Methods that assay protein foldedness with proteomics have generated censuses of apparent protein folding stabilities in biological milieu. However, different censuses poorly correlate with each other. Here, we show that the reason for this is that methods targeting foldedness through monitoring amino acid sidechain reactivity also detect changes in conformation and ligand binding, which can be a substantial fraction of the data. We show that the reactivity of only one quarter of cysteine or methionine sidechains in proteins in a urea denaturation curve of mammalian cell lysate can be confidently explained by a two-state unfolding isotherm. Contrary to that expected from unfolding, up to one third of the cysteines decreased reactivity. These cysteines were enriched in proteins with functions relating to unfolded protein stress. One protein, chaperone HSPA8, displayed changes arising from ligand and cofactor binding. Unmasking this hidden information using the approaches outlined here should improve efforts to understand both folding and the remodeling of protein function directly in complex biological settings.
The maturation of an active protein is typically reliant upon the nascent polypeptide folding into a complex topology. Folding involves a thermodynamic component, which describes the free energy difference between the folded state and unfolded state at equilibrium (folding stability, ΔG). Folding also involves other sequential processing steps, such as post-translational modifications and transport. Because folding is fraught with potential mishaps including misfolding and aggregation, in cells a proteostasis network oversees all steps related to synthesis, folding and degradation. In mammalian cells this network consists of several hundred proteins, including molecular chaperone families (e.g., heat shock protein families 40, 70 and 90), the ubiquitin-proteosome system, autophagy and stress response systems.

Proteostasis imbalance is implicated in diseases involving inappropriate protein aggregation, including neurodegeneration. As such, there has been extensive interest in determining how protein foldedness varies for proteomes inside cells in healthy and disease contexts. A canonical approach for measuring protein folding stability is a purified protein involving measuring the abundance of folded and unfolded states in different concentrations of chaotropes, such as urea or guanidine hydrochloride, or exposure to increasing temperature. This approach yields measures of ΔG or other correlates of ΔG such as chemical denaturation midpoint (Cm) or thermal melting midpoint (Tm) values that are informative in the case where the protein folds by a two-state equilibrium mechanism. Recent advances have allowed this canonical strategy to measure protein folding stabilities in biological extracts, thereby enabling the en masse determination of folding stabilities of proteins. Measurement strategies have targeted differences between the folded and unfolded states, such as aggregation propensity, sensitivity of solvent exposed amino acid sidechains to reactive chemicals, which we hereon refer to as residue labeling, and protease cleavage susceptibility.

While the main thrust of many proteomics studies employing these approaches has been to test the effect of ligand binding on protein stability rather than define the ΔG per se, it remains generally unclear how well the reported measures of stability actually relate to the thermodynamic stability of the protein. In biological settings measures of protein folding stability are affected by conformational change, ligand binding and protein network organization. For example, Tm values act as proxies for enzyme activity, DNA binding, and complex formation as well as posttranslational modifications during the cell cycle. In other work, ATP was shown to increase the resistance of thermal denaturation of positively charged, intrinsically disordered proteins, and this resistance depends on their localization to different membrane-less organelles. Limited proteolysis-based mapping of protein structure has also yielded information on metabolite-induced conformational changes of proteins. Because measures of folding stability can act as proxies for other distinct features of proteins, a strategy to more completely decipher whether the measured values of stability relate to the actual conformational stability versus a conformational change or ligand binding event would greatly aid the ability to interpret complex in situ denaturation datasets.

Here, we investigate this problem at two levels. First, we examined how well published datasets correlated with each other to establish whether the folding stabilities are indicative of genuine ΔG values. Second, we investigated how well the measured values of Tm, Cm and ΔG of individual cysteine and methionine residues within proteins correlate to the actual stability using residue labeling approaches. Our work provides a platform to quantify the extent through which these values report on additional complexity in the data. We find surprising information related to chaperone activity on a urea denaturation curve of mammalian lysozyme. The approaches we describe provide a pathway to unmask hidden information pertaining to protein function in complex biological milieu.

**Results**

**Methodological differences yield poorly correlated measures of thermodynamic proteome stability.** First, we examined 20 datasets from 12 studies that reported high-quality protein folding stability data, which used limited proteolysis, residue labeling and thermal profiling methods to assay foldedness (complete reference details are provided in Supplementary Table 1). These studies reported Tm or Cm values which, for two-state folding models indicate the conditions of equal populations of unfolded and folded protein (i.e., where ΔG equals zero). We hypothesized that if these values authentically report on two-state protein folding stability the different datasets should correlate with one another. To test this hypothesis, we first scaled each Tm or Cm dataset to range between 0 and 1 to account for the inherently different magnitudes of Tm and Cm values, and then performed pairwise comparisons between datasets. Linear regressions fitted to each comparison revealed a strong positive relationship in stabilities when comparing datasets derived from the same methodology, particularly among thermal profiling data (Fig. 1; lower diagonal datasets 7–20). This conclusion was not dependent on the species from which the protein stability was measured, suggesting that closely related proteins from different species behave similarly in terms of the Tm and Cm values. However, comparisons between the datasets derived from different methodologies, such as thermal profiling versus residue labeling, revealed poor correlations at best and none at worst. This lack of correlation was supported by Spearman’s correlation coefficients calculated for each comparison (Fig. 1; upper diagonal), whereby significant positive correlations were primarily observed between datasets derived from the same methodology. One notable exception was residue labeling dataset 417, which was moderately correlated with 14 of the 15 thermal profiling datasets. However, closer inspection of dataset 4 revealed that only 17% of the quantified data reported Cm values, because these were the only data that fitted well to a two-state unfolding curve. It thus follows that 83% of the data was disregarded because it did not fit to the two-state model (see Supplementary Table 1 for complete reference details). By contrast, between 66% and 99%, or 66% and 69% of proteins were reported to be well fitted to two-state unfolding isotherms in other thermal profiling and residue labeling datasets, respectively. Collectively, these findings suggested that the most canonical and simple patterns of unfolding, i.e., those that look like two-state unfolding curves were indeed the most likely to report on two-state-like protein unfolding behavior and to correlate with different apparent folding stability datasets. More intriguing however was that such two-state like stability values encompass only a fraction of the data available. Hence the remaining data likely was more applicably explained by complex unfolding mechanisms or mechanisms distinct to folding.

