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Deconvoluting the biology and druggability of protein lipidation using chemical proteomics

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Author Comments:	

Deconvoluting the biology and druggability of protein lipidation using chemical proteomics

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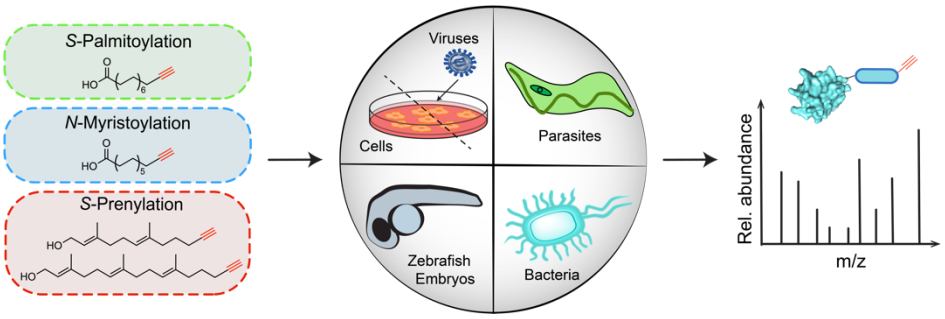
Abstract:

Lipids are indispensable cellular building blocks and their post-translational attachment to proteins make them important regulators of many biological processes. Dysfunction of protein lipidation is also implicated in many pathological states, yet its systematic analysis presents significant challenges. Thanks to innovations in chemical proteomics, lipidation can now be readily studied by metabolic tagging using functionalized lipid analogs, enabling global profiling of lipidated substrates using mass spectrometry that has spearheaded the first deconvolution of their full scope in a range of contexts, from cells to pathogens and multicellular organisms. Protein *N*-myristoylation, *S*-acylation and *S*-prenylation are the most well-studied lipid post-translational modifications due to their extensive contribution to the regulation of diverse cellular processes. In this review we focus on recent advances in the study of these post-translational modifications, with an emphasis on how novel mass spectrometry methods have elucidated their roles in fundamental biological processes.



Graphical abstract

Introduction:

The post-translational modification (PTM) of proteins provides vital and extensive proteome function in all organisms, amplifying the chemical diversity of the protein primary sequence by orders of magnitude. Protein lipidation stands unique as the only class of PTMs capable of promoting spontaneous partitioning to cell membranes; the addition of these hydrophobic groups modulates protein-membrane association events that are vital to cellular function in all organisms, including signaling pathways, protein-protein interactions, trafficking and secretion [1]. The importance of

lipidation is recapitulated in its involvement in disease, with dysregulation known to be essential to drive infectivity, cancer, inflammation, and various developmental diseases [2]. Lipid-modifying enzymes have been targeted in numerous drug discovery contexts; yet their substrate scope and dynamic interplay with other PTMs present formidable challenges of analysis, limiting comprehensive understanding of their roles in diverse contexts and their exploitation for therapeutic benefit.

The first tools to study protein lipidation were radiolabeled lipids, primarily employed in metabolic labeling assays to monitor lipidation states of enzyme substrates [3] (**Fig. 1a**). Development of selective enrichment tools was hampered by the lack of exploitable functional groups in lipids, as well as their

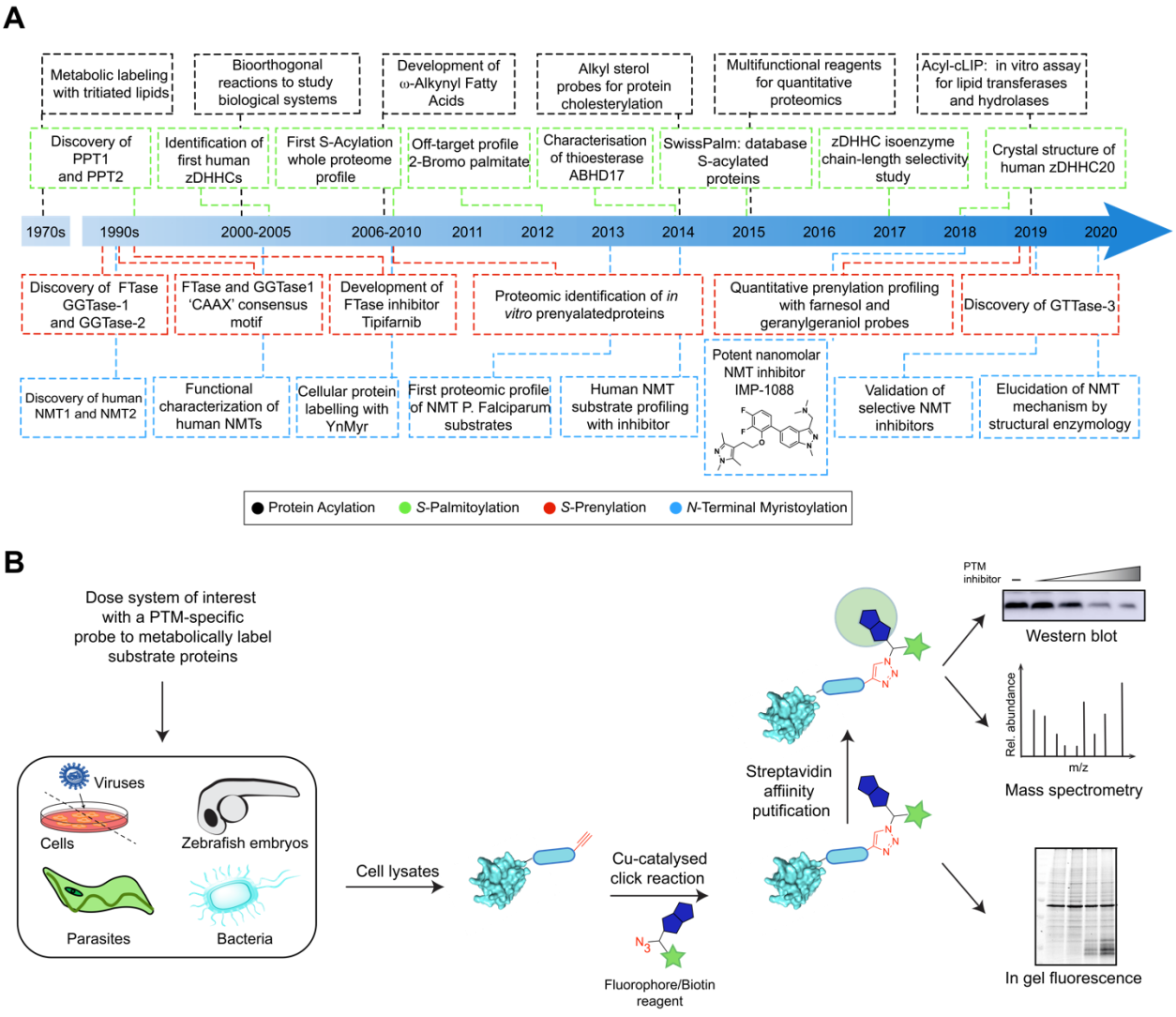


Figure 1. A) Timeline of key developments in the lipidation field with focus on *S*-acylation, *N*-terminal myristoylation and *S*-prenylation. **B)** Summary of the key steps present in common metabolic labelling workflows to identify substrates for each of the classes of lipidation. Through a copper-catalyzed click reaction, affinity tags and fluorophores enable the most common readouts: mass spectrometry, western blot and in-gel fluorescence analysis.

hydrophobicity, instability and low immunogenicity. Therefore, an important advance in the study of lipidation arrived with the development of biorthogonal reactions and the application of click chemistry *to chemical proteomics* [4] **(Fig. 1a).** Installation of alkyne or azide tags in endogenous lipid analogs enabled the global study of lipid modifications at the whole proteome level by way of quantitative proteomics, offering a system-wide view of how lipidation functions in complex cell environments for the first time [5–7.] Large-scale metabolic incorporation of these probes has yielded various orthogonal readouts (**Fig.1b**), including fluorescent tags and affinity handles which can be used to interrogate a range of isolated and native systems, with recent developments in mass spectrometry enabling higher standards of quantification and in vivo applications [8,9.] In tandem with genetic studies, inhibitor development and structural analyses, recent work has greatly widened the scope of what can be understood from the complex processes that govern lipidation. Historic developments in this field have been reviewed elsewhere, and are summarized in Figure 1a, including studies on probes for protein cholesterylation[10] and other classes of lipidation [1,2,11,12.] Whilst bacterial lipoproteins have previously been very successfully studied using lipid probes[13,14], the past few years has seen greatest research activity in the core eukaryotic lipid modifications, *N*-acylation, *S*-acylation, and *S*-prenylation, revealing new substrates, transferases, and roles in disease; including viral infections[15]. This review focuses on the recent advances in probes and proteomic methods for these modifications, which remain at the forefront of PTM drug discovery.

Protein *N*-myristoylation at the N-terminus and at lysine:

N-terminal myristoylation is the condensation of the myristate (C14) fatty acyl group from myristoyl-coenzyme A to the N-terminal glycine of substrate proteins, catalyzed by *N*-myristoyltransferases (NMT) [16.] Lower eukaryotes, where these PTMs were first described, harbor a single NMT, whereas higher eukaryotes possess two isozymes, NMT1 and NMT2. NMT1 is essential for cell survival whilst the essentiality for NMT2 in mammalian systems is contested, although some studies point to a level of functional redundancy between the two [17]. In most instances, *N*-myristoylation (Myr) is co-translationally catalyzed at the ribosome following initiator methionine cleavage by methionine aminopeptidases (MetAP), exposing the N-terminal Gly poised for modification **(Fig. 2a)** [16]. Internal post-translational myristoylation has also been described for caspase-cleaved proteins **(Fig. 2b)**, such as BID, during apoptosis [18]. This PTM is considered irreversible, and aside from specific bacteria effector proteases known to cleave after the *N*-myristoyl-glycine motif [19,20], there are no known host hydrolases for N-terminal myristoylation.

N-myristoylation modulates membrane affinity and specific protein interactions, impacting trafficking, signaling and stability of NMT substrates [16,21–24]. The *N*-myristoylated proteome is expansive, with around 400 potential co-translational NMT substrates recently proposed in humans [25]. Profiling

myristoylated substrates in native contexts posed a significant bottleneck prior to the development of chemical proteomic approaches; the invention of “clickable” functionalized probes azido-Myr (AzMyr) and alkyne-Myr (YnMyr) (**Fig. 2c**) provided a reliable means of profiling substrates for the first time [26]. Recently developed photoactivatable myristic acid analogues also offer the opportunity to identify myristoyl PTM-driven interactions, potentially revealing new biological roles and mechanisms for *N*-myristoylated proteins [27] (**Fig. 2d**), however, to date their application has been limited to model, cell-free systems.

Potent and selective NMT inhibitors can enhance quantitative analysis of the *N*-myristoylated proteome and have established NMT as a promising and tractable drug target, including in fungi and

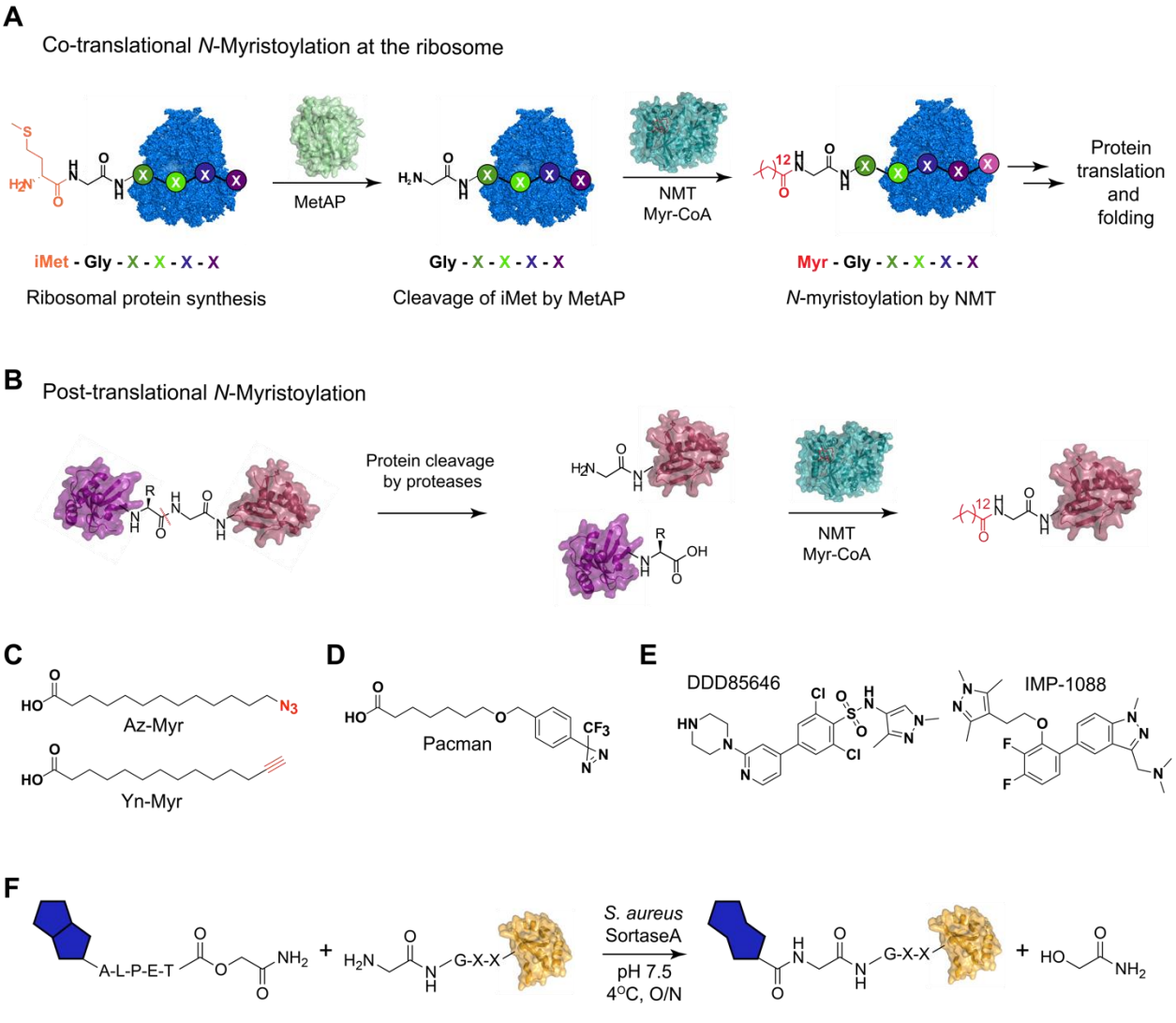


Figure 2. A) N-terminal myristoylation of proteins occurs primarily co-translationally at the ribosome. Methionine initiator residues are removed by methionine aminopeptidases (MetAPs), exposing a glycine residue to which N-myristoyltransferases (NMTs) can add a myristate group. **B)** N-terminal myristoylation can also occur post-

translationally upon endoproteolysis by proteases. **C)** Azido and alkyne myristic acid probes used for identification of *N*-myristoylated proteins with metabolic labelling approaches. **D)** First PhotoACtivatable Myristate ANalogue (Pacman) for the detection of myristate binding proteins. **E)** Validated potent and selective NMT inhibitors DDD85638 and IMP-1088 **F)** Schematic representation of the Sortase A reaction for the enrichment and identification of N-terminal glycine containing substrates.

parasites. Examples include DDD8564, targeting NMT in parasites causing human African trypanosomiasis [28] , and dual human NMT1/2 inhibitor IMP-1088 (**Fig. 2e**), a novel potential therapeutic for viral infections including poliovirus, foot-and-mouth disease virus, and human rhinovirus [29]. IMP-1088 was shown to block host-dependent *N*-myristoylation of viral capsid components, halting virion assembly whilst preserving host cell viability at low nanomolar concentrations; targeting the host cell machinery overcomes serotypic variation and viral resistance, delivering a new benchmark for targeting host biology in antiviral drug discovery.

