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ARTICLE

Label-free technologies for target identification and validation†

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Phenotypic screening is a powerful strategy for identifying active molecules with particular biological effects in cellular or animal disease models. Functionalized chemical probes have been instrumental in revealing new targets and confirming target engagement. However, substantial effort and resources are required to design and synthesize these bioactive probes. In contrast, label-free technologies have the advantage of bypassing the need for chemical probes. Here we highlight the recent developments in label-free methods and discuss the strengths and limitations of each approach.

Introduction

Target-based screening has been the mainstay of drug discovery over the past two decades in both pharmaceutical industry and academic translational research. However, recently, there has been a revival in phenotypic screening for drug discovery.[1, 2] Unlike target-based screens, phenotypic screens offer unbiased ways to find molecules that generate the desired biological effects in disease-relevant cellular or animal models. More importantly, such screens can potentially result in identifying unprecedented targets as well as uncovering novel mechanisms of action that may ultimately lead to the development of first-in-class drugs. Nonetheless, target deconvolution of small molecules is often a challenging and laborious task, and generally, the process would require systematic integration of multiple and complementary approaches. Lately, considerable progress has been made in the development of new technologies that have markedly expedited the workflow of target identification and validation.[3, 4]

Affinity chromatography is a classical approach for finding target proteins from a complex proteome. Briefly, small molecules of interest are either chemically conjugated to an affinity moiety or directly immobilized onto a solid support that can be used to isolate bound protein targets.[5] The method is mostly suitable for small molecules that possess high affinity for their relative high abundant protein targets. To overcome these limitations, newer approaches based on chemical or ultraviolet light-induced cross-linking have increased the likelihood of capturing low abundant proteins or those with low affinity for the small molecule binder. [6, 7] Additionally, the process can be accelerated through the development of library chemistry that incorporates useful functionality, such as photolabels.[8, 9] Although affinity-based methods have demonstrated great success in identifying certain protein targets of both natural and synthetic small molecules,[10, 11] distinguishing true hits from false positives remains a significant challenge to these methods. In addition, affinity-based techniques require extensive medicinal chemistry effort and significant resources to develop structure-activity relationships that satisfy pharmacological efficacy while incorporating functional handles that enable protein isolation and identification. On the contrary, label-free technologies do not require any chemical modification of small molecules, and thus there is no need to create functionalized chemical probes. In this review, we will focus on the recent advances in label-free methods and discuss their utilities for target identification and validation.

Cellular thermal shift assay (CETSA)

Over the past decade, the thermal shift assay (TSA) has been widely explored in early drug discovery for identifying small molecules that bind to their cognate

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protein targets using fluorescence- or light scattering-based approaches.[12,13,14] Although these methods have greatly facilitated drug discovery, their application is limited to purified or recombinant proteins. Recently, a new technology called Cellular Thermal Shift Assay (CETSA) was developed to broaden applicability by directly assessing small molecule-target engagement in a cellular context, thus preserving the native environment including subcellular localization, post-translational modification and protein-protein interactions. Similarly to TSA, the CETSA® method builds on the concept of thermal stabilization of target proteins upon ligand binding. In 2013, Martinez Molina *et al*. reported the utility of CETSA by measuring ligand-induced stabilization in more complex biological systems including cell lysates, intact cells and even tissues.[15] In a typical CETSA experiment, live cells are treated with either vehicle control or ligand, and then aliquots are heated to temperatures ranging from 37°C to 60-65°C. In general, as the temperature increases, proteins start to unfold and eventually aggregate and precipitate out of solution. Following cooling and cell lysis, the soluble protein fraction is separated from precipitated proteins and cell debris by centrifugation. Subsequently, the abundance of the native folded target proteins from the soluble fraction is quantified by western blotting using target specific antibodies. The melting temperature (T_m) of the vehicle- and ligand-treated cells can be derived from a thermal melting curve (or thermal aggregation curve) by plotting the relative level of soluble protein against temperature. If a ligand indeed engages its target, the T_m of the ligand-bound protein is typically higher than that of the unbound protein due to stabilization. It is worth pointing out that a ligand could also destabilize its target, which may lead to a lower T_m for the ligand-bound protein relative to the unbound protein (Figure 1). In order to measure target engagement potency, isothermal dose-response fingerprint ($ITDRF_{CETSA}$) can be generated where cells are exposed to different concentrations of ligand while keeping the temperature constant, at which a significant difference in the level of protein of interest is observed between the control and ligand-treated samples. Although the obtained $ITDRF_{CETSA}$ EC_{50} values often correlates well with the XC_{50} values obtained from other methods, sometimes the absolute value of $ITDRF_{CETSA}$ deviate. This deviation can explain why affinity does not translate into efficacy but it may also be a consequence of the temperature used for the isotherm, and further studies are needed to demonstrate this relationship in detail.[16] In 2014 the same group published detailed experimental procedures as well as the feasibility of developing assays in high-throughput formats.[17] As an example, the authors employed the AlphaScreen technology using a commercial SureFire kit against the protein kinase p38α. The technology is based on antibody pairs that recognize distinct epitopes on the same folded protein followed by binding of the antibodies to a protein A-conjugated acceptor bead and a streptavidin-coated donor bead, respectively.[18] Since the emergence of CETSA, researchers have successfully applied the method to diverse research programs to study target engagement, such as MTH1[19,20], Bcl-2/Bcl-xL[21], PARP1[22], Cdc-20[23], Menin[24], NQO2[25], EZH2[26], Mdm2/Mdm4[27], PRMT5[28] and eIF2B[29,30].