**Residue labeling techniques reveal nuanced and heterogeneous changes in protein conformation due to chemical denaturation.** To further examine which data can be appropriately explained in terms of two-state folding or not, we collected our own dataset of apparent folding stability using tetraphenylethene maleimide (TPE-MI) as a probe for unfolded proteins. TPE-MI reacts with exposed cysteine free thiols that are otherwise buried in the folded state. Free cysteine thiols are the least surface-exposed residue of all amino acids in globular proteins so provide an excellent target for examining protein foldness. We first performed a urea denaturation curve of purified β-lactoglobulin,
which is a model globular protein containing a single buried free thiol residue, and unfolds via two-state-like behavior (Supplementary Fig. 1). The rate of reaction of TPE-MI with β-lactoglobulin was proportional to the anticipated exposure of the buried thiol upon two-state unfolding. The relationship between rate of reaction and urea concentration yielded a \( C_m \) consistent with that obtained from intrinsic tryptophan fluorescence and in accordance with other published results on β-lactoglobulin folding19.

To use TPE-MI on a proteome-wide scale we created denaturation curves of cell lysate with urea (Fig. 2a). Lysates were prepared from mouse neuroblastoma cells (Neuro2a) subjected to Stable Isotope Labeling by Amino acids in Cell culture (SILAC) using light or heavy (\(^{13}\)C L-lysine and \(^{13}\)C,\(^{15}\)N L-arginine) isotopes for quantitation of reactivity. In essence, lysate from light-isotope labeled cells was used as the "native" control versus lysate from heavy-isotope labeled cells prepared with different concentrations of urea.

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Complete reference information is provided in Supplementary Table 1. Source data are provided as a Source Data file.
Fig. 2 Residue labeling methods reveal patterns of change inconsistent with two-state unfolding during chemical denaturation. a Schematic representation of the workflow used to detect conformational change using TPE-MI following chemical denaturation. The graphs show hypothetical data to illustrate the point. b Clustering of cysteine-peptide TPE-MI reactivity profiles as a function of urea concentration using fuzzy c-means. Families of individual peptide traces are shown where the color saturation reflects the cluster membership score for each peptide, such that the darkest traces are representative of the typical response for each cluster. Data (n = 3 biological replicates) were smoothed by significance scaling and loess smoothing. The proportion of total peptides assigned to each cluster is also indicated. c Histogram of goodness-of-fit among individual peptides following fitting to a two-state unfolding curve. d Cross-correlation analysis of peptides in each cluster to previously published stability datasets (list of datasets in Supplementary Table 1). Correlation heatmap is colored according to the Spearman’s correlation coefficient (R) and significance is denoted by * (p < 0.05) or ** (p < 0.01). Exact p values are provided in Supplementary Data 1. Datasets are ordered according to species of origin (human or mouse) and method of stability measure (limited proteolysis, residue labeling or thermal profiling). Missing points (gray line) indicate fewer than 5 proteins in common. e Corresponding clustering analysis of an independent published residue labeling dataset (dataset 4) that targeted oxidation of exposed Met residues. Data here is represented as per panel B. Source data are provided as a Source Data file.
labeled samples were each reacted with TPE-MI before mixing and quantification for the level of reactivity. Peptides modified with TPE-MI are not readily quantified via mass spectrometry due to unfavorable physicochemical properties. While we cannot distinguish TPE-MI from other covalent modifications of an individual cysteine, both report on a change in the local environment for the cysteine and result in a net loss of the unreacted peptide. Therefore, the extent of cysteine reactivity was determined from the change in abundances of peptides with unreacted cysteines normalized to the ratio of peptides from the same protein that lacked cysteine (Fig. 2a).

To determine the underlying trends in cysteine reactivity as a function of urea concentration we clustered the cysteine peptide reactivity profiles using an unbiased computational approach of fuzzy-c means. This analysis yielded four distinct patterns of cysteine response to urea titration (clusters 1–4) shown in Fig. 2b. Cluster 1 appeared to represent patterns lacking a systematic change in reactivity with urea concentration. Clusters 2 and 3 both appeared to represent patterns of increasing cysteine thiol reactivity, which was anticipated for greater exposure of buried thiols upon denaturation. Cluster 2 differed from cluster 3 by appearing to show the reactivity changes occurring first at higher urea concentrations whereas the changes occurred at lower urea concentrations for cluster 3. Cluster 4, representing one third of the identified cysteine peptides, appeared to represent decreases in reactivity upon increasing concentrations of urea. This was counter to the anticipated increase in reactivity expected from the exposure of buried cysteine residues induced by denaturation, suggesting that distinct processes were occurring in some proteins in response to urea titration that led to greater burial of exposed cysteine thiols (discussed in more detail below).

To determine which of the data were most consistent with a two-state folding mechanism, individual cysteine reactivity curves were fitted to a two-state unfolding curve and assessed for goodness of fit (Fig. 2c). Consistent with the greater reactivity upon urea denaturation, clusters 2 and 3 contained the most peptides with good fits defined by an $R^2 > 0.9$ (Fig. 2c). However, the peptides in cluster 2 were the only group that showed a significant correlation between the fitted $C_m$ values with those of existing census datasets described herein previously in Fig. 1 (Fig. 2d). Given that the published datasets were pre-filtered in the original studies to be consistent with two-state unfolding, this finding demonstrates an authenticity in this subset of data for tracking bona fide two-state unfolding events. The peptides in cluster 3 did not share this correlation with other datasets, and may be a diagnostic of reactivity reporting on non-two-state folding mechanisms such as multistate or non-reversible folding, or other non-folding related mechanisms (Fig. 2d).

To investigate whether the general conclusions made from the TPE-MI dataset can be drawn in datasets derived from other residue labeling methods for foldedness, we re-examined the pre-processed peptide quantitation from dataset 4 according to our clustering procedures. Dataset 4 monitored methionine exposure in the lysate of human cell line, HCA2-hTert, by a free radical oxidation approach in different concentrations of the denaturant guanidine hydrochloride. This dataset was therefore analogous to the TPE-MI approach but independent in multiple parameters of chemical denaturant (guanidine hydrochloride versus urea), species (human versus mouse), target residue for labeling (methionine versus cysteine) and research team (independent research labs). In addition, unlike TPE-MI, oxidized methionines are readily quantifiable, which offered us the opportunity to evaluate the applicability of our method to a directly measured modification.

