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Original article

Nuclear factor-erythroid-2 related transcription factor-1 (Nrf1) is regulated by O-GlcNAc transferase

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ABSTRACT

The Nrf1 (Nuclear factor E2-related factor 1) transcription factor performs a critical role in regulating cellular homeostasis. Using a proteomic approach, we identified Host Cell Factor-1 (HCF1), a co-regulator of transcription, and O-GlcNAc transferase (OGT), the enzyme that mediates protein O-GlcNAcylation, as cellular partners of Nrf1a, an isoform of Nrf1. Nrf1a directly interacts with HCF1 through the HCF1 binding motif (HBM), while interaction with OGT is mediated through HCF1. Overexpression of HCF1 and OGT leads to increased Nrf1a protein stability. Addition of O-GlcNAc decreases ubiquitination and degradation of Nrf1a. Transcriptional activation by Nrf1a is increased by OGT overexpression and treatment with PUGNAc. Together, these data suggest that OGT can act as a regulator of Nrf1a.

1. Introduction

O-GlcNAc transferase (OGT) is a highly conserved enzyme that catalyzes the addition of GlcNAc moiety through a β -glycosidic O-linkage to serine and threonine residues of target proteins [1,2]. Unlike other forms of glycosylation in cells that occurs within the secretory pathway, O-GlcNAc modifications occur in the nucleus and cytoplasm [3]. O-GlcNAc glycosylation has been shown to play a role in regulating protein function by influencing enzymatic activity, stability and sub-cellular localization of proteins [4–7]. O-GlcNAcylation is sensitive to the nutritional state of the cell as components used for synthesis of uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), the substrate for OGT, are generated from nutrients. O-GlcNAcylation also modulates regulatory pathways in response to cellular stress [8]. In addition, O-GlcNAcylation has been shown to mediate cell survival in response to increased ROS production [9]. OGT is able to associate with a diverse group of proteins through its TPR domain that mediates protein-protein interactions with numerous accessory proteins [10–13]. These accessory proteins include HCF1, TET proteins, TRAK1, BAP1, among others [14]. Interestingly, OGT also acts as a protease that catalyzes the cleavage and maturation of Host Cell Factor-1 (HCF1) [15].

HCF1 (also known as HCFC1) is a conserved protein originally identified as a required component for induction of immediate-early genes of herpes simplex virus type-1 [16,17]. However, HCF1 is now

recognized as an essential co-factor for various transcription factors, and it is involved in regulating a plethora of cellular processes including cell-cycle progression and gene expression [18–21]. HCF1 is synthesized as a large 220-kDa precursor protein that undergoes site-specific proteolysis mediated by OGT into N- and C-terminal fragments of protein ranging in size from 68 to 180 kDa that remain stably associated as a complex [15]. HCF1 has no detectable DNA-binding or enzymatic activity. Instead, it contains multiple protein-protein interaction domains such as the six-blade β -propeller Kelch domain related to the *Drosophila* Kelch protein that acts as a scaffold for different proteins through a tetrapeptide consensus sequence (D/E)HYX termed the HCF-binding motif (HBM) present in target proteins [22]. HCF1 interacts with multiple transcriptional regulator proteins including, LZIP, Zhangfei, Krox20 and PGC1 [20,23–25]. HCF1 also associates with O-GlcNAc transferase, the BAP1 deubiquitinating enzyme, and various chromatin modifying enzymes [21,26–29].

Nrf1 (Nuclear factor erythroid-derived 2-related factor-1, and also known as NFE2L1) belongs to the Cap-N-Collar (CNC) family of transcription factors that includes, Nrf2, Nrf3 and p45NFE2 [30–33]. Nrf1 is essential for the maintenance of cellular homeostasis. The global knockout of Nrf1 in mice leads to embryonic lethality, whereas conditional knockout of Nrf1 in various tissues indicate Nrf1 is important in the regulation against development of steatohepatitis and neurodegeneration in mice [34–36]. Nrf1 regulates expression of genes that

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Fig. 1. Nrf1a interacts with HCF1 and OGT. The interaction between Nrf1a and HCF1 was tested by co-immunoprecipitation. (a) HEK293 cells were transfected with 10 μ g V5-tagged Nrf1a, and cell lysate was immunoprecipitated using the anti-V5 mAb followed by western blotting using anti-HCF1 Ab and anti-OGT mAb. Western blotting with anti-V5 mAb was done as input control. (b) HEK293 cells were transfected with 10 μ g V5-tagged HCF1, and cell lysate was immunoprecipitated using the anti-V5 mAb followed by western blotting using antibodies as described in panel-A. (c) Schematic diagram showing the amino acid sequence and location of HCF1 binding motif (HBM) on Nrf1a. HEK293 cells were transfected with (d) 10 μ g Nrf1a Δ HBM-V5, a mutant Nrf1a containing a deletion of the HBM, or (e) 10 μ g Nrf1aHBM^{A4}-V5, a mutant Nrf1a in which the HBM sequence has been changed to four alanine residues, and Nrf1a was immunoprecipitated using the anti-V5 mAb followed by western blotting as described above. As previously reported, Nrf1a exists in two major forms that are detected here at approximately 130 and 110 kDa [45–47]. The p130 represents the ER-membrane embedded protein and the p110 is the soluble form (lacking the N-terminal peptide) that is processed from p130. OGT migrates at 110 kDa, and HCF1 is seen as multiple bands ranging in size from approximately 100 to 175 kDa.

