DHHC PROTEIN ACYLTRANSFERASES CATALYZES THE LYSINE PALMITOYLATION OF MEMBERS OF THE RAS SUPERFAMILY OF SMALL GTPASES

A Dissertation

Presented to the Faculty of the Graduate School

Of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

By

Xiao Chen

December 2018

DEDICATION

To my parents for their countless sacrifices that have allowed me the opportunity to pursue higher education. Everything I am and ever will be is a product of their selflessness to ensure the success of myself and my brothers. To my partner, Xin, this achievement is both yours and mine.

To Dr. Hening Lin, I am forever indebted and grateful for your mentorship, wisdom, and belief in our ability to succeed.

DHHC PROTEIN ACYLTRANSFERASES CATALYZES THE LYSINE PALMITOYLATION OF MEMBERS OF THE RAS SUPERFAMILY OF SMALL GTPASES

Xiao Chen

Cornell University 2018

Abstract

Protein fatty acylation is the attachment of a long-chain fatty acyl group, generally 14–16 carbons in length to the side chain of an amino acid. Cysteine palmitoylation or S-palmitoylation is best understood and widespread post-translational modification in this class, followed closely by N-terminal glycine myristoylation and lastly by O-serine fatty acylation. In recent years, a previously underappreciated member of this class, lysine N^{ε}- fatty acylation, has gained traction due to work by Jiang et al with their discovery that SIRT6 can defatty-acylate TNFα and regulate its secretion, providing the first example for the function of protein lysine fatty acylation in mammalian cells[1,2]. Additional substrates such as K-Ras4a, and RRAS2 further highlight the importance of this modification. However, while advances have been made in the identification and study of the erasers of this modification, little progress has been made in the identification of the writers of this modification. The goal of my thesis project is to identify the writers of lysine fatty acylation.

We report the identification of the first mammalian N^{ε}-lysine fatty acyl transferase. We identified the writer through screening various acyl transferases characterized as S-palmitoyl transferases, O-serine fatty acyl transferases, and lipid fatty acyl transferases, against substrates

identified to have lysine fatty acylation. Multiple family members of the DHHC class of S-palmitoyl transferases were identified as lysine fatty acyl transferases. Utilizing alkynyl-fatty acid labeling with in-gel fluorescence, we observed members DHHC2/3/7/15 of the DHHC family of S-palmitoyl transferase labeling the lysine residues of multiple GTPases. This acylation event is confirmed as lysine fatty acylation through the use of the SIRT2 in-vitro defatty-acylation assay and mutagenesis of the modification sites and P-32 NAD^+ assay. Knockdown of the zDHHC7 gene was able to perturb the lysine fatty acylation of KRAS4A. Screening additional GTPases, not known to have lysine fatty acylation, against DHHC7 identified additional substrates such as CDC42. We identified DHHC7 mediated lysine fatty acylation of CDC42 inactivates the GTPase and induces morphological change in a similar manner as a CDC42 inactive mutant. Expression of a lysine mutant of CDC42 which cannot be acylated by DHHC7 is able to rescue the cell and prevent DHHC7 induced cell rounding. The discovery of the novel lysine acyltransferase activity of the DHHC enzymes highlights the importance of lysine fatty acylation and provides insights to further understanding the function of DHHC enzymes and protein lysine fatty acylation.

BIOGRAPHICAL SKETCH

Xiao Chen was born on December 23, 1990 in Fuzhou City, China and grew up in multiple cities. Many years were spent in Lang Qi, China followed by consecutive five year stays in New York City, Osage (Iowa), and Apple Valley (MN). In 2008, he began undergraduate studies at Iowa State University of Science and Technology (ISU), majoring in the field of Chemistry and field of Genetics. In the spring of 2012 he graduated with a degree in Chemistry and a second degree in Genetics. In the fall of 2012, he moved to Ithaca, NY to pursue a Ph. D. in Chemical Biology from Cornell University. He studied under the guidance of Dr. Hening Lin and toiled to identify the writers of lysine fatty acylation.

ACKNOWLEDGEMENTS

I have been awarded the opportunity of a lifetime to pursue higher education at Cornell University with a group of intelligent and professional people who have fed my professional and intellectual growth. First and foremost, I thank my thesis advisor Dr. Hening Lin for the opportunity and continued support throughout my training. This work would not be possible without his scientific insight and guidance. I am forever grateful for his generosity and unwavering belief and support for his pupils. Thank you forever.

I would like to thank all former and current members of the Lin lab, for providing a rich environment in which my intellectual and professional skill flourished. In particular I would like to thank Dr. Sushabhan Sadhukhan for his sharing his immense chemical knowledge, patient assistance with my chemical synthesis and truly entertaining stories of his own graduate studies; Ornella Nelson for being the best office and lab co-worker one could ask for; Zhewang Lin for great technical tips; Nicole Spiegelman for synthesizing Alk14; Special thanks to Dr. Ji Cao for mentoring me, always being available to discuss ideas, and sharing his immense technical and scientific knowledge; this work would not have been possible without his help. In particular, Dr. Ji Cao discovered the CDC42 lysine fatty acylation phenotype and provided several pieces of critical biochemical data. I would also like to thank my thesis committee: Dr. Richard Cerione, Dr. Yimon Aye for their support, insight, and professional advice with special thanks to Dr. Maurine Linder for her immeasurable support throughout my studies into the DHHC enzymes.

Thanks to those who contributed to the completion of this study. Thanks to the Cornell University Biotechnology Resource Center (BRC) for providing support for on campus cloning sequencing, confocal imaging microscopy and mass spectrometry. Thanks to Xuan Lu and striving to create a caring, fun, and professional work environment.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2BP: 2-Bromopalmitate

Alk14: Palmitic Acid Alkyne

ABHD17: α/β-hydrolase domain 17

ACAT: Acyl-coenzyme A:cholesterol acyltransferase

ARF1: ADP-ribosylation factor 1

APT1: acyl protein thioesterase 1

CBB: Coomassie Brilliant Blue

CRD: cysteine-rich domain

COP: coatomer protein complex

DGAT: acyl-CoA:diacylglycerol acyltransferase

ER: endoplasmic reticulum

Erf2: effect on Ras function

FT: Farnesyltransferase

GGT-1: Geranylgeranyl transferase

GPI: glycosylphosphatidylinositol

Hhat: Hedgehog acyltransferase

HPLC: High Pressure Liquid Chromatography

IL-1α: Interleukin 1-α

LPAT: lysophospholipid acyltransferases

MBOAT: Membrane-bound O-acyltransferase

NMT: myristoyl-CoA:protein N-myristoyltransferase

PAT: protein acyltransferase

PM: Plasma Membrane

PORCN: Protein-serine O-palmitoleoyltransferase porcupine

Shh: Sonic Hedgehog

TMD: transmembrane domains

TNFα: Tumor Necrosis Factor α

Yck2: yeast casein kinase 2

CHAPTER ONE

INTRODUCTION

Protein post-translational modifications encompass a vast variety of functional groups that can be attached to the side chain of a multitude of amino acids, ranging from phosphorylation, acetylation, ubiquitination, and lipidation. The various lipidation events on proteins can be divided into several distinct types based upon the type of lipid attached: isoprenoids, sterols, glycosylphosphatidylinositol (GPI) anchors, phospholipids, and fatty acids. These lipidation events are now known to occur on over one thousand proteins[3], affecting many aspects of cellular physiology. In general, lipidation increases the hydrophobicity of the protein which promotes and regulates membrane-protein interactions. Surprisingly, some forms of lipidation can also regulate protein-protein interactions, protein stability, and enzymatic activities. Some lipidation events are highly dynamic such as S-palmitoylation with turnover rates in the seconds, while N-myristoylation and prenylation are irreversible. Over the years, the identification and extensive characterization of the writers and erasers of these modifications have greatly advanced the scientific community's appreciation of each particular form of lipidation. Unfortunately, certain lipid modifications have been overlooked and underappreciated such as lysine fatty acylation which was first reported in 1992 and 1993 on TNFα[4] and IL-1α[5], respectively. Only recently, as the erasers have been identified, has the community begun to recognize the importance of this forgotten modification.

Protein Prenylation. Prenylation is among the best understood and appreciated lipid post-translational modifications. The five carbon isoprene unit is the building block of prenylation. Farnesylation, formed by three isoprene blocks totaling 15 carbons, and geranylgeranylation,

formed by four isoprene blocks totaling 20 carbons, are the two forms of prenylation. The isoprene chain is attached through a thioether bond on the thiol side chain on a cysteine residue. Currently, no enzyme has been identified as capable of reversing this modification, leading to the general belief that the modification is irreversible. Farnesylation of Ras protein was first reported in 1989, and since then it has been well established that the Ras superfamily of proteins are initially prenylated on the cysteine found in a C-terminal CaaX (where C indicates cysteine, a indicates an aliphatic amino acid, X indicates any amino acid) motif. Proteolytic cleavage of the terminal aaX[6] and subsequent carboxymethylation of the prenylated cysteine[7] occur after the lipidation event. The modification is critical for the function of many Ras proteins as mutation of the farnesylated cysteine renders the protein inactive[8]. The prenylation functions as a lipid anchor, allowing the Ras proteins to interact with the membrane lipids, whereas non-prenylated Ras is generally dispersed throughout the cell.

The writers of prenylation in eukaryotes are well studied and characterized. Farnesyltransferase (FT) utilizes farnesyl diphosphate (FPP), while Geranylgeranyl transferase (GGT-1), and Rab geranylgeranyl transferase (RGGT) utilizes geranylgeranyl diphosphate (GGPP) as their donor molecules. Initially isolated from rat brain, crystal structures of FT and GGT-1 have been solved providing valuable insight into how the transferases achieve selectivity for FPP or GGPP[9,10]. In short, in the binding pocket of the GGT-1 β subunit the critical determining residue, Thr49, allows for a fourth isoprene chain to bind but the corresponding Trp102 in the FT β subunit is too bulky, preventing GGPP from binding. The substrate selectivity for FPP by FT can be switched by a single point mutation of the critical Trp102 to Thr[9].

Fatty acylation. This class of modification is the covalent addition of a short or long fatty acyl chain that varies in length from very short chains of two carbons[11] to longer chains such as

palmitoylation (C16) and stearic acid (C18)[12]. For the purpose of this thesis, subsequent references to fatty acylation only consider long chain fatty acyl chains. The degree of saturation can also vary as well as the stoichiometry of this modification whereas. N-terminal myristoylation and S-palmitoylation are the two most common and best-studied examples of protein fatty acylation. Less studied are O-fatty acylation and lysine $^{\varepsilon}$N-fatty acylation. [13,14].

N-terminal myristoylation is the addition of a myristate (C14) to the amino group of an N-terminal glycine. The resulting amide bond is considered stable and irreversible as there are no known mammalian erasers. Interestingly, a protein injected into host cells by *Shigella flexneri* during infection, called IpaJ, was shown cleave the peptide bond between the N-myristoylated glycine-2 and asparagine-3 of ARF1.[15] While not directly erasing the modification, IpaJ treatment offers an opportunity to study demyristoylated proteins. The writer of N-myristoylation is myristoyl-CoA:protein N-myristoyltransferase (NMT). NMT recognizes the following Gly-X-X-X-Ser/Thr consensus sequence obtained after cleavage of the initial methionine by methionine aminopeptidase[16]. In general, this modification functions similarly to prenylation and is important for membrane association, ensuring proper protein localization and consequently function. A classic example is the Src kinase whose N-myristoylation controls its localization, stability, and kinase activity[17,18].

O-fatty acylation is characterized by the attachment of a fatty acyl group to the hydroxyl group of a serine. Various acyl groups have been reported including palmitoleoyl (C16:1), palmitoyl (C16), and octanoyl (C8).[19-21] O-Palmitoleoylation and O-octanoylation are catalyzed by the membrane bound O-acyltransferase (MBOAT) family of PATs, while serine O-palmitoylation is catalyzed by LPCAT1 of the glycerol-3-phosphate acyltransferase (GPAT) family of glycerolipid acyltransferases. The MBOAT family is divided into three subfamilies,

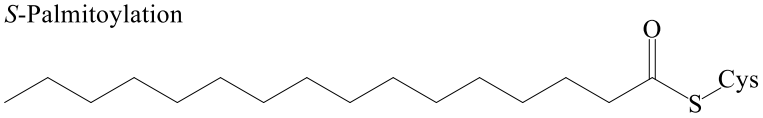
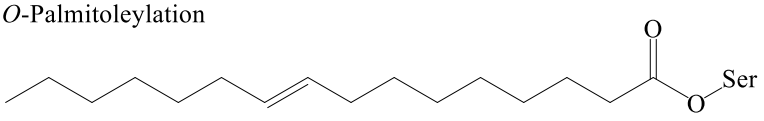
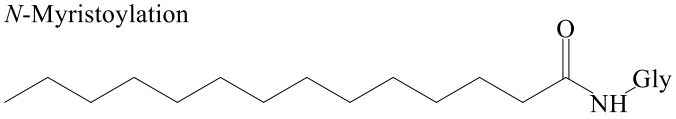
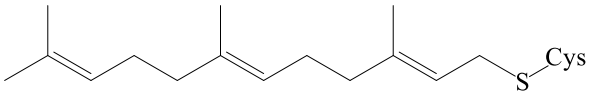
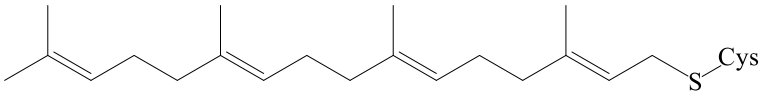
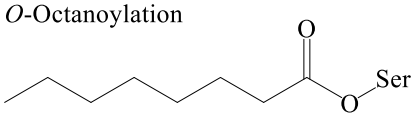
based upon substrate specificities. Class I enzymes are mainly involved in neutral lipid biosynthesis.[22] Class I members, acyl-CoA cholesterol acyltransferase 1 and 2 (ACAT1/2) esterifies cholesterol using oleoyl-CoA as the acyl donor, while diacylglycerol acyltransferase 1 (DGAT1) catalyzes the biosynthesis of retinyl esters, wax esters, and triacylglycerol.[23] Class II members acylate protein substrates and includes PORCN, Hhat, Hhat-like, and GOAT.[19,24-26] Class III is made up of lysophospholipid acyltransferases (LPAT), which remodel phospholipid using arachidonoyl-CoA.[27] Hhat is unique in that it catalyzes the –S to N transfer of palmitate of Hedgehog. The lipid is transferred from the cysteine side chain to its amine, resulting in N-palmitoylation of a cysteine. O-fatty acylation is considered to be a stable irreversible modification, and generally thought to remain on the protein substrate throughout its lifespan. The best-studied example is the O-palmitoleoylation of the Wnt proteins, which is crucial for the binding of Wt to its receptors and downstream signaling.[28,29]

Lysine $^{\varepsilon}$N-fatty acylation is characterized by the attachment of a myristate (C14) or palmitate (C16) to the $^{\varepsilon}$N-amine of a lysine.[4,30] The importance of lysine myristoylation has been demonstrated in bacteria with studies into *E. coli* hemolysin, a pore-forming toxin. The toxin requires lysine myristoylation for activity.[31,32] In mammalian systems, the function of lysine fatty acylation is poorly understood and underappreciated. Recently, the discovery that the sirtuin class of histone deacetylases are capable of efficiently removing the long-chain fatty acyl group from lysine has reignited interest in this modification.[1] This discovery has challenged the stability of this modification and suggests that lysine fatty acylation may have a regulatory function. Studies coupling sirtuins with the acylated substrates have revealed the importance of this modification. Furthermore these studies suggest that lysine fatty acylation may be more abundant than previously thought. Lysine $^{\varepsilon}$N-fatty acylation is also characterized by an amide bond, reminiscent of N-

terminal myristoylation, which suggest that the modification may also require an enzyme catalysis. An exogenous bacterial $^{\varepsilon}$N-fatty acyltransferase has been reported[33] to target host proteins, but there has been no mammalian $^{\varepsilon}$N-fatty acyltransferase reported to date.

S-palmitoylation is catalyzed by the DHHC family of proteins, which is a major focal point of this thesis and warrants extensive coverage and discussion which will be presented in the following sections.

Table 1. Protein lipidation

Lipidation	Writer Enzyme
S-Palmitoylation 	DHHC PATs
O-Palmitoleylation 	Protein-serine O-palmitoleoyltransferase
N-Myristoylation 	*N*-myristoyl transferase
S-Farnesylation 	Farnesyl Transferase
S-Geranylgeranylation 	Geranylgeranyl transferase I (GGT-1)
O-Octanoylation 	Ghrelin O-acyltransferase (GOAT)

PROTEIN S-PALMITOYLATION

S-palmitoylation is the addition of palmitate (C16) to the thiol on the side chain of a cysteine residue, from a palmitoyl-CoA. The modification was first observed in the early 1970's in bovine brain[34,35]. Although essential proteins such as GPCRs and Ras proteins were later demonstrated to be palmitoylated, it took another thirty years before the identification of the writers of the modification. The current dogma is that the majority of S-palmitoylation is catalyzed by an evolutionarily conserved family of protein acyltransferases (PATs), however non-enzymatic autoacylation has also been observed[36-38]. The palmitate from palmitoyl-CoA can, *in vitro*, spontaneously form a covalent bond on the same modified residues *in vivo*[38]. Interestingly, the autoacylation event requires the presence of detergent, and the reaction rate is limited by the detergent micelle concentration. This suggests that autoacylation is dependent on the interaction of the substrate with the detergent micelle where the palmitoyl-CoA is embedded. The leading admonition with these non-enzymatic autoacylation observations is the high, non-physiological concentrations of palmitoyl-CoA required (10 μM), whereas the cellular concentration of free palmitoyl-CoA is < 1 μM[39,40]. While these conditions are not representative of *in vivo* conditions, it raises the possibility that high, localized concentration of acyl-CoAs could potentially serve as an acylation mechanism *in vivo*. The first PAT was discovered and characterized in *S. cerevisiae* as an essential enzyme complex for the palmitoylation of Ras2. The complex comprised of two distinct proteins, Erf2 and Erf4. Erf2 carries the catalytically active residues, however, Erf2 alone could not palmitoylate Ras2. It is dependent on Erf4's stabilizing effects to form a stable enzyme complex. Another yeast protein, Akr1 was also identified to have PAT activity against yeast casein kinase, Yck2. Akr1 and Erf2 share a common sequence found within a cysteine-rich domain (CRD) generally referred to as an aspartic acid-histidine-histidine-cysteine (DHHC) motif. It was later

demonstrated that this DHHC domain is the signature of palmitoyltransferases, and used to identify the mammalian S-palmitoyltransferases. The first mammalian DHHC enzyme (GODZ, also known as DHHC3) was identified in 2004 by a yeast two-hybrid screen using the γ-aminobutyric acid A receptor γ2 subunit as a bait.[41] Subsequent co-expression studies demonstrated upon overexpression, DHHC3 increased the S-palmitoylation of the γ2 subunit. Genomic analysis of the human and mouse genomes for proteins with DHHC domains identified 23 family members in total (Table 2). Co-expression of several family members with PSD-95 increased the substrate's palmitoylation with ^{3}H-palmitate[42], indicating that the DHHC family of enzymes are protein palmitoyltransferases. These co-expression studies will later develop into the classical approach used to identify new enzyme-substrate pairs.