Later Savitski *et al.* extended the utility of CETSA from target engagement using a Western blot-based readout to an unbiased proteomic readout using multiplexed quantitative mass spectrometry (MS).[31] First, the authors demonstrated distinct differences in melting properties and drug responses between cell lysates and intact cells, suggesting that the disruption of cell integrity has many effects on protein stability. As a proof of principle, staurosporine was evaluated in the thermal proteome study and shown to exhibit either a positive or negative shift for more than 50 targets among the 7000 detected proteins. In comparison to the kinobead strategy[32], approximately 30% of the kinases observed in the kinobead assay did not show significant thermal shifts in the CETSA experiment. The limited degree of overlap could be explained by the fact that unique hits to the CETSA study may not bind to the immobilized ligands. At the same time, unique hits to the kinobead method could be missed in the thermal profiling due to the differences between cells and lysates and/or the absence of a ligand-induced effect. Taken together, it is possible to expect false positives and/or false negatives from these two methods, thus highlighting the complementary nature of both techniques.[16] Moreover, the authors showed that treatment of the BCR-ABL inhibitor, dasatinib, induced thermal shifts for known downstream effectors. Lastly, the CETSA-MS data also revealed that ferrochelatase, a heme biosynthesis enzyme, is a novel off-target for the BRAF inhibitor vemurafenib and the ALK inhibitor alectinib. Recently, the Superti-Furga lab reported a proteome-wide study of protein engagement by metabolites and drugs using a similar approach.[33] Besides soluble proteins, Reinhard *et al.*

reported that thermal proteome profiling also enabled detection of ligand-membrane protein interactions using a cell lysis method including carefully selected detergents.[34]

CETSA enables the direct measurement of target engagement inside live cells. Unlike traditional affinity-based methods, CETSA is label-free and does not require additional design-synthesis efforts for chemical probe development. Most importantly, CETSA may enable unbiased target or pathway deconvolution and off-target identification for hits generated from phenotypic screens. Recently this work has been reviewed extensively.[16] However, despite all these advantages, the method has some potential pitfalls. For instance, multi-domain proteins may give weak or no ligand-induced response, although the limitation in this regard has not been well-established. [17] Furthermore, if the Tm is in the range in which cell membranes rupture, it may limit the use of CETSA for clinical assays.[16] Finally, while the rate of false positives is suggested to be very low as shown in the first MS-CETSA study, it is possible to produce false negatives, since not all binding events change the thermal stability of the targets.

DARTS and LiP-SRM

Limited proteolysis (LiP) is a commonly used biochemical method to study protein structures and conformational changes. It is performed using a relatively low concentration of a protease with broad specificity in order to achieve partial proteolysis. The protease usually cleaves at exposed regions of a given protein, e.g. loops, flexible regions. Upon ligand binding, the target protein may undergo conformational changes and shield some of its LiP cleavage sites from the protease. The effect of ligand binding on limited proteolysis is the underlying principle for two label-free technologies.

