# Fat Chance! Getting a Grip on a Slippery Modification

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ABSTRACT: Protein palmitoylation describes the post-translational fatty acyl thioesterification of cellular cysteine residues and is critical for the localization, trafficking, and compartmentalization of a large number of membrane proteins. This labile thioester modification facilitates a dynamic acylation cycle that directionally traffics key signaling complexes, receptors, and channels to select membrane compartments. Chemical enrichment and advanced mass spectrometry-based proteomics methods have highlighted a pervasive role for palmitoylation across all eukaryotes, including animals, plants, and parasites. Emerging chemical tools promise to open new avenues to study the enzymes, substrates, and dynamics of this distinct post-translational modification.
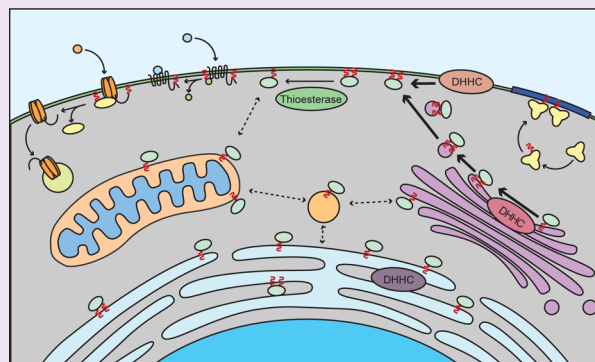
Covalent fatty acid modification of viral proteins was first identified more than 30 years ago after metabolic labeling experiments of virus-infected cells with tritiated palmitic acid.[1] This linkage occurred on cysteine residues through a labile, high-energy thioester bond.[2−4] It was quickly realized that protein palmitoylation is essential for intracellular signaling and critical for the function of Src-family kinases, Ras family GTPases, G-proteins, and G-protein coupled receptors.[5] Furthermore, many of these early annotated palmitoylated proteins are dynamically regulated. Oncogenic H-ras (G12 V) requires palmitoylation at each of two C-terminal sites to promote cellular transformation,[6] and the active GTP-bound form shows >15-times faster palmitoylation turnover.[7] Similarly, Gs(alpha) palmitoylation is rapidly hydrolyzed after adrenergic stimulation.[8] These examples suggest a unique mode of regulation, where activated signaling components are attenuated by rapid depalmitoylation and relocalized away from the plasma membrane. These early examples highlight the dynamic reversible nature of palmitoylation and imply additional regulation by enzymes that write (acyl transferases) and erase (thioesterases) this nonpolar post-translational modification.

While palmitoylation is crucial for many growth signaling pathways, the historical lack of robust analysis tools has, until recently, greatly impeded its study. Unlike phosphorylation and other post-translational modifications, palmitoylation has not been shown to be antigenic. There are no palmitoyl-specific antibodies, and early methods relied on [³H]-palmitate metabolic labeling, which requires days to weeks of film exposure time. Metabolic labeling with [¹²⁵I]-linked fatty acids greatly decreased development time[9] and was used in classic studies exploring the acyl length selectivity and lipid raft association of Src-family kinases.[10] These studies highlighted the acyl heterogeneity of protein acylation in cells and demonstrate that polyunsaturated fatty acids, including

arachadonic and eicosapentenoic acid, are thioesterified to native cysteines on proteins. Furthermore, different acyl chain lengths and degrees of unsaturation affect raft association and T-cell activation. Therefore, the term "palmitoylation" is not restricted to palmitic acid modifications but rather describes the heterogeneous long-chain fatty acyl modification of proteins. The extent and regulation of acyl heterogeneity is unknown but may play an important role in linking lipid metabolism with membrane protein microdomain compartmentalization.

The oncogene Ras is the most intensely studied palmitoylated protein. N-ras, H-ras, and the K-ras splice isoform K-ras4A are each palmitoylated and farnesylated on their C-terminal tails, which is critical for spatial organization and steady state distribution of palmitoylated Ras to the plasma membrane. Microinjection of semisynthetic fluorescent Ras analogues or overexpression of photoactive GFP-tagged Ras mutants has led to a self-organizing model of Ras localization.[11,12] High acyl-transferase activity at the Golgi drives palmitoylation of Ras, which is then trafficked through the secretory pathway to the plasma membrane. Depalmitoylation by protein thioesterases quickly redistributes Ras to endomembranes for random diffusion back to the Golgi. Therefore, Ras is spatially organized by the compartmentalization of palmitoyl transferase enzymes, flux through the secretory system, and a driving equilibrium toward the plasma membrane. The generality of this model deserves additional study, especially given the diversity of palmitoylated proteins.

There are no unique consensus sites for palmitoylation, yet only select cysteines are modified in target proteins as defined
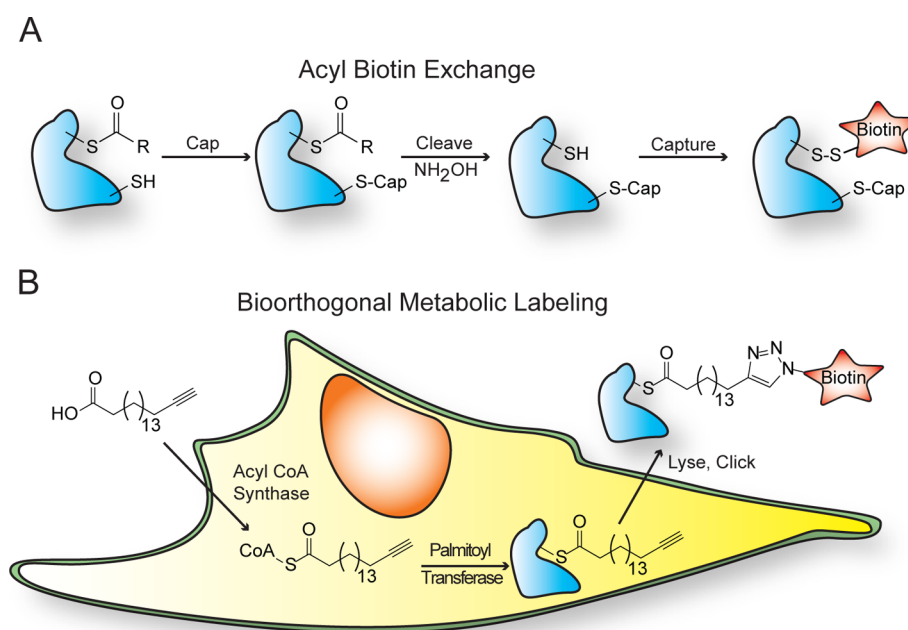
Figure 1. Chemo-proteomic methods for enriching palmitoylated proteins. (A) Acyl biotin exchange (ABE) describes the chemical hydrolysis and capture of thioesterified cysteines.[14] The modified acyl-RAC method uses activated thiol resin,[24] eliminating need for additional biotin capture steps. The thiol "cap" can be maleimide, iodoacetamide, or MMTS. (B) Bioorthogonal metabolic labeling describes the endogenous labeling of native palmitoylated proteins with the alkynyl palmitate analogue 17-octadecynoic acid (17-ODYA). After metabolic labeling, cells are lysed and conjugated to biotin-azide using click chemistry for streptavidin enrichment.[26]