THE TOPOLOGY OF DHHC ENZYMES.

The DHHC enzymes contain multiple transmembrane domains (TMD) with the conserved DHHC-CRD located on the loop between TMD2 and TMD3 facing the cytosol. The number of TMDs varies depending on the family member, with all members containing a minimum of four TMDs and up to six TMDs predicted for DHHC13, 17, and 23. The DHHC-CRD was characterized by the following consensus sequence[43], $CX_2CX_9HCX_2CX_2$ $CX_4DHHCX_5CX_4NX_3FX_4$. This sequence is similar to the C_2H_2 type of zinc finger motifs and predicted to function as a zinc-binding domain[44]. The cysteine in the DHHC motif is required for catalytic activity, whereas the other cysteine residues within the conserved sequence are involved in the structural folding of the enzyme. Generally, mutations of these conserved cysteines result in an unstable protein with no activity[45,46]. Although the vast majority of the DHHC PATs follow this consensus sequence, there are exceptions. DHHCs with zero, one or two zinc fingers are known, further indicating that the zinc finger motif is not involved in the catalytic step and is

instead vital for structural integrity. Surprisingly, structural integrity can be achieved without the actual ability to bind zinc. These DHHC PATs instead rely on hydrogen bonds[45] for structural integrity. The remaining resides within the DHHC-CRD form a binding pocket that controls acyl-CoA and substrate selectivity. In addition, the variable N- and C- termini also help determine substrate selectivity. These attributes will be covered below in the following section.

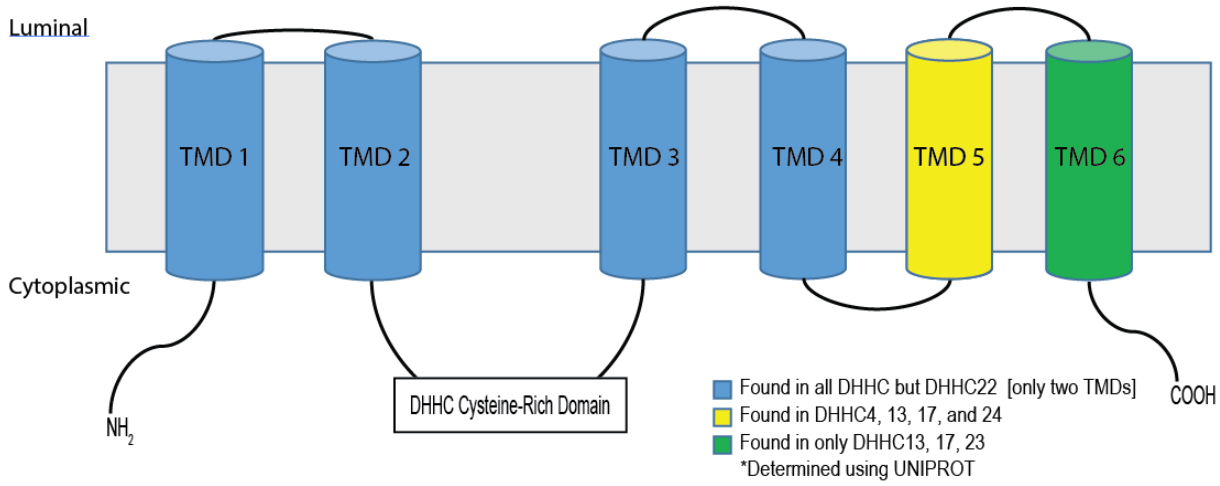


Figure 1. A cartoon representation of the topology of the DHHC enzymes[47].

SUBSTRATE SELECTIVITY.

As there is a large number of DHHC PATs known, understanding how each DHHC PAT recognize its substrates is imperative. Protein interacting domains, amino acids surrounding the modification site, subcellular localization and residues in the acyl-CoA binding pocket, all contribute to the acyl-CoA and substrate selectivity of a DHHC PAT. Enzyme-substrate pairs are generally identified through co-expression screening studies. In short, a substrate is co-expressed with a DHHC member and its fatty acylation level assessed by ^{3}H-palmitate radiography. Newer techniques such as acyl-biotin exchange (ABE), acyl-resin-assisted capture (acyl-RAC) and palmitate alkyne/azide orthologues have enhanced our ability to identify S-palmitoylated substrates and is discussed in detail in a later section. These classical DHHC screens are now known as the Fukata screen in honor of the first DHHC screen against PSD-95[42]. Occasionally only one DHHC member increases the acylation of a substrate[48-50], but more commonly observed is that multiple members increasing a substrate's fatty acylation. These screens highlight two traits of the DHHC family of enzymes. First is the broad specificities of several DHHCs such as DHHC3 and DHHC7, which share many overlapping substrates. Second is the inherent redundancies built into the DHHC PATs that allows for another family member to acylate a substrate in the event the primary PAT is compromised. These two traits also highlight the challenges in identifying true enzyme-substrate pairs.[42,51,52] Knocking down a DHHC, identified through the initial screening experiments, is not guaranteed to abolish a substrate's fatty acylation.[52,53] Complete abolishment of the substrate's palmitoylation may require multiple deletions, which can be challenging in mammalian systems.

The DHHC PATs have distinct cellular localization and can be found at the plasma membrane, endoplasmic reticulum, Golgi membranes, and endosomal membranes. In general,

179 the sorting mechanism of the DHHCs is not clear, but the sorting of several members are well
180 characterized. Both DHHC4 and DHHC6 contain a canonical dilysine motif that interacts with
181 coat protein complex 1 to restrict the PATs to the ER. The dilysine motif from DHHC4 or
182 DHHC6 when fused to the Golgi-specific DHHC3 is sufficient to relocalize it to the ER[54].
183 DHHC1 contains a classical KDEL sequence explaining its ER localization[55]. Swapping the C-
184 terminal portion of DHHC2 onto DHHC15 is sufficient to alter the localization of the chimeric
185 DHHC15 to intracellular membranes similar to that of WT DHHC2[56]. Interestingly, the
186 localization of DHHC2 is altered in response to external stimuli. In dendritic cells, blocking
187 synaptic activity relocalizes DHHC2 to the dendritic spine from the shaft. The relocalized
188 DHHC2 increases PSD-95 palmitoylation which in turns upregulates 2-amino-3-(hydroxy-5-
189 methyl-4-isoxazole) propionic acid type glutamate receptor activity to maintain homeostasis[57].
190 These localization studies have shortcomings as they utilized overexpressed DHHCs which may
191 not be properly localized. The overproduction of protein may cause it to spill out of its
192 endogenous compartment.[58] Endogenous DHHC3 was reported to be preferentially localized to
193 the cis-Golgi, while overexpressing the protein may have resulted in localization to non-
194 physiological cellular compartments[59]. The mislocalized DHHC enzyme likely retains its
195 catalytic activity which could acylate non-natural substrates resulting in false positive enzyme-
196 substrate pairs. This further highlights the challenge in understanding the relationship of the
197 DHHC enzyme-substrate pairs identified in the classical Fukata screens. It is interesting to note
198 that DHHC7, the closely related cousin to DHHC3 and also Golgi-specific, was shown to be
199 preferentially localized to the trans-Golgi compartments. Both enzymes are known to
200 palmitoylate the γ2 subunit of GABA type A receptors[60], however, palmitoylation of the γ2
201 subunit is not affected in DHHC7 KO mice[59]. These observations suggest that overexpressed

DHHC7 may leak into the cis-Golgi compartments. Furthermore this study reminds us to be cautious when attributing a substrate's palmitoylation to a specific DHHC identified through co-expression studies as their specificities may be narrower than previously thought.

The variable N- and C-termini of the DHHC PATs contain domains that confer substrate specificity.[61] DHHC17, also known as huntingtin-interacting protein 14 (HIP14), interacts with huntingtin through its ankyrin repeat domain[62]. A chimeric DHHC3 with the ankyrin repeat domain of DHHC17 gains the ability to interact with huntingtin and redistribute it to the perinuclear region through palmitoylation-dependent vesicular trafficking.[63] Several members have been reported to contain PDZ-interacting domains that allow for enzyme-substrate interactions, conferring substrate specificity[64,65]. A crystal structure[66] of the DHHC17 ankyrin repeat domain in complex with a truncated form of Snap25b showed that the interaction primarily involve hydrogen bonding and hydrophobic interactions centered on a previously identified ΨβXXQP.[67] This motif is present in all of the known DHHC17 substrates including huntingtin. The motif is dependent on the glutamine and proline (QP), whereas the other residues can vary. Mutation of the QP motif is sufficient to disrupt binding of huntingtin to DHHC17. The DHHC-CRD domain itself can also convey substrate selectivity. A chimeric DHHC15 containing the DHHC-CRD of DHHC3 is able to palmitoylate SNAP23, which is modified by WT DHHC3 but not WT DHHC15.[61] Swapping out the DHHC-CRD of yeast Swf1 with the DHHC-CRD domain of Pfa3, Pfa4, or Erf2 is unable to restore the function of Swf1.[68] These findings indicate that interactions between the catalytic domain and the substrate may also determine enzyme-substrate selectivity.

The amino acid sequence surrounding the palmitoylation site also has a role in substrate recognition by the DHHC-enzyme. The PSD-95 palmitoylation sites are surrounded by several

hydrophobic residues (Leu4, Ile6, and Val7), and mutation of these residues result in diminished palmitoylation levels and PSD-95 mislocalization. Mutation of surrounding hydrophilic residues Asp2, Thr8, or Thr9, did not alter the localization.[69] SNAP23 can be mutated into a DHHC15 substrate when its Cys79 is mutated to phenylalanine. The C79F mutant is highly similar to SNAP25b, which is a substrate of DHHC15.[61] Structural elements such as the secondary structure near the palmitoylation site have also been shown to be significant. Surprisingly, a 21 amino acid sequence, predicted to be an amphipathic α-helix near the palmitoylation site of a sodium-calcium exchanger (NCX), is capable of converting nonpalmitoylated cysteines into modification sites when placed adjacently[70]. In NCX, this α-helix appears to finely direct the DHHC to the correct cysteine to ensure proper palmitoylation. The surrounding amino acid sequence is not always involved in determining whether a cysteine can be modified[71]. Instead, the distance of the palmitoylated cysteine from the transmembrane domain can be the determining factor. Mutating the surrounding residues of the palmitoylated Cys100 of p63 and Cys36 of the H1 subunit of mammalian hepatic asialoglycoprotein receptor does not alter their palmitoylation.[71,72] Altering the cysteine residue position relative to the TMD could abolish palmitoylation.

Many palmitoylated proteins are integral membrane proteins, cytosolic proteins that undergo prenylation, or myristoylated proteins. Notably, the reported palmitoylation sites of the majority (>95%) of palmitoylated single-pass integral membrane proteins are located adjacent to or inside the annotated transmembrane domain. One hypothesis is that various DHHC-PATs prefer a particular set of substrates. There is some limited evidence supporting this. For example, Swf1 preferentially targets transmembrane proteins whereas, Akr1 targets mainly soluble proteins[52]. These trends are more difficult to identify among the mammalian DHHCs as the list

of enzyme-substrates are incomplete and associating a substrate's palmitoylation to a single DHHC member remains challenging.

Researchers have demonstrated that DHHCs also show broad specificity for the acyl-CoA donor molecules. While palmitoyl-CoA (C16) is the preferred substrate, myristoyl-CoA (C14) and stearoyl-CoA (C18) also serve as efficient substrates. DHHC2 has a broad acyl-CoA specificity, efficiently transfer acyl chains 14 carbons and longer[73]. Surprisingly, the promiscuous DHHC3 has a narrower acyl-CoA substrate selectivity, being only capable of transferring acyl chains no longer than 16 carbons. This may support the hypothesis that DHHC3 is a general PAT while other PATs may have unique substrates. Another interesting finding compared the acyl-CoA selectivity of DHHC7 to its cousin, DHHC3, and found that DHHC7 preferred longer acyl chains of 18 carbons compared to DHHC3's preference of 16 carbons[74]. The differences in acyl-CoA length preference are attributed to Ile182 in TM3 of DHHC3 which is bulkier than the corresponding serine found in DHHC7. This extra steric hindrance likely prevents the binding of the longer 18 carbon acyl chain. These observations are supported by the recent crystal structures of full-length human DHHC20 (hDHHC20) and zebrafish DHHC15 (zfDHHC15)[75]. The crystal structures contain a hydrophobic cavity, formed by the four transmembrane helices, where the acyl chain is bound. Near the tapering end of the hydrophobic cavity of hDHHC20, is Tyr181, which forms a hydrogen bond with Ser29 that closes off the cavity. Mutation of Tyr181 to a less bulky alanine, allows hDHHC20 to utilize the longer stearoyl-CoA (C18), while mutation of the serine to a bulky phenylalanine increases preference for a shorter chain acyl-CoA. It is important to note that the vast majority of studies assume that palmitate is the acyl group being attached by the DHHCs; however, the broad specificity of acyl-CoA efficiently transferred by the DHHCs suggest otherwise. While palmitate is the most likely,

there is a general lack of mass spectrometry (MS) data to validate the acyl group transferred by each DHHC. The variety of S-fatty acids reported[12,76-82] suggest that the DHHC PAT family are not just palmitoyl transferases. Instead it may be more correct to consider them as general fatty acyl transferases.

The high degree of redundancy associated with the DHHCs for their substrate proteins and acyl-CoA substrate selectivity is remarkable. Understanding how the cell ensures proper acylation of the right substrates with the right acyl group will require extensive research and provide the scientific community a fertile field for study.

DHHC MECHANISM OF PALMITOYLATION.

S-palmitoylation is characterized as a thioester bond with similar energy as to the precursor thioester bond of its donor molecule, palmitoyl-CoA. The reaction is energy neutral and does not require an energy source such as ATP. *In vitro* reconstitution assays require no energy sources[42]. The DHHC PATs themselves are autoacylated both *in vivo* and *in vitro*[41,42,83] and can occur in the absence of a substrate with only palmitoyl-CoA[83]. The autoacylation is dependent on an intact DHHC motif. If the cysteine is mutated to serine, the resulting enzyme cannot be autoacylated. This suggests that the enzyme is acylated on the cysteine of its DHHC motif. Incubating a ^{3}H-palmitoyl-CoA autoacylated DHHC2 or DHHC3 with myristoylated Gi_{α} results in the transfer of the acyl group to the substrate[73]. These experiments demonstrate both the chemical and kinetic competence of the acyl intermediate and indicates that the catalytic mechanism proceeds through a two-step ping-pong mechanism. The first step is a fast transfer of palmitate to the DHHC cysteine, followed by a slower second step, where the palmitate is transferred to the substrate. This mechanism assumes that the autoacylated residue is the DHHC cysteine, although it was not known for a long time whether the DHHC cysteine could indeed be

modified. A recent report identified, through MS, a palmitoylated DHHC peptide with the acyl group attached to the catalytic cysteine[46]. This result confirmed that DHHC cysteine can indeed carry the palmitoyl group and is likely the form of the acyl intermediate. This is also supported by a crystal structure of hDHHC20 alkylated on its catalytic cysteine by 2-BP.[75]

The function of the three other residues in the motif are less certain. Initial studies suggest that the first histidine is critical for the transfer of palmitate from the acyl intermediate to the substrate. Mutation of the first histidine abolishes PAT activity but does not prevent the formation of the acyl intermediate.[83] It is thought that the histidine is important for deprotonation of the substrate's thiol to make it more nucleophilic. Analysis of the hDHHC20 crystal structure suggests another function. The structure shows the aspartic acid and first histidine forming a hydrogen bond that can accept a hydrogen from the catalytic cysteine. This deprotonation of the DHHC cysteine enables its nucleophilic attack on the carbonyl thioester of the palmitoyl-CoA, forming the acyl intermediate. The histidine with its captured proton can then donate it to the carbonyl oxygen of the acyl-intermediate to activate it. This would allow the substrate's thiol to capture the palmitoyl group. This mechanism is reminiscent of the Cys-His-Asp catalytic triad found in cysteine proteases. The second histidine coordinates Zn^{+} to position the catalytic cysteine for its the nucleophilic attack, according to the structure.[84]

Interestingly, there are DHHC enzymes with mutations in the DHHC motif that retain activity. In particular, DHHC13 possesses a natural DQHC motif, instead of the canonical DHHC motif. As expected, DHHC13 has low levels of autoacylation[85] but is surprisingly able to palmitoylate huntingtin.[86] While glutamine can participate in hydrogen bonding with aspartic acid, it is unable to function as an acid-base catalyst.[87] This may explain the low autoacylation levels observed, but does not explain how DHHC13 is able to retain its PAT activity. Akr1,

Akr2, and Pfa2 has a tyrosine instead of a second histidine (DHYC) and still has palmitoyltransferase activity.[88]Also a DHHR mutant of Swf1 retains its ability to able to acylate its substrates.[89] How these variants transfer palmitate to their substrates is not clear, but is likely dependent on the nucleophilicity of their substrate's thiol. The nucleophilicity of a cysteine, which differs depending on its molecule environment[90,91], may be sufficient to capture the palmitoyl group from the DHHC acyl intermediate. Even without forming an acyl intermediate, DHHC enzymes may still bind both the palmitoyl-CoA and substrate at the same time. This could bring the two close enough for the acyl transfer to occur through a non-catalytic protein-assisted mechanism.[92] These observations again highlight the complexity that exists within the DHHC family and the need to further study these remarkable enzymes.

