcylation as wild-type Flag-eIF2α (Fig. 4E). Thus, we conclude that eIF2α is *O*-GlcNAcylated at three amino acids (Ser 219, Thr 239, and Thr 241), but not at Ser 51.

3.5. Disruption of the O-GlcNAcylation of eIF2α induces phosphorylation of Ser 51 and CHOP expression and ultimately leads to apoptosis

Hyper-*O*-GlcNAcylation decreased phosphorylation of eIF2α at Ser 51 under ER stress (Fig. 2). We hypothesized that if *O*-GlcNAcylation of eIF2α hinders phosphorylation of Ser 51, the interruption of *O*-GlcNAc sites of eIF2α would increase phosphorylation of Ser 51 under ER stress. To test this hypothesis, we overexpressed each of the three Flag-eIF2α constructs with point mutations in *O*-GlcNAc sites (Flag-S219A eIF2α, Flag-T239V eIF2α, and Flag-T241V eIF2α) in Hek293 cells and examined the level of phosphorylation at Ser 51 following DTT treatment for 0.5 or 1 h. All three point mutants exhibited enhanced phosphorylation at Ser 51 (Fig. 5A). The amount of p-eIF2α was normalized to the amount of exogenous eIF2α (Fig. 5B). DTT-induced phosphorylation of eIF2α at Ser 51 reduced *O*-GlcNAcylation of eIF2α (Fig. 3B), whereas hyper *O*-GlcNAcylation reduced phosphorylation of eIF2α at Ser 51 (Fig. 2). Taken together, these results demonstrate that *O*-GlcNAcylation of eIF2α affects its phosphorylation at Ser 51.

When eIF2α is phosphorylated under ER stress, global translation is blocked with the exception of that of specific mRNAs, such as ATF4, that induce stress-responsive genes including CHOP [4,10]. To determine whether *O*-GlcNAcylation of eIF2α is involved in CHOP expression, we first examined whether CHOP was expressed upon eIF2α overexpression. Induction of CHOP expression required DTT-induced ER stress and could not be achieved by eIF2α overexpression alone (Fig. 6A). Therefore, we overexpressed eIF2α with mutated *O*-GlcNAcylation sites in Hek293 cells, treated these cells with DTT for 6 h, and measured the level of CHOP. Overexpression of wild-type Flag-eIF2α increased the level of CHOP in comparison to empty vector transfection; therefore, we concluded that overexpressed eIF2α was working properly in the cell (Fig. 6A and B). Cells expressing each of the eIF2α point mutants (Flag-S219A eIF2α, Flag-T239V eIF2α, and Flag-T241V eIF2α) had higher levels of CHOP than wild-type Flag-eIF2α-expressing cells (Fig. 6A and C). These results indicate that *O*-GlcNAcylation of eIF2α is involved in CHOP expression during the ER stress response.

CHOP suppresses expression of the anti-apoptotic protein Bcl-2 and induces apoptosis under ER stress [12]. Therefore, we determined whether induction of apoptosis is enhanced in cells expressing Flag-eIF2α point mutants (Flag-S219A eIF2α, Flag-T239V eIF2α, and Flag-T241V eIF2α). As apoptosis markers, we checked the amounts of cleaved caspase 3 and cleaved PARP, which is a substrate for active caspase 3 [30]. To robustly induce apoptosis, we treated cells with DTT for 12 h. Under this condition, the level of CHOP was higher in cells expressing Flag-eIF2α point mutants than in cells expressing wild-type Flag-eIF2α. Levels of cleaved caspase 3 and cleaved PARP were up-regulated in cells expressing Flag-eIF2α point mutants (Fig. 7A). Upon DTT treatment, the level of cleaved caspase 3 and cleaved PARP were higher in wild-type Flag-eIF2α-expressing cells than in empty vector-expressing cells and were higher in cells expressing Flag-eIF2α point mutants than in wild-type Flag-eIF2α-expressing cells (Fig. 7B–E). Taken together, we conclude that *O*-GlcNAcylation of eIF2α protect cells under ER stress by blocking CHOP-induced apoptosis via regulation of eIF2α phosphorylation at Ser 51.

