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Label-free target identification in drug discovery via phenotypic screening

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Phenotypic screening has demonstrated its advantage in the discovery of first-in-class therapeutics, whereas target-based screening has showed strength for follower drugs. Owing to the unbiased nature of phenotypic screening, novel druggable proteins can be uncovered by target identification. Chemical label-free target identification methods can eliminate the functionalization step of an original bioactive compound. Herein, we summarize recent advances in the development of label-free target identification methods, which are based on changes in protein stability against proteolysis, and chemical and thermal denaturation. Owing to the increasing application of shift in thermal stability for protein analysis in live cells and tissues, we mainly focus on the cellular stability shift assay and its proteome-wide application for target identification.

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Introduction

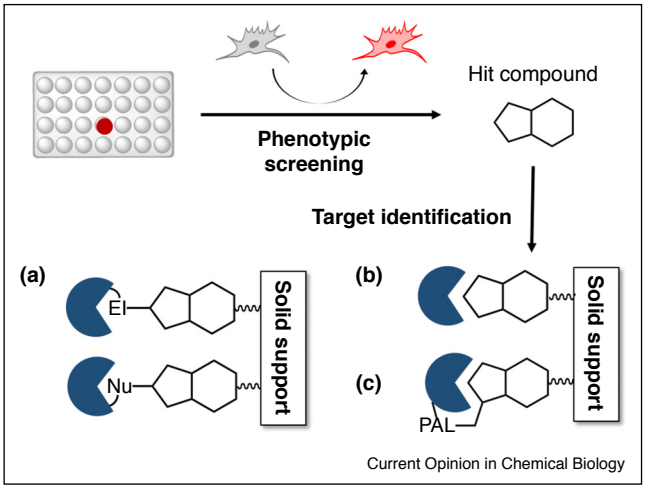
In the biological system, molecular interactions play pivotal roles in stimuli recognition and signaling cascade, and such molecular interactions form the basis of how therapeutics function. Small bioactive molecules have been perceived as effective therapeutics owing to their specific modulation capacity of pathologic conditions [1]. As the yearly approvals of new small molecule drugs has been relatively constant, diversifying target proteins of new classes is needed to enhance the discovery of novel chemical entities [2]. Compared with target-based screening, phenotype-based screening has exhibited considerably high success rate in terms of discovery of novel therapeutics with new modes of action [1,3,4]. As phenotypic screening is an empirical and unbiased approach that depends on disease-relevant phenotypic changes, hit compounds obtained by phenotypic screening might modulate novel signaling pathways via specific engagement with new protein targets [5]. However, the identification of target proteins has been recognized as a bottleneck in elucidating the underlying mechanism of bioactive small molecules [6].

Conventionally, pull-down assays have been widely used to identify potential binding partners by either activity-based or affinity-based proteome profiling (Figure 1) [7–10]. In this type of assays, bioactive compounds are immobilized on solid supports, and the proteins that bind to those solid supports are considered potential target proteins. The pull-down assays require chemical modification of an original compound to incorporate functional handles for the immobilization or attachment of affinity tags. However, the chemical modification is one of the major hurdles in target identification since extensive structure–activity relationship (SAR) study is required to identify the modification site without altering the biological activities of a compound. In case of tight SAR, where even a slight modification causes complete loss of original activities, the pull-down method is not applicable. Besides, SAR study and specific functionalization of complex natural products are highly difficult and sometimes impossible because of their limited quantity obtained by extraction from natural sources or chemical synthesis. Hence, there is a need for label-free target identification methods in order to eliminate the need for such chemical functionalization steps. Herein, we focus on recent progress in target identification by label-free methods that perturb proteins by protease activity, chemical denaturation, or thermal denaturation.

