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CETSA in integrated proteomics studies of cellular processes

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Abstract

The Cellular Thermal Shift Assay (CETSA) has recently emerged as a promising method to directly monitor functional modulations of protein interaction states in intact cells and tissue samples. Recent data support that the mass spectrometry–coupled proteome-wide implementation of CETSA (MS-CETSA) generates stringent information on a wide range of different interaction classes and is uniquely well suited to study the modulation of protein interaction states in cellular processes and during drug action. To expand the mechanistic insight of CETSA shifts, and to complement information from CETSA experiments, we outline how the integration of MS-CETSA with other proteomics techniques can provide a new platform for detailed, comprehensive, and interactive studies of the functional modulations of proteomes *in situ*.

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Cellular thermal shift assay (CETSA), Mass spectrometry, Proteomics, Protein interaction states (PRINTS), Cell states, Drug response.

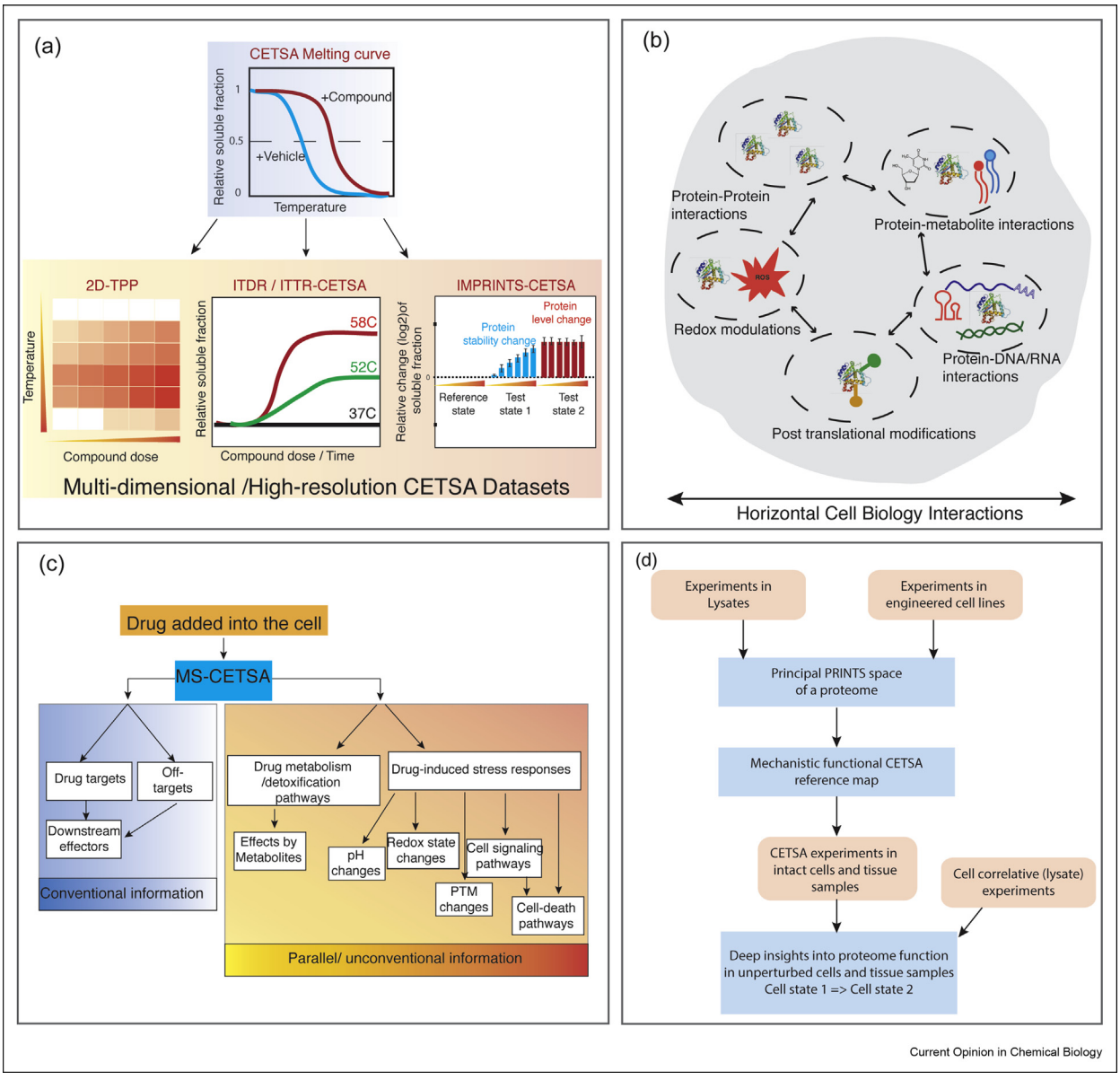
Introduction

A cell is a complex and dynamic environment controlled by numerous specific biochemical reactions and interactions between cellular molecules [1]. Proteins are key players in most cellular processes as well as targets of most therapeutic drugs. Our understanding of proteins comes from a multitude of experimental approaches where on the one hand, studies of individual purified proteins or protein complexes can provide detailed understanding of structure and mechanism, while on the other hand, mass spectrometry (MS)-based proteomics techniques can access functional aspects of a large fraction of a proteome in a single experiment.

MS-based proteomics have developed rapidly in recent decades and transformed our understanding of general aspects of cellular proteomes [2,3]. In addition to assessing protein levels in specific cell and tissue samples, different functional proteomics techniques now yield comprehensive information related to protein function by directly detecting interactions of proteins with other cellular molecules such as nucleic acids [4,5], proteins [6,7], and metabolites [9,10], or post-translational modifications (PTMs) [11,12] at the proteome level. Unfortunately, most of these functional proteomics techniques depend on engineered cells or chemical probes, and/or analyses of samples equilibrated in cell lysates before measuring interactions and therefore only partially reflect the situation in unperturbed cells. A conclusive understanding of proteins in cellular processes underlying disease and drug action, however, will arguably need both detailed and interactive information on the functional modulations of proteomes in their physiological environment in cells.