Despite these differences in parameters, the clustering procedures resulted in a strikingly similar grouping of the oxidized methionine peptides to the TPE-MI dataset (Fig. 2e – note however that the direction of change is inverted due to the nature of the measurements) that illustrates several fundamentally consistent conclusions. First was that the data formed 4 clusters with similar patterns of response. Second was that there were broadly consistent proportions of peptides in the different clusters. Most notably was the consistent cluster for increased protection upon denaturant titration that indicated changes inconsistent with unfolding.

Together the TPE-MI and methionine oxidation data indicated that with an unbiased clustering procedure only about one quarter of residue labeling data in chemical denaturation curves of mammalian proteomes can be confidently described as consistent with a two-state unfolding mechanism. Most notably, this independent dataset confirmed the observation that a substantial portion of the reactivity changes measured in the presence of denaturant cannot be satisfactorily explained by unfolding.

Residue labeling patterns earmark protein conformational rearrangement as well as unfolding. To investigate protein properties that explain the different patterns of response to urea titration, we first examined the physicochemical characteristics of the peptides in each cluster (and the proteins to which they belong to; Fig. 3a). We examined properties predicted from amino-acid composition that pertained to likely burial of the target residues in the folded state, including charge, hydrophobicity, likelihood to reside in regions of secondary structure and relative solvent exposure for individual peptides associated with each cluster. Several characteristics stood out. Notably the peptides in clusters 2 and 3, which we have thus far demonstrated to have best consistency with two-state folding mechanisms, were most likely to contain hydrophobic, and least likely to be in unstructured protein regions which is in accordance with this conclusion (Fig. 3b). In addition, cluster 2 were most likely to contain solvent-buried residues. For the parent proteins that have known structures (around 20% of the proteins identified), the extent of solvent exposure of the labeled cysteine residue further supported the conclusion that cysteines in cluster 2 were in buried regions of proteins and hence became exposed upon denaturation (Supplementary Fig. 2a). For completeness of analysis, we did not identify any enrichment for other protein features, such as whether the peptides resided in active sites, binding sites, functional motifs, disulphide bonds or annotated domains (Supplementary Fig. 2b–d). Another feature we considered was the presence of conserved protein domains, annotated using the PFAM database which assigns protein domains to families according to conserved structural or sequence features that have been aligned using hidden Markov models. While the proportion of residues located within annotated PFAM domains was relatively high (more than 70% in every cluster), this was anticipated due to the likelihood of free thiols being buried in the folded state of most proteins.

Because individual proteins may include multiple cysteine-containing peptides that fall into different clusters, we next grouped proteins into categories based on which cluster the peptides belonged to (Fig. 3a). More than half of the peptides belonged to proteins that contained at least one other cysteine peptide from a different cluster (Fig. 3c). Proteins with such peptides were considered multi-clustered and separated from proteins that contained cysteine peptides exclusively in one of the other four clusters (which we hereon call uni-clustered) for further analysis. Of the multi-clustered proteins, one-third had at least one cysteine peptide which decreased in reactivity (i.e., was in cluster 4) and one or more cysteine peptides for which reactivity increased in the presence of higher concentrations of...
urea, which suggested multimodal impacts on the protein during denaturation. It is reasonable to predict that such proteins are larger and multi-domain. Indeed, multi-clustered proteins were more likely to have a larger molecular mass and contain more annotated PFAM domains than uni-clustered proteins, consistent with this conclusion (Fig. 3d, e; two-sample t-test, p < 0.001). Also of note was the consistency in predicted physicochemical features for uni-clustered proteins whose peptides were associated with cluster 2, which featured elevated hydrophobicity and lower solvent exposure compared to the other uni-clustered and multi-clustered categories (Supplementary Fig. 2e). This result further supported the conclusion that proteins containing solely cluster 2 peptides were the most likely to display two-state unfolding and that the other proteins with mixed clusters displayed more complex unfolding or other non-folding changes in response to urea titration.

In addition to physicochemical properties, we also examined the molecular functions of proteins assigned to the uni- and...
multi-clustered protein categories. Protein-protein interaction analysis using the STRING database revealed no difference in the number of direct high-confidence protein binding partners between uni- versus multi-clustered proteins (Fig. 3f). However, the average node degree (the number of protein-protein interactions within each cluster) was up to four-fold higher in multi-clustered proteins than among uni-clustered proteins (Fig. 3g). This result was consistent with the anticipation that multi-clustered proteins are more likely to be multi-domained and larger in size. It therefore follows that such proteins would operate in larger functional networks, which display coordinated changes in cysteine reactivity where exposure of one protein of a complex yields exposure of its binding partner. By comparison, uni-clustered proteins, particularly those in cluster 2, were more likely to be poorly interconnected. This conclusion is consistent with an anticipation that these proteins were simpler globular proteins whereby the data reported solely on their foldedness and not their function in networks.

Gene ontology (GO) analysis was investigated for each of the protein categories to examine the possibility of a coordinated functional response corresponding to the cysteine reactivity changes (Fig. 3H). Of the 34 significantly enriched top-level GO terms in the multi-clustered proteins, half encompassed mechanisms pertaining to binding and protein complexes or proteostasis response mechanisms such as protein folding machinery. Of note, three of the GO terms (chaperonin-containing T-complex, GO:0005832; cellular response to heat, GO:0034605; and protein folding, GO:0006457) are associated with the stimulus of denaturation. These changes in thiol reactivity appeared to earmark changes in ligand binding or the conformation of select proteins that have functions in responding to unfolded proteins that accumulate at higher concentrations of urea. A parallel analysis of the methionine oxidation data (dataset 4) yielded nine identical GO terms (Fig. 3f) for common terms, Supplementary Fig. 3 for all dataset 4 specific terms. This result was further striking in that data from a distinct method, species and denaturant led to a conserved GO enrichment of terms related to responses to unfolded proteins, including identical terms of chaperonin-containing T-complex (GO:0005832) and protein folding (GO:0006457). These data therefore led us to conclude that multi-clustered proteins were likely to encompass functional responses to protein denaturation. By contrast, GO analysis of uni-clustered proteins showed no conserved terms between the two datasets (Supplementary Fig. 4).

