

Protein palmitoylation and cancer

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Abstract

Protein S-palmitoylation is a reversible post-translational modification that alters the localization, stability, and function of hundreds of proteins in the cell. S-palmitoylation is essential for the function of both oncogenes (e.g., NRAS and EGFR) and tumor suppressors (e.g., SCRIB, melanocortin 1 receptor). In mammalian cells, the thioesterification of palmitate to internal cysteine residues is catalyzed by 23 Asp-His-His-Cys (DHHC)-family palmitoyl S-acyltransferases while the removal of palmitate is catalyzed by serine hydrolases, including acyl-protein thioesterases (APTs). These enzymes modulate the function of important oncogenes and tumor suppressors and often display altered expression patterns in cancer. Targeting S-palmitoylation or the enzymes responsible for palmitoylation dynamics may therefore represent a candidate therapeutic strategy for certain cancers.

Keywords lipid; lipidation; post-translational modification; S-palmitoylation; tumor

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See the Glossary for abbreviations used in this article.

Introduction

Hallmark cancer cell phenotypes such as sustained proliferation, resistance to cell death, and increased metastasis result from the altered activity of intracellular signaling, metabolic, and gene regulatory networks [1,2]. The function of these networks, both in normal and cancer cells, is regulated by dynamic post-translational modifications (PTMs) that alter protein localization, stability, and function. Hundreds of distinct PTMs impacting thousands of proteins have been identified in mammalian cells [3,4]. Protein lipidation—the post-translational addition of a lipid to a protein—comprises one important and diverse class of PTMs [5]. Lipidated proteins generally have greater affinity for non-polar structures such as lipid bilayers, with important consequences for the localization, diffusion, and physical interactions of these proteins within the cell. Indeed, lipidation of key signaling proteins, such as Hedgehog, Wnt, and RAS, is essential for their function, both normally and in cancer [5–9].

Many protein lipidation events are thought to be essentially irreversible, including N-myristoylation, S-farnesylation, O-palmitoylation,

and N-palmitoylation. By contrast, protein S-palmitoylation (hereafter simply palmitoylation), which is the thioesterification of a sixteen-carbon saturated fatty acid (palmitate) to an internal cysteine residue, is a reversible modification. Indeed, proteins can cycle between palmitoylated and de-palmitoylated forms on time-scales that range from seconds to hours [10–12]. Dynamic palmitoylation can impact protein localization, accumulation, secretion, stability, and function by altering membrane affinity [5,9,13,14]. Understanding how protein palmitoylation influences the function of individual proteins in normal and cancer cells is an important driver of current research in this area.

Currently, several hundred mammalian proteins, many of which are cancer-related, have been discovered to be palmitoylated [4,15]. While certain proteins are mono-palmitoylated, examples of proteins with as many as five or six individual palmitoylated cysteine residues are known [16]. Some proteins spontaneously autopalmitoylate [17,18]. However, the majority of protein palmitoylation is catalyzed by the zinc finger DHHC-type containing (ZDHHC) family of palmitoyl S-acyltransferases (PATs), comprising 23 distinct proteins in mammals (Table 1) [19]. Most ZDHHC proteins are localized to the ER and Golgi apparatus, the primary sites of protein palmitoylation activity within mammalian cells [10,20], although at least three ZDHHC enzymes (ZDHHC5, ZDHHC20, ZDHHC21) appear primarily localized to the plasma membrane [21]. Protein deacylation can be catalyzed by acyl-protein thioesterases (APTs) 1/2 (APT1/2, LYPLA1/2) belonging to the α/β -hydrolase family of serine hydrolases [22]. Evidence links specific palmitoylated proteins and the function of individual PAT and APT enzymes to carcinogenesis and cancer cell growth, survival, and therapy resistance (Table 1, and see below). Thus, enzymes that modulate protein palmitoylation may be useful targets for anti-cancer treatments. The nature of protein palmitoylation, the enzymes involved, and the prospects for targeting these enzymes therapeutically are described below.

Protein S-palmitoylation: basic biochemical mechanisms

Studies of the *Saccharomyces cerevisiae* integral membrane proteins Erf2 (effect on ras function 2) and Akr1 (ankyrin-repeat containing 1) were the first to firmly establish the enzymatic basis of protein palmitoylation and provided the first insights into the structure and function of enzymes catalyzing this reaction [23–25] (Fig 1A). The recent crystal structure of human ZDHHC20 confirmed and extended these earlier biochemical studies [26]. ZDHHC20 adopts a

Glossary

APT	acyl-protein thioesterase
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3 related
cAMP	cyclic adenosine monophosphate
EGFR	epidermal growth factor receptor
Erf	effect on ras function
IP3R	inositol 1,4,5-triphosphate receptor
MC1R	melanocortin 1 receptor
PAT	palmitoyl S-acyltransferase
PTM	post-translational modification
ZDHHC	zinc finger DHHC-type containing

teepee-like structure (wide at the cytoplasmic side, narrow at the membrane-internal side) with the Asp-His-His-Cys (DHHC) enzyme active site located on the cytosolic linker between transmembrane domains 2 and 3. Here, it is positioned to interact with both palmitoyl-CoA and substrate proteins at the membrane–cytosol interface. Palmitoyl-CoA first reacts with the cysteine residue in the DHHC motif itself, forming an acyl-intermediate and releasing free CoA-SH (i.e., autopalmitylation). This intermediate is then transferred from the DHHC motif directly to substrate proteins in the cell.

Cysteine residues that lie near the catalytic DHHC motif coordinate two structural zinc atoms that are essential for proper enzyme folding and function, but do not play a catalytic role in palmitate transfer [26–28]. Despite overall amino acid similarity, the 23 human ZDHHC enzymes exhibit distinct abilities to autoacylate, implying differences in catalytic efficiency [29,30]. Of note, while often referred to in general as “palmitoylation”, this process can involve not only the addition of palmitate (C16:0), but also other fatty acids with different chain lengths (e.g., C18:0, stearoylation) [31]. Indeed, mammalian ZDHHC enzymes display distinct fatty acyl preferences [32,33], with the ability to preferentially accommodate particular fatty acyl chain lengths specified by amino acids that cap the hydrophobic cavity of the protein [26].

How ZDHHC enzymes select substrate proteins for modification is not entirely clear as there is no consensus palmitoylation sequence. Palmitoylated proteins are often substrates for more than one ZDHHC enzyme, but one particular ZDHHC enzyme often has a stronger effect than others on substrate palmitoylation in the cell (e.g. [29,34–36]). Chimeric ZDHHC proteins and structure–function studies have defined specific regions of individual ZDHHC enzymes that promote substrate interaction and palmitoylation, indicating that unique substrate-binding preferences could guide the palmitoylation of certain proteins [5]. For other proteins, palmitoylation requires an initial lipidation event (e.g., RAS C-terminal farnesylation, SRC-family N-terminal myristoylation) at an amino acid residue near the to-be palmitoylated Cys residue(s), which likely helps localize the substrate to ZDHHC-enriched membranes (e.g., the Golgi) [37–39]. Two ZDHHC enzymes, ZDHHC13 and ZDHHC17, contain unique C-terminal ankyrin-repeat domains that can bind certain proteins and enhance their membrane localization, facilitating palmitoylation by other ZDHHC enzymes [30,40]. Efficient palmitoylation by some DHHC enzymes (Erf2/ZDHHC9, ZDHHC6) requires an accessory protein (Erf4/GOLGA7, SELENOK), and speculatively, these accessory proteins could aid in substrate selection [24,41–44].