by several common themes.[5] Cysteine-rich motifs are commonly palmitoylated, as are cysteines close to the N- or C-termini. Palmitoylation is also commonly observed proximal to the cytosolic side of transmembrane domains or coupled with N-terminal myristoylation or C-terminal prenylation sites. Computational algorithms are also available as a starting point for characterizing and validating sites of palmitoylation.[13] Palmitoylation sites are routinely validated by cysteine mutagenesis and then assayed by fluorescence microscopy to visualize improper trafficking or biochemically by metabolic labeling.

PALMITOYLATION ENRICHMENT METHODS

The targets of protein palmitoylation have only recently begun to be annotated on a proteome-wide scale. This was accomplished first in yeast with the advent of a novel enrichment technique based on selective thioester hydrolysis by hydroxylamine, termed acyl biotin exchange (ABE)[14,15] (Figure 1A). In this multistep protocol, free thiols are first alkylated or capped, and then half the sample is treated with hydroxylamine to selectively cleave thioesters. The newly exposed cysteines are then disulfide-bonded to a biotin analogue, affinity purified, and digested into peptides, leaving the labeled peptides on the affinity beads, or the eluant is then analyzed by liquid chromatography—mass spectrometry (LC—MS) for protein identification. Using this approach, approximately 50 yeast palmitoylated proteins were qualitatively measured using spectral counting,[15] an analysis method shown to correlate with protein abundance over a limited dynamic range.[16] This approach has since been applied to mouse brain synaptosomes,[17] lipid rafts,[18] B-cells,[19] platelets,[20] endothelial cells,[21] and apicomplexans.[22,23] A simplified version of ABE, termed acyl-RAC (resin-assisted capture),[24] uses activated-thiol sepharose to eliminate the biotin enrichment steps to simplify

the purification scheme. Furthermore, individual sites of palmitoylation can be mapped by disulfide reduction and peptide elution for orthogonal alkylation and LC—MS proteomic identification. More importantly, ABE methods have essentially replaced radioactive metabolic labeling for gel-based analysis of palmitoylation. After ABE enrichment, eluted proteins are separated by SDS-PAGE for Western blot analysis of thioester-dependent enrichment (i.e., palmitoylation).[17] ABE enriches all cellular thioesters, including lipoic acid conjugated dehydrogenases, and long-lived acyl intermediates of ubiquitin ligases, which increases the false-positive rate and further complicates the final analysis.[25] Finally, the enrichment fidelity is totally dependent on complete reduction and alkylation to eliminate capture of nonpalmitoylated thiols.

A second labeling method uses metabolic labeling of bioorthogonal fatty acids for click chemistry conjugation for fluorescent detection or enrichment[26−28] (Figure 1B). The fatty acid analogue 17-octadecynoic acid (17-ODYA) is commercially available and inexpensive and demonstrates contrast significantly higher than that of analogous azido-fatty acids.[26] Cultured cells are metabolically labeled with 17-ODYA for several hours, then lysed, and conjugated to azide-linked reporters using standard click chemistry methods. This approach offers several unique advantages, most importantly temporal control of labeling, flexible choice of reporter azides, and a significant reduction in false positives. The simple labeling protocol is ideal for rapid fluorescent gel-based analysis of palmitoylation dynamics and provides a nonradioactive alternative to traditional [^{3}H]-palmitate pulse-chase methods. Biotin-azide conjugation facilitates straightforward streptavidin enrichment and mass spectrometry proteomics annotation. This approach was first used to annotate palmitoylated proteins in Jurkat T-cells[26] and later in mouse T-cell hybridomas,[29] dendritic cells,[30] neuronal stem cells,[31] and apicomplexans.[23]

Alkynyl fatty acids also rapidly incorporate into diverse cellular lipids, including phospholipids, triglycerides, and glycosylphosphatidylinositol-anchored proteins.[32] Depending on the cell type, alkynyl fatty acids are often shortened or lengthened by fatty acid synthase or β-oxidation.[26,32,33] Based on the rapid metabolism into both lipid and lipidation production, these are not direct tools for fluorescence microscopy of palmitoylated proteins.

Both 17-ODYA and ABE enrichment methods are now widely used for palmitoyl protein enrichment and mass spectrometry annotation. The recent implementation of isotopic labeling approaches dramatically increases the sensitivity of palmitoyl protein annotation by reducing the number of assigned fragmentation events required for quantification.[23,29,31] For example, light and heavy isotopically labeled neuronal stem cells were labeled with either 17-ODYA or palmitic acid, and lysates were mixed equally for click chemistry, enrichment, and quantitative mass spectrometry analysis.[31] Peptides selectively enriched >5-fold by 17-ODYA treatment or reduced >5-fold after hydroxylamine treatment were assigned as high confidence palmitoylated proteins. This led to the annotation of more than 300 palmitoylated proteins in neuronal stem cells and a similar but unique list of approximately 400 palmitoylated proteins in mouse T-cell hybridoma cells.[29] With each new proteomics data set, the number of annotated palmitoylated proteins continues to expand, stressing the ubiquitous role of protein palmitoylation across a large number of membrane localized proteins. As the analysis methods become more sensitive, such broad proteomics experiments do not directly address the stoichiometry of palmitoylation. Many of the newly identified palmitoylated proteins may represent only fractional site occupancy, possibly as the product of acyl-CoA thioester exchange. In order to understand the regulation of palmitoylated membrane proteins, new biochemical and mass spectrometry methods are needed to explore thioester occupancy at individual sites of protein palmitoylation.