Residue reactivity captures features of chaperone conformational change in lysate. To decipher the molecular mechanisms that underlie the functional responses to unfolded protein, we next focused on a class of proteins that we expected to have functions in engaging with unfolded proteins, i.e., molecular chaperones. 42 proteins annotated with the ontology term “chaperone-mediated protein folding” machinery (GO:0061077) were identified across the protein groups, particularly in the multi-clustered proteins (Supplementary Fig. 5).

One of these proteins HSPA8 (HSC70; P63017) is the cognate heat shock protein 70 (Hsp70), and was identified by 3 cysteine peptides assigned to two different clusters (Fig. 4a). HSPA8 binds to unfolded proteins in concert with J-domain protein co-chaperones and nucleotide exchange factors. Together they drive protein folding through ATP-dependent cyclical binding and release. A conserved structural feature of Hsp70 proteins are four modules: an N-terminal nucleotide binding domain (NBD), a substrate binding domain (SBDB), a helical lid domain (SBDa), and a disordered C-terminal tail of variable length. The disordered tail of HSPA8 comprises an EEVD motif that mediates interactions with cofactors such as J-domain protein DNAJB1. One peptide from the NBD, containing Cys17, became more reactive to TPE-MI at urea concentrations greater than 4 M (Fig. 4d). The other two peptides were from the SBDa domain, containing Cys574 and Cys603, and these both became more protected in concentrations of urea between 3 and 5 M (Fig. 4d). We also identified one peptide from DNAJB1, that contained two cysteine residues (Cys267, Cys269). These cysteines displayed a complex biphasic (but overall increased) reactivity profile upon exposure to increasing concentrations of urea (Fig. 4d). We therefore postulated that the changes in cysteine reactivity in HSPA8 and possibly cofactor DNAJB1 upon urea titration may arise due to allosteric conformational changes resulting from their binding to each other and/or upon the engagement with unfolded proteins.

To test this hypothesis, we examined thiol reactivity changes in a reconstituted HSPA8 system in vitro that comprised of purified human HSPA8, DNAJB1 and a well-characterized model client, malate dehydrogenase (MDH2) (Fig. 4a–c). To look specifically for changes resulting from interaction with denatured client, TPE-MI reactivity was compared between reconstituted systems containing native versus thermally denatured MDH2 using tandem mass tag (TMT) isotopic labeling (Fig. 4e).

First, we assessed the system without added ATP. Under this condition HSPA8 and DNAJB1 can bind to non-native MDH2 substrate to form a stable complex. More specifically, the SBDa domain of an Hsp70 protein (DnaK) has been shown to interact with substrate when the chaperone is in the ADP-bound state. We saw no change in reactivity of the HSPA8 NBD peptide containing Cys17 (Fig. 4f) suggesting that the increase in reactivity observed above 4 M urea titration in lysate was attributable to NBD unfolding. In contrast, the SBDa domain peptide containing Cys574 decreased in reactivity (one-sample t-test, p = 0.033). This result therefore suggested that the decrease in reactivity observed in the SBDa region during urea denaturation arose from substrate and/or ligand binding.

In DNAJB1, we observed two cysteine peptides in the reconstituted system. One peptide, containing Cys179, is located close to the hinge between two β-barrel-like subdomains (CTD1 and CTDII) that binds to substrates (Fig. 4a). The second, containing two closely adjacent cysteines (Cys267, Cys269), is in the homodimer interface. It is important to note that we could not ascribe reactivity changes to a single cysteine within this peptide, thus the changes represent an average across these residues. The peptide containing Cys267 and Cys269 became significantly more reactive under these conditions (Fig. 4f; one-sample t-test, p = 0.023). The central location of this peptide within the homodimer interface of DNAJB1 suggested that accumulated client-binding altered the conformation of DNAJB1 to expose the structure nearby these cysteines.

Next we examined the effect of adding supplemental ATP to the reconstituted system, which we predicted would fuel HSPA8 to undergo the full catalytic cycle and therefore release accumulated complexes of HSPA8 and DNAJB1 bound to client. The Cys574 peptide from HSPA8 became more reactive under these conditions, which is in agreement with HSPA8 disengaging client and/or DNAJB1. Intriguingly, the Cys179 peptide from DNAJB1 showed increased reactivity (Fig. 4f; one-sample t-test, p = 0.023). Of note, this peptide is close to the region of DNAJB1 shown to bind the EEVD motif of HSPA8, suggesting a level of deprotection when client-bound complexes of the HSPA8-DNAJB1 machinery are dissociated. While this is an attractive hypothesis, we could not exclude the possibility of other allosteric changes associated with DNAJB1 activity. Namely, intramolecular J-domain interactions with the hinge region occur near this residue which are modulated by DNAJB1 engagement with HSPA8. The peptide in DNAJB1
containing (Cys267, Cys269) was no longer exposed (Fig. 4f; one-sample t-test, \( p = 0.26 \)), which is consistent with the lack of accumulated substrate-chaperone complexes that would otherwise drive the conformational changes at the dimer interface described above.

We also examined the reconstituted chaperone experiment under conditions of urea denaturation to mimic the lysate denaturation experiment (Supplementary Fig. 6a). In this context, Cys17 in HSPA8 showed no change in reactivity (Supplementary Fig. 6a; one-sample t-test, \( p = 0.14 \) and \( p = 0.68 \)), which is consistent with the NBD being denatured and hence making Cys17 amenable to reaction irrespective of other ligands. This conclusion is supported by a recent study reporting a urea denaturation curve of HSPA8 that showed it displayed two-domain folding behavior\(^{33}\). One domain, likely the NBD, had a \( C_m \) around 1.5 M urea and the other domain a \( C_m \) around 3.6 M urea. The changes in the SBD also showed protection at Cys603 that was abolished by the addition of ATP (Supplementary Fig. 6a; one-sample t-test, \( p = 0.044 \) and \( p = 0.31 \) respectively). Hence this is consistent with residual binding activity in the presence of urea that protect the cysteine from reaction. DNAJB1 appeared to be mostly denatured at 6 M urea (Supplementary Fig. 6c) and accordingly the peptide Cys179 did not show any change in reactivity (Supplementary Fig. 6a; one-sample t-test, \( p = 0.22 \) and \( p = 0.12 \) in the absence or presence of exogenous ATP prior to TPE-MI labeling). Means \( \pm \) S.D. (\( n = 4 \) biological replicates) are shown, and deviations from the expected mean of 1 were tested using a one-sample t-test (* denotes \( p < 0.05 \), ns denotes \( p > 0.05 \)). Exact p values are provided in Supplementary Data 1. In all panels, cysteine residues are labeled and colored according to the cluster their respective peptides were assigned (orange, red, purple and blue correspond to clusters 1–4 respectively, black was not observed). Source data are provided as a Source Data file.

