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## Click Chemistry in Proteomic Investigations

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### Abstract

Despite advances in genetic and proteomic techniques, a complete portrait of the proteome and its complement of dynamic interactions and modifications remains a lofty, and as of yet unrealized objective. Specifically, traditional biological and analytical approaches have not been able to address key questions relating to the interactions of proteins with small molecules, including drugs, drug candidates, metabolites, or protein post-translational modifications. Fortunately, chemists have bridged this experimental gap through the creation of bioorthogonal reactions. These reactions allow for the incorporation of chemical groups with highly selective reactivity into small molecules or protein modifications without perturbing their biological function, enabling the selective installation of an analysis tag for downstream investigations. The introduction of chemical strategies to parse and enrich subsets of the “functional” proteome has empowered mass spectrometry (MS)-based methods to delve more deeply and precisely into the biochemical state of cells and its perturbations by small molecules. In this *Primer*, we discuss how one of the most versatile bioorthogonal reactions, “click chemistry,” has been exploited to overcome limitations of biological approaches to enable the selective marking and functional investigation of critical protein/small-molecule interactions and post-translational modifications in native biological environments.

### eTOC blurb

eToC blurb – Parker and Pratt

In this Primer, Pratt & Parker overview the biological applications of “click chemistry,” a class of small molecule reactions that can efficiently conjugate substrates to specific biomolecules. Focusing on proteomic investigations, they provide insight into the biological questions where it may be most useful, including studies of post-translational modifications and protein-protein, protein-drug, and protein-metabolite interactions.

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## Introduction

Explosive advances in genome sequencing technologies have greatly enhanced our understanding of the full protein complement of the genome (the proteome) and have provided valuable insight into numerous physiological processes and disease mechanisms. However, such methods alone do not capture the myriad post-translational events and molecular interactions that can affect protein function. This dramatic contextual and temporal variability of the proteome is part of what sets it apart from the relatively stable genome, limiting the reach of genome-based strategies to study proteins and their interactions in native environments.

Proteomic methods, on the other hand, enable the measurement of multiple properties for thousands of proteins, including their abundance, tissue distribution, subcellular localization, various post-translational modifications (PTMs), and other protein-protein interactions (Figure 1). Significant advances in enrichment techniques, ionization methods and instrument resolution have greatly facilitated the identification, characterization and quantitation of PTMs, while herculean proteomic studies have mapped networks rich with thousands of new candidate protein-protein interactions (PPIs) (Hein et al., 2015; Huttlin et al., 2015). However, despite these advances, traditional proteomic methods alone do not illuminate the complete network of interactions and modifications that proteins may undergo, nor do they evaluate protein activity or functional state.

Thus, a set of challenges emerged which have sparked shared interests between chemists and biologists: 1) the selective capture of dynamic interactions between proteins and small molecules, 2) direct assessment of the functional state of proteins in native environments, and 3) the selective enrichment of proteins with specific modifications. Here, we discuss the integration of chemistry with proteomic methods to address these challenges. In particular, we highlight how biorthogonal chemistry, specifically azide-alkyne click chemistry, has been exploited to selectively label, measure and identify proteins, their interactions and modifications in native environments.

## Bioorthogonal Chemistry

The ability to selectively label, enrich and detect proteins, their interactions and their modifications in their native systems is key to fundamental biological research as well as drug discovery. Traditional biological approaches, including the genetic incorporation of affinity tags and the creation of selective antibodies, have been transformative for the detection of proteins. However, for various reasons, both biophysical and technical, they struggle to address the interactions of proteins, particularly with small molecules, as well as many protein post-translational modifications. These challenges have prompted the development of biorthogonal reactions that enable the selective chemical modification of proteins in complex, native environments utilizing complementary functional groups that fulfil the following criteria: (1) functional groups are not present in living systems, (2) are inert towards native biological components, and (3) react with one another under biocompatible conditions (e.g., in aqueous environment, physiological pH and temperature, favorable kinetics).

These reactions involve a two-step labeling strategy. First, the selective tagging of any protein requires the introduction of a bioorthogonal functional group to directly onto the target(s) of interest (Figure 2, *step 1*). The relatively small size of the chemical groups required for a bioorthogonal reaction allows them to be incorporated into small molecules, like drugs or metabolites, without perturbing their ability to interact or modify proteins. Following this installation of a “probe”, the second step involves the use of a bioorthogonal reaction to selectively install a visualization or enrichment tag (Figure 2, *step 2*), enabling subsequent analysis (Figure 2, *step 3*) via gel-based assays, various imaging techniques, or liquid chromatography mass spectrometry (LC-MS) methods. There are well over 20 unique bioorthogonal transformations available (Figure 3), which offer a range of reactivities, orthogonality, and utility in various applications. These reactions and their various applications have been the topic of numerous comprehensive and recent reviews, and thus will not be detailed here (Lang and Chin, 2014b; McKay and Finn, 2014; Patterson et al., 2014b; Prescher and Bertozzi, 2005; Sletten and Bertozzi, 2009). Our objective is to discuss one of the most widely-used bioorthogonal transformations, the cycloaddition reaction between an alkyne and azide functional group (Figure 3E, and 3F), often referred to just as a click reaction, and its utility in proteomic investigations of small molecule-protein interactions and protein post-translational modifications.

**An Introduction to Click Chemistry – The Azide-Alkyne Cycloaddition—**The term click chemistry, coined by Sharpless and coworkers, is used to describe chemical reactions that are selective, modular, wide in scope and high yielding (Kolb et al., 2001). The Cu(I)-catalyzed “click” reaction between an azide and terminal alkyne to form a 1,2,3-triazole (Figure 3E) ranks as one of the most widely utilized bioorthogonal reactions to date. Although the reaction between these functionalities was first reported in the 1960s (Huisgen, 1963), its uncatalyzed form requires heat for it to proceed at observable rates, making it unsuitable for applications outside of the round-bottom flask. The copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) was first reported independently by Sharpless (Rostovtsev et al., 2002) and Meldal in 2002 (Tornøe et al., 2002), where it was discovered that Cu(I) dramatically enhances that reaction rate, enabling its use at room temperature. In 2003, Finn and coworkers reported the first implementation of CuAAC in biological systems, where it was used to modify purified Cowpea Mosaic Virus (CPMV) particles with fluorescent dyes (Wang et al., 2003). Soon after, Benjamin Cravatt and coworkers optimized CuAAC conditions for reactions in cell lysates, laying the groundwork for applications in more complex biological systems and for its use as a bioorthogonal reaction in proteomics (Speers et al., 2003).

**CuAAC Considerations—**A potential liability of CuAAC is the cytotoxicity of the Cu(I) catalyst, which initially limited the reaction to *in vitro* or *ex vivo* applications (e.g., cell lysates) and prompted the development various solutions to mitigate Cu-mediated toxicity. The development of several biocompatible ligands (Chan et al., 2004) has not only accelerated CuAAC reactions but also reduced the amount of Cu required for the click reaction to occur, minimizing unwanted side effects induced by Cu(I) and enabling the use of CuAAC in living cells and organisms (del Amo et al., 2010). Picolyl azide reagents have also been developed, which internally chelate Cu, thus accelerating CuAAC in the absence

of exogenous ligands (Uttamapinant et al., 2012). To obviate the need of Cu(I) altogether, Bertozzi and coworkers turned to ring strain to accelerate the reaction between alkynes and azides (Agard et al., 2004; Baskin et al., 2007). These click reactions, known as strain-promoted alkyne-azide cycloaddition (SPAAC, Figure 3F), capitalize on the inherent strain of cyclooctyne rings to promote the reaction of various cyclooctyne derivatives with azides under ambient conditions without any additional catalysts. Such improvements have enabled the labeling of proteins and other biological molecules with azido tags in live cells and organisms, including *C. elegans*, zebrafish and mice (Chang et al., 2009; Laughlin et al., 2008; Laughlin and Bertozzi, 2009), although cyclooctyne motifs are larger and synthetically more challenging to incorporate compared to terminal alkynes. We do not discuss them here, but other biorthogonal reactions (Figure 3) may have unique advantages over CuAAC for specific applications, such as those that would benefit from alternative or improved reaction speeds and those that may require *in situ* reactivity under physiological conditions such as live-cell imaging (Lang and Chin, 2014a; Murrey et al., 2015; Rutkowska et al., 2016). For example, inverse electron Diels-Alder reactions (Figure 3D) have gained popularity for a variety of applications due to their favorable kinetics, high selectivity, and catalyst-free nature (Oliveira et al., 2017). However, obstacles associated with their relatively large, hydrophobic reactive groups, which could influence biological interactions, and challenges associated with the synthesis of tetrazine and dienophile reactive groups limits their broad implementation. For simplicity in this article, click chemistry will be used to reference reactions between azide and alkyne functionalities to ligate two components together.

There are a few important technical considerations when performing the CuAAC reaction (Pickens et al., 2018). First, the choice of buffer system used to generate cell lysates can affect the efficiency of the reaction (Darabedian and Pratt, 2019). In general, buffers with primary amines, like Tris (tris(hydroxymethyl)aminomethane), should be avoided as they inhibit CuAAC. Accordingly, two of the most widely used buffers for CuAAC reactions are PBS and triethanolamine (TEA), while one of the best buffers is HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). Second, the orientation of the CuAAC reaction is key, as this reaction can be performed in two orientations: an azide-probe and alkyne-tag or an alkyne-probe and azide-tag. In order to increase the rate of the CuAAC reaction, the tags are used in large excess compared to the probe-labeled proteins. The mechanism of CuAAC involves first the activation of the alkyne, and when the alkyne-tag is present in excess, this can result in subsequent reaction with protein nucleophiles, most notably cysteine residues. Therefore, the ideal orientation contains an alkyne-probe and corresponding azide-tag to minimize non-specific proteomic labeling (Speers and Cravatt, 2004). Unfortunately, due to synthetic limitations, this may not always be achieved. If an azide-probe is to be used, either PBS or TEA should be used as a buffer, as HEPES results in significantly higher cysteine reactivity of alkyne-tags. Similarly, it has been shown that certain cyclooctyne SPAAC reagents can also react with cysteine residues, which can be avoided with thorough blocking of free thiols via alkylation (Kim et al., 2013; van Geel et al., 2012). Lastly, there are commercially available click-reaction kits; however, caution should be used as the components are often incompletely described and may contain reagents incompatible with desired workflows, and furthermore, premixed reagents can

deteriorate overtime, making these kits unreliable. Given the simplicity and robustness of this bioorthogonal reaction, we recommend acquiring the individual reagents and their fresh preparation prior to click-based applications.

## Proteomics

A significant portion of mass spectrometry (MS)-based proteomics utilize a bottom-up workflow, in which proteins are characterized and measured by the analysis of peptides generated from proteins after proteolysis (Zhang et al., 2013). When performed on a mixture of proteins (e.g. cell or tissue lysates), it is most commonly referred to as “shotgun” proteomics (Wolters et al., 2001). A typical shotgun proteomics experiment (Figure 4) begins with the preparation, extraction or isolation of the protein mixture derived from cells, tissue or biofluids. Considering proteins are part of a complex network of interacting biomolecules and sometimes are of low abundance, they often require isolation, separation or enrichment from their native environment. This is particularly true in instances where specific protein modifications or interactions are being investigated. Once isolated, proteins are subjected to selective digestion using a variety of proteases, often trypsin, resulting in the formation of a heterogenous mixture of proteolytic peptides. This digest is subsequently subjected to liquid chromatography to physically separate peptides, most often exploiting differences in peptide charge and hydrophobicity. This separation step enhances proteome coverage and increase the resolution of each peptide by reducing the likelihood of coeluting and co-ionizing species. The resulting separated peptides are then analyzed by MS and assigned by database searching against an appropriate database (e.g. SwissProt) with a search engine (e.g. Sequest or Mascot) in order to identify enriched proteins and permit a more in-depth analysis. Numerous methods, including isotopic labels (e.g. SILAC (Ong et al., 2002), ICAT (Gygi et al., 1999), iTRAQ (Ross et al., 2004), TMT (Dayon et al., 2008)) and various label free approaches (Cox et al., 2014; Gillet et al., 2012; Venable et al., 2004), have been developed for the relative quantitation of proteins across entire proteomes.

**Proteomics and Click Chemistry**—When integrated with proteomic methods, chemical tools, such as click chemistry, can be used to selectively label and enrich proteins for their subsequent identification and quantitation. Common applications of click chemistry include the mapping of small molecule-protein interactions as well as various post-translational modifications (PTMs). Although each of these applications of click chemistry are unique, the overall general workflow is similar to that of standard shotgun proteomics. Tissue, cells or lysates are first treated with a tag containing a click handle (either azide or alkyne) for the labeling of proteins of interest. This is most often done through direct chemical modification of endogenous proteins with chemical probes. Proteins may also be labeled through unnatural amino acid mutagenesis with functionalized amino acids or ligation of acceptor peptides with bioorthogonal motifs (Figure 2). Following protein labeling with a click moiety, cells or lysates are then subjected to click reaction conditions with a complementary click functional group linked to a tag for protein enrichment, such as biotin. Labeled proteins are subsequently enriched with an affinity matrix and washed extensively, typically with detergents, salts or denaturing agents. Enriched proteins are then subjected to the proteolysis, separation, and mass spectrometry workflow described above. In the sections

below, we will discuss strategies in which bioorthogonal labeling and click chemistry tools can be used to study protein-small molecule interactions and PTMs.