3.6. O-GlcNAcylation of eIF2α is increased and phosphorylation of eIF2α at Ser 51 and the level of CHOP are reduced in thiamet-G-injected mouse liver

To determine whether our results are reproducible in vivo, we examined *O*-GlcNAcylation of eIF2α in liver lysates of thiamet-G-

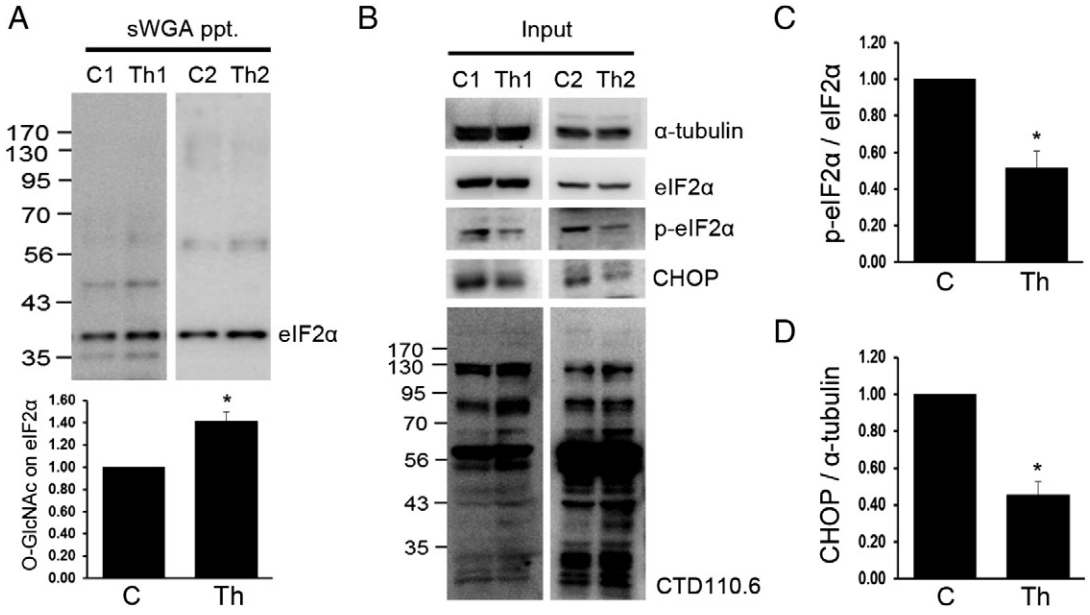

Fig. 8. Liver extracts of thiamet-G-injected mice exhibited increased *O*-GlcNAcylation of eukaryotic translation initiation factor 2α (eIF2α) and reduced phosphorylation of eIF2α at Ser 51 and C/EBP homologous protein (CHOP) expression. Vehicle (C1 and C2)- or thiamet-G (Th1 and Th2)-injected mouse livers were collected and lysates were obtained by homogenization. A. Endogenous eIF2α was precipitated with succinylated wheat germ agglutinin (sWGA) from liver lysates. Four milligrams of liver lysates was used for sWGA precipitation. The amount of eIF2α precipitated with sWGA was quantified and normalized to the amount of total eIF2α. B. Total eIF2α, eIF2α phosphorylated at Ser 51 (p-eIF2α), CHOP, *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc), and α-tubulin were immunoblotted in liver lysates. C. Quantification of p-eIF2α. The amount of p-eIF2α was normalized to the amount of total eIF2α. D. Quantification of CHOP. The amount of CHOP was normalized to the amount of total α-tubulin. Statistical significance was determined by two-tailed Student's t test (*$p < 0.05$). All results are given as the mean ± standard error based on at least three independent experiments.

injected mice. Vehicle (C1 and C2) or thiamet-G (Th1 and Th2) was injected into 8-week-old C57BL/6 mice every day for 1 week at a dose of 40 mg/kg/day. As expected, thiamet-G injection increased the level of total *O*-GlcNAcylation in liver lysates (Fig. 8B). Using sWGA precipitation, we confirmed that *O*-GlcNAcylation of eIF2α was enhanced in thiamet-G-injected mouse liver (Fig. 8A). Interestingly, levels of p-eIF2α and CHOP were reduced in thiamet-G-injected mouse liver (Fig. 8B–D). These results show that *O*-GlcNAcylation of eIF2α also occurred in vivo. In addition, they implied that *O*-GlcNAcylation of eIF2α affects its phosphorylation at Ser 51 and leads to decreased CHOP expression in vivo.