The Cellular Thermal Shift Assay (CETSA) was recently introduced as the first broadly applicable biophysical technique to study ligand binding to proteins in intact cells [13]. The technique was initially applied for studies of drug binding in mammalian cells, but it is now widely used to validate and quantify target engagement of drugs in cells and tissues from different species [14–16]. CETSA is based on the observation that protein melting curves can be measured in intact cells, after a heating step, by quantifying the amount of remaining soluble protein. CETSA measurements for many proteins are stringent when "the protein reports" and reflects the status of the protein in the cell, because the critical measurement step, that is, heating, is done while the cells are intact. Using MS techniques for quantification of the soluble fraction, MS-CETSA has been used to identify unknown targets of various drugs (e.g., orphan drugs [14], phenotypic screening hits [17], and toxicity-prone drugs [18,19]). However, since the introduction of high-resolution MS-CETSA techniques (2D-TPP [18], ITDR-CETSA [20], and IMPRINTS-CETSA

Figure 1



(a) The initial format of the CETSA (i.e., the melting curves) has been extended to several multidimensional or high-resolution formats such as 2D-TPP, ITDR-CETSA, and IMPRINTS-CETSA. **(b)** The data sets from the high-resolution CETSA formats can capture several of the "Horizontal Cell Biology Interactions" as opposed to the "Vertical" Cell Biology where levels of biomolecules are measured. Such data sets can capture a comprehensive picture of the physiological interactions of cellular proteins with other biomolecules such as metabolites, other proteins, and nucleic acids as well as protein modifications such as PTMs and redox modulations. **(c)** A snapshot of the physiological interactions captured with a high-resolution MS-CETSA experiment, after drug is added into cells. While the more conventional information such as direct drug binders and immediate downstream effectors are captured, several other parallel/unconventional information such as drug metabolism, detoxification, and drug-induced stress responses can also be visualized. **(d)** Integration of MS-CETSA data with other proteomics data, including experiments in lysates and on engineered cells, can yield insights into the biophysical origins of the shifts in the CETSA-responsive proteins and help to establish a mechanistic functional reference map of a cellular proteome.