### Click Chemistry to Investigate Small Molecule-Protein Interactions

Targeted gene disruption and editing methods have been employed with great success to investigate protein function. However, such approaches are often weighted towards loss-of-function (LoF) perturbations and chronic removal of proteins can lead to compensatory mechanisms, which can potentially confound functional investigations. Further, a delay is required prior to examining the effects of genetic manipulation, making such approaches unsuitable to investigate temporally sensitive processes. Selective chemical probes can serve as powerful tools to directly investigate and modulate protein interactions and functions (Arrowsmith et al., 2015). Chemical probes can produce LoF, gain-of-function (GoF) and even neo-functional outcomes on protein targets (Belshaw et al., 1996; Lai and Crews, 2017; Lu et al., 2014; Schreiber, 2018), and often they provide exquisite dose-dependent control over the magnitude and kinetics of perturbation. Finally, quality chemical probes can create paths for further clinical development (Blagg and Workman, 2017; Garbaccio and Parmee, 2016). Despite the utility of small molecules for the study of protein function in living systems, the vast majority of proteins still lack useful chemical probes, and indeed, only ~650 human proteins are currently targeted by small molecules therapeutically (Santos et al., 2017). Key to the development of chemical probes is the complete understanding of their protein targets in native biological systems and the identification of proteins that are tractable to small molecule-based approaches.

Investigations of small molecule-protein interactions using chemical proteomics can be divided into two general categories: (i) *protein-centric* strategies, which aim to broadly profile features of proteins, such as enzymatic activity or protein ligandability (i.e. the ability to interact with small molecules), through the use broad-spectrum chemical probes to map interactions across the proteome, or (ii) *chemical-centric* strategies, which focus on characterizing protein targets of individual bioactive small molecules, such as drugs or metabolites (Figure 5A). Both categories utilize chemical probes that are designed from the outset to contain: 1) a *recognition group* that promotes interaction with specific subsets of proteins in the proteome, 2) a *reactive group* (either electrophilic or photoreactive) for covalent bonding and capture of interacting proteins, and 3) a *reporter tag* (fluorophores, biotin, or click-chemistry enabled handles like alkynes and azides) for the detection, enrichment, and identification or visualization of interacting proteins. Major strengths of such chemical proteomic-enabled probes are: 1) efficient enrichment and identification of low-abundance and low-affinity probe-interacting proteins enabled by the covalent trapping of these interactions; and 2) probe-protein interactions can be trapped and ultimately identified in native environments such as living cells, or in some cases, *in vivo*, thereby preserving labile interactions that might be disrupted by cell lysis and processing protocols, or even missed during investigations which are limited to *in vitro* studies with purified protein.

Biological investigations using chemoproteomic-enabled probes are most often conducted in two different formats: *comparative* or *competitive* profiling (Figure 5B). In comparative

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profiling, multiple probes or conditions can be directly compared to understand differences in protein enrichment, which might result from differences in probe recognition or reactivity. In addition, various experimental conditions (e.g. cell type, genetic variations, growth conditions, etc.) can be directly compared upon treatment with a common probe in order to assess the influences of external perturbations on corresponding probe-protein interactions. Competitive profiling, on the other hand, involves treating a biological system with several candidate small-molecule ligands (e.g. inhibitors, drugs, metabolites) and comparing probe-protein interactions relative to an untreated systems. With this method, such ligands can be screened against numerous proteins simultaneously in native proteomes, cells, or even animal models (Cisar et al., 2018; Speers and Cravatt, 2004) (Leung et al., 2003), thereby providing information about ligand targets, potency and selectivity. There are numerous excellent reviews covering chemical proteomic platforms, such as ABPP (Roberts et al., 2017b; Sanman and Bogyo, 2014; Yang and Liu, 2015), chemical proteomic inhibitor discovery (Niphakis and Cravatt, 2014) and various compound-centric profiling applications in detail (Drewes and Knapp, 2018; Rix and Superti-Furga, 2009; Wright and Sieber, 2016). Below, we discuss strategies in which click-chemistry enabled probes have been used to illuminate small molecule-protein interactions and other biology.

### Protein-Centric Profiling

**Activity-Based Protein Profiling**—One of the earliest protein-centric profiling strategies, known as activity-based protein profiling (ABPP) pioneered by the Cravatt lab, aims to broadly profile the activities of enzymes in native biological systems. Applications of ABPP have historically involved the use of electrophilic chemical probes that mechanistically react with the active sites of members of related enzyme families, thereby serving as a reporter of their biochemical activity and as powerful tools for the discovery of new proteins with analogous enzymatic activity (Cravatt et al., 2008). Probe labeling is guided by active-site accessibility and the intrinsic reactivity of catalytic residues, properties that can be dynamically regulated within the cell. These probes enable global profiling of many enzymes in parallel, and in some cases, achieve family-wide coverage. Such broad coverage makes ABPP particularly useful for the discovery of dysregulated enzymes in disease settings via comparative profiling as well as for assessing the selectivity of inhibitors via competitive profiling. Numerous activity-based probes (ABPs) have been developed and deployed for many enzyme classes, including serine hydrolases (Kidd et al., 2001; Liu et al., 1999), cysteine proteases (Kato et al., 2005), metallohydrolases, phosphatases, deubiquinating enzymes (Hewings et al., 2018; Ward et al., 2016), kinases (Zhao et al., 2017), various oxidoreductases and others. Further, ABPP has been used to map deregulated protein activities in cancer (Jessani et al., 2002; Jessani et al., 2005; Nomura et al., 2010) and other diseases (Barglow and Cravatt, 2004), to discover as well as optimize both covalent and non-covalent inhibitors for numerous enzymes (Niphakis and Cravatt, 2014), and also assist in the identification of targets and off-targets of bioactive compounds (van Esbroeck et al., 2017).

Although ABPs often utilize reporter groups such as biotin or fluorophores for the direct enrichment or visualization of labeled proteins, bypassing the need for additional conjugation steps, such bulky tags can hamper cellular uptake and tissue distribution,

potentially prohibiting their use in living systems. The adoption of sterically minimized latent “clickable” tags can overcome these challenges and subsequently be reacted with probes, thereby providing a convenient modular system for detection, enrichment and identification of probe labeled protein targets directly from cells, tissues or even whole animals. The first class of enzymes targeted for ABPP studies were the serine hydrolases (SHs), a large and diverse family of enzymes that perform numerous roles in physiological and pathological processes. Although most ABPP studies for SHs are performed using biotin or rhodamine tagged fluorophosphonate (FP) (Simon and Cravatt, 2010), which mechanistically reacts with active-site serine residues, CuAAC has shown utility in the profiling of these enzymes with the generation of a “tag-free” SH probe (FP-alkyne, Figure 6) (Gillet et al., 2008; Tully and Cravatt, 2010). This probe was shown to label SHs with greater affinity than the reporter-tag functionalized FP probes, and importantly, the vastly improved cell permeability of FP-alkyne facilitates profiling of SH activity directly in living cells, enabling the development of a competitive ABPP platform to screen inhibitor selectivity directly within a cellular milieu instead of in lysates where information pertaining to subcellular localization is disrupted (Gillet et al., 2008).

Although SHs were subject of some of the earliest ABPP studies, CuAAC-enabled ABPs or “click-ABPs” are constantly being developed for diverse enzyme classes. Electrophilic click-ABPs have been developed for kinases (Zhao et al., 2017), protein arginine deiminases (PADs) (Nemmara et al., 2018; Slack et al., 2011), ubiquitin machinery (An and Statsyuk, 2013; Hewings et al., 2018), cytochrome P450 enzymes (Wright and Cravatt, 2007), and glycosidase enzymes (Tsai et al., 2013). For example, high-throughput methods for profiling kinase inhibitors have typically relied on enzymatic activity or competitive binding readouts on isolated kinases, removed from native environments. Recent chemical proteomic methods have been developed which quantify inhibitor binding to endogenous kinases in complex proteomes through the use of bead-immobilized kinase inhibitors (‘kinobeads’) (Bantscheff et al., 2007) or covalent ATP-biotin probes (Patricelli et al., 2007). These platforms enabled the profiling of 150-200 endogenous kinases in a single cell line, however, neither are cell permeable, requiring membrane dissolution and disruption of protein complexes during lysis, potentially altering kinase conformation and activity. To overcome this limitation, the Tauton lab, in a collaboration with colleagues at Pfizer, developed a cell-permeable sulfonyl fluoride-based click probe (Figure 6) that target a conserved lysine in the ATP binding site which captures 133 phylogenetically diverse kinases in cells, 50 of which were not captured by kinobeads, highlighting the utility and complementarity of click ABPs to other platforms (Zhao et al., 2017).

In addition to broad spectrum probes, which are suited for maximizing coverage across protein families, investigators can also tailor click ABPs to engage a limited number of proteins. By limiting probe reactivity with only a limited number of proteins the overall signal and resolution is increased, aiding in studies where broad reactivity can become confounding, such as in instances with proteins of low abundance which might be obscured by highly expressed proteins. Tailored probes are often derived from activity-based inhibitors discovered via competitive profiling with broad spectrum ABPs, which are subsequently derivatized with click groups (Figure 6). For example, a recent competitive ABPP screen against SHs led to the discovery of a selective and *in vivo* active covalent



inhibitor of palmitoyl protein thioesterase (PPT1), mutations of which cause infantile neuronal ceroid lipofuscinosis. Subsequent modification of this inhibitor with an alkyne yielded a tailored ABP for the direct detection and enrichment PPT1 from cells and tissues, enabling direct functional and biochemical investigation (Cognetta et al., 2015). Click-ABPs have also been developed for *in vivo* applications. Recently, highly potent and orally available activity-based inhibitors were developed for monoacylglycerol lipase (MGLL), an SH that controls the content and signaling of the endogenous cannabinoid 2-archidonoylglycerol (2-AG) in the central nervous system. To assess *in vivo* selectivity, the authors modified their lead inhibitor ABX-1431 with an alkyne, and showed with oral treatment, exquisite proteome-wide selectivity, with their click-ABP and lead inhibitor engaging only one off-target (Cisar et al., 2018). ABX-1431 is currently in clinical trials for the treatment of Tourette syndrome and chronic motor tic disorder.

**Activity-Based Protein Profiling of Amino Acid Reactivity**—In addition to enzyme activity profiling, ABPP applications have been extended beyond enzyme active sites towards the global analysis of amino acid reactivity via general electrophilic chemical probes that react with nucleophilic amino acid side chains, such as cysteine and lysine. These reactivity-based probes bypass the requirement of enzyme activity-mediated probe capture, dramatically expanding the scope of ABPP beyond catalytic sites on enzymes but to all sites on proteins with reactive side chains. One of the first strategies to integrate reactivity-based probes with proteomics was through the utilization of electrophiles to label and enrich alkylation-sensitive proteinaceous cysteines. Iodoacetamide, acrylamide and maleimide reagents had long been used for the alkylation of cysteine residues on purified proteins. However, Gygi and coworkers developed electrophilic probes with isotopically encoded affinity tags (called ICATs), composed of a biotin enrichment handle, an isotopically labeled linker and a cysteine reactive group (iodoacetamide or maleimide), enabling the selective enrichment and quantitation of labeled proteins from lysates (Gygi et al., 1999). Though, the large size and presence of biotin impeded profiling of more diverse electrophiles and limited cell permeability. The advent of click chemistry and its integration with ABPP enabled the characterization of more diverse electrophilic compounds (Backus, 2019). For example, Weerapana and colleagues used tandem orthogonal proteolysis-ABPP (TOP-ABPP) (Weerapana et al., 2007) for the simultaneous identification of protein targets and exact sites of modification by a diverse set of electrophilic alkyne containing click-probes (Weerapana et al., 2008). In these experiments, lysates were treated with various electrophilic click probes and labeled lysates are then conjugated via CuAAC to a bioorthogonally cleavable Tobacco Etch Virus (TEV)-biotin purification tag, enabling the enrichment and release of captured peptides for the subsequent identification of probe-labeled reactive residues. These studies revealed high selectivity of  $\alpha$ -halide electrophiles for cysteine thiols. In subsequent work, the use of isotopically encoded ‘heavy’ and ‘light’ TEV tags enabled quantitative profiling of reactive cysteines with an iodoacetamide alkyne (IAA, Figure 6) probe (Weerapana et al., 2010). This strategy, termed isotopic tandem orthogonal proteolysis – activity-based protein profiling (isoTOP-ABPP), has been used to identify thousands of reactive cysteines and to demonstrate that ‘hyper-reactive’ cysteines are highly enriched in protein functional sites. In a competitive format, isoTOP-ABPP has been used to identify the covalent modification of reactive cysteines with various

electrophiles or modifications. For example, competitive isoTOP-ABPP has been used to identify sites on proteins modified by lipid-derived electrophiles, such as oxidative stress product 4-hydroxy-2-nonenal (HNE), resulting in modulation of endogenous proteins, such as ZAK kinase (Wang et al., 2014). Competitive isoTOP-ABPP has recently been used to screen electrophilic fragments in proteomes to broadly identify ligandable, and therefore potentially druggable, cysteine residues. These efforts lead to the discovery over 700 ligandable cysteines across 600+ proteins, most of which previously lacked chemical probes (Backus et al., 2016). Initial broad-reactive fragments identified in such studies have been used to develop lead covalent ligands of protein targets, including compounds that selectively target pro-caspase-8 (Backus et al., 2016) and the transcriptional regulator NR0B1 (Bar-Peled et al., 2017) to investigate their physiological and pathophysiological roles. Competitive isoTOP-ABPP has also been used to identify the targets of bioactive compounds, including clinically approved drugs (Blewett et al., 2016; Zaro et al., 2019), herbicides (Ford et al., 2017), phenotypic screening hits (Bateman et al., 2017; Roberts et al., 2017a) and natural products (Grossman et al., 2017), providing direct insights into their mechanism of action and biological consequences. Additional broadly-reactive cysteine click probes have been developed to improve upon the biological properties and residue selectivity of IAA. For instance, although IAA is broadly reactive, its cytotoxicity precludes live cell labeling. Weerapana and coworkers recently developed photo-caged bromomethylketone (Abo and Weerapana, 2015) and iodomethyl ketone (Abo et al., 2017) click probes that show minimal cytotoxicity and provide spatial and temporal control of electrophile activation directly in living cells. In addition, Abegg *et al.* demonstrated that hypervalent iodine ethynyl benziodoxolone-based click probes exhibit improved selectivity for cysteine residues over other nucleophilic residues, compared to IAA (Abegg et al., 2015).