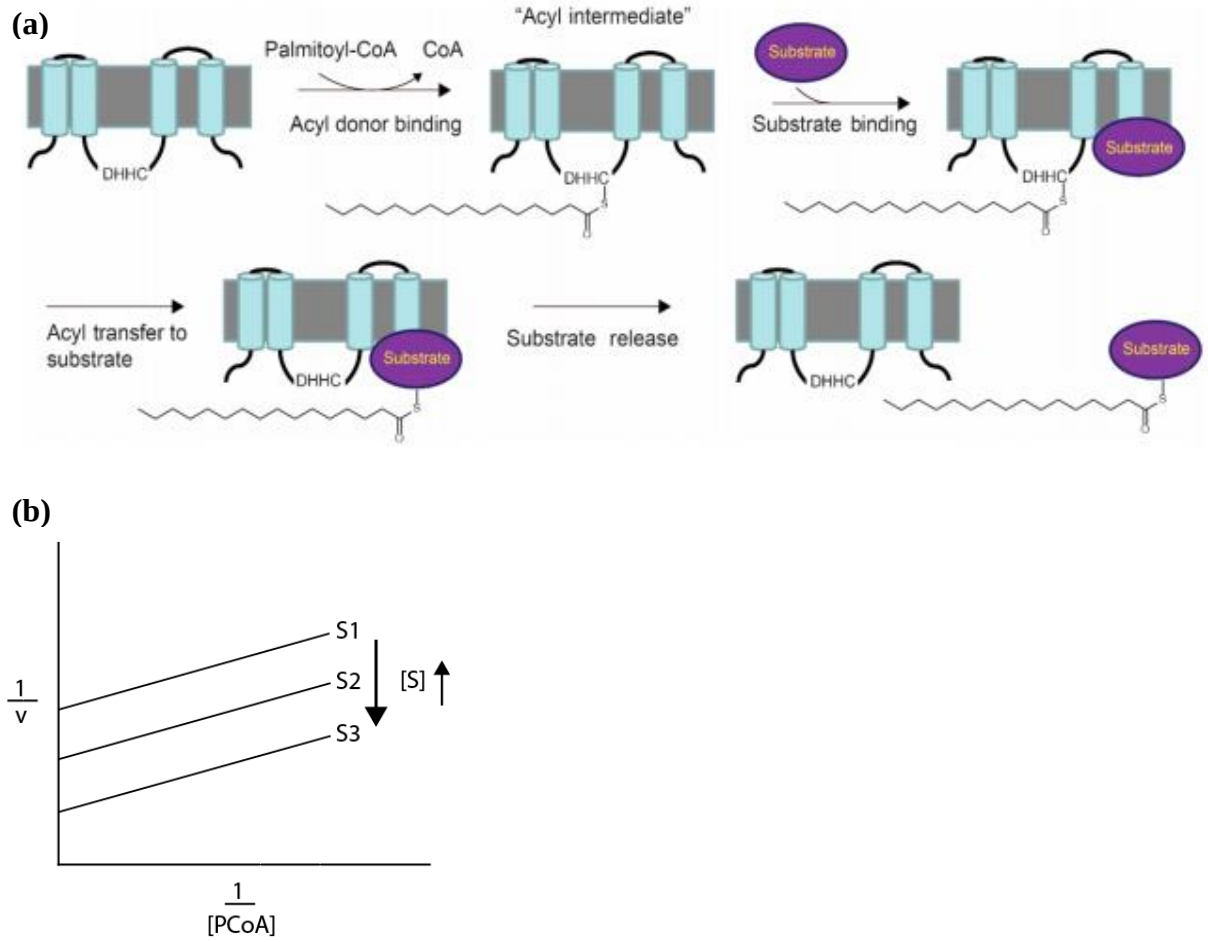
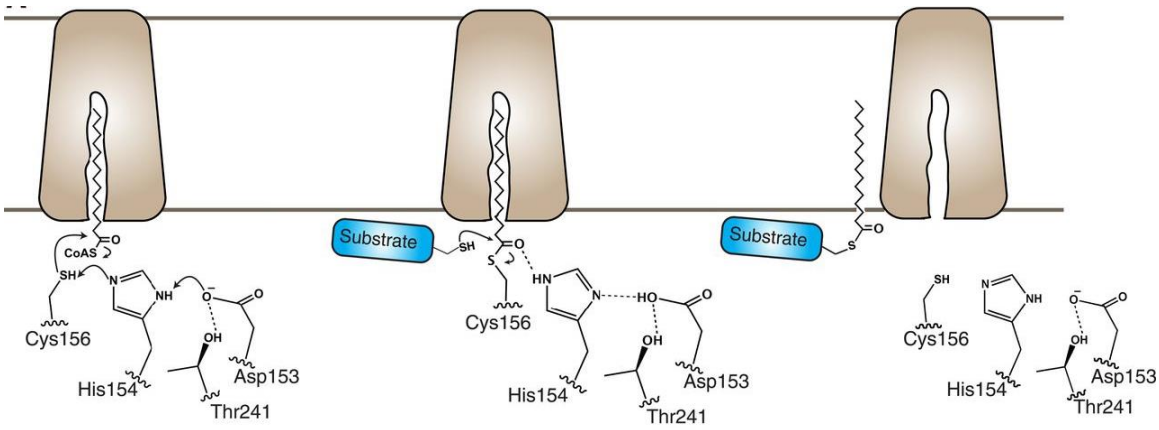


Figure 2. "Ping Pong" mechanism of DHHC-mediated cysteine palmitoylation[93].

(a) Cartoon representation of ping pong mechanism. (b) Example Line-weaver Burk plot of DHHC activity as a function of palmitoyl-CoA concentration in increasing protein substrate (S) concentration. The series of parallel lines obtained indicate a "Ping Pong" mechanism.



337

338 **Figure 3. Detailed reaction mechanism[94].**

339

340

The mechanism by which the DHHCs catalyze S-palmitoylation is generally well understood, but how this activity is regulated is poorly understood. DHHC6 was recently demonstrated to be a downstream substrate for the PAT DHHC16 and the depalmitoylase APT2[95]. Palmitoylated DHHC6 has detectable activity, whereas its depalmitoylated form does not. Interestingly, DHHC6 exists in multiple differentially palmitoylated states that have variable activity and stability. These variable states are reminiscent of protein phosphorylation. Phosphorylation of DHHC enzymes could potentially be a means by which the DHHCs are regulated. A phosphorylation-deficient DHHC3 was reported to have increased interaction with neural cell adhesion molecule (NCAM) and also increased its palmitoylation when compared to WT DHHC3[96]. Another study reported that DHHC2 and DHHC3 could form homodimers which reduced its enzymatic activity[97]. The oligomerization of the DHHC enzymes may potentially be a mechanism to regulate activity. Protein-protein interactions are one common mechanism by which enzymes are regulated and the DHHC9-GCP16, Erf2-Erf4 complexes demonstrate that the DHHCs could be regulated in such a manner. These interactions generally help to stabilize the DHHC proteins[49,98]. Whether other DHHCs form protein-protein complexes or have accessory subunits are not known. It is interesting to note that not all of the mammalian DHHCs form detectable acyl intermediates[85]. Perhaps those that do not, require an accessory subunit. Lastly, a cofactor which could regulate DHHC enzyme activity is zinc. While not all DHHCs bind zinc, those that do have reduced protein stability if they lose their ability to coordinate zinc. Reminiscent of intracellular Ca^{2+}, an important cofactor for many enzymes and their activities[99], differential levels of zinc could potentially affect the stability and conversely the DHHC-enzyme activity, however this requires further study.

FUNCTIONS OF S-PALMITOYLATION.

S-palmitoylation is now known to occur on a vast and diverse set of proteins, from cytosolic proteins, integral membrane proteins, and transmembrane proteins. Not surprisingly, S-palmitoylation has been reported to have functions other than membrane tethering. These reported functions will be covered in this section.

Regulation of protein trafficking. The hydrophobic nature of the palmitoyl group allows modified proteins to interact with membranes of various organelles and facilitates the trafficking of such proteins to and from these membranes. Among the best-studied examples of protein trafficking due to palmitoylation are members of the Ras Superfamily of GTPases, in particular, H-RAS, N-RAS, and K-RAS. The Ras proteins are initially prenylated at a C-terminal cysteine that is part of a CaaX motif at the ER. After prenylation, the –aaX amino acids are cleaved and carboxymethylated at the newly formed terminal –COOH[6,7]. Prenylation itself is not sufficient to anchor the modified protein to the plasma membrane where Ras is destined[100,101]. Something extra is needed; for H-RAS, NRAS, and K-Ras4a, that something extra is S-palmitoylation. S-palmitoylation occurs at the Golgi where the Ras protein translocates after carboxymethylation. The palmitoylated cysteine is located near the CaaX cysteine, and the stoichiometry differs as H-Ras has two cysteines which are dually palmitoylated[102]. Dually lipidated Ras is then trafficked to the PM via the secretory pathway. Whenever Ras is depalmitoylated, it will redistribute to all membranes until it is repalmitoylated at the Golgi and directed back to the PM. This cycle of palmitoylation, depalmitoylation helps to ensure accurate localization of the Ras proteins to the PM[103]. The K-Ras4b isoform of K-Ras, lacks an extra cysteine near the CaaX motif and consequently is not palmitoylated. This isoform instead utilizes the positive charge of eight

lysine residues found at the C-terminus to electrostatically interact with the negatively charged plasma membrane[104].

Regulation of Protein Stability. Another function of S-palmitoylation is its ability to regulate the stability of its protein substrates. A WT huntingtin protein typically contains a patch of 6-35 repeated glutamine residues, whereas, in Huntington's disease, the huntingtin protein has more than 40 repeated glutamines[105]. The extra glutamines cause the huntingtin protein to aggregate, and this aggregation is a biomarker of the disease. The huntingtin protein can be palmitoylated by DHHC17 (HIP14) and DHHC13 (HIP14L) on Cys214[106]. Overexpressing DHHC17 reduces huntingtin protein aggregation while knockdown of DHHC17 increases aggregation and induces neuronal cell death. Another well-studied example is Tlg1, a yeast protein. Tlg1 is involved in the regulation of protein recycling between endosomes and the Golgi[107,108]. Palmitoylation traps Tlg1 at the trans-Golgi membranes and prevents degradation. Mutation of its palmitoylation site or inactivation of its PAT, Swf1, leads to Tlg1 ubiquitination and subsequent degradation[109]. Here, S-palmitoylation prevents protein ubiquitination and therefore increasing protein half-life and stability. Surprisingly, palmitoylation-deficient Tlg1 has similar cellular localization as WT Tlg1, which supports the ability of S-palmitoylation to function in roles other than protein localization. Other examples where S-palmitoylation increases protein stability include HIV receptor C-C chemokine receptor type 5 (CCR5)[110], estrogen receptor-α[111], and regulator of G protein signaling 4 (RGS4)[112]. In these examples, S-palmitoylation also inhibits the substrate's ubiquitination; how S-palmitoylation prevents ubiquitination is not clear. S-palmitoylation can also stabilize a protein and promote its ER exit. A palmitoylation-deficient lipoprotein receptor-related protein 6 (LRP6) is trapped at the ER and is recognized by an ubiquitin-dependent ER quality control mechanism. Mutation of the

ubiquitinated lysines allows ER exit of a palmitoylation-deficient LRP6. Yeast chitin synthase Chs3[113], and amyloid precursor protein (APP)[114] also share a similar phenotype, in that blocking the substrate's palmitoylation result in ER retention of the palmitoylation-deficient substrates. A general hypothesis would be that during the translation process an improperly folded protein would not be palmitoylated and instead be ubiquitinated. Once the protein achieves the proper fold required for palmitoylation, this prevents ubiquitination and allow the protein to exit the ER.

Regulation of protein-protein interactions. S-palmitoylation can also affect protein-protein interactions. A demonstrated example is the palmitoylation of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor, GluR1. C-terminal palmitoylation of GluR1 reduces its interaction with the 4.1N protein which in turn reduces its internalization by endocytosis at the PM[115]. How palmitoylation affects these protein-protein interactions is not clear, but one likely explanation is that the lipidation affects the conformation of the modified protein. Palmitoylation and subsequent membrane integration could force the flanking residues into a closer membrane proximity. If these residues are involved in protein-protein interactions, then palmitoylation will inhibit these interactions. The modification could also potentially regulate protein-protein interactions by spatially segregating proteins to the proper membrane organelles and microdomains. Lastly, the modification itself could be recognized by another protein like that of PDE6δ recognizing the prenylation on K-Ras4b and other GTPases[116].

BIOLOGICAL FUNCTION AND DISEASE RELEVANCE OF THE DHHC ENZYMES.

The functional role of protein S-palmitoylation is generally well studied and understood; however, the role of the DHHC PATs, which mediate nearly all S-palmitoylation events, remain poorly understood. Various knockdown and genetic deletion studies have identified a multitude of phenotypes that can be attributed to a specific DHHC PAT. In general, these changes correlate

with neurodegenerative diseases such as Alzheimer's, Huntington's and schizophrenia. Diseases such as cancer and developmental defects have also been correlated and attributed to the DHHCs. The modified substrate proteins ultimately determine the biological phenotypes of the DHHCs, however in many cases, the substrates remain to be identified. As such, a complete understanding of the biological function of the DHHCs and their roles in the disease phenotypes is limited. Several challenges also impede the study of the biological function of the DHHCs. These challenges include but are not limited to redundancies among the DHHCs, difficulties purifying and reconstituting the activity *in vitro*, and a general lack of suitable antibodies against endogenous DHHCs.

Huntington's disease. The biological function of DHHC17 and DHHC13 in Huntington's disease is a well-studied case. DHHC17 and DHHC13 are respectively known as huntingtin interacting protein 14 (HIP14) and huntingtin interacting protein 14 like (HIP14L). They were initially discovered to interact with the huntingtin protein through their ankyrin repeat domains. Both DHHCs are also able to palmitoylate huntingtin. The Huntington's disease mutation expands the polyQ region (a patch of repeated glutamines) of huntingtin. This expansion reduces the interaction between the PATs and huntingtin; leading to lower levels of huntingtin palmitoylation. This ultimately results in cell death.[62] Interestingly, when WT huntingtin protein levels are low, DHHC17 itself has significantly reduced activity. In mice lacking one allele for huntingtin, the palmitoylation of SNAP25 and GluR1 is also reduced. SNAP25 and GluR1 are DHHC17 substrates. Deletion of the huntingtin gene using antisense oligos further reduces the palmitoylation. These phenotypes are further supported by the observation that mice deficient in DHHC17 share a neurological and behavioral phenotype similar to that of patients who have Huntington's disease[117]. DHHC17 and DHHC13 knockout mice also share a lethal embryonic

phenotype similar to that of huntingtin (-/-) embryos[118]. The critical role which DHHC17 plays in the progression of Huntington's disease is well established, but the exact mechanism of how DHHC17 contributes to the disease is not clear. It appears that the huntingtin protein acts as a protein scaffold to bring together DHHC17 and its substrates. As many DHHC17 substrates are part of neurological processes, loss of normal huntingtin or DHHC17 function could easily result in the observed neurological defects and disease progression. Another mechanism by which DHHC17 may contribute to Huntington's disease is through the palmitoylation of caspase-6, a cysteine protease involved in neurological disorders. Palmitoylation inhibits caspase-6 activity, and in the DHHC17 -/- mice, caspase-6 has increased activity which is required for the progression of Huntington's disease.[119]

Schizophrenia and X-linked mental retardation. Patients with schizophrenia often have a deletion located at the chromosome 22q11 region. The zDHHC8 gene is found here, and studies have linked the loss of zDHHC8 to this disease.[120,121] However, other studies have also argued that there is no link between the two.[122-125] Mutations in the zDHHC15 and zDHHC9 have also been associated with X-linked mental retardation.[126,127] The mutations in these studies generally implicate a loss of function of the PAT. How deficiencies in these two PATS cause the severe phenotype is not known, but it is not surprising as their substrates are involved in neural development (see TABLE1).

Other neurological disorders. Mice with zDHHC5 reduced function are deficient in contextual fear conditioning which is an indicator of defective hippocampal-dependent learning. Two substrates are potentially the link between DHHC5 and its role in postsynaptic function, learning, and memory. The first substrate is PSD-95 which is vital for normal neural development and function. DHHC5 interacts with PSD-95 through a PDZ3 domain found on

PSD-95.[64] The second substrate is the GPCR SSTR5, which is found in neural tissue.[128] The effect of palmitoylation near the C-terminus of SSTR5 is not evident but it is known that the C-terminus of GCPRs is essential for interaction with its downstream effectors[129,130] and palmitoylation could potentially regulate GPCR SSTR5 signaling.