Drug affinity responsive target stability

Huang *et al.* exploited proteolytic susceptibility of target proteins upon engagement of a bioactive compound to develop the drug affinity responsive target stability (DARTS) method for label-free target identification (Figure 2a) [11,12]. In this method, cell lysates were treated with proteolytic enzymes in the absence or presence of drug molecules. After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), protein bands that exhibited enhanced resistance to proteolysis were excised and analyzed by mass spectrometry to identify potential target proteins. When laurifolioside and dichloroacetate were used, live cells, instead of cell lysates, were treated with drug molecules to advance the DARTS method [13,14]. Recently, Ke *et al.* increased the resolution of

Figure 1



Target identification in drug discovery via phenotypic screening. Compounds that induce disease-relevant phenotypic changes are selected as hits. Binding proteins of the hit compounds are deconvoluted in the subsequent target identification step. Conventional label-based methods in chemical proteomics are illustrated. **(a)** Activity-based probes embedded with an electrophile (El) or a nucleophile (Nu). **(b)** Transient interaction by noncovalent modulators. (c) Covalent interaction by the incorporation of photoaffinity linker (PAL).

DARTS by 2-dimensional difference gel electrophoresis (2D-DIGE) for separation and visualization of target proteins [15,16]. Unlike the general assumption that the engagement of bioactive compound to its target proteins increase the stability toward protease activity, destabilization of target proteins against proteolysis was observed for heat shock protein 90 and nuclear protein localization protein 4 upon treatment with matrine and disulfiram, respectively [17,18].

Stability of proteins from rates of oxidation

Fitzgerald *et al.* proposed another label-free target identification method, the stability of protein from rates of oxidation (SPROX), which is based on the principle of thermodynamic changes in protein folding (Figure 2b) [19,20]. Briefly, in the absence or presence of a drug molecule, cell lysates were treated with various concentrations of guanidinium chloride—a protein denaturing agent. The methionine residues exposed by chemical denaturation were oxidized by hydrogen peroxide. Subsequently, proteins were digested to peptides, and the oxidized methionine residues in the peptide fragments were analyzed by mass spectrometry. Target candidates were identified by comparing curve shifts, which illustrate the amount of oxidized methionine versus the concentration of guanidinium chloride. However, the frequency of methionine in proteome is generally low; therefore, the proteins that lack methionine could not be observed. To overcome this limitation, other residues such as tryptophan and lysine and were modified with dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide and *S*-methyl thioacetimidate, respectively [21,22]. Because of the protein denaturation step, SPROX has been limited to studies involving drug engagement in cell lysates and has not been applied in live cells.

