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Exploring Metabolic Pathways and Regulation through Functional Chemoproteomic and Metabolomic Platforms

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Genome sequencing efforts have revealed a strikingly large number of uncharacterized genes, including poorly or uncharacterized metabolic enzymes, metabolites, and metabolic networks that operate in normal physiology, and those enzymes and pathways that may be rewired under pathological conditions. Although deciphering the functions of the uncharacterized metabolic genome is a challenging prospect, it also presents an opportunity for identifying novel metabolic nodes that may be important in disease therapy. In this review, we will discuss the chemoproteomic and metabolomic platforms used in identifying, characterizing, and targeting nodal metabolic pathways important in physiology and disease, describing an integrated workflow for functional mapping of metabolic enzymes.

One of the most provocative findings to come out of the Human Genome Project was the discovery of a large number of genes encoding proteins with unknown function, including many uncharacterized enzymes that participate in the metabolism of small-molecule metabolites (Venter et al., 2001). These data revealed that our knowledge of cellular metabolism was far less complete than we thought, and opened up the possibility for yet undiscovered landscapes of metabolites and metabolic pathways. Indeed, even our understanding of well-characterized enzymes and their metabolic functions in normal physiology remains largely incomplete, especially in pathological states where these pathways may be rewired or possess unique or novel functions. We are now faced with the grand challenge of deciphering these uncharacterized metabolic networks and disentangling the normal and disease roles of previously described metabolic pathways. This undiscovered metabolic space presents an exciting opportunity for discoveries in basic biology and opens up the potential for targeting unique or novel metabolic drivers of diseases related to dysregulated metabolism, such as obesity, diabetes, atherosclerosis, cancer, infection, and inflammatory diseases. Recent work has also demonstrated the regulatory importance of metabolite flux through a given pathway and the diverse roles of small biomolecules beyond classical metabolism, including signaling and epigenetic, transcriptional, and posttranslational regulation of critical cell functions.

In this review, we will describe how innovative metabolic mapping techniques have been used to successfully identify, characterize, and pharmacologically target nodal metabolic pathways important in mammalian physiology and disease. Specifically, we will discuss using chemoproteomic and metabolomic approaches to globally assess enzyme activities, developing chemical tools to interrogate enzyme function, and mapping the metabolic pathways and metabolite-driven regulation controlled by these enzymes.

Chemoproteomic Approaches to Assess the Functional State of Enzymes in Complex Biological Systems

One of the key challenges of studying enzyme function has been the ability to assay for explicit metabolic enzyme activities of specific proteins in complex biological systems, especially for enzymes with no known substrate or function. Developing a method for global assessment of enzyme functionality remains difficult because (1) enzymes can be regulated by posttranslational events in vivo, which are poorly detected with standard gene and protein expression profiling; (2) a substantial proportion of the proteome remains functionally uncharacterized, preventing the use of substrate-activity assays; and (3) the physicochemical properties of many enzymes complicate their analysis in biological samples (e.g., low abundance, difficulty in enrichment).

One powerful method developed to address these challenges is activity-based protein profiling (ABPP), a chemoproteomic platform that uses activity-based probes (ABPs) that measure the functional state of enzymes en masse in complex biological samples (Evans and Cravatt, 2006; Moellering and Cravatt, 2012; Nomura et al., 2010a). An ABP consists of a chemical group that covalently reacts with the active sites of enzymes across a particular enzyme class based on chemical reactivity within a conserved catalytic architecture, and an analytical handle that facilitates a simultaneous read out enzyme activities (Figure 1A). This analytical handle can be a fluorophore for depicting enzyme activities, or a biotin handle for enrichment, identification, and quantification of activities with mass spectrometry-based proteomics (Figure 1B). To date, there are ABPs for more than a dozen enzyme classes, including hydrolases, proteases, kinases, phosphatases, glycosidases, caspases, oxygenases, oxidoreductases, and nitrilases (Adam et al., 2001; Barglow and Cravatt, 2006; Kato et al., 2005; Kidd et al., 2001; Liu et al., 1999; Patricelli et al., 2007; Saghatelian et al., 2004a; Walls et al., 2009; Weerapana et al., 2008; Williams et al., 2006; Xiao et al., 2013).
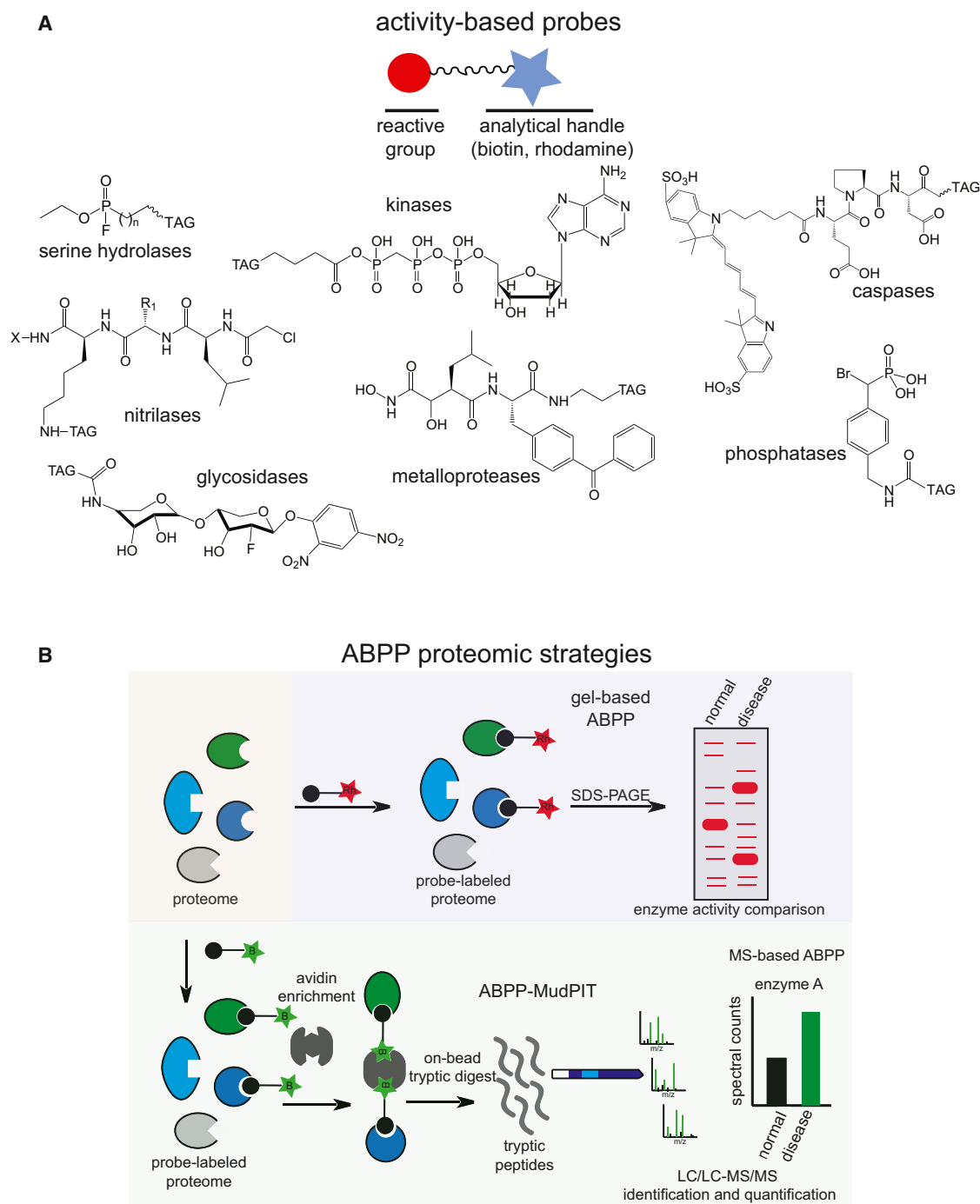
Figure 1. Activity-Based Protein Profiling
(A) Examples of activity-based probes.
(B) Gel-based ABPP and ABPP-MudPIT platforms for fluorescent and mass-spectrometry-based analysis of enzyme activities. Rh, rhodamine; B, biotin.