In 2009, Lomenick *et al.* first developed a method called Drug Affinity Responsive Target Stability (DARTS) for target identification and target validation.[35] Briefly, cell extracts are treated with or without drug. A protease, e.g. thermolysin, or a mixture of proteases (Pronase) is then added to trigger proteolysis. Target proteins would be enriched, which could be then analyzed either by immunoblot or mass spectrometry (Figure 2). Some proteins may not be sensitive towards thermolysin. Thus Pronase is preferred for DARTS, particularly for target identification work. As a proof of principle, the authors applied DARTS to confirm known drug-target interactions (Didemnin B – EF1-alpha, FK506 – FKBP12, Rampamycin –mTOR). In addition, Tif1 (yeast EIF4A) was identified to be the target of the longevity-enhancing natural product Resveratrol by using gel-based proteomics. The same group also used DARTS to identify ATP synthase subunit B as a novel binder of α-ketoglutarate that contributed the extended lifespan in *Caenorhabditis elegans*.[36] Recently, a similar proteomics approach was utilized by another group to study the mechanism of action of Grape Seed Extracts (GSE) that exhibit anti-cancer efficacy in pre-clinical *in vivo* models for lung, bladder and colon cancers. DARTS revealed that ER stress response proteins may be targeted by GSE, which was further supported by subsequent mechanistic studies.[37]

The DARTS technique is quite straightforward. However, significant efforts may be required during the optimization period. For instance, the amount of protease used and the time for protease treatment both have to be in a certain range so that the differences in proteolysis between control and drug treated samples can be captured. This optimization process could be relatively easy for target validation work, since immunoblotting can be used to rapidly assess a number of conditions. However, this could be challenging for target ID, as it is difficult to choose the optimal proteolysis condition for an unknown target. As a result, DARTS may have a high false negative rate. Another complication for DARTS proteomics experiments is that target proteins may only be partially digested. Protein fragments are simply too large to be filtered away (i.e. dialysis) after proteolysis. Consequently, it would be quite difficult to detect and quantify the small number of peptides derived from the proteolytically protected fragment in the high background of the other peptides from the same protein. Lomenick *et al.* proposed that fractionation of the samples based on molecular weights before mass spectrometry may help to alleviate this issue,[38] although no example is available yet. Additionally, DARTS can only be used with cell lysates, which do not always appropriately reflect physiological events.

Recently, Feng *et al*. developed a limited proteolysis-based method to globally study the protein

conformational changes between different growth conditions, namely Limited Proteolysis coupled Selected Reaction Monitoring (LiP-SRM).[39] This approach employed a double-digestion step. The first digestion was performed using protease with broad cleavage specificity (such as protease K, thermolysin, subtilisin) under non-denaturing conditions, at a low E/S ratio for a short period of time. This would generate some large protein fragments, which reflect the structural conformations of the proteins prior to proteolysis. The proteome was then fully digested by trypsin under denaturing conditions, followed by bottom-up proteomics. A control aliquot of the same proteome sample was taken for trypsinization only. All fully tryptic peptides would be quantified by SRM. By comparing the double-digested sample and the trypsin-only control, the fully tryptic peptides containing LiP cleavage sites would be identified, as they are less abundant or missing in the double-digested sample. Then a subsequent SRM measurement was adopted to identify the half-tryptic peptides in the double-digested sample corresponding to a specific protein conformation. These peptides are named "conformotryptic peptides". Different proteolysis patterns between two conditions reflect the structural differences induced by a perturbation.

The authors were able to detect and distinguish major structural changes of α-Syn (a monomeric unfolded form versus a β-sheet rich amyloid fibril form) as well as a subtle structural difference between holomyoglobin and apomyoglobin (one helix in myoglobin is ordered in its holo form, while disordered in the apo- form) after spiking the proteins into yeast proteome. They went further and simultaneously assessed the structural features of over 1000 proteins when yeast switched the carbon source from glucose to ethanol. Many glycolytic enzymes and two 14-3-3 proteins were found to undergo structural changes that have functional consequences. In addition, they demonstrated a potential application of LiP-SRM towards target identification by identifying yeast Fas1 as a novel interactor of fructose-1,6-biphosphate (FBP), which was further validated *in vitro* using purified fatty acid synthase protein complex. The information from a LiP-SRM experiment would be quite comprehensive, since downstream effects (e.g. PTMs) and PPIs may be captured as well. An additional benefit is that structural information related to compound binding can be potentially obtained. However, LiP-SRM appears to require a tremendous amount of instrument time, in addition to sophisticated data analysis and a necessary optimization step for proteolysis. In addition, similar to DARTS, it appears incompatible with live cells.

