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Thermal Proteome Profiling Reveals the O-GlcNAc-Dependent Meltome

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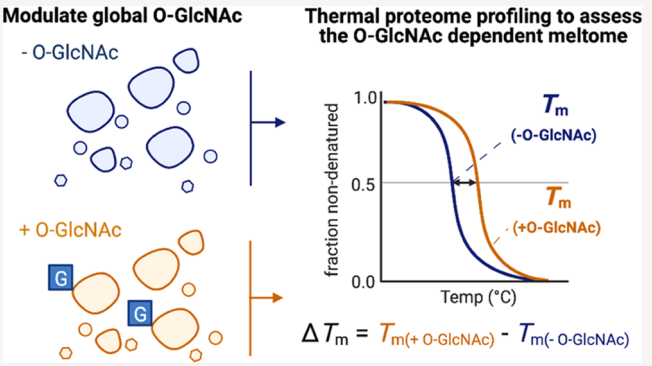
ABSTRACT: Posttranslational modifications alter the biophysical properties of proteins and thereby influence cellular physiology. One emerging manner by which such modifications regulate protein functions is through their ability to perturb protein stability. Despite the increasing interest in this phenomenon, there are few methods that enable global interrogation of the biophysical effects of posttranslational modifications on the proteome. Here, we describe an unbiased proteome-wide approach to explore the influence of protein modifications on the thermodynamic stability of thousands of proteins in parallel. We apply this profiling strategy to study the effects of O-linked *N*-acetylglucosamine (O-GlcNAc), an abundant modification found on hundreds of proteins in mammals that has been shown in select cases to stabilize proteins. Using this thermal proteomic profiling strategy, we identify a set of 72 proteins displaying O-GlcNAc-dependent thermostability and validate this approach using orthogonal methods targeting specific proteins. These collective observations reveal that the majority of proteins influenced by O-GlcNAc are, surprisingly, destabilized by O-GlcNAc and cluster into distinct macromolecular complexes. These results establish O-GlcNAc as a bidirectional regulator of protein stability and provide a blueprint for exploring the impact of any protein modification on the meltome of, in principle, any organism.



INTRODUCTION

There are more than 300 known posttranslational modifications (PTMs) found on proteins,[1] and they have diverse roles in regulating the structure and biochemical functions of proteins. Among these PTMs, some are present on relatively few proteins, whereas others are widespread. The widespread distribution of a specific PTM is generally perceived as suggesting that it serves important roles in cell function. One emerging role for such widely distributed PTMs is to alter the thermodynamic stability of proteins by modulating various intra- and intermolecular interactions.[2] Indeed, some PTMs have been shown to alter the conformational free-energy landscape of proteins impacting, for example, the stability of their native states,[3] intermediates along folding pathways,[4] their unfolded states,[5] or higher-order quaternary complexes.[6] From the perspective of cellular function, tuning the thermodynamic stability of proteins can serve as a means to control the levels of their properly folded forms, which, in turn, may impact the delicate balance between their synthesis and degradation—a process of central importance for cellular health known as protein homeostasis or "proteostasis".[7,8]

Despite their emerging importance as regulators of proteostasis, however, global methods to assess the role of PTMs in regulating the thermal stability of proteins are lacking. In recent years, rapid advances in high-throughput mass spectrometry (MS)-based proteomics have enabled interrogation of the thermal unfolding of thousands of proteins in parallel using thermal proteome profiling (TPP).[9−12] This strategy was originally developed to identify protein targets of small-molecule ligands within cells,[10,11] where even a small number of interactions can often significantly thermostabilize proteins.[13] Recently, TPP has revealed that the thermostability of individual proteins varies across the phases of the cell cycle,[11] and for certain proteins, this variation is synchronized with changes in phosphorylation levels during the mitotic transition. These observations suggest that PTMs, similar to small-molecule ligands, may be physiological regulators of protein thermodynamic stability within the cell through their ability to form stabilizing interactions.[11]

Recently, a method called Hotspot thermal profiling (HTP) was developed that enables studying the proteome-wide

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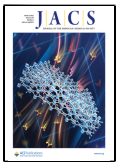

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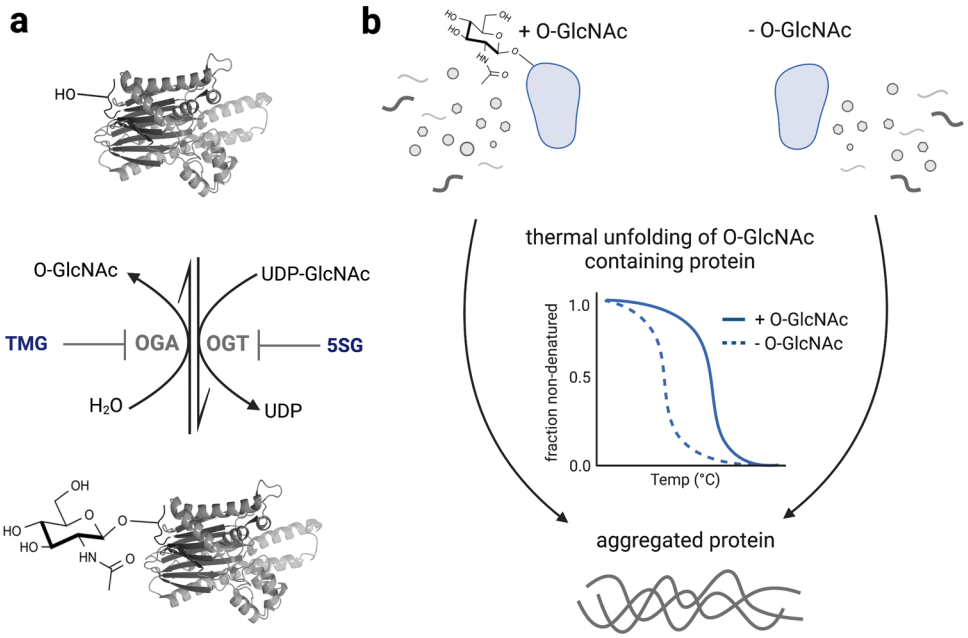

Figure 1. Exploring the O-GlcNAc-dependent meltome. (a) Installation of O-GlcNAc on proteins by OGT and its removal by OGA can each be antagonized within cells using the small-molecule inhibitors 5SG and TMG, respectively. (b) Hypothesis that O-GlcNAc stabilizes proteins against thermal unfolding. Abbreviations: 5SG, 2-acetamido-2-deoxy-5-thio-α-D-glucopyranose; TMG, thiamet-G; OGA, O-GlcNAc hydrolase; and OGT, O-GlcNAc transferase.

impact of phosphorylation on protein thermostability.[14] Following this pioneering study, subsequent phosphosite-specific methods were developed that build upon this proof-of-concept work to improve technical aspects.[14−17] This method enables simultaneous mapping and determination of thermal melt profiles for thousands of phosphopeptides in parallel. Though powerful, this method would be difficult to implement on labile PTMs, such as O-GlcNAc, that are refractory to MS/MS mapping. Furthermore, as the method relies on mapping individual phosphopeptides, it does not report on the thermostability of the complete ensemble of proteoforms of a given protein. This is relevant because PTMs are typically present in substoichiometric levels, often on a small overall fraction of protein sites.[18] Therefore, inspired by the high interest in this area and the needs of the field, we set out to develop a method for O-GlcNAc that reports on the global ensemble of proteoforms of a given protein. Notably, such a method could, in principle, be applied to any PTM and provide a complementary method to HTP.

Toward this goal, we envisioned a strategy that could leverage TPP to assess the effect of one type of PTM on the thermostability of proteins by applying it to samples in which global levels of a specific PTM were altered, an approach we call posttranslational modification-thermal proteome profiling (PTM-TPP). Ideally, such a method could enable determining the influence of one type of PTM on the stability of the population-averaged ensemble of peptides from a given protein. Such a method would avoid the analytically demanding requirement of mapping specific PTMs, which would, in turn, enable a greater depth of coverage of the overall proteome.