Selective NMT inhibitors can be combined with chemical proteomics to characterize inhibition across the proteome in cells, and to cross-validate putative NMT substrates. YnMyr has been used in tandem with DDD85646 in live parasites such as *Plasmodium*, *Leishmania*, and *Trypanosoma* species as well as in human cells, building a library of over 100 validated human substrates, with over 90% quantified for the first time at the endogenous level without substrate overexpression [22,24,30–32]. A similar approach substantiated dependence of rhinovirus capsid assembly on *N*-myristoylation and its consequent susceptibility to NMT inhibition using IMP-1088 [29], and a role for *N*-myristoylation in ferroptosis (iron-dependent necrotic cell death) whereby recruitment of *N*-myristoylated ferroptosis suppressor protein 1 (FSP) to the plasma membrane reduces the antioxidant CoQ to ubiquinol, thereby suppressing propagation of lipid peroxides that initiate ferroptosis [33,34]. Targeting FSP myristoylation is one of many emerging avenues by which this mode of inhibition might be exploited for the treatment of cancer. However, a striking majority of other reported NMT inhibitors have been shown to be unspecific or inactive; a recent comprehensive chemical proteomic and phenotypic evaluation showed inactivity against NMT in a cellular context, as well as problematic instability and off-target toxicities, for purported NMT inhibitors including 2-hydroxymyristic acid, D-NMAPPD, and Tris-DBA palladium [35]. This in-depth analysis highlights the capacity of chemical proteomics to uncover shortcomings in previous studies delineating roles for NMT and its substrates using non-inhibitors, and also validated DDD85646 and IMP-1088 as uniquely *bona fide* NMT inhibitors.

Whilst metabolic incorporation of lipid analogues is a powerful approach, it remains limited to metabolically active systems and can be challenging to apply *in vivo* despite previous successes profiling *N*-myristoylation during embryonic development [36]. A novel complementary approach to global *N*-myristoylation profiling was recently reported using a high-activity *S. aureus* Sortase A (SrtA) pentamutant to endogenously label and enrich free N-terminal Gly-containing peptides using a tagged depsipeptide SrtA substrate [37] (**Fig. 2f**). In combination with IMP-1088, this approach allows global

quantification of changes in NMT activity with no discernable bias towards specific N-terminal sequences across the proteome, and as samples are processed post-lysis it is readily amenable to analysis of tissue lysates, filling an important technical gap in the study of N-terminal modifications. A recent report using a large scale in-cell peptidome screen suggests that a quality control mechanism regulates failed *N*-myristoylation events [38] via a free N-terminal Gly degron recognized by E3 ligase complex components, resulting in increased degradation of potentially toxic proteins for which myristoylation has failed. SrtA-based proteomic approaches will be valuable in further exploring this potential quality control marker.

Long-chain fatty acylation has also been proposed to occur physiologically at ε-amino groups of lysine side chains in what is known as lysine fatty acylation [12,39]. A recent report describes IcsB, an intriguing bacterial virulence effector which modulates host cell trafficking by long-chain lysine acylation [39], suggesting potential for widespread physiological impacts; however, outside this specific instance long-chain Lys acylation has yet to be directly identified by mass spectrometry in a true endogenous model, and host cell enzymes catalyzing this attachment have yet to be identified or validated. Numerous other studies implicate SIRTs and HDACs as mediators of Lys-Myr hydrolysis, which would make this a reversible and dynamic modification [40–42]. For example, chemical proteomic approaches using lipid alkyne probes show changes in probe incorporation in SIRT6 knockout and mutant models that implicate R-Ras2 and KRas-4a as lysine fatty-acylated substrates, although these studies combine protein overexpression with high lipid probe concentrations, raising questions over whether these modifications are of sufficient stoichiometry to be physiologically relevant [40,43,44]. Given the propensity for this modification to be found at polybasic sites, it is plausible that non-enzymatic acylation by reactive thioesters similar to that proposed for lysine acetylation [45–47] or non-physiological transfer from protein thioesters during sample processing contribute toward identification of this modification under conditions of substrate overexpression.

Structural enzymology has recently revealed the details of catalysis by human NMT1 at atomic resolution, demonstrating new roles for both active site residues and an N-terminal extension [48]. Furthermore, this work characterizes catalytically competent enzyme-substrate complexes which permit *N*-myristoylation on the side chain of an N-terminal Lys containing peptide substrate, suggesting that they can theoretically be myristoylated by NMT in cells. However, the most plausible instance in which Lys-Myr occurs is following N-terminal Gly acetylation by *N*-acyltransferases (NATs), which is inherently disfavored due to NAT preference for negatively charged residues at the N-terminus [25]. However, a very recent study raises the possibility that NMT modulates ARF6 activation cycles by stimulating membrane association of the GTP-bound form via N-terminal Lys-Myr, while SIRT2 de-myristoylates ARF6 to promote GTP-loading [49]. Other studies that feature global profiling of human PTMs, as well as analyses of thousands of reported proteomic datasets, have failed to provide direct

evidence for the existence of lysine long-chain acylation in cellular contexts, including for ARF6, despite the direct detection of numerous N-terminal Gly myristoylation events [48,50].

NMT substrate specificity remains ill-defined beyond the requirement for N-terminal Gly, and may be partly determined at the co-translational level through ribosome-complex interactions similar to those described for NATs[29]. NMT and NATs are both members of the GNAT superfamily of acyl-CoA transferases, and have been found to compete co-translationally for N-terminal Gly [25], supporting analogous regulatory interactions proximal to the ribosome. This additional level of regulatory complexity emphasizes the need to determine physiological substrate profiles experimentally, rather than relying on bioinformatic or biochemical cell-free analyses.

Protein *S*-acylation at cysteine:

S-Acylation is the post-translational addition of fatty acids to cysteine residues which is often, if imprecisely, referred to as *S*-palmitoylation or simply 'protein palmitoylation' due to the prevalence of C-16 fatty acids in cellular lipid pools, even though incorporation of chain lengths from 14 up to 20 are commonly observed [51,52]. *S*-acylation in humans is mediated by 23 acyltransferases also known as zDHHC enzymes, named after their conserved zinc finger and Asp-His-His-Cys catalytic motif [53]; the corresponding enzymes in mouse are termed DHHCs. The first enzyme crystal structures were recently reported for human zDHHC20 and zebrafish zDHHC15, which revealed a conserved four transmembrane helix catalytic domain arranged in a conical fashion, creating a lipid cavity in their core into which the fatty acid substrate is loaded (**Fig. 3a**) [52]. The amino acid residues that shape the cavity also confer a degree of fatty acid chain-length selectivity. For example, Ile182 in zDHHC3 is responsible for a fatty acid chain-length preference of C14 and C16, which shifts towards C18 when Ile182 is mutated to serine[51]. Similarly, Tyr 181 in zDHHC20 has been linked to its preference to load mainly C16but also C14 and C18 fatty acids, most-likely due to the hydrogen-bond Tyr181-Ser29 which closes the lipid cavity [52]. Following *S*-acylation, zDHHC substrate proteins may be subject to the action of acyl thioesterases from the serine hydrolase superfamily [54], the best-characterized being lysosomal PPT1 and PPT2 [55,56], cytosolic acyl protein thioesterases APT1 and APT2 [57,58], and more recently the α/β hydrolase fold domain (ABHD) proteins ABHD10 and ABHD17A-C [59–61]. The combination of these two enzyme classes creates a highly reversible and dynamic catalytic cycle (**Fig. 3b**), that contrasts with the irreversibility of other lipid PTMs and creates an on/off switch that allows for *S*-acylation to participate in signaling cascades, mediating localization, stability and activity of a broad scope of substrates.

Studying *S*-acylation presents three challenging analytical hurdles: hydrophobicity, which prevents facile isolation and characterization of lipidated peptides; lability of the thioester bond, which may result in loss of the PTM during analysis or its transfer to other residues during sample preparation; and the extremely complex nature of the *S*-acylated proteome which corresponds to 10% of the total proteome and contains substrates harboring multiple *S*-acylation sites. Two proteomic approaches, 'thioester centric' and 'lipid centric' [1], have been developed to counter these challenges (**Fig. 3c**) and together, these have allowed the creation of a manually curated database for *S*-acylated substrates (SwissPalm©) [62]. The latter makes use of alkynyl or azide tagged fatty acids which are metabolically incorporated by cells into native palmitoylation sites and have proven to be good mimics of their untagged counterparts [51]. After a copper-catalyzed click reaction to biotin reagents, *S*-acylated proteins can be isolated by streptavidin affinity purification and analyzed via mass spectrometry (**Fig. 1b**). Addition of EDTA to quench the click reaction has recently been found

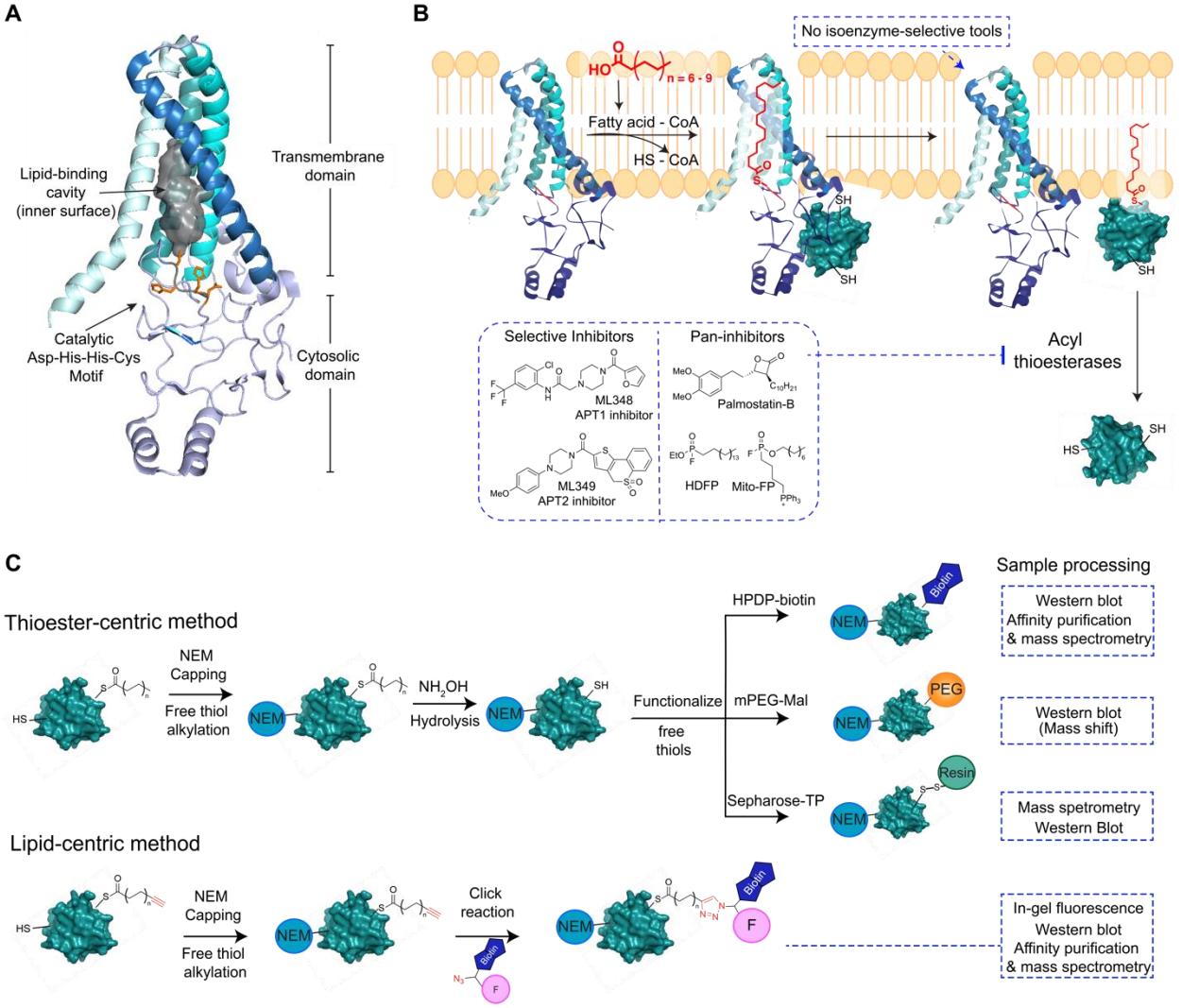


Figure 3. a) Crystal structure of human zDHHC20 with its four transmembrane helices, which form the transmembrane domain, and the cytosolic domain in light purple. In grey is the lipid cavity and in orange the catalytic

Asp-His-His-Cys motif (PDB ID: Q5W0Z9). **b) Proposed catalytic cycle for zDHHCs**. Fatty acids with chain lengths C14-C20 load onto the catalytic cysteine and reside transiently until substrate transfer through binding or complex formation with adaptor proteins. *S*-Acylation of the substrate alters its localization and increases its membrane affinity. Thioesterases hydrolyze the fatty acid reversing the action of zDHHCs and their activity can be chemically regulated through selective (ML384, ML389) or unselective inhibitors (Palmostatin-B, HDFP, MitoFP). **c) Workflows of the mass spectrometry methods to study *S*-acylation**. Includes the sample processing options for the analysis of *S*-acylated substrates. F (fluorophore), NEM (N-ethylmaleimide), PEG (Polyethylene glycol).

essential to prevent protein degradation [63].Early lipid-centric proteomic studies in dendritic cells linked palmitoylation to the cellular immune response, identifying the *S*-acylation of IFITM3 (Interferon Induced Transmembrane Protein 3) [64,65] and Toll-like receptors, involved in the response to viral and microbial pathogens respectively [66]. The viral proteome itself has been found to rely extensively on the host *S*-acylation machinery. Systematic proteomic experiments using alkyne palmitate allowed the parallel study of host and virus-encoded proteins upon viral infection with herpes simplex viruses (HSV), where approximately 10-15% of the viral proteome was found to be *S*-acylated, with host encoded S-acylation decreased for a number of proteins, including several tetraspanin family proteins and regulators of interferon.[15] More recently, alkyne palmitate proteomic analysis of HEK293T cells putatively demonstrated that *S*-acylation of transcription factor STAT3 modulates its transcriptional activity, however these findings have come into question in light of its recent subsequent retraction related to data implicating zDHHC19 as its specific modifier [67]. Interestingly, other pulse-chase metabolic labeling experiments in mammalian cells combined with a promiscuous lipase inhibitor have revealed that static metabolic labeling experiments do not always capture changes in substrates with fast turnover palmitoylation cycles, which readily reach steady state acylation levels [68]. Proteins with dynamic palmitoylation cycles are often involved in rapid signaling cascades that take part in cellular growth and their dysregulation is associated with cancer.