[21]; Figure 1a), it was obvious that a wide range of protein modulations with/by physiological ligands could be seen reflecting other processes in the cell, for example, downstream or stress effects of drugs (Figure 1b,c). In studies of the cell cycle, it was shown that MS-CETSA gives a comprehensive picture of physiological interactions along cell cycle transitions. We introduced two terms, PRINTS i.e., Protein Interaction States to describe the existing functional forms for individual proteins [15], and horizontal cell

biology to describe a new perspective about the modulations of such interactions during cell states transition, as a contrast to many studies only addressing "vertical" level changes of proteins and other cellular molecules [15].

Although not all interaction changes in the cell will yield CETSA shifts when binding energy or stoichiometry might not be sufficient to induce measurable thermal shifts, accumulated studies, including unpublished data from our laboratory, indicate that thousands of proteins in the human proteome will likely be responsive in MS-CETSA experiments and potentially show PRINTS modulations reflecting specific functional changes of proteins in the intact cell. These CETSA-responsive proteins will therefore allow us to detect which "cogwheels" of the cellular proteome machinery move during a specific cell transition, also capture functional changes of pathways in the absence of protein level changes. Hence, MS-CETSA provides a novel and more direct means to study protein function in cellular processes at the proteome level. However, to fully explore the method, further advances are needed. For example, one challenge is that the specific structural origins of detected shifts are often not known initially, especially when many proteins have multiple interaction partners. Also, it is not known whether a specific shift is activating or inactivating, whether it reflects the flux through an enzyme (by modulated substrate or product levels), or whether it reports on the relocalization of the protein in the cell. Although knowing which of the CETSA-accessible "cogwheels" of the cellular proteome machinery are moving or not, in a specific cell transition, will be very valuable *per se*, the integration of MS-CETSA data with other proteomics data can potentially yield much more detailed information and establish a mechanistic functional reference map of a cellular proteome where the biophysical origins of shifts in many CETSA-responsive proteins can be assigned (Figure 1d). This map will likely be applicable for studies of many cell types from the same organism (e.g., the human proteome) and be used as a reference to directly interpret complex CETSA data sets in the future, in terms of specific structural/interaction changes of individual proteins.

Complementarity of proteomics techniques

Different aspects of CETSA were recently reviewed in depth [15,22], and in this article, we will address the current state and emerging view of the information content in high-resolution MS-CETSA experiments and how such information can synergize with other MS-based proteomics approaches. In Table 1, we provide a summarized overview, indicating the applicability of different proteomics approaches and their complementarity to CETSA.

A number of proteomics techniques can identify "principal interactions," answering the question, "Which cellular molecule has the protein evolved to interact with?", that is, the available PRINTS for each protein in the proteome (Table 1). Such studies have most often been performed with detection in cell lysates, where proteins are no longer in their physiological environment. A range of nonproteomics approaches has also contributed to mapping the principal interactions of a proteome, including traditional focused studies as well as proteome-wide 2-hybrid interaction screens [23]. Although a very valuable initial roadmap for understanding the potential interactions made in a proteome has been established for a specific transition between two cell states, a significant fraction of these PRINTS is likely not present in either of the cell states and alternatively may not be modulated in the transitions between the two cell states, and hence, no CETSA shifts will be seen.

To better understand functional aspects of proteomes, we need to be able to capture interactive functional modulations of proteins in the cellular context. In many cases, PTM chemical modifications can be stabilized through rapid denaturation of proteins or inactivation of enzymes during lysis, minimizing the distorting effects. For noncovalent interactions with proteins, even in cases where experiments depend on the equilibration in the lysate phase, correlative differences can be detected on many proteins as a first approximation of cellular changes. Attempts to study cellular differences based on PRINTS have been made using (1) limited proteolysis (LiP) [24] and (2) stability of proteins from rates of oxidation (SPROX) [25] techniques, where potential functional/structural changes are probed subsequently in cell lysates by examing changes in the accessibility of specific sites on the protein to oxidation or proteolysis. In the next section, we discuss the ability of CETSA to capture different types of physiological interactions in intact cells and outline how information from other methods, as well as CETSA lysate experiments, can be integrated to better understand the horizontal cell biology of specific cellular processes in the future.