To develop such a strategy, we decided to explore as a model PTM the modification of select hydroxyl groups of serine and threonine residues of proteins with O-linked *N*-acetylglucosamine (O-GlcNAc, Figure 1A).[19] We selected this PTM because it is of high interest owing to its emerging roles in a range of physiological processes and because manipulation of O-GlcNAc has emerged as a therapeutic strategy with compounds having entered into the clinic.[20] In addition, O-GlcNAc is found within multicellular eukaryotes on over a thousand nuclear, cytoplasmic, and mitochondrial proteins. We were particularly intrigued by O-GlcNAc because it is known to be coordinated with the heat shock response (HSR)[21] and confers resistance to heat during development in ectotherms.[22] Additionally, genetic studies in *Caenorhabditis elegans* have illuminated O-GlcNAc-dependent stability differences for several cellular proteins.[22−24] Furthermore, several proteins are known to be stabilized against aggregation by O-GlcNAc, including polyhomeotic (PH-P), TGF-β-activated kinase 1 binding protein 1 (TAB-1), nucleotide-binding oligomerization domain receptors 1 and 2 (NOD1 and NOD2), tau (MAPT), and α-synuclein (SNCA).[25−30] Recently, O-GlcNAc has been shown to reduce phase separation and enhance condensate dynamics for the RNA-binding protein EWS (EWSR1),[31] providing a striking example of how O-GlcNAc can regulate intermolecular interactions of its target proteins. We also noted that other forms of glycosylation, such as N-linked glycosylation, have been shown to alter the thermodynamic stability of specific proteins.[32,33] Given these lines of reasoning, we hypothesized that O-GlcNAc could be a general regulator of the thermodynamic stability of a subset of proteins (Figure 1B) and we accordingly set out to harness TPP to explore this possibility. Furthermore, O-GlcNAc is notoriously challenging to map by MS/MS[34] and thus provides an ideal model for demonstrating the utility of our method that does not rely on site mapping.

Levels of protein O-GlcNAc are regulated by just two enzymes that act antagonistically. O-GlcNAc transferase (OGT, CAZy[35] glycosyltransferase family GT41) installs O-GlcNAc on target proteins and O-GlcNAc hydrolase (OGA, CAZy glycoside hydrolase family GH84) removes this modification. Regulation by just these two enzymes should

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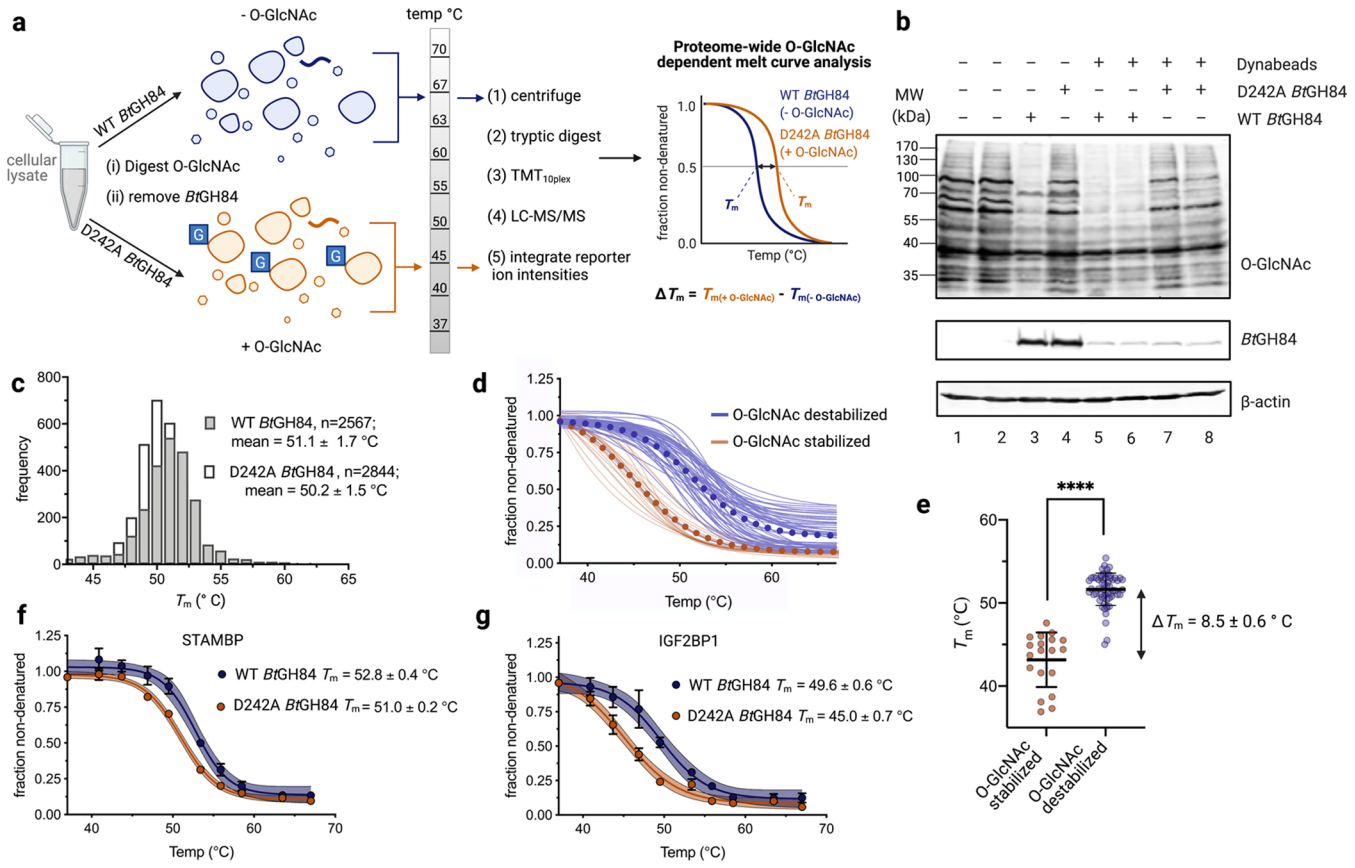

Figure 2. erPTM-TPP enables the global identification of proteins having O-GlcNAc-dependent thermostability. (a) Schematic outlining the erPTM-TPP method. (b) Immunoblot following the treatment of cellular lysates with WT or D242A *Bt*GH84. (c) Histogram of T_m values for all proteins that could be curve-fit in the WT and D242A *Bt*GH84-treated samples. (d) Overlay of melt curves for O-GlcNAc-stabilized and O-GlcNAc-destabilized proteins. Curves for both WT and D242A *Bt*GH84 conditions are shown. (e) Scatter plot comparing T_m values for O-GlcNAc-stabilized and -destabilized proteins from panel (d) with *P* values: two-tailed Student's *t* test assuming unequal variance; ****$P < 0.0001$. (f, g) Melt curves for select proteins comparing WT and D242A *Bt*GH84 conditions. Error bars correspond to SE from four experimental replicates. See also Figures S1−S6, Tables S1 and S2.

enable facile control over its global levels on proteins (Figure 1A). We reasoned that maximizing the difference in global levels of O-GlcNAc between samples would facilitate the successful application of TPP to study this modification. To vary levels of O-GlcNAc *in vitro*, we expected that we could simply add an exogenous O-GlcNAc hydrolase to remove the modification. Alternatively, to alter levels of O-GlcNAc within cells, one can use small-molecule inhibitors to vary endogenous enzyme activity or expression levels.[36] Because altering O-GlcNAc levels within cells induces a heat shock response[37] and heat shock conversely increases O-GlcNAc levels,[38] we expected that an *in vitro* approach using cell lysates would be both more simple and more direct.