encode antioxidant genes via cis-active sequences known as the antioxidant response element [37,38]. Nrf1 is also important in the proteotoxic stress response through its role in regulating expression of genes encoding the proteasome [35,39,40]. Aside from cellular stress response, Nrf1 has also been shown to regulate differentiation of osteoblast and odontoblast, and loss of Nrf1 function leads to genetic instability and the promotion of tumorigenesis [41–43].

Multiple isoforms of Nrf1 exist; these protein isoforms include, TCF11, Nrf1a, p65NRF1 (also referred to as LCRF1/Nrf1 β), Nrf1b, among others. TCF11, consisting of 772 amino acids, is the longest form of Nrf1 [44]. Nrf1a (742 aa) is generated through alternate splicing of exon 4 of the Nrf1 gene and lacks a Neh4 subdomain (aa 242–271 of TCF11) that contains a transactivation domain [44]. TCF11 and Nrf1a are anchored within the endoplasmic reticulum membrane through their N-terminal domain, and they undergo retro-translocation into the cytoplasm and processing prior to entry into the nucleus to promote gene expression [45–47]. Short isoforms of Nrf1 such as p65Nrf1 and Nrf1b lack the ER-membrane targeting domain. p65Nrf1 is made up of 453 amino acids, and it has been reported to possess weak activation function, as well as trans-dominant negative effects on gene activation [48–50]. The Nrf1b isoform is generated from an alternative transcript, through an alternate promoter, that contains a unique N-terminus encoded by an alternate first exon. Nrf1b encodes for a protein of 583 amino acids and it is also capable of mediating gene activation [51].

Our previous work indicated that Nrf1a is a target for post-translational modification by ubiquitination. We hypothesize that stabilization of Nrf1a in response to stress arises due to its post-translational modification through deubiquitination. Through screening for the putative regulators of Nrf1a, we found that Nrf1a interacts with HCF1 and

OGT. We show that O-GlcNAc modification by OGT leads to decreased ubiquitination and stabilization of Nrf1a, and HCF1 and OGT enhances transcriptional activation by Nrf1a.

2. Material and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), streptomycin, penicillin and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Bradford protein assay reagent was from BioRad (Hercules, CA). BioT was purchased from Bioland Scientific (Paramount, CA). Dual Reporter Assay Kit was from Promega (Madison, WI). ImmunoPure streptavidin, and enhanced chemiluminescence substrate kit was from Pierce Biotechnology (Rockford, IL). Sequencing grade trypsin was purchased from Promega Corp. (Madison, WI), and chymotrypsin was from Roche (Palo Alto, CA). Tunicamycin and PUGNAc were purchased from Sigma-Aldrich (St. Louis, MO). All other general chemicals for buffers and culture media were purchased from ThermoFisher Scientific and/or Sigma-Aldrich. Primary antibodies against Tubulin-A (3873, mouse mAb), HCF1 (50708), OGT (24083, Rabbit mAb), Flag-Tag (14793, Rabbit mAb), Myc-Tag (2276, mAb), and HA-Tag (2367, mAb) were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase linked anti-rabbit IgG (7074) and anti-mouse IgG (7076) antibodies were also from Cell Signaling Technology (Beverly, MA). Mouse monoclonal anti-V5 tag (MA5-15253) and RL2 antibodies (anti-O-GlcNAc; MA1-072) were from ThermoFisher. Antibodies from Cell Signaling Technology and ThermoFisher have been tested and validated on the lot level by

Fig. 2. Nrf1a is O-GlcNAc modified by OGT. O-GlcNAc modification of Nrf1a was examined by co-immunoprecipitation assays. (a) HEK293 cells were transfected with 10 μ g V5-tagged Nrf1a or V5-tagged HBM^{4A} mutant form of Nrf1a, and Nrf1a immunoprecipitated using the anti-V5 mAb followed by western blotting with anti-V5 as input control, and the anti-O-GlcNAc mAb, RL2 to detect O-GlcNAcylation of Nrf1a. HEK293 cells were transfected with 2 μ g (b) Nrf1a-V5 or (c) V5-tagged Nrf1aHBM^{4A}-V5 along with Flag-tagged OGT or control vector, Nrf1a was immunoprecipitated with anti-V5 (2 μ g) and analyzed for O-GlcNAc modification by Western blotting with RL2. Immunoblotting against V5 and Flag was done as input controls.

manufacturer's in-house scientists for specificity, sensitivity, and reproducibility. Rabbit antibody against endogenous Nrf1 was previously described, and has been validated for specificity using Nrf1 knockout

cells [47].