Reactivity-based click probes have also been developed for other nucleophilic amino acids. Lysine represents a particularly attractive target for click probe development due its abundance in proteins (~6% of all residues), particularly at enzyme active sites and PPIs as well as their frequent post translational modification. Over the past decades, numerous electrophiles have been shown to react with lysine residues, albeit to varying levels of reactivity and selectivity, however, it was only recently that lysine-reactive click probes were developed to assess lysine reactivity directly in human proteomes. Hacker et al. recently integrated isoTOP-ABPP with lysine profiling, finding that a sulfotetrafluorophenyl (STP, Figure 6) click probe displays exquisite selectivity for lysine over other side chains (Hacker et al., 2017). In this work, approximately 4,000 lysine residues were profiled and 100+ ligandable lysine residues were identified in competitive isoTOP-ABPP experiments with a small library of electrophiles. It was also shown that these interactions can be advanced to generate covalent inhibitors of enzymes and protein-protein interactions. Alternative electrophiles have been used for the broad profiling of lysine residues, including NHS ester-based click probes, however, these probes show substantial reactivity for other nucleophilic residues, such as serine, threonine, tyrosine, and cysteine (Ward et al., 2017). Various click probes have been used to profile other nucleophilic amino acid reactivity with aryl sulfonyl fluorides, which covalently react with various residues, including serine, lysine and tyrosine via sulfur (VI) fluoride exchange (SuFEx) reaction (Gu et al., 2013; Narayanan and Jones,

2015; Shannon et al., 2012). Recently, click probes with arylfluorosulfate electrophiles have been shown to have reduced proteomic reactivity relative to sulfonyl fluorides, and an apparent preference for proteinaceous tyrosine residues (Chen et al., 2016; Martin-Gago and Olsen, 2019). Highly selective click probes have recently been generated for intrinsically less nucleophilic methionine residues by cleverly utilizing its unique redox reactivity (Lin et al., 2017). Specifically, Lin and coworkers generated novel oxaziridine-based click probes (Figure 6) which were shown to achieve exceptional selective modification of methionine over all other nucleophilic residues and were used to investigate effects of methionine oxidation on protein function. Collectively, these studies highlight the impact of chemical intuition in the generation of selective reactivity-based probes for proteomic investigations.

**Proteomic Profiling of Non-Covalent Binding Interactions**—Not all ligandable sites on proteins possess nucleophilic residues that can be targeted by mechanistically-driven or electrophilic reactive groups and not all bioactive compounds possess protein-reactive moieties for covalent capture, thus necessitating the development of alternative modes of reactivity to covalently capture bound protein targets. Classical approaches to identify non-covalent small molecule-protein interactions, such as affinity chromatography, where compounds are immobilized to a solid support for enrichment from cell lysates, have been used to identify target proteins of bioactive compounds for decades (Rix and Superti-Furga, 2009). While affinity purification is well suited for identifying targets that are abundant and have a high affinity for corresponding immobilized small molecules ( $K_d$  in the nM range), such methods are ineffective for low affinity interactions, low abundance protein targets, and for the broad discovery of ligandable proteins in native contexts. Photoaffinity labeling provides a practical solution as it allows transient reversible interactions between a probe and protein to be fixed in a residue-agnostic fashion upon UV-irradiation. Applications of photoaffinity probes date to the early 1960's (Singh et al., 1962); however, recent pairing these reagents with modern chemical proteomics and their integration with ABPP principles has dramatically enhanced their impact on mapping small molecule-protein interactions and the discovery of ligandable proteins (Geurink et al., 2012; Lapinsky and Johnson, 2015; Smith and Collins, 2015).

There are many enzyme classes that do not possess protein-bound nucleophiles for catalysis. However, in certain instances, enzyme classes utilize conserved functional or structural features which may be exploited for ABP implementation. For example, metalloproteases (MPs), a large and diverse group of enzymes that play key roles in a variety of physiological and pathological processes, use a zinc-activated water molecule for catalysis. Considering this conserved mechanism, Saghatelian, Sieber and coworkers designed click ABPs (Figure 6) that specifically target MP active sites through the incorporation of a zinc-chelating hydroxamate group coupled to a photoactivatable benzophenone group for covalent capture and an alkyne handle for CuAAC mediated enrichment and detection of bound MPs (Saghatelian et al., 2004; Sieber et al., 2006). Similarly, histone deacetylases utilize zinc-activated water in their active sites, enabling the development of zinc chelating suberoylanilide hydroxamic acid (SAHA) based click ABPs to profile HDAC activity in cells (Salisbury and Cravatt, 2007, 2008). Additionally, cofactors as well as enzyme substrates or products can be used as recognition elements in photoaffinity based ABPs. For

example, Cisar and coworkers described the development of photoreactive GTP click probes to identify known and previously unknown proteins that utilize GTP in cells (Cisar et al., 2013). Horning et al. described a set of S-adenosyl homocysteine (SAH) photoaffinity (diazirine and benzophenone) probes with a high selectivity for methyltransferases (MTs) in cells as well as MT inhibitor discovery via competitive ABPP (Horning et al., 2016). Interestingly, in this study, it was discovered that the use of an alkyne handle resulted in substantial UV-independent labeling under CuAAC conditions, necessitating the use of biotin, rather than click-based, enrichment handles, highlighting the importance of careful control experiments in ABP development and the potential, albeit rare, drawbacks of CuAAC in some proteomic investigations.

Despite the immense success of ABPP and similar chemical proteomic platforms, the vast majority of proteins still lack chemical tools to study their function. This is due, in part, to the fact that not all protein families have conserved functional or structural features that can be selectively targeted with broad profiling chemical probes. To begin to address this challenge, recent chemical proteomic studies have aimed not to directly profile protein activity or reactivity but to agnostically explore protein ligandability, independent of protein function, cellular location or structure. For example, Kambe et al. generated a structurally diverse library (~60 members) of fully functionalized chemical probes which possess diazirine and alkyne functionalities for target capture and enrichment, respectively (Kambe et al., 2014). Chemical proteomic profiling of these drug-like probes in live human cells revealed that such probes achieved proteome-wide structure-activity relationships (SAR) and that numerous proteins (~25) were selectively enriched, including well-established druggable proteins, such as enzymes, as well as much less pharmacologically accessed targets, such as scaffolding and adaptor proteins. To more broadly identify ligandable, and therefore potentially druggable proteins in cells, we recently integrated chemical proteomics with fragment-based ligand discovery (FBLD) to broadly interrogate proteome ligandability (Parker et al., 2017a; Wang et al., 2019). Small molecule fragments efficiently explore broad chemical space and can serve as practical leads for subsequent synthetic elaboration. The development of fully-functionalized fragment (FFF) probes (Figure 6), which are structurally diverse drug-like fragments appended to a retrieval tag composed of an alkyne and photoactivatable diazirine, enabled the identification of more than 2000 reversible fragment-protein interactions in cells. It was demonstrated that FFF probes have proteome-wide binding preferences and can be used to map sites of ligandability on endogenous proteins in living cells through the identification of probe-modified peptides. It was further established that fragment-protein interactions could be used to generate selective ligands that modulate functions of proteins in cells. For example, simple fragment leads identified in comparative profiling studies were subsequently utilized in competitive profiling experiments (Figure 5B) to generate first-in-class inhibitors for prostaglandin reductase 2 (PTGR2) and the multi-pass transmembrane protein SLC25A20. Additional studies lead to the identification of a gain-of-function ligand for the poorly characterized transmembrane protein progesterone receptor membrane component 2 (PGRMC2), which facilitated the subsequent characterization of PGRMC2 as a heme chaperone critical for adipocyte function (Galmozzi et al., 2019). Although this strategy yielded an abundance of potentially ligandable protein targets, due to the simple nature of fragments, discerning specific

interactions (e.g. at a genuine binding pocket) from less/non-specific interactions required careful manual review. To overcome this challenge, a second generation library of FFF probes consisting of stereochemically matched fragments was constructed, which provide high confidence evidence of specific interactions between small molecule and proteins in cells (Wang et al., 2019).

### Chemical Centric Profiling

Chemical centric approaches (Figure 5) have been used for decades to deconvolute the targets of bioactive compounds, such as drugs, phenotypic screening hits, natural products and metabolites. An important feature of chemical centric proteomic profiling methods is that they are inherently target agnostic and functionally unbiased, minimizing the risk of overlooking unexpected off-targets that might be missed using target- or function-centric characterization assays (Drewes and Knapp, 2018). Classically, chemical centric proteomic methods have involved the immobilization of bioactive compounds to a solid support followed by incubation with cell or tissue lysates to capture and subsequently identify bound protein targets. Competition experiments are often conducted with free active and structurally similar but inactive control compounds to identify and prioritize selective protein targets over non-selective binders. Pioneering work using affinity based methods include the identification of the targets of macrocyclic immunosuppressants and the discovery of HDACs (Harding et al., 1989; Liu et al., 1991; Taunton et al., 1996). However, as discussed above, these approaches are limited when binders have lower affinities and such methods do not preserve intact cellular environments. The use of minimally perturbing, cell permeable click handles and covalent capture groups can mitigate these challenges.

Photoaffinity-based click probes are likely the most common form of chemical proteomic tools to capture and identify noncovalent targets of bioactive small molecules (Lapinsky and Johnson, 2015). Generation of these probes involves the synthetic installation of click groups (azide or alkyne) and photoaffinity groups (e.g. diazirines, benzophenone, aryl azides) in such a way that is minimally perturbing to the bioactivity of the compound, a process that can sometimes require extensive SAR investigations and even entire new synthetic routes. The recent development of modular “all-in-one” tags that contain i) functional groups for compound attachment, ii) an azide or alkyne for CuAAC and iii) photo-crosslinking groups for covalent capture, has assisted simple installation of these groups often in one synthetic step (Li et al., 2013; Li et al., 2014). Cell permeable click photoaffinity probes have been developed to characterize targets of various clinically approved drugs and inhibitors (Figure 6), including kinase inhibitors (Li et al., 2013; Shi et al., 2012),  $\gamma$ -secretase inhibitors (Ballard et al., 2014),  $\beta$ -secretase inhibitors (Zuhl et al., 2016), antibiotics (Eirich et al., 2011), nonsteroidal anti-inflammatory drugs (NSAIDs) (Gao et al., 2018), and epigenetic modulating compounds (Li et al., 2014; Montgomery et al., 2014) as well as natural products (Wright and Sieber, 2016). In many cases, these studies uncover targets that were previously unassociated with the compound’s phenotypic effects but turn out to be functionally relevant molecular targets. For example, photoaffinity click probes of a BACE1  $\beta$ -secretase inhibitor revealed that cathepsin D (CatD) is a common off-target of several clinical BACE inhibitors, which may contribute to observed ocular toxicity *in vivo* (Zuhl et al., 2016). Interestingly, the authors observed much greater CatD inhibition in cells

compared to cell-free assays, highlighting the importance of in-cell target engagement assays. In recent studies, a photoaffinity click probe made based on the diterpenoid ester ingenol mebutate (IngMeb), the active ingredient in the topical drug Picato, revealed 28 previously unknown IngMeb targets in cells, including the mitochondrial transporter SLC25A20, which IngMeb was found to functionally inhibit (Parker et al., 2017b). Photoaffinity click probes can also be used to reveal new protein interactions of important metabolites such as lipids (Niphakis et al., 2015) and sterols (Hulce et al., 2013). Given that metabolites likely engage functionally relevant sites on proteins, such metabolite-derived click probes can serve as valuable profiling tools to map drug interactions across the metabolite-interaction proteome and even serve as tools to develop modulators of metabolite-binding protein function (Niphakis et al., 2015). Numerous bioactive compounds can also engage protein targets covalently. As discussed above, broad reactive ABPs can be used in a competitive format to identify targets of the covalent compound that also overlap with the ABP. Alternatively, target retrieval probes can be generated by appending a click handles directly to the bioactive compound, enabling CuAAC enrichment of captured targets. For example, click probes have been used to assess the target landscape of covalent kinase inhibitors (Gushwa et al., 2012; Lannine et al., 2014; Niessen et al., 2017) and various bioactive natural products (Wright and Sieber, 2016). Click probes have also recently been used to identify covalent targets of reactive drug metabolites, including that of the fatty acid amide hydrolase (FAAH) inhibitor BIA 10-2474 (Figure 6), a clinical candidate that failed Phase I trials due to serious neurotoxicity which led to the death of one human volunteer (Bateman et al., 2013; Huang et al., 2019; van Esbroeck et al., 2017). This approach has even been extended to identify *in vivo* targets of the reactive metabolites of hepatotoxic drugs including troglitazone, clozapine, and acetaminophen (Whitby et al., 2017).

### Click Chemistry to Study Protein Post-Translational Modifications

The proteome is much more diverse than the corresponding genome as the result, in part, of PTMs. A wide range of both enzymatic and chemical modifications can be found on various amino acids and play key roles in nearly all biological processes. Therefore, identifying the protein substrates of PTMs, as well as the specific sites of modification when possible, is critical for a complete understanding of cellular biochemistry. Unfortunately, PTMs are typically very difficult to observe in shotgun proteomics experiments for several reasons. For example, PTMs are often substoichiometric and can be highly dynamic. Certain modifications are also chemically labile and can be lost during peptide ionization in the mass spectrometer, while others can inhibit peptide fragmentation and sequencing. Additionally, some PTMs are large and heterogeneous, making their recognition by proteomics-software challenging. Given these considerations, PTM selective enrichment is necessary prior to MS analysis. For certain PTMs, this can be accomplished using the biophysical properties of the modification or more traditional biological tools like antibodies. For example, phosphorylated peptides can be enriched using immobilized metal ion affinity chromatography (IMAC) or metal oxide matrices, and these technologies have allowed for the identification and quantitation of thousands of phosphorylation sites across the human proteome (Sharma et al., 2014). A similar number of lysine acetylation sites were identified after enrichment using an anti-acetyllysine antibody (Choudhary et al., 2009). Unfortunately,

the properties of many PTMs are not amenable to direct enrichment and many antibodies are not truly pan-selective and will therefore fail to enrich certain modified peptide sequences.