RESULTS

Given these various considerations, we first set out to assess the effects of O-GlcNAc on protein stability by treating cell lysates with a recombinant OGA homolog to selectively remove O-GlcNAc. In this strategy, which we term enzymatic removal (erPTM-TPP; Figure 2A), we exploited the *Bacteroides thetaiotaomicron* *Bt*GH84[39] glycoside hydrolase that is known to efficiently remove O-GlcNAc and digested cellular lysate obtained from human embryonic kidney cells HEK293T (Figure S1A−E). Following hydrolysis, *Bt*GH84 was removed using magnetic beads (Figure 2B) to generate the O-GlcNAc-deficient (−O-GlcNAc) sample. As a negative control, we used the same concentration of a catalytically dead variant of *Bt*GH84,[40] in which a key catalytic residue is mutated (D242A *Bt*GH84) to generate the O-GlcNAc-enriched (+O-GlcNAc) sample (Figures 2B and S1E,F). We confirmed that O-GlcNAc levels in both the +O-GlcNAc and −O-GlcNAc samples were altered as expected by immunoblot using a pan-specific anti-O-GlcNAc antibody (Figure 2B). Subsequently, we also confirmed that the WT and D242A *Bt*GH84 enzymes were successfully removed from these samples (Figure 2B). The resulting pair of quadruplicate samples was then subjected to the subsequent steps of the TPP workflow (Figure 2A). One potential complicating factor of our method was the possibility that D242A *Bt*GH84 may remove O-GlcNAcylated proteins from our lysate samples. This seemed possible since a different bacterial OGA homolog, rendered inactive by site-directed mutagenesis of a key active site residue, has been used to enrich O-GlcNAc-modified proteins.[41] To rule out this possibility, we showed that overall protein abundances remain consistent between both enzymatic treatments (Figure S1G). Furthermore, we observed no significant difference in the relative abundance of known O-GlcNAcylated proteins when comparing WT and D242A *Bt*GH84 treatments in our erPTM-TPP data set (Figure S2). The data from this erPTM-TPP experiment allowed us to

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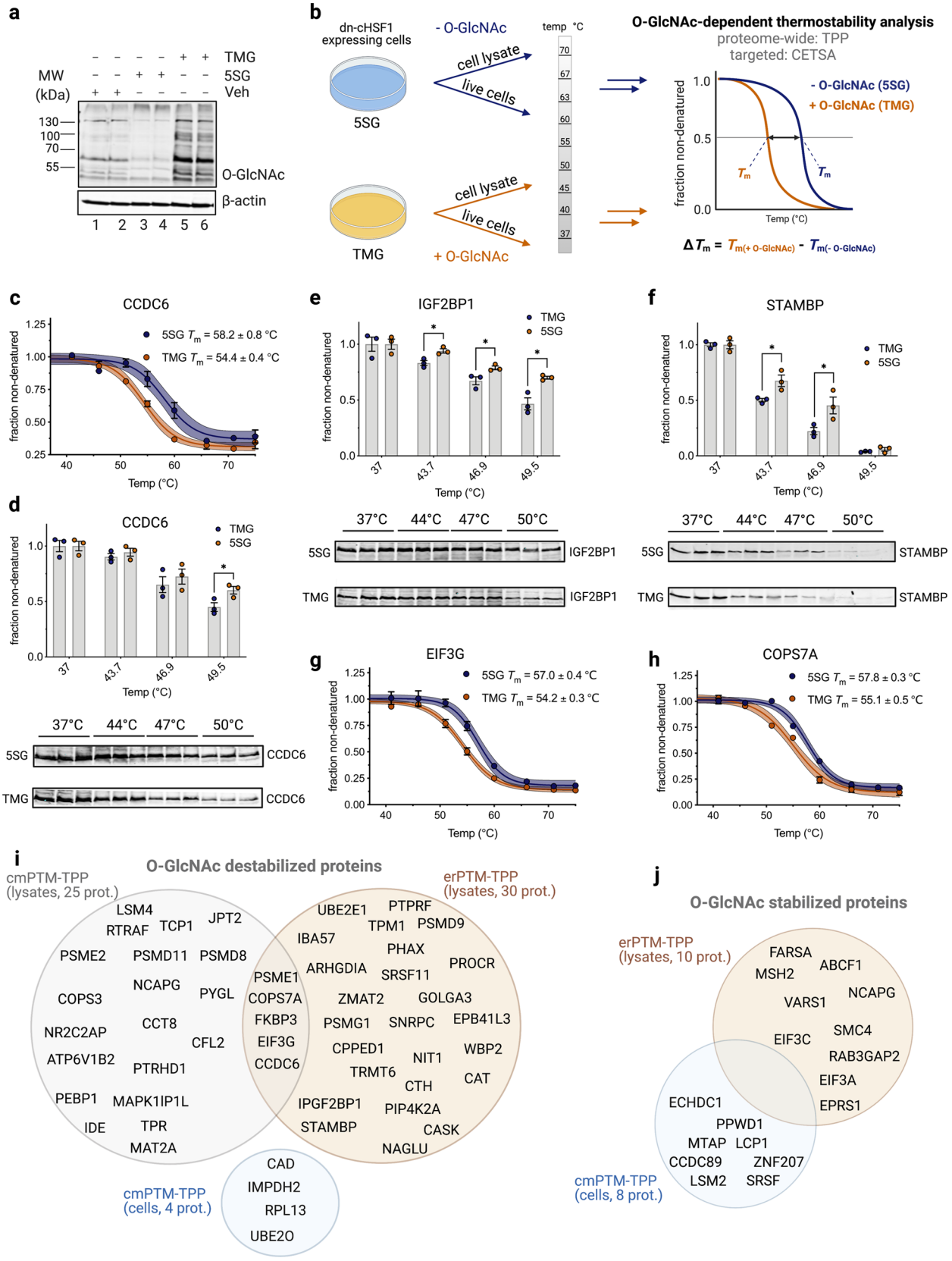

Figure 3. cmPTM-TPP validates a panel of proteins as being thermodestabilized by O-GlcNAc. (a) Immunoblot analysis of O-GlcNAc-modified proteins following the treatment of HEK293T-REx-dn-cHSF1 cells with TMG (70 nM), 5SG (250 μM), or vehicle control. (b) Schematic diagram outlining the cmPTM-TPP approach. (c, g, h) cmPTM-TPP melt curve profiles for select proteins in lysates. Error bars are SE from two experimental replicates. (d–f) Immunoblots and densitometry analysis for select proteins following cmPTM-CETSA on live cells. Error bars are SE from three experimental replicates, with *P* values: two-tailed Student's *t* test assuming unequal variance; $^*P < 0.05$. (i, j), Venn diagrams for proteins displaying O-GlcNAc-dependent destabilization and stabilization identified using different PTM-TPP methods.

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investigate the O-GlcNAc-dependent thermostability of a proteome comprising 3213 proteins. A histogram of the resulting T_m values revealed an almost identical mean global T_m for the O-GlcNAc-modified and unmodified conditions (Figures 2C and S1H), suggesting that O-GlcNAc has a minimal overall impact on the global stability of the proteome.

To identify candidate O-GlcNAc-modulated proteins, we used the following set of four stringent criteria. First, to be included for the analysis of potential O-GlcNAc-dependent stability differences, each protein needed to be identified in at least two experimental replicates from each experimental condition of + and −O-GlcNAc (see Table S5). This criterion enabled assessing errors for each identified thermal melt curve as done previously in general TPP experiments to avoid false positives.[42,43] A total of 2482 of the 3213 identified proteins met this first criterion. Second, for any given protein, the change in its average T_m ($\Delta T_{m(erPTM\text{-}TPP)} = |T_{m(D242A\ BtGH84)} - T_{m(WT\ BtGH84)}|$) needed to be greater than 1.0 °C, a shift that is commonly used as a selection criterion in TPP.[11,44] Third, the average ΔT_m value needed to be greater than its standard error. These three criteria were applied to the panel by fitting melt curves to each experimental replicate separately, as is typical in TPP.[10,11] This strategy enabled productively focusing our panel of candidates without inadvertently excluding potential hits and led, in the erPTM-TPP experiment, to the selection of 1392 proteins that met these three initial common TPP criteria. A histogram of all $|\Delta T_m|$ values for the complete panel of proteins analyzed revealed that numerous proteins displayed O-GlcNAc-dependent thermostability differences (Figure S3).

However, we found that individual melt curves were often skewed by poor curve-fits or missing data for certain experimental replicates. Therefore, we found that in selecting our final panel of candidates, it was valuable to introduce a fourth and final criterion that involved plotting the averaged data from all replicates for a given experimental condition and fitting this to a Boltzmann sigmoidal. We then leveraged these fitted data to test for our fourth criterion, which required that the 95% confidence intervals between the two experimental conditions did not overlap at the inflection point (Figures S4 and S6). We judged that this was an important stringent criterion that enabled detecting statistically robust stability effects. Notably, this rigorous analysis pipeline revealed 40 proteins in total that displayed clear O-GlcNAc-dependent thermostability differences within the erPTM-TPP data set (Figures S4, S6, Tables S1 and S2).