Cancer. It has been implicated that DHHC2 functions as a tumor suppressor. Reduced expression predicts a poor prognosis in gastric adenocarcinoma patients and is associated with lymph node metastasis.[131] Knocking down zDHHC2 reduces the palmitoylation levels of cytoskeleton-associated protein 4 (CKAP4), which regulates its interaction with antiproliferative factor (APF) and decreases the ability of APF to regulate cellular proliferation and tumorigenesis.[132] Gastric cancer tissue with elevated levels of DHHC14 are characterized with more aggressive tumor invasion *in vivo*. Overexpressing DHHC14 in TMK-1 cells resulted in increased migration and invasion while knockdown of zDHHC14 in HSC-44PE and 44As3 gastric cancer cells reduced invasiveness.[133] These observations link zDHHC14 to gastric cancer but the mechanism by which the PAT contributes to the disease is not known. Knockdown of zDHHC5 inhibits non-small cell lung cancer cell proliferation, colony formation, and cell invasion. Overexpression of WT DHHC5 can rescue the cell growth.[134]

Additional phenotypes. A loss of function mutation in the zDHHC21 gene is sufficient for hair loss in mice.[135] Fyn, an Src-family kinase involved in keratinocyte differentiation, is the linking substrate between hair loss and DHHC21. zDHHC21 deficient mice have also been reported to be more resistant to injury, linking DHHC21 to endothelial inflammation. The linking substrate is likely PLCβ1.[136] DHHC21 may also be involved in vascular function as it can palmitoylate the α1D adrenoceptor.[137] A nonsense mutation in the zDHHC13 gene results in amyloidosis, alopecia, and osteoporosis.[138] This osteoporosis phenotype is attributed to the

DHHC22 (zDHHC13) substrate membrane type-1 matrix metalloproteinase (MT1-MMP), a factor that controls skeletal development. zDHHC13's role in hair anchoring, skin barrier, and hair loss are likely mediated through its substrate cornifelin.[139] zDHHC13 knockout mice also show diminished mitochondrial function attributable to verified substrates such as malonyl-CoA-acyl carrier protein transacylase.[140] Lastly, zDHHC13 deficient mice are more susceptible to bacteria resulting in skin inflammation.[136] Mice deficient in zDHHC16 (Aph2) exhibit cardiomyopathy and cardiac defects. The mediating substrate is phospholamban (PLN). Palmitoylated PLN interacts with protein kinase A and protein phosphatase 1 to control the pentamer formation of PLN. In the zDHHC16 deficient mice, PLN has reduced phosphorylation which in turn inhibits its function.

The DHHCs highlighted above, have substrate pools smaller than that of DHHC3 or DHHC7. Surprisingly, genetic deletion of zDHHC3 or zDHHC7 in mice does not have apparent phenotypes.[59] Double knockout mice, however, do exhibit reduced body and brain mass and perinatal lethality. This is a strong indication of the functional redundancies for DHHC3 and DHHC7. Some studies have reported potential biological functions of DHHC7 in glucose tolerance, hyperglycemia[141], and tumorigenesis[142] and cell migration[143]. Viral proteins require palmitoylation for proper function, but viral transferases are not known[144]. It is more likely that viral proteins are capable of hijacking the cellular DHHCs to ensure virus survival. Promiscuous DHHCs such as DHHC3 are a likely target and indeed HSV-1 envelope protein UL20 was shown to interact with and serve as a substrate for DHHC3.[145] Cells expressing DHHS3 have lower viral titers and altered UL20 localization. The hijacking of host palmitoylation machinery is not limited to viruses. The GobX protein from *L. pneumophila* and SifA from *Salmonella* are

both palmitoylated once inside the host cell and requires palmitoylation for proper localization.[146,147]

The DHHCs have essential roles for healthy cellular functions and are involved in the progression of multiple neurological diseases and various cancers. Similar to the protein kinases, the DHHCs are part of signaling pathways, but the modifications they catalyze do not turn on their substrates, instead directing them to the correct cellular membranes. In order to understand and treat the diseases associated with the DHHCs, we must determine how their activities are regulated.

ERASERS OF S-PALMITOYLATION

The first enzyme discovered capable of deacylating palmitoylated substrates was palmitoyl-protein thioesterase (PPT1).[148] PPT1 was later shown to reside in the lysosome and remove S-palmitoyl groups from peptides and proteins during lysosomal degradation.[149,150] The localization of PPT1 prevents it from participating in the regulation of the dynamic S-palmitoylation of cytoplasmic proteins. That role would later be attributed to the cytosolic acyl protein thioesterase 1 (APT1), and APT2. Originally isolated from rat liver, APT1 has been demonstrated to be a protein defatty-acylase with substrates such as Ras[151], G protein α subunit[151], and SNAP23[152] through *in vitro* assays and *in vivo* experiments.[153] APT1 has an expected broad substrate specificity as it must catalyze the removal of S-palmitoylation from a multitude of substrates. This means there is a lack of a consensus sequence for APT1 recognition among its substrates. APT1 is capable of depalmitoylating substrates which are dually N-terminal myristoylated and cysteine acylated, substrates that are prenylated and acylated, and acylated integral membrane proteins. Its specificity is not with any discrimination as not all acylated proteins are APT1 substrates, for example, Caveolin.[154] Additionally, APT1 deacylates

546 Ga_i tenfold more efficiently than Ras. The governing factors behind this selectivity are not yet
547 apparent. APT1 belong to the α/β hydrolase superfamily of proteins and contains a conserved
548 catalytic triad.[155] This family also includes lipases, esterases, and dehalogenases. The homolog
549 APT2 functions similarly as a thioesterase and is capable of deacylating palmitoylated
550 substrates.[156] Both APT1 and APT2 are palmitoylated, and this modification is hypothesized to
551 target the thioesterases to membranes where they can then interact with and access their
552 substrates palmitoyl group, which is usually embedded in those membranes.[157] However, it has
553 also been suggested that autodepalmitoylated APT is the active form of the enzyme.[158]

554 The α/β hydrolase domain 17 (ABHD17) family of depalmitoylases were identified by
555 profiling novel serine hydrolase targets of the APT1/2 inhibitor, Palmostatin B.[159] The founding
556 member, ABHD17**,** catalyzes the depalmitoylation of PSD-95 and N-Ras. Other ABHD17 family
557 members were later demonstrated to be capable of deacylating PSD-95.[160] An increasing number
558 of cellular depalmitoylases suggest that like the DHHC PATs, there may be substrate selective
559 depalmitoylases while a few members exhibit a generally broad and redundant depalmitoylating
560 activity.

561 **TECHNIQUES USED TO DETECT PROTEIN S-PALMITOYLATION**

562 Radioactive isotope labeled palmitic acid. Commonly used isotope labeled palmitic acids
563 are ^{3}H-, ^{14}C-, and ^{125}I-palmitic acids.[21,49,161] Radioactive-isotope labeled palmitic acid was the
564 first reported method by which protein S-palmitoylated could be detected.[162] Radiolabeled
565 palmitic acid added to the cell culture media is readily taken up and converted to palmitoyl-CoA
566 inside the cell. This radiolabeled palmitoyl-CoA serves as a substrate for the DHHC PATs and is
567 readily used to modify their substrate proteins. Enrichment, by immunoprecipitation or
568 fractionation, allows for visualization of palmitoylated proteins. While ^{3}H- and ^{14}C-palmitic

569 acids are near perfect mimics of endogenous palmitate, the signal generated by these isotopes are
570 weak and generally require an exposure time that can extend from weeks into months. ^{125}I-
571 palmitic acid has a stronger radioactive signal; however, it is structurally different from palmitic
572 acid and may not give an accurate depiction of palmitoylation. Radiolabeled palmitic acid probes
573 operate via metabolic labeling, which may disrupt the metabolic state and normal cell functions
574 due to the addition of exogenous fatty acids. Conditions which affect the metabolic conversion of
575 the palmitate into palmitoyl-CoA and its ratio to unlabeled palmitate further decreases the
576 sensitivity of radioactive isotope labeling.

577 Acyl-Biotin Exchange. Acyl-biotin exchange (ABE) is a three-step procedure that
578 harnesses the sensitivity of the thioester bond of S-palmitoylation to cleavage by hydroxylamine
579 to indirectly visualize protein S-palmitoylation.[163,164] The first step utilizes *N*-ethylmaleimide to
580 block all the free thiols, including cysteines on proteins. The second step is treatment with
581 hydroxylamine which cleaves the thioester bond and removes the palmitoyl group from modified
582 cysteines. The third step is the addition of biotin-N-[6-(biotinamido) hexyl]-3′-(2′-pyridyldithio)
583 propionamide (biotin-HPDP), which reacts with the newly freed cysteines. The modified
584 proteins are then enriched, via streptavidin pull-down, and the modification site can be
585 determined via MS identification. ABE can be coupled with a precursor immunoprecipitation
586 step to enrich for a particular protein. Additionally, the biotin moiety can be detected by
587 streptavidin western blot to visualize the degree of palmitoylation. ABE does not operate via
588 metabolic labeling, which means it can be utilized to detect dynamic S-palmitoylation under any
589 conditions, including stress conditions. Another advantage is its applicability to animal studies in
590 which metabolic labeling is less feasible.[165] The ABE procedure is adaptable due to the
591 simplicity of the chemistry involved. Fluorescent dyes can replace the biotin moiety to allow for

592 in-gel fluorescence visualization of palmitoylation. Thiol-reactive sepharose beads can also be
593 directly added to the hydroxylamine treated proteins to simplify the procedure and reduce the
594 number of steps performed; this adapted procedure is also known as acyl-resin assisted capture
595 (acyl-rac).[166] Replacing biotin-HPDP with maleimide-functionalized polyethylene glycol
596 reagents induces a mobility shift on S-acylated proteins that can be detected by western blot, also
597 known as an acyl-PEG exchange (APE).[167] APE allows for the detection of multi-palmitoylated
598 states of a protein, such as the dually palmitoylated H-Ras, and provide insight into the
599 stoichiometry of protein palmitoylation.[167] The major drawback to ABE is the hydroxylamine
600 treatment step, which removes all forms of lipidation from cysteines (myristoylation,
601 palmitoylation, and other acyl groups). This means identification of the actual acyl group on the
602 cysteine is impossible via ABE. Additionally, not all S-palmitoyl modifications are equally
603 susceptible to hydroxylamine treatment; junction adhesion molecule C is reported to be S-
604 palmitoylated yet is surprisingly hydroxylamine resistant.[143] Also, an incomplete hydroxylamine
605 treatment can result in false negatives while incomplete alkylation of proteins during the first
606 step can result in false positives.

607 Bioorthogonal Palmitic Acid Probes. Bioorthogonal palmitic acid probes generally
608 contain a terminal azido or alkynyl group that allows the installation of a variety of handles via
609 click chemistry for downstream procedures.[168] Biotin can be installed to facilitate affinity
610 purification and subsequent protein identification by MS, or visualization by streptavidin western
611 blot. Fluorescent probes can also be installed for visualization by in-gel fluorescence. The
612 bioorthogonal palmitic acid probes function similarly to the radiolabel palmitic acid probes, in
613 that both utilize metabolic labeling to install the probes onto the protein substrates. While the
614 bioorthogonal probe suffers from similar drawbacks as radiolabel probes due to the nature of

metabolic labeling, it offers significant advantages and improves upon the radiolabel technique. These probes have improved sensitivity compared to their radiolabeled counterparts, are easier to handle (does not require a radiation safety permit), and allows affinity purification and identification of the palmitoylated substrates. In general, alkynyl fatty acids are the preferred probe as they are more structurally similar to palmitic acid and have reduced background labeling compared to their azido cousins.[169]

Mass spectrometry. Analysis of post-translational modifications by MS is the gold standard which provides quantitative information, direct visualization of the modified site and the real identity of the modification. Unfortunately, protein and peptide palmitoylation by MS is a challenging task due to the hydrophobicity of the modification and instability of the thioester bond under the ionizing conditions of MS. [170] While challenging, the task is not impossible and S-palmitoylated peptides have been identified and their corresponding protein sites assigned.[46,171,172] Improvements to the methodology and MS conditions will require further development for routine MS analysis.

Bioinformatics software to predict protein S-palmitoylation site. An increasing number of palmitoylated proteins identified by proteomic studies has led to the development of software designed to predict potential protein S-palmitoylation sites. CSS-Palm 1.0 (CSS: Clustering and scoring strategy) is the first of such programs. CSS-Palm 2.0 was used to successfully predict the palmitoylation sites of 16 known palmitoylated proteins, which were subsequently validated experimentally.[173] The software is currently in its 4.0 iteration. Additional software includes NBA-palm[174] (naive Bayes algorithm) and CKSAAP-Palm (composition of k-spaced amino acid pairs).[175]

Conclusion

Protein lipidation is a well-recognized post-translational modification that has significant impact on cell health and function. Among the variety of lipidations is the uniquely reversible S-palmitoylation. The significance of S-palmitoylation was revolutionized by the discovery of the DHHC family of enzymes. It allowed the scientific community to link a variety of pathologies to almost all the DHHC members. This certainly means that the DHHCs are an invaluable pharmacological target in our pursuit of health. Although significant progress has been achieved in the two decades since the discovery of the DHHCs, the field can still be considered in its infancy. S-palmitoylation and DHHCs will provide a rich field for study for years to come.

Another recently identified reversible lipidation modification is lysine $^{\varepsilon}$N-fatty acylation. Like S-palmitoylation in the early 2000's, lysine fatty acylation has been demonstrated to be important for the function of several GTPases. Although exogenous lysine $^{\varepsilon}$N-fatty acyltransferases have been reported, no mammalian lysine fatty acyltransferases has been identified. The need for a mammalian lysine fatty acyltransferase is unquestionable; without one, our understanding of lysine fatty acyltransferases will remain limited. One can imagine how the field will change with an identified transferase simply by looking at S-palmitoylation. My work will to contribute to the field by reporting the first mammalian lysine $^{\varepsilon}$N-fatty acyltransferase. Our finding that DHHC2/3/7/15 are capable lysine fatty acyltransferases will help push us into a new era in the study of lysine fatty acylation.

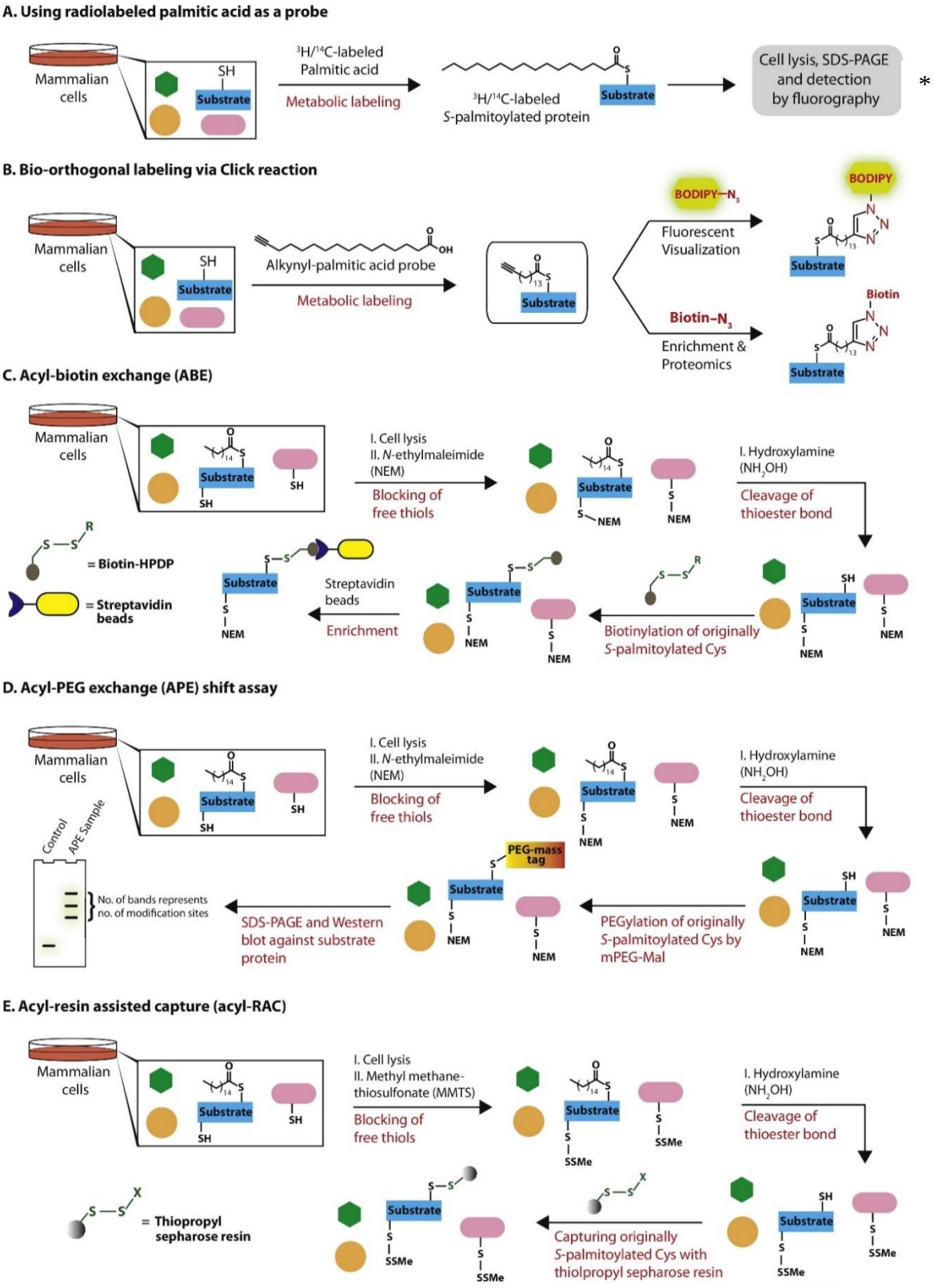


Figure 4. Different analytical methods to identify and characterize protein S-palmitoylation.[177]

661 *Readout for A should be autoradiography or scintillation counter, not fluorography.

