

REVIEW

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2, 1567Insights into auto-S-fatty acylation: targets,
druggability, and inhibitors

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Posttranslational S-fatty acylation (or S-palmitoylation) modulates protein localization and functions, and has been implicated in neurological, metabolic, and infectious diseases, and cancers. Auto-S-fatty acylation involves reactive cysteine residues in the proteins which directly react with fatty acyl-CoA through thioester transfer reactions, and is the first step in some palmitoyl acyltransferase (PAT)-mediated catalysis reactions. In addition, many structural proteins, transcription factors and adaptor proteins might possess such “enzyme-like” activities and undergo auto-S-fatty acylation upon fatty acyl-CoA binding. Auto-S-fatty acylated proteins represent a new class of potential drug targets, which often harbor lipid-binding hydrophobic pockets and reactive cysteine residues, providing potential binding sites for covalent and non-covalent modulators. Therefore, targeting auto-S-fatty acylation could be a promising avenue to pharmacologically intervene in important cellular signaling pathways. Here, we summarize the recent progress in understanding the regulation and functions of auto-S-fatty acylation in cell signaling and diseases. We highlight the druggability of auto-S-fatty acylated proteins, including PATs and other proteins, with potential *in silico* and rationalized drug design approaches. We also highlight structural analysis and examples of currently known small molecules targeting auto-S-fatty acylation, to gain insights into targeting this class of proteins, and to expand the “druggable” proteome.

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Post-translational protein fatty acylation covalently attaches a fatty acyl group to the amino acid residues of the protein. While several amino acid residues, such as lysine (Lys), serine (Ser), and cysteine (Cys), could be modified, S-fatty acylation on the cysteine residue is a major form of modification. The labile thioester bond enables dynamic cycling of protein acylation and de-acylation. Among the fatty acyl groups utilized in the modifications, the C16 palmitoyl group is the most common one. Therefore, S-fatty acylation is often referred to as S-palmitoylation. However, C14 myristoyl, C18 stearoyl, and other unsaturated acyl groups, such as C18:1 oleoyl, are also found in protein S-fatty acylation. Therefore, S-fatty acylation is a more accurate terminology to describe such modification and will be used in this review. Traditionally, S-fatty acylation has been known to regulate membrane association and trafficking of proteins by increasing their hydrophobicity. In addition, it has been shown that attachment of fatty acyl groups could regulate protein stability, co-factor binding and other properties. Therefore, such modifications could spatially and temporally fine-tune protein location, trafficking, stability, protein-protein interactions and activities.^{1–4} Such unique

features of S-fatty acylation make it indispensable and ubiquitous in highly orchestrated signaling networks.^{5,6}

S-Fatty acylation could be achieved through enzymatic and non-enzymatic processes. The evolutionarily conserved Asp-His-His-Cys (DHHC) domain containing palmitoyl acyltransferases (PATs)⁷ has been shown to mediate enzymatic S-fatty acylation of many membrane-bound proteins. Non-enzymatic S-fatty acylation, termed as auto-S-fatty acylation, involves a nucleophilic reaction between a Cys thiol group and a thioester moiety of fatty acyl-CoA.⁸ Previously, it has been suggested that the non-enzymatic S-fatty acylation is less likely to occur spontaneously under cellular physiological conditions, owing to low concentrations of free fatty acyl-CoA.⁹ However, such reactivity could be enhanced by increased concentration of fatty acyl-CoA, which could fluctuate due to the metabolic status of the cells. In addition, it has been shown that auto-S-fatty acylation is involved as the first step of several PAT-mediated catalysis reactions,¹⁰ in which fatty acyl-CoA reacts with the active site cysteine residue of the enzyme to form a thioester adduct *via* nucleophilic addition. Therefore, the unique nucleophilic properties of the cysteine residue in the protein micro-environment could also enhance auto-S-fatty acylation. Indeed, we and others have demonstrated that some proteins could undergo auto-S-fatty acylation similar to the first step of several PAT catalysis reactions upon binding to fatty acyl-CoA. Thus,

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important to understand and develop therapeutic agents to effectively modulate protein *S*-fatty acylation. However, the “druggability” of auto-*S*-fatty acylation has long been overlooked. Even for enzymatic *S*-palmitoylation, targeting the auto-*S*-fatty acylation step of several PATs would be a promising avenue for pharmacological intervention of these enzymes, which remain difficult to target with specific inhibitors. In this review, we will focus on the biological significance of auto-*S*-fatty acylation in human diseases and discuss the rationale of using small molecules to modulate auto-*S*-fatty acylation.