S-palmitoylation occurs on internal cysteine residues of substrate proteins, and within a given protein, only specific cysteine residues are S-palmitoylated. Despite progress in computational prediction [45,46], it remains difficult to determine *a priori* which specific cysteines within a given protein will be modified, and experimental trial-and-error remains essential to define palmitoylated residues (e.g. [47–49]). Due to the location and orientation of the DHHC motif, palmitoylation of integral membrane proteins occurs preferentially on cysteines located within eight angstroms of the membrane–cytosol interface, providing a structural constraint on the palmitoylation potential of certain residues [26,50]. For peripheral membrane proteins, the ZDHHC-substrate binding conformation appears to be important for effective palmitoylation to occur on specific residues; substrate mutations distal to the sites of palmitoylation can strongly impact the likelihood of modification [47,49]. How substrate conformation or binding influences palmitoylation remains to be worked out in detail.

The enzymatic removal of S-acyl modifications in mammalian cells is catalyzed by a family of serine hydrolases [51] (Fig 1B). The first enzyme, APT1, was reported to depalmitoylate the alpha subunit of G proteins and HRAS *in vitro* [52]. However, it appears that in cells APT2 is a cytosolic enzyme while APT1 is localized to the mitochondria, which may explain the selective effect of APT2 inhibition on the palmitoylation of cytosolic proteins [53,54]. APT1 and APT2 are not the only thioesterases in the cell and in fact do not impact the palmitoylation of key cancer-related proteins in the cell [11,54]; ABHD17A, ABHD17B, and ABHD17C are novel depalmitoylation enzymes that regulate the palmitoylation status of NRAS, for example [51,55,56]. Finally, palmitoyl protein thioesterases 1 and 2 (PPT1/2) localize to and deacylate proteins in the lysosome.

PAT and APT enzymes can exist within complex regulatory networks. ZDHHC5, ZDHHC6, and ZDHHC8 are palmitoylated at cysteine residues outside the catalytic DHHC motif [57], and these modifications are likely essential for normal enzyme localization and function. Recently, ZDHHC16 was shown to physically associate with and palmitoylate ZDHHC6 on Cys³²⁸, Cys³²⁹, and Cys³⁴³ residues found within the ZDHHC6 C-terminal Src homology 3 (SH3) domain [36] (Fig 2A). Modeling and experimental studies suggest that this palmitoylation “cascade”, from ZDHHC16 to ZDHHC6, enhances the overall stability and activity of ZDHHC6, although different configurations of modifications yield distinct activity and stability profiles. Palmitoylation of ZDHHC6 at these C-terminal cysteine residues is reversible by APT2, with turnover on Cys³²⁸ being especially rapid (Fig 2B). Adding further complexity to this network, APT1/2 and ABDH-family thioesterases are also themselves palmitoylated, and this modification is necessary for their proper localization and function [57,58]. For example, ABHD17A-C proteins have a highly conserved cluster of cysteines near the N-terminus that are required for proper localization to plasma and endosomal membranes and optimal catalytic activity [51]. PPT1 is also palmitoylated by ZDHHC3 and ZDHHC7, but in this case, palmitoylation decreases enzyme activity [59]. The function of both PAT (e.g., ZDHHC13, ZDHHC3) and APT (e.g., APT1) enzymes can also be regulated by phosphorylation in cancer and other cells, downstream of different signaling pathways [49,60,61]. Thus, a web of interconnected PTMs regulates both ZDHHC and thioesterase localization and activity, with

Table 1. List of ZDHHC genes, alternative names, associated properties, and links to cancer.

Gene	Alternative names	UniProt ID	Size (aa)	Localization	Cancer links
ZDHHC1		Q8WTX9	485	ER	High mRNA expression is a favorable prognostic marker in endometrial, renal, and pancreatic cancers (HPA)
ZDHHC2	REAM	Q9UIJ5	367	ER, Golgi	In a fusion gene in acute myeloid leukemia; in a region of deletion in hepatocellular carcinoma; high expression is a favorable prognostic marker in gastric cancer; high mRNA expression is a favorable prognostic marker in renal cancer (HPA)
ZDHHC3	GODZ	Q9NYG2	327	Golgi	High expression can be an unfavorable prognostic marker in breast carcinoma; in a region of recurrent loss in cervical cancer; high mRNA expression is a favorable prognostic marker in renal and colorectal cancer, but an unfavorable marker in liver cancer (HPA)
ZDHHC4		Q9NPG8	344	Golgi	High mRNA expression is a favorable prognostic marker in renal cancer, but an unfavorable marker in head and neck, urothelial, and cervical cancer (HPA)
ZDHHC5		Q9C0B5	715	Plasma membrane	High expression together with mutations in p53 are associated with reduced survival in glioma; high mRNA expression is an unfavorable prognostic marker in pancreatic cancer (HPA)
ZDHHC6		Q9H6R6	413	ER	–
ZDHHC7	SERZ-beta	Q9NXF8	308	Golgi	In a region of deletion in breast carcinoma; deleted in prostate and ovarian cancer; high mRNA expression is an unfavorable prognostic marker in renal and liver cancer (HPA)
ZDHHC8		Q9ULC8	765	Golgi	High mRNA expression is an unfavorable prognostic marker in renal and cervical cancer (HPA)
ZDHHC9	ZDHHC10, MMSA-1	Q9Y397	364	ER, Golgi	Required for palmitoylation of RAS proteins and Nras-driven leukemias; high expression in a range of cancers including breast and colorectal cancer, and multiple myeloma; high mRNA expression is an unfavorable prognostic marker in cervical, breast, and head and neck cancers (HPA)
ZDHHC11		Q9H8X9	412	ER	High expression in Burkitt lymphoma cells promotes proliferation; frequently deleted in hepatoblastoma; in a region of amplification in non-small-cell lung carcinoma and bladder cancers; high mRNA expression of the non-coding <i>ZDHHC11B</i> transcript is a favorable prognostic marker in lung cancer (HPA)
ZDHHC12		Q96GR4	267	ER, Golgi	–
ZDHHC13	HIP14L, HIP3RP	Q8IUH4	622	ER, Golgi	Required for protective function of MC1R against melanoma and to inhibit chemically induced papilloma
ZDHHC14		Q8IZN3	488	ER	Genomic deletions, mutations, and reduced expression in testicular germ cell tumor and prostate cancer; overexpression in scirrhous type gastric cancer and tongue squamous cell carcinoma; activating mutation (gene fusion) in acute biphenotypic leukemia; high mRNA expression is a favorable prognostic marker in pancreatic cancer (HPA)
ZDHHC15		Q96MV8	337	Golgi	–
ZDHHC16	Aph2	Q969W1	377	ER	–
ZDHHC17	HIP14, HIP3, HYPH	Q8IUH5	632	Golgi	High mRNA expression is an unfavorable prognostic marker in renal cancer (HPA)
ZDHHC18		Q9NUE0	388	Golgi	High mRNA expression is an unfavorable prognostic marker in renal cancer, liver cancer, and glioma (HPA)
ZDHHC19		Q8WVZ1	309	ER	–
ZDHHC20		Q5W0Z9	354	Plasma membrane	Elevated mRNA expression in ovarian, breast, kidney, colon, and prostate cancer tissue; high mRNA expression is an unfavorable prognostic marker in renal and pancreatic cancer (HPA)
ZDHHC21		Q8IVQ6	265	Plasma membrane	High mRNA expression is a favorable prognostic marker in urothelial and renal cancer (HPA)
ZDHHC22		Q8N966	263	ER, Golgi	–
ZDHHC23	NIDD	Q8IYP9	409	ER	High mRNA expression in B-precursor acute lymphoblastic leukemia; high mRNA expression is a favorable prognostic marker in renal cancer, but unfavorable in endometrial and thyroid cancer (HPA)