Table 2. Mammalian DHHC enzyme-substrates, localization, and disease association[178]

DHHC enzyme [Gene symbol]	**Cellular localization**	**Disease association**	**Known substrates**
DHHC1 [zDHHC1]	ER, extracellular vesicular exosome		NCDN[179*a*]
DHHC2 [zDHHC2]	plasma membrane, recycling endosome[*e*]	Lymph node metastasis and independently predicts an unfavorable prognosis in gastric adenocarcinoma patients; colorectal cancer.[180]	Lck,[181*a*] R7BP (RGS7),[182*a*] CKAP4/p63,[183*a*] CD9 and CD151,[184*ad*] PSD-95[42,57*ab*] and GAP43,[42*b*] SNAP23/25,[61*b*] eNOS,[185*bd*] gp78,[186*b*] AKAP79/150[187*a*]
DHHC3 [zDHHC3]	Golgi		(TARP γ-2, TARP γ-8, CNIH2, CaMKIIα, Syd-1, zyxin, TRPM8, TRPC1, HCRTR2),[179*b*] integrin α6/β4,[188*a*] TRAIL-R1,[189*a*] γ2 subunit of $GABA_{(A)}$,[41*bc*] NCDN,[179*a*] SNAP25/23,[61*b*] Gα,[190*a*] CSP,[191*b*] PI4KIIα,[192*a*] RGS4,[112*b*] STREX,[193*ac*] eNOS,[185*bd*] PICK1,[194*acd*] GluR1/2,[63*b*] PSD-95,[42*b*] CALHM1[195], PPT1[196*b*]
DHHC4 [zDHHC4]	Golgi, ER		No known substrates
DHHC5 [zDHHC5]	Plasma membrane, dendrite	Post-synaptic function affecting learning and memory.[64]	δ-catenin,[197*a*] FLOT2,[198*a*] SSTR5,[128*adc*] GRIP1b,[65*acd*] STREX,[193*ac*] PLM[199]
DHHC6 [zDHHC6]	ER		Calnexin,[50*a*] gp78,[186*b*] IP3R[200]

DHHC7 [zDHHC7]	Golgi		(TARP γ-8, CaMKIIα, Syd-1, NCDN)[179b] SNAP25/23,[61b] PSD-95,[42b] PI4KIIα,[192a] RGS4,[112b] Gα,[190a] CSP,[191b] estrogen[d], progesterone, and androgen receptors,[201a] STREX,[193ac] eNOS,[185bd] Glut4[141a], Fas[202b] JAMC[143a], Scribble,[142] PPT1,[196b] NMNAT2[203a]
DHHC8 [zDHHC8]	Golgi, Cytoplasmic vesicle, mitochondrion	Genetic deletion may be linked to susceptibility to schizophrenia. Synaptic regulation. ZDHHC8 knockdown enhances radio-sensitivity of mesothelioma cells.	PICK1,[194acd] GRIP1b,[65acd] ABCA1,[204b] paralemmin-1,[63 bd] eNOS[185bd]
DHHC9 [zDHHC9]	Golgi, ER, cytoplasm	Mutations in the gene are associated with X-linked mental retardation.	H-Ras,[49] N-Ras,[49] STREX[193ac]
DHHC10 [zDHHC11]	ER	Gain of ZDHHC11 gene may be a potential biomarker for bladder cancer and non-small cell lung cancer.	NCDN[179a]
DHHC11 [zDHHC23]	Plasma membrane		KCNMA1,[205a] gp78[186b]
DHHC12 [zDHHC12]	ER, Golgi	Alzheimer's disease[206]	No known substrates
DHHC13 [zDHHC24]	ER[58]		gp78[186b]
DHHC14 [zDHHC14]	ER[9]	Deletion may be linked to development delay[207]. Activation through chromosomal translocation in patients with acute biphenotypic leukemia.[208]	No known substrates
DHHC15 [zDHHC15]	Golgi	Mutations in the gene cause X-linked mental retardation type 91.[127]	CSP,[191b] SNAP25b,[61b] PSD-95,[42b] JNK3[209]
DHHC16 [zDHHC16]	ER, cytoplasm[210]		PLN,[211] DHHC6

DHHC17 [zDHHC17]	Golgi, Golgi-associated vesicle membrane, cytoplasmic vesicles[62]	Memory and synaptic deficits in KO mice.[212]	SNAP25/23,[61*b*] CSP,[191*b*] STREX,[193*ac*] ClipR-59,[213] PSD-95,[214*b*] GAD65,[214*b*] SYT1,[214*b*] huntingtin,[106*a*] MPP1/p55,[215*a*] LCK,[42*b*] JNK3,[216*bd*] GLUR1,[63*b*] GLUR2,[63*b*] Caspase-6[119]
DHHC18 [zDHHC18]	Golgi		H-Ras,[42*b*] Lck[42*b*]
DHHC19 [zDHHC19]	ER		R-Ras[48*b*]
DHHC20 [zDHHC20]	Plasma membrane		EGFR,[217] Peptide mimic of N terminal myristoylated proteins,[218] δ-catenin,[197*b*] CALHM1[195]
DHHC21 [zDHHC21]	Golgi, plasma membrane	Loss of protein function result in delayed hair shaft differentiation and hyperplasia of interfollicular epidermis and sebaceous glands.	Estrogen[*d*], progesterone, and androgen receptors,[201*a*] PECAM-1,[219*a*] Fyn,[135*bc*] eNOS[185*ad*]
DHHC22 [zDHHC13]	Golgi, Golgi-associated vesicle membrane[220], ER,	ZDHHC13 deficient mice develop alopecia, amyloidosis and osteoporosis[138] and have reduced bone mineral density.[221] Huntington's disease	MT1-MMP,[221*bc*] huntingtin,[86*b*] CNFN[139*a*]
DHHC24 [zDHHC22]	Golgi, ER		KCNMA1,[205*a*] gp78[186*b*]

a - knockdown evidence *b* - overexpression evidence *c* = murine orthologue *d* = protein/substrate interaction
e - localization may depend on cell type

CHAPTER TWO

DHHC protein acyltransferases catalyzes the lysine palmitoylation of members of the Ras superfamily of small GTPases

INTRODUCTION

Modification of protein lysine residues by long-chain fatty acyl groups has been known for over three decades. However very little had been known about its prevalence, regulation, and biological function.[31] In mammalian cells, the first two proteins reported to contain myristoyl lysine were tumor necrosis factor-alpha (TNFα)[4] and interleukin-1 alpha.[5] Lens integral membrane protein aquaporin-0 reportedly undergoes lysine palmitoylation and oleylation. In the past five years or so, studies on a class of enzymes called sirtuins have begun to shed light on the function of lysine long-chain fatty acylation. Sirtuins regulate numerous biological processes, including transcription, metabolism, genome stability, and aging.[222-224] They were initially known to be NAD^+-dependent protein lysine deacetylases,[225] but several of the seven mammalian sirtuins that lack efficient deacetylase activity were found to catalyze the hydrolysis of other acyl lysines efficiently.[226] Among these, several sirtuins have been reported to have the ability to remove long-chain fatty acyl groups.[1] In particular, SIRT6 can defatty-acylate TNFα regulates its secretion, providing the first example for the function of protein lysine fatty acylation in mammalian cells.[1]

Recently, several other proteins found to be regulated by reversible lysine fatty acylation[30,227] and sirtuins. The most exciting examples are two members of the Ras superfamily of proteins, K-Ras4a and R-Ras2.[227] The Ras superfamily of proteins contain more than 150

686 members in mammals and they play essential roles in various signaling events. The finding that
687 lysine fatty acylation regulates several Ras proteins provides compelling support to the
688 hypothesis that lysine fatty acylation has important regulatory functions in biology. Lysine fatty
689 acylation is likely more abundant than previously thought, as many sirtuins and zinc-dependent
690 histone deacetylase are capable of removing long-chain fatty acyl groups.[1,30,228,229]

691 These findings support that protein lysine fatty acylation is emerging as a physiologically
692 important protein post-translational modification. To gain further insights into its occurrence and
693 function, the enzymes that install the fatty acyl groups onto protein lysine residues must be
694 identified, if there are any. Recently, a bacterial toxin is reported to palmitoylate its host GTPase.
695 However, whether mammalian cells possess endogenous lysine palmitoyltransferases remains
696 unknown. Here we report the first mammalian lysine palmitoyltransferases.

697

RESULTS

DHHC2/3/7/15 expression can increase K-Ras4a lysine fatty acylation.

We started the work with the attempt to identify the acyltransferases responsible for the lysine acylation of K-Ras4a, which was recently reported to have lysine palmitoylation and regulated by SIRT2. We focused on the 23 members of the DHHC family of protein palmitoyltransferases. Overexpressing the 23 DHHCs in HEK293T cells showed that DHHC3, DHHC7, and DHHC15 were potential writers of K-Ras4a fatty acylation as increased fluorescent signals (an indication of fatty acylation level) were observed when these DHHCs were overexpressed. Surprisingly, after treating with 400 mM (2.5%) hydroxylamine, the increased K-Ras4a fatty acylation levels by DHHC3/7 expression could still be detected. Quantification and normalization of the fluorescent signal against the protein level indicated that DHHC3 was able to increase the hydroxylamine-resistant fatty acylation by approximately 64%* ± 38%, DHHC7 by 57%* ± 7% (Figure 5a). DHHC2 and DHHC8 did not express in our initial screens (Figure S1). Later on, we were able to express these two enzymes individually and observed that expression of DHHC2, but not DHHC8, was able to increase the hydroxylamine-resistant fatty acylation of K-Ras4a similarly as DHHC3 and DHHC7 (Figure S2a,b). Additionally, we observed DHHC15, closely related to DHHC2[230], is also capable of increasing K-Ras4a hydroxylamine-resistant fatty acylation to DHHC2/3/7 (Figure S3b).

The results from our screening experiments were unexpected as members of the DHHC enzymes are well known as cysteine palmitoyl transferases. We anticipated increases in hydroxylamine-sensitive fatty acylation (S-palmitoylation), not hydroxylamine-resistant fatty acylation. One possible explanation as to why expressing DHHCs could increase lysine fatty acylation could be that cysteine fatty acylation served as a signal for the lysine fatty

721 acyltransferase to modify the lysine residues. Thus by increasing cysteine fatty acylation through
722 the ectopic expression of the DHHC, it could increase lysine fatty acylation. Alternatively, lysine
723 fatty acylation could be the result of a Cys-to-Lys acyl shift as the palmitoylated lysine residues
724 in K-Ras4a are close to the palmitoylated Cys. To investigate this possibility we co-expressed
725 DHHC2/3/7/15 with the non-palmitoylated K-Ras4a mutant C180S. Surprisingly, we still
726 observed increased hydroxylamine-resistant labeling on K-Ras4a C180S mutant in cells
727 expressing DHHC2/3/7/15, suggesting that the DHHC enzyme is still able to modify this mutant
728 that cannot be Cys acylated (Figure 6b, c). The gain in fatty acylation is almost completely
729 hydroxylamine resistant giving us the first indication that the modification is likely attached to
730 K-Ras4a through an amide bond as hydroxylamine treatment readily removes S- and O-
731 acylation[231]. The hydroxylamine resistant and SIRT2-regulated palmitoylation on K-Ras4a has
732 previously been attributed to three lysine residues in the C-terminal region. When these three
733 lysine residues were mutated to arginine (the 3KR mutant), DHHC2/3/7/15 expression did not
734 increase the hydroxylamine-resistant fatty acylation, in contrast to what was observed with K-
735 Ras4a WT and the C180S mutant. This indicated that the endogenous lysine fatty acylation sites
736 were the modification sites for the overexpressed DHHC2/3/7/15.

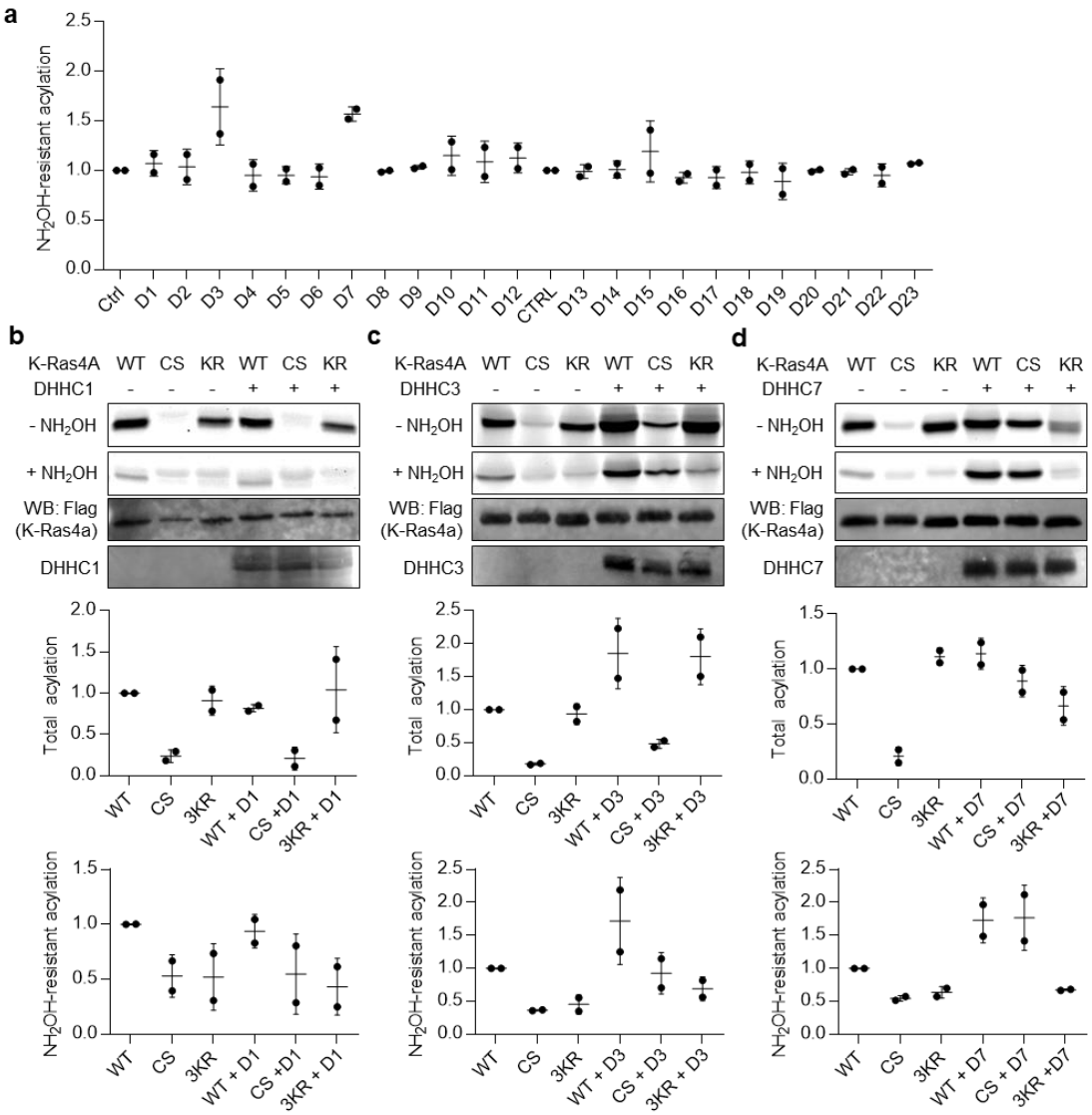
737 To rule out the possibility the fatty acylation is an artifact due to artificially high levels of
738 ectopic DHHC expression, DHHC1 was also overexpressed with the K-Ras4a WT, C180S, and
739 3KR mutants as a control. DHHC1 was expressed at similar or higher levels than DHHC2/3/7
740 (Figure S2a), but we did not observe any increases in fatty acylation. Thus, certain specificities
741 exist among the DHHCs on catalyzing the lysine fatty acylation of K-Ras4a.

742 The second isoform of K-RAS known as KRAS4B lacks a palmitoylated cysteine at the
743 C-terminal region and appears similar to our K-Ras4a C180S mutant. We wondered if this

744 isoform would serve as a lysine palmitoylation substrate for DHHC2/3/7. When we expressed
745 with DHHC7, K-Ras4b had a threefold increase in fatty acylation before and after
746 hydroxylamine treatment. The absolute fluorescence intensity, however, was lower than that with
747 for K-Ras4a, suggesting that K-Ras4b had lower levels of lysine fatty acylation. The combined
748 acylation data of the K-Ras4a C180S mutant and K-Ras4b with DHHC7 overexpression strongly
749 suggest that the DHHCs are capable of directly acylating the ε-amino group on a lysine side
750 chain and lysine palmitoylation is not a result of an S-to-N acyl shift.

751

752



753

754 **Figure 5. K-Ras4a fatty acylation is increased by ectopic expression of DHHC3/7 in**
755 **HEK293T cell**

756 (**a**) Quantification and normalized hydroxylamine-resistant fatty acylation of WT K-Ras4a with
757 over-expressed DHHC1 – 23 (n = 2). (**b**) Fatty acylation of K-Ras4a mutants with over-
758 expressed DHHC1 (n = 2) and quantifications. (**c**) Fatty acylation of K-Ras4a mutants with over-
759 expressed DHHC3 (n = 2) and quantifications. (**b**) Fatty acylation of K-Ras4a mutants with
760 over-expressed DHHC7 (n = 2) and quantifications. The individual plotted values are normalized
761 to the WT control sample before and after 0.4 M hydroxylamine treatment (error bars represent
762 S.D.)

763

764

Further confirmation that DHHC-mediated hydroxylamine-resistant acylation on K-RAS is lysine acylation.

We next set out to further confirm that the increased hydroxylamine resistant acylation when we co-expressed DHHC2/3/7/15 with K-Ras4a was due to lysine acylation. We previously reported certain cysteine fatty acylation was resistant to hydroxylamine treatment[143]. In that study, treatment of JAM-C was able to remove a significant amount of fatty acylation but could not remove all of the fatty acylation. The hydroxylamine resistant signal was thought to be on a lysine but mutation of multiple lysine residues did not affect the fatty acylation. Mutation of the cysteine acylation sites however could inhibit the fatty acylation which indicates that the acylation are indeed on cysteines. These observations suggest that the acylation are on a cysteine yet somehow hydroxylamine resistant. Thus, for this study it remains possible that the increased hydroxylamine resistant acylation we observed is located on a cysteine residue not previously reported to be modified. To address this, we mutated all of the cysteines found on K-Ras4a including the palmitoylated cysteine, but excluding the farnesylated cysteine (when the farnesylated cysteine is mutated, DHHC3/7 was unable to acylate this mutant), generating the mutant referred to as 4CS. When K-Ras4a 4CS was co-expressed with DHHC2/3/7, we observed increased acylation that was completely hydroxylamine resistant (Figure 6a). This gain of acylation was similar to that observed when the DHHCs were co-expressed with the C180S single mutant. This indicates the three other cysteines were not the modified sites by the ectopically expressed DHHC2/3/7.