SPROX

Stability of Proteins from Rates of Oxidation (SPROX) is another mass spectrometry-compatible target deconvolution technique that detects changes in protein thermodynamic stability between conditions for target identification.[40] The SPROX protocol has been previously described in detail.[41] In brief, the SPROX technique utilizes the chemical denaturant dependence of hydrogen peroxide-mediated oxidation of methionine side chains to probe the thermodynamic properties of proteins in different states (e.g. the presence and absence of ligand). The protocol involves dividing a protein mixture or lysate into a series of aliquots and adding increasing concentrations of a chemical denaturant such as guanidinium chloride or urea to each aliquot (Figure 3). Hydrogen peroxide-mediated oxidation at methionine side chains is initiated by addition of hydrogen peroxide. The oxidation reaction can be quenched using catalase or an excess of free methionine. The extent of hydrogen peroxide-mediated oxidation for a globally protected methionine is related to the unfolding equilibria for a given protein or protein domain. The relative amount of oxidation for each protein can be inferred from the ion intensity of oxidized or non-oxidized methionine containing proteins or surrogate peptides in a bottom up application. Chemical denaturation curves are generated from these data and shifts in the transition midpoints of these curves are ultimately used to identify potential targets. To achieve this, the experiment is repeated between conditions (i.e. cell type[42] or compounds[43-45]) and shifts in the midpoints of these curves indicate proteins which are thermodynamically stabilized or destabilized by the condition.

The data and information obtained from a SPROX experiment depend on the specific approach used for peptide quantification (Figure 3). Isobaric tags (e.g. tandem mass tag or isobaric tags for relative and absolute quantitation) enable pooling across denaturant concentrations in SPROX experiments. In this approach, the reporter ions from methionine

containing peptides (oxidized or non-oxidized) are used to generate points in a SPROX chemical denaturation curve. Proteins that show thermodynamic stabilization (i.e. where the transition midpoints are right shifted) in the presence of a compound can be indicative of a direct binding effect between compound and target. Thus the SPROX technique can be used for target identification (or off-target identification). An alternative approach uses duplex tags (e.g. SILAC or heavy and light hydrogen peroxide) to pool identical denaturant samples across conditions.[46-47] In this approach heavy/light ratios can be plotted for the denaturant concentrations used and deviations from linearity indicate a shift in protein thermodynamic stability. However, it is important to be aware of the possibility for thermodynamic stabilization in the absence of a direct interaction between a compound and protein, for example as a result of protein signaling pathways leading downstream of the direct target interaction. These 'hits' are termed indirect hits and can in theory help elucidate signaling pathways affected by the conditions tested in a SPROX experiment. Direct interactions can be separated from indirect interactions by follow up experiments on purified proteins in the absence of the cellular milieu. It is likely that thermodynamically destabilized proteins (i.e. the transitions midpoints are left shifted) also fall under the indirect hit category. However, different domains in multi domain proteins can have unique thermodynamic properties and it is important to keep this in mind when interpreting data from any mass spectrometry-compatible target deconvolution technique.

One advantage of the SPROX technique is the ability to distinguish thermodynamic properties at the domain level, as mentioned above. It is important to analyze and interpret SPROX data at the peptide level for this reason. In addition, it is possible to identify binding pockets based on the characteristics of SPROX denaturation curves from peptide hits.[48] A unique advantage inherent in the SPROX technique is the ability to generate quantitative affinity measurements based on the magnitude of the shift in transition midpoint. Although chemical oxidation as a chemical probe can limit the ability to accurately measure thermodynamic stability, it is possible to obtain quantitative information for direct protein-ligand interactions when oxidation does not perturb the ligand-binding properties of the target protein.[40] One of the disadvantages of the SPROX technique is the requirement that proteins must be detected using a proteomics platform. This limits the SPROX technique, and all mass spectrometry-compatible target deconvolution techniques, to relatively abundant proteins within a complex proteome. In addition, certain protein classes may not be amenable to chemical denaturation, for example membrane proteins where chemical denaturation is impeded by detergent or a lipid bilayer. The classic SPROX technique is limited to globally protected methionine containing peptides, however an extension of the SPROX technique was recently reported that enabled non-methionine containing peptides to report on the thermodynamic properties of proteins in a SPROX experiment.[47] Additional covalent modification reactions have extended the technique to amino acid side chains other than methionine.[49, 50]