Table 1. (continued)

Gene	Alternative names	UniProt ID	Size (aa)	Localization	Cancer links
ZDHHC24		Q6UX98	284	ER	High mRNA expression is an unfavorable prognostic marker in glioblastoma (HPA)

Localization data are from [21] and the UniProt database. Cancer links is defined broadly as any literature link between a ZDHHC-family member and a particular cancer, including large-scale and small-scale experimental studies, animal and cell culture studies, and clinical studies, whether functional or correlative. References: ZDHHC2 [70,129,130], ZDHHC3 [131–133], ZDHHC5 [87], ZDHHC7 [47,134], ZDHHC9 [89,92,135,136], ZDHHC11 [77,78,137,138], ZDHHC13 [49,73], ZDHHC14 [82–85], ZDHHC20 [81], ZDHHC23 [139]. HPA, Human Protein Atlas (www.proteinatlas.org/).

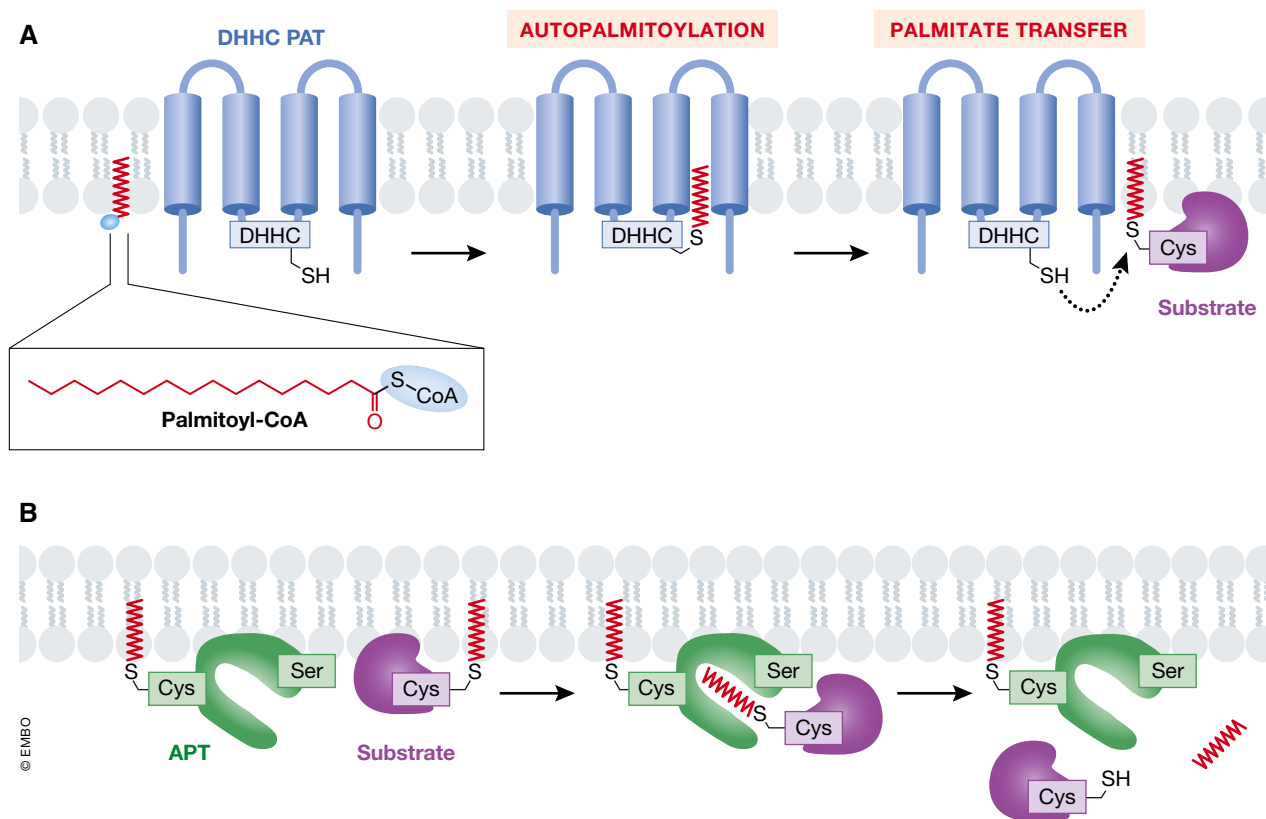


Figure 1. The biochemistry of protein palmitoylation.

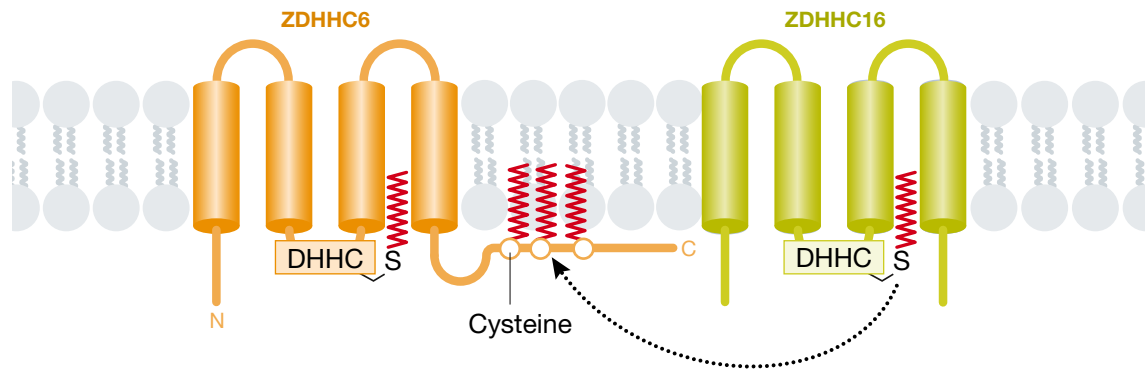
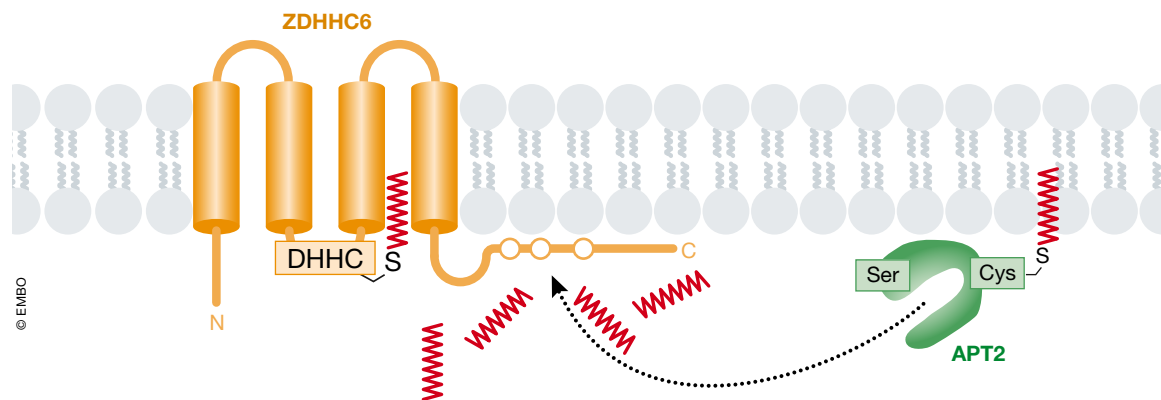
(A) Palmitate (derived from palmitoyl-CoA) can be thioesterified to substrate proteins by DHC (Asp-His-His-Cys)-family protein S-acyltransferases (PATs). DHC PATs are integral membrane proteins (blue) with the active site oriented toward the cytosol. These enzymes catalyze palmitoylation on internal cysteine (Cys) residues of substrate proteins (S-acylation). The DHC enzyme is first autopalmitylated on the DHC cysteine residue with the release of free coenzyme A (CoA), followed by a transfer of the palmitate group to the acceptor cysteine residue of a substrate protein (purple). (B) Acylprotein thioesterase (APT, green) can remove palmitate groups from palmitoylated proteins (purple). APT1/2 are themselves palmitoylated and contain a hydrophobic pocket to accept palmitoylated substrates and position the substrate palmitoylated cysteine near the active site serine (Ser) residue.