Protein–metabolite interactions and metabolomics by CETSA

When metabolites often bind proteins transiently and with lower affinities than drugs, it was initially not clear to what extent CETSA could detect metabolite binding in the cellular context. However, changes in PRINTS due to metabolite binding have now been seen in a number of in-cell CETSA experiments [21,27,28] (Figure 2a,b). To benchmark the stringency of CETSA, we studied a set of well-characterized nucleotides in a human cell lysate [20]. Most known interactions of these nucleotides could be captured, including short-lived substrate and product interactions. Only a small

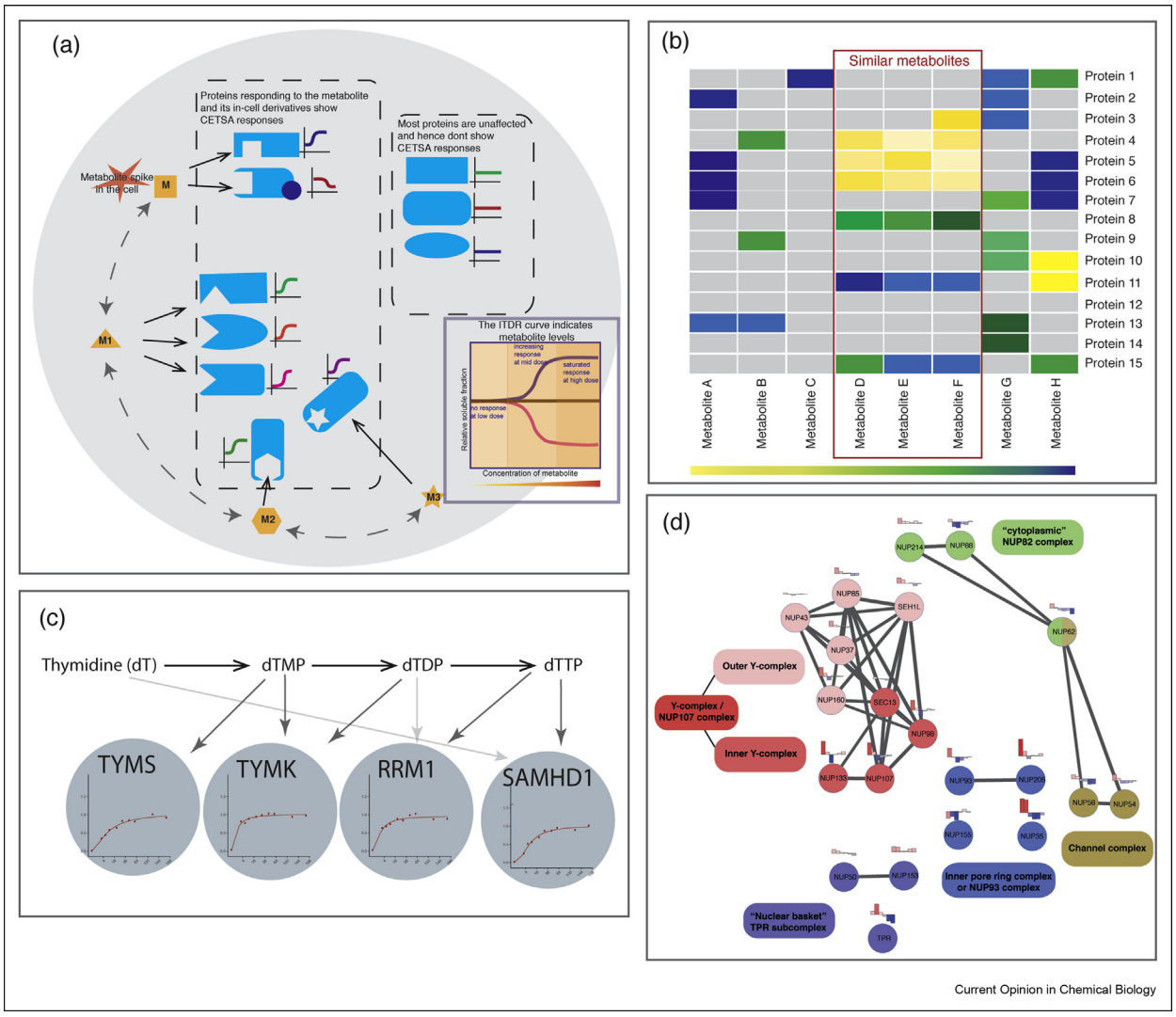
Table 1

A comparative summary of the different approaches currently available to address different questions as indicated; protein interactions with drugs/small molecules, protein–protein interactions, protein complexes, and proteome-wide protein states assessment.

Application	Technology*	Without engineering	In lysate	In intact cell	Applicability to membrane proteins	Caveats
Compound/small molecule–protein interaction, drug target deconvolution	Affinity chromatography chemical proteomics	No (customized probe needed)	Yes	No	Yes	High background
	Activity-based protein profiling (ABPP)	No (customized probe needed)	Yes	Yes	Yes	Only a few classes of enzyme proteins
	Limited proteolysis (LiP)-MS	Yes	Yes	No	Difficult	Challenging for low-abundance proteins
	Stability of proteins from rates of oxidation (SPROX)-MS	Yes	Yes	No	Difficult	Only methionine-containing peptides can be measured
	MS-CETSA	Yes	Yes	Yes	Difficult	Possibility of low/no T_m shift
Protein–protein interaction, protein complex	AP-MS	No (engineered protein constructs needed)	Yes	No	Yes	Loss of weak/transient interacting partners
	IP-MS	Yes	Yes	No	Yes	Loss of weak/transient interacting partners
	Proximity-dependent biotinylation (BioID/APEX)	No (engineered protein constructs needed)	No	Yes	Yes	Need proper control to discriminate real interacting proteins