Other technologies (TICC, SEC-TID)

Chan *et al.* introduced Target Identification by Chromatographic Co-elution (TICC), a label-free technique based on co-elution of compound-protein complexes during dual Ion Exchange (IEX) chromatography.[51] The protein targets are identified by bottom-up proteomics. TICC is able to detect known protein-target interactions over a relatively wide range in affinity (nM to μM). Moreover, the authors identified Erg6p (Delta(24)-sterol C-methyltransferase) as a novel target of an anti-fungal compound, while Asc1, Dak1, and dihydroxy acetone kinase as the off-targets of a dopamine receptor agonist . One potential limitation is the difficulty in identifying proteins for follow-up confirmation studies, since the resolution of HPLC is usually not high, and numerous proteins may be present in any single fraction.

More recently, Salcius *et al.* developed a similar approach, Size-Exclusion Chromatography for Target Identification (SEC-TID) which assesses binding of an underivatized compound to approximately 1000 individually purified proteins in a 384-well format. In short, small molecules are first incubated with purified proteins, and then size-exclusion chromatography is applied to resolve the interactions between small molecules and target proteins. Finally, the amount of small molecules bound to the target protein is quantified using LC-MS.[52] Since a collection of

recombinant proteins were used instead of cell lysate, SEC-TID greatly simplified the assay, and allowed the rapid validation and quantitative assessment of known small molecule target interactions as well as identification of novel protein interactors of XAV939, vadimezan and mefruside. Another feature of this technique is that proteins are in aqueous solution during the profiling, and thus may offer potential advantages over technologies where the proteins are immobilized, such as protein microarrays. It is also worth noting that for TICC and SEC-TID methods, hydrophobic compounds tend to be "sticky" and may co-elute with many proteins, which could lead to high false positive rates.

Conclusion and Future Perspective

Phenotypic screening is an appealing approach that identifies small molecules which can rescue the disease pathology at the outset, yet follow-up studies to determine the precise protein target(s) remain a daunting task for the drug discovery community. Even though the idea of affinity based methods seems simple and straightforward, any chemical modification may potentially affect the binding affinity of the small molecules to their targets. Recent progress in label-free technologies may improve the pace and workflow of target deconvolution. In this review, we have outlined the most recent advances in label-free methods including CETSA, DARTS and LiP-SRM, SPROX, as well as TICC and SEC-TID. As described above, each method has its own advantages and disadvantages (Table 1), and we hope the table serves as a reference guide when deciding which technology to use in a given project. However, it should be noted that label-free methods are sometimes labor intensive and require significant instrument time, which is another factor to take into consideration. Finally, label-free and chemical probe-based technologies are often complementary. Target engagement using chemical probe-based methods should be explored if label-free technology, such as CETSA, is unsuccessful as they can provide simplified opportunities to perform biotin-streptavidin enrichment if required, and additional avenues for target and binding site identification, such as imaging[53], can be readily investigated. We expect to see more examples of how these techniques are applied in drug discovery research in the near future.