Proteomic annotation of palmitoylation has stimulated our understanding of this dynamic, nonpolar modification. Individual sites of palmitoylation can be identified by ABE after reduction of the disulfide linkage and orthogonal alkylation for direct analysis.[18,24] This approach led to the assignment of several hundred sites of palmitoylation, yet the analysis is indirect and difficult to validate. Furthermore, both ABE and 17-ODYA ignore the native acyl chain attachment, either by metabolic replacement with an alkynyl analogue or by direct hydrolysis. Thioesters are stable to electrospray ionization, so where are the palmitoylated peptides in large-scale mass spectrometry proteomics data sets? It may be that standard C18 reverse phase chromatography and acetonitrile gradients are simply not optimized for analyzing highly nonpolar peptides and fail to efficiently resolve lipidated peptides under standard experimental conditions. The C-terminal farnesylated peptide from Rab3A elutes nearly 30 min after the elution of most HeLa cell lysate peptides and in the last few minutes of analysis.[34] Since palmitoylation generally occurs next to N-terminal myristoylation, C-terminal prenylation, or on adjacent cysteines, it is especially difficult to chromatographically resolve the majority of palmitoylated peptides with traditional methods. However, these properties alone may allow direct hydrophobic enrichment of native lipidated peptides. Optimization of sample preparation, columns, gradients, and analysis must be capable of addressing these issues and in time will establish new methods for direct analysis of palmitoylated peptides as well as the diversity and selectivity of acyl linkages on select proteins.

◼ PROTEIN ACYL TRANSFERASES

Protein palmitoylation is catalyzed by a conserved family of acyl-CoA dependent protein acyl transferases (PATs).[35] The enzyme family has four to six transmembrane domains encompassing a cytosolic cysteine-rich catalytic domain harboring the conserved Asp-His-His-Cys (DHHC) catalytic motif. Initial annotation named the gene family ZDHHCs, under the assumption the cysteine-rich active site contained zinc finger motifs. After an extended search for palmitoylation enzymes, two-dozen human PATs were identified in the past decade.[36−38] These enzymes are now referred to as DHHC proteins, although the original ZDHHC nomenclature remains as the formal identifiers. DHHC enzymes are numbered DHHC1 through DHHC24, although the number system does not include DHHC10. This is accounted for because there are two genomic loci encoding duplicated copies of DHHC11 (DHHC11a and DHHC11b), yielding a common protein product. Members vary in length from 29 to 81 kDa, suggesting a minimal catalytic motif with elaborated protein−protein interaction or regulatory domains. DHHC3, 5, 8, 14, and 23 have putative C-terminal PDZ binding motifs, while DHHC13 and 17 have N-terminal ankyrin repeats. Most DHHC enzymes are localized in the ER and golgi compartments, although DHHC5, 20, and 21 are localized to the plasma membrane in transfected cells.[39]

Genetic models are quickly highlighting the conserved functional importance of this recently annotated enzyme family in development and disease. DHHC13$^{-/-}$ (HIP14L) and DHHC17$^{-/-}$ (HIP14) mice have Huntington-like neurodegeneration,[40−42] possibly orchestrated through palmitoylation of vesicular proteins and synaptic proteins. A *N*-ethyl-*N*-nitrosourea mutagenesis-derived mutant of DHHC13 is truncated after the ankyrin repeats but before the DHHC catalytic domain.[43] These mice display alopecia, osteoporosis, and early death due to systemic amyloidosis. None of these phenotypes are observed in DHHC13$^{-/-}$ mice generated by gene-trap insertion in the first intron, suggesting that isolated expression of the N-terminus can act as a dominant negative. These results are especially interesting given the recent finding that DHHC17 also interacts *via* its ankyrin motifs with JNK to mediate neuroinflammation. This affect can be ameliorated by delivery of a synthetic competitive peptide fused to a protein transduction domain.[44] The hair loss mouse mutant *depilated* (*dep*) is caused by a point mutation in DHHC21,[45] which leads to hair follicle disregulation, as well as a loss in PCAM-1 surface expression in endothelial cells.[21] A Drosophila homologue most similar to DHHC9 and 14 is linked to planar cell polarity defects,[46] and the human homologues are disregulated in several human cancers. DHHC5, 8, 9, 12, 13, 15, and 17 have been linked to neuronal and cognitive deficits,[41,47−52] while DHHC2, 3, 9, 11, 14, 17, and 20 are thought to contribute to the onset or regulation of oncogenesis and metastasis.[53−59] Understanding the mechanism, substrates, and regulation of this diverse family of acyl transferases will require new genetic, analytical, and pharmacological tools.

Large quantities of select detergent-solubilized active PAT enzymes are readily purified from yeast or baculovirus expression systems, allowing more detailed kinetic analysis.[60,61] The enzymes are believed to function in a two-step "ping-pong"
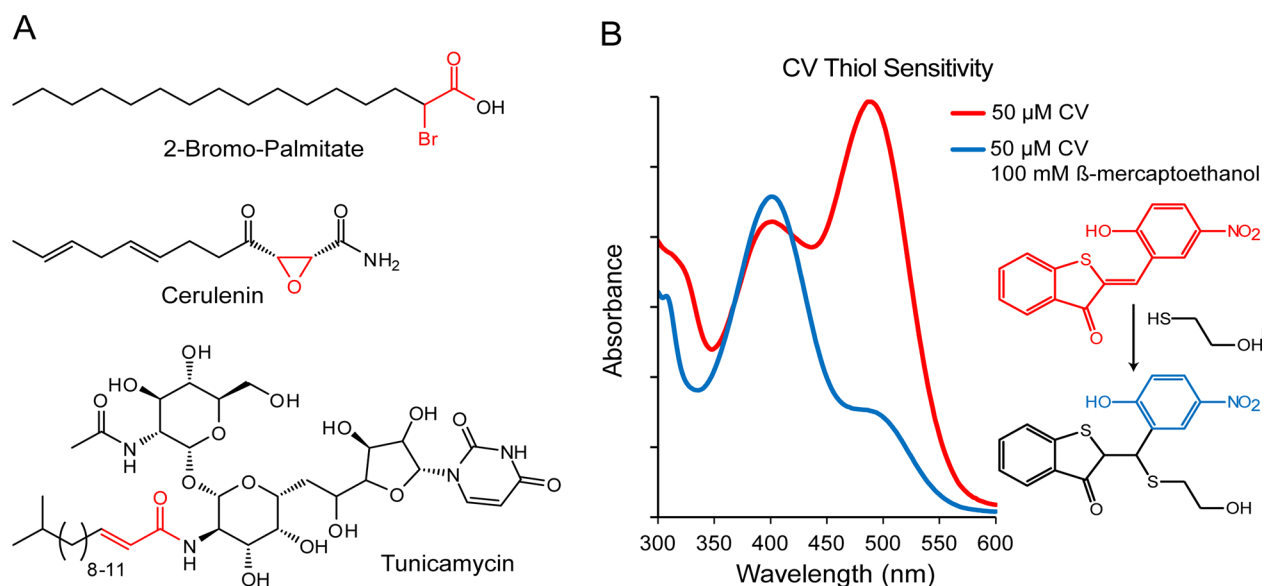
Figure 2. Small molecule inhibitors of DHHC protein acyl transferases. (A) 2-Bromo-palmitate, cerulenin, and tunicamycin are reported generic inhibitors of palmitoylation.[63−65] Electrophilic functional groups (red) facilitate irreversible alkylation. (B) The *p*-nitrophenyl benzylidine inhibitor, Compound V (CV),[68] absorbs visible light. The benzylidine Michael acceptor reacts with excess *β*-mercaptoethanol, breaking the conjugation and shifting the absorbance to the *p*-nitrophenyl chromophore (unpublished data). Experiments were performed at RT in phosphate-buffered saline at the concentrations listed.