Cellular thermal shift assay

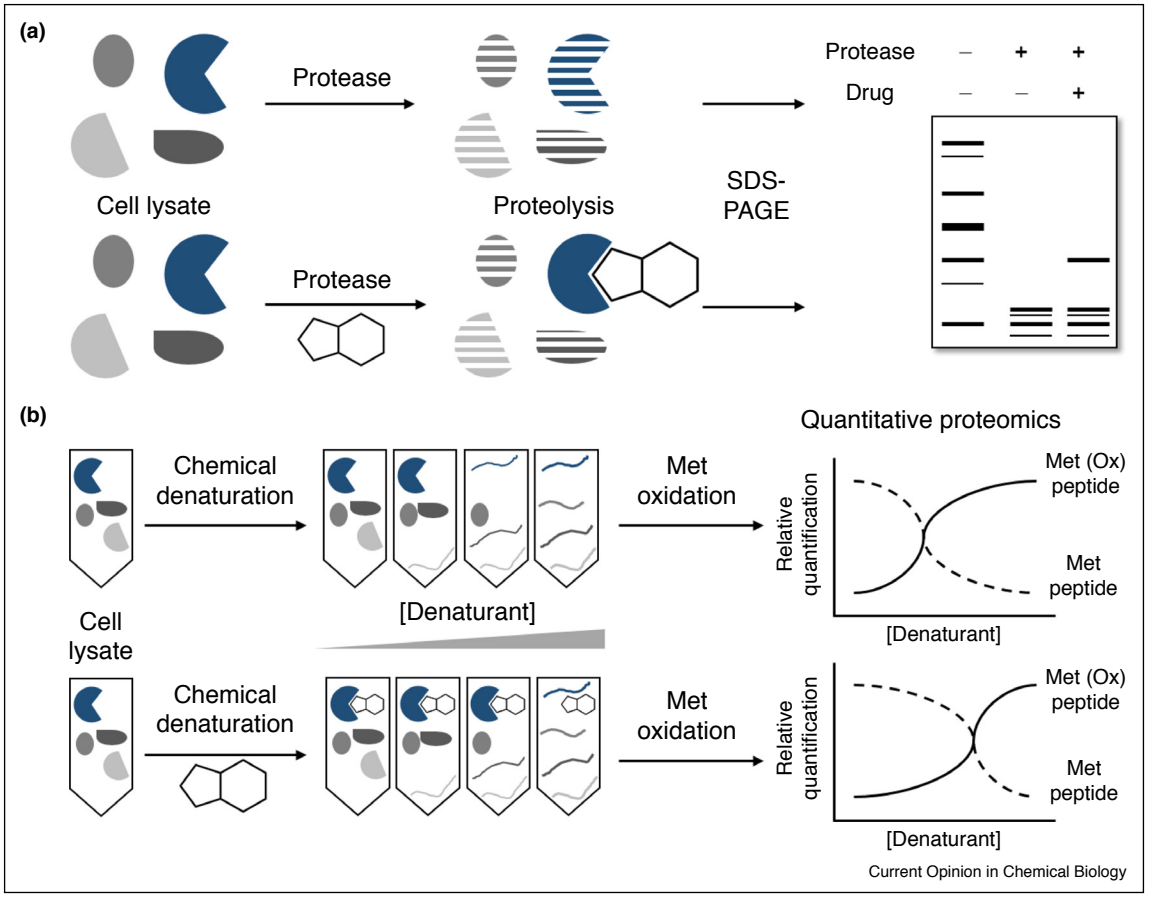
In the thermal stability assay (TSA), the thermal stability shift is utilized to validate the ligand engagement [23]. For proper functioning in cells, native proteins should be in a thermodynamically favorable folded structure with minimal energy. When a protein is heated above its activation energy, it is denatured via the domination of entropy factor [24,25]. The denatured proteins tend to aggregate, and then precipitate irreversibly. As individual proteins have unique thermal stability against heat denaturation under a given buffer condition, the engagement of a ligand to a protein might change the thermal stability of a ligand–protein complex. This leads to a shift in thermal denaturation curve of the complex when compared with that of its apo protein. However, the applicability of TSA has been limited to purified proteins *in vitro*.

Recently, Nordlund *et al.* developed a cellular thermal shift assay (CETSA), in which the TSA is expanded to examine target engagement of drug molecules in cells and tissues (Figure 3a and b) [26••,27]. In this method, intact cells were heated to various temperatures, in the absence or presence of drug molecules. Subsequently, the cells were lysed, and the soluble fraction was collected. Western blotting revealed that the proteins are denatured in a temperature-dependent manner, and that the melting curve of some proteins is shifted upon engagement of drug molecules in live cells. In the isothermal dose-response fingerprint ($ITDRF_{CETSA}$) experiment—a modified version of CETSA, cells are treated with various concentrations of drug molecules at a constant heating temperature [26••]. The $ITDRF_{CETSA}$ further validates drug–target engagement in a dose-dependent manner. Both CETSA and $ITDRF_{CETSA}$ have been rapidly adopted for drug discovery as they can reliably confirm the engagement of drug molecules to cognate targets or off-targets in the cellular system [28].

Thermal proteome profiling

In CETSA, specific antibodies are used to examine the shift in thermal stability of target proteins. Therefore, this method can be ideal for the target validation of cognate target proteins in a biased manner. However, CETSA cannot be applied for the identification of unknown target proteins. To develop an unbiased target identification method, the principle of CETSA has been extended to proteome-wide applications. In 2014, Savitski *et al.* reported thermal proteome profiling (TPP) as a label-free