important implications for the regulation of cancer-associated substrates.

Protein palmitoylation and cancer: connecting the dots

Several important cancer-associated proteins are palmitoylated. A classic example is provided by the RAS family of small GTPases [62,63]. In addition to being irreversibly farnesylated at a conserved C-terminal CaaX sequence, HRAS, NRAS, and the KRAS-4A isoform (but not the KRAS-4B isoform) are reversibly S-palmitoylated at one (NRAS, KRAS-4A) or two (HRAS) internal cysteine residues. RAS

that is both farnesylated and palmitoylated has a 100-fold greater affinity for membranes than RAS that is only farnesylated [64]. Only the dually lipidated (i.e., farnesylated and palmitoylated) forms of HRAS, NRAS, and KRAS-4A are properly localized to the plasma membrane and capable of transforming cells, with the distinct patterns of lipid modification found between these closely related GTPases dictating trafficking and signaling properties [55,65–68]. Looking more broadly, intersecting a recent list of 299 validated cancer driver genes [69] with the results of fifteen palmitoylome studies currently available in SwissPalm suggests that 78 (26%) of the encoded proteins may be palmitoylated, highlighting the relevance of this modification to cancer (Fig 3). For most

A Palmitoylation cascade**B Specific removal of regulatory palmitoylation events****Figure 2. A highly connected web of palmitoylation events.**

(A) ER-resident ZDHHC6 can autopalmitylate, like other DHHC family members. In addition, three cysteine residues within the C-terminal tail of ZDHHC6 are palmitoylated by a distinct enzyme, ZDHHC16, representing a palmitoylation cascade. (B) The acylprotein thioesterase APT2, which itself requires palmitoylation to function normally, can remove the C-terminal palmitoyl groups on ZDHHC6.

cancer-relevant palmitoylated proteins, the responsible ZDHHC enzyme(s) is not known.

The role of protein palmitoylation in cancer can also be approached from the direction of the PAT and APT enzymes, many of which display associations with cancer. Here, we focus on ZDHHC enzymes that are linked to cancer as candidate oncoproteins, tumor suppressors, or prognostic markers (Table 1). For example, low *ZDHHC2* expression is found in gastric adenocarcinoma and associated with lymph node metastasis [70], and low *Zdhhc2* expression is found in highly metastatic murine colorectal adenocarcinoma clones [71,72]. Truncation mutations in *Zdhhc13* (*Hip14l*) increase susceptibility to chemically induced skin cancer in mice, possibly through enhanced NF- κ B signaling [73]. Thus, ZDHHC2 and ZDHHC13 could act as tumor suppressors, but links between these enzymes, candidate substrates [74–76], and tumor suppression are unclear.

A similar situation exists in relation to ZDHHC enzymes that may act as oncoproteins. *ZDHHC11* is found on chromosome 5 in a small region of recurrent amplification in non-small-cell lung and high-grade bladder cancers [77,78]. *ZDHHC17* (also known as HIP14) is upregulated in several tumor types, including breast and colon, and overexpression of this enzyme can also transform NIH

3T3 cells [79,80]. Overexpression of *ZDHHC20* can likewise transform NIH 3T3 cells [81]. The specific substrate protein(s) that link increased *ZDHHC11*, *ZDHHC17*, or *ZDHHC20* expression to cancer are not known.

In some cases, there are conflicting associations between ZDHHC expression and cancer. Many testicular germ cell tumors contain genomic deletions, mutations, or other alterations that reduce *ZDHHC14* expression, suggesting that this enzyme could act as a tumor suppressor in this disease [82]. Indeed, overexpression of *ZDHHC14* can slow xenograft tumor formation and induce apoptosis in cultured HEK 293 cells [82]. However, studies of scirrhous type gastric cancer, tongue squamous cell carcinoma, and a patient with acute biphenotypic leukemia indicate that expression and/or activation of ZDHHC14 could be oncogenic in these diseases through enhanced cell migration or reduced terminal differentiation [83–85]. In other cases, functional studies have disclosed a role for a particular ZDHHC enzyme in cancer development without any obvious change in gene or protein expression level. In lung cancer, ZDHHC5 expression levels are not associated with overall survival, but short interfering RNA (siRNA)-mediated silencing of *ZDHHC5* leads to reduced cell proliferation, colony formation, cell migration, and xenograft tumor growth [86]. By contrast, in glioma cells, high

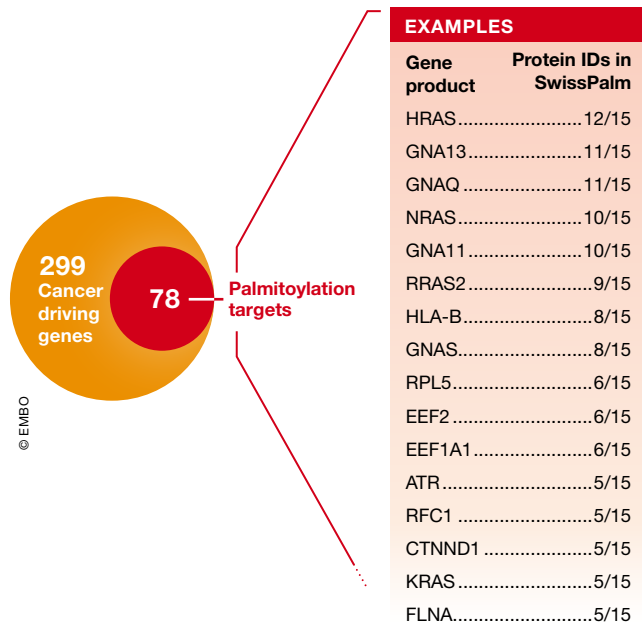


Figure 3. Many cancer drivers are palmitoylated.