ABPP overcomes many of the traditional challenges facing enzyme activity assessment in complex samples. First, these probes selectively and simultaneously label only active, but not inactive, enzymes in a class, revealing changes in enzyme activity distinct from alterations in protein or transcript expression level (Jessani et al., 2005; Kidd et al., 2001). Second, ABPs

enable enzyme activity assessment of uncharacterized enzymes because these probes react with active sites based on class-wide conserved chemical reactivity (Bachovchin et al., 2010; Chiang et al., 2006; Weerapana et al., 2008). Third, ABPs allow enrichment of specific classes of enzymes based on shared functional properties, facilitating characterization of enzymes
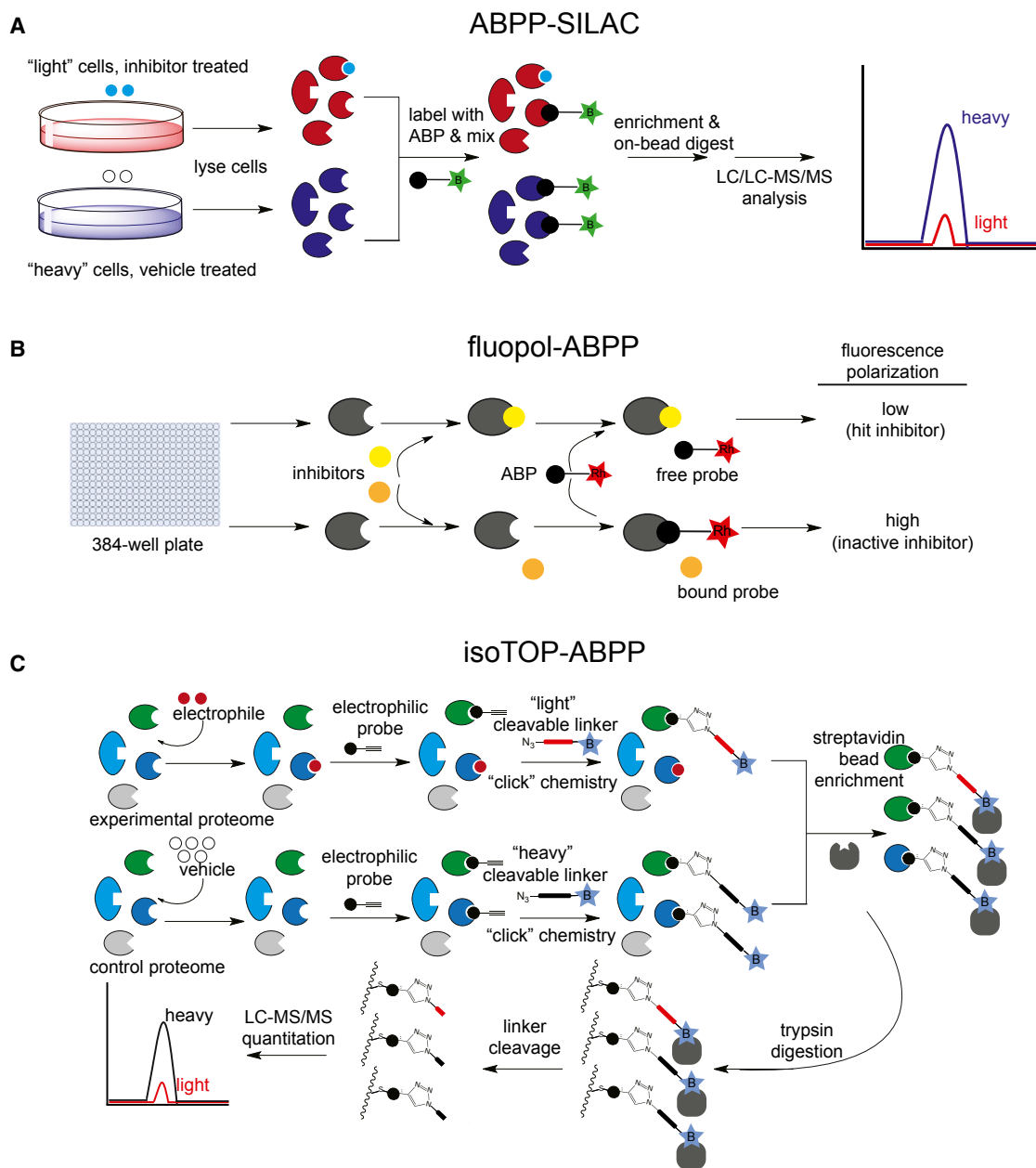
Figure 2. Competitive ABPP Platforms
(A) ABPP-SILAC.
(B) Fluopol-ABPP.
(C) Isotope-ABPP.

that may be in low abundance or are embedded in a membrane (Bachovchin et al., 2010; Weerapana et al., 2010).

Since the initial development by Cravatt and Bogyo, ABPP platforms have incorporated chemical and analytical methods that enable detecting enzyme activities in cells or in vivo, mapping sites of probe labeling in the proteome, and quantitative assessment of enzyme activities. One of the most significant advances in ABPP platforms has been the complementation with bioorthogonal "click chemistry" methods (Speers et al., 2003). Bioorthogonal ABPs bearing an alkyne handle (instead of rhoda-

mine or biotin) can be treated in vitro, in situ, or even in vivo to label active enzymes, facilitating target identification by subsequently appending an analytical handle (e.g., rhodamine-azide or biotin-azide) in vitro through copper-catalyzed "click chemistry" (Speers et al., 2003). Another advancement of the method involves coupling ABPP with stable isotopic labeling of cells (ABPP-SILAC; Figure 2A) for quantitative proteomic analysis of enzyme activities (Adibekian et al., 2011). To improve the throughput of gel-based or mass spectrometry-based ABPP, Bachovchin and colleagues adapted a method

for high-throughput screening using fluorescence polarization (fluopol-ABPP; Figure 2B), primarily used for inhibitor screening (Bachovchin et al., 2009). Recently, Weerapana and colleagues developed an ABPP platform called tandem orthogonal proteolysis ABPP (TOP-ABPP; Weerapana et al., 2008) to identify hyperreactivity and functionality of specific amino acid hotspots within the proteome. In TOP-ABPP, alkyne-bearing ABPs or reactivity-based probes (RBPs) are "clicked" to azide-bearing tags that contain chemically or TEV protease cleavable linkers and a biotin handle, allowing enrichment of probe-bound proteins and subsequent release of probe-bound peptides upon tandem digestions with trypsin and TEV protease. This TOP-ABPP can be adapted for quantitative proteomics by incorporating an isotopically "heavy" labeled valine into the linker and measuring the ratio of heavy to light labeling on a protein or at a particular residue, a platform called isotopic TOP-ABPP (iso-TOP-ABPP; Figure 2C; Weerapana et al., 2010). These ABPP platforms have been successfully used to identify and characterize enzyme activities in various human diseases, including cancer, obesity, neurodegenerative diseases, and microbial infection (Blais et al., 2010; Dominguez et al., 2014; Nomura et al., 2010b, 2011a; Sadler et al., 2012; Singaravelu et al., 2010).