Auto-S-fatty acylation of non-enzymatic proteins and PATs

The public database SwissPalm predicts that roughly 10% of the human proteome are putative *S*-fatty acylated proteins.¹⁵ Recent evidence has well documented and linked the dysfunctions of protein *S*-fatty acylation with various diseases, including cancers¹⁶ and neurological diseases.^{17,18} Therefore, it is

Proteins	Assay	Other evidence	Comments	References
Bet 3	<i>In vitro</i> S-palmitoylation	Palmitoylated when purified from a PAT-deficiency system A hydrophobic pocket bound with palmitate in crystal structure	Strong evidence	19–21
TEADs	Cell-based ZDHHCs screening <i>In vitro</i> S-palmitoylation	Palmitoylated when purified from a PAT-deficiency system Mass spectrometry showed TEAD2 was modified by palmitoyl group <i>in vitro</i> A hydrophobic pocket bound with palmitate in crystal structure	Strong evidence	13 and 89
RFX3	<i>In vitro</i> fatty acylation Cell-based ZDHHCs screening	N/A	Strong evidence	22
SFV p62	<i>In vitro</i> S-palmitoylation	N/A	High concentration of palmitoyl-CoA Lack of cell-based PATs screening	23
Rhodopsin	<i>In vitro</i> S-palmitoylation	N/A	High concentration of palmitoyl-CoA Lack of cell-based PATs screening Contradictory <i>in vitro</i> results	24 and 25
Myelin proteolipid protein	<i>In vitro</i> S-palmitoylation	N/A	High concentration of palmitoyl-CoA	26
Myelin Po Glycoprotein			Lack of cell-based PATs screening	27
Gα				28
Erf2P	<i>In vitro</i> S-palmitoylation	N/A	Consistent evidence	29 and 31
Akr1p	<i>In vitro</i> S-palmitoylation			30
ZDHHC2	<i>In vitro</i> S-palmitoylation	Palmitoylated ZDHHC2 and 3 transfer palmitoyl group to substrates <i>in vitro</i>	Strong evidence	32
ZDHHC3	<i>In vitro</i> S-palmitoylation	A hydrophobic pocket bound with 2-bromopalmitate (2-BP) in crystal structure	Strong evidence	11
ZDHHC20	<i>In vitro</i> S-palmitoylation			
Zebrafish ZDHHC15		A hydrophobic pocket bound palmitate in modelled crystal structure		

[³H]-palmitoyl-CoA at physiological conditions *in vitro*.²⁰ A recent palmitoylated proteome study in yeast has confirmed that *S*-palmitoylation of Bet3 is not affected by the deficiency of any ZDHHC.²¹ Taken together, these results strongly support that Bet3 could undergo auto-*S*-fatty acylation (or auto-*S*-palmitoylation). Auto-*S*-fatty acylation has also been reported in two classes of transcriptional factors, TEADs and RFXs. Incubation of recombinant TEAD2 YBD (YAP-binding domain) with palmitoyl-CoA *in vitro* leads to strong *S*-palmitoylation,¹³ which was also confirmed by mass spectrometry analysis. Similarly, recombinant RFX3 protein could be auto-*S*-fatty acylated *in vitro*, and prefers the C18 stearoyl group for the modification.²² These reactions happen at physiologically relevant fatty acyl-CoA concentrations and neutral pH, supporting that auto-*S*-fatty acylation could happen under normal physiological conditions. Overexpression of each of the ZDHHC family of PATs failed to significantly enhance their fatty acylation levels, confirming that TEADs and RFX3 are auto-*S*-fatty acylated proteins. In addition, in the crystal structures of TEADs, a deep lipid-binding pocket has been identified to accommodate the fatty acyl chain, and the fatty acylated cysteine residue is located right at the entrance to the pocket, supporting its reactivity towards palmitoyl-CoA.