acyl transfer reaction.[60,62] First, the active site nucleophilic cysteine attacks palmitoyl-CoA, transferring the palmitate as a thioester linkage. After the autopalmitoylation step, the substrate thiol attacks the active-site thioester, exchanging and palmitoylating the substrate. The DHHC active site encompasses a conserved cysteine-rich domain, yet mutation of the central cysteine (DHH**C**) to the corresponding serine (DHH**S**) completely abolishes autopalmitoylation. This does not exclude the possibility that other residues directly contribute to catalysis or whether acyl-transfer occurs between other active site catalytic cysteines. Without a detailed atomic structure, the precise mechanism of palmitoylation remains speculative. Interestingly, DHHC2 can autoacylate and transfer a variety of shorter and longer acyl chains, whereas DHHC3 has reduced autoacylation and acyl transfer of chains longer than palmitate.[60] This suggests discrimination by DHHC proteins for acyl-CoA substrates and potentially enzymatically programmed patterns of substrate acyl heterogeneity. Second, these findings point to possible mechanisms for selective inhibition of different DHHC proteins based on this substrate discrimination. These important studies set a foundation for the identification and design of novel DHHC inhibitors and chemical probes.

■ DHHC INHIBITORS

The most widely used inhibitor of protein palmitoylation is 2-bromo-palmitate (2BP),[63] an electrophilic *α*-brominated fatty acid. Other electrophilic palmitoylation inhibitors include the epoxide fatty acid synthase inhibitor cerulenin[64] and the *α,β*-unsaturated ketone N-glycosylation inhibitor tunicamycin[65] (Figure 2A). PATs contain an active site cysteine nucleophile that is thought to transfer palmitate from palmitoyl-CoA to protein substrates *via* an acyl-enzyme intermediate. Like iodoacetamide, 2BP is a highly reactive toward thiols and has long been known to alkylate many membrane-bound proteins and noncompetitively inhibit many thiol-dependent enzymes.[66] Nonetheless, it has become embedded into the literature as a

"specific" inhibitor of palmitoylation. The IC$_{50}$ of 2BP is 10−15 μM[67] and has been used in hundreds of papers to demonstrate PAT-dependent palmitoylation and trafficking of cellular proteins. Ironically, [^{14}C]-bromo-palmitate and [^{14}C]-bromo-palmitate-CoA were shown to nonselectively and covalently alkylate and inactivate numerous membrane-bound enzymes more than 20 years ago.[66] On the basis of these critical yet often overlooked findings, we strongly suggest that 2BP should not be used as direct proof of enzymatic palmitoylation but may be useful as complementary evidence along with DHHC knockdowns, point mutations, and mass spectrometry annotation. Given the potential off-target effects of 2BP inhibition, new selective inhibitors are essential research tools for dissecting the functional role of DHHC enzymes in the regulation of protein palmitoylation.

The first effort to discover drug-like inhibitors of palmitoylation used a cell-based high-throughput screen to identify several lead compounds.[68] This screen used a cell-permeable myristoylated or farnesylated palmitoylation substrate peptide linked to a NBD environmentally sensitive fluorophore. Upon palmitoylation and membrane recruitment, the substrate becomes highly fluorescent.[69] The fluorescent reporter peptides are presumably palmitoylated by several DHHC enzymes, and thus any active compounds are likely to demonstrate broad selectivity. After screening a commercial library of 17,000 compounds, a new palmitoylation inhibitor, 2-(2-hydroxy-5-nitro-benzylidene)-benzo[*b*]thiophen-3-one, or Compound V (CV), was identified with an observed IC$_{50}$ of 0.5 μM.[68] CV blocks the autoacylation of several purified DHHC enzymes[61] and inhibits cancer cell proliferation with IC$_{50}$ values as low as 2 μM in MCF7 cells and 9 μM in MDA-MB-231 cells.[68] Upon closer inspection, CV possesses an *α,β*-unsaturated ketone that may react with thiols through Michael addition. Solutions of CV are visibly red, suggesting the extended conjugation absorbs light in the visible spectrum. The compound shows a major absorbance peak at 487 nm and a lesser absorbance peak at 401 nm (the same as the absorbance
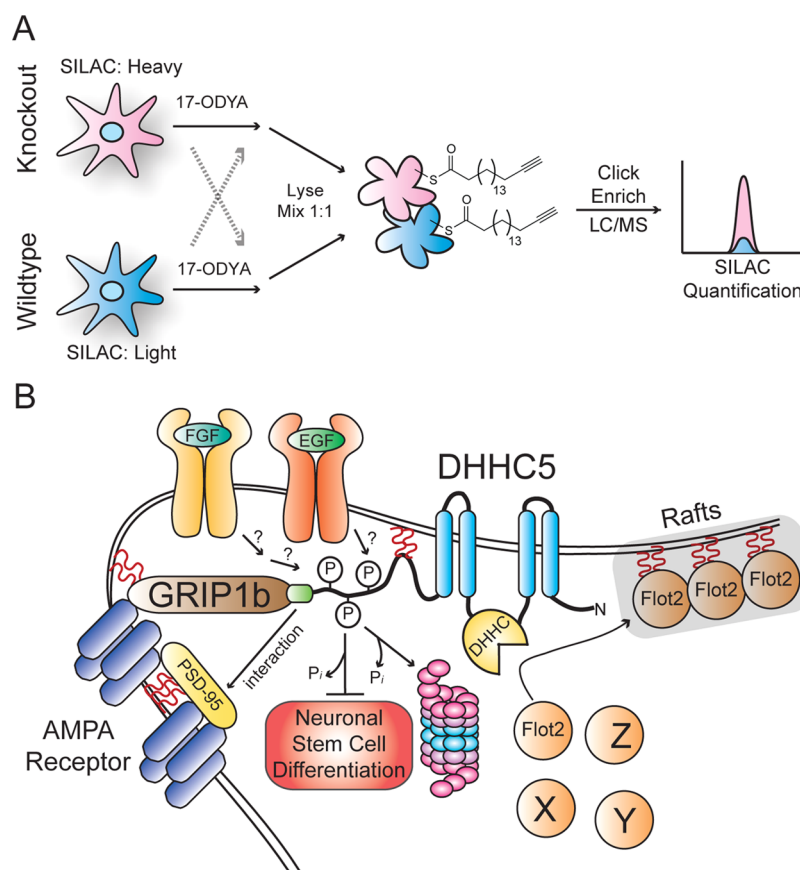
Figure 3. DHHC protein acyl transferase substrate identification by SILAC quantitative proteomics. (A) Knockout and wild type ZDHHC5 neuronal stem cells were grown in heavy and light SILAC media and metabolically labeled with 17-ODYA. Cells were mixed with either light (wt)/heavy (ko) or light (ko)/heavy (wt) in 1:1 ratios for click chemistry, enrichment, and quantitative LC−MS analysis.[31] (B) DHHC5 has distinct regulatory roles in neurons and neuronal stem cells. Flotillin2 is palmitoylated by DHHC5, leading to oligomerization and microdomain localization.[31] EGF and FGF signaling is required for DHHC5 stabilization,[31] likely mediated by multiple C-terminal sites of phosphorylation.[75] In the absence of growth factors, DHHC5 is rapidly degraded by the proteasome.[31] DHHC5 knockout neuronal stem cells fail to differentiate to neurons in culture.[31] The C-terminal PDZ-binding motif of DHHC5 facilitates interactions with GRIP1b and PSD-95.[47,74] GRIP1b is palmitoylated by DHHC5, which contributes to effective activity-dependent AMPA receptor recycling.[74]