Thioester-centric methods are based on acyl-exchange, whereby unmodified thiols are capped with *N*-ethylmaleimide (NEM) and thioester-bound fatty acids are hydrolyzed with hydroxylamine (HA). The resulting free thiols are isolated by reaction with functionalized resins for acyl-RAC (Resin-Assisted Capture) or biotin reagents followed by affinity purification on streptavidin coated resins for Acyl-Biotin Exchange (ABE). Acyl-exchange protocols are routinely applied to study the *S*-palmitoylated proteome in biological samples where metabolic labeling is not readily applied; for example, it was recently used to identify functional *S*-acylation of the sodium-calcium exchanger (NCX1) in cardiac muscle, brain, and kidney tissues [69] and more recently of PRDX3 and PRDX5 [61], and endogenous 5-HT1A receptors in rodent and human brains [70]. Nevertheless, cysteine-centric methods suffer from high background and noise due to off-target binding from unprotected thiol residues and cysteine oxidation during processing, which hinder the identification of low-abundance proteins. Uncomplete reaction of thiol residues with NEM, has benefited from an extra thiol quenching step with 2,2′-dithiodipyridine (DTPD), which has been shown to reduce falsely identified *S*-acylated proteins by 70% [71]. The use of EDTA has proven crucial for the identification of *S*-acylated proteins; it chelates metals involved in cysteine

oxidation, which otherwise would prevent their binding to functionalized resins [72]. The combination of acyl-RAC with stable isotopic cysteine labelling (SILAC) allows a direct quantification of the *S*-acylated proteome by filtering acylated peptides and reducing spectral complexity [73]. Acyl-exchange methods are also employed to study endogenous palmitoylation levels of individual substrates by acyl-PEG, causing mobility shifts by SDS-PAGE, or through ABE by comparing the amount of protein pulled down to that in the input lysate [72].

Direct identification of acylation sites with standard mass spectrometry protocols has proved challenging, though it may be achieved by exchanging labile *S*-acyl groups with alkylating reagents, such as iodoacetamide or chloroacetamide after a streptavidin affinity purification and the hydrolysis of the resin-bound thioester bond [63], since the resulting carbamidomethyl bond is stable under standard mass spectrometry conditions. Furthermore, validation of specific *S*-acylation sites as modulators of protein activity paves the way for the design of small molecule inhibitors targeting those sites. For example, STING (stimulator of interferon genes) mutants lacking *S*-acylated cysteine sites cannot activate the type I IFN response, part of the host defense mechanism against viral infections [74]. Nitro fatty acids, which are natural anti-inflammatory mediators, have been shown to covalently modify STING at its two palmitoylation sites and have been proposed as treatment for STING-dependent inflammatory disorders [75]. Putative small molecule covalent inhibitors targeting these sites have also been reported; however, the whole proteome target profile of these agents has not been investigated, and their specificity and mode of action remains unclear [76].

The lack of chemical inhibitors to study zDHHC enzymes has impeded progress in understanding the roles and potential druggability of *S*-acylation in cellular pathways. To the detriment of the field, 2-bromopalmitate continues to be incorrectly used as a zDHHC inhibitor, even though accumulated evidence from multiple studies clearly demonstrates that it interferes widely not only with lipid-metabolizing enzymes but also reacts covalently and promiscuously with dozens of membrane-associated proteins at a higher rate than any zDHHC [77]. Without isoenzyme-selective chemical tools, deciphering the substrate scope of an individual zDHHC in a high throughput manner remains a difficult challenge in the field. This is complicated by compensatory effects observed upon knockdown or knockout of a specific zDHHC, a consequence of hypothesized overlapping substrate scope in the family. For example, *S*-acylation of IFITM3 was found to be unaffected upon knockdown of any single zDHHC [78]. To date, overexpression experiments have been used to identify enzyme-substrate pairs even though enzyme concentrations above endogenous level are likely to force non-physiologically relevant *S*-acylation. The identification and analysis of individual zDHHC substrates might unravel isoenzyme-specific consensus sequences, as contrary to other lipid PTMs no consensus sequence has been identified for *S*-acylation. Indeed, it may well be that ‘exosite’ enzyme-protein interactions outside the catalytic site together with enzyme-substrate organelle distribution and tissue expression

patterns are the only important drivers of substrate selectivity [79,80]. The crosstalk between S-acylation and other lipid PTMs remains an understudied subject, yet co-translational *N*-myristoylation regulates downstream post-translational *S*-acylation, and *N*-myristoylated proteins are frequently identified in *S*-acylation datasets, typically at a neighboring cysteine residue, supporting a co-evolutionary mechanism by which *S*-acylated proteins are efficiently localized to their cognate transferases at the membrane [25].

In summary, progress in the field of *S*-acylation ought to be directed towards the design of tools that allow the understanding of the activity of individual isoenzymes, the identification of specific zDHHC-substrate pairs and study of the functional relevance of substrate *S*-acylation sites, with an emphasis on disease-associated proteins.

Protein *S*-prenylation at cysteine:

Protein *S*-prenylation is the irreversible attachment of a farnesyl (C15) or geranylgeranyl (C20) isoprenoid via a thioether linkage at C-terminal cysteines [81] (**Fig. 3a**). Farnesyl transferase (FTase) and two of the three geranylgeranyltransferases (GGTase-1, and the recently discovered GGTase-3) prenylate substrate proteins at a canonical C-terminal motif, referred to as a "CaaX" box, where 'a' is an aliphatic residue. Some selectivity arises from the X residue, which can putatively predict farnesylation or geranylation of a substrate [82], however these trends do not translate to exclusive specificity in cells, complicating analysis of the interplay between these two modifications [83]. Each prenyltransferase consists of an alpha and a beta subunit, with the active site formed at the interface of these proteins (**Fig. 3b**). CaaX substrates often undergo further processing through proteolytic cleavage of the "aaX" tripeptide by Ras-converting CaaX endopeptidase-1 (RCE-1) and subsequent methylation of the revealed cysteine carboxy group by isoprenylcysteine carboxyl methyltransferase (ICMT) [81]. These additional steps modulate membrane interactions of prenylated substrates and have been the subject of several drug discovery efforts[81,84,85].

Rab proteins are prenylated by a single enzyme, RabGGTase (also known as GGTase-2), which singly or doubly attaches geranylgeranyl isoprenoids to a lesser conserved C-terminal double cysteine motif (CC, CXC, CXXX), and requires Rab escort proteins (REP1/2) to facilitate recruitment and trafficking of its substrates [81]. GGTase-3 was recently described to consist of the Rab-GGTase β-subunit in a heterodimer complex with orphan protein prenyltransferase alpha subunit repeat containing 1 (PTAR1) [86,87]. Synaptobrevin homolog (Ykt6), a SNARE protein involved in Golgi trafficking and autophagosome–lysosome fusion, was shown to be geranylgeranylated at a cysteine upstream from initial farnesylation and CaaX box processing, giving rise to an unprecedented heterogenous double prenylation modification [83]. While the full substrate scope of GGTase-3 remains to be explored, these findings shift our current understanding of prenylation pathways and substrate selectivity.

S-prenylation is a prominent drug target by virtue of its known substrate scope, which includes Ras, Rho and Rab families of small GTPases, nuclear lamins, as well as kinases and phosphatases [83,86–88]. Though prenylation remains irreversible within the mammalian context, some bacterial effectors have been described to remove prenylated motifs of host substrates, promoting microbial pathogenesis[89]. More commonly, disruption of prenylation dynamics is implicated in human pathologies, and prenyltransferase inhibitors (PTIs) have been extensively investigated in the context of viral infection (hepatitis D virus, which encodes a substrate for human FTase), progeria (premature aging), and cancer [90–93]. However, despite decades of drug development no PTIs have passed clinical trials owing to cytotoxicity or unforeseen lack of efficacy, due in large part to limited understanding of prenylation dynamics and substrate scope. PTI treatment can alter endogenous prenylation dynamics

by driving substrates to switch between farnesylation and geranylgeranylation through a poorly understood compensatory rescue mechanism, rendering treatment ineffective whilst potentially alleviating side-effects induced *S*-prenylation is a prominent drug target by virtue of its known substrate scope, which includes Ras, Rho and Rab families of small GTPases, nuclear lamins, as well as kinases and phosphatases [83,86–88]. Disruption of prenylation dynamics is implicated in human pathologies, and prenyltransferase inhibitors (PTIs) have been extensively investigated in the context of viral infection (hepatitis D virus, which encodes a substrate for human FTase), progeria (premature aging), and cancer [90–93]. However, despite decades of drug development no PTIs have passed clinical trials owing to cytotoxicity or unforeseen lack of efficacy, due in large part to limited understanding of prenylation dynamics and substrate scope. PTI treatment can alter endogenous prenylation dynamics by driving substrates to switch between farnesylation and geranylgeranylation through a poorly understood compensatory rescue mechanism, rendering treatment ineffective whilst potentially alleviating side-effects induced

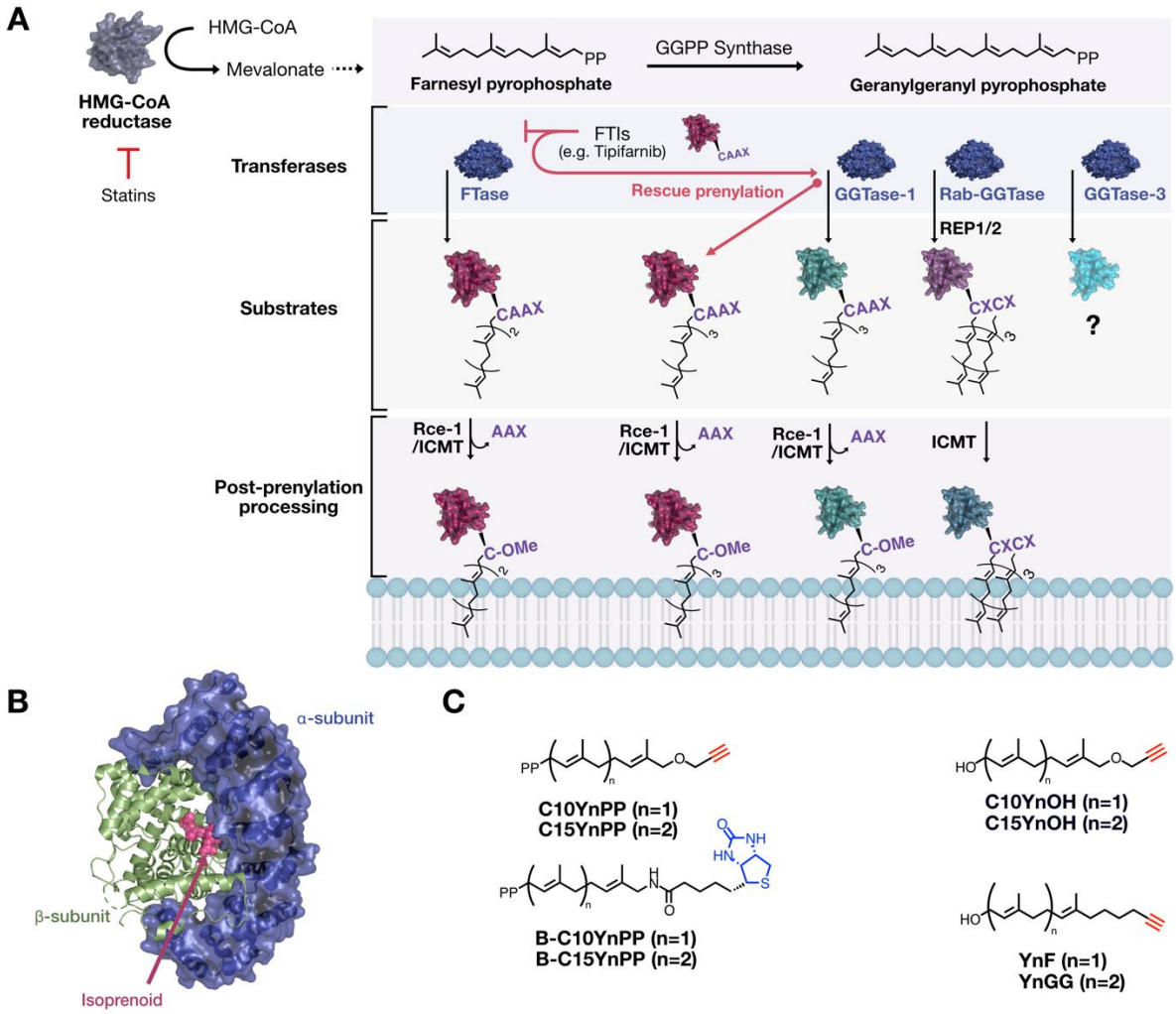


Figure 4: a) Summary of *S*-prenylation. Isoprenoid biosynthesis is initiated by HMG-CoA reductase, which is inhibited by statins. Farnesyltransferase (FTase) and geranylgeranyltransferase-1 (GGTase-1) append a single farnesyl or geranylgeranyl, respectively, to the Cys of the CaaX motif of substrates, which may undergo further processing by Rce-1 and ICMT. FTIs cause mislocalization of substrates that may undergo rescue prenylation by GGTase-1. RabGGTase (GGTase-2) adds two geranylgeranyl groups to the Rab C-terminus and requires the aid of REP1/2 chaperone proteins. GGTase-3 performs atypical prenylation with a substrate scope which includes Ykt6 and FBXL2. Fully processed prenylated proteins are then trafficked to their target membrane. **b) Crystal structure of human farnesyltransferase.** Two subunits make up the general structure of FTase and GGTase, with the α-subunit shared between the two prenyltransferases. The β-subunit determines specificity for the isoprenoid (shown: farnesyl pyrophosphate, magenta). PDB ID: 2F0Y **c) Chemical probes for *S*-prenylation.** Yn-prenyl-PPs and B-Yn-prenyl-PPs are unselective between prenyltransferases and have poorly characterized cell permeability due to their pyrophosphate groups. Applications of B-Yn-prenyl-PPs are limited to lysate labeling and are exclusive to RabGGTase. YnOHs show improved permeability but are not selective for individual prenyltransferases. YnF and YnGG are the only *S*-prenylation probes known to be fully compatible with the prenylation cascade and to exhibit specificity for their cognate transferases.

by complete Ras inhibition[48]. Recent findings also suggest that prenylation can mediate inhibition of Rho GTPase-effector interactions that if left unrestrained lead to hyperactivation of inflammatory pathways, suggesting that a specific balance of prenylated and non-prenylated Rac1 regulates the inflammasome [91]. This might partially account for why GGTase inhibitors (GGTIs) in particular cause adverse effects *in vivo* that have contributed to their failure in the clinic [48]. Interestingly, *Yersinia* bacterial effector YopT cleaves upstream of the prenylated C-terminus of select Rho GTPases,

including Rac1, to inhibit phagocytosis, effectively de-prenylating substrates to hinder immune clearance by macrophages [89]. The capacity to de-prenylate substrates remains unique to bacterial effectors, although together this might suggest a host-mediated combative mechanism against events disrupting prenylated substrate stoichiometry. Nevertheless, the concept of targeting prenylation remains attractive; for example, competitive neo-isoprenoid substrates that harbor an electrophilic moiety at the farnesyl pyrophosphate bridge can cause mislocalization of target substrates such as KRas by complete Ras inhibition[48]. Recent findings also suggest that prenylation can mediate inhibition of Rho GTPase-effector interactions that if left unrestrained lead to hyperactivation of inflammatory pathways, suggesting that a specific balance of prenylated and non-prenylated Rac1 regulates the inflammasome [91]. This might partially account for why GGTase inhibitors (GGTIs) in particular cause adverse effects *in vivo* that have contributed to their failure in the clinic [48]. Nevertheless, the concept of targeting prenylation remains attractive; for example, competitive neo-isoprenoid substrates that harbor an electrophilic moiety at the farnesyl pyrophosphate bridge can cause mislocalization of target substrates such as KRas[93]. The concept of using these neo-substrates remains in its infancy, however the therapeutic use of modified isoprenoids is liable to non-specifically perturb prenylated substrates, causing unfavorable off-target toxicity.