Although there are other proteins (Table 1) being reported as auto-*S*-fatty acylated, the evidence supporting such conclusions is relatively weak. Almost 40 years ago, the Schmidt group described that the Semliki Forest virus (SFV) glycoprotein precursor p62 is auto-*S*-fatty acylated in a cell-free system.²³ Unfortunately, the lack of genetic evidence made the conclusion ambiguous. The GPCR protein rhodopsin has been shown to undergo *S*-palmitoylation without any enzyme *in vitro*.²⁴ Interestingly, it was later reported that *S*-palmitoylation of rhodopsin could be dramatically enhanced by partially purified PAT (10 times higher than initial rate),²⁵ which suggests that enzyme-mediated *S*-palmitoylation of rhodopsin also occurs. However, it is not clear whether the auto-*S*-fatty acylation and enzymatic *S*-fatty acylation occur at the same site or not. It is possible that they are responsible for different sites or they are complementary for the same site *in vivo*. In addition, it is possible that such a surface reactive cysteine could have low basal activity for “auto-*S*-fatty acylation”, but certain PATs could significantly enhance their *S*-fatty acylation. Several other proteins, including the myelin proteolipid protein,²⁶ myelin P0-glycoprotein,²⁷ and G protein alpha subunit (Gα),²⁸ have also been reported to be auto-*S*-fatty acylated *in vitro*. However, the use of high palmitoyl-CoA concentrations and high pH values of the reaction buffer, as well as the lack of solid cellular functional evidence, makes such claims less convincing.

In addition to these non-enzymatic proteins, several PATs have been shown to be auto-*S*-acylated (Table 1), based on fatty acyl adduct formation on the active site cysteine residues. Almost two decades ago, Deschenes and Davis independently reported the identification of palmitoyl acyltransferases (PATs) in yeast, Erf2p/Erf4p and Akr1p respectively. The former is responsible for *S*-palmitoylation of Ras2,²⁹ whereas the latter mediates Yck2p *S*-palmitoylation.³⁰ These enzymes were also

observed to be *S*-palmitoylated, suggesting the formation of fatty acyl-enzyme adduct in the reactions. Auto-*S*-fatty acylation (*S*-palmitoylation) of Erf2 has been demonstrated by Deschenes *et al.* by using the [³H]-palmitoyl-CoA radioactivity assay in purified recombinant protein.³¹ This is the earliest observation that some PATs might initiate their catalysis through auto-*S*-fatty acylation. A two-step mechanism was proposed to depict the catalytic process for Erf2. In the first step, a deprotonated cysteine thiol group in the DHHC-domain attacks palmitoyl-CoA to generate a palmitoyl-enzyme intermediate, which then transfers the fatty acyl group to the substrates.

With the advance of protein purification techniques, more PATs have been shown to be auto-*S*-fatty acylated in biochemical assays. The Linder group³² detected significant signals of *S*-palmitoylation of ZDHHC2 and ZDHHC3, when purified enzymes were incubated with only palmitoyl-CoA *in vitro*. Palmitoylated ZDHHC2 and ZDHHC3 immediately transfer the palmitoyl moiety to the corresponding substrates after mixing with substrates, supporting a two-step ping-pong mechanism. Recently, the Banerjee group showed that treatment of human ZDHHC20 and zebrafish ZDHHC15 with palmitoyl-CoA *in vitro* would release free CoA, suggesting that ZDHHC20 undergoes auto-*S*-fatty acylation *in vitro*.¹¹ Auto-*S*-fatty acylated intermediates were not always detected for some PATs, and different models and assays could lead to confusing and contradicting results. For example, it has been shown that auto-*S*-fatty acylation of ZDHHC17³³ was below the detection limit in gel electrophoresis analysis. However, mass spectrometry studies suggested that ZDHHC17 is *S*-palmitoylated at the active site.³⁴ Taken together, it has been confirmed biochemically that ZDHHC2, ZDHHC3, ZDHHC15, and ZDHHC20 could undergo auto-*S*-fatty acylation as part of their catalytic processes. It is speculated that other ZDHHC proteins could also be auto-*S*-fatty acylated;¹⁰ however, more evidence is still needed to confirm that auto-*S*-fatty acylation is a conserved mechanism involved in all PATs-mediated catalysis.

Auto-*S*-fatty acylation in diseases

Auto-*S*-fatty acylation activities of PATs in diseases

The critical role of ZDHHCs in diseases has been extensively explored in cancers¹⁶ and neural diseases^{17,18} (Fig. 1). In addition, metabolic disorders, infectious diseases and inflammation³⁵ (such as viral infection and sepsis) have also been linked to the deregulation of ZDHHCs. To date, some of the PATs have been shown to have deregulated auto-*S*-fatty acylation levels in diseases, which will affect their PAT activities and lead to substrate dysfunction (Fig. 1).