Using this stringent data analysis pipeline, 10 proteins from erPTM-TPP met our strict criteria for O-GlcNAc-dependent thermostabilization ($\Delta T_{m(erPTM\text{-}TPP)}$; 1.7−6.8 °C; Figure S4 and Table S1) and, remarkably, 30 displayed O-GlcNAc-dependent destabilization ($\Delta T_{m(erPTM\text{-}TPP)}$; −1.2 to −7.0 °C; Figures 2F,G, S5, S6 and Table S2). Among the 40 O-GlcNAc-modulated proteins, only 17 have been identified as being O-GlcNAc-modified (Tables S1 and S2). Notably, O-GlcNAc-stabilized proteins had a significantly lower mean T_m as compared to O-GlcNAc-destabilized proteins ($\Delta T_m = T_{m(destabilized)} - T_{m(stabilized)} = 8.5 \pm 0.6$ °C; Figure 2D,E). The large majority of O-GlcNAc-modulated proteins identified using erPTM-TPP displayed no significant difference in protein abundance between experimental conditions (Tables S1, S2, and S6), indicating that differences in protein abundances were not a significant factor impacting our data. These data showed that erPTM-TPP allows interrogation of a large fraction of the proteome and enabled identification of candidate proteins, which may have their stability regulated by O-GlcNAc.

Having identified a panel of O-GlcNAc-modulated proteins using erPTM-TPP, we next aimed to validate this set using a different approach to alter global PTM levels using chemical-modulators (cmPTM-TPP). We envisioned that this strategy could be applied in live cell culture and might also identify a unique subset of O-GlcNAc-regulated proteins since the melting of proteins would occur within cells in the presence of a range of potential binding partners. To increase global O-GlcNAc levels, we turned to the potent and selective OGA inhibitor thiamet-G (TMG).[45] Modification levels were decreased using an established metabolic inhibitor of OGT, 2-acetamido-2-deoxy-5-thio-α-D-glucopyranose (5SG)[46] (Figures 1A and3A). Both inhibitors are frequently used to modulate O-GlcNAc levels within cells.[46,47] We recognized, however, that a complicating consequence of increasing O-GlcNAc levels *in cellulo* would be induction of the heat shock response (HSR) via activation of the master transcriptional regulator heat shock factor 1 (HSF1).[37,48] The resulting increase in chaperone expression could confoundingly enhance O-GlcNAc-dependent thermostabilization of proteins and/or mask destabilization. To circumvent this issue, we turned to a chemically inducible cell line that stably expresses a potent dominant negative form of HSF1 (dn-cHSF1) that suppresses cytoplasmic heat shock response in a doxycycline-dependent fashion.[49−51] We confirmed by immunoblot that dn-cHSF1 was robustly induced in this cell line upon the addition of doxycycline and that its induction suppressed HSP70 and HSP40 expression even after exposure to various stressors (Figure S7A). Importantly, we also showed that when dn-cHSF1 is induced HSP levels were similar in all samples treated with vehicle, TMG, or 5SG (Figure S7B). Notably, both OGA and OGT interact with cellular protein partners.[52−54] Recently, Savitski and colleagues showed that proteins that are part of a complex tend to comelt and that small molecules can impact the overall stability of these protein complexes.[55] Therefore, to avoid potential false positives, we diluted residual OGA and OGT inhibitors following cell culture to avoid potential impact on the thermostability of OGT and OGA and their protein partners in cmPTM-TPP experiments (Figure S8). These data show this cell system and probes are suitable for interrogating the effects of altering O-GlcNAc on protein stability directly within cells.

We next applied our TMG- and 5SG-treated cells to the cmPTM-TPP workflow (Figure 3B), enabling us to assess O-GlcNAc-dependent thermostability [$\Delta T_{m(cmPTM\text{-}TPP)} = T_{m(TMG)} - T_{m(5SG)}$] of 2473 proteins in samples where temperature treatments were applied to lysates derived from these cells as well as for 2393 proteins where the temperature treatments were instead applied directly to live cells. Global average melt curves for 5SG and TMG treatments were similar when comparing results for live cells and lysates (Figure S9), indicating that there was no significant thermostability effect on the bulk proteome, which was consistent with our erPTM-TPP experiments using cell lysates. Detailed analyses of the melting curves using the same criteria as defined for erPTM-TPP, however, revealed 25 proteins displaying O-GlcNAc-dependent destabilization within lysates and four within live cells (Tables S3, S4 and Figures S10−S12). All O-GlcNAc-modulated candidates identified using the cmPTM-TPP experiment displayed no significant difference in protein abundance at the lowest temperature point between the

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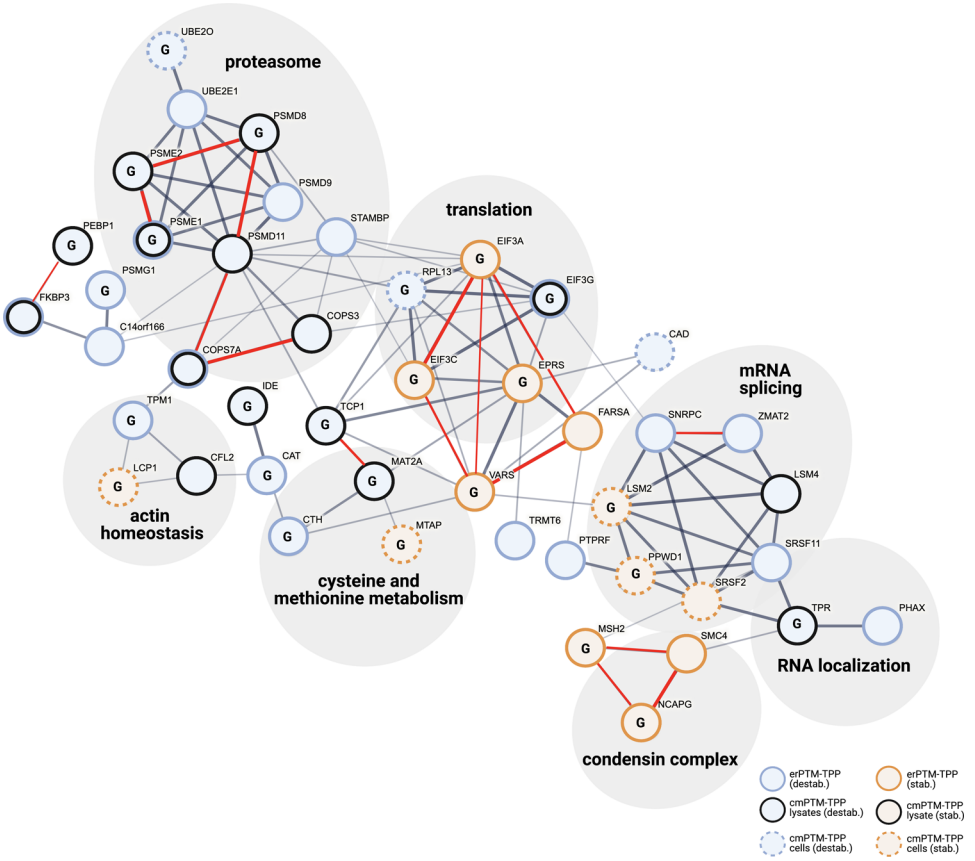

Figure 4. Proteins displaying O-GlcNAc-dependent thermostability cluster into distinct protein complexes. STRING protein interaction network of proteins displaying O-GlcNAc-dependent thermostability. Nodes represent individual proteins coded by stability difference and PTM-TPP method(s). The network was constructed using the STRING v11 server.[57] Line thickness connecting nodes reflects the interaction score as outlined in the Supporting Information, and proteins are grouped into cellular components (gene ontology, false discovery rate <0.001). Proteins displaying evidence for direct interaction in thermal proximity coaggregation (TPCA) analysis ($P < 0.1$) have a red line connecting nodes (see Supporting Information). Known O-GlcNAcylated proteins are labeled (G).