	Biochemical fractionation	Yes	Yes	No	Difficult	Loss of weak/transient interacting proteins
	Cross-linking mass spectrometry (XL-MS)	Yes	Yes	Yes	Yes	Challenging for data analysis, limited measurement depth
	MS-CETSA, TPCA	Yes	Yes	Yes	Difficult	Possibility of low/no T_m shift
Proteome-wide protein states assessment	LiP-MS	Yes	Yes	No	Difficult	Challenging for low-abundance proteins
	SPROX-MS	Yes	Yes	No	Difficult	Only methionine-containing peptides can be measured
	MS-CETSA	Yes	Yes	Yes	Difficult	Possibility of low/no T_m shift

The asterisk mainly indicates the two molecules being studied for interactions

Figure 2



(a) A schematic of metabolomics by CETSA. Small subsets of proteins are sensitive to the level of a specific metabolite within physiological concentration range, and these can be used to study intracellular metabolite levels indirectly by using the protein responders as a surrogate. **(b)** Specificities and relative affinities of metabolites to a set of responding identified proteins in an isothermal dose response (ITDR) experiment. Color scale depicts the minimal dose threshold (MDT) [20] with yellow being the lowest and blue being the highest MDT. Similar structured metabolites tend to have similar profiles. **(c)** Isothermal time response (ITTR) of thymidine in an in-cell experiment where thymidine was spiked into the cell culture and the protein responses were chased for 30 min. Different proteins respond at various times indicating the conversion of thymidine into several different phosphorylated derivatives [20]. **(d)** IMPRINTS-CETSA profile of nuclear pore complex (NUP) member-proteins, when cells transit to prometaphase compared to the G1/S phase [20]. The subcomplex assignment follows the one summarized by Beck and Hurt [56]. The experimentally confirmed protein interactions indicated by the edge between protein nodes were retrieved from String database (http://string-db.org/).