We review several representative examples of how ABPP has been used to discover metabolic drivers of disease. In particular, ABPP has been widely used to study the serine hydrolase superfamily of enzymes using the serine hydrolase-directed ABPs fluorophosphonate (FP)-rhodamine and FP-biotin that covalently phosphorylate the active site serine of nearly all of the >200 serine hydrolase enzymes (Bachovchin et al., 2010; Kidd et al., 2001; Liu et al., 1999). The serine hydrolase family is one of the largest metabolic enzyme classes in the mammalian genome, though many of the family members are poorly or incompletely characterized (Long and Cravatt, 2011). This class encompasses many types of enzymes, including hydrolases, esterases, lipases, proteases, thioesterases, and peptidases (Long and Cravatt, 2011). Through mining serine hydrolase activities with ABPP, several key metabolic or proteolytic drivers and biomarkers of cancer have been identified, including KIAA1363, monoacylglycerol lipase (MAGL), and retinoblastoma-binding protein 9 (RBBP9; Chiang et al., 2006; Jessani et al., 2002; Nomura et al., 2010b; Shields et al., 2010). Serine hydrolase profiling was also used to discover that mutations in the poorly characterized alpha/beta-hydrolase domain-containing 12 (ABHD12) in patients with a neurodegenerative disease known as PHARC (polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataracts) encoded a functionally inactive ABHD12. Despite the lack of functional information regarding ABHD12, Blankman and colleagues used ABPP to reveal inactive ABHD12 in PHARC tissues and followed with metabolomic techniques (discussed later in this review) to ascertain the function of ABHD12 and its role in PHARC pathogenesis (Blankman et al., 2013). Serine hydrolase ABPs have also been used to identify important enzyme activities in bacterial and viral infections such as carboxylesterase 1 (CES1) as an upregulated enzyme activity in hepatitis C virus-infected hepatoma cells critical in maintaining viral replication (Blais et al., 2010).

Many other ABPs have been generated and validated in complex proteomes, including but not limited to (1) 2-oxoglutarate-dependent oxygenase probes that employ a hydroxyquinoline template coupled to a photoactivatable crosslinking group and biotin handle (Rotili et al., 2011), (2) S-adenosylmethionine (SAM)-dependent methyltransferase probes that consist of S-adenosylhomocysteine analogs with amino linkers attached to scaffolds containing photo-crosslinkers and a biotin handle (Dalhoff et al., 2010), (3) a suite of bioorthogonal cytochrome P450 ABPs against a wide cross-section of human P450s (Wright and Cravatt, 2007; Wright et al., 2009), and (4) pargyline and deprenyl-based bioorthogonal ABPs for monoamine oxidase (Krysiak et al., 2012).

Bogyo and colleagues have been on the forefront of using ABPP in depicting enzyme activities in cancer, generating ABPs to track cysteine protease activity in cancer cell progression and proteosomal substrate specificity (Greenbaum et al., 2000; Nazif and Bogyo, 2001). They have used quenched near-infrared fluorescent ABP (qNIRF-ABP) to image cysteine protease activities in tumor xenografts in vivo in mice (Blum et al., 2007) and have also developed a highly selective aza-peptidyl asparadinyl epoxide qNIRF-ABP probe for legumain, a lysosomal cysteine protease upregulated in multiple human cancers, to depict tumors. Recently, they generated a caspase-directed ABP to depict and quantify dexamethasone-induced apoptosis in the thymus and Apomab-induced apoptosis in tumor xenografts in vivo in mice (Edgington et al., 2009).

ABPP platforms have also been extended to map the endogenous reactivity of the proteome with reactive electrophile-based RBPs. Carroll and colleagues developed bioorthogonal dimedone and sulfenome RBP probes that selectively react with sulfenic acid cysteine modifications in the proteome and used these probes to identify redox regulated pathways such as the cysteine sulfenic acid-modified Gpx3 regulation of Yap1 in yeast, involved in regulating epidermal growth factor receptor tyrosine kinase activity (Leonard et al., 2009; Paulsen and Carroll, 2009; Paulsen et al., 2012; Reddie et al., 2008; Seo and Carroll, 2011). Weerapana and colleagues used isoTOP-ABPP to perform a massive quantitative proteomic profiling effort to comprehensively profile hyperreactive cysteines in complex proteomes, in which cells were first labeled with the cysteine-reactive iodoacetamide-alkyne bioorthogonal probe. Proteomes were then subjected to click chemistry with an isotopically-labeled azide-linked cleavable biotin linker for enrichment and release of cysteine-labeled peptides for subsequent analysis by quantitative proteomics (Weerapana et al., 2010). The authors uncovered a wide range of hyperreactive cysteines in the proteome that were enriched in functional cysteines involved in a wide range of activities, including nucleophilic and reductive catalysis and sites of oxidative modification for both characterized and uncharacterized proteins across many different protein classes, including some metabolic enzymes (Weerapana et al., 2010). As demonstrated, isoTOP-ABPP is a broad and quantitative approach capable of assessing the specific sites involved in catalytic and regulatory function of large numbers of proteins and metabolic enzymes.

As described above, ABPP has proven to be a powerful technology in probing enzyme activities across a wide range of enzyme classes and across many physiological and cellular contexts, although like any approach, this technology is not without its limitations. While many ABPs and RBPs have been developed by the chemical biology community, there are still many

metabolic enzyme classes that cannot be assayed with ABPP-based methods. Furthermore, whereas higher throughput ABPP methods have been developed to screen large numbers of compounds against one enzyme, the broad profiling of large numbers of enzyme activities is still medium to low throughput using gel-based and proteomic-based methods. These limitations notwithstanding, as described here, ABPP is a versatile platform can be used not only for target identification of enzymes important in various diseases, but also for characterization of unknown enzymes, for depicting enzyme activities, and even uncovering hyperreactivity and functionality across the proteome.

Chemoprotomics for Developing Selective Small-Molecule Inhibitors for Metabolic Enzymes

In addition to target discovery and imaging applications, ABPP can also be used in a competitive mode to screen for enzyme inhibitors (Moellering and Cravatt, 2012). Inhibitor screening by competitive ABPP exhibits several advantages over conventional substrate assays. First, enzymes can be tested in native proteomes without the need for recombinant expression or purification (Adibekian et al., 2011; Chang et al., 2011; Chiang et al., 2006; Long et al., 2009), and second, inhibitors can be developed for uncharacterized enzymes without prior knowledge of endogenous substrates (Adibekian et al., 2011; Bachovchin et al., 2010; Chang et al., 2011; Chiang et al., 2006; Li et al., 2007). Because inhibitors are tested against many enzymes in parallel, inhibitor potency and selectivity can be simultaneously assessed, enabling subsequent medicinal chemistry efforts to develop highly specific and effective enzyme inhibitors (Chang et al., 2011; Long et al., 2009; Bachovchin et al., 2009, 2011; Adibekian et al., 2011). Thus, competitive ABPP provides a universal assay for inhibitor discovery applicable to any enzyme regardless of existing knowledge of its function as long as there is a cognate ABP or RBP for the enzyme. Competitive ABPP has emerged as a powerful approach for developing potent and selective small-molecule inhibitors for both characterized and uncharacterized enzymes, which have then been used to inform the functions of metabolic enzymes in complex systems. As described previously with standalone ABPP approaches, competitive ABPP can be employed in a low-throughput/high resolution mass spectrometry-based proteomics format with biotin-tagged activity-based probes using a multidimensional protein identification technology (ABPP-MudPIT), a medium-throughput gel-based format with fluorescent activity-based probes (gel-based ABPP), or a high-throughput screening format using fluorescence polarization with ABPs against large compound libraries (fluopol-ABPP; Adibekian et al., 2011; Bachovchin et al., 2009, 2010; Chang et al., 2011).