TMG and 5SG conditions (Tables S3, S4, and S6), indicating that protein abundance was not a major factor influencing our data. Notably, five of these O-GlcNAc-modulated proteins were destabilized in both erPTM-TPP and cmPTM-TPP experiments in lysates including coiled-coil domain-containing protein 6 (CCDC6; $\Delta T_{m(erPTM\text{-}TPP)} = -2.0 \pm 0.4$ °C, $\Delta T_{m(cmPTM\text{-}TPP)} = -3.8 \pm 0.9$ °C), Cop9 signalosome 7A (COPS7A; $\Delta T_{m(erPTM\text{-}TPP)} = -2.3 \pm 0.5$ °C, $\Delta T_{m(cmPTM\text{-}TPP)} = -2.7 \pm 0.6$ °C), proteosome activator subunit 1 (PSME1; $\Delta T_{m(erPTM\text{-}TPP)} = -1.4 \pm 0.4$ °C, $\Delta T_{m(cmPTM\text{-}TPP)} = -5.5 \pm 1.0$ °C), elongation initiation factor 3G (EIF3G; $\Delta T_{m(erPTM\text{-}TPP)} = -1.2 \pm 0.5$ °C, $\Delta T_{m(cmPTM\text{-}TPP)} = -2.8 \pm 0.5$ °C), and peptidyl-prolyl cis−trans isomerase 3 (FKBP3; $\Delta T_{m(erPTM\text{-}TPP)} = -1.7 \pm 0.3$ °C, $\Delta T_{m(cmPTM\text{-}TPP)} = -2.8 \pm 0.9$ °C) (Figures 3C,G–J, S10 and Table S3). Using commercially available antibodies that we assessed as being of high quality, we performed targeted cellular thermal shift assay (CETSA) using cells treated with chemical modifiers (cmPTM-CETSA). In this way, we validated three proteins as having their stability influenced within cells by O-GlcNAc including STAM binding protein (STAMBP) and insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1), both of which were also destabilized by O-GlcNAc in erPTM-TPP (Figure 3E,F,I), as well as CCDC6 (Figure 3C,D,I), which was destabilized by O-GlcNAc in all experiments. Of these seven proteins, only EIF3G, PSME1, and CCDC6 are known to be directly O-GlcNAc-modified.[38,56] We leveraged the O-GlcNAc database to identify known O-GlcNAcylated proteins from within our complete list of candidates from all three PTM-TPP experiments. This analysis revealed a total of 34 known O-GlcNAcylated proteins from within our panel of 72 O-GlcNAc-modulated proteins (Tables S1–S4).

Among our observations, there were two aspects we found particularly surprising. One is that many proteins appear to be destabilized by O-GlcNAc and the second is that the majority of O-GlcNAc-modulated proteins identified were not known to be O-GlcNAcylated. These observations suggested to us that some of the effects of O-GlcNAc on thermostability likely arise through perturbation of protein−protein interactions. We therefore assessed whether members from our panel of proteins interact with one another. We inputted all 72 O-GlcNAc-modulated proteins into the STRING server to build a protein interaction network using a combination of experimental and computational sources to score interactions as described previously.[57] This analysis uncovered 34 high-confidence interactors, several of which form complexes (Figure 4). To assess whether O-GlcNAc broadly affected protein complexes, we inputted the full list of proteins identified in our erPTM-TPP experiment using our first selection criterion into the STRING server to build a comprehensive protein interaction network. This analysis revealed a total of 984 different high-confidence protein

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complexes, associated with different biological gene ontology terms, that were identified as not being modulated by O-GlcNAc (Table S7). This analysis supports the specificity of the effect of O-GlcNAc modulation observed on the set of seven protein complexes identified using this high-throughput proteomic analysis.

Interestingly, members from several complexes were identified within different PTM-TPP experiments. Notably, several complexes are parts of macromolecular machines including the proteasome, translation initiation machinery, and the spliceosome—all of which have been reported to have at least one node that is O-GlcNAcylated and to also be functionally regulated by O-GlcNAc[58−60] (Tables S1−S4 and Figure 4). Strikingly, all proteasomal proteins displayed matched O-GlcNAc-dependent destabilization (Figure 4). In contrast, other complexes such as the translation initiation machinery and spliceosome had a mixture of stabilized and destabilized nodes. A closer inspection of these mixed-stability complexes revealed that subsets of connected nodes identified within the same experiment generally had matched stability shifts (Figure 4), a finding that is unlikely to occur simply by chance and supports their joint regulation by O-GlcNAc. Recently, a method called thermal proximity coaggregation (TPCA) was developed to detect protein−protein interactions within TPP data sets.[55] This method relies on the premise that when two proteins interact, they tend to comelt. Applying this TPCA method to our data revealed that several protein partners within our protein interaction network display clear evidence for coaggregation (Figures 4, S13, Tables S8 and S9), supporting our proposed network. Importantly, each identified protein interaction cluster contained at least one protein that is known to be O-GlcNAcylated[61] (Figure 4). Taken together, these data suggest that perturbations in O-GlcNAc likely impact the stability of protein complexes.

CONCLUSIONS

Despite the crucial role of PTMs in regulating protein structure and stability, high-throughput methods to interrogate their effect on the thermodynamic stability of the proteome are lacking. Here, we advance a simple thermal profiling approach, PTM-TPP, to interrogate the effect of O-GlcNAc on the thermostability of the population-averaged ensemble of proteoforms of thousands of proteins within the human proteome. We leverage two strategies, enzymatic removal and chemical modulation, to alter global O-GlcNAc levels and illustrate how these orthogonal approaches can complement and cross-validate one another. A recent report elegantly adapted TPP to study the thermostability effect of protein phosphorylation by HTP.[14] In contrast to our method, HTP involves prior enrichment of phosphorylated peptides to permit simultaneous phosphosite mapping and thermal melt determination for proteoforms modified with phosphorylation in the same experiment. Accordingly, HTP focuses on PTM-modified peptides that are generally substoichiometric.[14] By comparison, our PTM-TPP method is complementary to HTP in that it elucidates the effect of PTMs on the thermodynamic stability of the total endogenous protein pool rather than substoichiometric pools of modified proteoforms. In addition, PTM-TPP has high accuracy in its relative quantitation due to the ability to average reporter ion intensities across multiple identified peptides within a given protein. Importantly, because PTM-TPP examines effects on the global proteome, we believe that it allows for the detection of robust effects that are likely to mediate cellular processes. These features accordingly enable the robust ranking of proteins for downstream study and detection of indirect effects such as those occurring through PTM-regulated protein−protein interactions. Moreover, though here we use direct chemical inhibitors of the PTM-processing enzymes, it is equally feasible to use alternative strategies. For example, transient genetic perturbations can be used to target such enzymes *in cellulo*, or alternatively, PTM levels can be varied by altering flux through biosynthetic pathways responsible for generating a key precursor,[62] or chemical mimetics of PTMs could be used.[63] In addition, PTM-TPP is simple in that it does not require the purification of PTM-modified peptides and therefore it is particularly useful for certain PTMs for which no enrichment strategies exist. As such, we envision that PTM-TPP can be conveniently pursued in a variety of contexts and will prove to be a useful complement to HTP. Thus, PTM-TPP will be valuable for exploring the effects of any PTM on the thermodynamic stability of proteomes.