Taken together, these considerations highlight the importance of prenylated proteome deconvolution. Enzymatic assays and *in vitro* labelling approaches have previously enabled limited investigations into prenyl substrate scope through recombinant protein reactions [94,95]. More recent techniques include selective palladium-catalyzed chemical ligation of isoprenoids to recombinant proteins that may facilitate structural analysis of prenylated substrates [96], and a novel universal lipidation screen to validate substrates of an individual transferase, allowing high-throughput *in vitro* investigation of lipidation kinetics, including prenylation [97,98]. However, these techniques are restricted to isolated systems and do not lend themselves to prenylated proteome profiling.

Functionalized geranylgeranyl pyrophosphate probes have been used for in-lysate labeling with a recombinant transferase; similar to the Sortase A assays noted above, these can be useful to study inhibition in metabolically inactive samples such as *ex vivo* tissue [99]. However, this approach is limited by substrate specificity and the non-physiological context of a cell or tissue lysate. Various alkynyl C15 pyrophosphate derivatives (YnC10PP and YnC15PP) have been employed in metabolic labeling and MS-based proteomic profiling of prenylated substrates in mammalian cells, and more recently to define the first prenylated proteome of the human malaria parasite *Plasmodium falciparum* [100] (**Fig. 3c**). A more recent study used alkynyl farnesol (YnC15OH) probes which more readily traverse the cell membrane compared to previous pyrophosphate derivatives, to demonstrate FTase inhibition in parasites [101]. These probes and others have also been extended to a range of orthogonal analytical techniques, including confocal imaging, 2D-gel analysis, and flow cytometry [102]. Importantly however,

these probes do not differentiate between farnesylation and geranylgeranylation, limiting their applications in studying prenylation switch dynamics or C-terminal processing [103]. Moreover, they generally require depletion of endogenous isoprenoids via statin treatment to favor incorporation of exogenous probe, presumably due to their divergence from the structure of endogenous prenyl metabolites, biasing lipid equilibrium in the cell and altering expression of various prenylated proteins, giving rise to pleotropic non-physiological effects [104].

To this end, a new generation of probes, termed YnF and YnGG, were recently developed to more closely mimic endogenous farnesol (FOH) and geranylgeraniol (GGOH), and retain the native chain length and hydrophobicity of each respective isoprenoid [98]. A proteomic comparison against published isoprenoid probes demonstrated that YnF and YnGG exhibit high-fidelity incorporation in a range of cell lines and are selectively processed by their cognate transferases without the use of statins, capturing 80 substrates, 64 of these for the first time at an endogenous level of expression. These probes enabled direct detection of CaaX-processed peptides for the first time at the whole proteome level, including non-canonically C-terminal processed Rab peptides, a novel finding that implicates post-translational regulation of Rab prenylation stoichiometry, demonstrating that these probes are uniquely compatible with the post-prenylation modification cascade. Furthermore, YnGG enabled the first quantitative analysis of the role of REP1, a co-regulator of RabGGTase; loss of REP1 underlies the retinal degenerative disease Choroideremia, and renders Rab prenylation fully dependent on REP2, quantitatively altering the balance of Rab prenylation. A number of substrates were shown to accept both YnGG and YnF probes, indicating that mixed prenylation may hold a regulatory role in the cell for some proteins. Consistent with the subsequent discovery of farnesylation-dependent geranylgeranylation of Ykt6 by GGTase-3, Ykt6 was found in both YnF and YnGG datasets, and both types of prenylation were inhibited by FTI treatment [87]. However, the exact role of this atypical modification, and whether other proteins undergo similar mixed prenylation events, is not yet known.

These probes are also the first to quantify prenylation rescue responses to PTIs at the whole proteome scale by measuring YnF:YnGG incorporation ratios. Interestingly, only KRas, NRas and RRas2 were shown to switch to geranylgeranylation in response to Tipifarnib, a well-known FTI that has been at the forefront of several clinical trials, suggesting that this compensatory mechanism is unique to a small subset of the prenylated proteome. The dual probe methodology both validates the prenylation switching phenomenon for known substrates and highlights proteins that remain susceptible to FT inhibition, such as HRAS; indeed, FTIs are currently being re-evaluated in the context of HRas-dependent cancers [105,106]. However, the mechanisms and substrate criteria that drive alternative prenylation remain unknown and could be a bottleneck to understand the scope of FTIs in clinical settings. For example, recent work indicates that some small GTPases form complexes with mutant

Rab-like proteins or chaperone proteins that may promote prenylation events in a context-dependent manner [107–109].

Detection of natively prenylated substrates has also been recently described using a thiopropyl-functionalized chromatographic resin that can enrich for proteins with intact prenyl groups [110]. The mechanism by which this capture technique works has yet to be determined, and it lacks the flexibility and quantitative precision of metabolic tagging; it thus remains limited by the poor analytical properties of prenylated peptides, harbors interaction bias towards specific amino acids that flank the prenylation site, and is difficult to apply quantitatively. However, it has the distinct advantage of being amenable to studies of *in vivo* prenylation and directly identifies heterogenous prenylation and C-terminal processing events, complementing data from biomimetic dual prenylation probes [98] and further supporting potential regulatory roles that rely on the balance between differentially prenylated species. In the future, relative quantification might be achieved by way of tandem-mass tags (TMT) or similar, which could then be applied to study changes in prenylation levels across a range of conditions. Together, these methods will continue to deliver valuable insights into prenylation dynamics, and potentially reveal novel prenylation-dependent vulnerabilities in disease.

Perspectives:

Protein lipidation remains a highly investigated field in which chemical proteomics has emerged as a key technology, and stable biomimetic lipid probes and transferase-selective small molecule inhibitors are now indispensable tools for the study of these PTMs. Cross-species and system-based analyses have provided increasing evidence for dynamic and context-dependent regulation of lipidation events, and the combination of new chemical tools with those already available will guide future resolution of outstanding questions in protein lipidation:

Deconvoluting transferase activity within a class of lipid modification: new lipid probes amenable to the study of PTM-specific enzyme dynamics, such as the dual probe system for studying prenylation dynamics [98], offer a holistic deconvolution of transferase and PTM-interplay in multiple cellular contexts, including pathological models. Similar approaches can be envisioned for *S*-acylation, although design of selective lipid probes will be more challenging due to the higher number of isoenzymes present in the family (23 in humans). Identification of selective small-molecule inhibitors or substrates designed by exploiting isoenzyme or transferase family structural differences or genetic manipulation will be decisive tools to decipher individual substrate scope and functional enzymatic redundancies.

Dependencies amongst different post-translational modifications: advances in metabolic labelling approaches in combination with mass spectrometry present an opportunity to study the interplay

between different lipid and non-lipid PTMs. Mixed or double lipidation, such as dual farnesylation and geranylgeranylation of Ytk6 [86], and *S*-acylation dependencies on *N*-myristoylation [25], *S*-prenylation[111] and phosphorylation[112] suggest a largely unexplored level of spatiotemporal complexity mediated by PTM crosstalk across the cell; these phenomena may be addressed by combining technologies, allowing for multiplex analysis of interdependent combinatorial PTMs and substrate profiles.

Exploiting post-lipidation processing events in drug discovery: post-lipidation protein processing is well-described for prenylation, and recent evidence for non-canonical Rab C-terminal processing suggests a novel mode of regulation for lipidated substrate stoichiometry[62]. Such processing events, including the machinery catalyzing post-lipidation events, may be exploited in drug discovery approaches. Similar to interdependent regulation of lipidation with other PTMs, these PTM cascades warrant further investigation using multi-probe systems or resin-based enrichment strategies that offer tools for proteomic detection and quantification of post-lipidation processing, such as those described for *S*-prenylation [62,75].

Elucidating lipid transferase substrate criteria: the scope and regulation of lipidation transcends primary sequence specificity, and the drivers of selectivity are still not fully understood. For example, we are only at the beginning of understanding how REP1 and REP2 modulate Rab prenylation. Other studies have also implicated various adapter proteins as regulators of *S*-prenylation which may be disease-dependent[72-74], and similar regulatory interactions have been found to govern substrate specificity for *S*-acylation [111,113], for which there is no known consensus sequence. Similarly, proposed NMT sequence recognition patterns do not recapitulate the full scope of the *N*-myristoylated proteome, nor address the roles of the different NMT isozymes in higher eukaryotes. Investigation of factors beyond primary sequence such as ribosomal interactions with transferases may offer new insights into regulation of co-translational lipidation. Identifying and modulating substrate-specific co-regulators of lipidation will shed light on substrate specificity and hold merit as a therapeutic approach complementary to transferase inhibition, which remains a challenging endeavor due to the breadth of substrates affected.

Lipidation as a target in microbial pathogenesis: various bacterial effectors have evolved to irreversibly "de-lipidate" host substrates by specifically recognizing and proteolytically cleaving lipidated motifs; such effectors have been described to act on host *S*-prenylated[89] and *N*-myristoylated substrates[19,20], releasing them from cognate membranes and perturbing host immune responses. For example, effector RavZ in *Legionellla* has been shown to cleave the phosphatidylethanolamine-lipidated C-terminal Gly of autophagy-related proteins, inhibiting host-mediated autophagy [114] . With the removal of lipidated motifs, as well as lipid attachment at Lys, bacterial effectors have evolved the capacity to alter host cell lipidation in a way that is inaccessible to mammalian cell machinery, enabling microbial pathogenesis. The types and breadth of these lipid modifications have not yet been fully explored due

to challenges associated with convergent evolution of the bacterial enzymes that carry out these processes. However, there lies opportunity to profile these bacterial effectors and their substrates by way of chemical proteomics, similar to what has been undertaken by recent studies[19,20], and may lead to the identification of new druggable targets in infection.

Contextualizing lipid modifications in different biological settings: assessing the importance of the cellular context on lipidation has been limited by inefficient metabolic labeling strategies, particularly in complex settings with multiple interacting cell types. As chemical probes for lipidation are further refined, systematic lipidated proteome profiling enhanced by cell-selective or interactome proteomics becomes possible [106,107]. Interactome mapping of lipidated substrates, differential essentiality of lipid transferases across cell types, and the impact on lipidation of changes in lipid metabolism or exogenous factors imparted by tumor microenvironments are areas for future exploration.

Progression towards refined chemical lipid probes in recent years has yielded new insights into the complexity and dynamism of protein lipidation but has also shown that we have only scratched the surface of the roles played by these widespread PTMs. As we strive towards more comprehensive profiles of global lipidation, questions of regulation and crosstalk across different types of lipidation are at the forefront, alongside the allure of identifying exploitable mechanisms for therapeutic intervention. It is notable that even with these advancements, the systemic study of lipidation has not yet been undertaken as intensely as the development of inhibitors, leaving questions of drug mode of action largely unanswered, and ripe for investigation. The development of integrative proteomic workflows that utilize complementary chemical tools and omics analyses likely hold the greatest potential for deconvoluting transferase specificities, substrate scopes, and how alternative or atypical lipidation events occur in disease contexts -- all of which will harbor important insights into druggable vulnerabilities in protein lipidation.

Abbreviations:

ABHD	α/β hydrolase fold domain
ABE	Acyl-biotin Exchange
AzMyr	Azido myristic acid
Cys	Cysteine
DTPD	2,2′-dithiodipyridine
EDTA	Ethylenediaminetetraacetic acid
FSP	Ferroptosis suppressor protein 1
Gly	Glycine
GNAT	GCN5-related N-acetyltransferase
HA	Hydroxylamine
IFITM3	Interferon Induced Transmembrane Protein 3
MetAP	methionine aminopeptidase
NAT	*N*-acetyl transferase
NCX1	Sodium-calcium exchanger
NMT	*N*-myristoyltransferase
PEG	Polyethylene glycol
PTI	Prenyltransferase inhibitor
PTM	Post-translational modification
RAC	Resin-assisted Capture
REP	Rab escort protein
SNARE	Soluble N-ethylmale-imide-sensitive factor-attachment protein receptor
SrtA	Sortase A
STING	Stimulator of interferon genes
SILAC	Stable isotope labelling

MYR	*N*-myristoylation
YKT6	SNARE protein Ykt6
YnMyr	Alkyne myristic acid
YnF	Alkyne farnesol
YnGG	Alkyne geranylgeraniol

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† These authors contributed equally to this work.