2.2. Plasmids

Nrf1a-HTBH was generated by cloning Nrf1a cDNA into the NotI and PacI sites of the HTBH expression plasmid, which contains two different affinity (streptavidin and hexahistidine) purification tags [52]. OGT-Flag and HCF1-Myc plasmids were from Addgene (Danvers, MA). HCF1-V5 was a gift from Dr. Thomas Kristie (NIH), and HA-tagged Ubiquitin expression plasmid was a gift from Dr. Peter Kaiser (University of California, Irvine). Nrf1a-V5 was previously described. Nrf1a Δ HBM and Nrf1aHBM^{4A} were generated by inverse PCR using the Nrf1a-V5 as template with the primers Nrf1a Δ HBM F/R and Nrf1aHBM^{4A} F/R, respectively (Nrf1a Δ HBM-F: TACAACATGGCACCC-AGTGCC; Nrf1a Δ HBM-R: CACGTGCTCCAGGT-AGGG; Nrf1aHBM^{4A}-F: GCAGCCAACATGGCACCCAG-TGCCCTG; Nrf1aHBM^{4A}-R: AGCTGCGT-GGCCCCACGTGCTCC-AGGTA).

2.3. Tandem affinity purification and mass spectrometric analysis of Nrf1-interacting proteins

Purification and LC-MS/MS analysis of Nrf1 complex were done as previously described [52,53]. In brief, HEK293 cells stably expressing either Nrf1a-HTBH or the HTBH tag were grown to confluence, and collected by trypsinization. Cell pellets were lysed in buffer A [100 mM sodium chloride, 50 mM sodium phosphate, 10% glycerol, 5 mM ATP, 1 mM DTT, 5 mM MgCl₂, 1 $\times$ protease inhibitor, 1 $\times$ phosphatase inhibitor, and 0.5% NP-40 (pH 7.5)]. Lysates were cleared by centrifugation at 13,000 rpm for 15 min, and the supernatant was incubated at 4 $^{\circ}$ C overnight with streptavidin resin. The streptavidin beads were then washed with 20 bed volumes of buffer A, followed by a final wash with 10 bed volumes of TEB buffer [50 mM Tris-HCl (pH 7.5)] containing 10% glycerol. Beads were then incubated in 2 bed volumes of TEB buffer with 1% TEV at 30 $^{\circ}$ C for 1 h. Nrf1a protein was eluted from the beads with TEB. The final eluate was concentrated and subjected to mass spectrometric analysis.

2.4. Western Blotting

Cells were lysed in cold RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1X Protease Inhibitor). Lysates were cleared by centrifugation for 15 min at 4 $^{\circ}$ C, and protein concentrations were determined by Bradford assay. An equal volume of 2 $\times$ SDS sample buffer (100 mM Tris, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 10% 2-mercaptoethanol) was added to cell lysates, and the mixture was boiled for 5 min. Proteins were electrophoresed on SDS-PAGE gels and transferred onto nitrocellulose membranes. Membranes were then blocked in 5% skim milk in TBS-T (150 mM NaCl, 50 mM Tris-HCl pH 8.0, and 0.05% Tween 20) at room temperature for one hour, and then incubated with the indicated primary antibodies at 1:1000 dilution (unless otherwise indicated) overnight at 4 $^{\circ}$ C followed by a incubation with 1:2000 dilution of horseradish peroxidase-conjugated secondary anti-rabbit, or anti-mouse antibody. Antibody-antigen complexes on the blots were detected using chemiluminescent detection system. Densitometric analysis was performed using the Un-Scan-It Gel Analysis software (Orem, UT) for normalization.

2.5. Transfection and luciferase assays

Cells were seeded onto a 24-well plate twenty fours before transfections. DNA transfections were done using BioT reagent according to the manufacturer's protocol. Cellular extracts were prepared 48 h after transfection, and Firefly- and Renilla-luciferase activities measured using Dual Reporter Assay Kit.