Our application of PTM-TPP uncovered 72 proteins with O-GlcNAc-regulated stability, seven of which were observed over more than one experiment. Our various PTM-TPP methods use different approaches to modulate global O-GlcNAc levels and, thus, are expected to differentially affect certain O-GlcNAc sites on different proteins. This could lead to variation in O-GlcNAc-dependent stability between these varied methods for certain proteins, as was observed for NCAPG (Tables S1 and S3). Nevertheless, the panel of seven proteins that were congruent across experiments validates our approach and provides key leads for follow-up by the field. We note that the remaining proteins that were uniquely identified by a single independent method, though potentially important, should be further validated prior to conducting detailed follow-up studies. Nevertheless, our complete panel of identified proteins demonstrates that endogenous O-GlcNAcylation, though typically substoichiometric on proteins,[64] can substantially impact the overall thermodynamic stability of a subset of the proteome. Notably, O-GlcNAc-thermostabilized proteins had a significantly lower average T_m compared to the meltome average, consistent with O-GlcNAc conferring physiologically relevant stabilization on certain proteins. This observation is in keeping with the previous work, whereby O-GlcNAc was also found to hinder aggregation of various proteins including polyhomeotic (Ph), tau, and α-synuclein.[25−27] Unexpectedly, however, despite being generally viewed as a stabilizing modification,[25−30] our unbiased method revealed O-GlcNAc as having a predominantly destabilizing effect on this panel of proteins. Notably, the majority of O-GlcNAc-modulated proteins identified here are not known to be glycosylated, raising the possibility of indirect regulation. Following on this line of reasoning, several of these O-GlcNAc-modulated proteins interact with one another as part of large macromolecular assemblies, members of which can be modified by O-GlcNAc. These observations suggest that O-GlcNAc may mediate these destabilizing effects by perturbing protein−protein interactions, as seen for PKM2 where its tetramerization was hindered by O-GlcNAc.[65] This analysis provides a valuable starting point to explore the thermostability effects of O-GlcNAc on protein complexes. However, targeted downstream studies are needed to further validate and explore the detailed molecular effects of O-GlcNAc on these complexes. Our results accordingly indicate that O-GlcNAc can act as a bidirectional regulator of protein thermostability,

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an attribute that is in keeping with its emerging role as a regulator of proteostasis that can act on protein complexes.[58−60,66] We believe that these observations should open new lines of inquiry into how O-GlcNAc may both stabilize and destabilize proteins and protein complexes perhaps, in the latter case, for example, by impairing protein−protein interactions. More generally, these findings highlight the potential for PTMs to regulate thermostability of macromolecular complexes in a similar fashion to small-molecule effectors.[55] Notably, this new PTM-TPP method enables detecting both direct and indirect effects of PTMs on thermostability. Future studies combining this approach with HTP should enable pinpointing those modification sites having the greatest effects on the stability of proteins and protein complexes. We also envision that combining PTM-TPP with TPCA,[55] which allows identifying protein−protein interaction partners on the proteome-wide scale, could help identify protein complexes regulated by PTMs. Finally, we anticipate that the adaptation and application of PTM-TPP to other PTMs should yield new insights into how PTMs regulate the stability of both proteins and protein complexes—illuminating how the diverse set of protein modifications influences cell function in a proteome-wide manner.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c10621.

Methods, supplementary figures, and tables (PDF)

Source data for erPTM-TPP and cmPTM-TPP MS-proteomics experiments (XLSX)

Fold abundance source data for erPTM-TPP and cmPTM-TPP MS-proteomics experiments (XLSX)

Protein interaction network analysis (XLS)

TPCA analysis (XLSX)

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Notes

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■ REFERENCES

(1) Bagwan, N.; El Ali, H. H.; Lundby, A. Proteome-wide profiling and mapping of post translational modifications in human hearts. *Sci. Rep.* **2021**, *11*, No. 2184.

(2) Kumar, A.; Narayanan, V.; Sekhar, A. Characterizing Post-Translational Modifications and Their Effects on Protein Conformation Using NMR Spectroscopy. *Biochemistry* **2020**, *59*, 57−73.

(3) Bah, A.; Vernon, R. M.; Siddiqui, Z.; Krzeminski, M.; Muhandiram, R.; Zhao, C.; Sonenberg, N.; Kay, L. E.; Forman-Kay, J. D. Folding of an intrinsically disordered protein by phosphorylation as a regulatory switch. *Nature* **2015**, *519*, 106−109.

(4) Bachman, A. B.; Keramisanou, D.; Xu, W.; Beebe, K.; Moses, M. A.; Vasantha Kumar, M. V.; Gray, G.; Noor, R. E.; van der Vaart, A.; Neckers, L.; Gelis, I. Phosphorylation induced cochaperone unfolding promotes kinase recruitment and client class-specific Hsp90 phosphorylation. *Nat. Commun.* **2018**, *9*, No. 265.

(5) Shental-Bechor, D.; Levy, Y. Effect of glycosylation on protein folding: a close look at thermodynamic stabilization. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 8256−8261.

(6) Duan, G.; Walther, D. The roles of post-translational modifications in the context of protein interaction networks. *PLoS Comput. Biol.* **2015**, *11*, No. e1004049.

(7) Balch, W. E.; Morimoto, R. I.; Dillin, A.; Kelly, J. W. Adapting Proteostasis for Disease Intervention. *Science* **2008**, *319*, 916−919.