of *p*-nitrophenol) (Figure 2B). In order to explore the reactivity and electrophilicity of CV, we measured the absorbance before and after treatment with 100 mM β-mercaptoethanol. Upon the addition of excess β-mercaptoethanol, the 487 nm peak is lost, consistent with covalent modification of the inhibitor by disruption of the conjugated benzylidine linkage. CV is a promising first step in the path toward developing selective chemical probes for the DHHC enzyme family and suggests that a focus on tuned electrophiles may yield probes with high affinity and selectivity.

■ ASSIGNING DHHC SUBSTRATES

To understand the pathways that regulate palmitoylation and validate select DHHC proteins as therapeutic targets, it is important to link individual DHHC enzymes to their corresponding substrates.[70] The most common approach is to cotransfect cDNAs for a select palmitoylated protein separately with each of the 23 DHHC enzymes and assay for enhanced palmitoylation. This approach was first used to assign DHHC2, 3, 7, and 15 as candidate PSD-95 palmitoylating enzymes[37] but cannot be considered comprehensive. DHHC9 requires coexpression of Gcp16 to reconstitute activity,[61,71] and several DHHC enzymes express poorly and do not autopalmitoylate upon heterologous expression.[72] More recently, RNA interfer-

ence has emerged as a more direct path to measure the DHHC contributions,[73] but variable knockdown efficiency and compensation further complicate substrate assignment. Thus, direct enzyme substrate relationships have been difficult to assign to distinct DHHC enzymes.

DHHC3, 5, 8, 14, and 23 have PDZ interaction motifs that are thought to facilitate protein−protein interactions that direct palmitoylation of distinct substrates. Of these proteins, DHHC5 is localized at the plasma membrane, is highly expressed in the brain, and is enriched in the postsynaptic density membrane fraction.[47] Two-hybrid analysis identified several PDZ-domain proteins that interact with DHHC5, including the synaptic scaffolding protein PSD-95 and the AMPA receptor trafficking protein Grip1.[47] Surprisingly, DHHC5 does not contribute to PSD-95 palmitoylation, suggesting a different mechanism of synaptic regulation. In mature cultured neurons, DHHC5 and DHHC8 are the major neuronal DHHC enzymes responsible for palmitoylating GRIP1b, which facilitates accelerated activity-dependent AMPA receptor recycling at dendritic endosomal vesicles.[74] Mice engineered to express a hypomorphic allele of DHHC5 are born at half the expected rate and show an approximate 90% reduction in DHHC5 protein levels.[47] Viable mice homozygous for this allele demonstrate a significant deficit in

Figure 4. Shared homology of *H. sapiens* (black) and *P. falciparum* (red) DHHC protein acyl transferases. Malaria parasites express 12 DHHC proteins, and humans express 23 DHHC proteins.

contextual fear conditioning, suggesting perturbed hippo-campal-dependent learning. Forebrain neuronal stem cells were isolated and passaged in media supplemented with heavy (+10 Arg, +8 Lys) or light media.[31] Cells were labeled in parallel with 17-ODYA, mixed, and analyzed by quantitative mass spectrometry to identify DHHC5-dependent changes in palmitoylation enrichment (Figure 3A). These data were compared with unenriched membrane proteome digests to correct for differential expression induced by DHHC5 reduction. In total, about 50 proteins demonstrated reduced 17-ODYA enrichment in DHHC5 hypomorphic cells, including the AMPA receptor subunit Gria4, neuronal cell adhesion molecule 2 (Ncam2), and phospholipase D1(Pld1).[31] Surprisingly, each protein shows a corresponding reduction in total protein abundance, signifying either DHHC5-dependent stabilization or expression. Flotillin-2 is a myristoylated and palmitoylated protein that was found to be a direct substrate of DHHC5 (Figure 3B). Alternatively, Flotillin-1 palmitoylation and/or expression are dependent on DHHC5 levels. This example demonstrates a critical role of DHHC enzymes in the palmitoylation and stabilization of unique substrates in cells. Therefore, DHHC inhibitors may be useful to selectively target the degradation of a subset of palmitoylated membrane proteins. Importantly, this study also highlights a potential caveat to assigning DHHC substrates by proteomics using genetic systems with prolonged (germline) knockout of specific DHHC proteins. The observed destabilization and reduction in abundance may be avoidable by more rapid DHHC ablation, either by conditional knockouts, RNA interference, or through chemical inhibition.