ZDHHC9 has been identified as a critical regulator of neurological functions, and ZDHHC9 deficiency in hippocampal cultures leads to shorter dendritic arbors and fewer inhibitory synapses.^{36,37} Indeed, ZDHHC9 could activate the ERK pathway through mediating Ras *S*-palmitoylation.³⁸ Palmitoylated Ras locates to the plasma membrane and activates downstream MAPK signaling, leading to dendritic growth. On the



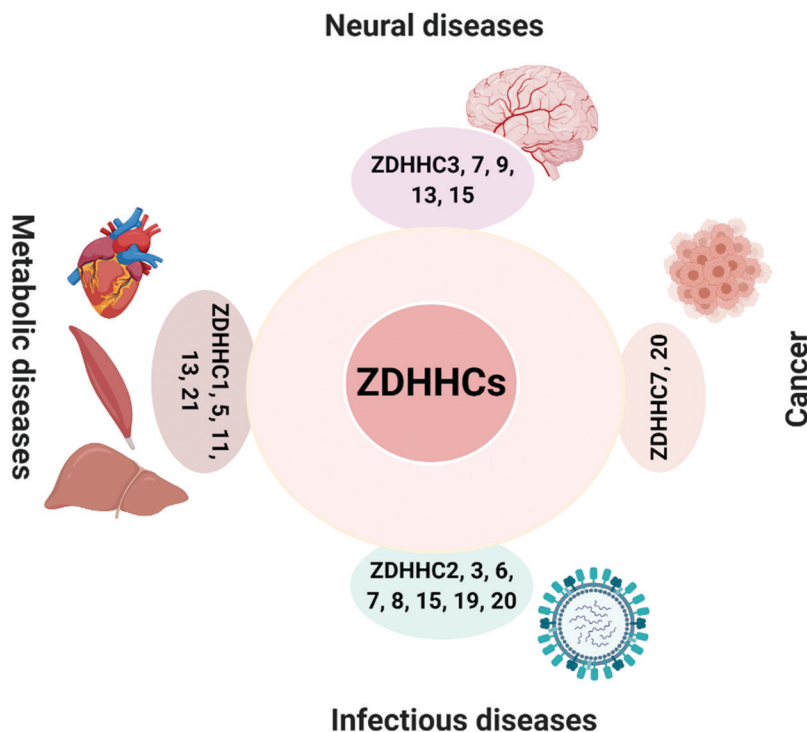


Fig. 1 ZDHHC-family of PATs in various human diseases. PATs are involved in neurological diseases, cancers, infectious diseases and metabolic diseases. ZDHHC3, 7, 9, 13 and 15 are involved in the pathogenesis of neurological diseases; ZDHHC7 and 20 are involved in cancers; ZDHHC1, 5, 11, 13 and 21 are involved in metabolic diseases; and ZDHHC2, 3, 6, 7, 8, 15, 19 and 20 are involved in infectious diseases. PATs, palmitoyl acyltransferases. The figure was created using BioRender.com.

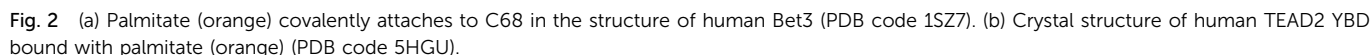
other hand, ZDHHC9 also fine-tunes inhibitory synapse formation by palmitoylating GTPase TC10 which is required for its localization at the plasma membrane and then motivating gephyrin clustering, and inhibitory synapse formation.³⁸ Thus, ZDHHC9 balances the excitatory and inhibitory signaling in neurological networks. Its loss-of-function mutations are implicated in neurology related diseases, such as X-linked intellectual disability (XLID) and epilepsy. These mutations impair its PAT enzyme activity by affecting its auto-S-fatty acylation levels, highlighting the importance of auto-S-fatty acylation in ZDHHC9 functions.³⁹ Consistently, disruption of *zdhhc9* in C57BL/6 mice leads to neurological impairment.⁴⁰