Figure 2

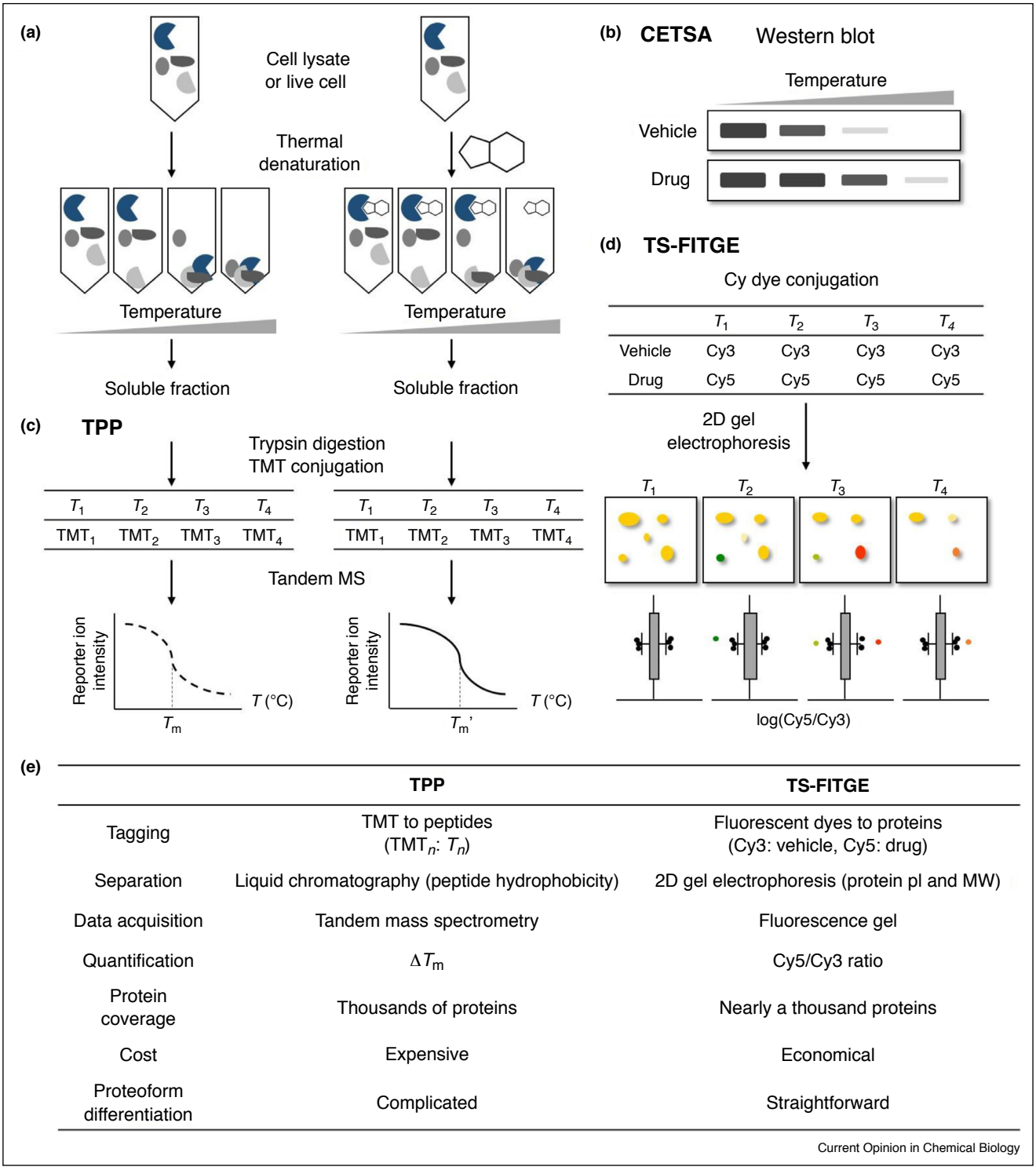


Target identification by label-free methods. **(a)** Drug affinity responsive target stability (DARTS) based on proteolysis susceptibility. **(b)** Stability of proteins from rates of oxidation (SPROX) based on the resistance to chemical denaturation.