Competitive ABPP platforms have been remarkably successful in generating small-molecule inhibitors of serine hydrolases using the fluorophosphonate ABP. Chang and colleagues developed the compound JW480, a highly selective, irreversible, in vivo efficacious, and orally bioavailable inhibitor of the previously uncharacterized enzyme KIAA1363 that selectively inhibited KIAA1363 activity in various tissues and in tumor xenografts, and impaired cancer cell migration and in vivo tumor growth (Chang et al., 2011). Bachovchin and colleagues generated a library of >140 serine hydrolase inhibitors based on the carbamate scaffold and tested all of these inhibitors against a

library of >70 recombinantly expressed serine hydrolases in a "library versus library" screening effort, and successfully identified lead inhibitors for >40% of enzymes tested (Bachovchin et al., 2010). Competitive ABPP platforms have also been used to generate selective inhibitors for the serine hydrolases MAGL and fatty acid amide hydrolase (FAAH), which degrade the endocannabinoid signaling lipids 2-arachidonoylglycerol (2-AG) and anandamide, respectively. MAGL inhibitors found through a competitive ABPP screen of a structurally diverse carbamate library and subsequent optimization led to the development of the carbamate JZL184 as the first potent, selective, and in vivo active MAGL inhibitor (Long et al., 2009). As described later in this review, JZL184 has been used extensively to characterize the biochemical functions of MAGL using metabolomics approaches, in the process revealing this enzyme as a therapeutic target for cancer, inflammation and inflammatory diseases, neurodegenerative diseases, anxiety, and pain. Ahn and colleagues have also used ABPP to generate the highly selective and in vivo efficacious biaryl ether piperidine urea FAAH inhibitor PF-3845. The authors also generated a bioorthogonal analog of PF-3845 bearing an alkyne handle to show that PF3845-yne only inhibited FAAH in vivo (Ahn et al., 2009).

Adibekian and colleagues used competitive ABPP-SILAC platforms to show that the 1,2,3-triazole urea scaffold is ideal for generating irreversible serine hydrolase inhibitors. The authors generated a library of triazole urea inhibitors and optimized the highly selective inhibitors AA74-1, AA39-2, and AA44-2 for acyl peptide hydrolase, platelet activating factor acetylhydrolase 2, and uncharacterized hydrolase ABHD11, respectively (Adibekian et al., 2011). Using this scaffold, Hsu and colleagues generated the triazole urea inhibitors KT109 and KT172 for the 2-AG-synthesizing enzyme diacylglycerol lipase and confirmed selectivity of these inhibitors in situ with ABPP-SILAC and in vivo with ABPP-MudPIT (Hsu et al., 2012).

Screening large inhibitor libraries via high-throughput fluopol-ABPP has led to identification of several small-molecule inhibitors of metabolic enzymes. These include selective inhibitors of anticancer targets protein methyl esterase 1, glutathione transferase omega, and RBBP9, as well as an inhibitor for the anti-inflammatory target protein arginine deaminase 4 (Bachovchin et al., 2009, 2011; Knuckley et al., 2010; Tsuboi et al., 2011).

As demonstrated above, competitive ABPP platforms are very useful in developing inhibitors for metabolic enzymes. The ability to assess inhibitor selectivity and target occupancy of inhibitors in cells or even in vivo has been a particularly useful feature of this approach toward providing highly specific chemical tools for further biological discovery, leads for clinical development, and biomarkers for inhibitor efficacy (Moellering and Cravatt, 2012).

Metabolomics to Annotate the Functions of Uncharacterized Metabolic Enzymes

Chemoproteomic strategies such as ABPP have greatly facilitated efforts to assess metabolic enzyme activities and developing chemical tools to disrupt these activities in complex biological systems. However, these strategies still need to be integrated with functional metabolomic approaches to decipher the metabolites that are regulated by these enzymes and how these metabolites and their associated metabolic pathways are involved in (patho)physiology. The metabolome is generally
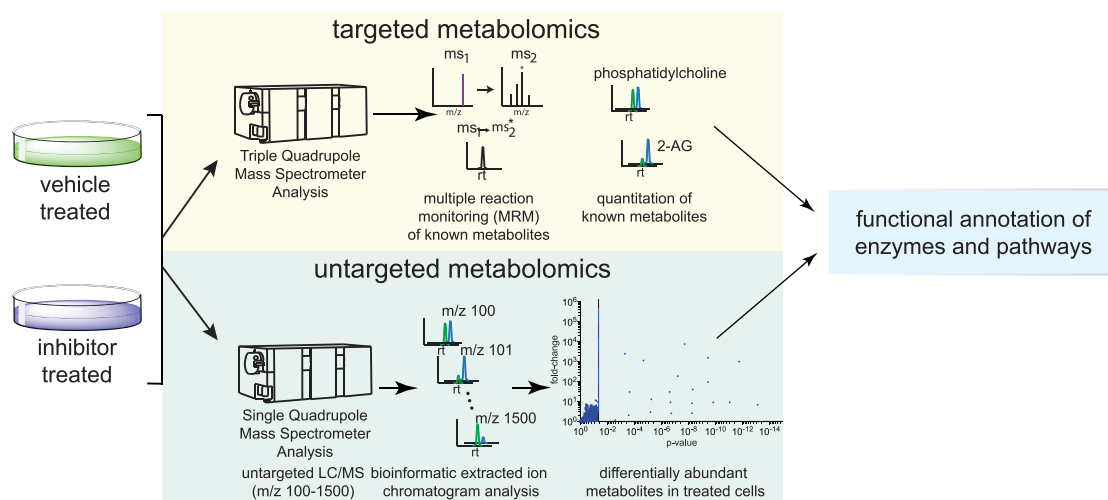
Figure 3. Targeted and Untargeted Metabolomic Profiling Platforms
Targeted metabolomic approaches oftentimes are performed by multiple reaction monitoring-based liquid chromatography-tandem mass spectrometry methods. Untargeted metabolomic approaches are performed through collecting all mass spectra and using bioinformatics tools (e.g., XCMS) to identify, integrate, and compare ions among comparison groups.

considered a collection of small-molecule metabolites that include nutrients and their biosynthetic intermediates to provide biomass (nucleic acids and DNA/RNA, amino acids and proteins, fatty acids, and membrane lipids) and energy for cell growth and function. However, this view is rapidly expanding to encompass diverse metabolite constituents that serve as intracellular and extracellular signaling molecules influencing physiological processes such as neurotransmission (e.g., acetylcholine, glutamate, 2-AG, anandamide; Fisher and Wonnacott, 2012; Hassel and Dingledine, 2012; Kohnz and Nomura, 2014), inflammation (e.g., sphingosine-1-phosphate, prostaglandins; Wymann and Schneiter, 2008), and cancer (e.g., eicosanoids and lysophosphatidic acid; Mills and Moolenaar, 2003; Wang and Dubois, 2010), as well as endogenous nuclear hormone receptor ligands that influence transcriptional regulation (Evans and Mangelsdorf, 2014), and metabolites that confer posttranslational and epigenetic regulation onto the proteome and genome (e.g., UDP-GlcNAc and glycosylation, acetyl-coA and acetylation; Wellen and Thompson, 2012). The metabolome is the functional output of enzymes that generate, degrade, or convert biomolecules. Thus functional metabolomic strategies, which uncover metabolite changes that occur upon disruption of a specific metabolic enzyme, are essential in deciphering biochemical functions and (patho)physiological roles of enzymes in complex living systems.