Bailey *et al* [69] identified a set of 299 cancer drivers, 78 of which are annotated in SwissPalm (Dataset 3) as being palmitoylated in at least one of fifteen proteomics studies. A subset of the most frequently identified proteins are shown in the box, indicating how many times they have been discovered as palmitoylated in high-throughput studies (15 total).

ZDHHC5 expression is predictive of poorer prognosis, and this overexpression appears to be driven by oncogenic mutations in the tumor suppressor protein p53 [87].

These results suggest that individual ZDHHC enzymes can act as either oncoproteins or tumor suppressors in a tissue-specific and/or cancer type-specific manner. These opposing roles may be explained by differences in the relative expression of ZDHHC enzymes and one or more key substrates between tissues. The effect of deleting or overexpressing a single ZDHHC gene on cancer phenotypes could also be masked in a given tissue due to functional redundancy. Because of this complexity, how the expression of individual ZDHHC enzymes alters substrate palmitoylation to influence cancer phenotypes is often difficult to establish. To most clearly illustrate the different ways in which protein palmitoylation can impact cancer, we describe below in greater detail several recent examples where enzyme–substrate–phenotype “triads” have been more firmly established.

Regulation of oncogenic RAS signaling from the plasma membrane

Palmitoylation of HRAS, NRAS, and KRAS-4A at the Golgi ensures the proper delivery of these proteins to the plasma membrane via the secretory system [62]. *In vivo*, NRAS-driven leukemogenesis is palmitoylation-dependent: mice transplanted with bone marrow cells expressing oncogenic human NRAS^{G12D} die of leukemia-like diseases within 3 months, while those transplanted with cells expressing an activated but non-palmitoylatable mutant (NRAS^{G12D,C181S}) remain healthy for up to 2 years [88]. Mechanistically, inhibition of palmitoylation does not impair GTP loading of NRAS but rather prevents

its normal plasma membrane localization and the hyperactivation of downstream signaling effectors such as Akt, Erk1/2, and Ral [88]. This clearly demonstrates that oncogenic NRAS signaling requires palmitoylation-dependent plasma membrane localization. Similarly to *S. cerevisiae* Ras2, which is palmitoylated by the Erf2-Erf4 PAT complex, mammalian NRAS and HRAS are palmitoylated by an orthologous Golgi-localized complex comprising ZDHHC9 and GOLGA7 [42,89] (Fig 4A). Interestingly, mammalian GOLGA7 is itself palmitoylated by an unknown ZDHHC enzyme and this modification is essential for its stability and localization to the Golgi apparatus, as well as for ZDHHC9 function [42,90]. Indeed, GOLGA7 has been identified as a selective dependency in NRAS-mutant human AML cell lines, highlighting the importance of accessory subunits in ZDHHC function [91]. In mice lacking *Zdhhc9*, *Nras*^{G12D}-driven T-cell acute lymphocytic leukemia and chronic myelomonocytic leukemia are both significantly attenuated but not completely prevented [92]. The incomplete phenotypic suppression implies that additional PATs likely palmitoylate *Nras in vivo* [92].

Regulation of tumor suppressor localization to the plasma membrane

Another example of palmitoylation-dependent control over protein localization is provided by Scribble (SCRIB). SCRIB is a tumor suppressor critical for regulating epithelial cell polarity [93,94]. Loss of SCRIB inactivates the Hippo kinase cascade, leading to the accumulation of unphosphorylated Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) in the nucleus, and increased mitogen-activated protein kinase (MAPK) and AKT pathway activity [47,54,95,96]. To activate the Hippo kinase cascade and repress MAPK and AKT signaling, SCRIB must be localized to the plasma membrane. This localization is dependent upon palmitoylation of Cys⁴ and Cys¹⁰ in the N-terminus of SCRIB, most likely by ZDHHC7, but with other enzymes potentially contributing to this process [47,54] (Fig 4B). Overexpression of the transcription factor SNAIL promotes intracellular mislocalization of SCRIB, likely through the coordinate downregulation of multiple ZDHHC enzymes and upregulation of APT2, leading to loss of protein palmitoylation [54]. Treatment with the specific APT2 inhibitor ML349 results in stabilization of SCRIB at the plasma membrane by enhancing the palmitoylation of this protein [54]. While the effects of this treatment on SCRIB-dependent tumor cell growth *in vivo* have not been examined, the expectation is that increased membrane retention would result in decreased tumorigenicity.

Regulation of G protein-coupled receptor activity

In melanocytes, ZDHHC13-dependent palmitoylation of the plasma membrane α -melanocyte-stimulating hormone (α -MSH) receptor, melanocortin-1 receptor (MC1R), at Cys³¹⁵ is required for MC1R tumor suppressor function [49]. Specifically, only properly palmitoylated MC1R can stimulate sufficient cyclic adenosine monophosphate (cAMP) production to induce a protective transcriptional cascade in response to UV irradiation and consequent DNA damage. Cancer-sensitizing mutations (e.g., R151C and R160W, found in people with red hair or fair skin, termed red hair color (RHC) variants) reduce Cys³¹⁵ palmitoylation and attenuate cAMP production (Fig 4C). Whether this attenuation of signaling is due to mislocalization of the RHC-variant MC1R or due to loss of MC1R activity