Given these challenges, click chemistry has been particularly powerful for the analysis of PTMs (Chuh and Pratt, 2015) (Chuh et al., 2016) (Grammel and Hang, 2013). In general, these approaches can be divided into three basic categories (Figure 7). The first relies on the unique chemical reactivity of certain PTMs or protein residues for the direct installation of the click probe. Second, cellular metabolism can be exploited to “sneak” azides or alkynes into PTMs using endogenous biosynthetic pathways, an approach we have termed metabolic chemical reporters (MCRs). Finally, enzymes can be exploited to modify certain PTMs *ex vivo* with click groups. In the metabolic and enzymatic strategies, the use of click chemistry is particularly important and often necessary. More specifically, the biosynthetic machinery and enzymes responsible for PTM installation or elaboration are able to tolerate small structural changes, like the addition of an azide or alkyne, but not larger groups like biotin that would allow for direct detection.

**Glycosylation**—Glycosylation is one of the most common protein PTMs, involving the covalent addition of carbohydrates with various sizes and compositions (Varki et al., 2015). In metazoans, proteins destined for the lumen of the secretory pathway, the cell surface, or the extracellular space can be subjected to a variety of different types of glycosylation. By far the most common of these forms are N-linked (Aebi, 2013; Schwarz and Aebi, 2011; Weerapana and Imperiali, 2006) and mucin O-linked glycosylation (Hang, 2005 145; Jensen, 2010 163; Tran, 2013 131). N-linked glycans are first biosynthesized as an oligosaccharide on a dolichol lipid before an *en bloc* transfer to the protein substrate in the endoplasmic reticulum (ER) lumen. Specifically, the carbohydrate-protein linkage can be made to the side chain of asparagine in the sequence motif Asn-Xxx-Ser/Thr, where Xxx is any amino acid except proline. As the glycoprotein moves through the ER and Golgi apparatus, the oligosaccharide can be trimmed and then elaborated with additional carbohydrates to generate a range of large and heterogeneous structures. N-linked glycans are critical for protein folding and intracellular protein trafficking, as well as mediating various interactions at the cell surface and the function and stability of circulating proteins. Mucin O-linked glycosylation first involves the addition of an *N*-acetylgalactosamine through an  $\alpha$ -linkage to the side chains of serine or threonine residues of proteins transiting the Golgi. Like N-linked glycans, this core modification can then be elaborated through the addition of more carbohydrates. Mucin O-linked glycosylation plays key roles in cell-cell contacts, protective barrier formation, and the biophysics of the cell surface. Intracellular proteins in animals can be subjected to O-GlcNAc modification, the addition of *N*-acetylglucosamine through a  $\beta$ -linkage to serine or threonine residues (Yang and Qian, 2017). Unlike the major forms of glycosylation on secreted proteins, O-GlcNAc is not further elaborated. However, it is dynamically cycled on and off proteins and therefore acts as a signaling modification. Thousands of protein substrates of O-GlcNAc have been identified and this PTM can affect protein-protein interactions, enzymatic activity, and compete with other modifications like phosphorylation.

Traditional methods for studying glycoproteins relied on lectins and antibodies. Lectins are natural carbohydrate-binding proteins found in many organisms. Unfortunately, they suffer

from a lack of complete specificity for particular carbohydrate but rather recognize structural “themes” that can be found in several different types of glycans. Additionally, they typically bind glycan structures with relatively low affinity. Some carbohydrate antibodies have been generated, but some of them display preference for the underlying peptide sequence, preventing them from being truly pan-selective reagents. Therefore, the field of glycobiology has benefited greatly from bioorthogonal approaches to the visualization and enrichment of glycoproteins. The first such method was pioneered by the Bertozzi lab, termed metabolic oligosaccharide engineering, involves the use of metabolic chemical reporters (MCRs) - analogs of naturally occurring monosaccharides bearing bioorthogonal functional groups (Mahal et al., 1997; Saxon and Bertozzi, 2000). These MCRs, can be accepted by living cells or even animals where they are biosynthetically converted to high-energy sugar donors and then directly incorporated into glycans by endogenous enzymes. For example, one of the first MCRs was an azide-containing analog of N-acetylmannosamine, Ac<sub>4</sub>ManNAz, with the hydroxyl groups masked as acetate esters to allow for passive diffusion into cells (Table 1) (Saxon and Bertozzi, 2000). Once internalized, the O- acetates are removed by esterases and the resulting ManNAz can be biosynthetically transformed into an azide-containing sialic acid monosaccharide that is then added to the termini of N-linked and mucin O-linked glycans. It is important to point out that in the case of the MCR method, the use of bioorthogonal chemistry is absolutely critical. Unlike some reactive probes that can be directly conjugated to tags without largely compromising their ability to covalently label their targets, direct tagging of monosaccharides would result in a large modification that would not be tolerated by the cellular biosynthetic enzymes, resulting in little or no labeling. Since this initial study using Ac<sub>4</sub>ManNAz, several other azide- or alkyne-containing MCRs have been developed, including analogs of N-acetylglucosamine (Vocadlo et al., 2003; Zaro et al., 2011), N-acetylgalactosamine (Hang et al., 2003), and fucose (Kizuka et al., 2016; Rabuka et al., 2006) (Table 1). Additionally, other bioorthogonal groups like alkenes (Niederwieser et al., 2013; Späte et al., 2014) and cyclopropenes (Cole et al., 2013; Patterson et al., 2014a; Patterson et al., 2012) that can undergo inverse-demand Diels-Alder reactions have been incorporated into monosaccharides.

Not surprisingly, MCRs of glycans have been extensively used for the proteomic identification of glycoproteins in a variety of contexts. To facilitate the identification of glycosylation sites, the Bertozzi lab recently developed isotope-targeted glycoproteomic (IsoTaG) reagents (Woo et al., 2017). These biotinylated tags generate a unique isotopic signature on glycosylated peptides post bioorthogonal tagging, facilitating the detection of low-abundance glycopeptides. For example, combining an IsoTag with Ac<sub>4</sub>GalNAz, an azide-containing analog of N-acetylgalactosamine, enabled the identification of over 2,000 potential O-GlcNAcylated peptides from primary human T-cells (Woo et al., 2018). Unfortunately, several glycan MCRs suffer from a lack of glycan selectivity. In retrospect this is not surprising, as individual monosaccharides are found in multiple types of glycans. Additionally, cells have evolved to interconvert different monosaccharides, resulting in potentially undesirable interconversion of MCRs as well (Boyce et al., 2011). In an attempt to address these selectivity issues, we and others have synthesized various structural analogs of MCRs that could bias the utilization of these reporters by different glycosylation pathways (Chuh et al., 2017; Chuh et al., 2014; Li et al., 2016; Shen et al., 2017; Zaro et al.,



2017). Notably, several MCRs that are selective for O-GlcNAcylated proteins, suggesting that this strategy may be applicable for other classes of glycans. It is also important to note that because MCRs compete with natural monosaccharides, their incorporation levels do not necessarily reflect the natural amounts of protein glycosylation. Finally, recent evidence has suggested that some amount of signal generated by MCRs is likely due to either direct chemical labeling of proteins or their breakdown into intrinsically reactive metabolites (Qin et al., 2018); however, other data from the same labs indicates that this “background” labeling may be a minor component (Qin et al., 2017).

Complementary to these metabolic strategies, chemoenzymatic methods have been developed. The most well-established of these approaches for proteomics is in the area of O-GlcNAcylation and was inspired by early work on the enzymatic radiolabeling of native O-GlcNAc modifications in cell or tissue lysates. Specifically, terminal GlcNAc moieties can be labeled using a combination of radioactive UDP-galactose (UDP-Gal) and a  $\beta$ 1,4-galactosyltransferase (GalT), which generates a Gal-GlcNAc disaccharide on O-GlcNAcylated proteins that can be detected by autoradiography (Torres and Hart, 1984). Replacing the native GalT with a mutant (Y289L) enabled the enzymatic transfer of *N*-azidoacetyl galactosamine (GalNAz) from UDP-GalNAz to generate a disaccharide bearing an azide for subsequent bioorthogonal chemistry (Table 1) (Clark et al., 2008). This technology has been transformative for the confirmation of potential O-GlcNAcylated proteins identified using MCRs, as well as the direct identification of modified proteins. For example, when combined with a photo-cleavable biotin enrichment-tag, this technique enabled the identification of 458 O-GlcNAc sites on 278 proteins from mouse cerebocortical brain tissue (Alfaro et al., 2012) and subsequently 1094 sites on 530 proteins from human brain samples (Wang et al., 2017). Building upon this idea, several other UDP-azido-sugars and enzymes have been developed for the incorporation of bioorthogonal handles onto different cell surface glycans (Wen et al., 2018; Wu et al., 2018). However, to our knowledge, they have not yet been applied to proteomics analysis. Since these methods modify native glycosylation, they necessarily enrich endogenous modifications and can enable overall quantification.

**Lipidation**—Lipidation, the attachment of a variety of fatty acids, is a PTM that controls the localization and thereby function of proteins (Jiang et al., 2018). More specifically, this modification dynamically regulates the position of proteins throughout a sophisticated network of membranes and organelles that are critical to cellular function. Two of the most common types of lipidation are the addition of saturated fatty acids. Myristoylation typically occurs co-translationally on the N-terminus of proteins through the formation of an amide bond to the 14-carbon myristic acid. Likewise, palmitoylation is the reversible and often highly dynamic addition of the 16-carbon palmitic acid to cysteine side chains, resulting in fatty acid thioesters. Unsaturated lipids can also be irreversibly added as thioethers to cysteines through a PTM termed S-prenylation. More specifically, two different isoprenoids, a 15-carbon farnesyl or 20-carbon geranylgeranyl, can be found at one or two cysteines typically at the extreme C-terminus of certain proteins. Together these PTMs control the subcellular localization of proteins with a variety of biological consequences. For example, the combination of S-prenylation and dynamic palmitoylation control the positioning of

activated Ras to the cellular membrane and therefore the subcellular localization of the associated signaling pathways.

In contrast to their complex biology, lipids have chemically uniform structures and lack essentially any functional groups. This makes the creation of traditional enrichment tools like antibodies extremely difficult. Therefore, visualization of protein lipidation largely relied on the metabolic incorporation of radioactive lipids. This method suggested that, like the carbohydrate pathways discussed above, lipid MCRs bearing small bioorthogonal functionalities might also be tolerated and incorporated onto proteins. Again, the use of small bioorthogonal handles was absolutely required for this technology, as these relatively small modifications can be tolerated by the cellular metabolic and transferase enzymes. This possibility was first confirmed through the metabolic labeling of lipidated proteins with myristic- and palmitic-acid analogs bearing azides at the omega end of the lipid chain (Hang et al., 2007). These original lipid MCRs were quickly followed by the development of analogous alkyne-lipids that take advantage of the better CuAAC orientation described above (Table 1) (Charron et al., 2009). These alkynyl-lipids have enabled a variety of transformative proteomics experiments. For example, 13-tetradecynoic acid (Alk-12) was used as a myristic acid analog to identify myristoylated proteins from the *Plasmodium falciparum*, the malaria pathogen (Wright et al., 2014). This same reporter was also combined with SILAC to identify myristoylation events that were sensitive to an *N*-myristoyltransferase inhibitor (Thinon et al., 2014). Likewise, the palmitic acid analog 17-octadecynoic acid, or Alk-16, was used to identify critical palmitoylation events that control the antiviral activity of the protein IFITM3 (Yount et al., 2010). The unique nature of metabolic incorporation of Alk-16 was subsequently exploited in combination with SILAC to perform a pulse-chase experiment and catalog the dynamics of different palmitoylation events across the cell (Martin et al., 2012). And, this probe has been successfully applied to profile palmitoylated proteins in pathogens, like *Toxoplasma gondii* (Foe et al., 2015). Similarly to the saturated fatty acids, azide and alkyne analogs of isoprenoids have also been developed that can be metabolically incorporated into S-prenylation by cells (Table 1) (Charron et al., 2013; DeGraw et al., 2010; Storck et al., 2019). Again, when combined with proteomic strategies, these reporters have enabled the discovery of key S-prenylation events that control antiviral activity of proteins and the profiling of S-prenylation changes in disease.

**Acetylation**—Acetylation of the  $\epsilon$ -amine of lysine is a PTM best known for critical regulation of gene transcription through specific modification patterns on chromatin (Shahbazian and Grunstein, 2007) but can also play other important roles throughout the cell (Yang and Seto, 2008). Through capping of lysine, acetylation can directly alter the charge state of a protein and directly compete with other modifications like methylation, ubiquitination, and SUMOylation. Acetate is added to proteins by a family of acetyltransferases that use the high-energy acetyl-CoA cofactor, and the modification can be removed by another family of deacetylases. Anti-acetyl lysine antibodies have been particularly powerful for the identification of almost 5,000 modification sites across the mammalian proteome. However, these antibody tools cannot easily read out on the dynamics of protein acetylation or distinguish between the enzymatic modification of proteins by

acetyltransferases versus the direct chemical modification by the reactive acetyl-CoA thioester. For example, a metabolic chemical reporter of acetylation can be used in a similar fashion to radioactive or bioorthogonal amino acids to perform pulse and/or pulse-chase experiments. As a first step towards using bioorthogonal chemistry to address these issues, 4-pentynoic acid was tested as an MCR and was shown to label a range of proteins in living cells, including known acetylated proteins (Table 1) (Yang et al., 2010). The utility of this short alkyne acid as an MCR for lysine acetylation was then bolstered by the fact that purified p300 acetyltransferase can utilize synthetic 4-pentynonyl-CoA to label proteins in cell lysates (Yang et al., 2011). In addition to acetylation, longer fatty acids and modified acetate groups, including propinylation, butyrylation, malonylation, and succinylation have been found on lysine side chains (Lin et al., 2012). This encouraged the more recent development of an MCR for protein malonylation, 2-propargyl malonate (Mal-yne) (Table 1) (Bao et al., 2013). Much like the monosaccharide MCRs described above, the carboxylic acids of Mal-yne were protected as acetoxymethyl esters to enable diffusion into living cells, enabling the identification of almost 400 proteins from HeLa cells, including 14 known malonylated proteins. As mentioned directly above, it is unclear how many lysine acetylation events occur enzymatically versus chemically, but bioorthogonal reactions have also provided an approach to answer this question through the development of a direct-modifying probe. More specifically, an alkyne-containing thioester was synthesized that mimics acetyl-CoA but cannot be used by acetyltransferases (Table 1) (Kulkarni et al., 2017). Profiling of cells using this reporter allowed for the identification of ~100 potential protein targets of direct chemical acylation and enabled the characterization of malonyl-CoA as a particularly reactive thioester for this type of reaction.