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Protein lipidation; *N*-myristoylation; *S*-prenylation; *S*-acylation; chemical proteomics; biorthogonal labeling; click chemistry; drug discovery; viral proteome; bacterial effectors; parasite proteome

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Deconvoluting the biology and druggability of protein lipidation using chemical proteomics

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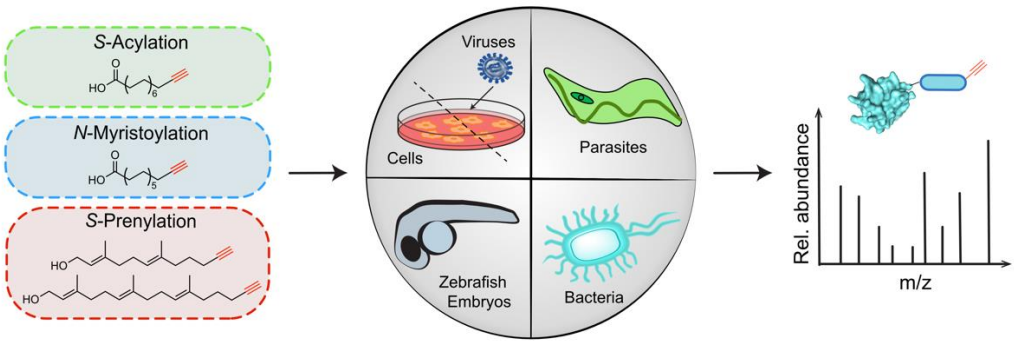
Abstract:

Lipids are indispensable cellular building blocks and their post-translational attachment to proteins make them important regulators of many biological processes. Dysfunction of protein lipidation is also implicated in many pathological states, yet its systematic analysis presents significant challenges. Thanks to innovations in chemical proteomics, lipidation can now be readily studied by metabolic tagging using functionalized lipid analogs, enabling global profiling of lipidated substrates using mass spectrometry. This has spearheaded the first deconvolution of their full scope in a range of contexts, from cells to pathogens and multicellular organisms. Protein *N*-myristoylation, *S*-acylation and *S*-prenylation are the most well-studied lipid post-translational modifications due to their extensive contribution to the regulation of diverse cellular processes. In this review we focus on recent advances in the study of these post-translational modifications, with an emphasis on how novel mass spectrometry methods have elucidated their roles in fundamental biological processes.



Graphical abstract

Introduction:

The post-translational modification (PTM) of proteins provides vital and extensive proteome function in all organisms, amplifying the chemical diversity of the protein primary sequence by orders of magnitude. Protein lipidation stands unique as the only class of PTMs capable of promoting spontaneous partitioning to cell membranes; the addition of these hydrophobic groups modulates protein-membrane association events that are vital to cellular function in all organisms, including

signaling pathways, protein-protein interactions, trafficking and secretion [1]. The importance of lipidation is recapitulated in its involvement in disease, with dysregulation known to drive infectivity, cancer, inflammation, and various developmental diseases [2]. Lipid-modifying enzymes have thus been targeted in numerous drug discovery contexts, yet their substrate scope and dynamic interplay with other PTMs present formidable challenges of analysis, limiting comprehensive understanding of their roles in diverse contexts and their exploitation for therapeutic benefit.

The first tools to study protein lipidation were radiolabeled lipids, primarily employed in metabolic labeling assays to monitor lipidation states of enzyme substrates [3] (**Fig. 1a**). Development of selective enrichment tools was hampered by the lack of exploitable functional groups in lipids, as well as their

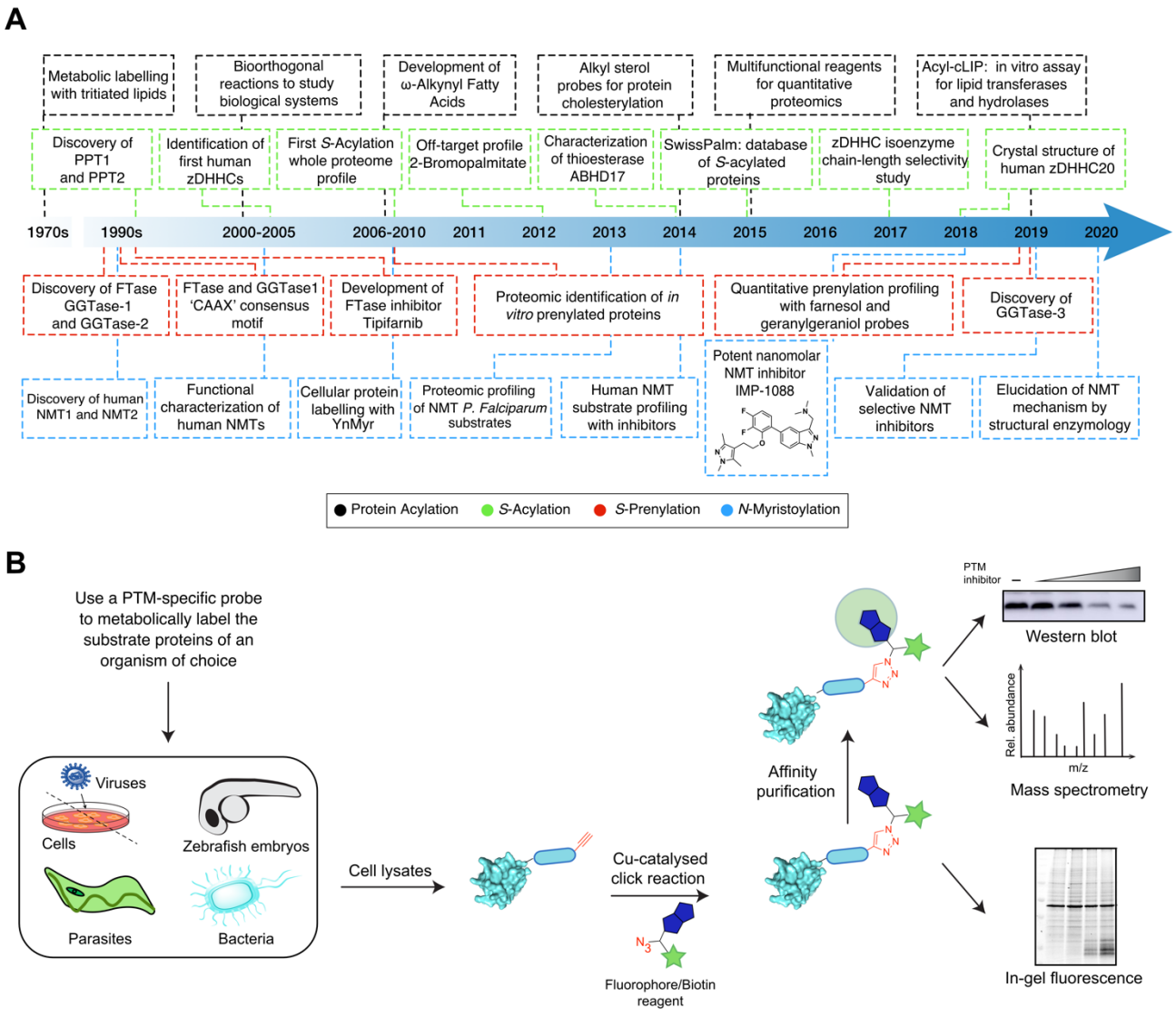


Figure 1. A) Timeline of key developments in the lipidation field with focus on *S*-acylation, *N*-terminal myristoylation and *S*-prenylation. **B)** Summary of the key steps present in common metabolic labelling workflows to identify substrates for each of the classes of lipidation. Through a copper-catalyzed click reaction, affinity tags and fluorophores enable the most common readouts: mass spectrometry, western blot and in-gel fluorescence analysis.

hydrophobicity, instability and low immunogenicity. Therefore, an important advance in the study of lipidation arrived with the development of biorthogonal reactions and the application of click chemistry to chemical proteomics [4] **(Fig. 1a).** Installation of alkyne or azide tags in endogenous lipid analogs enabled the global study of lipid modifications at the whole proteome level by way of quantitative proteomics, offering a system-wide view of how lipidation functions in complex cell environments for the first time [5–7]. Large-scale metabolic incorporation of these probes has yielded various orthogonal readouts (**Fig.1b**), including fluorescent tags and affinity handles which can be used to interrogate a range of isolated and native systems, with recent developments in mass spectrometry enabling higher standards of quantification and *in vivo* applications [8,9]. In tandem with genetic studies, inhibitor development, and structural analyses, recent work has greatly widened the scope of what can be understood from the complex processes that govern lipidation. Historic developments in this field have been reviewed elsewhere, and are summarized in **Figure 1a**, including studies on probes for protein cholesterylation[10] and other classes of lipidation [1,2,11,12]. Whilst bacterial lipoproteins have previously been very successfully studied using lipid probes[13,14], the past few years has seen greatest research activity in the core eukaryotic lipid modifications, *N*-acylation, *S*-acylation, and *S*-prenylation, revealing new substrates, transferases, and roles in disease, including viral infections[15]. This review focuses on the recent advances in chemical probes and proteomic methods related to these modifications, which remain at the forefront of PTM drug discovery.

Protein *N*-myristoylation at the N-terminus and at lysine:

N-terminal myristoylation is the condensation of the myristate (C14) fatty acyl group from myristoyl-coenzyme A to the N-terminal glycine of substrate proteins, catalyzed by *N*-myristoyltransferases (NMT) [16]. Lower eukaryotes, where these PTMs were first described, harbor a single NMT, whereas higher eukaryotes possess two isozymes, NMT1 and NMT2. NMT1 is essential for cell survival whilst the essentiality for NMT2 in mammalian systems is contested, although some studies point to a level of functional redundancy between the two [17]. In most instances, *N*-myristoylation (Myr) is co-translationally catalyzed at the ribosome following initiator methionine cleavage by methionine aminopeptidases (MetAP), exposing the N-terminal Gly poised for modification **(Fig. 2a)** [16]. Internal post-translational myristoylation has also been described for caspase-cleaved proteins **(Fig. 2b)**, such as BID, during apoptosis [18]. This PTM is considered irreversible, and aside from specific bacteria effector proteases known to cleave after the *N*-myristoyl-glycine motif [19,20], there are no known host hydrolases for N-terminal myristoylation.

N-myristoylation modulates membrane affinity and specific protein interactions, impacting trafficking, signaling and stability of NMT substrates [16,21–24]. The *N*-myristoylated proteome is expansive, with around 400 potential co-translational NMT substrates recently proposed in humans [25]. Profiling myristoylated substrates in native contexts posed a significant bottleneck prior to the development of chemical proteomic approaches; the invention of “clickable” functionalized probes azido-Myr (AzMyr) and alkyne-Myr (YnMyr) (**Fig. 2c**) provided a reliable means of profiling substrates for the first time [26]. Recently developed photoactivatable myristic acid analogues also offer the opportunity to identify myristoyl PTM-driven interactions, potentially revealing new biological roles and mechanisms for *N*-myristoylated proteins [27] (**Fig. 2d**) - however, to date their application has been limited to model, cell-free systems.

Potent and selective NMT inhibitors can enhance quantitative analysis of the *N*-myristoylated proteome and have established NMT as a promising and tractable drug target, including in pathogenic fungi and

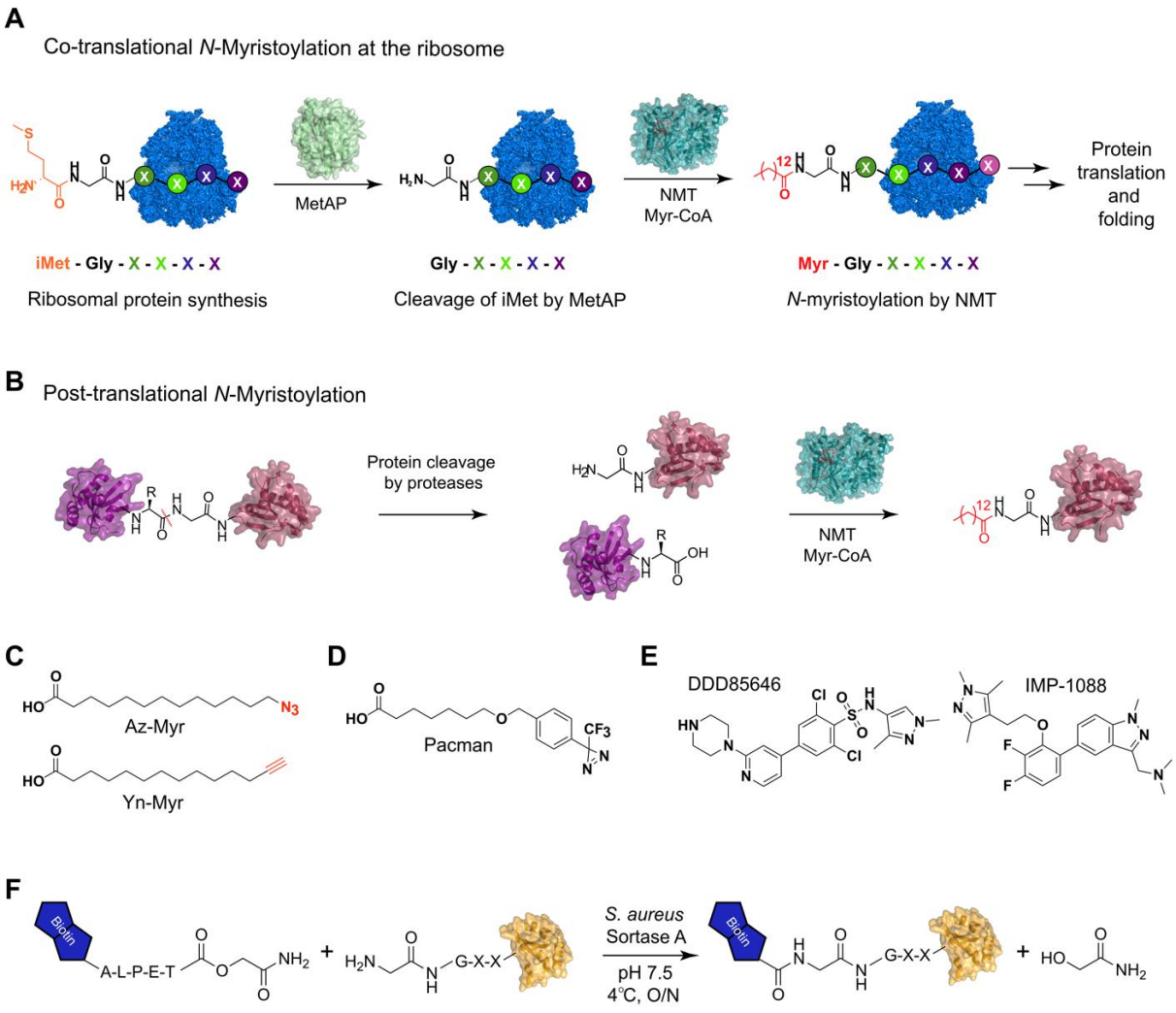


Figure 2. A) N-terminal myristoylation of proteins occurs primarily co-translationally at the ribosome. Methionine initiator residues are removed by methionine aminopeptidases (MetAPs), exposing a glycine residue to which N-myristoyltransferases (NMTs) can add a myristate group. **B)** N-terminal myristoylation can also occur post-translationally upon endoproteolysis by proteases. **C)** Azido and alkyne myristic acid probes used for identification of *N*-myristoylated proteins with metabolic labelling approaches. **D)** First PhotoACtivatable Myristate ANalogue (Pacman) for the detection of myristate binding proteins. **E)** Validated potent and selective NMT inhibitors DDD85646and IMP-1088 **F)** Schematic representation of the Sortase A reaction for the enrichment and identification of N-terminal glycine containing substrates.

parasites. Examples include DDD85646, targeting NMT in parasites causing human African trypanosomiasis [28] , and dual human NMT1/2 inhibitor IMP-1088 (**Fig. 2e**), a novel potential therapeutic for viral infections including poliovirus, foot-and-mouth disease virus, and human rhinovirus [29]. IMP-1088 was shown to block host-dependent *N*-myristoylation of viral capsid components, halting virion assembly whilst preserving host cell viability at low nanomolar concentrations; targeting the host cell machinery overcomes serotypic variation and viral resistance, delivering a new benchmark for targeting host biology in antiviral drug discovery.