Fig. 3. Expression of HCF1 and OGT leads to increased Nrf1a stability. Cycloheximide chase was done to test the effects of HCF1 and OGT on Nrf1a stability. HEK293 cells were transfected with 1 μ g (a) Nrf1a-V5 or (b) Nrf1aHBM^{Δ4}-V5 along with 1 μ g HCF1-Myc or control vector. After 24hr, cells were treated with cycloheximide (50 μ g/mL), and harvested at the indicated time points for western blotting using anti-V5 and anti-Myc antibodies. HEK293 cells transfected with 1 μ g (c) Nrf1a-V5 or 1 μ g (d) Nrf1aHBM^{Δ4}-V5 along with 1 μ g OGT-Flag or control vector. After 24hr, cells were treated with cycloheximide (50 μ g/mL), and harvested at the indicated time points for western blotting using anti-V5 and anti-Flag antibodies as described above. Loading of the lanes was determined by western blotting against alpha-tubulin. (e and f) Graphs show protein levels determined by densitometric quantification of band intensities in western blots normalized to alpha-tubulin. Each point represents the mean $\pm$ SEM of remaining protein for 3 independent experiments, *P value < 0.05, Student's *t*-test.

2.6. Co-Immunoprecipitation

Subconfluent HEK293T cells were transfected with 5–10 μ g of expression vectors using BioT according to manufacturer's protocol. Cells were lysed in buffer containing 2% SDS, 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM DTT, and then boiled for 5 min to disrupt protein-protein interactions. Lysates prepared in SDS-containing buffer were diluted fivefold with buffer lacking SDS. Diluted lysates were subjected to pre-clearing with protein-G Sepharose beads by incubation in the cold for 1-h. The protein samples were incubated with 2–5 μ g of primary antibodies or IgG as a control overnight at 4 °C. After overnight incubation, protein-G Sepharose beads were added and incubated at 4 °C for 1 h. Beads were then collected by brief centrifugation and washed extensively with RIPA buffer. Proteins were eluted in 1 $\times$ SDS sample buffer and heated at 95 °C for 5 min. The samples were separated by SDS-PAGE and transferred onto a nitrocellulose membrane, followed by immunoblotting with indicated primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Detection of peroxidase signal was performed using the chemiluminescence method.

2.7. Cycloheximide chase assays

HEK293T cells expressing various constructs indicated in the figures were incubated with 50 μ g/mL cycloheximide in DMEM at 37 °C. At the times indicated in the figures, cells were harvested and lysates prepared for immunoblotting.

2.8. Statistical analysis

Data are expressed as means $\pm$ SEM. Statistical analyses using Student's *t*-test were done with Microsoft Excel (Redmond, WA). * indicates *p* values < 0.05, and considered significant.

3. Results

3.1. HCF and OGT interact with Nrf1a

To identify novel Nrf1-interacting proteins that may modulate Nrf1 functionality, streptavidin-tagged Nrf1a purified from HEK293 cells was analyzed using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). HCF1 and OGT were repeatedly identified in three independent affinity purifications and LC-MS/MS analysis (data not shown). To validate the mass spectrometry data, we conducted immunoprecipitation and Western blot analysis of lysates prepared from HEK293 cells expressing Nrf1a-V5 fusion protein. Both HCF1 and OGT were detected (Fig. 1a). Next, we conducted co-immunoprecipitation assays using HEK293 cells expressing V5-tagged HCF1. Western blot analysis detected both endogenous Nrf1a and OGT (Fig. 1b). Proteins that have shown to interact with HCF1 bind via a tetrapeptide sequence - (D/E)HXY, designated the HCF1-binding motif (HBM). Sequence analysis revealed a consensus HBM (NHTY) in human Nrf1a, and is conserved in other species (Fig. 1c). To determine the significance of the HBM in Nrf1a in HCF1 binding, we prepared two HBM mutants for co-immunoprecipitation assays. The deletion of the NHTY sequence (Nrf1a-V5ΔHBM) completely abrogated the interaction between Nrf1a and HCF1 (Fig. 1d). Similar results were obtained with

Fig. 4. Ubiquitination on Nrf1a is modulated by HCF1 and OGT. Investigation of the effects of HCF1 and OGT on Nrf1a ubiquitination was done by immunoprecipitation assays. HEK293 cells transfected with 1 μ g HA-Ub and 2 μ g (a and c) Nrf1a-V5 or 2 μ g (b and d) Nrf1aHBM^{A4}-V5 along with (a and b) 5 μ g HCF1-Myc or (c and d) 5 μ g OGT-Flag. Cell extracts were then prepared 24 h after and immunoprecipitated with anti-V5 mAb and Nrf1a analyzed for their ubiquitin content by immunoblotting with an anti-HA Ab. Expression of HCF1 and OGT was verified by western blotting using anti-Myc and anti-Flag antibodies.

Nrf1a-V5HBM^{A4}, in which the NHTY sequence has been changed to four alanine residues (Fig. 1e). In addition, both HBM mutants failed to precipitate OGT (Fig. 1d and e), suggesting Nrf1a associates with OGT in vivo through its interaction with HCF1. Based on these results, we conclude that HCF1 and OGT interact with Nrf1a.