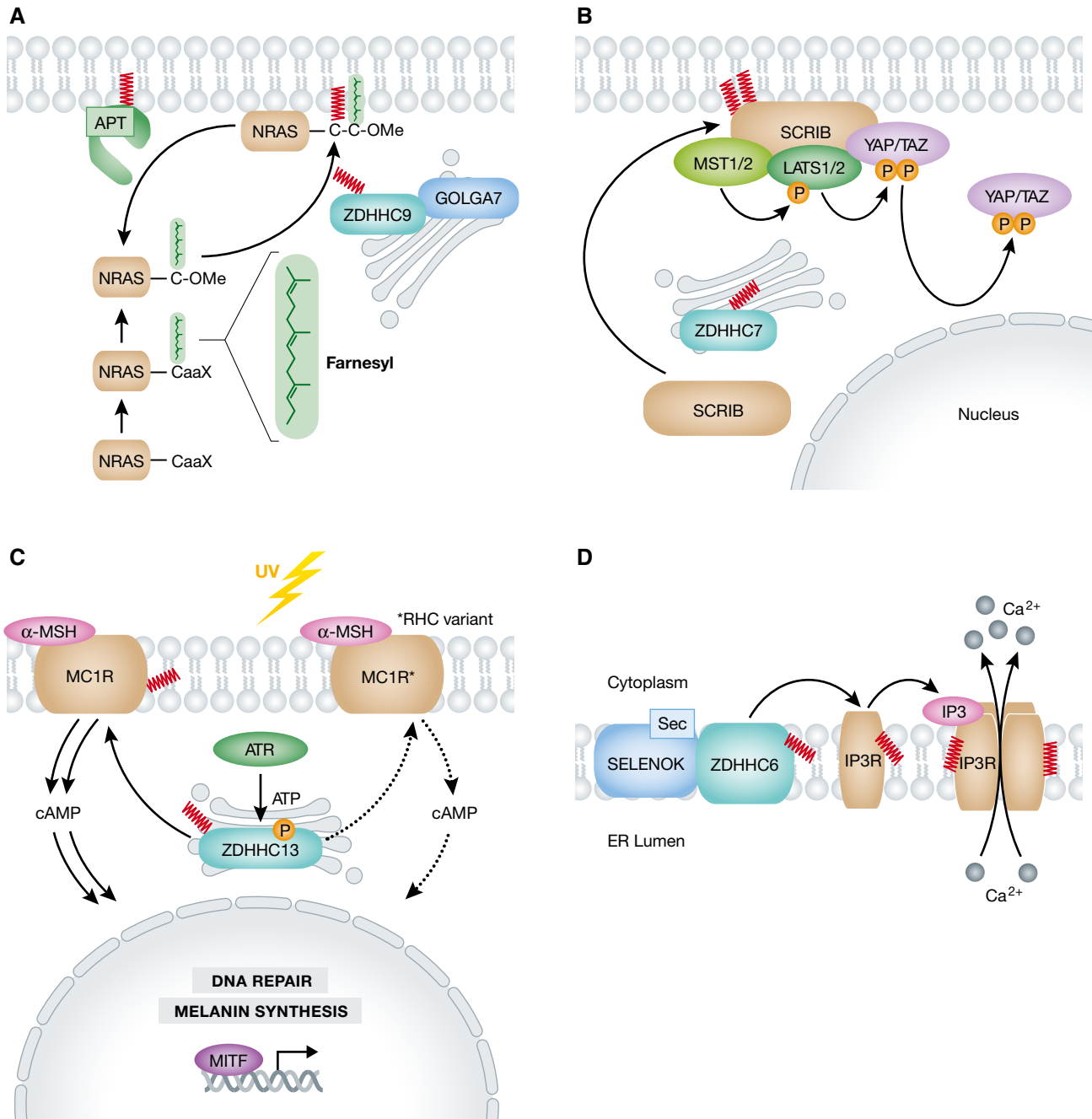


Figure 4. Examples of protein palmitoylation and cancer.
 (A) The NRAS GTPase has a conserved C-terminal CaaX motif that is farnesylated and O-methylated (OMe), then palmitoylated by the Golgi-resident ZDHHC9-GOLGA7 complex. Dually lipidated NRAS is properly anchored in the plasma membrane. (B) SCRIB is localized to the plasma membrane by ZDHHC7-catalyzed palmitoylation. SCRIB regulates the Hippo kinase cascade, ultimately phosphorylating YAP and TAZ, occluding them from the nucleus. (C) In response to ultraviolet (UV) radiation-induced DNA damage, ATR phosphorylates ZDHHC13, activating it. ZDHHC13 palmitoylates MC1R, which can then drive cAMP-dependent MITF transcriptional activation of melanin synthesis and DNA repair to mitigate DNA damage. Certain MC1R alleles, termed RHC variants, are found in cancer-sensitive populations and have mutations that reduce MC1R palmitoylation. This results in downregulation of the DNA damage response and sensitization of the cells to damage. (D) Inositol-1,4,5-triphosphate (IP3) activates the receptor IP3R, a tetrameric Ca²⁺ channel, to stimulate calcium release. IP3R monomers are palmitoylated by a ZDHHC6-SELENOK complex, with the selenocysteine (Sec) residue of SELENOK being essential to stabilize the autoacylated ZDHHC6 intermediate.

remains unclear. Regardless, this signaling defect can be rescued by overexpression of ZDHHC13 or treatment with the thioesterase inhibitor palmostatin B, which restores MC1R-RHC palmitoylation and attenuates the tumorigenic effects of UV irradiation in a

mouse BRAF^{V600E}-driven cancer model [49]. Thus, inhibition of depalmitoylation is a potential prophylactic strategy for individuals with certain cancer-prone mutations affecting palmitoylation of MC1R.

Regulation of ER calcium homeostasis and proliferation

Selenoprotein K (SELENOK) is a cofactor for ZDHHC6 that promotes its stability and activity [43]. One important substrate of the ER-resident ZDHHC6-SELENOK complex is the inositol 1,4,5-triphosphate receptor (IP3R, ITPR1) Ca²⁺ channel [44]. Silencing of *SELENOK* or *ZDHHC6* expression reduces palmitoylation of IP3R on at least two cysteine residues (Cys⁵⁶, Cys⁸⁴⁹), leading to protein destabilization, decreased IP₃-stimulated Ca²⁺ release from the ER to the cytosol, loss of Ca²⁺-stimulated calcineurin activity, and reduced cell adhesion and migration [44,97] (Fig 4D). In a mouse model of spontaneous melanoma driven by transgenic expression of glutamate receptor 1, deletion of *Selenok* reduced tumor formation on both ears and tail to control levels and also attenuated the appearance of metastatic cells within the lymph nodes [97]. This particular melanoma model is not lethal, and therefore, it was not possible to assess the effects of SELENOK inactivation on animal survival, but the results are consistent with the possibility that inhibition of ZDHHC6-SELENOK function could be beneficial in this cancer type. Whether this effect on tumor growth *in vivo* is mediated through palmitoylation of IP3R remains to be established. One interesting possibility is that this complex might, in addition to IP3R, also regulate the palmitoylation of other ER-resident proteins involved in Ca²⁺ handling and cancer metabolism, such as thioredoxin-related transmembrane protein 1 (TMX1) [98,99].

Targeting protein palmitoylation for cancer treatment

Inhibition of protein lipidation can be toxic to normal cells and was a main reason for the clinical failure of farnesyltransferase inhibitors (FTIs) developed to inhibit the farnesylation of RAS oncoproteins [100]. Notwithstanding this cautionary note, several lines of evidence provide a strong rationale for targeting PAT enzymes as a means to treat cancer. As described above, oncoproteins such as RAS-family GTPases require palmitoylation to promote tumor formation. Certain ZDHHC enzymes and cofactors (e.g., *Zdhhc9* [92], *Selenok* [97]) are essential for tumor formation and cancer progression but can be deleted in mice without gross effects on animal development. This suggests that cancer cells may develop non-oncogene addiction [101] to the function of specific ZDHHC enzymes and cofactors, such that normal tissues are much less sensitive to inhibition of these enzymes. In turn, this should translate into a therapeutic window enabling ZDHHC enzyme inhibition to arrest the growth of or kill cancer cells without adverse effects elsewhere in the body. RAS GTPases also provide an example of a protein that itself is a challenging drug target. For RAS and other potentially undruggable proteins, inhibiting oncoprotein function by blocking a key post-translational modification is an attractive strategy [102].

Currently, there are no potent and specific ZDHHC inhibitors. Broad-spectrum protein palmitoylation inhibitors like 2-bromopalmitate (2-BP) have been used in pre-clinical studies to validate the concept that ZDHHC protein inhibition can promote cancer cell death (e.g. [48,103,104]). However, 2-BP is not selective for individual ZDHHC enzymes and off-target acylation of other intracellular proteins makes it unsuitable as a drug lead or candidate therapeutic [105,106]. A screen of 30 million compounds using a scaffold ranking library approach for inhibitors of the yeast

ZDHHC9 ortholog Erf2 identified several bis-cyclic piperazines as candidate inhibitors of this enzyme with low micromolar activity in yeast [107]. Whether these lead compounds or derivatives thereof can inhibit mammalian ZDHHC enzymes in cancer cells remains to be determined.