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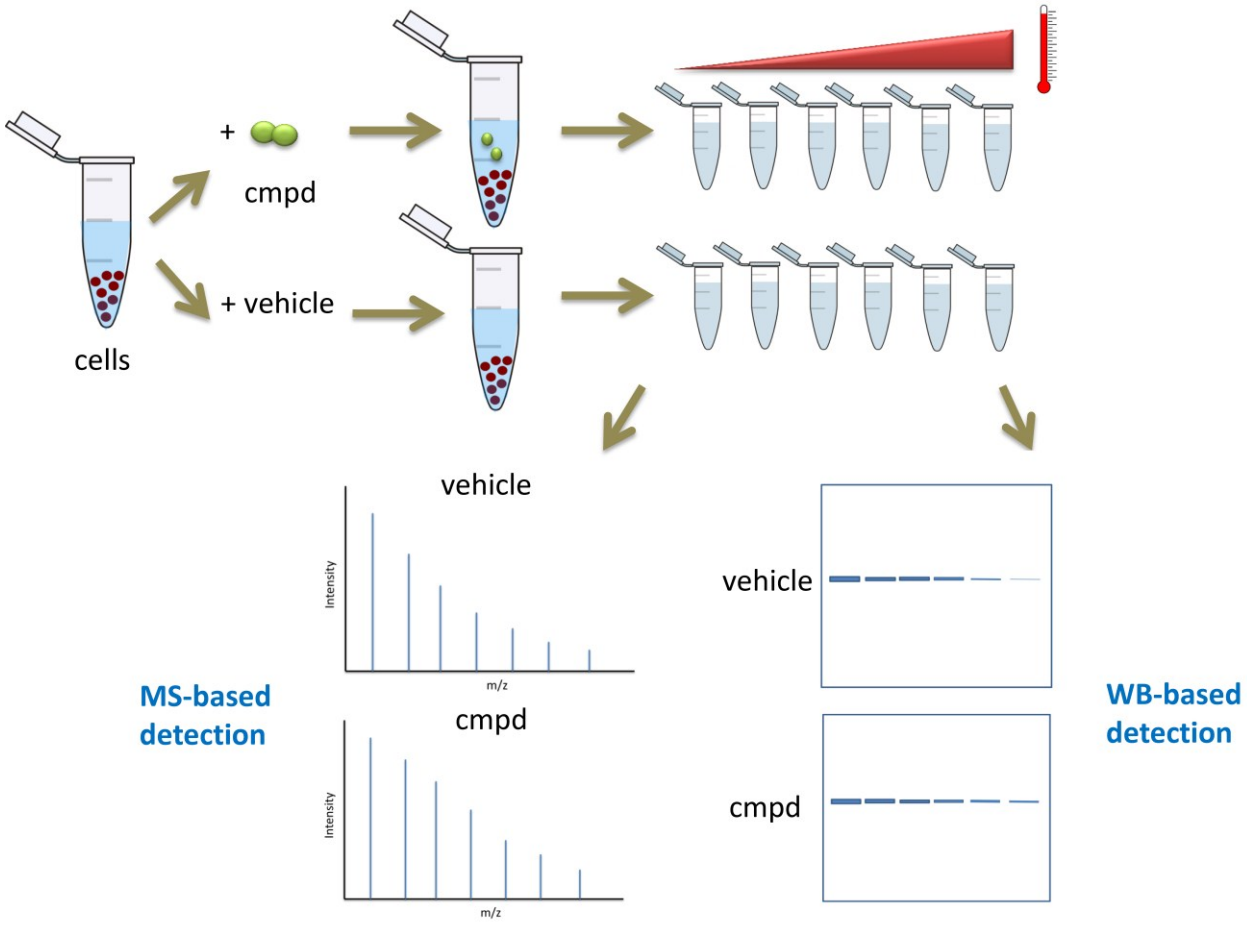

Figure 1: Schematic illustration of the CETSA protocol

Cells are treated with either drug or vehicle, and aliquots are subjected to heating. After cooling, the cells are lysed, and the soluble protein fraction is separated from precipitated proteins and cell debris by centrifugation. The abundance of the native folded target proteins from the soluble fraction can be analyzed by either antibody-based or MS-based approaches.