ZDHHC13 has been shown to play a critical role in maintaining anxiety-related behaviors, motor function, and brain bioenergetics,⁴¹ which is associated with a nonsense mutation in *zdhhc13*. In mice, this nonsense mutation results in a premature stop codon (L203X) and a truncated ZDHHC13 without the DHQC domain (generally shown as classic DHHC in PATs).⁴² Consistently, DHQC deletion abolishes its auto-S-fatty acylation activity, thereby exhibiting a loss of PAT activity. Such loss-of-function mutations of ZDHHC13 lead to hypo-S-palmitoylation of many substrates, including Drp1, resulting in the dysfunction of mitochondria morphology and distribution. In addition, dysfunction of ZDHHC13 has also been linked to metabolic and degenerative diseases. For example, *zdhhc13* with R425X mutation results in amyloidosis, alopecia, and osteoporosis in mice, which is probably associated with the

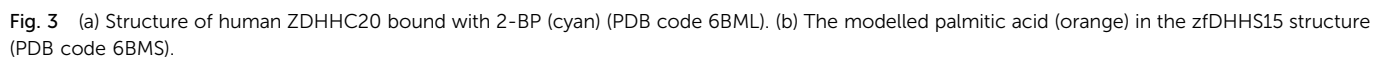
loss of its PAT enzyme function, as it is predicted that R425X mutation leads to a truncated protein lacking active site cysteine (C456).⁴³ Mechanistic studies showed that loss of ZDHHC13-mediated MT1-MMP S-palmitoylation was implicated in human osteoporosis.⁴⁴ ZDHHC13 (R425X) mutation leads to decreased S-palmitoylation of MT1-MMP and impaired association with VEGF and osteocalcin expression in chondrocytes and osteoblasts. In mouse hepatocytes, decreased ZDHHC13 auto-S-fatty acylation leads to liver dysfunctions, lipid abnormalities, and hypermetabolism.⁴⁵

ZDHHC3 and ZDHHC7 have been identified as palmitoylating enzymes for the neural cell adhesion molecule (NCAM).⁴⁶ S-palmitoylation of NCAM is required for its translocation to lipid rafts, where NCAM interacts with focal adhesion kinase (FAK) and fyn kinase to transiently activate MAPK/ERK signaling, thereby modulating fibroblast growth factor 2 (FGF2)-mediated neurite outgrowth, synaptogenesis, and synaptic plasticity. Interestingly, the FGF receptor (FGFR) and Src kinases phosphorylate ZDHHC3, and phosphorylation suppresses ZDHHC3 auto-S-fatty acylation and subsequent S-palmitoylation of NCAM,⁴⁷ revealing a potential feedback regulation of PAT activity. Caspase-6 (CASP6) plays a critical role in neurodegenerative diseases, such as Alzheimer's and Huntington's diseases.⁴⁸ Caspase-6 (CASP6) cleaves its substrates, amyloid precursor protein (APP) and the microtubule-associated protein tau, which may destabilize the microtubule and result in tangle formation. Therefore, activation of CASP6





reaction. The microenvironment surrounding the active cysteine might also contribute to the reactivity by deprotonating the cysteine thiol group, and stabilizing the transition state intermediate, thereby facilitating the departure of a leaving group. Furthermore, it is speculated that such lipid-binding pocket might be conserved among other auto-*S*-fatty acylated proteins, providing a basis for potential small molecule targeting. A similar lipid-binding pocket also exists in Bcr-Abl, a fusion oncoprotein implicated in chronic myelogenous leukemia (CML).⁷⁰ *N*-Myristoylation and binding of myristoyl group in Abl kinase lead to auto-inhibition of the kinase activities. Recently, small molecules binding to this myristoylation pocket, an allosteric site distinct from the ATP pocket, have been shown to overcome the resistance of inhibitors targeting the ATP site. In addition, some other proteins, such as UNC119⁷¹ and NOTUM,^{72,73} could be also targeted through



Smith and co-workers developed cell-based screening assays to look for inhibitors of PATs.⁸⁰ From a high throughput screening, they identified five compounds (I–V) as potential inhibitors of *S*-palmitoylation-related processes. Only compound V (Fig. 5a) was subsequently confirmed to suppress auto-*S*-fatty acylation of PATs in a reversible manner,⁸¹ which is validated with ZDHHC2 *in vitro*. However, compound V inhibits PAT activities of all four ZDHHCs tested, including yeast pfa3, yeast Erf2/Erf4, human ZDHHC2 and human ZDHHC9/GCP16. Despite these limitations, compound V might serve as a starting point for structure–activity relationship studies to develop more selective and potent PATs inhibitors, if its binding site on ZDHHCs can be determined. To further develop a more sensitive high throughput screening (HTS), Mitchell and co-workers exquisitely explored the detection of CoASH,⁸² which is released in the auto-*S*-fatty acylation step. The production of CoASH is monitored *via* a coupled fluorogenic assay catalyzed by α -ketoglutarate dehydrogenase complex (α -KDH), where α -ketoglutarate is converted to succinyl-CoA along with the formation of fluorescent NADH (Fig. 6).