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**Fig. 4 Detection of distinct HSPA8 conformations by residue labeling approaches.**

- **a** Ribbon structure of HSPA8 (models built with PDB 4H5R61, 3AGY29 and 4KBO62). Nucleotide binding domain (NBD; ruby), substrate binding domains (SBD\( \beta \) green, SBD\( \alpha \) dark-gray) and cofactor binding motif (EEVD motif; teal) are shown on protein backbones. J-domain (salmon) and HSPA8 binding region (teal) are shown on protein backbones, and C-terminal domains (CTDI and CTDII) are indicated by brackets.

- **b** Ribbon structure of DNAJB1 (model built with PDB 3AGZ29 and 1HDJ63), with dimer composed of two monomers colored dark and light gray respectively. J-domain (salmon) and DNAJB1 binding domain (teal) are shown on protein backbones, and C-terminal domains (CTDI and CTDII) are indicated by brackets.

- **c** MDH2, with Cys285, Cys93 and Cys89 shown and Cys212 (black) not observed. Source data are provided in Supplementary Data 1. In all panels, cysteine residues are labeled and colored according to the cluster their respective peptides were assigned (orange, red, purple and blue correspond to clusters 1–4 respectively, black was not observed).

- **d** Schematic for recombinant client-binding assay. Human HSPA8 and DNAJB1 were incubated with native (above line) or heat-denatured (below line) client, MDH2. Nucleotide binding domain (NBD; ruby), substrate binding domains (SBD\( \beta \); green and SBD\( \alpha \); gray) and cofactor interaction region (IR; teal) are shown on protein backbones. In the case of DNAJB1, dimer is shown with second monomer desaturated. After incubation, samples were labeled with TPE-MI then prepared for mass spectrometry.

- **e** Changes in thiol reactivity of HSPA8 and DNAJB1 peptides in Neuro2a lysate titrated with urea. The changes in the SBD also showed protection at Cys603 which was abolished by the addition of ATP (Supplementary Fig. 6a; one-sample t-test, \( p = 0.12 \) in the absence or presence of ATP respectively). The DNAJB1 peptide containing Cys267 and Cys269 showed no change in reactivity in the absence of ATP (Supplementary Fig. 6a; one-sample t-test, \( p = 0.15 \)) but there was a significant protection in the presence of ATP (Supplementary Fig. 6a; one-sample t-test, \( p = 0.015 \)). This protection was surprising and there is no simple interpretation but given the changes we observed in these regions of DNAJB1 with the thermally denatured MDH1, the results may underly poorly understood conformational changes in that region of DNAJB1.

To determine which, if any, protein-protein interactions may be responsible for the protection we observe in HSPA8 in lysate, we repeated the urea denaturation curve of lysate with cells grown in the absence and presence of the specific HSP70 inhibitor, VER155008 (Fig. 5a). In this experiment we compared the individual peptide abundances from control-derived lysate to...
VER155008-derived lysate at matching concentrations of urea, such that any peptides whose reactivity was not affected by the presence of VER155008 would have a ratio of 1. After correcting for any differences in expression of individual proteins using the mean ratio for non-cysteine-containing peptides for each protein, we determined potential hit cysteine peptides as those whose corrected ratio exceeded the standard deviation of the corrected per-protein non-cysteine peptide ratio. The graphs show hypothetical data to illustrate the point. Exemplar peptide traces for hit proteins CCT5 (P80316), PTGES3 (Q9R0Q7) and MAP1B (P14873). Gray band represents the cumulative S.D. of non-cysteine peptides across all urea concentrations. Interaction networks were validated by the STRINGdb (v11.0, medium confidence score >0.4) and manually grouped according to direct (edge distance = 1) and indirect (edge distance >1) interaction with an HSP70. Source data are provided as a Source Data file.

Last, we examined the other chaperone proteins in cluster 4 for clues on hidden patterns related to substrate binding. Major chaperone classes were represented in cluster 4, including the Hsp40, Hsp70 and Hsp90 families, the Bcl-2 associated anathogene cochaperone (BAG) family and the T-complex chaperonin. Many of the chaperones showed decreased cysteine reactivity in protein sequences previously associated with chaperone function (Supplementary Table 2). This includes Cys530 of HSP90AA1 (Hsp90α; P07901), which is located in the middle domain and is spatially close to residue cluster (E528, Y529, Q532) required for client binding and chaperone function (Fig. 6a). Thirty-three unique cysteine-containing peptides were observed across all eight subunits of
TRiC (TCP1; CCT2-CCT8). Of note was that four peptides derived from CCT2, CCT3, CCT6 and CCT8 had decreased reactivity at high urea concentrations (Fig. 6b). These peptides correspond to the apical or intermediate-equatorial hinge regions that are known to bind to substrate, or have conformational changes associated with substrate binding.35,36. These chaperones provide a wealth of candidates with well-characterized structure-function relationships for further investigation of the denaturation-induced protection phenomena.