target identification method using quantitative mass spectrometry with isobaric mass tags (Figure 3c and e) [29••,30•]. Briefly, in the absence or presence of drug molecules, live cells were heated to 10 different temperatures; the resulting proteomes were digested to peptides, which were then conjugated with 10 different tandem mass tags (TMT). The TMT-conjugated peptide samples were pooled and separated by liquid chromatography according to the peptide hydrophobicity. The peptides were then identified and quantified by tandem mass spectrometry, where the abundance of peptides at each temperature was determined according to their reporter ions. The melting curve of each protein is inferred from the comprising peptides, and the melting temperature (T_m) of the protein is compared in the absence and presence of drug molecules. Proteins with significant T_m shift were then selected as target candidates. In their study, the thermal stability of global proteins was profiled using pan-kinase inhibitors, and clinically relevant off-target proteins as well as known target kinases were identified. Shifts in the thermal stability of proteins also indicated the downstream effectors or proteins that interacted with the target proteins. Direct targets and indirect effectors could be distinguished by comparing the thermal stability shifts in live cells and cell extracts.

During the first phase of CETSA and TPP, membrane proteins were not included in the analysis, because detergents were excluded from lysis buffer. Follow-up studies demonstrated that the inclusion of mild detergents such as NP-40 was compatible with quantitative mass spectrometry analysis in TPP, enabling the analysis of thermal stability shift in transmembrane proteins by drugs and cell metabolites [31•,32•]. Becher *et al.* combined the two variables in thermal stability shift, heating temperature and drug dosage, to expand TPP to a two-dimensional TPP (2D-TPP) [33]. They showed that with 2D-TPP target proteins can be identified with higher sensitivity by

Figure 3



(e)

	TPP	TS-FITGE
Tagging	TMT to peptides (TMT_n: T_n)	Fluorescent dyes to proteins (Cy3: vehicle, Cy5: drug)
Separation	Liquid chromatography (peptide hydrophobicity)	2D gel electrophoresis (protein pI and MW)
Data acquisition	Tandem mass spectrometry	Fluorescence gel
Quantification	ΔT_m	Cy5/Cy3 ratio
Protein coverage	Thousands of proteins	Nearly a thousand proteins
Cost	Expensive	Economical
Proteoform differentiation	Complicated	Straightforward

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Label-free target identifications based on thermal stability shift in cells and their proteome-wide application. **(a)** Scheme of sample preparation including compound treatment and thermal denaturation. **(b)** Cellular thermal stability shift assy (CETSA) by Western blotting. **(c)** Thermal proteome profiling (TPP) by quantitative mass spectrometry (MS) with tandem mass tag (TMT). **(d)** Thermal stability shift-based fluorescene difference in two-dimensional gel electrophoresis (TS-FITGE). **(e)** Different characteristics between TPP and TS-FITGE.

simultaneously monitoring proteins based on thermal stability shift and dose-dependency.

Thermal proteome profiling was applied to identify unknown target proteins of the natural product brusatol [34] and the synthetic anticancer agents, a131 [35] and TH1579 [36]. Target identification by ligand stabilization (TILS) showed that the principle of TPP could be applied for bacterial proteins by analyzing precipitates rather than soluble fraction after thermal denaturation [37]. The TPP method has also been extended to investigate the shift in thermal stability caused by changes in the mammalian cell cycle [38] and by targeted protein degradation [39]. Furthermore, TPP, without drug treatment, was applied to monitor protein complex dynamics in the thermal proximity co-aggregation (TPCA) approach, and the results revealed that interacting proteins have similar melting curves as they co-aggregate by thermal denaturation [40].