Compared to the genome and the proteome, the metabolome presents unique challenges for global analysis due to the significant physicochemical diversity in metabolite size, molecular weight, hydrophobicity, chemical stability, charge, volatility, abundance, and ionization inherent in biological samples. Several types of technologies and methodologies have been used in metabolomic profiling to attempt near global detection and analysis. Nuclear magnetic resonance, gas chromatography-mass spectrometry, and liquid chromatography-mass spectrometry are the most common techniques used for metab-

olomic profiling (Patti et al., 2012), and metabolomics analysis is often performed using "targeted" or "untargeted" methods that capture complementary information (Figure 3). Using targeted metabolomic methods, a series of known metabolites are quantitatively measured, in which a mass spectrometer targets a list of known metabolites by their mass-to-charge ratio (m/z) and/or the transition of the "parent" m/z of metabolites to their respective ms2 fragment "daughter" ions (known as multiple reaction monitoring). Targeted metabolomics enables sensitive, simultaneous quantification of hundreds of known metabolites based on availability of standards, and is very useful in studying defined metabolic pathways and quantifying specific, very low abundance metabolites (Patti et al., 2012). However, targeted metabolomic detection is restricted to quantifying known metabolites for which there are existing standards. Untargeted metabolomics can prove especially useful for uncovering the function(s) of uncharacterized enzymes, when deciphering unique and novel roles of previously characterized enzymes, or identifying new metabolites. Untargeted metabolomic analyses in which the mass spectrometer is set to scan a wide m/z range and collect all mass spectra are used as a complementary approach to targeted analyses to improve metabolome coverage (Patti et al., 2012; Saghatelian and Cravatt, 2005; Saghatelian et al., 2004b; Vinayavekhin et al., 2010). This large amount of collected mass spectral data can then be analyzed with bioinformatic platforms such as XCMS or MAVEN to identify, integrate, and compare all detectable ions to identify those ions that are changed between comparison groups (Clasquin et al., 2012; Patti et al., 2012; Smith et al., 2006; Tautenhahn et al., 2012). This data can then be used to identify potentially novel metabolites altered in abundance between groups using metabolomic databases like METLIN, HMDB, and Lipid Maps and traditional analytical chemistry methods (Fahy et al., 2007; Nikolskiy et al., 2013; Smith et al., 2005; Wishart et al., 2013).

Both targeted and untargeted functional metabolomic approaches have recently revealed the functions of previously uncharacterized enzymes. Blankman and colleagues used untargeted metabolomics to uncover the function of the previously uncharacterized enzyme α/β-hydrolase domain-containing 12 (ABHD12), a serine hydrolase mutationally inactivated in patients with PHARC, as a lysophosphatidylserine (LPS) hydrolase (Figure 4A; Blankman et al., 2013). Blankman and colleagues showed that ABHD12-deficient mice show elevated levels of the toll-like receptor agonist LPS, leading to neuroinflammation and motor and auditory defects reminiscent of PHARC (Blankman et al., 2013).

Inhibitors developed through competitive ABPP platforms have been used to interrogate the functions of uncharacterized enzymes, which then led to insights into their pathophysiological roles. Using untargeted metabolomics and the selective KIAA1363 inhibitor AS115 developed by competitive ABPP efforts, Chiang and colleagues discovered that KIAA1363 is a 2-acetyl-monoalkylglycerol (2-acetyl MAGE) hydrolase that generates monoalkylglycerol ether (MAGE) leading to the generation of the oncogenic signaling lipid alkyl-lysophosphatidic acid, which in turn fueled cancer cell pathogenicity and tumor growth (Chiang et al., 2006).

Both targeted and untargeted metabolomics have been used in conjunction with genetic manipulation to reveal the functions of bacterial metabolic enzymes. Baran and colleagues used the combined metabolomic platforms to characterize and validate genes related to specific metabolite utilization in bacteria by profiling libraries of mutant strains in *Escherichia coli* and *Shewanella oneidensis* MR-1. Through this approach, the authors identified genes with known functions as well as novel transport proteins and enzymes required for utilization of tested metabolites. Specifically, they uncovered a predicted ABC transporter encoded by genes *SO1043* and *SO1044* required for citrulline utilization and a predicted histidase encoded by the gene *SO3057* required for ergothioneine use by *S. oneidensis* (Baran et al., 2013).

Metabolomics to Reveal Unique and Novel Roles for Previously Characterized Enzymes

Whereas many "characterized" metabolic enzymes have putative biochemical functions, often these enzyme functions have only been determined in vitro or may be described solely based on sequence homology or by association with an enzyme family, and may not have been validated in in vivo systems. Furthermore, enzymes may play alternate or additional roles or are linked to different metabolic pathways depending on the tissue or cell type, or in dysregulated and rewired disease states. Functional metabolomics has proven critical in mapping rewired, re-tasked, or novel functions of enzymes in tissue- or cell-specific or disease-specific contexts.

De Carvalho and colleagues used an untargeted metabolomic approach to describe the *Mycobacterium tuberculosis* enzyme Rv1248c, which was characterized at the time as a thiamine diphosphate-dependent α-ketoglutarate decarboxylase. Using metabolomic approaches, the authors found that Rv1248c was misannotated and that its actual function was to catalyze the conjugation of α-ketoglutarate and glyoxylate to yield 2-hydroxy-3-oxoadipate, which decomposes to 5-hydroxylevulinate,

possibly involved in glyoxylate detoxification, glutamine metabolism, or heme biosynthesis (de Carvalho et al., 2010).

Targeted and untargeted metabolomics were also used to show the tissue-specific and disease-specific roles of MAGL in coordinating multiple lipid signaling pathways that underlie inflammation, pain, mood, and cancer (Figure 4B). Competitive ABPP was used to generate JZL184, the first selective MAGL inhibitor, subsequently used to show that MAGL blockade caused large elevations in the levels of the endocannabinoid 2-AG in mouse brain, leading to cannabinoid receptor type 1 (CB1)-dependent antinociceptive, anxiolytic, and anti-inflammatory effects (Kinsey et al., 2009, 2010, 2011; Long et al., 2009; Sciolino et al., 2011). Using both targeted and untargeted metabolomic approaches, subsequent studies showed that MAGL blockade in specific tissues such as brain, liver, and lung also decreased arachidonic acid and arachidonic acid-derived pro-inflammatory eicosanoids such as prostaglandins and thromboxanes. This resulted in neuroprotective and hepatoprotective effects in degenerative and inflammatory diseases through suppressing inflammation, thereby linking anti-inflammatory endocannabinoid signaling to pro-inflammatory eicosanoid signaling through MAGL (Long et al., 2009; Nomura et al., 2008a, 2008b, 2011a; Schlosburg et al., 2010; Chen et al., 2012; Piro et al., 2012).

Metabolomics has also been fundamental in understanding how cancer cells alter their metabolism to fuel their pathogenic properties. In a very unique discovery of a neomorphic function for a mutated enzyme, Dang and colleagues used untargeted metabolomic profiling to show that a mutant form of the tricarboxylic acid cycle enzyme isocitrate dehydrogenase 1 (IDH1), IDH1 R132H, found in multiple types of cancers, generated a novel oncometabolite 2-hydroxyglutarate (2-HG) (Dang et al., 2009), which in turn caused epigenetic changes that fuel cancer progression (Xu et al., 2011).