(8) Sebastian, R. M.; Shoulders, M. D. Chemical Biology Framework to Illuminate Proteostasis. *Annu. Rev. Biochem.* **2020**, *89*, 529−555.
(9) Mateus, A.; Kurzawa, N.; Becher, I.; Sridharan, S.; Helm, D.; Stein, F.; Typas, A.; Savitski, M. M. Thermal proteome profiling for interrogating protein interactions. *Mol. Syst. Biol.* **2020**, *16*, No. e9232.
(10) Franken, H.; Mathieson, T.; Childs, D.; Sweetman, G. M.; Werner, T.; Togel, I.; Doce, C.; Gade, S.; Bantscheff, M.; Drewes, G.; Reinhard, F. B.; Huber, W.; Savitski, M. M. Thermal proteome profiling for unbiased identification of direct and indirect drug targets using multiplexed quantitative mass spectrometry. *Nat. Protoc.* **2015**, *10*, 1567−1593.
(11) Savitski, M. M.; Reinhard, F. B.; Franken, H.; Werner, T.; Savitski, M. F.; Eberhard, D.; Martinez Molina, D.; Jafari, R.; Dovega, R. B.; Klaeger, S.; Kuster, B.; Nordlund, P.; Bantscheff, M.; Drewes, G. Tracking cancer drugs in living cells by thermal profiling of the proteome. *Science* **2014**, *346*, No. 1255784.
(12) Becher, I.; Andres-Pons, A.; Romanov, N.; Stein, F.; Schramm, M.; Baudin, F.; Helm, D.; Kurzawa, N.; Mateus, A.; Mackmull, M. T.; Typas, A.; Muller, C. W.; Bork, P.; Beck, M.; Savitski, M. M. Pervasive Protein Thermal Stability Variation during the Cell Cycle. *Cell* **2018**, *173*, 1495−1507.
(13) Niesen, F. H.; Berglund, H.; Vedadi, M. The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. *Nat. Protoc.* **2007**, *2*, 2212−2221.
(14) Huang, J. X.; Lee, G.; Cavanaugh, K. E.; Chang, J. W.; Gardel, M. L.; Moellering, R. E. High throughput discovery of functional protein modifications by Hotspot Thermal Profiling. *Nat. Methods* **2019**, *16*, 894−901.
(15) Smith, I. R.; Hess, K. N.; Bakhtina, A. A.; Valente, A. S.; Rodriguez-Mias, R. A.; Villen, J. Identification of phosphosites that alter protein thermal stability. *Nat. Methods* **2021**, *18*, 760−762.
(16) Potel, C. M.; Kurzawa, N.; Becher, I.; Typas, A.; Mateus, A.; Savitski, M. M. Impact of phosphorylation on thermal stability of proteins. *Nat. Methods* **2021**, *18*, 757−759.
(17) Stein, B. D.; Huang, J. X.; Wu, D.; Cantley, L. C.; Moellering, R. E. *Diverse Hotspot Thermal Profiling Methods Detect Phosphorylation-Dependent Changes in Protein Stability*; bioRxiv, 2021.
(18) Larsen, M. R.; Thingholm, T. E.; Jensen, O. N.; Roepstorff, P.; Jorgensen, T. J. Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol. Cell. Proteomics* **2005**, *4*, 873−886.
(19) Alteen, M. G.; Tan, H. Y.; Vocadlo, D. J. Monitoring and modulating O-GlcNAcylation: assays and inhibitors of O-GlcNAc processing enzymes. *Curr. Opin. Struct. Biol.* **2021**, *68*, 157−165.
(20) Selnick, H. G.; Hess, J. F.; Tang, C.; Liu, K.; Schachter, J. B.; Ballard, J. E.; Marcus, J.; Klein, D. J.; Wang, X.; Pearson, M.; Savage, M. J.; Kaul, R.; Li, T. S.; Vocadlo, D. J.; Zhou, Y.; Zhu, Y.; Mu, C.; Wang, Y.; Wei, Z.; Bai, C.; Duffy, J. L.; McEachern, E. J. Discovery of MK-8719, a Potent O-GlcNAcase Inhibitor as a Potential Treatment for Tauopathies. *J. Med. Chem.* **2019**, *62*, 10062−10097.
(21) Zachara, N. E.; O'Donnell, N.; Cheung, W. D.; Mercer, J. J.; Marth, J. D.; Hart, G. W. Dynamic O-GlcNAc modification of nucleocytoplasmic proteins in response to stress. A survival response of mammalian cells. *J. Biol. Chem.* **2004**, *279*, 30133−30142.
(22) Radermacher, P. T.; Myachina, F.; Bosshardt, F.; Pandey, R.; Mariappa, D.; Muller, H. A.; Lehner, C. F. O-GlcNAc reports ambient temperature and confers heat resistance on ectotherm development. *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111*, 5592−5597.
(23) Love, D. C.; Ghosh, S.; Mondoux, M. A.; Fukushige, T.; Wang, P.; Wilson, M. A.; Iser, W. B.; Wolkow, C. A.; Krause, M. W.; Hanover, J. A. Dynamic O-GlcNAc cycling at promoters of *Caenorhabditis elegans* genes regulating longevity, stress, and immunity. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 7413−7418.
(24) Wang, P.; Lazarus, B. D.; Forsythe, M. E.; Love, D. C.; Krause, M. W.; Hanover, J. A. O-GlcNAc cycling mutants modulate proteotoxicity in *Caenorhabditis elegans* models of human neurodegenerative diseases. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 17669−17674.
(25) Yuzwa, S. A.; Shan, X.; Macauley, M. S.; Clark, T.; Skorobogatko, Y.; Vosseller, K.; Vocadlo, D. J. Increasing O-GlcNAc slows neurodegeneration and stabilizes tau against aggregation. *Nat. Chem. Biol.* **2012**, *8*, 393−399.
(26) Levine, P. M.; Galesic, A.; Balana, A. T.; Mahul-Mellier, A. L.; Navarro, M. X.; De Leon, C. A.; Lashuel, H. A.; Pratt, M. R. alpha-Synuclein O-GlcNAcylation alters aggregation and toxicity, revealing certain residues as potential inhibitors of Parkinson's disease. *Proc. Natl. Acad. Sci. U.S.A.* **2019**, *116*, 1511−1519.
(27) Marotta, N. P.; Lin, Y. H.; Lewis, Y. E.; Ambroso, M. R.; Zaro, B. W.; Roth, M. T.; Arnold, D. B.; Langen, R.; Pratt, M. R. O-GlcNAc modification blocks the aggregation and toxicity of the protein alpha-synuclein associated with Parkinson's disease. *Nat. Chem.* **2015**, *7*, 913−920.
(28) Gambetta, M. C.; Muller, J. O-GlcNAcylation prevents aggregation of the Polycomb group repressor polyhomeotic. *Dev. Cell* **2014**, *31*, 629−639.
(29) Drake, W. R.; Hou, C. W.; Zachara, N. E.; Grimes, C. L. New use for CETSA: monitoring innate immune receptor stability via post-translational modification by OGT. *J. Bioenerg. Biomembr.* **2018**, *50*, 231−240.
(30) Lim, K. H.; Chang, H. I. O-linked N-acetylglucosamine suppresses thermal aggregation of Sp1. *FEBS Lett.* **2006**, *580*, 4645−4652.
(31) Nosella, M. L.; Tereshchenko, M.; Pritisanac, I.; Chong, P. A.; Toretsky, J. A.; Lee, H. O.; Forman-Kay, J. D. O-Linked-N-Acetylglucosaminylation of the RNA-Binding Protein EWS N-Terminal Low Complexity Region Reduces Phase Separation and Enhances Condensate Dynamics. *J. Am. Chem. Soc.* **2021**, *143*, 11520−11534.
(32) Culyba, E. K.; Price, J. L.; Hanson, S. R.; Dhar, A.; Wong, C.; Gruebele, M.; Powers, E. T.; Kelly, J. W. Protein Native-State Stabilization by Placing Aromatic Side Chains in N-Glycosylated Reverse Turns. *Science* **2011**, *331*, 571−575.
(33) Chen, M. M.; Bartlett, A. I.; Nerenberg, P. S.; Friel, C. T.; Hackenberger, C. P.; Stultz, C. M.; Radford, S. E.; Imperiali, B. Perturbing the folding energy landscape of the bacterial immunity protein Im7 by site-specific N-linked glycosylation. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 22528−33.
(34) Thompson, J. W.; Griffin, M. E.; Hsieh-Wilson, L. C. Methods for the Detection, Study, and Dynamic Profiling of O-GlcNAc Glycosylation. In *Methods in Enzymology*; Academic Press, 2018; Vol. *598*, pp 101−135.
(35) Lombard, V.; Golaconda Ramulu, H.; Drula, E.; Coutinho, P. M.; Henrissat, B. The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res.* **2014**, *42*, D490−D495.
(36) Yang, A.; Cho, K.; Park, H. S. Chemical biology approaches for studying posttranslational modifications. *RNA Biol.* **2018**, *15*, 427−440.
(37) Kazemi, Z.; Chang, H.; Haserodt, S.; McKen, C.; Zachara, N. E. O-linked beta-N-acetylglucosamine (O-GlcNAc) regulates stress-induced heat shock protein expression in a GSK-3beta-dependent manner. *J. Biol. Chem.* **2010**, *285*, 39096−39107.
(38) Martinez, M. R.; Dias, T. B.; Natov, P. S.; Zachara, N. E. Stress-induced O-GlcNAcylation: an adaptive process of injured cells. *Biochem. Soc. Trans.* **2017**, *45*, 237−249.
(39) Shen, D. L.; Gloster, T. M.; Yuzwa, S. A.; Vocadlo, D. J. Insights into O-linked N-acetylglucosamine ([0-9]O-GlcNAc) processing and dynamics through kinetic analysis of O-GlcNAc transferase and O-GlcNAcase activity on protein substrates. *J. Biol. Chem.* **2012**, *287*, 15395−1408.
(40) Dennis, R. J.; Taylor, E. J.; Macauley, M. S.; Stubbs, K. A.; Turkenburg, J. P.; Hart, S. J.; Black, G. N.; Vocadlo, D. J.; Davies, G. J. Structure and mechanism of a bacterial beta-glucosaminidase having O-GlcNAcase activity. *Nat. Struct. Mol. Biol.* **2006**, *13*, 365−371.
(41) Selvan, N.; Williamson, R.; Mariappa, D.; Campbell, D. G.; Gourlay, R.; Ferenbach, A. T.; Aristotelous, T.; Hopkins-Navratilova, I.; Trost, M.; van Aalten, D. M. F. A mutant O-GlcNAcase enriches