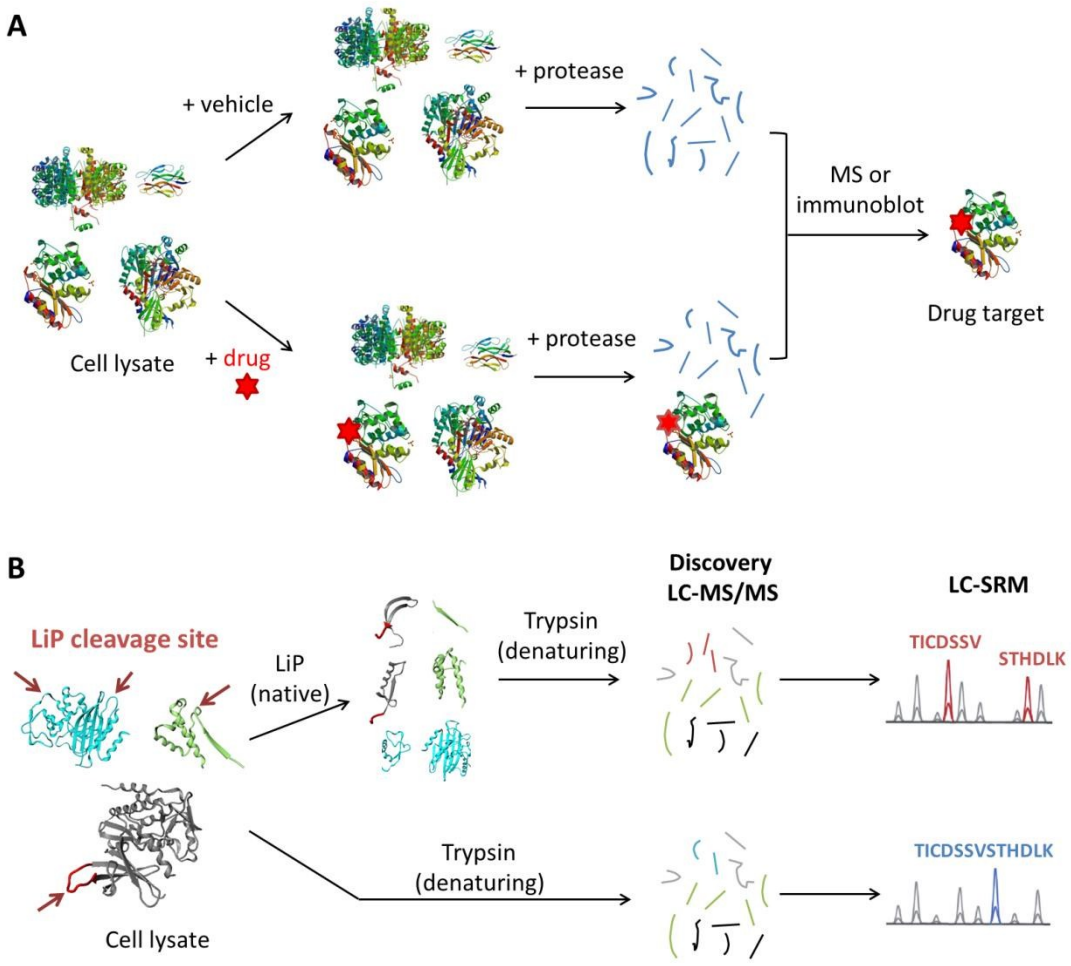

Figure 2: Label-free technologies based on limited proteolysis. (A) DARTS workflow. Cell lysates are treated with vehicle or drug, and then subjected to limited proteolysis. Target proteins would be stabilized against proteolysis, and thus enriched. The samples are then analyzed by either immunoblot (target validation) or mass spectrometry (target identification). (B) LiP-SRM workflow (adapted from ref.[1]). Red arrows indicate the LiP cleavage sites. Cell extracts are subjected to limited proteolysis under native conditions, generating large protein fragments reflecting structural conformations. The mixture is further digested by trypsin under denaturing conditions. In parallel, an aliquot of the same cell extract is digested by trypsin only under denaturing conditions. The fully tryptic peptides containing the LiP cleavage sites would be identified (shown in blue) with quantitative MS. Furthermore, protein structural differences of two differently treated samples (e.g. drug versus DMSO, different growth conditions) can then be detected by comparing LiP patterns.