Nomura and colleagues used untargeted metabolomic platforms to find that MAGL plays a distinct role in regulating fatty acid release for the generation of fatty acid-derived lysophospholipids and eicosanoids that drive aggressive features in cancer (Nomura et al., 2010c, 2011b). In another example, Benjamin et al. used targeted and untargeted metabolomic approaches to show that inactivating the ether lipid-generating enzyme alkyl-glycerone phosphate synthase (AGPS) in aggressive cancer cells dramatically reduced structural and oncogenic signaling ether lipid levels. AGPS inactivation also diverted the flux of arachidonic acid away from other tumor-promoting signaling lipids such as prostaglandins, and toward structural acylglycerophospholipids, leading to impaired cancer pathogenicity and tumorigenesis (Benjamin et al., 2013; Figure 4C).

Looking further at cancer metabolism, Locasale and Possemato independently showed phosphoglycerate dehydrogenase (PHGDH) is a critical metabolic node in cancer cells, diverting glucose metabolism into serine and glycine metabolism (Locasale et al., 2011; Possemato et al., 2011). Locasale and colleagues used heteronuclear single quantum coherence spectroscopy NMR and isotopic tracing using targeted liquid chromatography-mass spectrometry-based metabolomics of ^{13}C-glucose labeled cells to show significant ^{13}C incorporation into 3-phosphoserine and serine pathways through PHGDH (Locasale et al., 2011). Using functional metabolomics, Locasale and colleagues found that inactivating PHGDH in melanoma

A ABHD12 is a lysophosphatidylserine hydrolase that regulates neuroinflammation
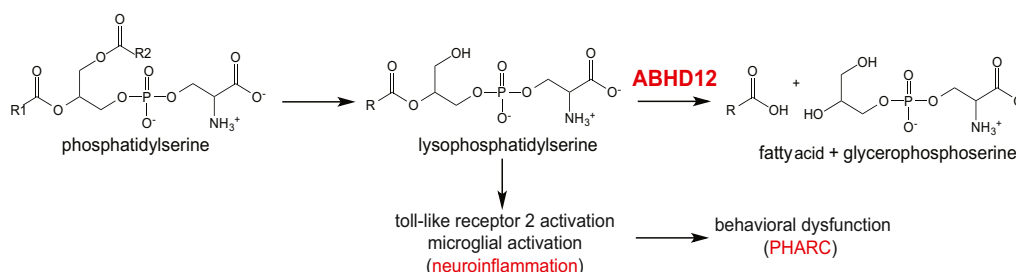
B MAGL controls a fatty acid signaling network to reduce pain, inflammation, and oncogenic signaling lipids
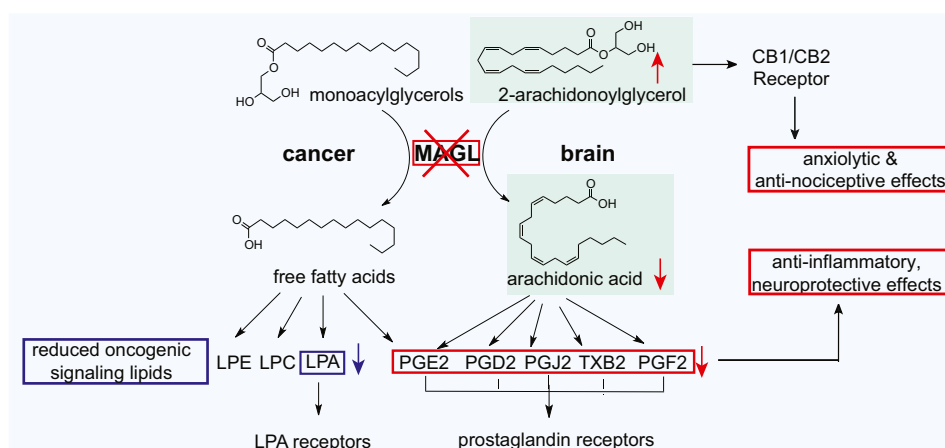
C AGPS regulation of ether lipids controls oncogenic signaling lipids to affect cancer aggressiveness
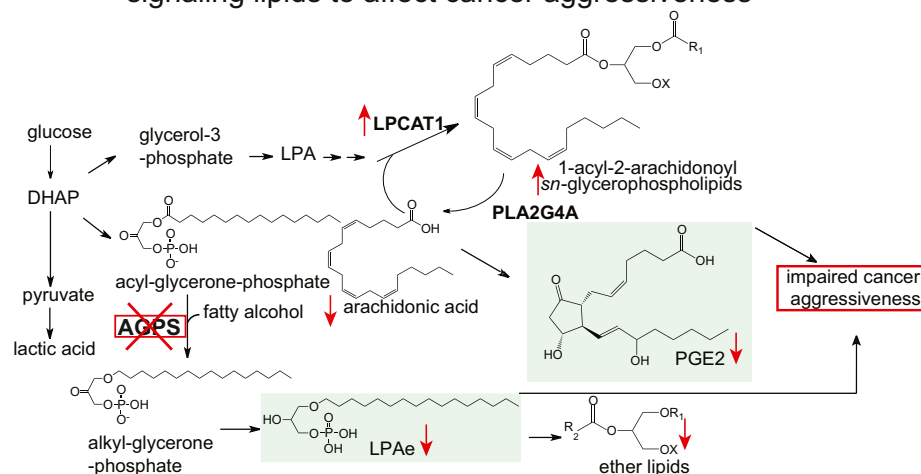
Figure 4. Examples of Metabolic Pathways Elucidated by Metabolomic Profiling

(A) ABHD12 was characterized as a LPS hydrolase. ABHD12 deficiency leads to an accumulation in LPS, activates toll-like receptor 2, induces neuroinflammation, and causes a neurodegenerative disease known as PHARC (retinitis pigmentosa, hearing loss, ataxia, cataract, and polyneuropathy).

(B) MAGL was shown to play critical roles of controlling endocannabinoid and eicosanoid signaling in the brain and fatty acid and fatty acid-derived oncogenic signaling lipids in cancer. MAGL blockade in the brain leads to elevations in 2-AG endocannabinoid signaling and anxiolytic and antinociceptive effects, while also lowering the primary arachidonic acid precursor pool for pro-inflammatory eicosanoid production, leading to reduced inflammation, and neuroprotection against neurodegenerative diseases. In cancer, MAGL blockade leads to reduced fatty acids and fatty acid-derived signaling lipids such as prostaglandins and lyso-phosphatidic acid, which impairs cancer pathogenicity.

(C) In cancer, AGPS was shown to not only control ether lipid synthesis, but also fatty acid metabolism that balances structural lipids with signaling lipids that fuel cancer. AGPS knockdown in cancer cells leads to reductions in the tumor-promoting lipid lysophosphatidic acid-ether, leading to a diversion of arachidonic acid away from oncogenic prostaglandins and toward structural lipids, leading to a net impairment in cancer aggressiveness.