An orthogonal approach to the development of ZDHHC inhibitors could exploit the recent crystal structure of ZDHHC20 to develop inhibitors using structure-guided methods [26]. However, structures of additional ZDHHC enzymes may be necessary to enable inhibitors specific for one enzyme over another to be found. In some cases, the process of discovering specific inhibitors could be facilitated by targeting unique domains. For example, the ankyrin-repeat domains unique to ZDHHC13 and ZDHHC17 recognize and bind consensus short linear motifs in normally unstructured regions of substrate proteins [30,40]. A small molecule could conceivably inhibit these protein–protein interactions, which as noted above are necessary for substrate palmitoylation by a different set of ZDHHC enzymes.

Many proteins can be palmitoylated by more than one ZDHHC enzyme [29,34,35]. This functional redundancy suggests that specific inhibitors of individual ZDHHC enzymes may not be as clinically useful as molecules that inhibit multiple family members, akin to the polypharmacology that is central to the efficacy of many kinase inhibitors used in cancer therapy [108]. One potential solution to this problem is to search for pan-ZDHHC inhibitors that can block the function of several enzymes required for the palmitoylation of a single target. For example, membrane preparations from Madin–Darby canine kidney (MDCK) cells were used as a source of PAT activity in a miniaturized screening assay looking for specific inhibitors of NRAS palmitoylation [109]. It remains to be seen whether this approach will yield any candidate inhibitors, but this approach is an exciting illustration of how technical challenges in this area are being overcome to develop useful screening assays. A complementary ZDHHC-agnostic approach is to identify compounds that directly prevent substrate palmitoylation via irreversible covalent modification of individual cysteine residues. For example, cell-based screening and structure–activity relationship analysis have yielded a series of compounds that covalently modify the stimulator of interferon genes (STING) protein in mouse and human cells at one specific cysteine residue (Cys⁹¹), blocking the normal function of this protein in promoting inflammation [110]. Identifying covalent modifiers for specific palmitoylated cysteine residues on cancer-associated proteins would eliminate the problem of functional redundancy between individual ZDHHC enzymes.

Rather than inhibiting protein palmitoylation, in some cases it may be more effective to prevent depalmitoylation. APTs in general are highly druggable targets [55,63], and as described above, inhibition of APT enzymes can suppress tumor formation by promoting the proper localization of SCRIB and enhancing the activity of MC1R [47,49,54]. This paradigm could be extended to other situations where reduced PAT expression or substrate palmitoylation leads to loss of a tumor-suppressive effect. For example, inhibition of ZDHHC7 function lowers the palmitoylation and membrane localization of the FAS death receptor, reducing the sensitivity of colorectal cancer cells to FAS ligand-induced cell death [111]. Inhibition of the appropriate thioesterase could enhance FAS receptor cell surface expression and the receipt of a lethal tumor-suppressive signal. However, the potential toxicity associated with long-term inhibition

of acylprotein thioesterases is an important consideration that would need to be addressed.

Beyond inhibition of APT1/2 to regulate tumor suppressor function, inhibition of lysosomal PPT1-mediated depalmitoylation may lead to a more direct toxic effect in cancer cells. Dual inhibition of PPT1 and eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)-dependent protein synthesis by the natural product didemnin B can trigger apoptosis in certain “exceptional responder” cell lines [112]. Here, apoptosis is caused by loss of the rapidly degraded pro-survival protein MCL1 and inhibition of PPT1, which may inhibit lysosomal acidification. Intriguingly, dimeric quinacrine compounds (e.g., DQ661) were recently described to inhibit PPT1 function and cause the concurrent inhibition of both mTOR signaling and autophagy, leading to protein synthesis inhibition, lysosomal membrane permeabilization, and apoptosis induction [113]. DQ661 inhibits the mTOR pathway by preventing RAGC, MTOR, and other key molecules from associating with the lysosomal surface. How this molecule blocks the adaptive catabolic process of autophagy, which is normally active in response to mTOR inhibition, is unclear, but could be as simple as an increase in lysosomal pH, as observed with didemnin B. The ability of DQ661 to simultaneously inhibit mTOR and autophagy is remarkable and may underlie the promising activity of this compound in models of melanoma, pancreatic, and colon cancers [113]. Identification of PPT1 substrates will contribute to a better understanding of the mechanism(s) by which DQ661 or didemnin B might cause disruption of lysosome function, which could occur through the inhibition of PPT1 and/or the accumulation of excess palmitoylated proteins.

How PPT1 inhibition can trigger cell death selectively in cancer cells is unclear. One possibility is that cancer cells have a higher number of palmitoylated proteins, or greater flux through the PPT1/lysosomal degradation pathway, rendering cells hypersensitive to any accumulation of palmitoylated proteins upon PPT1 inhibition. This would be analogous to how certain cancer cells are killed by the accumulation of reactive oxygen species (ROS) due to an already high pre-existing ROS load [114]. Interestingly, the small molecule caspase-independent lethal 56 (CIL56) triggers cancer cell death through an unknown pathway that is inhibited by deletion of *ZDHHC5* [115,116]. One possibility is that like didemnin B and DQ661, CIL56 inhibits PPT1 or a related thioesterase, resulting in *ZDHHC5*-driven accumulation of palmitoylated proteins to toxic levels. Further characterization of compounds that exhibit lethal mechanisms tied to specific *ZDHHC* enzymes could help unravel the function of these proteins and suggest new ways to target palmitoylation pathways.

Protein palmitoylation as a modulator of anti-cancer treatment efficacy

Above, we described how directly targeting PAT or APT/PPT enzymes could be beneficial for cancer prevention or treatment. Below, we describe how targeting protein palmitoylation could enhance the therapeutic effects of existing anti-cancer agents.

Activating mutations and amplifications of epidermal growth factor receptor (EGFR) are observed in non-small-cell lung, breast, and other cancers, leading to the development of targeted inhibitors such as gefitinib [117,118]. In non-small-cell lung carcinoma

cells, palmitoylation of EGFR on Cys⁷⁹⁷ by *ZDHHC1*, *ZDHHC2*, and/or *ZDHHC21* is essential for the stability, membrane localization, dimerization, and activation of this receptor [104]. Inhibition of EGFR palmitoylation using 2-BP therefore enhances the lethal potency of gefitinib [104]. In a similar vein, but through a distinct mechanism, *ZDHHC20*-dependent palmitoylation of EGFR at Cys¹⁰²⁵ within the C-terminal tail physically anchors this region of the receptor to the plasma membrane, preventing phosphorylation and activation of downstream ERK and AKT signaling pathways [48]. An EGFR mutant that cannot be palmitoylated at Cys¹⁰²⁵ has hyperactive EGFR pathway activity and exhibits a heightened dependency on (i.e., addiction to) EGFR signaling, such that the lethal potency of gefitinib is increased [48]. However, this mechanism is cell type specific, and is modulated by other mutations (e.g., *KRAS*), which means it may not be universal [103]. Though they describe different mechanisms, these reports show that combining an EGFR-specific kinase inhibitor with *ZDHHC* inhibitors could result in a useful anti-cancer synergy. Inhibition of fatty acid synthase (FASN), which causes a reduction in *de novo* palmitate synthesis and EGFR palmitoylation, could provide an alternative approach to blocking EGFR activity [119]. While inhibition of *de novo* palmitate synthesis is likely to have additional effects on the cell beyond those attributable to protein palmitoylation (e.g., *de novo* phospholipid synthesis and re-acylation), the availability of existing drugs targeting FASN (e.g., orlistat, Ref. 119) is one advantageous aspect of this approach.