Selective NMT inhibitors can be combined with chemical proteomics to characterize inhibition across the proteome in cells, and to cross-validate putative NMT substrates. YnMyr has been used in tandem with DDD85646 in live parasites such as *Plasmodium*, *Leishmania*, and *Trypanosoma* species as well as in human cells, building a library of over 100 validated human substrates, with over 90% quantified for the first time at the endogenous level without substrate overexpression [22,24,30–32]. A similar approach substantiated the dependence of rhinovirus capsid assembly on *N*-myristoylation and its consequent susceptibility to NMT inhibition using IMP-1088 [29]. This compound was further used to reveal the role of *N*-myristoylation in ferroptosis (iron-dependent necrotic cell death), whereby recruitment of *N*-myristoylated FSP1 to the plasma membrane reduces the antioxidant CoQ to ubiquinol, thereby suppressing propagation of lipid peroxides that initiate ferroptosis [33,34]. Targeting FSP myristoylation is one of many emerging avenues by which this mode of inhibition might be exploited for the treatment of cancer. However, a striking majority of other reported NMT inhibitors have been shown to be unspecific or inactive; a recent comprehensive chemical proteomic and phenotypic evaluation showed inactivity against NMT in a cellular context, as well as problematic instability and off-target toxicities, for purported NMT inhibitors including 2-hydroxymyristic acid, D-NMAPPD, and Tris-DBA palladium [35]. This in-depth analysis highlights the capacity of chemical proteomics to uncover shortcomings in previous studies delineating roles for NMT and its substrates using non-inhibitors, and also validated DDD85646 and IMP-1088 as uniquely *bona fide* NMT inhibitors.

Whilst metabolic incorporation of lipid analogues is a powerful approach, it remains limited to metabolically active systems and can be challenging to apply *in vivo* despite previous successes

profiling *N*-myristoylation during embryonic development [36]. A novel complementary approach to global *N*-myristoylation profiling was recently reported using a high-activity *S. aureus* Sortase A (SrtA) pentamutant to endogenously label and enrich free N-terminal Gly-containing peptides with a tagged depsipeptide SrtA substrate [37] (**Fig. 2f**). In combination with NMT inhibition using IMP-1088, this approach allows global quantification of changes in NMT activity with no discernable bias towards specific N-terminal sequences across the proteome, and as samples are processed post-lysis it is readily amenable to analysis of tissue lysates, filling an important technical gap in the study of N-terminal modifications. Relevantly, a recent report using a large scale in-cell peptidome screen suggests that a quality control mechanism regulates failed *N*-myristoylation events [38] via a free N-terminal Gly degron recognized by E3 ligase complex components, resulting in increased degradation of potentially toxic proteins for which myristoylation has failed. SrtA-based proteomic approaches will be valuable in further exploring this potential quality control marker.

Long-chain fatty acylation has also been proposed to physiologically occur at ε-amino groups of lysine side chains in what is known as lysine fatty acylation [12,39]. A recent report describes IcsB, an intriguing bacterial virulence effector which modulates host cell trafficking by long-chain lysine acylation [39], suggesting potential for widespread physiological impacts; however, barring this specific instance, long-chain Lys acylation has yet to be directly identified by mass spectrometry in a true endogenous model, and host cell enzymes catalyzing this attachment have yet to be identified or validated. Numerous other studies implicate SIRTs and HDACs as mediators of Lys-Myr hydrolysis, which would make this a reversible and dynamic modification [40–42]. For example, chemical proteomic approaches using lipid alkyne probes show changes in probe incorporation in SIRT6 knockout and mutant models that implicate R-Ras2 and KRas-4a as lysine fatty-acylated substrates, although these studies combine protein overexpression with high lipid probe concentrations, raising questions over whether these modifications are of sufficient stoichiometry to be physiologically relevant [40,43,44]. Given the propensity for this modification to be found at polybasic sites, it is plausible that non-enzymatic acylation by reactive thioesters similar to that proposed for lysine acetylation [45–47] or non-physiological transfer from protein thioesters during sample processing contribute toward identification of this modification under conditions of substrate overexpression.

Structural enzymology of human NMT1 has recently revealed the mechanistic details of catalysis at atomic resolution, demonstrating new roles for both active site residues and an N-terminal extension [48]. Furthermore, this work has characterized catalytically competent enzyme-substrate complexes which permit *N*-myristoylation on the N-terminal Lys of peptide substrates, suggesting this modification could occur in cells. Nevertheless, the most plausible instance in which Lys-Myr occurs is following N-terminal Gly acetylation by *N*-acyltransferases (NATs), which is inherently disfavored due to NAT preference for negatively charged residues at the N-terminus [25]. However, a very recent study raises

the possibility that NMT modulates ARF6 activation cycles by stimulating membrane association of the GTP-bound form via N-terminal Lys-Myr, while SIRT2 de-myristoylates ARF6 to promote GTP-loading [49]. Importantly, several studies that feature global profiling of human PTMs, as well as analyses of thousands of reported proteomic datasets, have failed to provide direct evidence for the existence of lysine long-chain acylation in cellular contexts, including for ARF6, despite the direct detection of numerous N-terminal Gly myristoylation events. Inevitably, this casts doubt on whether this modification occurs under physiological conditions [48,50].

Profiling atypical *N*-myristoylation events is further complicated by a poorly defined NMT substrate specificity beyond the requirement for N-terminal Gly. It remains possible that substrate specificity may partly arise at the co-translational level through ribosome-complex interactions similar to those described for NATs[29]. NMT and NATs are both members of the GNAT superfamily of acyl-CoA transferases, and have been found to compete co-translationally for N-terminal Gly [25], supporting analogous regulatory interactions proximal to the ribosome. This additional level of regulatory complexity emphasizes the need to determine physiological substrate profiles experimentally, rather than relying on bioinformatic or biochemical cell-free analyses.

Protein *S*-acylation at cysteine:

S-Acylation is the post-translational addition of fatty acids to cysteine residues which is often, if imprecisely, referred to as *S*-palmitoylation or simply ‘protein palmitoylation, due to the prevalence of C16 fatty acids in cellular lipid pools, even though incorporation of chain lengths from 14 up to 20 are commonly observed [51,52]. *S*-acylation in humans is mediated by 23 acyltransferases also known as zDHHC enzymes, named after their conserved zinc finger and Asp-His-His-Cys catalytic motif [53]. The first enzyme crystal structures were recently reported for human zDHHC20 and zebrafish zDHHC15, which revealed a conserved four transmembrane helix catalytic domain arranged in a conical fashion, creating a lipid cavity in their core into which the fatty acid substrate is loaded (**Fig. 3a**) [52]. The amino acid residues that shape the cavity also confer a degree of fatty acid chain-length selectivity. For example, Ile_{182} in zDHHC3 is responsible for a fatty acid chain-length preference of C14 and C16, which shifts towards C18 when Ile182 is mutated to serine[51]. Similarly, Tyr_{181} in zDHHC20 has been linked to its preference to load mainly C16 but also C14 and C18 fatty acids, most-likely due to the hydrogen-bond Tyr_{181}-Ser_{29} which closes the lipid cavity [52]. Following *S*-acylation, zDHHC substrate proteins may be subject to the action of acyl thioesterases from the serine hydrolase superfamily [54], the best-characterized being lysosomal PPT1 and PPT2 [55,56], cytosolic acyl protein thioesterases APT1 and APT2 [57,58], and more recently the α/β hydrolase fold domain (ABHD) proteins ABHD10 and ABHD17A-C [59–61]. The combination of these two enzyme classes creates a highly reversible and dynamic catalytic cycle (**Fig. 3b**), that contrasts with the irreversibility

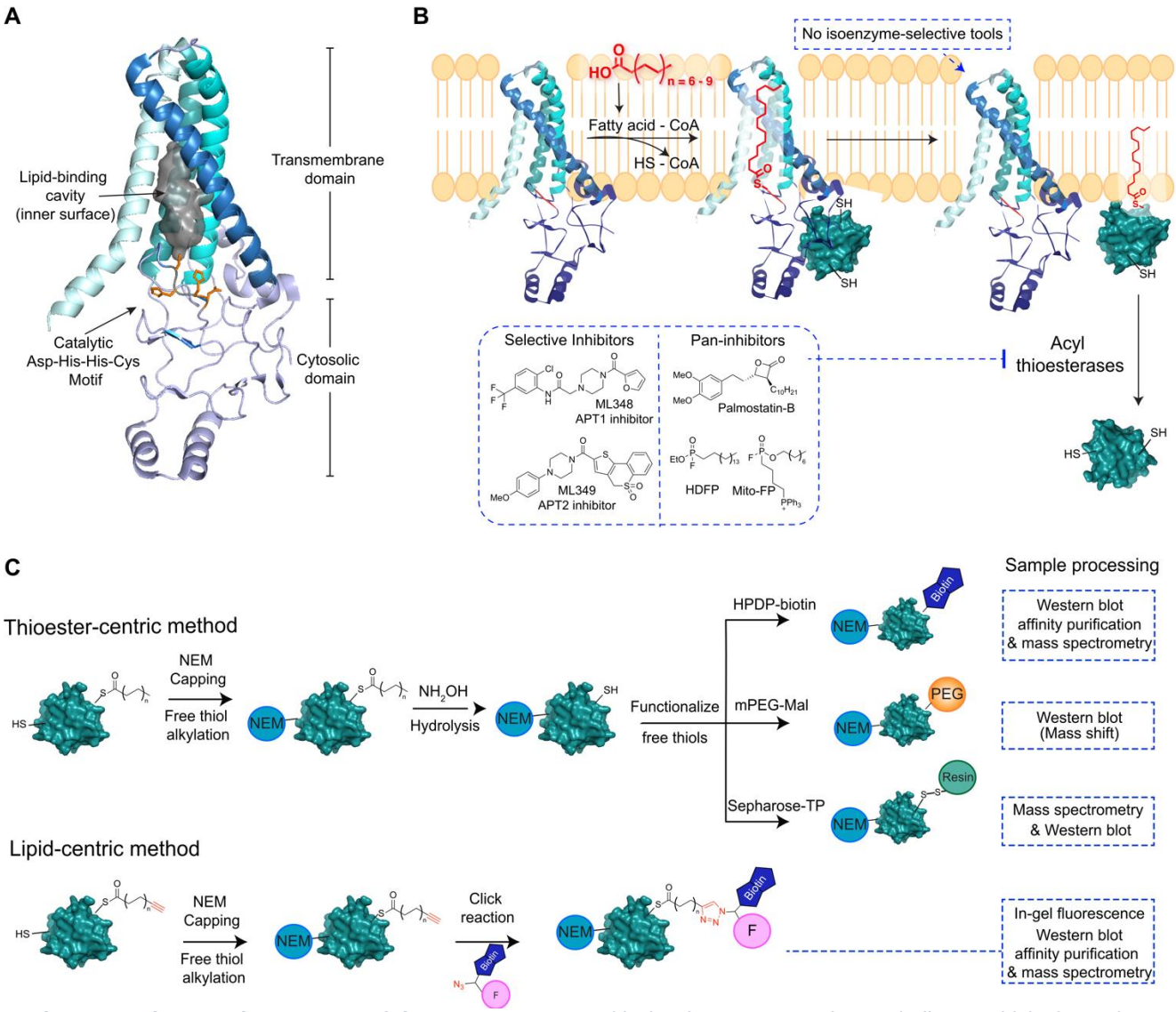


Figure 3. a) Crystal structure of human zDHHC20 with its four transmembrane helices, which form the transmembrane domain, and the cytosolic domain in light purple. In grey is the lipid cavity and in orange the catalytic Asp-His-His-Cys motif (PDB ID: Q5W0Z9). **b) Proposed catalytic cycle for zDHHCs**. Fatty acids with chain lengths C14-C20 load onto the catalytic cysteine and reside transiently until substrate transfer through binding or complex formation with adaptor proteins. *S*-Acylation of the substrate alters its localization and increases its membrane affinity. Thioesterases hydrolyze the fatty acid reversing the action of zDHHCs and their activity can be chemically regulated through selective (ML384, ML389) or unselective inhibitors (Palmostatin-B, HDFP, MitoFP). **c) Workflows of the mass spectrometry methods to study *S*-acylation**. Includes the sample processing options for the analysis of *S*-acylated substrates. F (fluorophore), NEM (N-ethylmaleimide), PEG (Polyethylene glycol).

of other lipid PTMs and creates an on/off switch that allows for *S*-acylation to participate in signaling cascades, mediating localization, stability and activity of a broad scope of substrates.

Studying *S*-acylation presents three challenging analytical hurdles: hydrophobicity, which prevents facile isolation and characterization of lipidated peptides; lability of the thioester bond, which may result in loss of the PTM during analysis or its transfer to other residues during sample preparation; and the extremely complex nature of the *S*-acylated proteome, which corresponds to 10% of the total proteome

and contains substrates harboring multiple *S*-acylation sites. Two proteomic approaches, described as either 'thioester centric' and 'lipid centric' [1], have been developed to counter these challenges (**Fig. 3c**), and together have allowed the creation of a manually curated database for *S*-acylated substrates (SwissPalm©) [62]. The latter approach makes use of alkynyl or azide tagged fatty acids which are metabolically incorporated by cells into native palmitoylation sites and have proven to be good mimics of their untagged counterparts [51,63]. Following a copper-catalyzed click reaction to biotin-containing reagents, *S*-acylated proteins can be isolated by affinity purification and analyzed via mass spectrometry (**Fig. 1b**). Importantly, to prevent protein degradation, the addition of EDTA to quench the click reaction has recently been found essential [63]. Studying *S*-acylation presents three challenging analytical hurdles: hydrophobicity, which prevents facile isolation and characterization of lipidated peptides; lability of the thioester bond, which may result in loss of the PTM during analysis or its transfer to other residues during sample preparation; and the extremely complex nature of the *S*-acylated proteome, which corresponds to 10% of the total proteome and contains substrates harboring multiple *S*-acylation sites. Two proteomic approaches, described as either 'thioester centric' and 'lipid centric' [1], have been developed to counter these challenges (**Fig. 3c**), and together, these have allowed the creation of a manually curated database for *S*-acylated substrates (SwissPalm©) [62]. The latter approach makes use of alkynyl or azide tagged fatty acids which are metabolically incorporated by cells into native palmitoylation sites and have proven to be good mimics of their untagged counterparts [51,63]. Dually functionalized fatty acids with an additional diazirine functional group have been employed to specifically map interactors of S-acylated substrates, yet their use in the field is not widespread [65]. S-acylated substrates can then be isolated by streptavidin affinity purification, after a copper-catalyzed click reaction to biotin-containing reagents, and analyzed via mass spectrometry (**Fig. 1b**). Importantly, to prevent protein degradation, the addition of EDTA to quench the click reaction has recently been found essential [64].