**Methylation**—Similar to acetylation, lysine side chains can also be subjected to methylation, which also plays important epigenetic roles through chromatin biology and can more broadly affect the function of a variety of proteins (Bedford and Clarke, 2009; Biggar and Li, 2015; Greer and Shi, 2012). However, this PTM is somewhat more versatile by virtue of its ability to add one, two, or three methyl groups to the same lysine, as well as methylation of arginine side chains in three different patterns. Methylation is added to proteins by distinct lysine- and arginine-methyltransferases that all use S-adenoyl-L-methionine (SAM) as a cofactor. The modification can then be removed by two families of demethylases that are defined by their differential enzymatic mechanisms. Methylation affects proteins by altering the size of lysine and arginine side chains and antagonizing other modifications. Antibodies that recognize methylated lysines and arginines are available, but bioorthogonal chemistry has made important contributions to the characterization of this PTM. As a first step, two different analogs of SAM with short alkyne modifications were synthesized (Binda et al., 2011; Peters et al., 2010). *In vitro* analysis demonstrated that these SAM analogs were accepted by a subset of the methyltransferases tested. These results suggested that pairs of bioorthogonal SAM reporters and methyltransferase mutants could be identified using a “bump-hole” strategy. In other words, larger alkyne- or azide-modifications of SAM or “bumps” might be tolerated by specific enzymes that have had their active site mutated to generate “holes” that would accommodate the modifications. This goal was subsequently realized through rational engineering of the methyltransferase active site, enabling the labeling and identification of methyltransferase-specific substrates

(Table 1) (Islam et al., 2012; Islam et al., 2013). Unfortunately, these SAM analogs are not cell permeable and therefore can only be used in cell lysates. To address this shortcoming, the SAM biosynthetic pathway was altered to accept alkyne-modifications in living mammalian cells (Wang and Luo, 2013). In this case, cells can be treated with the alkyne-modified analog of methionine that will subsequently act as an MCR in the engineered pathway to generate the corresponding alkyne-SAM. While a success, this engineering effort highlights both the fundamental limitation of the MCR-strategy, the acceptance of a bioorthogonal reporter by multiple enzymes, and the utility of the minimal bioorthogonal azide and alkyne handles that are best capable of sneaking through these pathways.

**ADP-Ribosylation**—Protein ADP-ribosylation results from the enzymatic transfer of ADP-ribose to a range of amino acid side chains, including lysine, asparagine, glutamine, serine and cysteine in mammals (Cohen and Chang, 2018; Palazzo et al., 2019). In humans, a family of 17 ADP-ribosyltransferases, termed poly-ADP-ribose polymerases (PARPs), can add this modification. Despite their name, the majority of PARPs appear to be mono-ADP-ribosyltransferases while a subset can generate poly-ADP-ribose through the extension of the ADP-ribose chain. This modification can also be rendered dynamic through removal by ADP-ribose hydrolases. ADP-ribosylation has been shown to play important roles in a variety of cellular processes, in particular stress response and DNA damage repair, making this PTM an intriguing therapeutic target in a variety of diseases (Curtin and Szabo, 2013). The structurally complex and chemically labile nature of poly-ADP-ribosylation has made it challenging to study and bioorthogonal probes have made important contributions to the global and enzyme specific analysis of substrate proteins. The ability of PARPs to accept modified analogs of ADP-ribose was first explored using 6- or 8-alkyne-NAD<sup>+</sup> molecules (Table 1), which enabled the labeling and identification of PARP1 substrates when incubated with cell lysates (Chang et al., 2009; Du et al., 2009a). A bump-hole strategy similar to that used for SAM analogs described above was also exploited to create probes for specific PARP family members. More specifically, a hole could be generated in PARPs that could accept bumps at the C5- position of the nicotinamide on NAD<sup>+</sup>, allowing for the identification of specific substrates of PARP1 and PARP2 (Carter-O’Connell et al., 2014). Although powerful, these approaches rely on synthetic NAD<sup>+</sup> analogs that are not cell permeable and therefore must be performed in cell lysates. To address this limitation, an MCR of ADP- ribosylation was recently developed (Westcott et al., 2017a). More specifically, cells can biosynthesize alkyne-containing NAD<sup>+</sup> from N<sup>6</sup>-propargyl adenosine, resulting in labeling of ADP-ribosylation in living cells and characterization of Hras modification. Again, the small size of the alkyne was key to transiting the NAD<sup>+</sup> biosynthetic pathway. More recently, a similar reporter bearing the alkyne at the 2-position of adenosine was shown to display similar cellular labeling and improved detection of protein targets by proteomics (Kalesh et al., 2019).

**Cysteine redox modifications**—The electron rich thiol group of cysteine has unique nucleophilic properties as outlined above but can also undergo a broad range of oxidative reactions to generate unique functional groups, including S-sulfenylation (-SOH), S-sulfinylation (-SO<sub>2</sub>H), S-sulfonylation (-SO<sub>3</sub>H), S-nitrosylation (-SNO), S-sulfhydration (-SSH), and S- glutathionylation (-SSG). Most thiol redox modifications can be formed by the

non-enzymatic reactions of reactive oxygen/nitrogen/sulfur species (ROS/RNS/RSS) with protein thiols, which has led to the widespread notion that these types of modifications are randomly distributed across the cysteine proteome. However, emerging evidence suggests that thiol redox modifications are well-controlled, site-specific cellular events, which play important roles in regulation of diverse protein functions, including catalytic or ligand binding activities, protein-protein interactions, and protein stability (Antelmann and Helmann, 2011). Identification of protein targets of thiol redox modifications is crucial to understanding their roles in biology and disease; however, proteome-wide analysis of thiol redox modifications is challenging due to the lack of selective biological reagents (e.g. antibodies) and is further complicated by the labile and highly dynamic nature of thiol redox forms. Not surprisingly, bioorthogonal chemistry has also provided solutions to these limitations (Yang et al., 2016). The first such method relied on the selective reaction between a sulfenylated cysteine and dimedone (5,5-dimethyl-1,3-cyclohexanedione) nucleophiles for the selective installation of a azide- or alkyne-tags as a stable thioethers that could then be subjected to bioorthogonal reaction with visualization or affinity tags (Table 1) (Leonard et al., 2009; Paulsen et al., 2011; Yang et al., 2014). Notably, this probe was used to identify a sulfenylated cysteine in the active site of the epidermal growth factor receptor (EGFR) that increases its kinase activity (Paulsen et al., 2011). More recently, sulfenylated cysteines were shown to selectively react with noroborene probes to give stable sulfoxide products (Table 1) (Alcock et al., 2019). The unique reactivity of electrophilic diazenes with  $-SO_2H$  was likewise exploited to generate a alkyne-containing probe of sulfinylated cysteines termed DiaAlk (Table 1) (Akter et al., 2018). In the case of these PTMs, like some of the enzymatic activity profiling experiments we described above, the exploitation of bioorthogonal chemistry is not absolutely necessary but is rather a key feature that can be exploited for multiple types of analyses. The chemical mechanisms exploited to enable these selective labeling reactions are shown in Figure 8.

**Examples of biological insights into PTM function gained using click chemistry**—Cataloging PTMs is in itself an important goal, reminiscent of RNA sequencing and global proteomics experiments. However, they have also enabled a range of important biological discoveries. For example, in the case of protein glycosylation, chemoenzymatic modification of mouse or human brain tissues followed by proteomics identified several O-GlcNAc modification sites on the Parkinson's disease-causing protein  $\alpha$ -synuclein (Alfaro et al., 2012; Wang et al., 2017). With this information, we subsequently used synthetic protein chemistry to generate site-specifically O-GlcNAcylated  $\alpha$ -synuclein and showed that this modification inhibits the amyloid aggregation and toxicity of this protein (Levine et al., 2019). As another example, the Hang lab recently used a metabolic reporter of short-chain fatty acids to profile modified proteins in *Salmonella* (Zhang et al., 2020). They found that several virulence proteins are butyrylated and showed that site-specific modification of the transcription factor HilA inhibits its activity and attenuates bacterial entry into epithelial cells. Similarly, the same lab used the  $N^6$ -propargyl adenosine metabolic reporter to discover that the critical signaling protein Hras is ADP-ribosylated under oxidative stress in cells and that this modification inhibits its activity (Westcott et al., 2017b). As a final example, the Carroll lab used their probe, alkyne-diazine, and quantitative proteomics to profile protein sulfinylation in wild-type and sulforedoxin-null cells, allowing

them to directly identify numerous unknown substrates of this critical enzymatic regulator of oxidative stress (Akter et al., 2018). In all of these cases, these insights would have been impossible or at least difficult to obtain using traditional biological tools like enrichment with anti-PTM antibodies, which either do not exist or do not recognize the entire repertoire of modified proteins. For example, using our synthetic proteins we found that the two most commonly used pan-selective anti-O-GlcNAc antibodies only recognize one out of four modification sites on  $\alpha$ -synuclein.

### **Extracting Biological Information from Click Chemistry Enabled Proteomic Investigations**

At this stage, click chemistry-facilitated proteomics is robust, and proteomic lists of proteins captured or modified by small-molecules are routinely published. However, it is still challenging to identify the interactions and modifications that are most likely to affect protein biochemistry and thus mediate important biology. Therefore, it is important to narrow the list of potential proteins for follow-up biochemical or functional studies. Unfortunately, there are no silver bullets to accomplish this, and each investigation often necessitates unique solutions. In the case of understanding functional consequences of small molecule-protein interactions, the use of structurally similar but functionally inactive control probes are essential to prioritize biologically relevant protein targets, which can require additional medicinal chemistry effort. In addition, although click probes can reveal potential protein targets, often such experiments do not directly provide relative levels ligand-protein stoichiometry, thus competitive experiments with underivatized bioactive analogs can facilitate the identification of selective and high- stoichiometry targets. Furthermore, careful validation of identified small molecule-protein interactions is necessary to home in on proteins of functional relevance. Secondary target engagement assays should be used to confirm small-molecule target interactions and genetic perturbation strategies (knock out/down, overexpression, etc.) should be employed to assess whether genetic modulation of identified proteins mimic or alters the activity of the bioactive compound. In the case of PTMs, we recommend several approaches. All proteomics lists should be entered into biological pathway analysis programs that can provide clues to the overall biochemical pathways that might controlled by a PTM of interest. This algorithm guided analysis should be paired with a working knowledge of the literature surrounding a PTM, with an emphasis on the previously characterized phenotypes and biochemistry. If the specific site of a PTM is identified, genetics can also be a guide. For example, if a given modification site is conserved evolutionarily, it is more likely to be functionally relevant. Additionally, if a site is mutated in human disease (e.g., a mutation consistently found in cancer) the modification may also be playing an important role. Once these approaches have been used to narrow down the list of potentially interesting proteins, the modification stoichiometry of some PTMs can be determined on a case-by-case basis (Darabedian et al., 2018; Percher et al., 2016; Rexach et al., 2010), as it is more likely that proteins with a high fraction of modification will have altered biochemistry.

### **Considerations and Looking Forward**

Bioorthogonal click chemistry has enabled the development of a wide range of chemical probes and reporters. In many cases, the use of small bioorthogonal reactive groups is essential for the application of probes to study living systems and, in all cases, increases

probe versatility through the divergent attachment of a various enrichment and analysis tags. Despite the many successes of numerous clickable probes, some challenges still remain. Due in part to the diversity of biological applications, there is no standardized protocol covering the different aspects of probe treatment, click chemistry conditions, or analysis. This results in a choice of a variety of different methods that may seem overwhelming or arbitrary to the newcomer, thus the sharing of detailed protocols from expert labs remains an important goal. Additionally, while the commercial availability of both probes and tags has exploded in the past decade, some reagents and most chemical probes require synthetic chemistry skills to prepare. Therefore, there is an important need for the continued close collaboration between biology and chemistry labs to ensure the application of the best probes to the most important biological questions. Finally, the proteomic analyses used downstream of certain probe applications generates a large volume of data that needs to be analyzed and curated, which again requires some non-standard expertise, proper controls, and rigorous statistical evaluation.

In the case of illuminating small molecule interactions, despite the utility of protein-centric and chemocentric click probes, the vast majority of proteins still lack useful chemical tools to interrogate their function. ABPP and similar chemical proteomic platforms have aimed to identify chemical probes for functionally related protein families which can be used in complex native environments; however, there currently exists no general capability to identify chemical probes or small molecule ligands for *all* ligandable proteins (Schreiber, 2019). Proteome-wide fragment-based ligand discovery (Backus et al., 2016; Parker et al., 2017a; Wang et al., 2019) and other methods (Franzini and Randolph, 2016) represent promising strategies to systematically yet agnostically identify small molecule protein ligands. Furthermore, click handles enable the enrichment of protein targets; however, their presence can often perturb molecular interactions and biological activity. Recent advances in “label-free” proteomic methods, such as thermal proteome profiling (TPP) and drug affinity responsive target stability (DARTS), represent powerful complementary strategies to map small molecule-protein interactions, bypassing the requirement of enrichment handles such as click functional groups (Lomenick et al., 2009; Savitski et al., 2014). However, these methods rely on ligand-induced stabilization towards heat or proteolytic degradation, for example, which are irreversible processes that can be influenced by other complicating factors including PTMs and other interaction partners, and, furthermore, the lack of an enrichment step can lead to substantial false negative readouts.