**Discussion**

Collectively, our findings provide a means to reveal hidden complexity in proteome-wide datasets targeting foldedness with residue labeling approaches. Namely, we demonstrate that a substantial component of the changes seen in residue labeling datasets applied to study proteome denaturation by chemical denaturants are better explained by changes in protein conformation and ligand interactions than unfolding. These findings have two implications of note. First is that analysis of the peptides (and proteins) that generate denaturation curves most similar to two-state unfolding curves provides the most consistent correlation between methodological approaches and provide a more robust core census list of stability measurements. While the absolute stability measurements may not be important if they are used as internal reference points for the effect of a particular treatment such as drug binding, their use for in situ absolute measures of folding stability needs to be carefully considered in any retrospective analysis of whole proteome stability datasets because it highlights that upwards of two thirds of the data previously fitted to a measure of folding stability may be more appropriately explained by changes in conformation or ligand association. Other proteomics approaches have drawn general conclusions that are in agreement with our findings here, namely how changes in proteome solubility encode information on rewired protein interaction networks.37–39. Others have also shown that ligand interactions can modulate the thermal melting profiles of proteins in lysate40,41, and recent commentary speculates this is one amongst a range of biophysical effects that could contribute to a protein’s observed thermal stability.42. The workflow presented here provides a useful strategy to delineate changes arising from unfolding from these other attributes of proteins.

The second implication is that the ability to determine subtle changes in tertiary and quaternary conformation with domain-level resolution distinguishes amino-acid specific methods from thermal melt and aggregation-based techniques. As such the approaches extend beyond other approaches, especially limited proteolysis, for such domain-level resolution of conformational changes.16. Subtle changes in protein conformation mediate protein-protein interactions underlying many cellular functions. However, the dynamic and often transient nature of these interactions can make them challenging to quantify in cells en masse and requires sensitive but non-specific conformational probes capable of distinguishing domain-specific changes. Overall, the data presented here support the ability of residue labeling methodologies such as TPE-MI to fill this void, providing quantitative insight into aspects of protein conformation beyond stability and unfolding. Like all residue labeling approaches, we acknowledge there are potential caveats with chemical labeling proteins such as perturbation of protein structure upon reaction. Such modifications have the potential to affect long-range...
conformational rearrangements\(^{13,44}\). And with the TPE-MI method the absence of directly-quantifiable modified peptides means we must infer modification based on loss of the non-modified form. However, one big advantage is the capacity to probe protein structures in live cells, which we anticipate to open the door for studies on proteome structure and function in natural integral biological settings.

**Methods**

**Materials.** All materials were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise. The mouse neuroblastoma cell line Neuro2a was obtained from lab cultures originating from the American Type Culture Collection and were used at passages 2-4. Neuro2a cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, ThermoFisher Scientific) supplemented with 10% (v/v) fetal bovine serum (ThermoFisher Scientific) and 1 mM L-glutamine (ThermoFisher Scientific).

In the case of isotopically labeled cultures (SILAC), cells were cultured in DMEM (Silantes) supplemented with either unlabeled (light) or 13C L-lysine and 13C,15N L-arginine, along with 10% (v/v) dialyzed fetal bovine serum (ThermoFisher Scientific) and 1 mM L-glutamine. 13C L-lysine was fed to SILAC cells for at least 8 doublings prior to use. Cells were maintained at 37°C in a humidified incubator with 5% atmospheric CO2 and were reseeded into fresh culture flasks once at 80% confluency following mechanical dissociation. For plating, cell count and viability were automatically determined using a Countess trypan blue assay (ThermoFisher Scientific). Cells were seeded in 6 or 12 well plates (Corning) and grown for at least 18 h before treatment.

**Cell culture.** Neuro2a cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; ThermoFisher Scientific) supplemented with 10% (v/v) fetal bovine serum (ThermoFisher Scientific) and 1 mM L-glutamine (ThermoFisher Scientific). In the case of isotopically labeled cultures (SILAC), cells were cultured in DMEM (Silantes) supplemented with either unlabeled (light) or 13C L-lysine and 13C,15N L-arginine, along with 10% (v/v) dialyzed fetal bovine serum (ThermoFisher Scientific) and 1 mM L-glutamine. Cells were cultured in isotopically labeled media for at least 8 doublings prior to use. Cells were maintained at 37°C in a humidified incubator with 5% atmospheric CO2 and were reseeded into fresh culture flasks once at 80% confluency following mechanical dissociation. For plating, cell count and viability were automatically determined using a Countess trypan blue assay (ThermoFisher Scientific). Cells were seeded in 6 or 12 well plates (Corning) and grown for at least 18 h before treatment.

**Lysate preparation and chemical denaturation.** Following treatment, cells were washed once in PBS before being mechanically harvested in fresh PBS and centrifuged at 150 x g for 5 min. Cell pellets were then resuspended in lysis buffer (50 mM Tris, pH 8.0, 0.8 % (v/v) IGEPAL CA-630, 1.5 mM MgCl\(_2\)) containing 50 mM HEPES-KOH, pH 7.5, 300 and 100 mM KCl, 5 mM MgCl\(_2\), 1 mM β-mercaptoethanol, 10% (v/v) glycerol was performed. Protein was eluted with 300 mM imidazole in the corresponding low salt buffers. Dialysis was performed overnight at 4°C in the presence of 4 mg His-tagged Upl1 per mg substrate protein for proteolytic cleavage of the 6x-His-Smt3 tag. The 6x-His-Smt3 tag and His-Upl1 were removed by incubating the dyalized proteins in Ni-IDA matrix for 60 min at 4°C and the proteins were then further purified by gel filtration chromatography using a Superdex 200 column (GE Healthcare).

**Correlation of published proteome stability datasets.** Published basal proteome stability datasets were collected as follows. First 813 articles were collected from PubMed keyword searches performed on July 31st 2020 for “thermal proteome profiling”, “thermal proteome unfolding”, “folding stability proteome”, “limited proteolysis proteome”, “proteome denaturation labeling”, “proteome unfolding label” and “SPROX proteome”. Abstracts were filtered manually for those which (i) reported primary experimental data for proteome-wide protein stability under control conditions, (ii) were derived from either human or mouse samples, and (iii) provided a publicly-accessible summary of the dataset. A total of 12 papers were found to meet these criteria. Datasets were then assigned into one of three categories, (i) were derived from human or mouse samples, and (iii) datasets were collected from the relevant supplementary materials analyzed limited proteolysis labeling (i.e., to account for different scales for thermal denaturation values (T\(_{50}\) and chemical denaturation values (C\(_{50}\)). Spearman’s correlation coefficients and p values were calculated in a pairwise manner for all proteins found to be commonly quantified in a given pair of datasets.