Our lab previously reported SIRT2 is capable of removing the endogenous lysine acyl modification on K-Ras4a, but not cysteine acylation. We sought to utilize the SIRT2 deacylation assay to confirm that the DHHC-catalyzed fatty acylation of K-Ras4a was on a lysine. K-Ras4a

C180S was co-expressed with DHHC2/3/7 to generate the hydroxylamine-resistant fatty acylation as we previously described in Figure 5. This C180S mutant has only hydroxylamine-resistant fatty acylation so when treated with SIRT2 there would be no interference from cysteine fatty acylation. As expected when treated with SIRT2 and NAD^+ *in vitro*, the hydroxylamine-resistant signal was completely removed (Figure 6b). When NAD^+ was omitted in the control reaction, the acylation remained. As SIRT2 is only able to remove acyl groups from lysine residues and not cysteine residues, we concluded that the increased fatty acylation signal on K-Ras4a by DHHC2/3/7 expression was indeed lysine fatty acylation.

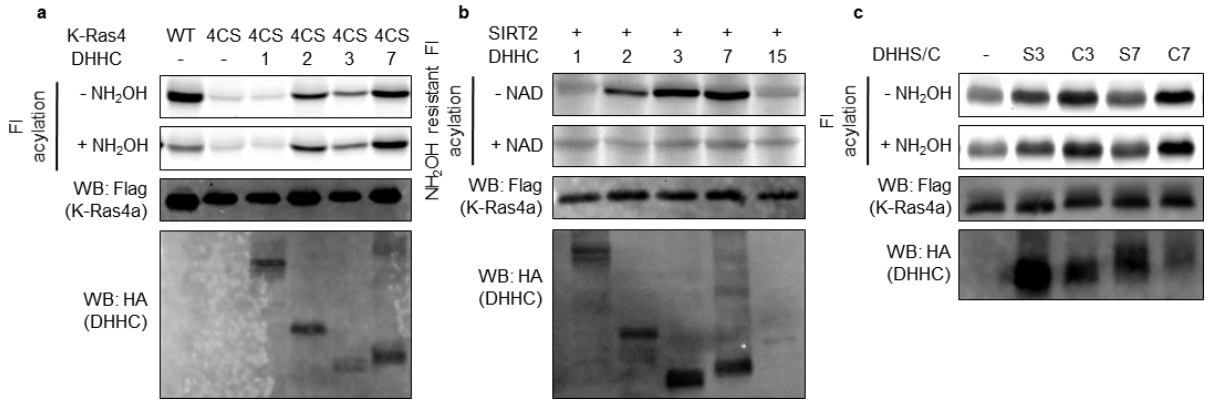


Figure 6. Further confirmation DHHC mediated hydroxylamine resistant acylation on K-Ras4a is lysine fatty acylation.

(a) Fatty acylation of the additional cysteine mutant of K-Ras4a (4CS) co-expressed with DHHC2/3/7 shows almost exclusive increases in NH_2OH resistant fatty acylation. **(b)** The increased hydroxylamine-resistant fatty acylation of K-Ras4a C180S by DHHC2/3/7 co-expression can be removed by purified SIRT2 enzyme. SIRT2 in the presence of NAD^+ in the *in vitro* deacylation reaction completely removed the DHHC mediated hydroxylamine resistant fatty acylation signal. **(c)** Fatty acylation of K-Ras4a C180S co-expressed with DHHC3/7 and the respective catalytically inactive DHHS mutants.

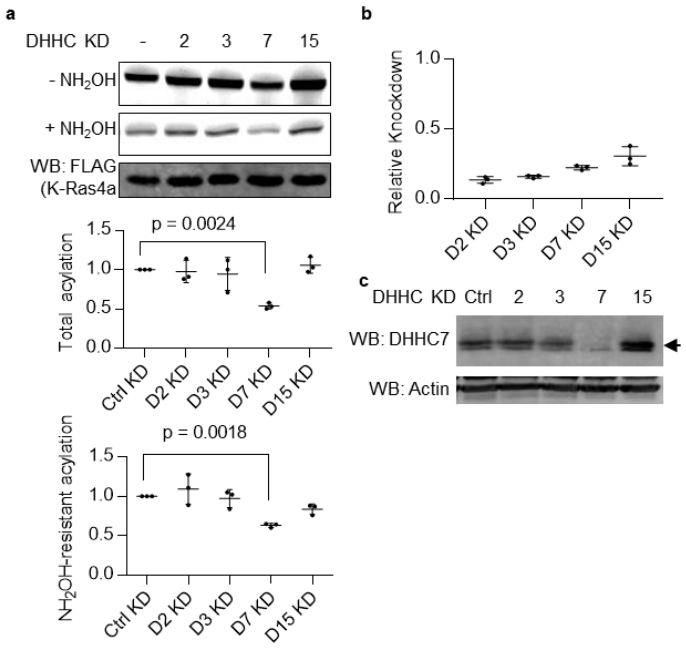
DHHC-mediated lysine acylation of K-Ras4a is dependent on DHHC catalytic activity.

The fatty acylation of lysine residues on K-Ras4a was dependent on the catalytic activity of the DHHC enzyme as overexpression of the DHHS3/7 mutant, where the catalytic cysteine was mutated to serine, dramatically reduced the hydroxylamine-resistant fatty acylation on K-Ras4a C180S (Figure 6c). The DHHC canonical cysteine fatty acylation activity proceeds through a ping pong mechanism[73] where the acyl donor is thought to first modify the catalytic cysteine of the DHHC forming a DHHC-acyl intermediate followed by a subsequent transfer of the acyl group from the intermediate to the cysteine on the substrate. The same DHHC-acyl intermediate is likely also utilized for the lysine fatty acyl transfer. Instead of deprotonation of a cysteine residue on the substrate, deprotonation of a lysine residue on the substrate through the same histidine of the DHHC motif could generate the required nucleophile to capture the acyl group from the catalytic cysteine residue.

DHHC7 knockdown perturbs K-RAS lysine fatty acylation

Our co-expression data strongly suggest that DHHC2/3/7/15 possess lysine fatty acylation activity. To further confirm this, we used shRNAs (Sigma Aldrich) to generate lentiviral particles to obtain DHHC2/3/7/15 stable knockdown cells in the HEK293T cells. We chose this cell line as we are able to easily detect the basal lysine fatty acylation of overexpressed K-Ras4a without overexpression of a DHHC. The knockdown efficiencies are verified by qPCR, as antibodies for endogenous DHHC proteins are, in our hands, generally unreliable. On average we obtained an average of 70% reduction in DHHC mRNA level over multiple independent repeats. When WT K-Ras4a is expressed in the DHHC7 stable knockdown cells, we were able to observe a 37% decrease in hydroxylamine resistant fatty acylation (Figure 7a). We did not observe a significant effect on K-Ras4a lysine acylation in the DHHC2 and the

DHHC3 stable knockdown cells. DHHCs have been demonstrated to share overlap substrates. This redundancy makes it challenging to confirm the substrate of DHHCs by knockdown. None the less, our data at least showed that endogenous DHHC7 is capable of acylating the lysine residues of K-Ras4a.



837

838 **Figure 7. DHHC7 knockdown perturbs K-Ras4a lysine fatty acylation.**

839 **(a)** Representative fluorescence gel of WT K-Ras4a overexpressed in DHHC2/3/7/15 stable
840 knockdown HEK293T cells with quantification (n = 3). The individual plotted values are
841 normalized to the WT control sample before and after 0.4 M hydroxylamine treatment. **(b)**
842 Relative mRNA level of DHHC2/3/7/15 in stable knockdown cells compared to the control
843 knockdown sample determined by qPCR (n=3). **(c)** Western blot against endogenous DHHC7 in
844 the DHHC2/3/7/15 stable knockdown HEK293T cells. Arrow denotes endogenous DHHC7.

845

846

847

DHHC3/7 ectopic expression promotes the lysine acylation of various GTPases

Next, we asked if other small GTPases could serve as lysine acylation substrates for DHHC3 or DHHC7 when ectopically expressed together in HEK293T cells. We screened several small GTPases from different subfamilies and found a few different outcomes. TC10 and Rac1 overall acylation were not affected by DHHC7 overexpression (Figure S4). RalA total fatty acylation is increased upon DHHC7 overexpression, but no differences were observed post hydroxylamine treatment. CDC42, which lacks a palmitoylated cysteine is similar to K-Ras4b and our K-Ras4a C180S mutant, had increased acylation before and after hydroxylamine treatment when overexpressed with DHHC3 and DHHC7. The acylation was little affected by hydroxylamine, which suggests that the acylation is located on a lysine residue. It is important to note that RALA in our hands served as a robust S-palmitoylation only substrate for DHHC7. This was somewhat surprising in contrast to our results with K-Ras4a, so we further screened RALA against all four DHHCs. DHHC2/3/7/15 increased the total acylation and did not affect the hydroxylamine resistant acylation, which indicates this gained acylation is on a cysteine (Figure S5). Additionally, we were able to confer lysine fatty acylation onto RALA by mutation of the arginine residue nearby the palmitoylated cysteine. This RALA arginine to lysine mutant when overexpressed with DHHC3/7 but not DHHC2/15 had hydroxylamine resistant fatty acylation. Overall, the acylation of RALA by DHHC2/3/7/15 serves a control to our K-Ras4a acylation results as it demonstrates there is a degree of selectivity regarding the protein substrate which can be lysine fatty acylated by the DHHCs. It further demonstrates that this novel enzymatic activity is not as promiscuous as one may think, particularly when working with DHHC3. The factors which govern which GTPase may serve as a DHHC lysine fatty acyl substrates require further investigation.

For later studies, we chose to focus on CDC42, which allow us to investigate the function of its lysine fatty acylation without the complications of potential cysteine-palmitoylation**.**

DHHC3/7 promotes lysine acylation of CDC42 in a manner similar to K-Ras4a.

CDC42 does not have cysteine palmitoylation and in our screen, exhibited a robust increase in hydroxylamine resistant fatty acylation when DHHC3/7 was overexpressed (Figure 8a). This increase in acylation is dependent on the enzymatic activity of the DHHC3/7 as the corresponding DHHS mutants had little activity (Figure 8b, c). The increased fatty acylation could also be removed by SIRT2 in an NAD^+ dependent manner (Figure 8d) similar to K-Ras4a's lysine fatty acylation. Through mutagenesis, we identified Lys184 as the major acceptor residue of the DHHC-dependent acylation as the K184R mutant could not be fatty acylated by DHHC3/7 while the K183R mutant could still be partially fatty acylated by DHHC3/7 (Figure 8e, f). Mutation of both lysine residues also blocked the effects of DHHC3/7 overexpression on CDC42 fatty acylation. Taken together, these results demonstrate that CDC42 is similar to K-Ras4b and K-Ras4a C180S as a lysine fatty acyl substrate for the DHHCs.

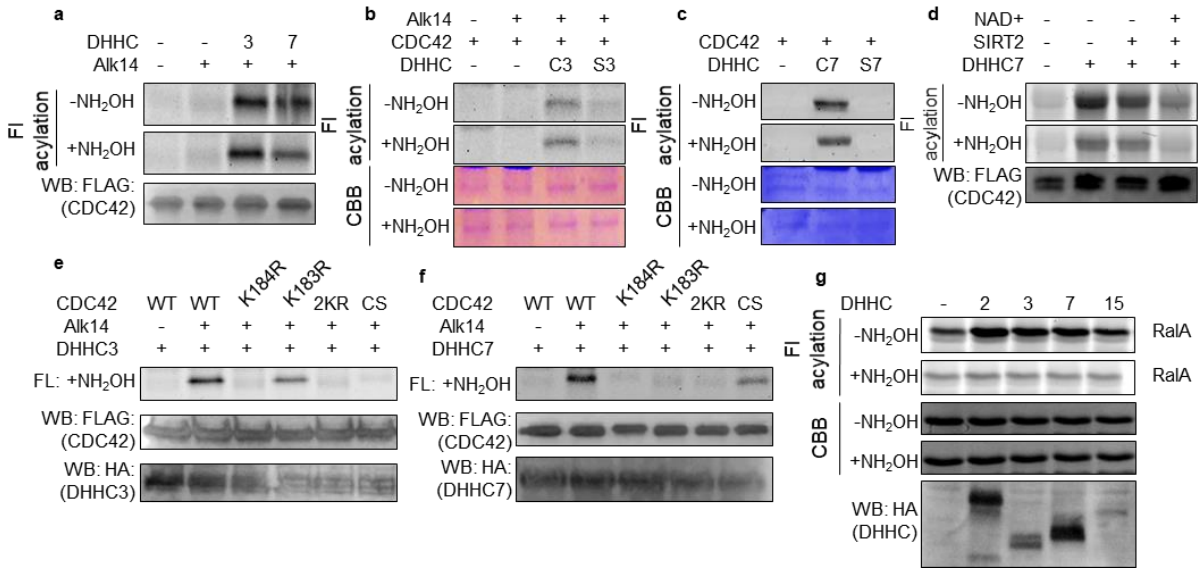


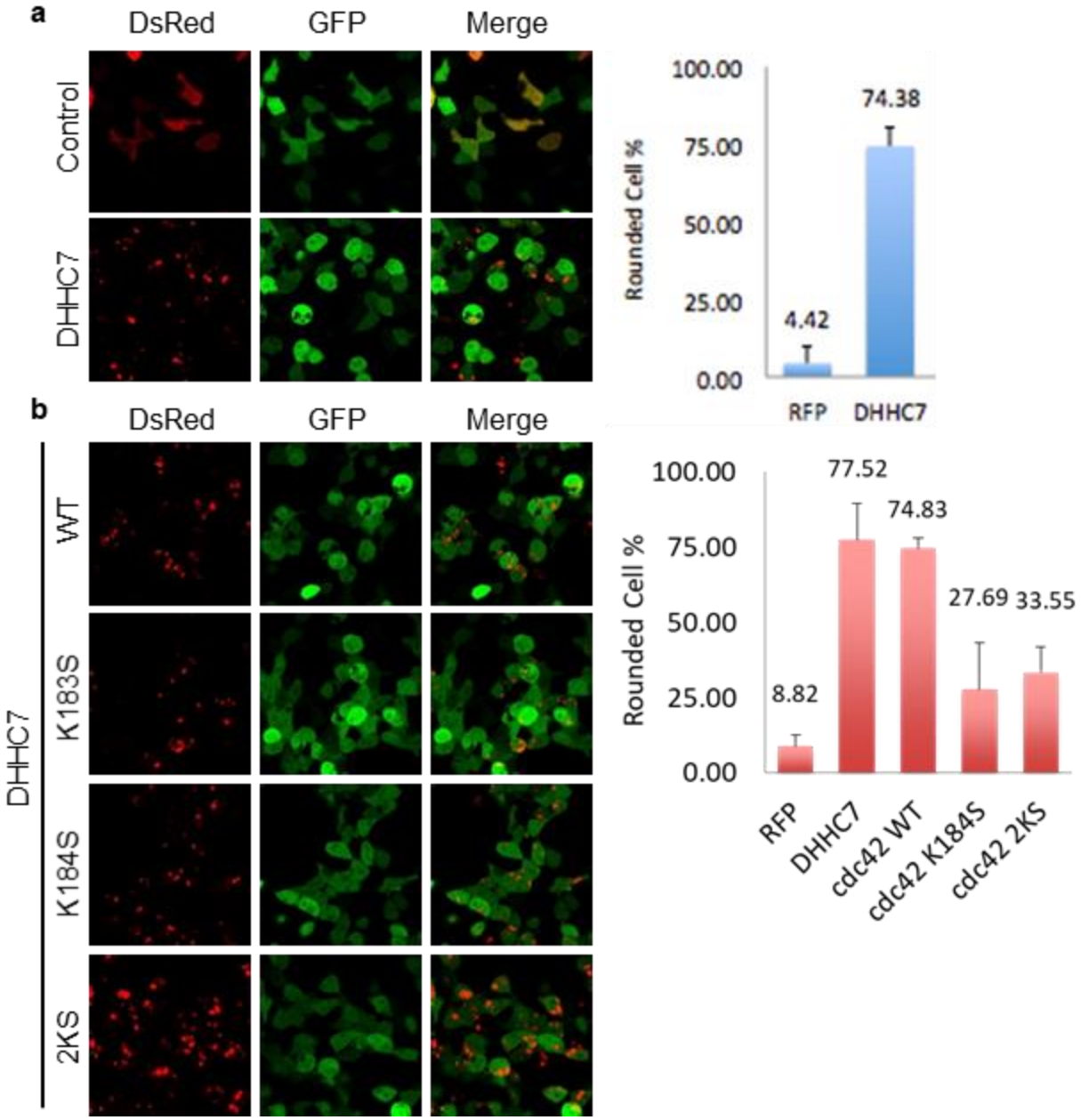
Figure 8. DHHC3/7 promotes lysine fatty acylation of CDC42.

(a) DHHC3/7 promotes pCDC42 NH_2OH-resistant fatty acylation **(b, c)** Increased CDC42 fatty acylation by DHHC3/7 is dependent on the DHHC catalytic cysteine. **(d)** DHHC7-dependent NH_2OH-resistant fatty acylation on CDC42 can be removed by SIRT2 *in vitro.* **(e, f)** K184 is the major acceptor site of the DHHC3/7 dependent fatty acylation on CDC42. **(g)** DHHC2/3/7/15 overexpression increases RALA NH_2OH-susceptible fatty acylation but not NH_2OH resistant acylation.

Ectopic expression of DHHC7 induces cell rounding through the lysine fatty acylation of CDC42

CDC42 is known to regulate cytoskeleton and thus cell migration and morphology[232]. We thus decided to examine whether DHHC3/7 expression could regulate cell morphology through the lysine acylation of CDC42. Rounded cells are a classical phenotype observed when CDC42 is either deleted or loses its function due to being locked in its GDP bound form.[233,234] Using live cell confocal imaging, we observed that ectopic expression of a DsRed tagged DHHC7 in HEK293T resulted in a majority (74%) of the cells adopting a round shape (Figure 9a). GFP is co-expressed with DHHC7 to highlight the shape of the cell.