It has been proposed that some PAT-mediated catalysis reactions require two steps, and it would be possible to have two strategies to modulate the activities of PATs (Fig. 4). One is to target the first auto-*S*-fatty acylation step, and the other focuses on the second step of acyl transfer to the substrates. For the second strategy, blocking acyl transfer could be achieved through inhibiting interaction between PATs and substrates. However, the substrate binding site of PATs is less defined for small molecule binding, and the presence of a hydrophobic cavity for lipid binding makes the former strategy more feasible. Although the hydrophobic pockets are conserved among different PATs, there are still significant differences of residues lining inside the pocket, suggesting that selectivity could be achieved. For example, at the end of the cavity of human ZDHHC20, Tyr181 interacts with Ser29 *via* a hydrogen bond to close off the cavity, whereas Ile182-Phe53 in ZDHHC3 and Ser185-Leu56 in ZDHHC7 act as homologous pair of Tyr181-Ser29 in hDHHC20. Similar to kinase-mediated phosphorylation, a common cofactor (ATP) might bind to pockets of similar shape in different proteins, but selectivity can still be successfully achieved. It is likely that selectivity could also be achieved for lipid-binding pockets of PATs and other auto-*S*-fatty acylated proteins. For example, TEAD inhibitor (MGH-CP1) does not inhibit the ZDHHC-family of proteins.⁷⁵ Furthermore, the intrinsic active cysteine in this pocket makes it possible to design covalent modulators, which expands the space for medicinal chemistry manipulation.

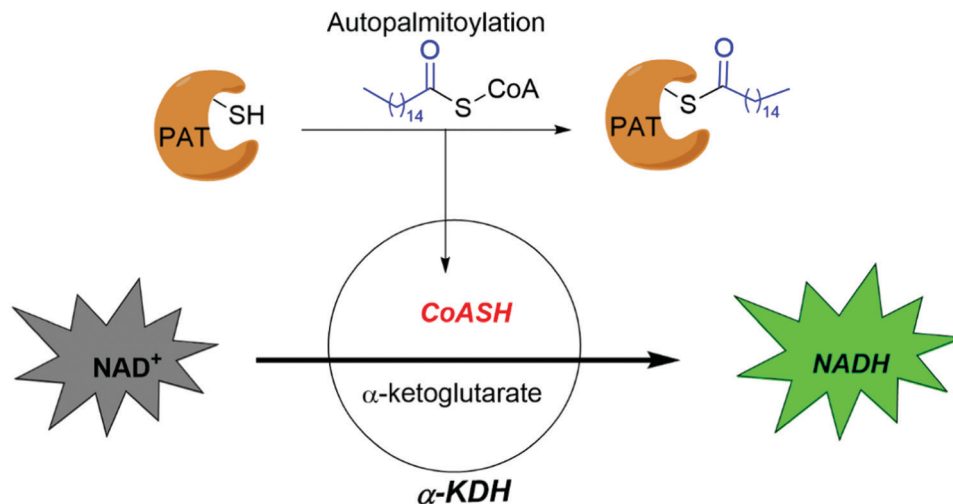


Fig. 6 High throughput assay developed by Mitchell *et al.*

different PATs in cells requires further confirmation. Given that Erf2 is the homolog of ZDHHC9, such reports might provide useful information for the discovery of ZDHHC9 inhibitors in the future. While there are other *S*-palmitoylation inhibitors discovered recently, such as inhibitors of Ras⁸⁴ and dual leucine-zipper kinase⁸⁵ (DLK) *S*-palmitoylation, we will not discuss them in this part due to their obscure mode-of-action.