3.2. Nrf1a O-GlcNAcylation is increased by OGT over expression

To determine the significance of OGT-Nrf1a interaction, we examined if Nrf1a is O-GlcNAcylated. Nrf1a-V5 transfected into HEK293 cells was immunoprecipitated and probed with RL2, an O-GlcNAc-specific antibody. O-GlcNAc-reactive bands corresponding to Nrf1a were detected in the immunoprecipitate from cells expressing Nrf1a-V5, but not in the immunoprecipitate from cells expressing the HBM^{A4} mutant of Nrf1a (Fig. 2a). Next, we examined whether Nrf1a undergoes O-GlcNAcylation by OGT. HEK293 cells were transfected with OGT-Flag along with Nrf1a-V5 or Nrf1aHBM^{A4}-V5 mutant, and the effects of OGT on O-GlcNAcylation of Nrf1a was assessed by co-immunoprecipitation and immunoblotting. As shown in Fig. 2b, OGT overexpression significantly augmented levels of O-GlcNAc-reactive bands in cells transfected with wild type Nrf1a, but not HBM^{A4} mutant Nrf1a (Fig. 2c). From these results, we conclude that Nrf1a can undergo O-GlcNAcylation by OGT.

3.3. Expression of HCF1 and OGT increases the half-life of Nrf1a

Next, we sought to examine whether HCF1 modulates the stability of Nrf1a protein. HEK293 cells were transfected with Nrf1a-V5 along with Myc-tagged HCF1, or vector-control. Chase assays were then performed and Nrf1a stability was assessed over a period of 120 min in the presence of cycloheximide, an inhibitor of protein synthesis. Western blotting showed that while Nrf1a-V5 levels rapidly diminished in cells transfected with empty vector, the half-life of Nrf1a-V5 was increased from 30 min to approximately 90 min by HCF1-Myc (Fig. 3a, e). In contrast, expression of HCF1-Myc had no effect on Nrf1a-HBM^{A4}-V5 (Fig. 3b, e). We then assessed whether an excess of OGT would also affect Nrf1a stability. Cycloheximide chase assays were carried out on HEK293 cells transfected with Nrf1a-V5 along with OGT-Flag expression construct, or vector-control. OGT-Flag expression also increased the half-life of Nrf1a-V5 by approximately 3-fold in HEK293 cells (Fig. 3c, f). However, Western blotting showed that OGT-Flag overexpression did not alter the half-life of Nrf1a-HBM^{A4}-V5 (Fig. 3d, f). It is also interesting to note that both the membrane-bound and processed form of Nrf1a is stabilized by HCF1 and OGT. Based on these results, we conclude that the Nrf1a protein is stabilized by HCF1 and OGT.

Fig. 5. The half-life of Nrf1a is increased by PUGNAc. HEK293 cells were transfected with 5 μ g (a) Nrf1a-V5 or (b) Nrf1aHBM^{A4}-V5 along 1 μ g HA-Ub. After 24 h, cells were treated with 100 μ M PUGNAc for 24 h to inhibit OGA-mediated removal of O-GlcNAc from proteins in cells. Cell extracts were then prepared and immunoprecipitated with an anti-V5 mAb and ubiquitin content on Nrf1a was analyzed by immunoblotting with anti-HA antibody. O-GlcNAc on Nrf1a was analyzed by immunoblotting with RL2. (c) HEK293 cells were transfected with 1 μ g Nrf1a-V5 or 1 μ g Nrf1aHBM^{A4}-V5, and after 24 h, cells were treated with 100 μ M PUGNAc for 24 h and followed by cycloheximide (50 μ g/mL). Cells were then harvested at the indicated time points for western blotting using anti-V5 mAb. Loading in each lane was determined by immunoblotting against alpha-tubulin. (d) Graph shows protein levels determined by densitometric quantification of band intensities in western blots normalized to alpha-tubulin. Each point represents the mean $\pm$ SEM of remaining protein for 3 independent experiments, *P value < 0.05, Student's *t*-test.