A   4-HNE oxididation of ZAK kinase senses oxidative stress through JNK
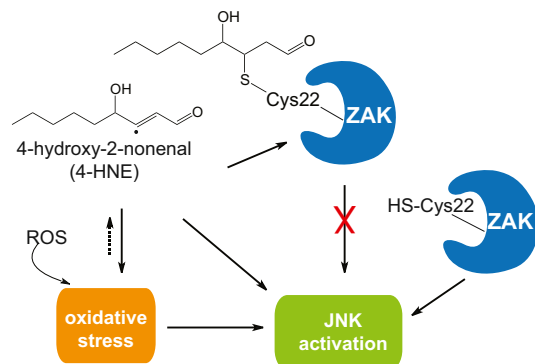
B   post-translational regulation of glycolysis by pgK
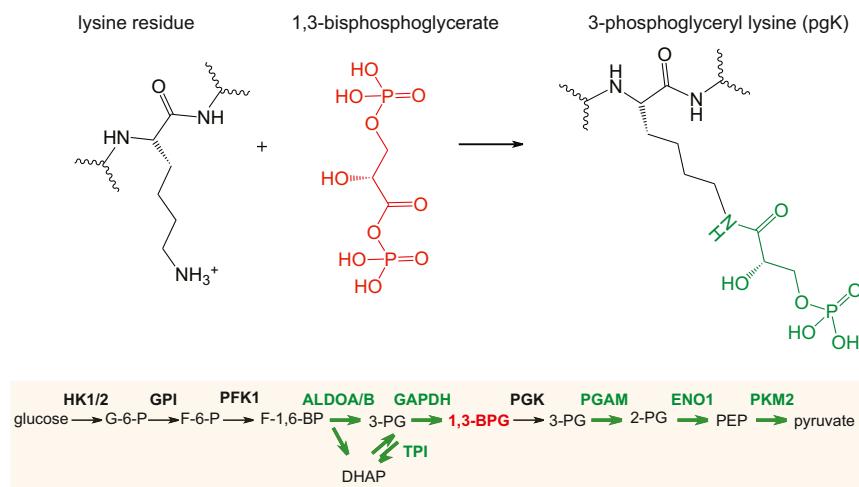
Figure 5. Examples of Posttranslational Modifications Regulated by Specific Metabolites

(A) isoTOP-ABPP was used to map distinct hyperreactive cysteines that were particularly sensitive to modification by the endogenously produced reactive electrophile 4-hydroxy-2-nonenal. (B) Posttranslational regulation by a glycolytic product 1,3-bisphosphoglycerate that modifies lysines on glycolytic enzymes and inhibits their activity.

settings. These techniques have proven useful in discovering the mechanisms through which these enzymes control disease progression and in elucidating the therapeutic potential of manipulating specific metabolic pathways.

Posttranslational and Epigenetic Regulation of the Proteome and Genome by Metabolic Pathways

Whereas small-molecule metabolites have long been known to confer posttranslational and epigenetic modifications onto the proteome and genome, these types of regulation have been considered to be primarily regulated by the enzymes directly involved in adding or removing these modifications. These metabolites, their modifications, and their respective enzymes include acetyl-CoA and acetylation/deacetylation by acetyltransferases and deacetylases, SAM and methylation/demethylation by methyltransferases and demethylases, and ATP and phosphorylation/dephosphorylation by kinases and phosphatases (Prabakaran et al., 2012). However, recent studies have shown that the metabolic enzymes and metabolic fluxes that generate these cofactors may play an important role in regulating the levels of posttranslational and epigenetic modifications (Wellen and Thompson, 2012). This realization brings forth the exciting prospect of controlling metabolic, signaling, and transcriptional networks simultaneously through directly manipulating metabolic pathways. Here, we provide some recent examples of metabolite-driven protein and gene regulation in controlling pathophysiological processes.

Chemoproteomic platforms can also be used to identify metabolite-driven posttranslational modifications in the proteome. Wang and colleagues used competitive isoTOP-ABPP platforms to globally map the targets of 4-hydroxy-2-nonenal (HNE), a common lipid product of lipid peroxidation, whereby HNE was competed against iodoacetamide-alkyne labeling in cells. The authors showed that a unique cysteine in ZAK kinase was the most sensitive target to HNE, and suggested that ZAK is a special node in MAPK signaling that is sensitive to oxidative stress (Wang et al., 2014; Figure 5A).

Moellering and colleagues recently discovered a novel posttranslational modification, 3-phosphoglyceryl-lysine (pgK),

cancer cells lowered phosphoserine levels and caused an accumulation in glycolytic intermediates (Locasale et al., 2011). In breast cancer cells, Possemato and colleagues used functional metabolomics to show that nearly half of α-ketoglutarate was derived from the serine pathway and that PHGDH inactivation in breast cancer cells reduced the levels of multiple tricarboxylic acid cycle metabolites, leading to impaired cancer pathogenicity (Possemato et al., 2011).
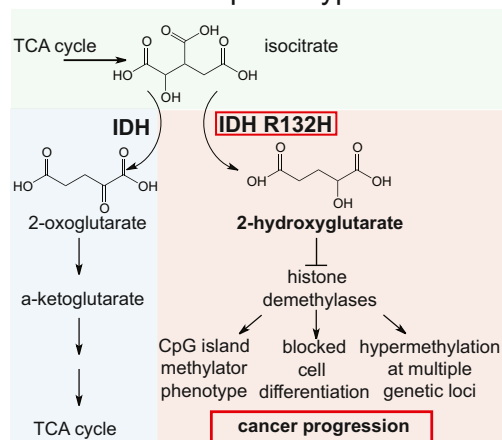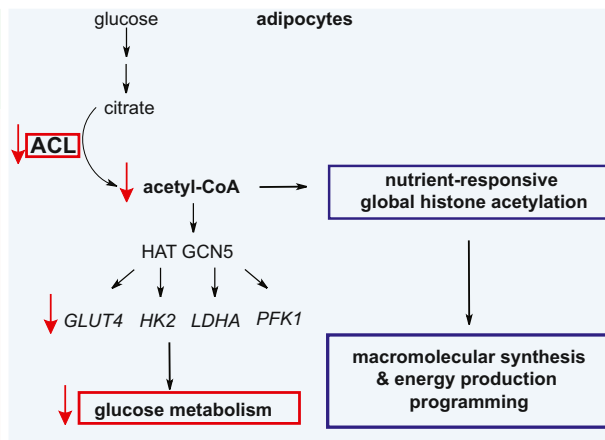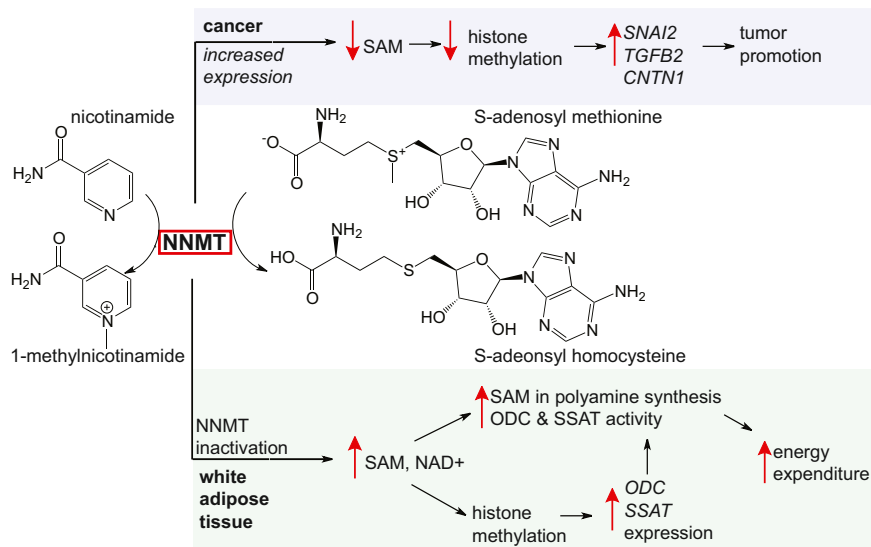
Metabolomics has also been useful in defining metabolic drivers of viral infection. Grady and colleauges used an siRNA screen to show that argininosuccinate synthase 1 (ASS1) knockdown increased virus yield. They subsequently used metabolomic profiling to show that ASS1 inactivation resulted in a metabolic signature that closely resembled herpes simplex virus-1 infection, in which levels of aspartate, carbamoyl-aspartate, one of the first committed metabolites on the pathway to nucleotide synthesis, and nucleotides and their precursors were reduced (Grady et al., 2013).