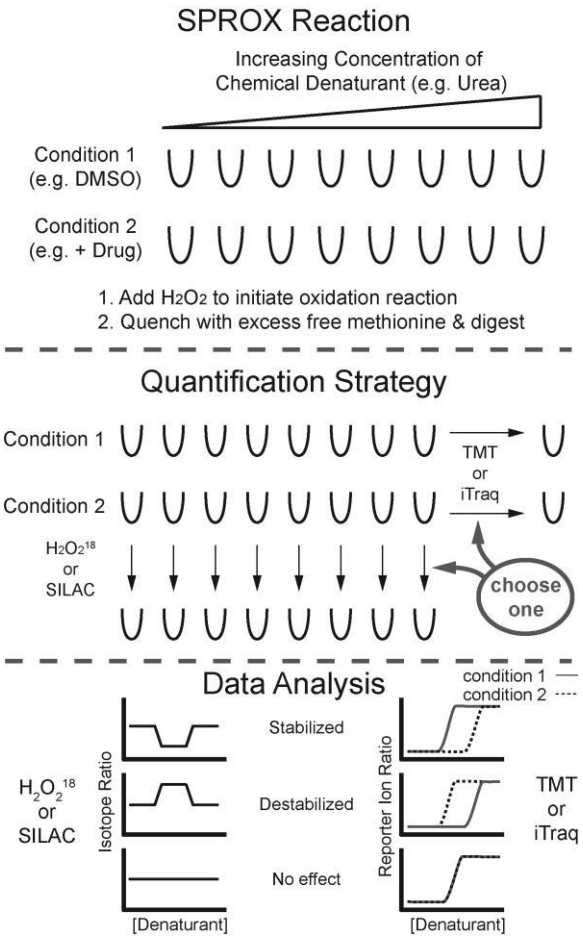

Figure 3: An outline of the SPROX protocol for comparing two conditions (e.g. a control and in the presence of a drug). The reaction uses hydrogen peroxide (H_2O_2) to oxidize methionine side chains. A series of denaturant concentrations are used to shift the unfolding equilibria of the protein population which exposes globally protected methionine side chains to oxidation. Two quantification strategies have been reported for the SPROX technique. One quantification strategy uses isobaric tagging to pool at the condition level. The other uses heavy hydrogen peroxide or SILAC to pool at the denaturant level. The method for data analysis will depend on the quantification strategy chosen. Theoretical data for stabilized, destabilized and no effects are shown for both strategies. The nature of the data and shape of the plots will depend on the oxidized state of a given peptide.

Table 1. Label-free technologies

	Compatible with cell lysate	Compatible with live cells	Binding affinity measurement (rank ordering)	Throughput
CETSA	Yes	Yes	Yes[b]	Low [c***] (IB/ proteomics) High [d] (Alpha Screen)
DARTS	Yes	No	Likely [a]	Low [c **]
LiP-SRM	Yes	No	Likely [a]	Low [*** c]
SPROX	Yes	No	Yes[b]	Low [*** c]
TICC	Yes	No	No	Low [c*]
SEC-TID	No	No	Likely [a]	Medium [* e]

[a] Although it has not been demonstrated in the literature, the technology is likely to help measure binding affinity or rank ordering of compounds based on its technical features.

[a b] A wide range of binding affinities (nM-μM) have been reported for these techniques. The detectable binding affinity for SPROX and CETSA both depend on the ligand concentration and the difference in denaturant concentration between consecutive buffers or temperatures.

[c] Less than 5 compounds can be processed in parallel.

[d] AlphaScreen-based CETSA assay is suitable for 96- and 384-well plate and allows for industrial scale high-throughput screening.

[e] It is compatible with 384-well plate screen. But the overall assay speed is limited by mass spectrometry.

[*]low personnel and instrument time [**]medium personnel and instrument time [***]high personnel and instrument time

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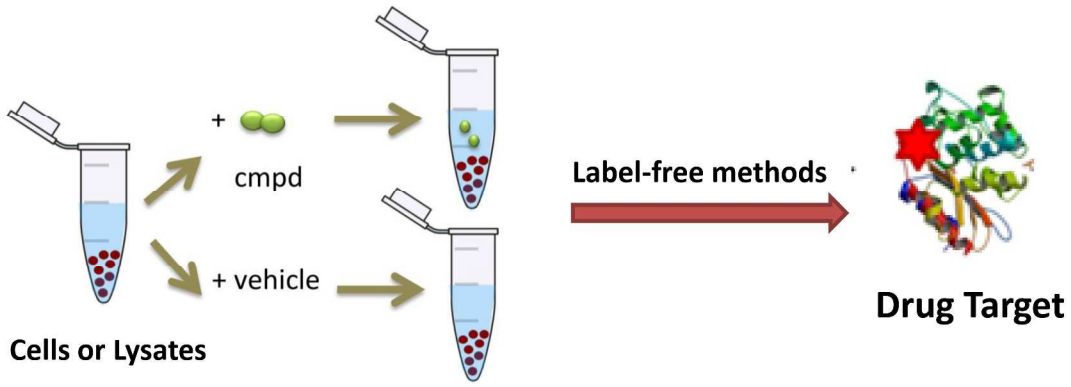

Phenotypic screening is a powerful strategy for identifying active molecules with particular biological effects in cellular or animal disease models. Functionalized chemical probes have been instrumental in revealing new targets and confirming target engagement. However, substantial effort and resources are required to design and synthesize these bioactive probes. In contrast, label-free technologies have the advantage of bypassing the need for chemical probes. Here we highlight the recent developments in label-free methods and discuss the strengths and limitations of each approach.