In addition to drug sensitivity, protein palmitoylation impacts sensitivity to radiation therapy. In response to radiation, cells will arrest proliferation to repair the damage. Those cells unable to arrest the cell cycle are more likely to undergo apoptosis due to mitotic catastrophe. HEK 293 cells exhibit increased sensitivity to x-ray irradiation when *ZDHHC8* is silenced, similar to what is observed when genes encoding the DNA damage response kinases ATM or ATR or the cell cycle regulatory protein p21^{CIP1/WAF1} are silenced [120]. Further analysis in mesothelioma cell lines pinpointed a specific defect in G₂/M cell arrest and the formation of micronuclei following irradiation [120,121]. In a mouse xenograft model, injection of siRNAs targeting *ZDHHC8* increased the lethal potency of X-ray irradiation toward sarcomatoid mesothelioma cells [121]. The specific target(s) of palmitoylation that are involved in regulating cell cycle progression following irradiation remain unclear. These results suggest that selective inhibition of *ZDHHC8* could be useful in combination with irradiation for the treatment of certain cancers.

Concluding remarks

The full complement of mammalian PAT enzymes is now well-established, the biochemical mechanism of reversible protein palmitoylation is increasingly well-understood, and our knowledge of the palmitoylome has increased substantially in recent years [9,122–124]. Looking ahead, one immediate goal is to link this information together to better understand how different *ZDHHC* and APT enzymes regulate specific substrates to impact cancer, especially *in vivo*. Amazingly, even for well-established palmitoylated oncoproteins such as *KRAS-4A*, the identity of the *ZDHHC* palmitoyltransferase(s) responsible for this modification *in vivo* is not clear.

Box 1: In need of answers

- (i) What is the full complement of palmitoylated proteins and how does this vary between normal and cancer cells? Can we link the palmitoylation of specific proteins to the activity of one or more ZDHHC enzymes in each case? How do proteins with altered palmitoylation contribute to hallmark cancer phenotypes such as growth, proliferation, metastasis, or evasion of cell death?
- (ii) How can the same ZDHHC enzyme act as an oncogene in one cancer and a tumor suppressor in another? What cell-type or context-specific differences account for these distinct functions? This ties in with the question of how individual ZDHHC enzymes select substrates at the biochemical level.
- (iii) Can we identify specific inhibitors for individual ZDHHC-family enzymes? Will such inhibitors offer a means to selectively inhibit the function of important and difficult-to-drug oncoproteins such as NRAS?
- (iv) Does the palmitoylation state of individual cysteine residues within a given protein vary over time in normal and cancer cells? Are there interactions at individual cysteine residues between different types of post-translational modifications, including disulfide formation, glutathionylation, and oxidation.
- (v) How are protein palmitoylation cascades integrated within the broader signaling and metabolic networks of the cell? ZDHHC and APT function appears to be regulated by phosphorylation and ubiquitination—how prevalent are post-translational modifications and how do they impact enzyme function? How do the common cancer-induced alterations in cellular lipid metabolism impact the import, *de novo* synthesis, compartmentalization, and degradation of palmitate or other free fatty acids used as substrates for palmitoylation?

Another question, given that high *ZDHHC* expression is a negative prognostic marker in several cancers (Table 1), is to understand biochemically how high ZDHHC enzyme expression promotes tumorigenesis. Experimental and modeling studies indicate that proteins are rarely fully palmitoylated at all possible sites and that ZDHHC overexpression can enhance substrate palmitoylation [36,50]. Thus, one possibility is that ZDHHC overexpression could produce populations of more fully palmitoylated substrate proteins that exhibit enhanced (oncogenic) activity.

Even in the absence of ZDHHC overexpression, it is conceivable that alterations in global lipid metabolism could directly influence the breadth or stoichiometry of protein palmitoylation in a given cell. For example, in cancer cells, boosting FASN expression or activity could result in increased *de novo* palmitate synthesis and palmitoyl-CoA levels, thereby enhancing the modification of key oncoproteins such as EGFR [119,125]. This would be akin to how enhanced glycolysis in cancer can lead to changes in the production of acetyl-CoA, a substrate for histone modification affecting gene expression [126]. A similar effect could possibly even be achieved by changes in the dietary uptake of fatty acids: Stearoylation of the transferrin receptor in *Drosophila melanogaster* is sensitive to dietary stearate consumption [31]. While speculative, diets high in saturated fats could conceivably promote cancer by enhancing the palmitoylation of certain oncoproteins. Interestingly, the glucose transporter GLUT4 is a target for ZDHHC7-mediated palmitoylation in adipose cells [127], and endoplasmic reticulum Ca^{2+} levels and cancer cell metabolism are regulated by the palmitoylation of ER-/mitochondrial-associated membrane-resident TMX1 [98]. Thus, enhanced or inhibited palmitoylation of

metabolic enzymes could directly reinforce or maintain the altered metabolic state of cancer cells.

Finally, much work remains to understanding how ZDHHC and APT/PPT enzyme expression and activity are integrated with and regulated by oncogenic pathways. In NIH 3T3 cells, overexpression of NRAS or KRAS can increase the levels of ZDHHC17 (HIP14) [79]. Likewise, growth factors (e.g., EGF, FGF) can prevent the proteolytic destruction of plasma membrane-localized ZDHHC5 [128]. Thus, oncogenic signaling pathways likely alter protein palmitoylation dynamics by affecting ZDHHC enzyme stability. Less directly, overexpression of transcription factors such as SNAIL can reduce the expression of several ZDHHC enzymes while increasing expression of APT2, resulting in coordinate net depalmitoylation of many proteins [54]. APT enzymes are also targets that can be engaged by oncogenic networks to promote disease progression. In melanoma cells, Wnt5a-driven phosphorylation of APT1 reduces its dimerization and increases its depalmitoylation activity, leading to enhanced metastasis [60]. Tumor suppressor pathways also engage ZDHHC enzymes. In melanoma cells, ZDHHC13 activity is positively regulated by ATR-dependent phosphorylation at Ser⁸, which promotes association between ZDHHC13 and its substrate MC1R, increasing palmitoylation of the latter and generating a protective anti-tumor response [49]. How phosphorylation promotes this association is not known, but hints that in some cases ZDHHC substrate selection and activity could both hinge on phosphorylation-dependent structural modifications. Adding further potential complexity, ATR itself may be palmitoylated (Fig 3) suggesting the potential for reciprocal regulation of ATR localization or activity in some contexts by ZDHHC function. Understanding these functional relationships and other important questions provides many avenues for future research in this field (see also Box 1).

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Conflict of interest

The authors declare that they have no conflict of interest.

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