Collectively, targeted and untargeted metabolomic platforms can be used to functionally characterize not only the substrate/product relationships of metabolic enzymes, but also the metabolic networks that these enzymes control in (patho)physiological

Figure 6. Metabolic Control of Epigenetic Features

(A) Mutant IDH1 R132H generates 2-HG, inhibiting histone demethylases to fuel cancer progression through multiple mechanisms.

(B) ACL activity controls acetyl-CoA levels to confer nutrient-responsive histone acetylation and gene expression, altering glucose metabolism and cellular programming of macromolecular synthesis and energy production.

(C) Increased expression of NNMT in cancer decreases histone methylation, increasing expression of tumor-promoting genes. In white adipose tissue, inactivation of NNMT increases polyamine synthesis and histone methylation, elevating gene expression and activity of enzymes critical for high energy expenditure.

generated by nonenzymatic covalent lysine modifications through the glycolytic intermediate 1,3-bisphosphoglycerate (1,3-BPG), the product of glyceraldehyde 3-phosphate dehydrogenase (Figure 5B). The authors showed that this modification accumulated on several glycolytic enzymes in cells exposed to high glucose, leading to inhibition of activity and redirection of glycolytic intermediates to biosynthetic pathways that support cancer cell pathogenicity (Moellering and Cravatt, 2013).

We described in the previous section how mutant IDH1 in cancer cells generate the oncometabolite 2-HG that drives cancer pathogenicity. Studies have shown that IDH1 mutations

are correlated with hypermethylation at specific loci known as the CpG island methylator phenotype in glioma and that mutant IDH1 causes hypermethylation at many genetic loci (Figure 6A; Turcan et al., 2012). Work by Xu and colleagues and Lu and colleagues have since shown that 2-HG acts as a competitive inhibitor of multiple α-KG-dependent dioxygenases, and that IDH1 mutations can impair histone demethylation resulting in a block to cell differentiation (Lu et al., 2012; Xu et al., 2011). A mutation in IDH1 generates the novel oncometabolite 2-HG, which then confers large-scale epigenetic alterations altering the expression of large numbers of genes, thus linking

central carbon metabolism to gene regulation and cancer pathogenicity.

Recent studies have also highlighted the importance of acetyl-CoA levels in histone acetylation and transcriptional regulation. Tu and colleagues used 2D gas chromatography-mass spectrometry-based metabolomic profiling to show changes in acetyl-CoA levels periodic with yeast cell cycle phase, suggesting that acetyl-coA levels may control temporal regulation of cell cycle processes (Tu et al., 2007). Subsequently, Cai and colleagues showed that an increase in acetyl-CoA levels led directly to the Gcn5p/SAGA-catalyzed acetylation of histones at genes important for growth, thus promoting growth transcriptional programming in yeast and serving as a metabolic rheostat to initiate cell growth through acetylation of specific histones (Cai and Tu, 2011). Similarly, Wellen and colleagues showed in mammalian cells that ATP-citrate lyase (ACL) influences cell growth and differentiation through controlling acetyl-CoA levels, driving nutrient-responsive histone acetylation and selective gene expression prompting growth factor-induced increases in nutrient metabolism and reprogramming of intracellular metabolism to utilize glucose for ATP production and macromolecular synthesis (Figure 6B; Wellen et al., 2009).

In another example, Ulanovskaya showed that N-methyltransferase (NNMT) can also influence the histone methylation and epigenetic regulation that drives cancer aggressiveness through methylating nicotinamide to generate N-methylnicotinamide (Figure 6C). This leads to reduced SAM levels, thus diverting SAM methylation away from histone methylation. The authors showed that NNMT overexpression leads to upregulation of many tumor-promoting gene products including *SNAI2*, *TGFB2*, and *CNTN1*, via the altered epigenetic landscape of cancer cells (Ulanovskaya et al., 2013). Interestingly, Kraus and colleagues showed that NNMT also alters epigenetic and metabolic landscapes in white adipose tissue (Kraus et al., 2014). Through these mechanisms, the metabolic enzyme NNMT regulates metabolism and epigenetic regulation to drive both cancer and obesity.

Future Challenges

The resurgence of interest in metabolism has spurred a rapid pace of advancements in the decade following the completion of the human genome project, providing insight into fundamental biochemistry as well as revealing mechanistic details of diseases with metabolic bases such as cancer, infection, and obesity and diabetes. Development of innovative chemoproteomic and metabolomic platforms has enabled the characterization and description of enzyme function in complex living systems, the metabolic pathways that these enzymes regulate, and even metabolic pathway-driven posttranslational and epigenetic regulation of the proteome and genome. Although these technologies have revealed previously uncharacterized aspects of metabolism and clarified existing ones, the majority of the metabolic map still remains obscured. Higher throughput technologies for metabolomics and improved genetic or pharmacological manipulation of enzyme activities in complex systems are required to decode the function of the currently concealed metabolic genome.

Furthermore, major challenges still exist in elucidating enzyme function. While we have provided successful examples of characterizing enzymes in complex living systems, there are at least an equal number of examples from personal experience where inactivating or overexpressing an enzyme causes no detectable metabolomic changes upon metabolomic profiling. There are many reasons that may account for this apparent metabolomic intransigence including technical and methodological limitations such as (1) extraction procedures and LC/MS chromatography conditions that confer metabolite instability; (2) the metabolites regulated by the enzyme are too low abundance or are otherwise undetectable by traditional metabolomic methods (e.g., does not ionize well, not volatile, or does not have a derivatization method that improves detection); and (3) the metabolite change may be localized to a specific cell type in a tissue or a specific intracellular compartment and the metabolite changes are masked by the remainder of the extracted metabolome. Elucidating enzyme function is also complicated by (4) mischaracterized enzymes that may be described as acting on a specific class of small-molecule metabolites but may actually be operating on alternative metabolite classes or even protein, peptide, or genomic substrates that may not be amenable to analysis by metabolomic platforms; and (5) enzymes that may be acting on a yet unknown posttranslational protein modification.

The information quality challenge of mischaracterized enzymes is likely a larger problem than currently appreciated, as many enzymes are named based on sequence homology to other proteins, but may not share similar substrate specificity. Metabolomic approaches are not amenable for substrate profiling of larger substrates such as protein, peptides, and posttranslational modifications. Nonetheless, innovative protease and peptidase substrate profiling methods have been developed to functionally define metabolic enzymes that may have protein and peptide substrates, including subtiligase-mediated degradomic strategies, protein topography and migration analysis platform, and peptidomic profiling methods (Dix et al., 2008, 2012; Kim et al., 2012; Mahrus et al., 2008; Nolte et al., 2009; Tinoco et al., 2010). Although there are innovative proteomic methods for mapping well-characterized posttranslational modifications such as phosphorylation and acetylation by phosphoproteomic or acetylomic methods (Choudhary and Mann, 2010), unfortunately, there are currently few to no methods for characterizing the functions of enzymes that act on unknown posttranslational modifications, because there are currently no proteomic strategies for globally identifying novel or unknown protein modifications across the proteome.

What is quite clear is that achieving the goal of large-scale functional characterization and description of metabolic enzymes in complex physiological and disease systems will require integrating multidimensional metabolic mapping technologies. This will likely include the chemoproteomic and functional metabolomic approaches described here in addition to newly developed chemical strategies that will expand our access into protein function, our ability to generate pharmacological tools, and our capacity to accelerate throughput of these analyses. These advances, coupled with increased resolution and depth of analytical platforms will support our drive to interrogate the unexplored aspects of the metabolome, proteome, and peptidome. The complex interplay between enzyme function, metabolomic landscape, posttranslational and epigenetic regulation, and metabolite-protein-signaling networks described in this review presents

a great challenge for scientists, but also offers exciting opportunities to understand complex biology and treat disease by understanding fundamental metabolic function.

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