3.4. Ubiquitination of Nrf1a is decreased by expression of HCF1 and OGT

Because Nrf1a has been previously demonstrated to be a short-lived protein degraded through the ubiquitin–proteasome pathway (56), we speculated that HCF1 and OGT may promote stabilization by inhibition of ubiquitination of Nrf1a. To test this idea, *in vivo* ubiquitination assays were done. HEK293 cells were transfected with HA-tagged ubiquitin expression vector along with expression constructs encoding Myc-tagged HCF1 and Nrf1a-V5. Lysates were immunoprecipitated with anti-V5 antibody followed by immunoblotting using anti-HA antibody. Levels of ubiquitinated Nrf1a were markedly reduced by expression of HCF1 (Fig. 4a, compare middle panels). Next, we examined the effects the HBM mutation of Nrf1a on deubiquitination *in vivo*. Western blotting of lysates immunoprecipitated from HEK293 cells transfected with Nrf1a-HBM^{A4}-V5 showed that levels of ubiquitinated Nrf1a was not diminished by HCF1 expression (Fig. 4b, compare middle panels). We then assessed the effects of OGT overexpression on Nrf1a ubiquitination. HEK293 cells were transfected with Ub-HA and Nrf1a-V5 along with OGT-Flag, or empty vector-control. Lysates were then immunoprecipitated with anti-V5 antibody followed by western blotting with anti-HA antibody. Similar to the effects of HCF1, a reduction in ubiquitinated Nrf1a levels was observed in cells transfected with OGT-Flag (Fig. 4c). However, this effect was not seen in the absence of HCF1 binding site when the HBM^{A4} mutant form of Nrf1a-V5 was used (Fig. 4d). From these results, we conclude that Nrf1 ubiquitination is

reduced by HCF1 and OGT.

3.5. O-GlcNAcylation promotes stabilization of Nrf1

Next, we asked whether the O-GlcNAc modification of Nrf1a is of functional significance. We tested the hypothesis that O-GlcNAcylation stabilizes the Nrf1a protein. To do this, we first examined the effects of PUGNAc, an inhibitor of O-GlcNAcase (OGA) enzyme that catalyzes removal of O-GlcNAc from proteins, on Nrf1a ubiquitination. To determine whether O-GlcNAcylation modulates Nrf1a ubiquitination, we carried out an *in vivo* ubiquitination assay. HA-tagged ubiquitin expression vector was transfected into HEK293 cells along with Nrf1a-V5 or Nrf1aHBM^{A4}-V5. Cells were then treated with DMSO-control, or PUGNAc, and lysates were prepared for immunoprecipitation and immunoblot analysis. A marked decrease in ubiquitinated Nrf1a-V5 levels was observed with PUGNAc treatment (Fig. 5a). However, ubiquitination of the HBM^{A4} mutant of Nrf1a was not diminished by PUGNAc-mediated inhibition of OGA (Fig. 5b). Next, we examined the effects of PUGNAc on Nrf1a-V5 protein levels in HEK293 cells. Cycloheximide chase analysis revealed that the half-life of Nrf1a-V5 protein was increased from 30 min in controls to approximately 120 min in PUGNAc-treated cells (Fig. 5c). By contrast, PUGNAc treatment had no effect on the half-life of Nrf1aHBM^{A4}-V5 (Fig. 5c). From these results, we conclude that O-GlcNAc levels modulate the ubiquitination and stabilization of Nrf1 protein.

Fig. 6. Oxidative stress stimulates O-GlcNAc modification of Nrf1a. HEK293 cells were transfected with Nrf1a-V5, and 24 h after transfection, cells were treated with (a) arsenic (5 μ M), (b) TBHQ (100 μ M) or DMSO for 24 h. Cell lysates were then prepared and Nrf1a was immunoprecipitated with anti-V5 and analyzed for O-GlcNAc modification by western blotting with RL2. As input control, immunoprecipitates were subjected to immunoblotting against V5-tag.

3.6. O-GlcNAcylation of Nrf1 is elevated by oxidative stress

Induction of oxidative stress by arsenic and tert-butylhydroquinone (TBHQ) has been shown to up-regulate Nrf1a protein expression [54]. Hence, we wondered whether oxidative stress affected O-GlcNAc modifications of Nrf1a. To do this, Nrf1a-V5 transfected HEK293 cells were treated with arsenic and TBHQ, and whole cell lysates were subjected to immunoprecipitation with anti-V5 antibody, and followed by examination of O-GlcNAc modification of Nrf1a-V5. As shown in Fig. 6, western blot analysis of cellular extracts from treated and untreated cells with RL2, an O-GlcNAc-specific antibody, indicates that total O-GlcNAc modified protein content was not substantially altered by arsenic or TBHQ. Next, immunoprecipitates were western blotted against V5, and similar amounts of Nrf1a-V5 were detected in treated and untreated cells indicating that expression of transfected Nrf1a-V5 was not altered by drug treatment. In contrast however, western blots showed O-GlcNAc modification of immunoprecipitated Nrf1a-V5 was increased by TBHQ, and especially by arsenic. Taken together, these findings suggest that O-GlcNAc modification of Nrf1a is enhanced by arsenic and TBHQ.