4. Discussion

In this study, DTT treatment changed the level of total cellular *O*-GlcNAcylation in HepG2 cells. DTT induced-phosphorylation of eIF2α at Ser 51 decreased as the level of total cellular *O*-GlcNAcylation increased (Fig. 1). Therefore, we wondered whether *O*-GlcNAcylation is an important post-translational modification that regulates phosphorylation of eIF2α. To investigate this, we examined phosphorylation of eIF2α at Ser 51 in the hyper-*O*-GlcNAcylation condition induced by thiamet-G treatment or OGT overexpression. DTT-induced phosphorylation of eIF2α at Ser 51 was reduced in the hyper-*O*-GlcNAcylation condition (Fig. 2). Using 1DLC–MS/MS and mutagenesis analyses, we showed that eIF2α was directly *O*-GlcNAcylated at Ser 219, Thr 239, and Thr 241. *O*-GlcNAcylation of eIF2α decreased when it was highly phosphorylated at Ser 51 by DTT treatment and was recovered as dephosphorylation occurred (Figs. 3 and 4). Mutation of the *O*-GlcNAc sites of eIF2α increased levels of phosphorylation at Ser 51 (Fig. 5), CHOP expression (Fig. 6), and apoptosis (Fig. 7). *O*-GlcNAcylation of eIF2α was also observed in extracts of thiamet-G-injected mouse liver, in which levels of p-eIF2α and CHOP were lower than in wild-type mouse liver (Fig. 8). Taken together, we demonstrated that *O*-GlcNAcylation of eIF2α is important to protect cells against ER stress-induced apoptosis because it affects phosphorylation of eIF2α at Ser 51 and controls CHOP expression.

In cells treated with ER stress inducers such as tunicamycin [23], DTT, and thapsigargin (Fig. 1), total *O*-GlcNAcylation was increased (Fig. 1). However, the mechanism by which total cellular *O*-GlcNAcylation is enhanced under ER stress is unclear. Recently, Hill's group reported that the UPR activates the HBP via Xbp1-dependent transcription of rate-limiting enzymes of this pathway [31]. This could explain how total cellular *O*-GlcNAcylation increases under ER stress.

O-GlcNAcylation of proteins can regulate their phosphorylation status by influencing the access of kinases or phosphatases. For instance, *O*-GlcNAcylation of Akt at Thr 305 and Thr 312 blocks the interaction between Akt and PDK1, which results in inhibition of phosphorylation at Thr 308 [32]. Ataxia-telangiectasia mutated (ATM) kinase is *O*-GlcNAcylated, and enhanced global *O*-GlcNAcylation dissociates ATM from protein phosphatase type 2A [33]. Under ER stress, active PERK phosphorylates eIF2α at Ser 51 [4,5], expression of growth arrest and DNA damage-inducible protein (GADD34) is promoted, and GADD34 recruits protein phosphatase 1 to form a functional holophosphatase complex that dephosphorylates eIF2α in a negative feedback loop [34]. In this study, we reported that *O*-GlcNAcylation of eIF2α affects its phosphorylation at Ser 51 (Figs. 1, 2, 3, and 5). Under our experimental conditions, the *O*-GlcNAc sites found in this paper seemed to be unphosphorylated (Supplementary Fig. 1). Therefore, similar to Akt and ATM, *O*-GlcNAcylation of eIF2α might affect the interaction between eIF2α and PERK (or a phosphatase complex) under ER stress, thereby regulating phosphorylation of Ser 51. However, further experiments are required to confirm this.

Changes in total cellular *O*-GlcNAcylation act as a pro-survival mechanism to protect cells in response to various cellular stresses [21–23]. However, how *O*-GlcNAcylation affects ER stress-induced cell death has not been determined. In this study, disruption of the *O*-GlcNAcylation of eIF2α increased its phosphorylation at Ser 51 (Fig. 5) and CHOP expression (Fig. 6), which determines whether cells undergo apoptosis in response to ER stress [11]. The levels of cleaved caspase 3 and cleaved PARP were up-regulated in cells expressing Flag-eIF2α with point mutations in the *O*-GlcNAc sites under ER stress (Fig. 7). These results imply that *O*-GlcNAcylation of eIF2α inhibits apoptotic cell death under ER stress.

5. Conclusions

In this paper, we found that eIF2α is *O*-GlcNAcylated at Ser 219, Thr 239, and Thr 241. *O*-GlcNAcylation of eIF2α affects its phosphorylation at Ser 51, controls CHOP expression, and ultimately hinders apoptotic cell death upon ER stress. Understanding how phosphorylation of eIF2α is regulated by its *O*-GlcNAcylation will be important to broaden our knowledge of ER stress-induced cellular responses.

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Conflicts of interest

The authors declare they have no competing interests.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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