In the case of PTMs, it still remains challenging to identify the exact site of modification on the protein and, in the case of heterogenous modifications like glycosylation, the exact structure of the modification. Improvements in mass spectrometry techniques, instrumentation, and data analysis will play key roles in the probe-related discoveries in the future. Additionally, many of the probes for PTMs require cellular metabolism to result in protein labeling. This reliance on interconnected biosynthetic pathways can result in the metabolic transformation of these reporters into “off target” pathways. Therefore, important future goals should be a more comprehensive understanding of the metabolism of probes and the generation selective reporters that are funneled down the PTM pathway of interest. Finally, continued chemical and biochemical insights are needed for the development of probes for currently untargeted PTMs, an ever expanding number in all kingdoms of life.

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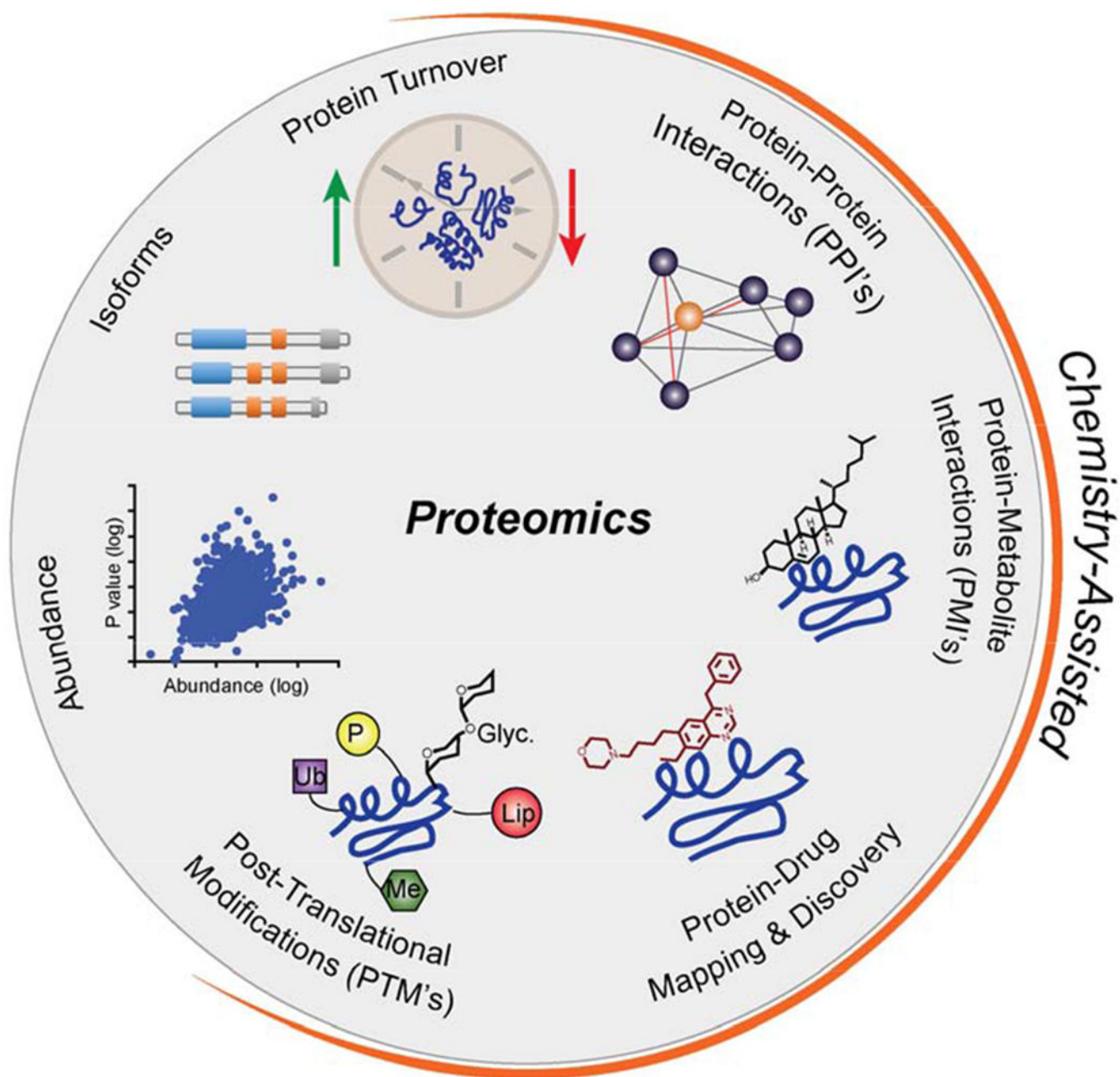


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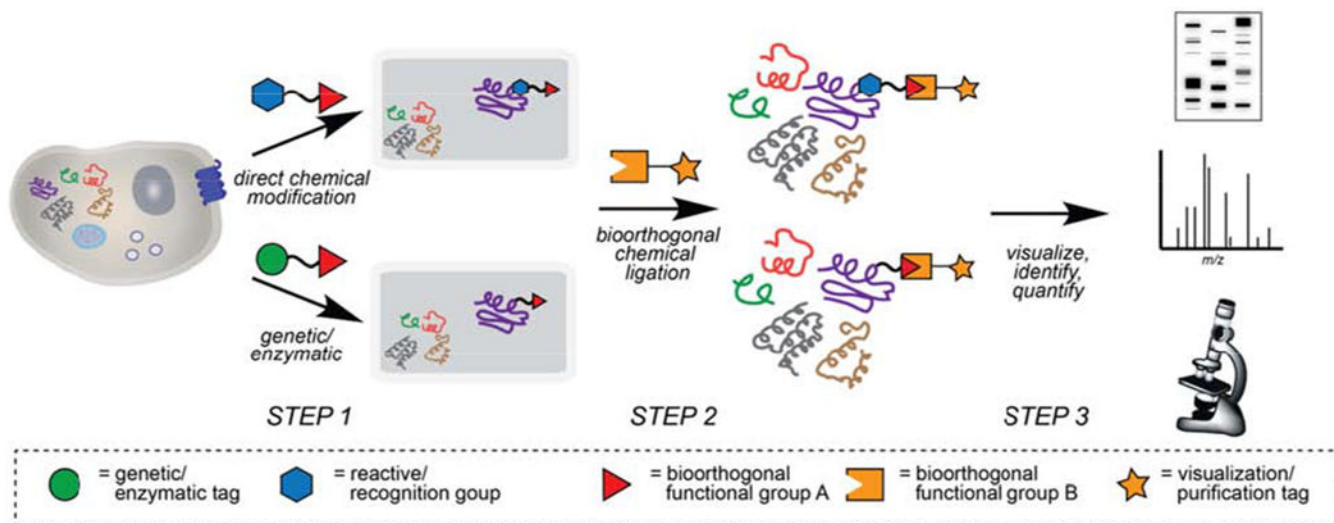
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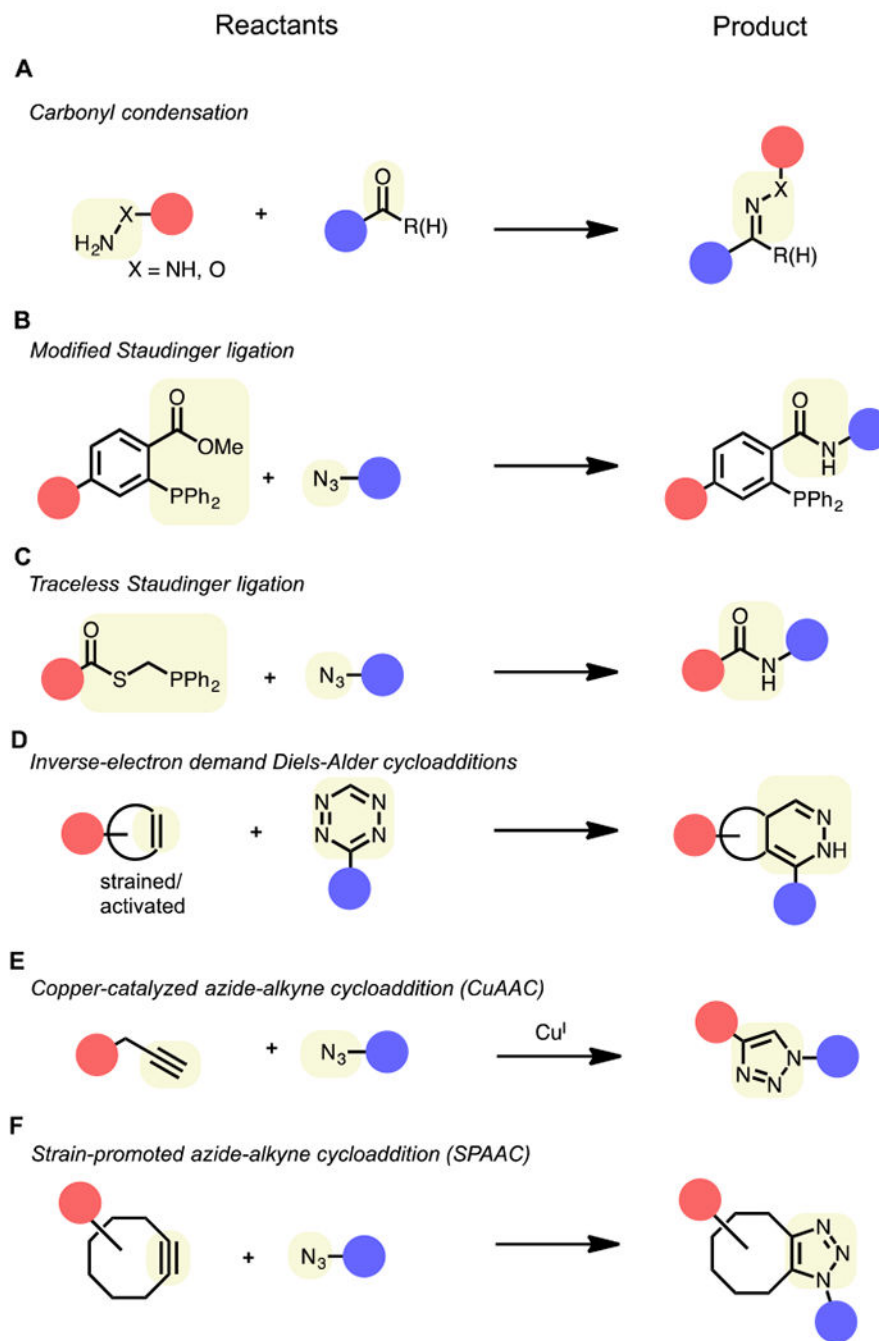
**Figure 1. Biological studies enabled by proteomics.**

Highlighted in orange are applications that can be integrated with chemical methods to aide in proteomic investigations