Neuronal stem cells are cultured in serum-free culture by addition of EGF, FGF2, and heparin. Growth factor removal triggers neuronal differentiation and is coincident with rapid degradation of DHHC5[31] (Figure 3B). Surprisingly, DHHC5 is eliminated by proteasome-dependent degradation within minutes of growth factor withdrawal. These kinetics suggest DHHC5 is regulated directly by growth factor signaling, presumably *via* phosphorylation at the more than 60 annotated sites distal to the DHHC catalytic domain.[75] Furthermore, DHHC5 hypomorphic neuronal stem cells differentiate to astrocytes and are blocked from neuronal differentiation. These findings suggest that extracellular signals can directly regulate the stability of the palmitoylation machinery to modulate the localization, stability, and function of distinct sets of palmitoylated proteins. Furthermore, several DHHC enzymes are implicated in oncogenesis, which may be mediated by growth factor-dependent DHHC stabilization, resulting in aberrant palmitoylation of key oncogenic signaling modules. These findings suggest multiple models of DHHC regulation, which in turn modulate the profile of protein palmitoylation in many aspects of physiology.

INFECTION AND PALMITOYLATION

Protein palmitoylation is critical for the infection and replication of many human pathogens, including parasitic, fungal, bacterial, and viral infection. Eukaryotic pathogens orchestrate their own cellular palmitoylation. *Saccharomyces cerevisiae* and other fungi encode several DHHC palmitoyl transferases with significant compensatory roles in palmitoylation of at least 50 palmitoylated proteins.[15] The parasitic protozoa *Toxoplasma gondii* encodes 18 DHHC genes, and the malaria parasite *Plasmodium falciparum* encodes 12 DHHC genes. These parasitic DHHC proteins are significantly homologous with the 23 human DHHC proteins and represent essentially each branch of homology, suggesting conserved roles for palmitoylation across highly distinct organisms (Figure 4). Quantitative proteomic analysis of the asexual stage of *P. falciparum* identified more than 400 palmitoylated proteins with diverse functions in signaling, adhesion, invasion, and development.[23] This approach used metabolic labeling with isotopic amino acids (SILAC) and utilized both ABE and 17-ODYA enrichment methods. These complementary mass spectrometry data sets demonstrate the utility of combining analysis from both approaches to increase the confidence of annotation. Interestingly, the expression profiles of *P. falciparum* DHHC proteins are altered in different developmental stages, suggesting differential palmitoylation dynamics and substrates throughout the infectious lifecycle.

Prokaryotic and viral pathogens exploit the host palmitoylation machinery for infection and replication. Most human-targeted viral glycoproteins are palmitoylated, including human influenza virus hemagglutinin (HA), Ebola virus glycoprotein, the fusion (F) protein of measles virus, retrovirus (HIV) and filovirus (Ebola) glycoproteins, as well as other spike proteins from togaviruses (Sinbis), rhabdovirus (VSV), herpesvirus (CMV), and coronavirus (SARS).[76] These palmitoylated surface glycoproteins require palmitoylation for efficient spike-catalyzed membrane fusion. Additionally, many viruses

encode for palmitoylated viraporins and other peripheral membrane palmitoylated proteins critical for raft localization and virus budding. Little is known about the cellular enzymes that catalyze the palmitoylation of these infectious proteins. Palmitoylation of the Salmonella type III secretion system effector proteins SspH2 and SseI were also shown to be critical for bacterial effector functions.[77] Overexpression of several DHHC enzymes enhanced their palmitoylation, suggesting diverse DHHC proteins facilitate bacterial effector protein palmitoylation. Overall, the cellular palmitoylation machinery plays a significant role beyond regulating normal cellular function but also is critical for the infectious life cycle of most human pathogens.

■ PROFILING DYNAMIC PALMITOYLATION

Protein palmitoylation is distinct from other post-translational modifications, as it is inherently unstable due to its labile thioester linkage. Interestingly, several proteins, such as N-Ras, H-ras, G-proteins, and PSD-95, all undergo rapid, stimulus-dependent depalmitoylation.[7,8,78] These results suggest there are signals and enzymes that regulate thioester hydrolysis, which in turn regulates the membrane association and function of signaling proteins. Overexpression and microinjection studies have revealed rapid depalmitoylation and redistribution of palmitoylated Ras,[11,12] but more evidence is needed to support the generality of these findings on other native proteins. Palmitoylation turnover is generally assayed using radioactive pulse-chase methods but has more recently taken advantage of nonradioactive bioorthogonal labeling. In this approach, 17-ODYA is added to cells for 30 min to 2 h (pulse), and then cells are washed and incubated with 10-fold excess unlabeled palmitic acid for varying times (chase).[79] Based on this method, the rate of Lck depalmitoylation is enhanced after pervanadate stimulation in Jurkat T-cells, yet stabilized in the presence of the nonselective serine hydrolase inhibitor methyl arachidonyl fluorophosphonate (MAFP).[79] This data points to the presence of specific serine hydrolase(s) responsible for stimulus-dependent depalmitoylation. Furthermore, C-terminal proline isomerization accelerates H-ras depalmitoylation,[80] demonstrating an orthogonal mode of conformational regulation in promoting depalmitoylation.

In order to profile the global dynamics of palmitoylation stability, bioorthogonal pulse-chase labeling was combined with stable isotope labeling for quantitative proteomic analysis.[29] The generic lipase inhibitor hexadecylfluorophosphonate (HDFP) targets cellular lipases without inhibiting peptidases, proteases, and other small molecule hydrolases.[29] In order to assign dynamically palmitoylated proteins, light and heavy isotopically labeled cells were pulse labeled with 17-ODYA and harvested immediately or chased with excess palmitic acid for 4 h. In parallel, a second experiment used pulse-chase labeling to identify palmitoylation stabilized in the presence of HDFP. In combination, these unbiased experiments pointed to several key findings. First, the majority of palmitoylated proteins demonstrate similar half-lives, yet the turnover of a small subset of G-proteins, Ras-family GTPases, MAGUK-family PDZ scaffolding proteins, and LAP-family polarity proteins is significantly faster. Second, each protein with accelerated palmitoylation turnover kinetics is stabilized by HDFP treatment, and hence these proteins are direct enzymatic targets of HDFP-sensitive hydrolases. These studies do not analyze specific sites of palmitoylation and thus highlight proteins with fewer, simultaneously regulated sites of palmitoylation. The longer incubation times necessary for sensitive proteomic quantification prevent efficient washout of 17-ODYA yet surprisingly corroborate each reported dynamically regulated protein, while discovering new targets of enzymatic regulation. Overall, these experiments establish a new focused map of protein thioesterase targets and highlight a general mechanism for regulating the membrane association of key oncogenic and metastatic signaling pathways.