**Recombinant β-lactoglobulin denaturation.** A stock solution of recombinant β-lactoglobulin was prepared in PBS (pH 7.4), before being diluted to a final concentration of 250 µM in triplicate in urea prepared at concentrations ranging from 0 to 6 M. Samples were then equilibrated in denaturant for 4 h at 25°C, before being labeled with TPE-MI (or the vehicle control DMSO) at a final concentration of 30 µM. Immediately after addition of the labeling reagent, samples were transferred to clear-bottom 96-well U96 Star plate (Grenier BioOne). TPE-MI (150-20 nm ex, 465/20 nm em) fluorescence was read every 60 s for 60 min using a CLARIOstar (BMG Labtech) with shaking at 200 rpm for 5 s prior to each cycle. In the case of TPE-MI, the first 5 min of linear increase in fluorescence were fitted with linear regression to derive the Timpl (minimum gradient) of the first 5 min. The linear gradient of the first 5 min was then normalized to the maximum gradient across all samples, before being fit via non-linear least squares regression to a two-state unfolding curve. In the case of intrinsic tryptophan fluorescence, the raw fluorescence intensity values at the assay endpoint were normalized to the maximal intensity across all samples, then fit via a nonlinear least squares regression to a two-state unfolding model. This model accounts for the pre- and post-folding transition baselines, which are underlying linear relationships commonly seen at extreme denaturation concentrations among experimental measures of foldness. These baselines are unrelated to the actual process of folding, and thus must be extrapolated to evaluate the equilibrium constant within the transition region. The following equation, which accounts for these states, was used to fit denaturation curves:

\[
[y_f + m_y (D)] + [y_i + m_y (D)] \times \frac{1 - e^{-\frac{D}{cD}}} {1 + e^{-\frac{D}{cD}}}
\]

where \(y_f\), \(y_i\), \(y_m\) correspond to the intercept and slope of the pre- and post-transition states respectively, \(D\) corresponds to denaturation concentration, \(m\) is the fitted slope of the transition region, and \(c\) is the concentration at which 50% of the protein is unfolded. \(T\) and \(R\) correspond to the temperature (298.15 K) and gas constants, respectively.
B Prior to the next analysis. All spectra were acquired in positive ionization mode with full scan MS acquired from m/z 400–1500 in the FT mode at a mass resolving power of 120000 after accumulating to an AGC target value of 5.00e6, with a maximum accumulation time of 50 ms. Lockmass of 445.12002 was used. Data-dependent HCD MS/MS of charge states ≥1 was performed using a 3 s scan method, at an AGC target value of 1.00e6, a maximum accumulation time of 54 ms, a normalized collision energy of 35%, and with spectra acquired at a 7500 mass resolving power of 15,000.

Dynamic exclusion was used for 45 s.

In the case of TMT-labeled samples, data were obtained on an Orbitrap Eclipse Tridrib mass spectrometer using nanoESI-LC parameters as described above. All spectra were acquired in positive mode with full scan mode full scan MS from m/z 300–1600 in the FT mode at a mass resolving power after accumulating to a target value 5.00e6 and with maximum accumulation time of 50 ms. Lockmass of 445.12002 was used. A preferred inclusion list containing doubly and triply masses of tryptic peptides belonging to HSPA8 (P11142), DNAJB1 (P25685) and MDH2 (P00346) was created to increase the coverage of identifiable peptides from these proteins. Data-dependent HCD MS/MS of precursors that match the inclusion list then other charge states ≥1 were performed using a 3 s scan method, at 0.7 m/z isolation width, target value of 5.00e6, a maximum accumulation time of 54 ms, a normalized collision energy of 35% and at a 30,000 mass resolving power (with TurboTMT mode) to resolve the low mass TMT reporter mass. Dynamic exclusion was used for 45 s.

**Peptide identification.** Initial data analysis of raw data generated during this study was carried out using Proteome Discoverer (v 2.1; ThermoFishier Scientific) or MaxQuant (v 1.6.3.4) against the Swissprot Mus musculus database (downloaded 04/07/2016; containing 16,795 entries). Searches were conducted with 20 ppm mass tolerance for MS, and 0.2 Da for MS/MS, with one missed cleavage allowed and match between runs enabled. Variable modifications included methionine oxidation, N-terminal protein acetylation, N-terminal methionine cleavage and SILAC Lys6, Arg10, while the carbamidomethylcysteine modification was fixed. The false discovery rate maximum was set to 0.005% at the peptide identification level (actual was 0.005% for each replicate) and 1% at the protein identification level. All other parameters were left as default.

**Ratio correction and scaling.** Further analysis was performed with custom Python scripts. The logic was as follows. First, the common contaminant protein keratin was removed, leaving 23326 unique peptides associated with 2977 unique proteins. Quantified proteins were considered as those identified by at least two unique peptides, one of which contained a cysteine residue. Although this would allow a protein to be quantified by only a single non-cysteine-containing peptide, the vast majority (90%) of proteins for which a cysteine peptide was identified were associated with more than one non-cysteine peptide. In addition, there was no significant difference in the median non-cysteine abundance ratio between single-non-cysteine- and multi-cysteine-containing peptides. The average peptide abundance ratio (heavy-labeled/ light-labeled peptide abundance) for the non-cysteine-containing peptides was calculated for each protein at each urea concentration. The mean non-cysteine abundance ratio was then used to correct the corresponding cysteine-containing peptide(s) for any change in overall protein abundance caused by the treatment, yielding the corrected cysteine ratio. The corrected cysteine ratio was normalized to the native sample (0 M Urea), such that no change resulting from urea denaturation would yield a ratio of 1.

The resulting data was then scaled with a p value weighted correction. This correction weights the mean corrected cysteine ratio of biological replicates (n = 3) according to the relative confidence with which it deviates from the expected value (in this case, 1) as per:

\[
R = \frac{1 - m}{20^p}
\]

where m corresponds to the mean of the corrected cysteine ratios and p corresponds to the p value derived from a one-sample t-test of the corrected cysteine ratios against the expected value at a single denaturant concentration. To improve confidence in the trends of the corrected peptide ratios across urea concentrations, the data were subsequently smoothed with Locally Estimated Scatterplot Smoothing (LOESS). The resultant curves for the peptide ratios across urea concentrations were clustered by using fuzzy-c means, where the optimal number of clusters was first estimated using the kmeans package before manual inspection of ≥2 correct clusters to achieve minimal residual in clustered patterns. For all subsequent bioinformatic analyses, peptides were then assigned to clusters with the highest membership score.