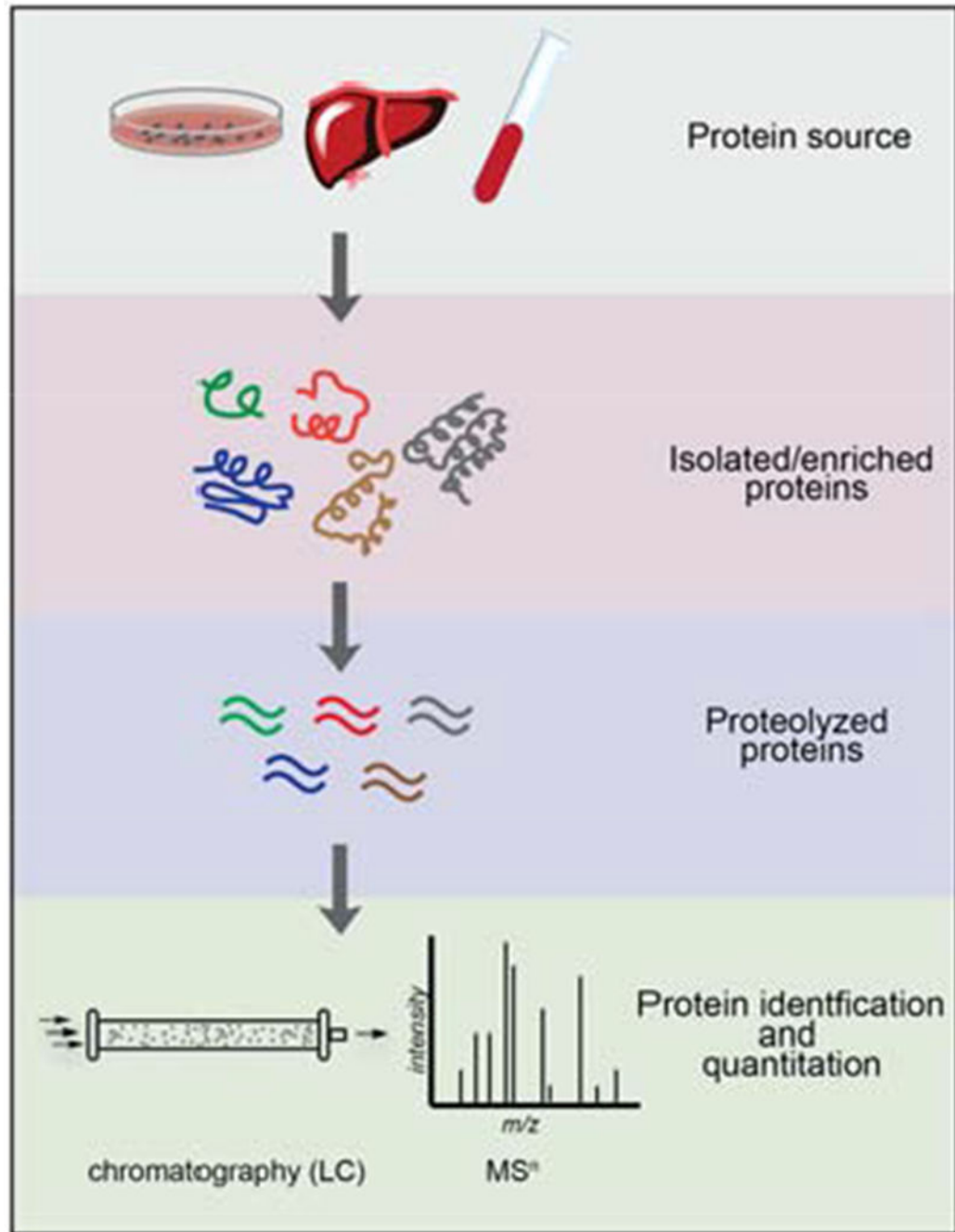


**Figure 2. General strategies to introduce bioorthogonal functionality to study proteins.**

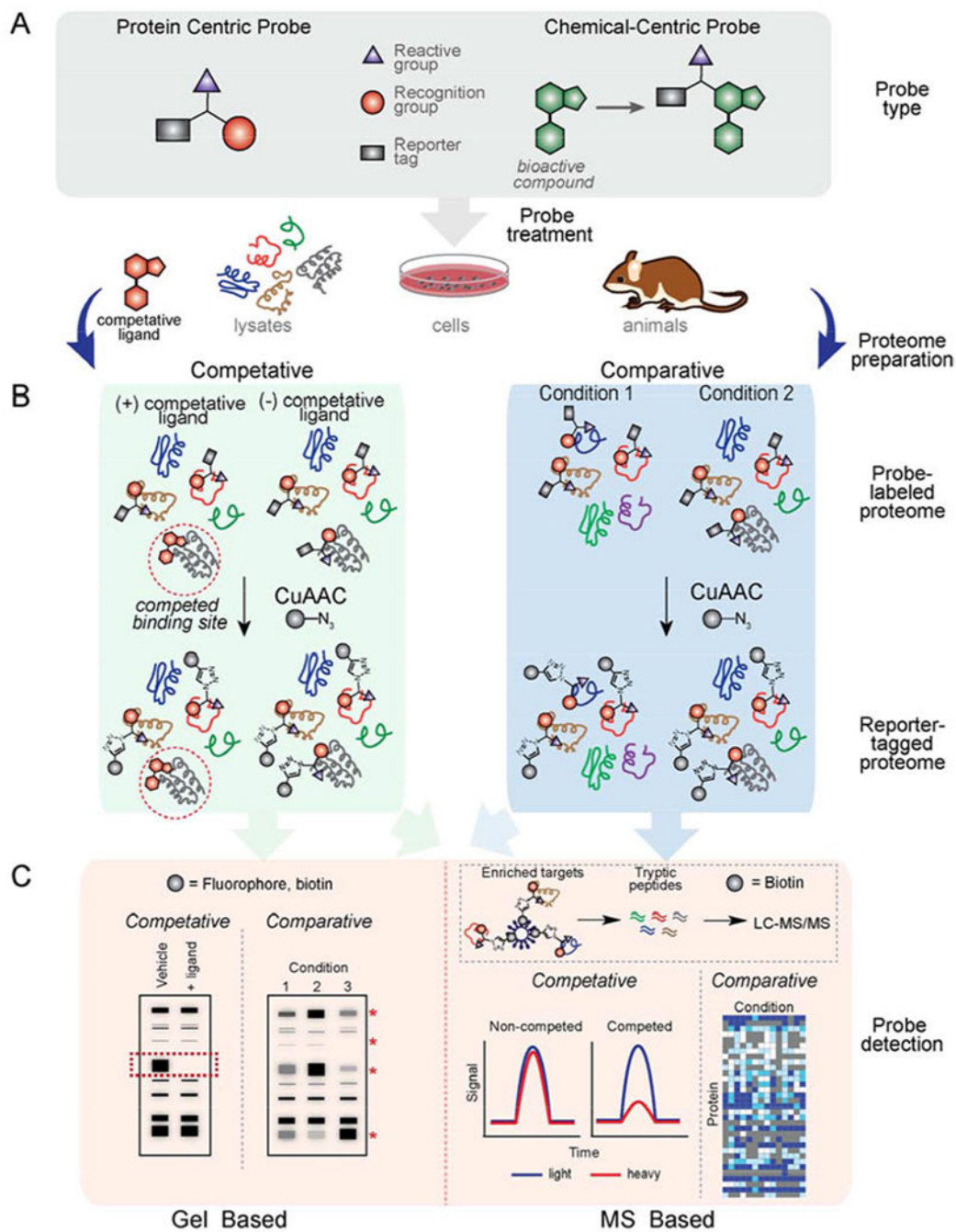
*Step 1)* Bioorthogonal reactive groups are first introduced onto protein targets through one of two general strategies, either i) direct chemical modification of endogenous proteins with chemical probes (top path), or ii) through a genetic tag, such as unnatural amino acid mutagenesis with functionalized amino acids and orthogonal tRNA/AARS pairs or enzymatic ligation of acceptor peptides with bioorthogonal motifs on engineered enzymes (*bottom path*). *Step 2)* Chemical tags possessing complementary bioorthogonal groups are selectively ligated to modified proteins. *Step 3)* Marked proteins are subsequently visualized, quantified and/or identified using a variety of techniques.



**Figure 3.** Common bioorthogonal chemical reactions. Red and blue circles correspond to the two “reactants” of a bioorthogonal reaction: 1) proteins labeled with a bioorthogonal reactive group and 2) complementary reactive tag for protein identification and/or visualization. Highlighted in yellow are the bioorthogonal reactive groups (left) and corresponding ligation product (right).

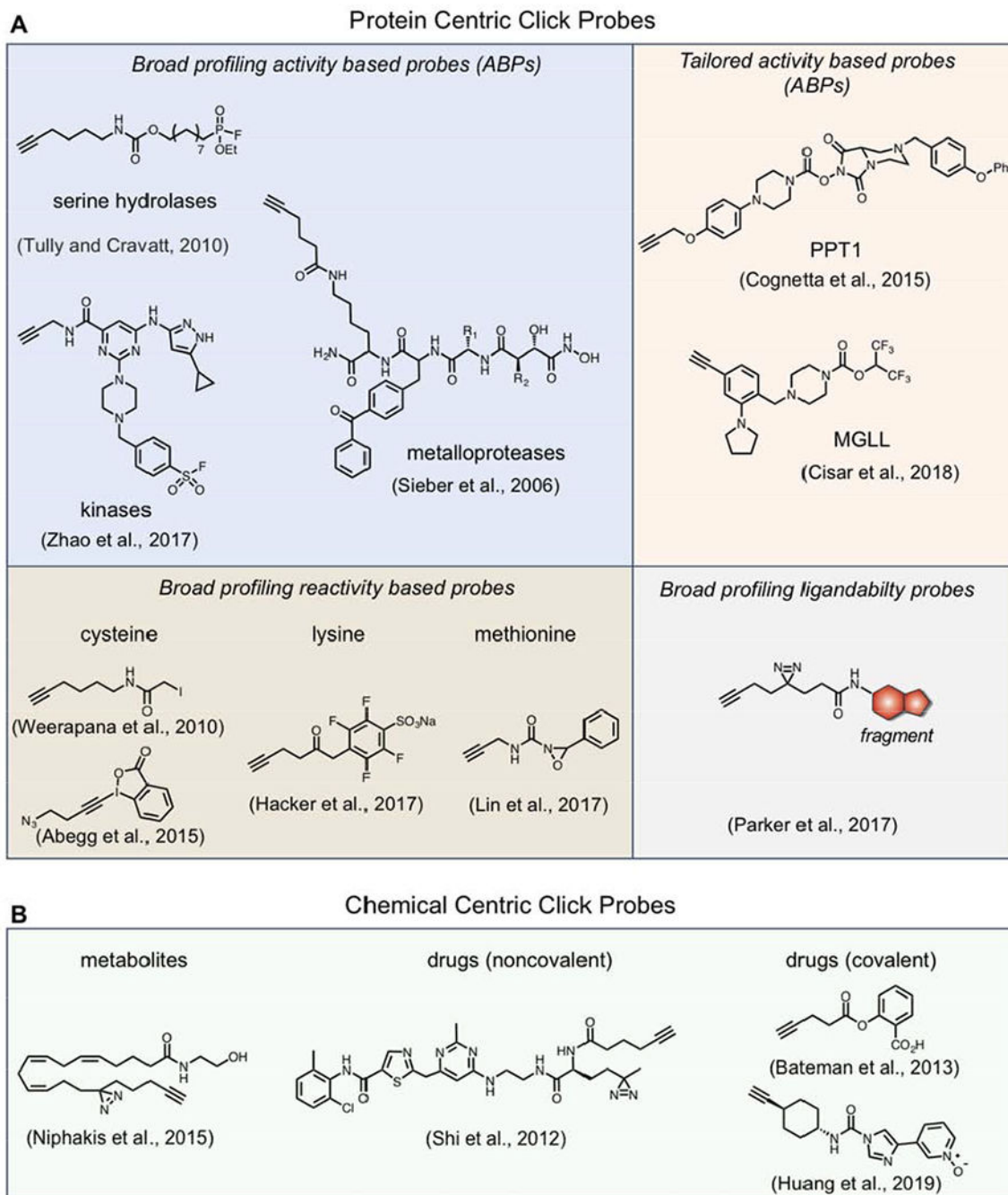


**Figure 4.**  
General “bottom-up” proteomics experimental workflow.

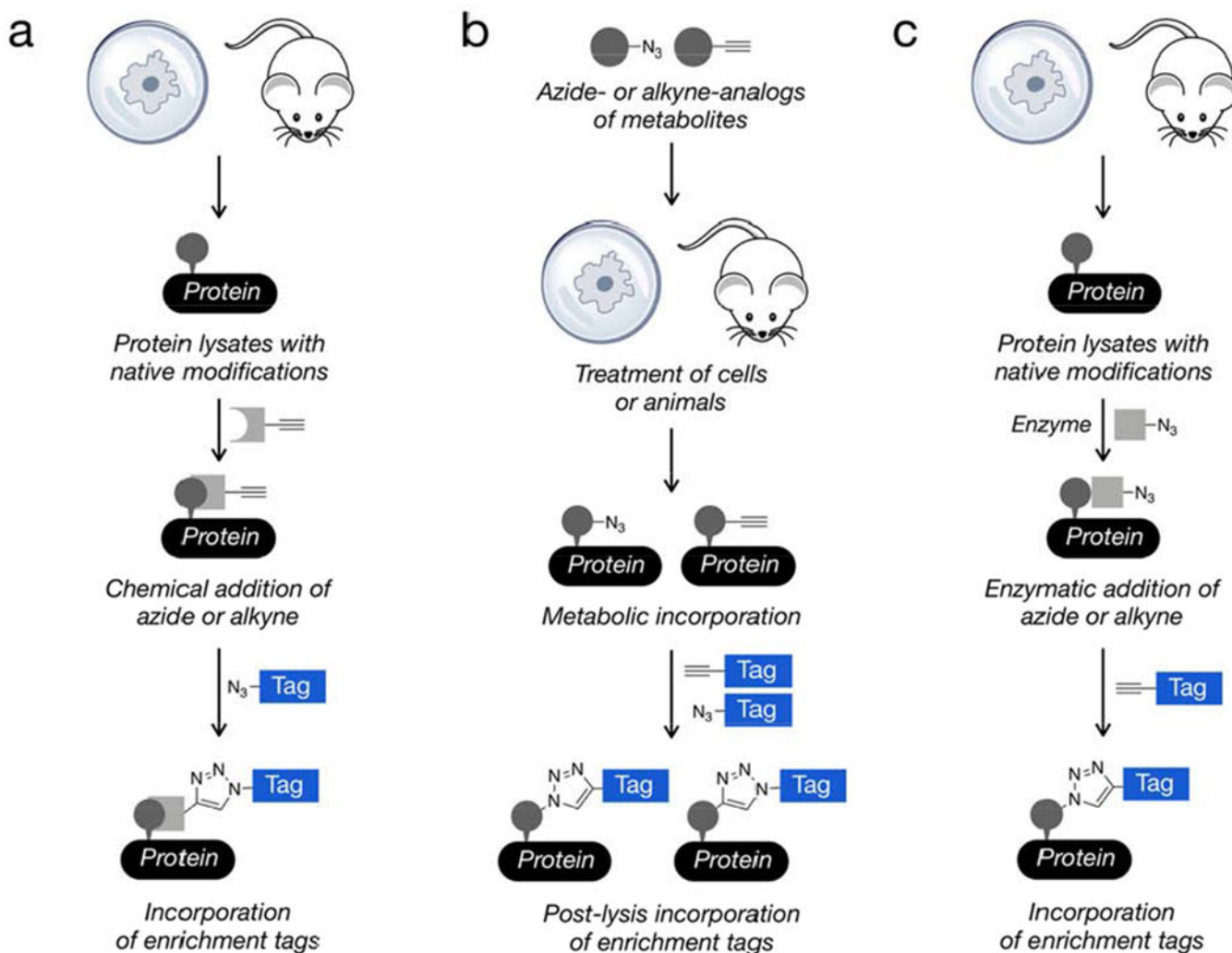


**Figure 5.** Chemoproteomic methods to map features of proteins or to characterize molecular interactions of bioactive compounds using chemical probes. (A) General design of click probes used in protein-centric or chemical-centric proteomic investigations. (B) Schematic of proteomic workflows. (C) Various readouts of chemoproteomic investigations.