We then investigated whether the cell rounding effect was due to the increased lysine fatty acylation on CDC42. In cells expressing GFP and DHHC7, we examined whether overexpression of a WT CDC42 or the lysine mutant could rescue the cell rounding phenotype. The addition of a K184S or a 2KS mutant reduced the amount of rounded cells, while the addition of WT CDC42 showed similar levels of rounded cells (Figure 9b Our observations indicate that the DHHC7 mediated lysine fatty acylation of CDC42 inhibits its normal function resulting in the rounded morphology observed. Introduction of the K184S mutant of CDC42 "rescues" this phenotype as it remains functional because it cannot be acylated.



916

917 **Figure 9. DHHC7 induced cell rounding is reversed by a nonacylated K184S CDC42.**

918 **(a)** DHHC7 (DsRed-tagged) ectopic expression induces cell rounding in HEK293T cells. GFP is
919 overexpressed to highlight cell morphology by confocal microscopy. Quantification shows
920 average percentage of rounded cells from 3 independent repeats. **(b)** The non-acylatable CDC42
921 K184S and 2KS rescues DHHC7 induced cell rounding in HEK293T cells. Quantification shows
922 average percentage of rounded cells from 3 independent repeats.

923

DISCUSSION

We report the first mammalian enzyme capable of catalyzing lysine fatty acylation. Surprisingly, we identified multiple DHHC proteins that are well established to function as cysteine fatty acyl transferases can also transfer fatty acyl groups to lysine. In particular, the promiscuous and robust cysteine fatty acyltransferases DHHC3 and DHHC7 were shown to possess novel lysine fatty acyltransferase activity against K-Ras and CDC42, members of the GTPase super family. Additionally the closely related cousins to DHHC3/7, DHHC2 and DHHC15 were also observed to possess this novel enzymatic activity (albeit weaker, but may due to the lower protein expression level). As K-Ras4a has previously been reported to possess lysine fatty acylation that can be removed by SIRT2, we confirmed that the modification placed onto K-Ras4a is indeed lysine residues a by utilizing the *in vitro* SIRT2 deacylation assay. Only knockdown of DHHC7 was able to perturb the lysine fatty acylation of K-Ras4a, while knockdown of DHHC2/3/15 did not affect K-Ras4a fatty acylation. This is likely due to the substrate redundancy commonly found within the DHHC family as multiple DHHC enzymes are able to acylate the same substrate.

Our study identified a physiological consequence of the novel lysine fatty acyltransferase activity. DHHC7 mediated lysine fatty acylation of CDC42 produces a phenotype reminiscent of CDC42 null cells and of inactive CDC42 cells. Similar to K-Ras4a, CDC42 is fatty acylated by DHHC7 at its C terminal hyper variable region on two adjacent lysine residues. HEK293T cells overexpressing DHHC7 were more rounded than controls cells. Overexpression of WT CDC42 was unable to revert the cell morphology while the K184S mutant could.

The importance of lysine fatty acylation has become clearer in recent years due to the finding that the sirtuin class of HDACs and several HDACs themselves have robust defatty-

947 acylase activity. Several defatty-acylated substrates of these erasers have been identified while
948 the writers of these modifications remain elusive. Our study identifies the DHHC family of
949 cysteine palmitoyl acyltransferases as enzymes capable of catalyzing both cysteine and lysine
950 fatty acylation, opening the field to identify new lysine fatty acylated substrates and their writers.
951

METHODS AND MATERIALS

Antibodies and reagents

Fugene®6 transfection reagent (Promega) was used for cell transfection with at a 3:1 DNA:Fugene ratio. Monoclonal anti-FLAG® (SIGMA) M2-peroxidase (HRP) produced in mouse was used for all FLAG western blots (catalogue A8952). Anti-FLAG® M2 affinity gel was used for immunoprecipitation (catalogue A2220). Anti-DHHC7 (Assay Biotech R12-3691) antibody was used at 1:1000 dilution. Anti-DHHC3 (Abcam ab31837) was used at 1:1000 dilution. Anti-HA antibody HRP conjugate (sc-7392 HRP) and other HRPconjugated secondary antibodies were purchased from Santa Cruz Biotechnology. Alkyne-14 was synthesized and kindly provided by Nicole Spiegelman as previously described[236]. Hydroxylamine solution (SIGMA) was diluted to 4 M in water and pH adjusted to ~9-10.

Plasmids and shRNAs

K-Ras4a and mutants was obtained as previously describe.[30] DHHC1 – 23 murine plasmids were kindly gifted by Professor Maurine Linder with permission from Professor Masaki Fukata. K-Ras4b, CDC42, and RalA plasmid were gifted by Professor Maurine Linder. DHHS2/3/7/15 clones and CDC42 mutants were generated by site-directed mutagenesis[237]. K-Ras4a 4CS mutant was generated using site-directed mutagenesis. Ds-Red DHHC7 plasmid was obtained by cloning human zDHHC7 from cDNA library into the dsRed2-N1 vector (Clontech) using the NheI and BamHI restriction sites. shRNAs used to generate DHHC stable knockdown cells were purchased from Sigma (catalogue numbers DHHC2 TRCN0000143212, DHHC3 TRCN0000133710, DHHC7 TRCN0000122203, DHHC15 TRCN0000145091, with non-mammalian shRNA SHC002 as the control).

Cell culture and generation DHHC stable knockdown cells

Cell lines used are HEK-293T from ATCC. HEK-293T cells were cultured in DMEM (gibco) media with 10% fetal bovine serum (life technologies). Cell were cultured at 37^{o}C in a humidified incubator supplemented with 5% CO_2. DHHC stable knockdown cells were generated as previously described[143].

Western blotting

Cells were washed once with ice cold PBS and lysed in 1% Nonidet P-40 (NP-40) lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% (v/v) NP-40 (SIGMA), and 10% glycerol with protease inhibitor cocktail (Sigma P8340)). 20 µg of whole cell lysate as determined by Bradford assay (Pierce^{TM} Coomassie Protein Assay Kit) was separated by 12% SDS-PAGE and subsequently transferred to a PVDF membrane (Bio-Rad) for 120 minutes at 350 mA. The membrane was blocked using 5% milk (SCBT) in TBST (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween 20) and incubated for 1 hour at room temperature for hrp conjugated primary antibodies or overnight at 4^{o}C for other primary antibodies. Three washes with TBST are performed before imaging or incubation with secondary antibodies (in TBST) for 1 hour at room temperature. Three more washes are performed after incubation with secondary antibodies. The membranes are developed using ECL-plus reagent (GE Healthcare). The chemiluminescent signal was visualized using a Typhoon 9400 variable mode imager with the 457 nm excitation and 526 nm detection settings with a photomultiplier setting of 600 V. The signal was analyzed and quantified using ImageJ.

Alkyne labeling of K-Ras4a and CDC42 fatty acylation.

In brief, 1 µg or 3 µg of plasmid with or without an equal amount of DHHC plasmid or empty vector was transfected into a single well of a 6-well plate or a 10 cm dish, respectively, using Fugene®6. After 24 hours, Alk14 was added to a final concentration of 50 µM and cultured for 6 hours. Cells were washed with PBS and lysed in 1% NP-40 lysis buffer and 20 µg of whole cell lysate was used for FLAG and HA western blot, which served to normalize protein expression level. Approximately 500 µg of whole cell lysate is added to 10 µL of anti-FLAG affinity gel slurry and brought up to a volume of 500 µL. The mixture is incubated at 4°C for a minimum of 2 hours with gentle agitation. The beads are centrifuged and washed three times with 0.1% NP-40 buffer at 500 *g*. After the last wash, all remaining buffer is removed using a gel loading tip. A click chemistry master mix is prepared with the final concentrations of 100 µM BODIPY azide (Active Motif, catalogue 16317), 600 µM Tris(benzyltriazolylmethyl)amine (TBTA) (Cayman chemicals), 2 mM $CuSO_4$. 19 uL of the master mix is added to the beads and 1 ul of 40 mM tris(2-carboxyethyl)phosphine is added to initiate the reaction at room temperature for 1 hour in the dark. After the click chemistry, 10 µL of 6x protein loading dye was added and the mixture boiled at 95°C for 5 minutes. The supernatants were divided in two equal portions, with one half treated with 50 mM Tris pH 8.0 and the other half with 0.4 M hydroxylamine. Both halves were incubated at 95°C for 5 minutes. The samples were then separated on a 12% SDS-PAGE gel and 1.5 µL of the click reaction were reserved to run a western blot to serve as the loading control. Thee fluorescence signal was then visualized with a Typhoon 9400 (GE Healthcare) variable mode imager using 526 nm excitation and 520 nm detection filter with a photomultiplier setting of 600 V. The signal was analyzed and quantified using ImageJ.

SIRT2 deacylation assay

The assay was performed as previously described.[30] In brief, K-Ras4a with or without DHHC was transfected into HEK-293T cells for 24 hours and subsequently treated with 50 μM of Alk14 for 6 hours before collecting and lysis in 1% NP-40 lysis buffer. K-Ras4a was immunoprecipitated using anti-FLAG beads for 2 hours at 4^{o}C. After three washes with 0.1% NP-40 buffer, the beads are suspended in reaction buffer (50 mM Tris pH 8.0, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT) with 5 μM SIRT2. NAD^+ is omitted or added to initiate the reaction at 37^{o}C for 1 hour. The reaction was stopped by removing the reaction mixture and washing three times with 1 mL of 0.1% NP-40 buffer. The beads were then used for on-bead click chemistry and fluorescence imaging as described above.

Confocal imaging

HEK293T cells were seeded in glass bottom dishes the night before transfection with the necessary plasmids. 1 ug of plasmid per protein was transfected in with 3 uL of Fugene 6. After 24 hours, cells were incubated in the Live Cell Imaging Solution (A14291DJ, Thermo Fisher Scientific) for live cell imaging. Cells were imaged with a Zeiss LSM710 confocal/multiphoton inverted microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) in a humidified metabolic chamber maintained at 37°C and 5% CO_2. EGFP was expressed to show the shape of the cell and demonstrate rounding of the cell.

Statistical analysis

Quantification values were obtained using ImageJ[238] and are expressed as mean ± s.d (standard deviation). Student's two tailed t-test were used to analyze differences between two groups of values with *$P < 0.02$, **$P < 0.01$, ***$P < 0.001$.

*It is important to note that due to the size of our K-Ras4a construct, it overlaps very well with the antibody light chain, which affected our ability to precisely quantify the fluorescence without interference from the light chain. The light chain can also at times be detected on the fluorescence image due to a combination of its high levels of protein and weak fluorescence signal from the substrate.

Acknowledgements

This work is supported in part by grants (R01GM121540-01A1 and R01DK107868) from NIH. HJ is a Howard Hughes Medical Institute International Student Research Fellow. We thank Dr. Maurine Linder for providing invaluable advice throughout the study. We thank the Cornell University Biotechnology Resource Center (BRC) Imaging Facility for help with the confocal microscopy, which is supported by NIH S10RR025502.

CHAPTER THREE

CONCLUSIONS

Protein post-translational modifications vastly increase the complexity and diversity of the proteome, often in rapid response to external and internal stimuli. These modifications ranging from phosphorylation to acetylation are critical for cellular function and pathogenesis. It cannot be understated how important it is to identify and understand protein post-translational modification. The work performed by our lab has pushed our collective understanding of post-translational modifications through the discovery of lysine succinylation and lysine long-chain fatty acylation. More importantly, our lab discovered the ability of sirtuins to remove these modifications. However without the writers our understanding will always be limited. To achieve a complete understanding of the role of these novel modifications, I sought to identify the writers. When I began my studies, little information was available to hint at the potential identity of these novel modifications. Several studies further suggested that these modifications were non-enzymatic events which made the identification of these hypothetical writers an unattractive and challenging endeavor. Progress was further hindered as few were substrates available to screen potential transferases against. For lysine long-chain fatty acylation, TNF-α was the only known substrate to be regulated by lysine fatty acylation. Breakthrough research by fellow lab members identified new substrates belonging to the GTPase family of proteins. This vastly increased the pool of substrates available to screen against my list of potential transferases. This led to the discovery that the DHHC family of protein-palmitoyl transferases were able to affect the lysine fatty acylation levels of these new substrates. Of particular interest was the ability of the DHHC enzymes to directly fatty acylate the substrate's lysines in the absence of a palmitoylated cysteine residue. The nature of this acylation was confirmed to be lysine fatty

acylation by the SIRT2 in-vitro defatty acylation assay. Overexpression of DHHC2, 3, 7, and 15 with a variety of GTPases showed varied effects ranging from, increased cysteine palmitoylation only, increased lysine fatty acylation only, increased cysteine and lysine fatty acylation and GTPases whose acylation was not affected at all. These findings reveal a novel enzymatic activity of DHHC family of enzymes as lysine fatty acyltransferases in addition to its reported function as cysteine fatty acyltransferases.

The lysine fatty acylation modification of TNFα, K-Ras4a, and R-Ras2 established the precedent that this modification has a biological impact. Our work identified the DHHC mediated lysine fatty acylation of CDC42 also has a biological impact. The mechanism by which the DHHC mediated lysine fatty acylation elicits this effect is currently under study. It is well established that a loss of CDC42 function, by point mutation or gene deletion, results in a morphological change resulting in rounded cells. Overexpression of DHHC7 increases CDC42 lysine fatty acylation, leading to the rounding of HEK293T in the absence or presence of overexpressed WT CDC42. Expressing the lysine fatty acylation deficient mutant of CDC42 (KR) diminished the effects of DHHC7 induced cell rounding. This finding is the first to demonstrate a biological impact of DHHC7 mediated lysine fatty acylation of CDC42.

The work here contributes to our understanding of lysine fatty acylation and the function of the DHHC family of enzymes. Several members of the DHHC family have yet to be characterized as cysteine fatty acyltransferases and our work suggests that it is possible these DHHCs may instead function as lysine fatty acyltransferases. The pathologies linked to the DHHCs highlight the importance of their cysteine palmitoyltransferase activity for disease progression. Now with our finding, the stage is set to determine the function of the DHHC's

lysine fatty acyltransferase activity in these pathologies and if this activity can be exploited to develop treatments.

FUTURE DIRECTIONS

IDENTIFY RESIDUES CRITICAL FOR LYSINE FATTY ACYLATION.

Our finding demonstrates that lysine fatty acylation activity of the DHHC is dependent on the catalytic cysteine of the DHHC motif. This is identical to its cysteine transferase activity, which is also dependent on the catalytic cysteine. Of particular interest is the observation that DHHC7 overexpression with WT K-Ras4a resulted mostly increases in lysine fatty acylation whereas its cousin DHHC3 increased in both cysteine and lysine fatty acylation. DHHC3 and DHHC7 share 71% amino acid homology which further increases when examining the DHHC-CRD. It would be exciting and highly informative to determine the residues that result in this differing effect. Even more exciting would be to obtain a mutant DHHC enzyme that performs only one of the two possible activities, i.e., a DHHC enzyme capable of lysine fatty acylation but not cysteine fatty acylation or vice versa.

While this study is not challenging as the potential resides critical for this activity may be readily identified, the investigation itself is time and material consuming. Site-directed mutagenesis can provide all the mutants, and the activities tested using a non-palmitoylated substrate such as K-Ras4a C180S, CDC42 or K-Ras4b. This allows for the detection of a lysine fatty acyltransferase capable or defective DHHC enzyme by looking for a gain or no gain of fatty acylation, respectively. Subsequent comparison against a lysine fatty acylation deficient substrate and a WT substrate would reveal the cysteine fatty acylation ability of the DHHC mutant. Obtaining a DHHC capable of only one activity would greatly help in dissecting the importance of each modification in the various pathologies associated with the DHHCs.

Our data demonstrated that a RalA 2RK mutant gained the ability to serve as a lysine fatty acylation substrate for DHHC7. This initial observation suggests we may be able to investigate the contributing factors on the substrate that determine which lysines may serve as fatty acylation site. These factors also appear to confer selectivity as to which DHHC may fatty acylate the substrate as DHHC2 and DHHC15 were not able to fatty acylate this RalA 2RK mutant, whereas these two DHHCs readily acylated K-Ras4a and WT RalA.

INVESTIGATE HOW LYSINE FATTY ACYLATION ACTIVITY OF THE DHHCS IS REGULATED

In general, how the DHHCs are regulated is poorly understood both at the activity and protein level. How the cell regulates the DHHCs is an important question that remains to be answered. Some studies have begun to provide insight into this critical question[95], and it would be interesting to determine if different mechanisms regulate the DHHC's lysine and cysteine fatty acylation. Several DHHCs have already been demonstrated to require an accessory protein for proper cysteine fatty acyltransferase activity. One potential mechanism to control whether a DHHC prefers to function as a cysteine or lysine fatty acyltransferase is the association of an "adapter" protein that precisely controls the conformation of the enzyme-substrate complex to position either the lysine or cysteine residue closer to the fatty acyl intermediate. Both an amine and thiol are capable of serving as a nucleophile, and the preference of one over the other is likely determined in the enzyme-substrate complex by their proximities to the acyl-thiol intermediate.

This investigation can be accomplished using mass spectrometry to identify DHHC interacting proteins in the presence of a lysine fatty acylated substrate. Deletion of these potential

adapter proteins may abolish the ability of the DHHC to function as a lysine fatty acyltransferase and can be added to the *in vitro* reconstitution assays to drive the lysine fatty acylation activity of the DHHCs. We also observed that low levels of expressed DHHC protein do not readily increase the substrate's fatty acylation, hinting at a dose-dependent effect. How does the cell increase DHHC protein level to replicate the effects of overexpressed DHHC will be critical to understanding the importance of DHHC mediated lysine fatty acylation.

INVESTIGATE THE EFFECTS OF DHHC MEDIATED LYSINE FATTY ACYLATION ON CDC42'S CELL TRANSFORMING ABILITY.

It has been reported that a fast cycling F28L CDC42 (effectively constitutively active) mutant transforms cells with cancer like phenotypes. This ability reportedly requires CDC42 to interact with the COP complex, specifically the γ subunit.[239] Additionally, the mentioned interaction is dependent on the dilysine motif found at the C-terminus of CDC42. Mutation of the two lysines to serine abolishes the interaction with the γCOP subunit and subsequently prevents the F28L CDC42 mutant from transforming NIH 3T3 cells into cancer like cells. These two lysines are the same lysine residues reported by our lab to be the acceptor sites by which DHHC7 fatty acylate CDC42. Our data also suggest that the DHHC7 mediated lysine fatty acylation of WT CDC42 can inhibit its normal function, whereas DHHC7 does not inhibit the dilysine mutant.