**Peptide and protein properties.** The curves for the peptide ratios across urea concentrations were fitted with a sigmoidal two-state unfolding model as per Eq. (3):

\[
y = \frac{a + b}{1 + \frac{G}{G + G_0}}
\]

where a and b correspond to the top and bottom plateaus respectively, C_m corresponds to the denaturant concentration at which both the folded and unfolded states are equally populated at equilibrium (assuming two-state protein folding), |d| corresponds to denaturant concentration, and m corresponds to the slope. T and G correspond to the temperature (298.15 K) and gas constants, respectively. Fits were filtered according to the following criteria; (1) R^2 > 0.75, (2) absolute value of a and b less than 0.1, (3) within the range of denaturant concentrations tested, (4) relative error in C_m less than 0.5, and (5) value at 0 M urea in greater than at 6 M urea. The fitted C_m values were then compared against the published datasets as described above.

**Physicochemical properties for individual cysteine residues, peptides and proteins of interest were compiled from various databases and extraction/prediction platforms, including UniProt [https://www.uniprot.org/], PAMF [https://pfam.xfam.org/], Protein Data Bank [https://www.ebi.ac.uk/pdb/], DSSP [https://swift.cmbi.umcn.nl/dssp/], IUPred2A [https://supreze.ela.eu/], STRINGdb [https://string-db.org/], Panther, and iFeature [https://ifeature.eric monash.edu/].**

**HS707 client denaturation assay.** Pig heart 1-malate dehydrogenase (MDH2; Roche, catalog # 10127256001) and recombinant human HS708 were prepared at HEPES buffer (50 mM HEPES, pH 7.5, 50 mM KCl, 5 mM MgCl2, 2 mM DTT) to a final concentration of 5 µM and 2 µM, respectively, in the presence or absence of recombinant human DNAJB1 (1 µM). In the case of heat-denatured samples, MDH2 was for 10 min in a heating block at 37 °C. The collected spectra were searched against a custom database containing sequences for HSPA8 (P11142), DNAJB1 (P25685) and MDH2 (P00346), then filtered according to the following criteria: (1) absolute value of fit > 0.75, (2) absolute value of fi > 0.75, (3) relative error in C_m < 0.75, (4) fi > 0.75, (5) value at 0 M urea in greater than at 6 M urea.

**HS707 inhibition assay.** Neuro-2a cells were seeded and grown for at least 18 h and described above. Culture media was then removed and replaced with fresh media containing VER15508 (Sigma) to a final concentration of 20 µM, or an equivalent amount of DMSO in the case of the vehicle control. Cells were then incubated for a further 18 h before harvesting, lysis, denaturation and TPE-MI labeling as described above. Samples, including a pooled control sample, were prepared for mass spectrometry using the Preomics iST-NHS (Preomics, catalog # P0756S) and TMT 11-plex labeling (ThermoFisher, catalog # A37725) kits according to the manufacturer’s protocol. The pooled channel was added to each biological replicate to support efficient normalization between replicates. Resultant peptides were analyzed using a TMT based MS methodology as described above.

The collected spectra were searched against a custom database containing sequences for HSPA8 (P11142), DNAJB1 (P25685) and MDH2 (P00346), with sequences downloaded from UniProt. The search was conducted as above, with the following alterations: the MS reporter ion was set to TMT 11-plex, isotopic distribution correction applied according to the product data sheet and the fixed carboxymethylcysteine was replaced with the Preomics alkylation (1-113,084 Da).

**Filtering and further analysis of the dataset was then carried out with custom Python scripts. The logic was as follows. First, the common contaminant protein keratin was removed, leaving 23326 unique peptides associated with 2977 unique proteins. Quantified proteins were considered as those identified by at least two unique peptides, one of which contained a cysteine residue. Although this would allow a protein to be quantified by only a single non-cysteine-containing peptide, the vast majority (90%) of proteins for which a cysteine peptide was identified were associated with more than one non-cysteine peptide. In addition, there was no significant difference in the median non-cysteine abundance ratio between single-non-cysteine- and multi-cysteine-containing peptides. The average peptide abundance ratio (heavy-labeled/light-labeled peptide abundance) for the non-cysteine-containing peptides was calculated for each protein at each urea concentration. The mean non-cysteine abundance ratio was then used to correct the corresponding cysteine-containing peptide(s) for any change in overall protein abundance caused by the treatment, yielding the corrected cysteine ratio. The corrected cysteine ratio was normalized to the native sample (0 M Urea), such that no change resulting from urea denaturation would yield a ratio of 1.**
Statistical analysis. Statistical analyses were performed either using the scipy module in python or using GraphPad Prism (v 8.4.3). The exact p values, raw values and statistical details are provided in the Source Data and Supplementary Data 1.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support this study are available from the corresponding author upon reasonable request. Details for the published datasets examined in this study are summarized in Supplementary Table 1. The mass spectrometry proteomics data generated in this study have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository database under accession codes PXD023587 (HSP70 client denaturation), PXD022640 (cell lyase) and PXD030567 (HSP90 inhibition). Protein structures presented in Fig. 4, Fig. 6 and Supplementary Fig. 1 are available via the PDB: ICJ5, 4H5R, 3AGY, 4K8Q, 3AGZ, 1HDD, 6NRC. All other data used in this study are available from Zenodo via the https://doi.org/10.5281/zenodo.4280620 [https://doi.org/10.5281/zenodo.4280620]. In addition, select summary datasets are provided in Supplementary Data 2, Supplementary Data 3, Supplementary Data 4 and Source Data files. Source data are provided with this paper.

Code availability

Custom python analysis scripts used in this study are available from Zenodo via the https://doi.org/10.5281/zenodo.4287766 [https://doi.org/10.5281/zenodo.4287766].

Received: 15 February 2021; Accepted: 25 March 2022;
Published online: 14 April 2022

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Acknowledgements
We thank Professor Pierre Goloubinoff from University of Lausanne for provision of...

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information
The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-29661-2.

Correspondence
and requests for materials should be addressed to Danny M. Hatters.

Peer review information
Nature Communications thanks Marcus Bantscheff, Lukasz Joachimik and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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