number of novel interactions were, however, identified for these highly studied nucleotides, which is logical. We also showed that in intact cells exposed to exogenous thymidine, MS-CETSA could monitor time and concentration-dependent buildup of metabolite adducts in sequential metabolic pathways (Figure 2c). Lysate studies of ATP have yielded hundreds of hits where a significant number corresponds to known or annotated ATP-binding proteins [29,30]. The direct modulation of ATP production in cells by two different drugs identified a small set of ATP-binding proteins that appear highly sensitive to distortions of ATP/ADP ratios within the physiological concentration range [31]. We suggested that this small subset could serve as a protein ensemble, reporting on physiological changes of the ATP/ADP ratio. Similarly, the CETSA-responsive thymidine metabolic enzymes can serve as an ensemble, reporting on changes in dTXP metabolites (Figure 2c), and we have in our laboratory seen highly sensitive protein ensembles for other types of

metabolites. Such small ensembles of proteins, sensitive to levels of a specific metabolite within physiological concentration range, can therefore be used as intracellular probes for changes in metabolite levels, that is, metabolomics by CETSA (Figure 2a).

Protein–protein interactions and complexes

Modulation of binary protein–protein interactions can often yield thermal shifts, and in the two cell cycle studies, such PRINTS modulations were e.g. suggested for cyclin binding to cyclin-dependent kinases [15,21,27]. Somewhat surprisingly, CETSA can capture structural changes for many large protein complexes including the ribosome and proteasome. We called the basis for this phenomenon, thermal proximity co-aggregation (TPCA) [32], when the likely biophysical basis is the concerted precipitation of the complex driven by the unfolding of the most thermal sensitive protein component(s) [15], The more structurally homogenous and stoichiometric the complex is, the more similar (correlated) the melting curves will be for the members of a TPCA-accessible complex. Therefore, when protein complexes show higher correlation in a TPCA analysis, they are likely to be present in a more activated form, albeit specific inhibitor complexes can also become more homogenous. Using high-resolution MS-CETSA methods, concerted stabilizations/destabilizations are also seen for many protein complexes, directly reflecting changes in ligand binding to these complexes. This has allowed, for example, the monitoring of PRINTS modulations of complexes involved in chromatin remodeling, transcription, and translation during the cell cycle [21,27], likely also reflecting modulations of interactions with nucleic acids (Figure 2d). Although TPCA analysis adds some statistical significance to predictions of novel protein complexes [32], the analysis has so far been dependent on the use of well-validated protein complexes (i.e., CORUM database [33]) as reference.

Other proteomics methods have yielded comprehensive information on the principal protein complex compositions in lysates, initially from various formats of pull-down assays coupled to MS [34]. More recently, other approaches such as biochemical fractionation methods have been used to discover novel protein complexes [35,36]. These lysate experiments constitute the core of our understanding of the repertoire of existing protein complexes, but it is anticipated that such strategies will not capture transient or less stable members of complexes. A TPCA analysis revealed that only two-thirds of the statistically significant complexes in cells were seen in lysates [32].

BioID and related methods constitute new exciting means to identify novel, spatially proximal, or interacting protein partners in intact cells [37,38]. The combination of cross-linking with mass spectrometry (XL-MS) is starting to be applicable to complex biological systems such as mammalian cells for probing conformational dynamics as well as for identifying novel potential protein–protein interactions [39]. When both BioID and XL-MS are applicable for studies in cells, to assess protein neighborhood or topology, they will provide valuable orthogonal information that can synergize with CETSA data. Similarly, protocols for rapid cross-linking of transcription factors and chromatin proteins to specific DNA sequences can directly reflect cellular processes and be correlated with CETSA shifts of such proteins [4,5]. Recently, cross-linking methods have also been described for studying RNA–protein interactions on the proteome-wide scale [40,41].

Post-translational and redox modifications

The chemical stability of PTMs in cell signaling such as phosphorylation, methylation, and acetylation sites vary [42]. However, in optimized experiments, relevant information can often be obtained for cellular changes of specific PTMs [43], and such studies have been critical for our understanding of PTM-based signaling. When many modifications, for example, of phosphorylation sites, are in flexible loops, they might not affect protein stability directly, while others are in a structural context where they will. It is often uncertain which fraction of a cellular protein is modified by PTM, and if only a small part of the protein population is modified, it will typically not yield a shift in a standard MS-CETSA experiment. We provided evidence that the CETSA shifts in the RB1 protein during the cell cycle are promoted by RB1 phosphorylation [21]. A recent study described the use of CETSA-based method termed as Hotspot Thermal Profiling, in the global detection of stability changes in response to phosphorylation [26]. Many other PTM sites will likely yield CETSA shifts, but the extent of these remains to be determined.