■ MUTUALLY COMPETITIVE MODIFICATIONS

After depalmitoylation, what is the fate of these newly exposed cysteines? Many signaling pathways reversibly modify cysteines by selective oxidation or palmitoylation. For example, growth factors receptors are coupled to NADPH oxidase (Nox), which upon stimulation triggers the local enzymatic generation of H_2O_2. This pool of H_2O_2 reacts with nearby cellular cysteines, which include the cysteine nucleophiles of tyrosine and lipid phosphatases.[81] The net effect is a reduction in phosphatase activity and sustained phosphorylation and activation. The reactive probe dimedone selectively reacts with sulfenylated cysteines (R-SOH) and has been coupled to biotin or alkynyl probes for enrichment and annotation of sulfenylation sites.[81] Recent evidence that mice fed high fat, high glucose diets have reduced H-ras palmitoylation (down 62%) caused by cysteine oxidation at sites of palmitoylation.[82] This competition may be caused by increased Nox signaling or elevated metabolism leading to unbalanced oxidant levels.

Protein nitrosylation also directly competes with palmitoylation at select cysteines.[83] Upon synaptic stimulation, the MAGUK family scaffolding protein PSD-95 is released from the postsynaptic density following rapid palmitoyl thioester hydrolysis. Next, activity-dependent calcium influx activates neuronal nitric oxide synthase (nNOS), which is stably associated with PSD-95 by PDZ interactions. This locally generated flux of NO directly nitrosylates the newly deacylated thiols, blocking further palmitoylation and membrane association. The mutually competitive modification of PSD-95 cysteines orchestrates synaptic release through an exchange of specific cysteine post-translational modifications. This example demonstrates a fundamentally new mode of protein regulation by mutually competitive cysteine modifications[83] but also raises the question of whether this is unique for PSD-95, or if many proteins undergo dynamic exchange of cysteine PTMs. Accordingly, the pulse-chase proteomics approaches may select for alternatively modified proteins, rather than absolute palmitoylation turnover kinetics. The functional interplay between reversible cysteine modifications will likely emerge as a common theme across many important signaling pathways.

■ PROTEIN THIOESTERASES

LYPLA1, or acyl-protein thioesterase 1 (APT1), was first described as a lysophosolipase[84] yet was later identified in soluble tissue fractions as a candidate H-ras depalmitoylating enzyme.[85] Deletion of LYPLA1 in yeast has no effect on viability or mating,[86] which rely on Ras and G-protein dependent signaling pathways. Overexpression of LYPLA1 accelerates depalmitoylation,[87] demonstrating the potential for *in vivo* regulation of depalmitoylation. Interestingly, the neuronal microRNA mi138 represses LYPLA1 translation at the synapse, and APT1 knockdown reduces synaptic spine volume.[88] The microRNA translational repression is released following activity-dependent proteosomal degradation of
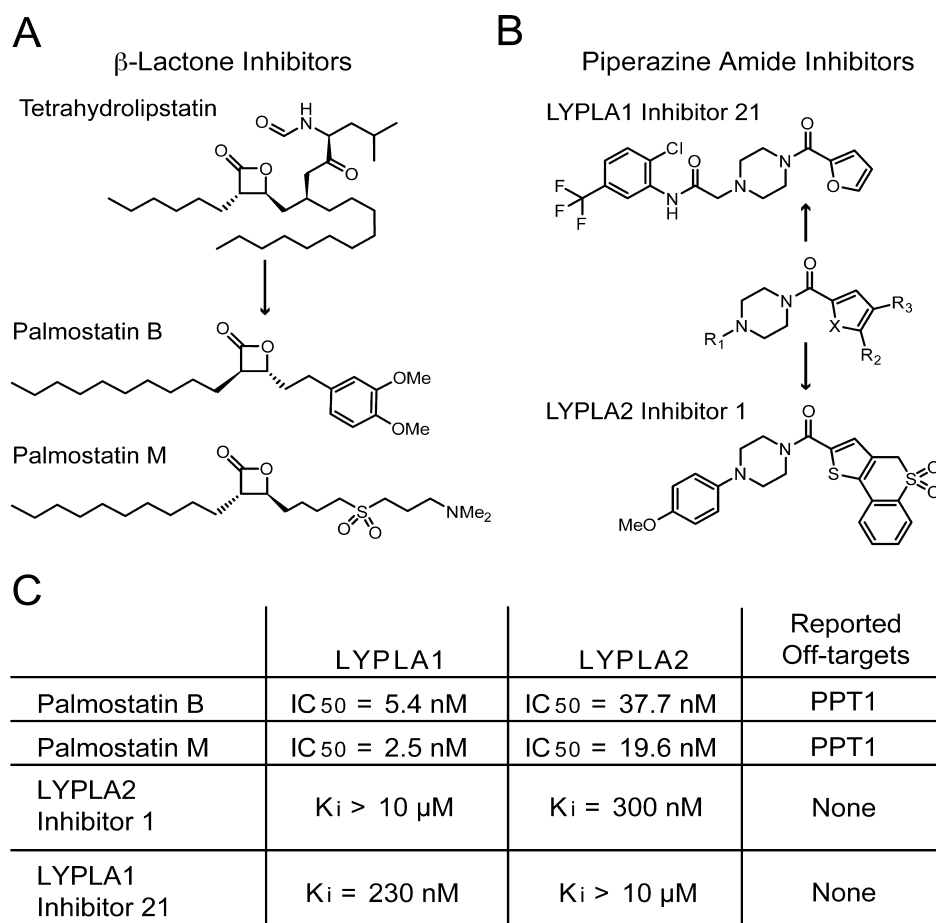
A β-Lactone Inhibitors

Tetrahydrolipstatin

Palmostatin B

Palmostatin M

B Piperazine Amide Inhibitors

LYPLA1 Inhibitor 21

LYPLA2 Inhibitor 1

C

	LYPLA1	LYPLA2	Reported Off-targets
Palmostatin B	$IC_{50} = 5.4$ nM	$IC_{50} = 37.7$ nM	PPT1
Palmostatin M	$IC_{50} = 2.5$ nM	$IC_{50} = 19.6$ nM	PPT1
LYPLA2 Inhibitor 1	$K_i > 10$ µM	$K_i = 300$ nM	None
LYPLA1 Inhibitor 21	$K_i = 230$ nM	$K_i > 10$ µM	None