3.7. Nrf1 mediated transactivation of ARE-genes is enhanced by OGT

Our results indicate that OGT plays a role in modulating the turnover rate of Nrf1a. To determine the biological implication of this finding, we examined the effects of OGT on Nrf1a-mediated activation of antioxidant response element (ARE)-driven targets. To test this, Nrf1a and OGT were co-expressed in HEK293 cells, and their effects on the ARE-driven luciferase reporter were analyzed. Co-expression of OGT potentiated Nrf1a-mediated activation of luciferase reporter

(Fig. 7a). In contrast, activation of luciferase expression by Nrf1a Δ HBM-V5, which is incapable of interacting with OGT, was not enhanced by co-transfection of OGT expression plasmid. Next, we evaluated the effect of PUGNAc-mediated elevation of O-GlcNAcylation on Nrf1-dependent luciferase reporter expression. As shown in Fig. 7b, PUGNAc treatment increased the reporter activation by Nrf1a-V5, but not by Nrf1a Δ HBM-V5. To further confirm the role of protein O-GlcNAcylation on Nrf1a-mediated transcriptional activation, we examined the effects of PUGNAc treatment on expression of endogenous Nrf1-target genes in cells. Nrf1 $^{-/-}$ MEF cells were transduced with Nrf1a or empty vector. Cells were then treated with DMSO, or PUGNAc, and mRNA levels of various known Nrf1-regulated genes were measured using RT-qPCR. As shown in Fig. 7c, PUGNAc treatment resulted in a 1.5–2-fold induction of GCLC, GCLM, GSTA4, PSMB2, PSMC5, and PSMD12 in cells expressing Nrf1a. In contrast, induction was not observed in vector transduced Nrf1 $^{-/-}$ cells treated with PUGNAc. Together, these results suggest that O-GlcNAcylation can modulate ARE-mediated gene transcription in cells through Nrf1a.

4. Discussion

The Nrf1 transcription factor regulates expression of cytoprotective and detoxification genes, and genes involved in development and other cellular processes. In this study, we identified OGT and HCF1 as cellular partners of Nrf1a, and OGT interacts with Nrf1a through HCF1. We provide evidence that Nrf1a undergoes O-GlcNAc modification by OGT, and O-GlcNAcylation enhances the stability and transcriptional activity of Nrf1a.

A growing body of evidence demonstrates that O-GlcNAcylation occurs in response to diverse forms of damaging agents resulting from normal metabolic processes, as well as environmental factors, suggesting that O-GlcNAc modifications of protein is a component of the stress response program in cells [9,55–58]. For instance, oxidative stress induced by hydrogen peroxide treatment leads to increased FOXO4 O-GlcNAcylation and enhanced transcriptional activity [59]. The FOXO1 transcription factor, which also has an important role in the oxidative stress response, is also known to undergo O-GlcNAc modification [59,60]. Our data shows that O-GlcNAc modification increases Nrf1a stability, and treatment to induce oxidative stress promotes O-GlcNAc modification of Nrf1a. Furthermore, PUGNAc treatment, which increases O-GlcNAc modification of proteins, resulted in increased reporter gene activation by Nrf1a. These findings suggest that O-GlcNAc also regulates ARE-mediated gene expression by Nrf1a. While our findings here suggest that increased activation by Nrf1a takes place as a result of increased protein stability, we cannot rule out from the current studies that O-GlcNAcylation may also affect the cellular localization or activity of Nrf1a. Additional experimentation is required to address these possibilities.

The control of protein stability plays an important role in regulation of numerous transcription factors. We have previously shown Nrf1a is a short-lived protein degraded through the ubiquitin–proteasome pathway [61]. We show that overexpression of HCF1 and OGT, as well as PUGNAc treatment decreases steady-state ubiquitination of Nrf1a. Based on this data, we surmise that O-GlcNAcylation modulates the ubiquitination and subsequent degradation of the Nrf1a protein. Although the mechanism by which this occurs is not known currently, O-GlcNAcylation exerting an effect on ubiquitination has also been reported for other proteins [62–65]. One possibility is that O-GlcNAc modified residues serves as docking sites for deubiquitinase enzymes that then removes ubiquitin from the protein. For instance, O-GlcNAcylation of PGC-1a has been suggested to recruit BRCA1 Associated Protein-1 (BAP1), a ubiquitin hydrolase, which cleaves ubiquitin from PGC-1a, thus rescuing the protein from UPS-mediated degradation [66]. It would be of interest to investigate whether O-GlcNAcylation of Nrf1 also promotes interaction with deubiquitinating enzyme such as BAP1, or other ubiquitin hydrolases, and to study how such interactions