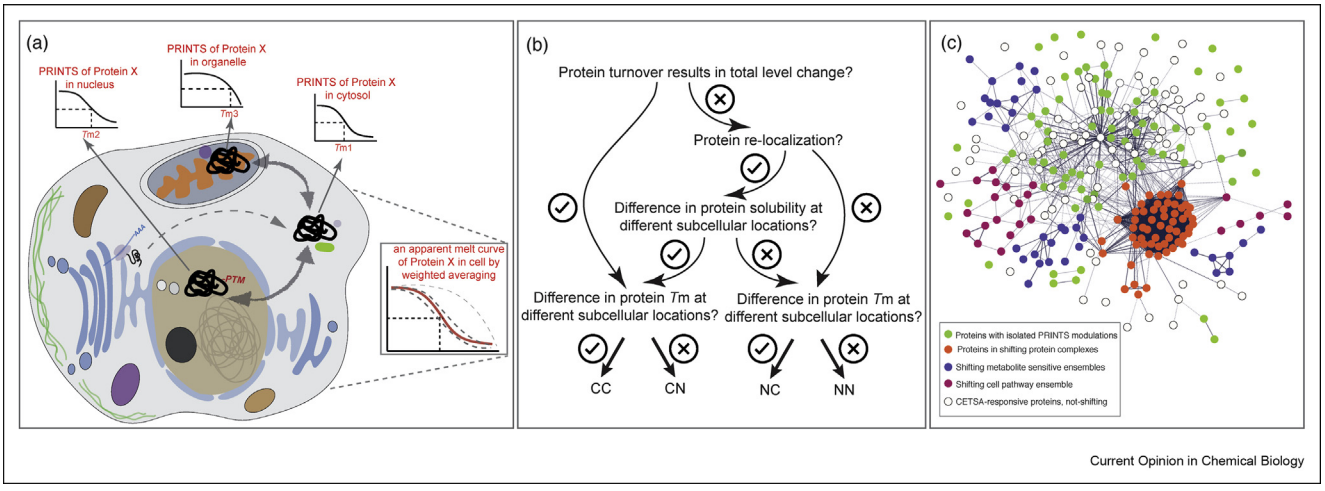
Reactive oxygen species (ROS)-induced redox modifications are often expected on reactive cysteine residues and proteome-wide studies accessing such modifications have been done both in lysate and cell contexts [44–46]. Owing to the intrinsic chemical instability of many modified cysteine residues, competition-labeling strategies have been used to map binding in lysates as well as cells [47–49]. In a CETSA study of redox stress relevant modifications, we found that many proteins with known reactive cysteines showed stability shifts due to glutathione-mediated and hydrogen peroxide–mediated ROS modifications [31]. Also, many novel preliminary candidates for ROS sensitive proteins were found. These stability shifts are likely due to many reactive cysteine residues being in the structural context of active sites or metal binding sites, where the chemical modifications of these cysteines induce some

structural changes. This is consistent with some structural domain families being enriched in shifting proteins [31]. More comprehensive and parallel proteomics studies of PTMs and CETSA in cell state transitions, as well as in lysate studies, can provide clues for which PTM sites can potentially yield shifts in MS-CETSA experiments.