Figure 5. Covalent and reversible inhibitor of protein thioesterase LYPLA1. (A) The tetrahydrolipstatin β-lactone derivatives Palmostatin B and Palmostatin M are mechanism-based, slowly reversible inhibitors of LYPLA1 and have been shown to redistribute N-Ras in cultured cells.[92,95] (B) The piperazine amide scaffold was discovered by high-throughput screening, and selective compounds were identified that inhibit LYPLA1 and LYPLA2.[98] (C) The potency of each inhibitor for both LYPLA1 and LYPLA2 are listed, along with reported off-targets.[94,98,100] IC_{50} values are shown for Palmostatin derivatives, due to the covalent mechanism. K_i values are reported for the piperazine amide reversible inhibitors.

MOV10,[89] a component of the RISC complex. MOV10 knockdown induces the accumulation of many synaptic RNAs, and surprisingly, some of the greatest changes occurred in LYPLA1 and the palmitoyl transferases DHHC2, DHHC15, and DHHC17. More experiments are needed to validate if activity-dependent translation of these palmitoylation regulators is important for synaptic function. While no genetic data is available for the close homologue LYPLA2 (68% homology), mutations nearby to the distant homologue LYPLAL1 (34% homology) locus are highly correlated with elevated visceral fat in women.[90] More experimental data is needed to explain the metabolic link between LYPLAL1 expression and fat deposition.

The FDA approved weight loss drug tetrahydrolipstatin (Orlistat) nonselectively inhibits digestive lipases and prevents fat absorption. This natural product has an electrophilic β-lactone that covalently inactivates certain serine hydrolases[91] yet is reversible following slow hydrolysis of the covalent acyl intermediate. Careful optimization of this electrophilic scaffold led to the stereospecific LYPLA1 inhibitor, Palmostatin B ($IC_{50} = 5$ nM)[92] (Figure 5A). Palmostatin B prevents depalmitoylation of semisynthetic or fluorescent protein fusions of H-Ras and N-Ras in cells, leading to redistribution and accumulation at the Golgi. Overnight incubation with 50 µM Palmostatin B re-established E-Cadherin plasma membrane distribution and

led to a partial phenotypic reversion to a more polarized phenotype, although at such high concentrations there is a greater opportunity for nonselective inhibition of other serine hydrolases. Similar experiments in Ras transformed myeloid cells show Palmostatin B selective growth inhibition of palmitoylated Ras isoforms (N-, H-Ras), but not K-ras4B.[93] Further optimization of Palmostatin based on lysophosphatidylcholine mimicry led to the discovery of Palmostatin M, with enhanced solubility and potency ($IC_{50} = 2$ nM).[94] The inhibition profile of Palmostatin M was profiled using a Palmostatin-derived activity-based probe and a competitive profiling strategy.[95] Proteomic analysis identified LYPLA1, LYPLA2, and the lysosomal thioesterase palmitoyl protein thioesterase 1 (PPT1) as potent targets of Palmostatin B and M. PPT1 resides in the lumen of the lysosomal-endosomal system and is responsible for the degradation of yet unannotated small cysteine derived thioester metabolites.[96] Inactive PPT1 leads to the human disease infantile neuronal ceroid lipofuscinosis, characterized by accumulation of waxy autofluorescent lysosomal storage material, neurodegeneration, and early childhood death.[97] Inhibiting LYPLA1 may have significant potential in the suppression of oncogenic Ras signaling but will require more selective compounds with more desired drug-like properties.

Fluorophosphonate (FP) activity-based probes are powerful tools for assaying inhibitor potency and selectivity in native proteomes and are now common tools for serine hydrolase inhibitor development. In order to identify new selective inhibitors of LYPLA1 and LYPLA2, the competitive activity-based fluorescence polarization assay (FluoPol-ABPP) was used to screen the 315,004 compound NIH Molecular Library.[98] Inhibitor active site occupancy competes with FP-rhodamine labeling, resulting in attenuated fluorescence polarization. This simple high-throughput assay demonstrates robust time-dependent inhibition analysis and is ideal for assaying soluble serine hydrolases.[99] Separate full deck assays were performed for both LYPLA1 and LYPLA2, and compounds were filtered on the basis of reported cross-reactivity with other serine hydrolases RBBP9 and PME1. Of the top 250 compounds, nearly 1/3 contained a piperazine amide motif, highlighting a unique convergence on a common noncovalent, drug-like chemotype (Figure 5B). LYPLA1 and LYPLA2 react exceptionally fast with FP-rhodamine, reducing the effective kinetic window for analyzing reversible inhibitor active site competition. By switching to less reactive activity-based probes, potent and selective inhibitors were identified that retained *in vivo* activity and selectivity in mice. Importantly, these inhibitors are exceptionally selective between LYPLA1 (Inhibitor 21, K_i = 300 nM) and LYPLA2 (Inhibitor 1, K_i = 230 nM), providing the first pharmacological tools to individually dissect the functions of these related protein thioesterases. Overall, these new pharmacological tools are critical for *in vivo* analysis of dynamic protein palmitoylation (Figure 5C).

CONCLUSIONS

Protein palmitoylation has emerged as a ubiquitous post-translational modification important for the folding, trafficking, and function of a large fraction of membrane proteins. This reversible modification is enzymatically regulated by protein palmitoyl transferases and thioesterases. These enzymes have emerged from both biochemical and genetic studies as important regulators of mammalian development and physiology. New chemo-proteomics enrichment strategies are quickly defining the breadth of palmitoylated proteins and provide an analytical platform for proteome-wide profiling of dynamic palmitoylation. Mouse knockout models have been reported for 5 DHHC enzymes, and each strain exhibits distinct phenotypes, including cognitive defects, neurodegeneration, hair loss, and amyloidosis. These few examples emphasize the broad significance of the DHHC enzyme family and warrant further genetic, pharmacological, and structural exploration of this conserved enzyme family. Furthermore, the introduction of selective LYPLA1 and LYPLA2 inhibitors opens new opportunities to define the distinct contributions of distinct protein thioesterases in signal-dependent dynamic palmitoylation and mutually competitive cysteine modification.

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Notes

The authors declare no competing financial interest.

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