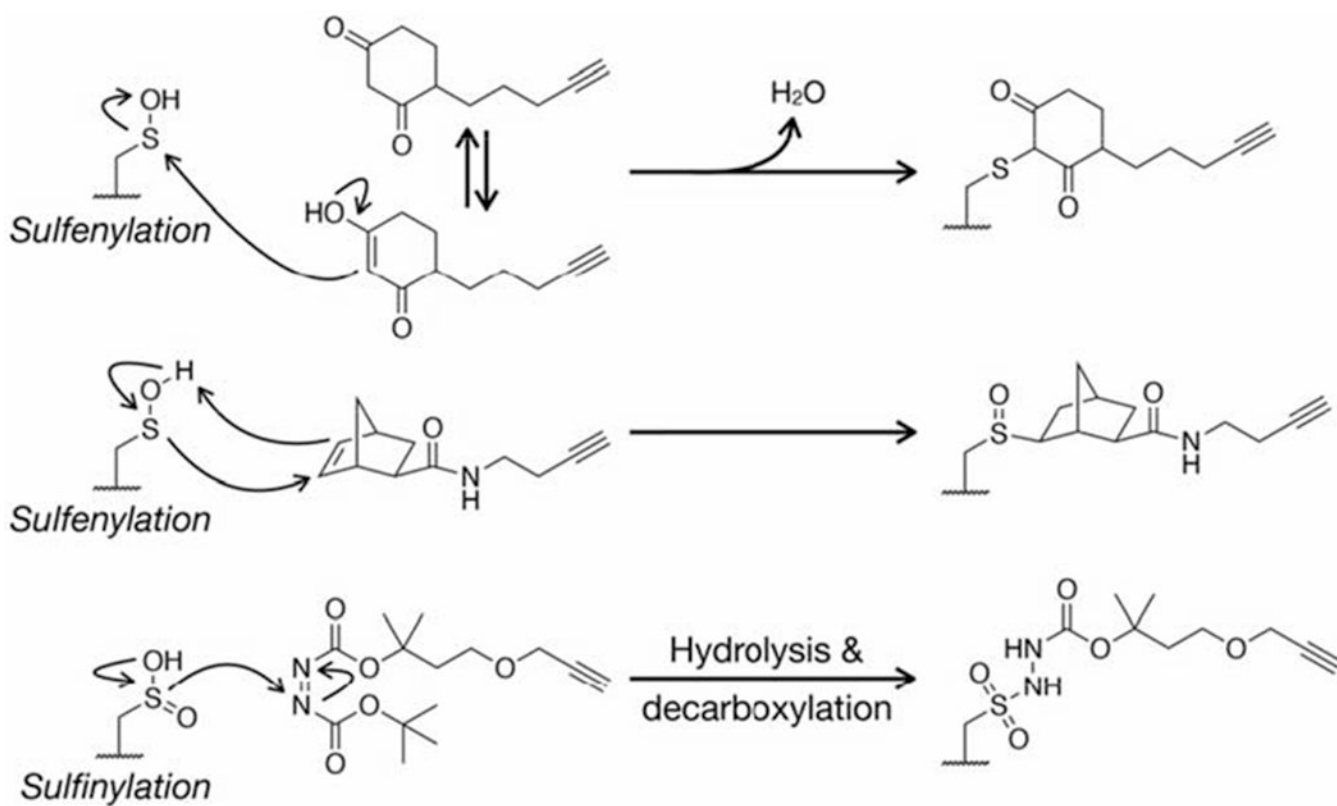




**Figure 6.**  
Examples of click probes chemical proteomic investigations



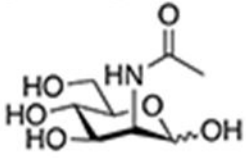
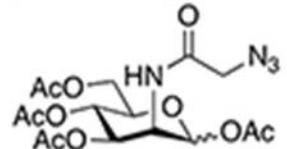
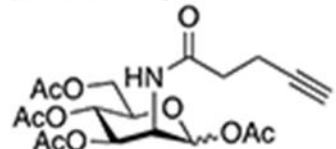
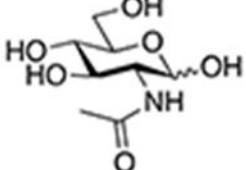
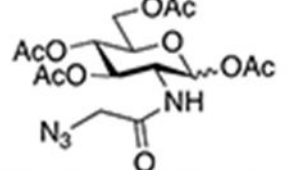
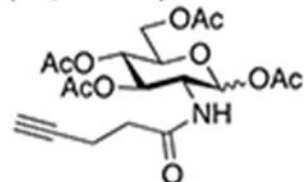
**Figure 7.** General classes of click-chemistry methods for the identification of post-translational modifications. a) The chemical reactivity of certain PTMs allows them to be reacted directly and selectively with bioorthogonal probes. b) The metabolism of living cells or animals can be exploited for the introduction of chemical probes with click-chemistry handles. c) Other PTMs can be modified enzymatically to install click-chemistry handles.

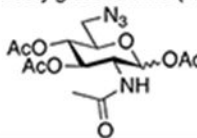
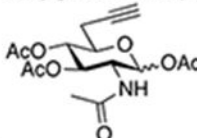
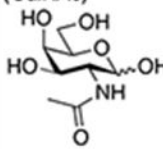
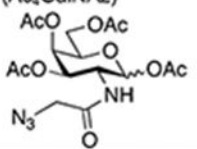
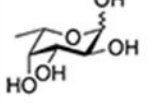
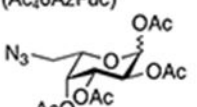
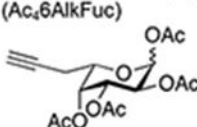
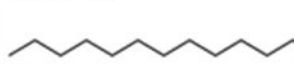
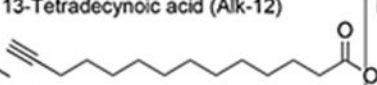
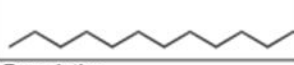
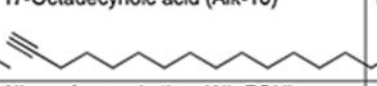
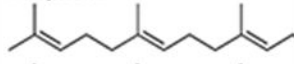
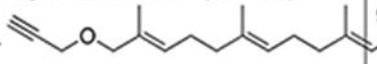
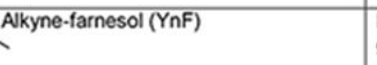


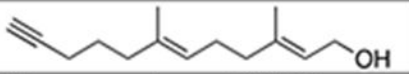
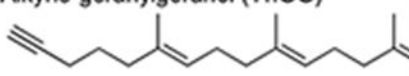

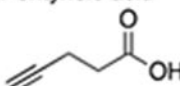
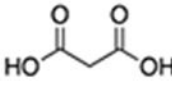
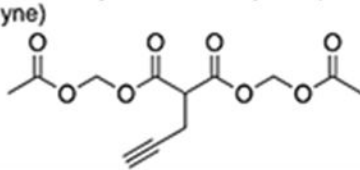
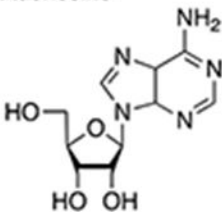
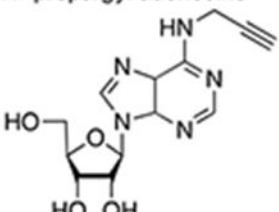
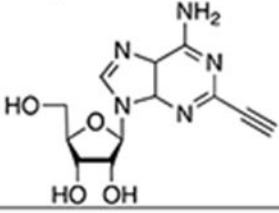
**Figure 8.** Chemical mechanisms responsible for the selective reaction of sulfenylated and sulfinylated cysteine residues with their respective probes.

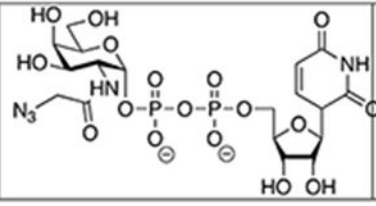
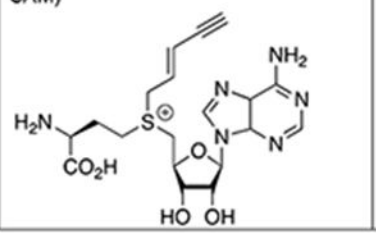
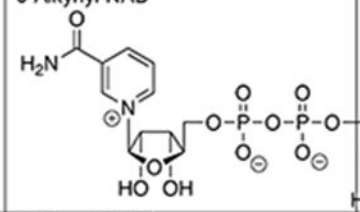
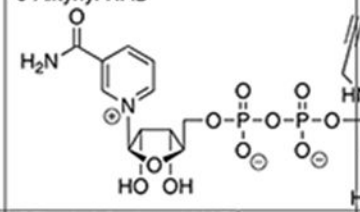
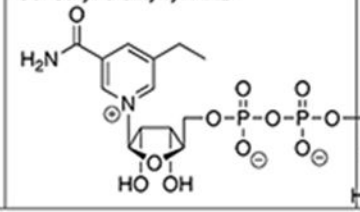
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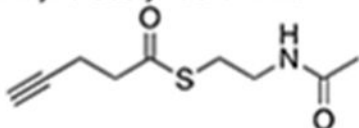
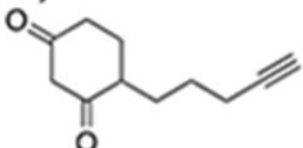
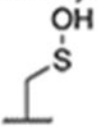
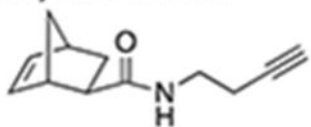
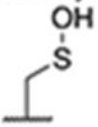
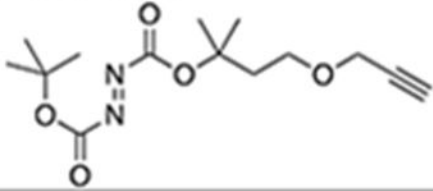
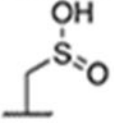
Chemical Reporters of PTMs

Natural Metabolite	Reporter	PTM	Reference
<i>Glycosylation</i>			
N-Acetylmannosamine (ManNAc) 	Per-O-acetyl-N-azidoacetylmannosamine (Ac <sub>4</sub> ManNAz) 	Sialylated cell surface glycoproteins	(Saxon and Bertozzi, 2000)
	Per-O-acetyl-N-pentynylmannosamine (Ac <sub>4</sub> ManNAz) 	Sialylated cell surface glycoproteins	Hsu, 2007 193
N-Acetylglucosamine (GlcNAc) 	Per-O-acetyl-N-azidoacetylglucosamine (Ac <sub>4</sub> GlcNAz) 	O-GlcNAc and cell surface glycoproteins	(Vocadlo et al., 2003)
	Per-O-acetyl-N-pentynylglucosamine (Ac <sub>4</sub> GlcNAk) 	O-GlcNAc and cell surface glycoproteins	(Zaro et al., 2011)

	Per-O-acetyl-6-azido-6-deoxy-N-acetylglucosamine (Ac <sub>3</sub> 6AzGlcNAc)	O-GlcNAc	(Chuh et al., 2014)
			
	Per-O-acetyl-6-alkynyl-6-deoxy-N-acetylglucosamine (Ac <sub>3</sub> 6AlkGlcNAc)	O-GlcNAc	(Chuh et al., 2017)
			
N-Acetylgalactosamine (GalNAc)	Per-O-acetyl-N-azidoacetylgalactosamine (Ac <sub>4</sub> GalNAz)	O-GlcNAc and cell surface glycoproteins	(Hang et al., 2003)
			
Fucose (Fuc)	Per-O-acetyl-6-azido-fucose (Ac <sub>4</sub> 6AzFuc)	Fucosylated cell surface glycoproteins	(Rabuka et al., 2006)
			
	Per-O-acetyl-6-alkynyl-fucose (Ac <sub>4</sub> 6AlkFuc)	Fucosylated cell surface glycoproteins	(Kizuka et al., 2016)
			
<b>Lipidation</b>			
Myristic acid	13-Tetradecynoic acid (Alk-12)	Myristoylation	(Charron et al., 2009)
			
Palmitic acid	17-Octadecynoic acid (Alk-16)	Palmitoylation	(Charron et al., 2009)
			
Prenylation	Alkyne-farnesol ether (Alk-FOH)	Farnesylation/geranylgeranylation	(Charron et al., 2013)
			
	Alkyne-farnesol (YnF)	Farnesylation/geranylgeranylation	(Storck et al., 2013)
			

			2019)
	Alkyne-geranylgeranol (YnGG)	Farnesylation/geranylgeranylation	(Storck et al., 2019)
			
<b>Acetylation</b>			
Acetate	Pentynoic acid	Acetylation	(Yang et al., 2010)
			
Malonic acid	O-Acetoxy-malonate-alkyne (MalAM-yne)	Malonylation	(Bao et al., 2013)
			
<b>ADP-ribosylation</b>			
Adenosine	N <sup>6</sup> -propargyl adenosine	ADP-ribosylation	(Westcott et al., 2017a)
			
	2-alkyne adenosine	ADP-ribosylation	<u>Kalesh et al., 2019</u>
			
<b>Enzymatic Modification</b>			
Enzyme	Substrate	PTM	Reference
<b>Glycosylation</b>			
Engineered galactosyltransferase	Uridine-diphosphate azidoacteylgalactosamine (GalNAz)	N-(UDP-	O-GlcNAc (Clark et al., 2008)

			
<b>Methylation</b>			
Engineered methyltransferase	Alkyne-S-adenosylmethionine (Alk-SAM)	Methylation	(Islam et al., 2013)
			
<b>ADP-Ribosylation</b>			
PARP1	6-Alkynyl-NAD <sup>+</sup>	ADP-ribosylation	(Du et al., 2009b)
			
PARP1	8-Alkynyl-NAD <sup>+</sup>	ADP-ribosylation	(Du et al., 2009b)
			
Engineered PARP	C5'-ethyl-6-alkynyl-NAD <sup>+</sup>	ADP-ribosylation	(Carte r-O'Connell et al., 2014)
			
<b>Direct Chemical Modification</b>			
Chemical probe		PTM	Refer

		ence
<b>Acetylation</b>		
Alkyne-acetyl-CoA mimic 	Chemical Acetylation	(Kulkarni et al., 2017)
<b>Cysteine Oxidation</b>		
Alkyne-dimedone 	Sulfenylation 	(Leonard et al., 2009)
Alkyne-noroborene 	Sulfenylation 	(Alcock et al., 2019)
Alkyne-diazine 	Sulfinylation 	(Akter et al., 2018)