Inhibitors of non-enzymatic auto-*S*-fatty acylation

The best-characterized example to target auto-*S*-fatty acylation of non-enzymatic proteins is the development of TEAD inhibitors (Fig. 5b). Deregulation of TEAD–YAP activity is involved in many human cancers.^{63,64} Developing inhibitors to disturb their interaction has been proposed as a promising strategy for drug discovery. However, given that the interface between TEAD and YAP is shallow and spans a large surface area, developing direct protein–protein interaction inhibitors might be difficult. The crystal structure of TEADs revealed that there is a highly conserved hydrophobic pocket near the active cysteine, where auto-*S*-fatty acylation occurs to stabilize TEAD structures and facilitate TEAD–YAP association. From a Pharmakon library screening, flufenamic acid (FA) (Fig. 5b) has been shown to bind to TEAD2 YBD.¹² The crystal structure of TEAD2 YBD bound to FA indicated that FA primarily binds to the *S*-palmitoylation pocket, suggesting that this pocket is targetable. However, no significant inhibition of TEAD–YAP binding by FA was detected by Meroueh *et al.* using the fluorescence polarization (FP) assay.⁸⁶ To improve the efficacy, they utilized the inherent active cysteine, which provides a strong foundation for covalent inhibitor discovery. In line with this hypothesis, the carboxylic group was replaced with an electrophilic chloromethyl ketone moiety as a warhead to irreversibly inhibit TEAD auto-*S*-fatty acylation by alkylating the active cysteine residue. TED-347 (Fig. 5b) was reported to form the expected covalent bond within palmitate-binding pocket. After treatment with TED-347 at 10 μM, viability of patient-derived glioblastoma cells, grown as three-dimensional spheroids, was inhibited by 30%.

In contrast, more than 100 μM temozolomide, the standard of care for glioblastoma, was required to reach comparable efficiency.

Similarly, the Luo group also succeeded by elaborately taking advantage of the active cysteine adjacent to the lipid binding pocket. Molecular docking-based virtual screening was first performed to characterize reversible binders, which are then modified with a reactive warhead. Finally, a series of vinylsulfonamide derivatives were developed to irreversibly inhibit TEAD auto-*S*-fatty acylation.⁸⁷ DC-TEADin02 attenuated TEAD4 auto-*S*-fatty acylation with an EC₅₀ of 197 ± 19 nM *in vitro*. Likewise, starting from FA, rational installation of a warhead by the Jänne and Gray groups leads to MYF-01-37 (Fig. 5b),⁸⁸ a covalent inhibitor of TEAD. TEAD–YAP is highly activated in EGFR-mutant non-small cell lung cancer (NSCLC) treated with EGFR (Osimertinib) and MEK inhibitor (Selumetinib), leading to acquired drug resistance. Co-treatment of MYF-01-37, Osimertinib and Selumetinib significantly boosts apoptosis. Recently, we reported that MGH-CP1 (Fig. 5b),⁷⁵ featured a phenylcarbamothioate scaffold, could reversibly and potently inhibit TEADs auto-*S*-fatty acylation *in vitro* with submicromolar IC₅₀. Blockade of the TEAD–YAP complex by MGH-CP1 leads to inhibition of downstream target genes in cells. In an APC-mutant intestine, MGH-CP1 treatment synergized with Last1/2 deletion to efficiently suppress epithelial over-proliferation and induce apoptosis, providing a new approach to treat APC-mutant cancers.

Although inhibition of TEAD *S*-fatty acylation is thought to disrupt TEAD–YAP association, as demonstrated by small molecules mentioned above, the detailed mode-of-action at the molecular level has not been fully elucidated. Recently, compound 2 (Fig. 5b), discovered by researchers at Genentech, suppressed downstream transcriptional activities of TEADs.⁸⁹ It is reported that compound 2 disrupts TEAD homeostasis, leading to inhibition of its binding to chromatin, thereby slowing down YAP-dependent tumor growth in a mouse xenograft model. Instead of blocking TEADs–YAP interactions,



compound 2 surprisingly surrogates palmitic acid to stabilize the cellular level of exogenous TEADs in HEK293T cells. However, endogenous pan-TEAD levels in several cancer cells did not show significant change after treatment of compound 2. These contradictory results would need further studies to be clarified. In addition, the results showing the association of TEADs and chromatin using *in vitro* biochemical and cell-based ChIP-seq experiments are also inconsistent. Therefore, the detailed mechanisms of how compound 2 inhibits TEAD are still elusive.⁹⁰ The therapeutic utilities of TEAD inhibitors are promising with accumulating evidence of TEAD inhibitors in cancer models, including VT103,⁹¹ which shows strong anti-tumor activity in NF2-deficient mesothelioma models *in vivo*.