Thermal stability shift-based fluorescence difference in two-dimensional gel electrophoresis

We developed another label-free method for target identification, thermal stability shift-based fluorescence difference in two-dimensional gel electrophoresis (TS-FITGE), which is based on the shift in thermal stability during protein–drug engagement, and it utilizes fluorescence signal differences on a 2-dimensional (2D) electrophoresis gel (Figure 3d and e) [41••]. In brief, cells were heated to various temperatures in the absence or presence of drug molecules, and soluble fractions of the cell lysate were extracted. Proteins, instead of peptides, were conjugated with different fluorescent dyes in TS-FITGE: Cy3 to vehicle-treated and Cy5 to drug-treated groups. The two groups were then mixed and separated by 2D gel electrophoresis according to their isoelectric point and molecular weight. Meanwhile, heat-untreated whole proteomes were conjugated with Cy2 dye and spiked into each gel as an internal standard for localization and quantification across different gels. The ratio of Cy5 to Cy3 signals was profiled and visualized in a box plot. Protein spots with outlier ratio were considered as target candidates and excised for protein identification by mass spectrometry. To demonstrate the necessity of label-free target identification, TS-FITGE was used to identify target proteins of the natural products, bryostatin 1 and hordenine, for which chemical modifications are difficult owing to structural complexity and simplicity, respectively. With respect to bryostatin 1, we could monitor thermal stability shift of different proteoforms of protein kinase C by TS-FITGE, probably because of the different post-translational modification states. It is worth mentioning that the separation of different proteoforms is a unique feature of TS-FITGE, which enables the identification of a proteoform-specific interaction with bioactive compounds.

Although both TPP and TS-FITGE are based on thermal stability shift, they differ in some technical aspects such as quantification tags, analyte separation, data acquisition, quantification, and proteomic profiling (Figure 3e). Using the HeLa cell-specific cytotoxic compound SB2001, which was discovered by phenotypic screening with our in-house small molecule library [42], we recently pursued label-free target identification of SB2001 by both TPP and TS-FITGE [43]. Interestingly, the top-ranked target candidates were complementary with a certain overlap between TPP and TS-FITGE. We assumed that their technical differences caused this difference because proteomic data are not ideally complete owing to the limited number of replicates considering their complexity in proteomics. Besides, it is noteworthy that a higher extent of shift in thermal stability does not guarantee that a protein is the target protein. When comparing a series of small-molecule derivatives sharing the same binding mode with a certain protein, the shift in thermal stability of that protein indicates the relative binding affinity of bioactive molecules [23]. However, in a proteome-wide comparison, the innate thermal stability of each protein and the binding mode of ligand to the proteins are highly diverse. Thus, various proteins with different thermodynamics cannot be directly compared based on the extent of thermal shift. Hence, the target candidates should be prioritized by functional relevance to the observed phenotypes.

Conclusions and perspectives

Phenotypic screening contributes to the discovery of first-in-class therapeutics by providing an opportunity to modulate previously undruggable proteins. Target identification can facilitate the development of small-molecule drugs by elucidating their binding proteins and mechanisms of action. There has been a need for label-free methods for target identification in order to exclude the need for chemical modifications of bioactive compounds. Recently, several unbiased proteomic tools such as DARTS, SPROX, TPP, and TS-FITGE were introduced as label-free methods. Particularly, TPP and TS-FITGE could dissect target engagement in cellular context via thermally denaturing proteomes in live cells, whereas DARTS and SPROX perturbed proteomes after cell lysis.

A common limitation of label-free target identification methods is the lack of target enrichment. In contrast to the pull-down methods, where bound proteins are enriched on solid supports, less abundant proteins are difficult to be identified by label-free methods. This problem can be overcome by improving instrument sensitivity and analysis protocols. In addition, although several successful methods have been demonstrated, no single method is equipped to cover the entire proteome. For example, the limitation of thermal stability shift-based target identification method is that the shift in thermal stability may not be detected even

though the target proteins are specifically engaged with the drug compounds. This phenomenon is apparent when the protein has endogenous ligands, where it forms a large protein complex or it is embedded in membrane. An appropriate label-free target identification method should be carefully chosen based on the available information, considering the objective of the study, type of analyte (live cells or cell lysates; prokaryotes or eukaryotes), observed cellular phenotypes, required protein coverage, allowed expense and time, accessible instrumentation, and available core facility or collaborators. It is also recommended to use multiple, complementary methods that are based on different working principles because a combination of diverse target identification methods can increase the success rate of identifying target proteins based on subtle changes in stability.

Conflict of interest statement

Nothing declared.

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