Early studies using tagged fatty acids enabled quantitative profiling of S-acylated proteins in cells and identified, with the use of thioesterase inhibitors, a cluster of proteins with faster *S*-acylation cycles where the tight interplay of zDHHCs and thioesterase enzymes plays a significant regulatory role [66]. Later lipid-centric proteomic studies in dendritic cells linked palmitoylation to the cellular immune response, identifying the *S*-acylation of IFITM3 (Interferon Induced Transmembrane Protein 3) [67,68] and Toll-like receptors, involved in the response to viral and microbial pathogens respectively [69]. The viral proteome itself has been found to rely extensively on the host *S*-acylation machinery. Systematic proteomic experiments using alkyne palmitate allowed the parallel study of host and virus-encoded proteins upon viral infection with herpes simplex viruses (HSV), where approximately 10-15% of the viral proteome was found to be *S*-acylated, with host encoded S-acylation decreased for a number of proteins, including several tetraspanin family proteins and regulators of interferon.[15] More

recently, alkyne palmitate proteomic analysis of HEK293T cells putatively demonstrated that *S*-acylation of transcription factor STAT3 modulates its transcriptional activity, however these findings have come into question in light of its recent subsequent retraction related to data implicating zDHHC19 as its specific modifier [70]. Interestingly, other pulse-chase metabolic labeling experiments in mammalian cells combined with a promiscuous lipase inhibitor have revealed that static metabolic labeling experiments do not always capture changes in substrates with fast turnover palmitoylation cycles, which readily reach steady state acylation levels [71]. Proteins with dynamic palmitoylation cycles are often involved in rapid signaling cascades that take part in cellular growth and their dysregulation is associated with cancer.

Thioester-centric methods are based on acyl-exchange, whereby unmodified thiols are capped with *N*-ethylmaleimide (NEM) and thioester-bound fatty acids are hydrolyzed with hydroxylamine (HA). The resulting free thiols are isolated by reaction with functionalized resins for acyl-RAC (Resin-Assisted Capture) or biotin reagents followed by affinity purification for Acyl-Biotin Exchange (ABE). Acyl-exchange protocols are routinely applied to study the *S*-acylated proteome in biological samples where metabolic labeling is not readily applied; for example, it was recently used to identify functional *S*-acylation of the sodium-calcium exchanger (NCX1) in cardiac muscle, brain, and kidney tissues [72] and more recently of PRDX3 and PRDX5 [61], and endogenous 5-HT1A receptors in rodent and human brains [73]. Nevertheless, cysteine-centric methods suffer from high background and noise due to off-target binding from unprotected thiol residues and cysteine oxidation during processing, which hinder the identification of low-abundance proteins. Incomplete reaction of thiol residues with NEM is partially resolved by an extra thiol quenching step with 2,2′-dithiodipyridine (DTPD), which has been shown to reduce falsely identified *S*-acylated proteins by 70% [74]. The use of EDTA has proven crucial for the identification of *S*-acylated proteins; it chelates metals involved in cysteine oxidation, which otherwise would prevent their binding to functionalized resins [75]. The combination of acyl-RAC with stable isotopic cysteine labelling (SILAC) allows a direct quantification of the *S*-acylated proteome by filtering acylated peptides and reducing spectral complexity [76]. Acyl-exchange methods are also employed to study endogenous palmitoylation levels of individual substrates by acyl-PEG, causing mobility shifts by SDS-PAGE, or through ABE by comparing the amount of protein pulled down to that in the input lysate [75].

Direct identification of acylation sites with standard mass spectrometry protocols has proved challenging, though it may be achieved by exchanging labile *S*-acyl groups with alkylating reagents, such as iodoacetamide or chloroacetamide after a streptavidin affinity purification and the hydrolysis of the resin-bound thioester bond [64], since the resulting carbamidomethyl bond is stable under standard mass spectrometry conditions. Furthermore, validation of specific *S*-acylation sites as modulators of protein activity paves the way for the design of small molecule inhibitors targeting those

sites. For example, STING (stimulator of interferon genes) mutants lacking *S*-acylated cysteine sites cannot activate the type I IFN response, part of the host defense mechanism against viral infections [77]. Nitro fatty acids, which are natural anti-inflammatory mediators, have been shown to covalently modify STING at its two palmitoylation sites and have been proposed as treatment for STING-dependent inflammatory disorders [78]. Putative small molecule covalent inhibitors targeting these sites have also been reported; however, the whole proteome target profile of these agents has not been investigated, and their specificity and mode of action remains unclear [79].

The lack of chemical inhibitors to study zDHHC enzymes has impeded progress in understanding the roles and potential druggability of *S*-acylation in cellular pathways. To the detriment of the field, 2-bromopalmitate continues to be incorrectly used as a zDHHC inhibitor, even though accumulated evidence from multiple studies clearly demonstrates that it interferes widely not only with lipid-metabolizing enzymes but also reacts covalently and promiscuously with dozens of membrane-associated proteins at a higher rate than any zDHHC [80]. Without isoenzyme-selective chemical tools, deciphering the substrate scope of an individual zDHHC in a high throughput manner remains a difficult challenge in the field. This is complicated by compensatory effects observed upon knockdown or knockout of a specific zDHHC, a consequence of hypothesized overlapping substrate scope in the family. For example, *S*-acylation of IFITM3 was found to be unaffected upon knockdown of any single zDHHC [81]. To date, overexpression experiments have been used to identify enzyme-substrate pairs even though enzyme concentrations above endogenous level are likely to force non-physiologically relevant *S*-acylation. The identification and analysis of individual zDHHC substrates might unravel isoenzyme-specific consensus sequences, as contrary to other lipid PTMs no consensus sequence has been identified for *S*-acylation. Indeed, it may well be that ‘exosite’ enzyme-protein interactions outside the catalytic site together with enzyme-substrate organelle distribution and tissue expression patterns are the only important drivers of substrate selectivity [82,83]. The crosstalk between *S*-acylation and other lipid PTMs also remains understudied, yet recent evidence shows that co-translational *N*-myristoylation regulates downstream post-translational *S*-acylation, and *N*-myristoylated proteins are frequently identified in *S*-acylation datasets, typically at a neighboring cysteine residue, supporting a co-evolutionary mechanism by which *S*-acylated proteins are efficiently localized to their cognate transferases at the membrane [25]. Deconvoluting these dependencies may offer better insight into substrate scope and selectivity of both lipid PTMs.

In summary, progress in the field of *S*-acylation ought to be directed towards the design of tools that allow the understanding of the activity of individual isoenzymes, the identification of specific zDHHC-substrate pairs, and study of the functional relevance of substrate *S*-acylation sites, with an emphasis on disease-associated proteins.

Protein *S*-prenylation at cysteine:

Protein *S*-prenylation is the irreversible attachment of a farnesyl (C15) or geranylgeranyl (C20) isoprenoid via a thioether linkage at C-terminal cysteines [84] (**Fig. 4a**). Farnesyl transferase (FTase) and two of the three geranylgeranyltransferases (GGTase-1, and the recently discovered GGTase-3) prenylate substrate proteins at a canonical C-terminal motif, referred to as a "CaaX" box, where 'a' is an aliphatic residue. Some selectivity arises from the X residue, which can putatively predict farnesylation or geranylation of a substrate [85], however these trends do not translate to exclusive specificity in cells, complicating analysis of the interplay between these two modifications [86]. Each prenyltransferase consists of an alpha and a beta subunit, with the active site formed at the interface of these proteins (**Fig. 4b**). CaaX substrates often undergo further processing through proteolytic cleavage of the "aaX" tripeptide by Ras-converting CaaX endopeptidase-1 (RCE-1) and subsequent methylation of the revealed cysteine carboxy group by isoprenylcysteine carboxyl methyltransferase (ICMT) [84]. These additional steps modulate membrane interactions of prenylated substrates and have been the subject of several drug discovery efforts[84,87,88].

Rab proteins are prenylated by a single enzyme, RabGGTase (also known as GGTase-2), which singly or doubly attaches geranylgeranyl isoprenoids to a lesser conserved C-terminal double cysteine motif (CC, CXC, CXXX), and requires Rab escort proteins (REP1/2) to facilitate recruitment and trafficking of its substrates [84]. GGTase-3 was recently described to consist of the Rab-GGTase β-subunit in a heterodimer complex with orphan protein prenyltransferase alpha subunit repeat containing 1 (PTAR1) [89,90]. Synaptobrevin homolog (Ykt6), a SNARE protein involved in Golgi trafficking and autophagosome–lysosome fusion, was shown to be geranylgeranylated at a cysteine upstream from initial farnesylation and CaaX box processing, giving rise to an unprecedented heterogenous double prenylation modification [86]. While the full substrate scope of GGTase-3 remains to be explored, these findings shift our current understanding of prenylation pathways and substrate selectivity.

S-prenylation is a prominent drug target by virtue of its known substrate scope, which includes Ras, Rho and Rab families of small GTPases, nuclear lamins, as well as kinases and phosphatases [86,89–91]. Though prenylation remains irreversible within the mammalian context, some bacterial effectors have been described to remove prenylated motifs of host substrates, promoting microbial pathogenesis[92]. More commonly, disruption of prenylation dynamics is implicated in human pathologies, and prenyltransferase inhibitors (PTIs) have been extensively investigated in the context of viral infection (hepatitis D virus, which encodes a substrate for human FTase), progeria (premature aging), and cancer [93–96]. However, despite decades of drug development no PTIs have passed clinical trials owing to cytotoxicity or unforeseen lack of efficacy, due in large part to limited understanding of prenylation dynamics and substrate scope. PTI treatment can alter endogenous

prenylation dynamics by driving substrates to switch between farnesylation and geranylgeranylation through a poorly understood compensatory rescue mechanism, rendering treatment ineffective whilst potentially alleviating side-effects induced by complete Ras inhibition[48].

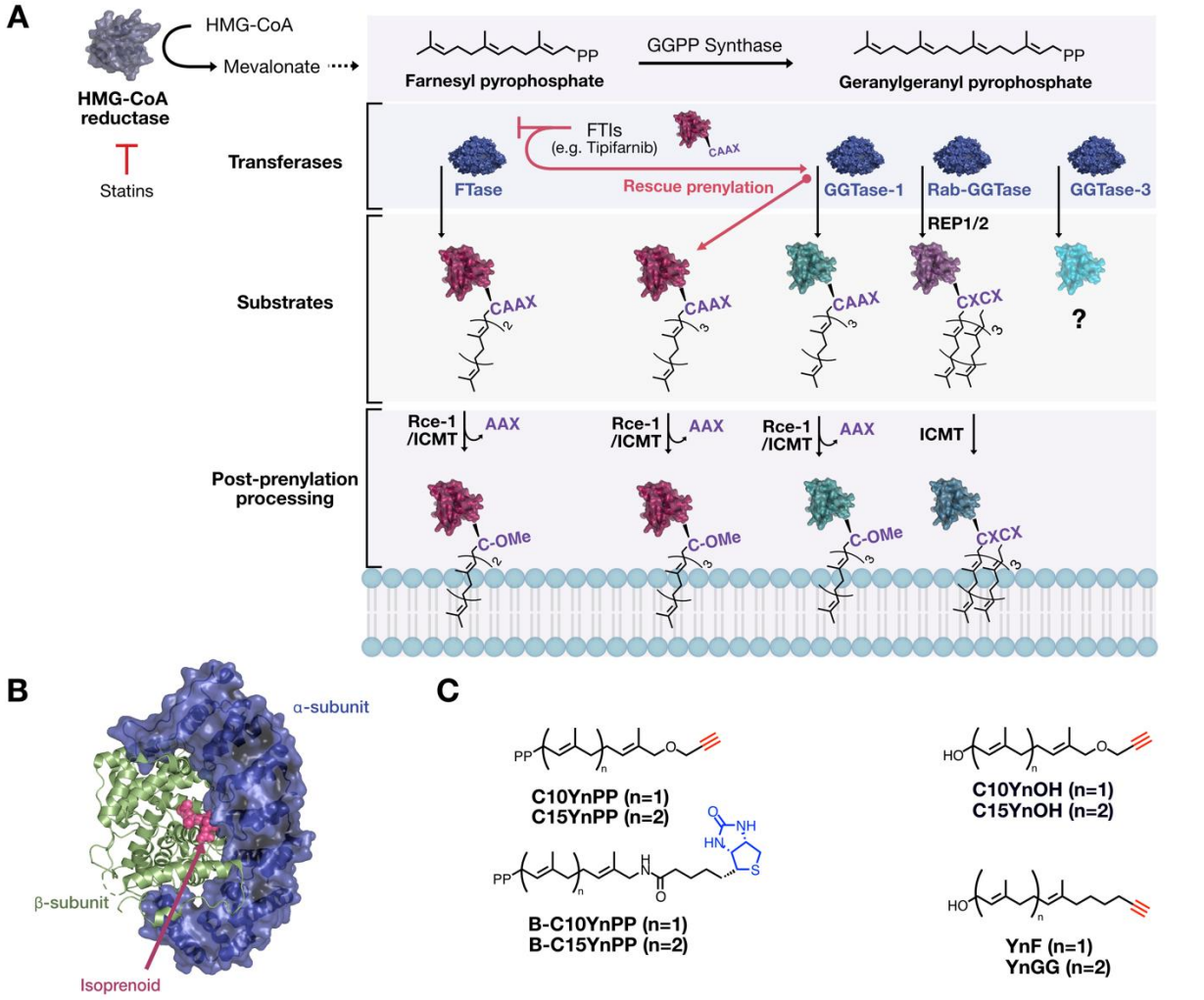


Figure 4: a) Summary of *S*-prenylation. Isoprenoid biosynthesis is initiated by HMG-CoA reductase, which is inhibited by statins. Farnesyltransferase (FTase) and geranylgeranyltransferase-1 (GGTase-1) append a single farnesyl or geranylgeranyl, respectively, to the Cys of the CaaX motif of substrates, which may undergo further processing by Rce-1 and ICMT. FTIs cause mislocalization of substrates that may undergo rescue prenylation by GGTase-1. RabGGTase (GGTase-2) adds two geranylgeranyl groups to the Rab C-terminus and requires the aid of REP1/2 chaperone proteins. GGTase-3 performs atypical prenylation with a substrate scope which includes Ykt6 and FBXL2. Fully processed prenylated proteins are then trafficked to their target membrane. **b) Crystal structure of human farnesyltransferase.** Two subunits make up the general structure of FTase and GGTase, with the α-subunit shared between the two prenyltransferases. The β-subunit determines specificity for the isoprenoid (shown: farnesyl pyrophosphate, magenta). PDB ID: 2F0Y **c) Chemical probes for *S*-prenylation.** Yn-prenyl-PPs and B-Yn-prenyl-PPs are unselective between prenyltransferases and have poor cell permeability due to their pyrophosphate groups. Applications of B-Yn-prenyl-PPs are limited to lysate labeling and are exclusive to RabGGTase. Yn-prenyl-OH derivatives show improved permeability but are not selective for individual prenyltransferases. YnF and YnGG are the only *S*-prenylation probes known to be fully compatible with the prenylation cascade and to exhibit specificity for their cognate transferases.