Outlook

Non-enzymatic auto-*S*-fatty acylation has been largely overlooked over the years. It is understandable that enzyme-mediated process is more likely to be regulated and could be directly targeted for drug discovery.⁹² However, emerging evidence supports that some PATs are also auto-*S*-fatty acylated as the first step of the catalysis, implying that targeting auto-*S*-fatty acylation is one potential approach to modulate PATs. In addition, the functions of some important non-enzymatic proteins, such as TEADs and RFX3, are regulated by their auto-*S*-fatty acylation, and intracellular fatty acyl-CoA levels could directly affect their functions.

The crystal structures of auto-*S*-fatty acylated proteins all revealed a hydrophobic pocket to accommodate lipid binding. It is speculated that such hydrophobic pocket could be a common feature of many auto-*S*-fatty acylated proteins. This unique structural feature provides a basis for rational drug design. More importantly, the inherent active cysteine near this hydrophobic pocket allows the development of irreversible inhibitors. Although there is still no approved drug targeting auto-*S*-fatty acylation, small molecule inhibitors targeting auto-*S*-fatty acylation have been successfully shown to intervene in the functions of the proteins. This approach opens a door to target previously “undruggable” targets to expand the “druggable” proteome. Moreover, these compounds provide novel chemical tools to decipher the functions of protein auto-*S*-fatty acylation in complex biological networks.

One of the outstanding questions is how we can advance technological methods for an in-depth understanding of auto-*S*-fatty acylation. In recent years, progress in chemical biology approaches has accelerated profiling of auto-*S*-fatty acylation, such as identification of new auto-*S*-fatty acylated proteins. However, our knowledge of how auto-*S*-fatty acylation is regulated in cell signaling is still lacking. Considering that a large number of non-membrane bound proteins are *S*-fatty acylated in a biological system, a significant number of these proteins could be “auto-*S*-fatty acylated” as they are not normally co-localized with the membrane-bound ZDHHC-family of PATs. Therefore, designing new chemical probes with higher specificity and various reactivities would have a significant impact.

In addition, a lack of robust and sensitive HTS assays, such as the assays developed for kinases,⁹³ further hinders the discovery of potent inhibitors of auto-*S*-fatty acylation. Traditional auto-*S*-fatty acylation assay relies on gel-based methods, which are extremely low throughput and time-consuming. While the throughput has been improved with the enzyme-linked assay by Mitchell *et al.*, it still lacks sufficient sensitivity because of the low fluorescence signal of NADH. Incorporating more sensitive fluorophores or even luminogenic materials will probably increase the detection sensitivity. A high false positive rate also hinders its application. For example, the formation of CoASH might come from hydrolysis of palmitoyl-CoA. In addition, compounds which inactivate the α -KDH-catalyzed reaction will be annotated false-positively as inhibitors of auto-*S*-fatty acylation. Direct and precise assessment of auto-*S*-fatty acylation in a more robust, sensitive and high throughput fashion would have a significant impact on drug discovery.

Another outstanding question is how to specifically target auto-*S*-fatty acylation of an individual PAT. Known inhibitors, such as 2-BP, cerulenin and compound V, are pan-inhibitors and non-specific, and have many off-target effects. It is theoretically feasible to design selective small molecules against each PAT. First, the geometry of the hydrophobic pocket in ZDHHCs is slightly different, evidenced by the fact that ZDHHCs have a preference for lipids with different chain lengths.⁹⁴ Secondly, the residues in the palmitoyl binding pockets have significant diversity, as evidenced in human ZDHHC20 and zebrafish DHHS15. Current research indicates that some PATs mediate *S*-palmitoylation of more than one substrate. For example, ZDHHC13 could palmitoylate HTT, as well as proteins in the NF- κ B pathway. It is essential to understand the on-target adverse effects of PAT inhibitors. In addition to developing inhibitors of *S*-fatty acylation, allosteric activation of *S*-fatty acylation or inhibition of depalmitoylation could be important strategies as well. For example, hypoactivation of auto-*S*-fatty acylation of HIP14/ZDHHC17 and RFX3 is linked to diseases. Small molecules mimicking the binding and function of palmitate in the pocket might serve as activators of these proteins. Indeed, a small molecule activator of TEADs, which binds to the lipid binding site, has been reported.⁹⁵ Furthermore, the inhibition of de-fatty acylating enzymes, such as APT1/2 and ABHD proteins, might rescue the loss of *S*-fatty acylation in disease conditions.

Conflicts of interest

X. W. is the inventor of a patent application of TEAD auto-*S*-palmitoylation inhibitors (WO2017053706A1).

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