Fig. 7. Nrf1a mediated transactivation of ARE-genes is enhanced by O-GlcNAcylation. (a) Luciferase activity in HEK293 cells transfected with 10 ng GCLM-luciferase (a luciferase reporter driven by the human GCLM gene promoter that contains an antioxidant response element), and 10 ng Nrf1a-V5, or Nrf1a-ΔHBM-V5 expression vector, along with 10 ng or 20 ng OGT expression vector and 0.2 ng RLTK control. Firefly luciferase activity was normalized to Renilla luciferase activity. Luciferase activity in the absence of OGT expression plasmid was set as 1-fold activation. Data is presented as means $\pm$ SEM for 3 independent experiments each containing 3 replicates. *P value < 0.05, Student's *t*-test. (b) HEK293 cells were transfected with 10 ng GCLM-luciferase, along with 10 ng Nrf1a-V5, or Nrf1a-ΔHBM-V5 and 0.2 ng RLTK control. After 24hr, cells were treated with PUGNac (100 μ M or 200 μ M) or DMSO vehicle control, and after overnight incubation, luciferase activity was normalized to Renilla luciferase activity. Data represents means $\pm$ SEM of three independent experiments. *P value < 0.05 (Student's *t*-test) relative to DMSO treated cells. (c) Nrf1^{-/-} MEF cells transduced with control, or Nrf1a retrovirus was cultured with DMSO or PUGNac (100 μ M) for 24 h; and analyzed for expression of known Nrf1 target genes. Dot-plots depict relative expression of indicated genes. P values were calculated by *t*-test (*n* = 3).

might function in gene regulation events during cellular stress response.

O-GlcNAc modification is a dynamic process that responds to various cellular cues. O-GlcNAc sites on proteins can also undergo phosphorylation, and it is accepted that there is extensive crosstalk between these two forms of modifications in the regulation of cell signaling pathways [67,68]. The phosphorylation site can be at the same location as the O-GlcNAcylation site, or at nearby serine and threonine residues in the protein. Several studies have shown that Nrf1 is regulated by phosphorylation. Glycogen Synthase Kinase 3 (GSK3) was previously demonstrated to negatively regulate Nrf1a stability [69]. Phosphorylation of Nrf1a facilitated by GSK3 was shown to recruit Fbw7, the F-box containing ubiquitin ligase, for proteasomal degradation of Nrf1a. Future studies aimed at determining if O-GlcNAcylation by OGT plays a role in modulating GSK3-dependent, FBW7-mediated Nrf1a degradation are warranted.

While this manuscript was in preparation, another group reported

that OGT negatively regulates expression of another Nrf1 isoform, TCF11 [70]. In their analysis, they observed that inhibition of OGT function by alloxan and siRNA-mediated knockdown led to increased TCF11 expression and function as measured by reporter gene activation. These findings contrast sharply with our results on Nrf1a. The reason for this discrepancy is unclear. The conflicting results observed could conceivably be explained by some contribution due to the technical nature of the different experiments. Although we do not know the possible reasons for the discrepancy about the consequence of OGT knockdown, the use of a pharmacological inhibitor of OGT may not be selective, as alloxan has also been shown to be more potent in inhibiting OGA than OGT [71]. It is also conceivable that OGT may regulate Nrf1a differently than TCF11. Nrf1a is generated through alternate splicing of exon 4 of the Nrf1 gene and lacks a Neh4 subdomain (aa 242–271 of TCF11). TCF11 expression in mammals is speculated to be a relatively recent evolutionary event as TCF11 expression has only been identified

in human cells thus far. Contrarily, Nrfla is expressed in nearly all vertebrates. Therefore, we speculate that TCF11 somehow has attained an opposite OGT mediated regulatory mechanism as it began to be expressed later in phylogeny. In this regard, it is interesting that SKN1, an Nrfl ortholog present in *C. elegans*, was recently shown to be positively regulated by an OGT interaction similar to that with Nrfla shown in our investigation [72]. As of now however, there are no studies elucidating the functional difference between TCF11 and Nrfla. Nevertheless, this data hints that TCF11 may have acquired novel regulatory mechanisms that oppose previous mechanisms controlling Nrfla stability. OGT-mediated regulation of TCF11 may have diverged from that of Nrfla due to the presence of an additional transactivation domain in Neh4 [49]. Such a possibility of differential regulation of protein isoforms is not without precedence. For example, the ARD1 (Arrest defective 1 protein) gene encodes two isoforms—ARD1²³⁵ and ARD1²²⁵ are expressed in mouse, while ARD1²³⁵ is expressed only in humans [73]. It was shown that both of these isoforms are regulated by different mechanisms in a species-specific manner suggesting that they have different functions in the cell.

In summary, our present study reports that Nrfl is O-GlcNAcylated, and this modification occurs in response to oxidative stress, thereby regulating Nrfl stability and Nrfl-mediated gene activation. These findings suggest that post-translational modification via addition of O-GlcNAc plays a role in stress responses of Nrfla.

Author contribution

JYC conceived and wrote the paper. JLV and JWH performed the studies in Figs. 1 through 7. CSL and DVH generated the Nrfl expression construct and purified the protein for mass spec analysis by XW and LH. HMK provided technical assistance and contributed to the preparation of figures. All authors reviewed the results and approved the final version of the manuscript.

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