Recent findings also suggest that prenylation can mediate inhibition of Rho GTPase-effector interactions that if left unrestrained lead to hyperactivation of inflammatory pathways, suggesting that a specific balance of prenylated and non-prenylated Rac1 regulates the inflammasome [94]. This might partially account for why GGTase inhibitors (GGTIs) in particular cause adverse effects *in vivo*, contributing to their failure in the clinic [48]. Interestingly, *Yersinia* bacterial effector YopT cleaves upstream of the prenylated C-terminus of select Rho GTPases, including Rac1, to inhibit phagocytosis, effectively de-prenylating substrates to hinder immune clearance by macrophages [92]. The capacity to de-prenylate substrates appears unique to bacterial effectors, although together this might suggest a host-mediated combative mechanism against events disrupting prenylated substrate stoichiometry. Nevertheless, the concept of targeting prenylation remains attractive; for example, competitive neo-isoprenoid substrates that harbor an electrophilic moiety at the farnesyl pyrophosphate bridge can cause mislocalization of target substrates such as KRas[96]. Development of these neo-substrates is currently in its infancy, however the therapeutic use of modified isoprenoids is liable to non-specifically perturb prenylated substrates, causing unfavorable off-target toxicity.

Taken together, these considerations highlight the importance of prenylated proteome deconvolution. Enzymatic assays and *in vitro* labelling approaches have previously enabled limited investigations into prenyl substrate scope through recombinant protein reactions [97,98]. More recent techniques include selective palladium-catalyzed chemical ligation of isoprenoids to recombinant proteins that may facilitate structural analysis of prenylated substrates [99], and a novel universal lipidation screen to validate substrates of an individual transferase, allowing high-throughput *in vitro* investigation of lipidation kinetics, including prenylation [100,101]. However, these techniques are restricted to isolated systems and do not lend themselves to prenylated proteome profiling.

Functionalized geranylgeranyl pyrophosphate probes have been used for in-lysate labeling with a recombinant transferase; similar to the Sortase A assays noted above, these can be useful to study inhibition in metabolically inactive samples such as *ex vivo* tissue [102]. However, this approach is limited by substrate specificity and the non-physiological context of a cell or tissue lysate. Various alkynyl C15 pyrophosphate derivatives (YnC10PP and YnC15PP) have been employed in metabolic labeling and MS-based proteomic profiling of prenylated substrates in mammalian cells, and more recently to define the first prenylated proteome of the human malaria parasite *Plasmodium falciparum* [103] (**Fig. 4c**). A more recent study used alkynyl farnesol (YnC15OH) probes, which more readily traverse the cell membrane compared to previous pyrophosphate derivatives, to demonstrate FTase inhibition in parasites [104]. These probes and others have also been extended to a range of orthogonal analytical techniques, including confocal imaging, 2D-gel analysis, and flow cytometry [105]. Importantly however, these probes do not differentiate between farnesylation and geranylgeranylation, limiting their applications in studying prenylation switch dynamics or C-terminal

processing [106]. Moreover, they generally require depletion of endogenous isoprenoids, via statin treatment, to favor incorporation of exogenous probes, presumably due to their divergence from the structure of endogenous prenyl metabolites. Consequently, this biases lipid equilibrium in the cell and alters expression of various prenylated proteins, giving rise to pleotropic non-physiological effects [107].

To this end, a new generation of probes, termed YnF and YnGG, were recently developed to more closely mimic endogenous farnesol (FOH) and geranylgeraniol (GGOH), and retain the native chain length and hydrophobicity of each respective isoprenoid [101]. A proteomic comparison against published isoprenoid probes demonstrated that YnF and YnGG exhibit high-fidelity incorporation in a range of cell lines and are selectively processed by their cognate transferases without the use of statins, capturing 80 substrates, 64 of these for the first time at an endogenous level of expression. These probes enabled direct detection of CaaX-processed peptides at the whole proteome level in an unprecedented manner, including non-canonically C-terminal processed Rab peptides. This novel finding reveals previously unknown post-translational regulation of Rab prenylation stoichiometry, and in turn demonstrates that these probes are uniquely compatible with the post-prenylation modification cascade. Furthermore, YnGG enabled the first quantitative analysis of the role of REP1, a co-regulator of RabGGTase; loss of REP1 underlies the retinal degenerative disease Choroideremia, and renders Rab prenylation fully dependent on REP2, quantitatively altering the balance of Rab prenylation. Moreover, a number of substrates were shown to accept both YnGG and YnF probes, indicating that mixed prenylation may hold a regulatory role in the cell for some proteins. Consistent with the subsequent discovery of farnesylation-dependent geranylgeranylation of Ykt6 by GGTase-3, Ykt6 was found in both YnF and YnGG datasets, and both types of prenylation were inhibited by FTI treatment [90]. However, the exact role of this atypical modification, and whether other proteins undergo similar mixed prenylation events, is not yet known.

These probes are also the first to quantify prenylation rescue responses to PTIs at the whole proteome scale by measuring YnF:YnGG incorporation ratios. Interestingly, only KRas, NRas and RRas2 were shown to switch to geranylgeranylation in response to Tipifarnib, a well-known FTI that has been at the forefront of several clinical trials, suggesting that this compensatory mechanism is unique to a small subset of the prenylated proteome. The dual probe methodology both validates the prenylation switching phenomenon for known substrates and highlights proteins that remain susceptible to FT inhibition, such as HRAS; indeed, FTIs are currently being re-evaluated in the context of HRas-dependent cancers [108,109]. However, the mechanisms and substrate criteria that drive alternative prenylation remain unknown and could be a bottleneck to understand the scope of FTIs in clinical settings. For example, recent work indicates that some small GTPases form complexes with mutant

Rab-like proteins or chaperone proteins that may promote prenylation events in a context-dependent manner [110–112].

Detection of natively prenylated substrates has also been recently described using a thiopropyl-functionalized chromatographic resin that can enrich for proteins with intact prenyl groups [113]. The mechanism by which this capture technique works has yet to be determined, and it lacks the flexibility and quantitative precision of metabolic tagging; it thus remains limited by the poor analytical properties of prenylated peptides, harbors interaction bias towards specific amino acids that flank the prenylation site, and is difficult to apply quantitatively. However, it has the distinct advantage of being amenable to studies of *in vivo* prenylation and directly identifies heterogenous prenylation and C-terminal processing events, complementing data from biomimetic dual prenylation probes [101] and further supporting potential regulatory roles that rely on the balance between differentially prenylated species. In the future, relative quantification might be achieved by way of tandem-mass tags (TMT) or similar, which could then be applied to study changes in prenylation levels across a range of conditions. Together, these methods will continue to deliver valuable insights into prenylation dynamics, and potentially reveal novel prenylation-dependent vulnerabilities in disease.

Perspectives:

Protein lipidation remains a highly investigated field in which chemical proteomics has emerged as a key technology, and stable biomimetic lipid probes and transferase-selective small molecule inhibitors are now indispensable tools for the study of these PTMs. Cross-species and system-based analyses have provided increasing evidence for dynamic and context-dependent regulation of lipidation events, and the combination of new chemical tools with those already available will guide future resolution of outstanding questions in protein lipidation:

Deconvoluting transferase activity within a class of lipid modification: new lipid probes amenable to the study of PTM-specific enzyme dynamics, such as the dual probe system for studying prenylation dynamics [101], offer a holistic deconvolution of transferase and PTM-interplay in multiple cellular contexts, including pathological models. Similar approaches can be envisioned for *S*-acylation, although design of selective lipid probes will be more challenging due to the higher number of isoenzymes present in the family (23 in humans). Identification of selective small-molecule inhibitors or substrates designed by exploiting isoenzyme or transferase family structural differences or genetic manipulation will be decisive tools to decipher individual substrate scope and functional enzymatic redundancies.

Dependencies amongst different post-translational modifications: advances in metabolic labelling approaches in combination with mass spectrometry present an opportunity to study the interplay

between different lipid and non-lipid PTMs. Mixed or double lipidation, such as dual farnesylation and geranylgeranylation of Ytk6 [89], and *S*-acylation dependencies on *N*-myristoylation [25], *S*-prenylation[114] and phosphorylation[115] suggest a largely unexplored level of spatiotemporal complexity mediated by PTM crosstalk across the cell; these phenomena may be addressed by combining technologies, allowing for multiplex analysis of interdependent combinatorial PTMs and substrate profiles.

Exploiting post-lipidation processing events in drug discovery: post-lipidation protein processing is well-described for prenylation, and recent evidence for non-canonical Rab C-terminal processing suggests a novel mode of regulation for lipidated substrate stoichiometry[62]. Such processing events, including the machinery catalyzing post-lipidation events, may be exploited in drug discovery approaches. Similar to interdependent regulation of lipidation with other PTMs, these PTM cascades warrant further investigation using multi-probe systems or resin-based enrichment strategies that offer tools for proteomic detection and quantification of post-lipidation processing, including those described for *S*-prenylation [62,75].

Elucidating lipid transferase substrate criteria: the scope and regulation of lipidation transcends primary sequence specificity, and the drivers of selectivity are still not fully understood. For example, we are only at the beginning of understanding how REP1 and REP2 modulate Rab prenylation. Other studies have also implicated various adapter proteins as regulators of *S*-prenylation which may be disease-dependent[72-74], and similar regulatory interactions have been found to govern substrate specificity for *S*-acylation [114,116], for which there is no known consensus sequence. Similarly, proposed NMT sequence recognition patterns do not recapitulate the full scope of the *N*-myristoylated proteome, nor address the roles of the different NMT isozymes in higher eukaryotes. Investigation of factors beyond primary sequence such as ribosomal interactions with transferases may offer new insights into regulation of co-translational lipidation. Identifying and modulating substrate-specific co-regulators of lipidation will shed light on substrate specificity and hold merit as a therapeutic approach complementary to transferase inhibition, which remains a challenging endeavor due to the breadth of substrates affected.

Lipidation as a target in microbial pathogenesis: various bacterial effectors have evolved to irreversibly "de-lipidate" host substrates by specifically recognizing and proteolytically cleaving lipidated motifs; such effectors have been described to act on host *S*-prenylated[92] and *N*-myristoylated substrates[19,20], releasing them from cognate membranes and perturbing host immune responses. For example, effector RavZ in *Legionellla* has been shown to cleave the phosphatidylethanolamine-lipidated C-terminal Gly of autophagy-related proteins, inhibiting host-mediated autophagy [117] . With the removal of lipidated motifs, as well as lipid attachment at Lys, bacterial effectors have evolved the capacity to alter host cell lipidation in a way that is inaccessible to mammalian cell machinery, enabling

microbial pathogenesis. The types and breadth of these lipid modifications have not yet been fully explored due to challenges associated with convergent evolution of the bacterial enzymes that carry out these processes. However, there lies opportunity to profile these bacterial effectors and their substrates by way of chemical proteomics, similar to what has been undertaken by recent studies[19,20], and may lead to the identification of new druggable targets in infection.

Contextualizing lipid modifications in different biological settings: assessing the importance of the cellular context on lipidation has been limited by inefficient metabolic labeling strategies, particularly in complex settings with multiple interacting cell types. As chemical probes for lipidation are further refined, systematic lipidated proteome profiling enhanced by cell-selective or interactome proteomics becomes possible [106,107]. Interactome mapping of lipidated substrates, differential essentiality of lipid transferases across cell types, and the impact on lipidation of changes in lipid metabolism or exogenous factors imparted by tumor microenvironments are areas for future exploration.

Progression towards refined chemical lipid probes in recent years has yielded new insights into the complexity and dynamism of protein lipidation, but has also shown that we have only scratched the surface of the roles played by these widespread PTMs. As we strive towards more comprehensive profiles of global lipidation, questions of regulation and crosstalk across different types of lipidation are at the forefront, alongside the allure of identifying exploitable mechanisms for therapeutic intervention. It is notable that even with these advancements, the systemic study of lipidation has not yet been undertaken as intensely as the development of inhibitors, leaving questions of drug mode of action largely unanswered, and ripe for investigation. The development of integrative proteomic workflows that utilize complementary chemical tools and omics analyses likely hold the greatest potential for deconvoluting transferase specificities, substrate scopes, and how alternative or atypical lipidation events occur in disease contexts -- all of which will harbor important insights into druggable vulnerabilities in protein lipidation.

Abbreviations:

ABHD	α/β hydrolase fold domain
ABE	Acyl-biotin Exchange
AzMyr	Azido myristic acid
Cys	Cysteine
DTPD	2,2′-dithiodipyridine
EDTA	Ethylenediaminetetraacetic acid
FSP	Ferroptosis suppressor protein 1
Gly	Glycine
GNAT	GCN5-related N-acetyltransferase
HA	Hydroxylamine
IFITM3	Interferon Induced Transmembrane Protein 3
MetAP	methionine aminopeptidase
NAT	*N*-acetyl transferase
NCX1	Sodium-calcium exchanger
NMT	*N*-myristoyltransferase
PEG	Polyethylene glycol
PTI	Prenyltransferase inhibitor
PTM	Post-translational modification
RAC	Resin-assisted Capture
REP	Rab escort protein
SNARE	Soluble N-ethylmaleimide-sensitive factor-attachment protein receptor
SrtA	Sortase A
STING	Stimulator of interferon genes
SILAC	Stable isotope labelling

MYR	*N*-myristoylation
YKT6	SNARE protein Ykt6
YnMyr	Alkyne myristic acid
YnF	Alkyne farnesol
YnGG	Alkyne geranylgeraniol

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Protein lipidation; *N*-myristoylation; *S*-prenylation; *S*-acylation; chemical proteomics; biorthogonal labeling; click chemistry; drug discovery; viral proteome; bacterial effectors; parasite proteome

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Response to reviewers' comments

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Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Edward Tate is a founder, shareholder and Director of Myricx Pharma Ltd.