https://doi.org/10.1021/jacs.1c10621
J. Am. Chem. Soc. 2022, 144, 3833−3842

Drosophila developmental regulators. *Nat. Chem. Biol.* **2017**, *13*, 882−887.
(42) Peck Justice, S. A.; McCracken, N. A.; Victorino, J. F.; Qi, G. D.; Wijeratne, A. B.; Mosley, A. L. Boosting Detection of Low-Abundance Proteins in Thermal Proteome Profiling Experiments by Addition of an Isobaric Trigger Channel to TMT Multiplexes. *Anal. Chem.* **2021**, *93*, 7000−7010.
(43) Perrin, J.; Werner, T.; Kurzawa, N.; Rutkowska, A.; Childs, D. D.; Kalxdorf, M.; Poeckel, D.; Stonehouse, E.; Strohmer, K.; Heller, B.; Thomson, D. W.; Krause, J.; Becher, I.; Eberl, H. C.; Vappiani, J.; Sevin, D. C.; Rau, C. E.; Franken, H.; Huber, W.; Faelth-Savitski, M.; Savitski, M. M.; Bantscheff, M.; Bergamini, G. Identifying drug targets in tissues and whole blood with thermal-shift profiling. *Nat. Biotechnol.* **2020**, *38*, 303−308.
(44) Saei, A. A.; Beusch, C. M.; Sabatier, P.; Wells, J. A.; Gharibi, H.; Meng, Z.; Chernobrovkin, A.; Rodin, S.; Nareoja, K.; Thorsell, A. G.; Karlberg, T.; Cheng, Q.; Lundstrom, S. L.; Gaetani, M.; Vegvari, A.; Arner, E. S. J.; Schuler, H.; Zubarev, R. A. System-wide identification and prioritization of enzyme substrates by thermal analysis. *Nat. Commun.* **2021**, *12*, No. 1296.
(45) Yuzwa, S. A.; Macauley, M. S.; Heinonen, J. E.; Shan, X.; Dennis, R. J.; He, Y.; Whitworth, G. E.; Stubbs, K. A.; McEachern, E. J.; Davies, G. J.; Vocadlo, D. J. A potent mechanism-inspired O-GlcNAcase inhibitor that blocks phosphorylation of tau in vivo. *Nat. Chem. Biol.* **2008**, *4*, 483−490.
(46) Liu, T. W.; Zandberg, W. F.; Gloster, T. M.; Deng, L.; Murray, K. D.; Shan, X.; Vocadlo, D. J. Metabolic Inhibitors of O-GlcNAc Transferase That Act In Vivo Implicate Decreased O-GlcNAc Levels in Leptin-Mediated Nutrient Sensing. *Angew. Chem., Int. Ed.* **2018**, *57*, 7644−7648.
(47) Macauley, M. S.; Vocadlo, D. J. Increasing O-GlcNAc levels: An overview of small-molecule inhibitors of O-GlcNAcase. *Biochim. Biophys. Acta, Gen. Subj.* **2010**, *1800*, 107−121.
(48) Hamiel, C. R.; Pinto, S.; Hau, A.; Wischmeyer, P. E. Glutamine enhances heat shock protein 70 expression via increased hexosamine biosynthetic pathway activity. *Am. J. Physiol.: Cell Physiol.* **2009**, *297*, C1509−C1519.
(49) Moore, C. L.; Dewal, M. B.; Nekongo, E. E.; Santiago, S.; Lu, N. B.; Levine, S. S.; Shoulders, M. D. Transportable, Chemical Genetic Methodology for the Small Molecule-Mediated Inhibition of Heat Shock Factor 1. *ACS Chem Biol.* **2016**, *11*, 200−210.
(50) Phillips, A. M.; Ponomarenko, A. I.; Chen, K.; Ashenberg, O.; Miao, J.; McHugh, S. M.; Butty, V. L.; Whittaker, C. A.; Moore, C. L.; Bloom, J. D.; Lin, Y. S.; Shoulders, M. D. Destabilized adaptive influenza variants critical for innate immune system escape are potentiated by host chaperones. *PLoS Biol.* **2018**, *16*, No. e3000008.
(51) Liebelt, F.; Sebastian, R. M.; Moore, C. L.; Mulder, M. P. C.; Ovaa, H.; Shoulders, M. D.; Vertegaal, A. C. O. SUMOylation and the HSF1-Regulated Chaperone Network Converge to Promote Proteostasis in Response to Heat Shock. *Cell Rep.* **2019**, *26*, 236−249.
(52) Ma, J.; Hou, C.; Li, Y.; Chen, S.; Wu, C. OGT Protein Interaction Network (OGT-PIN): A Curated Database of Experimentally Identified Interaction Proteins of OGT. *Int. J. Mol. Sci.* **2021**, *22*, No. 9620.
(53) Martinez, M.; Renuse, S.; Kreimer, S.; O'Meally, R.; Natov, P.; Madugundu, A. K.; Nirujogi, R. S.; Tahir, R.; Cole, R.; Pandey, A.; Zachara, N. E. Quantitative Proteomics Reveals that the OGT Interactome Is Remodeled in Response to Oxidative Stress. *Mol. Cell. Proteomics* **2021**, *20*, No. 100069.
(54) Groves, J. A.; Maduka, A. O.; O'Meally, R. N.; Cole, R. N.; Zachara, N. E. Fatty acid synthase inhibits the O-GlcNAcase during oxidative stress. *J. Biol. Chem.* **2017**, *292*, 6493−6511.
(55) Tan, C. S. H.; Go, K. D.; Bisteau, X.; Dai, L.; Yong, C. H.; Prebhu, N.; Ozturk, M. B.; Lim, Y. T.; Sreekumar, L.; Lengqvist, J.; Tergaonkar, V.; Kaldis, P.; Sobota, R. M.; Nordlund, P. Thermal proximity coaggregation for system-wide profiling of protein complex dynamics in cells. *Science* **2018**, *359*, 1170−1177.
(56) Zhang, W.; Liu, T.; Dong, H.; Bai, H.; Tian, F.; Shi, Z.; Chen, M.; Wang, J.; Qin, W.; Qian, X. Synthesis of a Highly Azide-Reactive and Thermosensitive Biofunctional Reagent for Efficient Enrichment and Large-Scale Identification of O-GlcNAc Proteins by Mass Spectrometry. *Anal. Chem.* **2017**, *89*, 5810−5817.
(57) Szklarczyk, D.; Gable, A. L.; Lyon, D.; Junge, A.; Wyder, S.; Huerta-Cepas, J.; Simonovic, M.; Doncheva, N. T.; Morris, J. H.; Bork, P.; Jensen, L. J.; Mering, C. V. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* **2019**, *47*, D607−D613.
(58) Zhang, F.; Su, K.; Yang, X.; Bowe, D. B.; Paterson, A. J.; Kudlow, J. E. O-GlcNAc Modification Is an Endogenous Inhibitor of the Proteasome. *Cell* **2003**, *115*, 715−725.
(59) Li, X.; Zhu, Q.; Shi, X.; Cheng, Y.; Li, X.; Xu, H.; Duan, X.; Hsieh-Wilson, L. C.; Chu, J.; Pelletier, J.; Ni, M.; Zheng, Z.; Li, S.; Yi, W. O-GlcNAcylation of core components of the translation initiation machinery regulates protein synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **2019**, *116*, 7857−7866.
(60) Tan, Z. W.; Fei, G.; Paulo, J. A.; Bellaousov, S.; Martin, S. E. S.; Duveau, D. Y.; Thomas, C. J.; Gygi, S. P.; Boutz, P. L.; Walker, S. O-GlcNAc regulates gene expression by controlling detained intron splicing. *Nucleic Acids Res.* **2020**, *48*, 5656−5669.
(61) Wulff-Fuentes, E.; Berendt, R. R.; Massman, L.; Danner, L.; Malard, F.; Vora, J.; Kahsay, R.; Olivier-Van Stichelen, S. The human O-GlcNAcome database and meta-analysis. *Sci. Data* **2021**, *8*, No. 25.
(62) Gloster, T. M.; Zandberg, W. F.; Heinonen, J. E.; Shen, D. L.; Deng, L.; Vocadlo, D. J. Hijacking a biosynthetic pathway yields a glycosyltransferase inhibitor within cells. *Nat. Chem. Biol.* **2011**, *7*, 174−181.
(63) Chuh, K. N.; Batt, A. R.; Pratt, M. R. Chemical Methods for Encoding and Decoding of Posttranslational Modifications. *Cell Chem. Biol.* **2016**, *23*, 86−107.
(64) Darabedian, N.; Thompson, J. W.; Chuh, K. N.; Hsieh-Wilson, L. C.; Pratt, M. R. Optimization of Chemoenzymatic Mass Tagging by Strain-Promoted Cycloaddition (SPAAC) for the Determination of O-GlcNAc Stoichiometry by Western Blotting. *Biochemistry* **2018**, *57*, 5769−5774.
(65) Wang, Y.; Liu, J.; Jin, X.; Zhang, D.; Li, D.; Hao, F.; Feng, Y.; Gu, S.; Meng, F.; Tian, M.; Zheng, Y.; Xin, L.; Zhang, X.; Han, X.; Aravind, L.; Wei, M. O-GlcNAcylation destabilizes the active tetrameric PKM2 to promote the Warburg effect. *Proc. Natl. Acad. Sci. U.S.A.* **2017**, *114*, 13732−13737.
(66) Guo, B.; Liang, Q.; Li, L.; Hu, Z.; Wu, F.; Zhang, P.; Ma, Y.; Zhao, B.; Kovacs, A. L.; Zhang, Z.; Feng, D.; Chen, S.; Zhang, H. O-GlcNAc-modification of SNAP-29 regulates autophagosome maturation. *Nat. Cell Biol.* **2014**, *16*, 1215−1226.

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