Protein level changes and cellular localization

In eukaryotic cells, the compartmentalization constitutes an important layer of regulation of cellular processes, where the appropriate spatial segregation allows for the right control of protein functions at the right place [50]. Comprehensive subcellular mapping of protein localization has enabled the study of organelle composition and dynamics, particularly via coupling the gradient- or differential centrifugation–based cell fractionation with quantitative proteomics [51–54]. Compartmentalization is often coupled to modification of protein interactions and activation states, which could be reflected in stability changes (Figure 3a). Systematic analyses of protein stability shift between different localizations have not yet been reported, although the first proteome-wide CETSA study has suggested the physiological context effects on the melting profiles of proteins [19]. In IMPRINTS-CETSA and 2D-TPP formats, where both protein level and protein stability information is acquired [21], it was noted that for some proteins and protein complexes, the level changes measured in the CETSA experiments are very different from those obtained in classic proteomics experiments (Figure 3b). This indicates that in the CETSA experiment, the protein has changed its interactions/localization, affecting its propensity to stay in the soluble fraction. This likely reflects relocalization to/from membranes or organelles; the latter are typically not fully solubilized in CETSA experiments. Analyses of protein solubility transitions, as carried out in two mammalian cell cycle CETSA studies [21,27], suggest that extra information regarding the specific proteins could be extracted, such as a prominent effect of lamina and nuclear pore complex proteins during the breakdown of the nuclear envelope in mitosis. The combination of MS-CETSA experiments with subcellular fractionation or BioID type proximity experiments might now be able to resolve in more detail the physical basis for some subsets of CETSA-responsive proteins where either level or stability is modulated.

Figure 3



(a) Complexity of apparent CETSA signals for multiple-site localized proteins in the cell. The mammalian cell consists of many highly organized compartments, such as the nucleus, various organelles (including but not limited to the endoplasmic reticulum, Golgi apparatus, mitochondrion, endosome, lysosome, and peroxisome), and cytoplasm. The posttranslational translocation of proteins dictates cellular processes, as well as possibly complicates the CETSA signal; in this case, the observed CETSA melt curve is a weighted average of all the different subpopulations (i.e., different PRINTS) at different subcellular locations. **(b)** A decision tree scheme of understanding the hit categories from an IMPRINTS-CETSA cell biology study. We have introduced a simple two-letter coding system to refer to the different response groups of proteins observed in IMPRINTS-CETSA data set, where the first letter (C or N) indicates whether there is CETSA protein level change or not, while the second letter indicates whether there is CETSA stability change. In this decision tree, we point out that in the complex cell biology system, the CETSA practitioner should always consider different aspects of protein behavior such as turnover (synthesis and degradation), relocalization, context-dependent extraction, and thermal stability. **(c)** A STRING plot from a hypothetical high-resolution CETSA data set showing how different proteins behave in an IMPRINTS experiment in the cell. Proteins can either not shift (remain unaffected), in spite of being CETSA-responsive (white nodes), or show isolated PRINTS modulations (green nodes). Furthermore, proteins in shifting protein complexes (orange nodes) tend to shift together. In addition, some cellular pathways (pink nodes) and metabolite-sensitive ensembles (blue nodes) are shown.

Summary and future perspective

As outlined previously, MS-CETSA now provides a unique strategy to study protein function in intact cells, and thousands of proteins in each mammalian cell types are likely to be CETSA-responsive, that is, report on the modulation of one or more interactions, involving physiological ligands such as metabolites, other proteins, metals, and nucleic acids. At the systems biology level, MS-CETSA will report on which proteins have modulated interactions during cell state transition including drug treatment, that is, which cogwheels in the proteome machinery are turning (Figure 3c). On a similarnote, out of the known CETSA-responsive proteins, as a consequence, it will also indicate which cogwheels are not moving. A future challenge will be to relate the CETSA-responsive proteins with interacting ligands and structural mechanisms for the stability changes, where signals can be determined to be reflecting activation or inactivation, metabolic flux, or subcellular relocalization. The integration of MS-CETSA experiments with other proteomics techniques, as discussed previously, and also metabolomics and existing bioinformatics resources such as the Reactome [55] will be key to deriving such relationships. Integration with other proteomics techniques will also provide orthogonal information not directly accessible with CETSA, such as many modulations of PTM sites and DNA/RNA binding. As the mechanistic model of the CETSA-responsive human proteome emerges, we predict that CETSA-based studies of cell state transitions will allow for detailed, comprehensive, and highly relevant information to be derived for the better understanding of functional modulation of proteomes in intact cells and tissue samples.

Conflict of interest statement

P.N. is the inventor of patents related to the CETSA method and the co-founder of Pelago Bioscience AB. All the other authors declare no competing interests.

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