It would be interesting to overexpress or elevate DHHC7 protein or activity levels in NIH 3T3 cells that are overexpressing the F28L mutant of CDC42. If the cell transforming ability of CDC42 can be inhibited as determined by the soft agar colony formation assay, then a potential treatment may be developed. In essence, to treat cancer cells with a constitutively active CDC42

background, one would exploit the novel DHHC7 mediated lysine fatty acylation activity to inhibit CDC42. While the soft agar colony formation assay is a standard lab experiment and relatively simple to perform, the challenge remains with elevating and maintain a high DHHC protein level to affect the transforming ability over the extended period required for the assay. Another approach would be to search for a condition or treatment in which the DHHC protein level would be upregulated; however, little is known about how DHHC7 is regulated.

RECONSTITUTION OF DHHC LYSINE FATTY ACYLTRANSFERASE ACTIVITY.

Preliminary attempts at reconstituting the lysine fatty acyltransferase activity of DHHC7 produced results not reflective of the activity observed in cells. Whereas in cells, we observe little to no increase in cysteine fatty acylation (NH_2OH susceptible fatty acylation) with DHHC7 overexpressed with K-Ras4a, *in vitro* reconstitution assays show DHHC7 increasing the cysteine fatty acylation of K-Ras4a in addition to the lysine fatty acylation. The difference between the in cell and reconstitution assay is likely attributed to three factors. First, the *in vitro* assay does not represent the biological environment in which the actual acylation event would occur in; in particular, both the DHHC enzyme and substrate should be interacting with a lipid membrane which the DHHC enzyme is embedded and the substrate tethered to via its farnesylation. The *in vitro* assay is an in-solution assay which is more representative of reactions with soluble proteins. Second, because the *in vitro* reconstitution uses only purified enzyme and substrate, an important adapter protein may be missing which could facilitate the lysine fatty acylation activity of the DHHC. The first factor may be addressed by utilizing lipid nanodiscs which allow the enzyme and substrate to interact within a lipid membrane environment. The second factor is more challenging as the adapter protein is not known, but reconstitution of the enzyme activity in

the presence of whole cell lysate may provide sufficient levels of this hypothetical adapter protein to drive the reaction. Lastly, while palmitoyl-CoA is generally to be the acyl-donor in this reaction, the acyl group may actually come from some other source such as a fatty acylated phospholipid or an acyl carrier protein. Screening various acyl donors may enhance the lysine fatty acyl transferase activity of the DHHC while palmitoyl-CoA may serve as the acyl donor for its cysteine palmitoyltransferase activity.

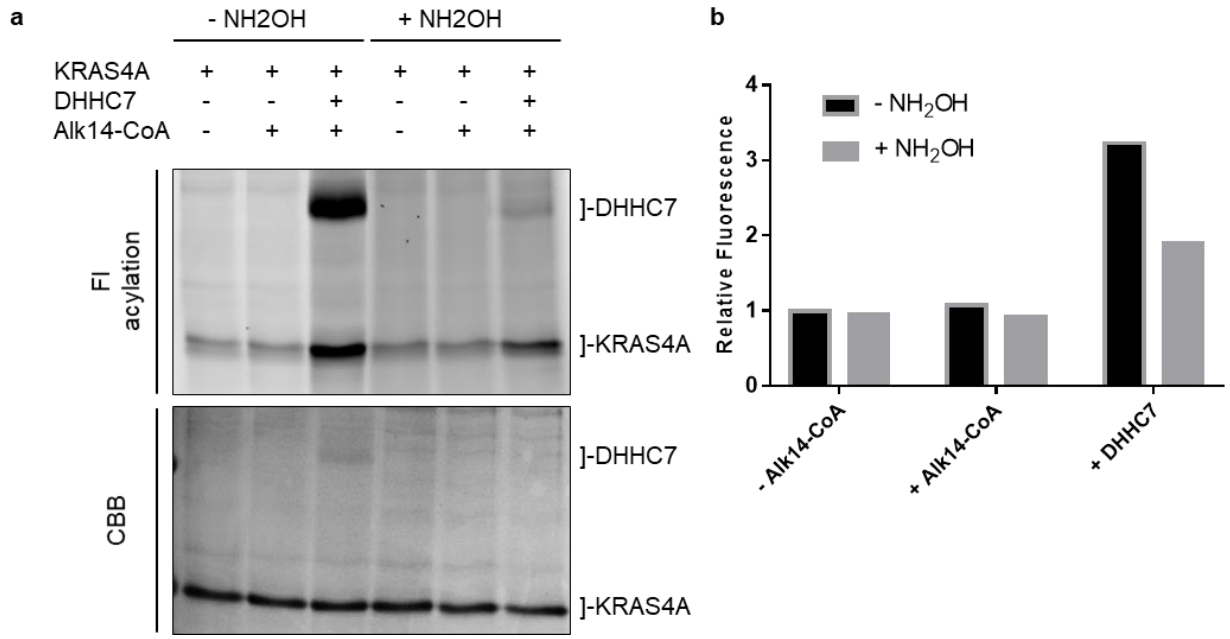


Figure 10. ***In vitro*** **reconstitution of DHHC7 lysine fatty acylation with purified K-Ras4a**

a) K-Ras4a was transfected into HEK-293T cells for 24 hours and then collected and lysed in 1% NP-40 lysis buffer. 10 µL of anti-FLAG slurry is used to immunoprecipitate K-Ras4a per 1 mg of whole cell lysate. The beads are washed 3X with 0.1% NP-40 buffer. K-Ras4a is eluted from the anti-FLAG beads using three separate "washes" of 300 µg/mL of 3X FLAG peptide (Sigma F4799) in enzyme dilution buffer (EDB) (50 mm MES pH 6.4, 100 mm NaCl, 10% glycerol, 0.1% DDM). The K-Ras4a concentration was determined by SDS-PAGE gel with a linear BSA curve. 1 µg of K-Ras4a is then incubated with 0.7 ug of DHH7 in 50 µL of EDB with 3.34 uM Alk14-CoA (Cayman Chemicals 15968) at 37ºC for 1 hour. The reaction is stopped by methanol/chloroform precipitation with 10 µg of BSA added to assist in pellet formation. The pellet is dissolved using 4% SDS lysis buffer (4% SDS, 50 mM triethanolamine pH 7.4, and 150 mM NaCl). Click chemistry is performed to attach fluorophore to visualize the acylation at room temperature for 1 hour. K-Ras4a is precipitated once more to remove excess fluorophore and dissolved in a 4% SDS in water only solution and treated with hydroxylamine, and gel imaged. **b)** Quantification of fluorescence gel and divided by K-Ras4a CBB signal then normalized to lane 1. Half of the fluorescent remains after hydroxylamine treatment. That is the lysine fatty acylation signal.

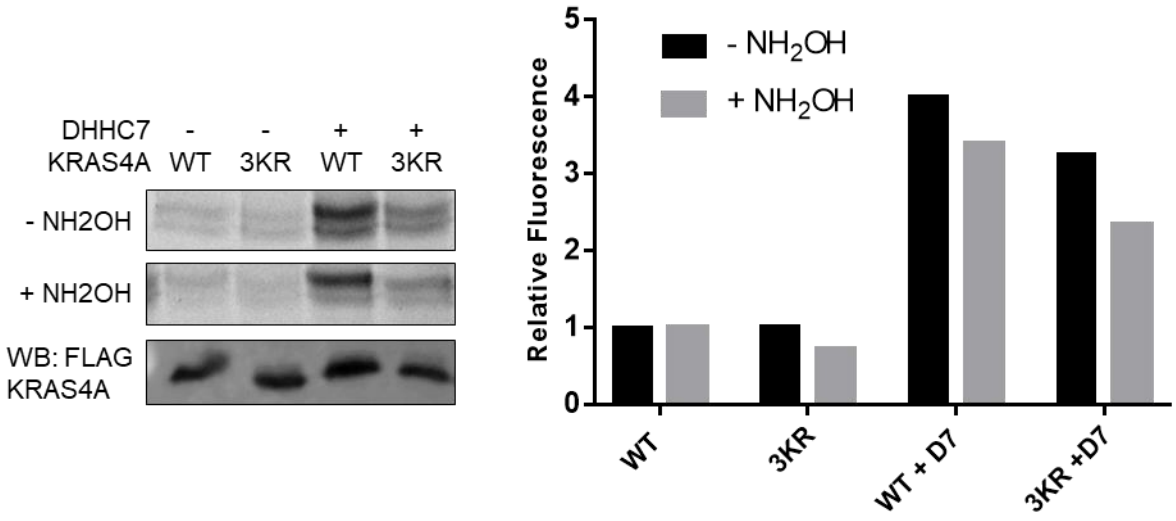


Figure 11. *In vitro* reconstitution of DHHC7 lysine fatty acylation with purified K-Ras4a 3KR

K-Ras4a WT and 3KR are purified as described above. Acylation experiment is identical as described above, except the K-Ras4a sample was dissolved in the 4% SDS lysis buffer at the last step instead of the 4% SDS in water only solution, and then boiled for five minutes at 95^{o}C. 3KR sample shows less acylation particularly post hydroxylamine treatment than WT sample. The weak reduction in fluorescent signal compared to above is due to the reduced total acylation before hydroxylamine treatment. Here, we observe less cysteine fatty acylation because the sample was boiled in the 4% SDS lysis buffer solution. The 3KR sample has reduced acylation because these lysine sites are modified by DHHC7 as observed in the in cell data. Extra signal leftover may be due to additional lysine sites being modified due to loss of selectivity in the *in vitro* assay.

INVESTIGATION OF NOVEL FATTY ACYLATION EVENT ON K-Ras4a DUE TO 2-BP TREATMENT

In the course of linking the DHHC enzymes to the basal lysine fatty acylation of K-Ras4a, I serendipitously discovered that WT K-Ras4a treated with 2-BP does not completely inhibit its fatty acylation as predicted. Although this initially suggests that the DHHC enzymes are not responsible for K-Ras4a's lysine or cysteine fatty acylation, closer analysis revealed that this remaining fatty acylation observed on K-Ras4a is entirely different from that of untreated K-Ras4a. The fatty acylation itself is entirely hydroxylamine resistant, and the fluorescent band is shifted higher. Furthermore, mutation of the seven lysine residues in the hypervariable region did not affect this acylation event. Only when the palmitoylated cysteine is mutated is the 2-BP induced acylation event abolished. Furthermore, this acylation cannot be removed by SIRT2, pfSir2 or HDAC11 in the *in vitro* deacylation assay. The combined observations suggest that 2-BP treatment of K-Ras4a's alk14 labeling does inhibit its fatty acylation, both lysine and cysteine, through inhibition of the DHHCs, but additionally at the same time, generates a novel PTM on K-Ras4a. This is surprising as 2-BP treatment is known to inhibit the various steps in long-chain fatty acid metabolism from fatty acid synthase (FA) to the DHHC transferases and APT depalmitoylases. How the cell generates this long-chain fatty acylation event in the presence of 2-BP is an interesting question.

The nature of this acylation is not clear and which residue it is found on is also not known. Mass spectrometry may help in identifying the acylation site and the nature of the acylation, however, may still prove challenging due to difficulties in the recovery of hydrophobic peptides from digested proteins and simply because we do not know what or where to look for the modification. However, as the modification is hydroxylamine resistant, it is likely to be on a

lysine residue, but even this is not certain as sirtuin and HDAC11 *in vitro* treatment was not able to remove the modification. A simple and straightforward approach to shed some light on this unusual acylation event is to mutate relevant residues until the acylation is perturbed.

This acylation event may be an artifact but interestingly also observed when other substrate proteins with cysteine palmitoylation are subjected to the same experimental conditions as K-Ras4a. Whether this is a general indication of a novel PTM or an artifact warrants further investigation. Furthermore, these observations suggest an intriguing hypothesis where 2-BP alkylates the substrate's palmitoylated cysteine through its brominated α carbon. This would lock the substrate into a constitutively acylated state which cannot be removed by conventional means, and the cell then uses this unusual acylation event to help restore function or lead to degradation or clearance of the damaged protein. Additionally, perhaps the Alk14 is interacting with the covalently bonded 2-BP, and that allows us to detect the modification and grants it hydroxylamine resistance and HDAC resistance.

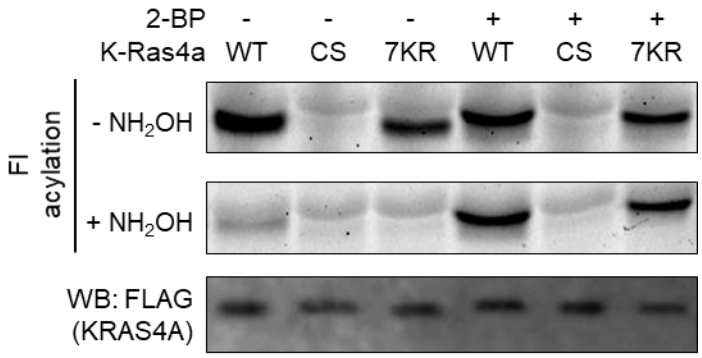


Figure 12. K-Ras4a and its mutants Alk14 fatty acylation in the presence of 2-BP.

K-Ras4a and mutants were overexpressed for 24 hours in HEK293T cells followed by one-hour pre-incubation with 100µM of 2-bromopalmitate and then six hours of combined 2-BP and 50µM Alk14 treatment. The cells were lysed and processed for in-gel fluorescence detection. Bottom membrane shows loading by western blot.

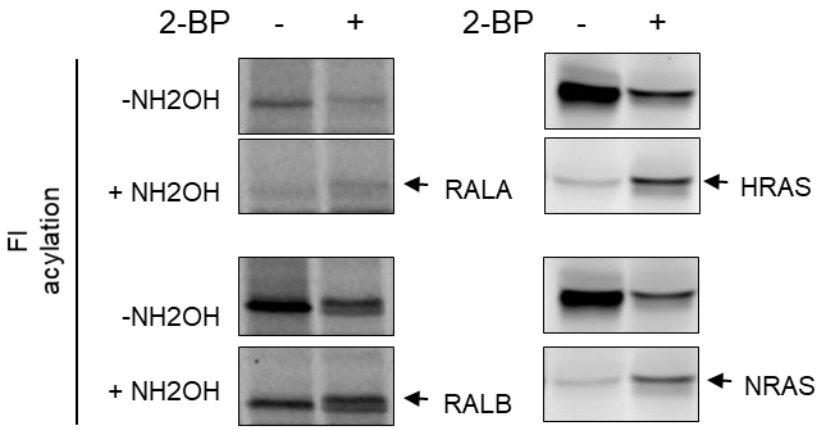


Figure 13. HRAS, NRAS, RALA, RALB Alk14 fatty acylation in the presence of 2-BP.

GTPases were overexpressed for 24 hours in HEK293T cells followed by one-hour pre-incubation with 100µM of 2-bromopalmitate and then six hours of combined 2-BP and 50µM Alk14 treatment. The cells were lysed and processed for in-gel fluorescence detection. Bottom membrane shows loading by western blot.

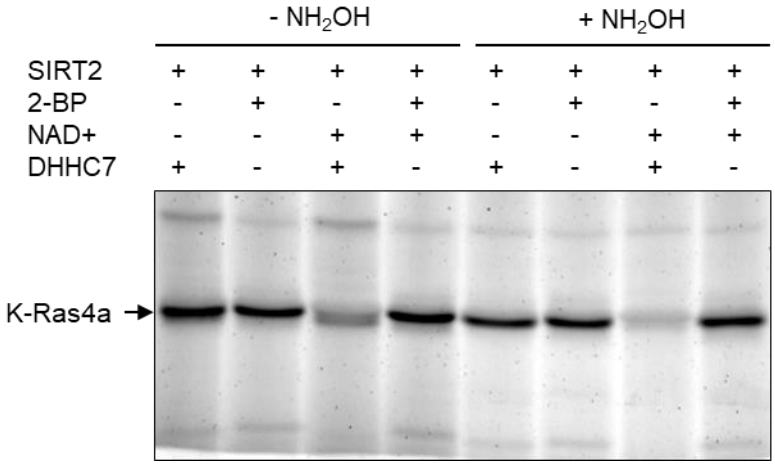


Figure 14. WT K-Ras4a fatty acylation with 2-BP and Alk14, in the SIRT2 in-vitro deacylation assay.

WT K-Ras4a fatty acylation with 2-BP and Alk14, in SIRT2 in-vitro deacylation assay. K-Ras4a was overexpressed with DHHC7 (as the positive control) for 24 hours in HEK293T cells followed by one-hour pre-incubation with 100μM of 2-bromopalmitate and then six hours of combined 2-BP and 50μM Alk14 treatment. The cells were lysed and processed for in-gel fluorescence detection. Bottom membrane shows loading by western blot. DHHC7 mediated fatty acylation of K-Ras4a is removed while 2-BP treated K-Ras4a fatty acylation cannot be removed when NAD^+ is added.

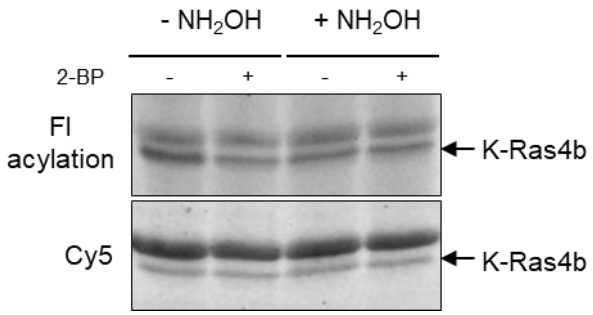


Figure 15. K-Ras4b fatty acylation in the presence of 2-BP.

K-Ras4b was overexpressed for 24 hours in HEK293T cells followed by one-hour pre-incubation with 100µM of 2-bromopalmitate and then six hours of combined 2-BP and 50µM Alk14 treatment. The cells were lysed and processed for in-gel fluorescence detection. Bottom gel shows loading by CBB. There is no gain in fluorescence signal (unusual fatty acylation event) when treated with 2-BP.

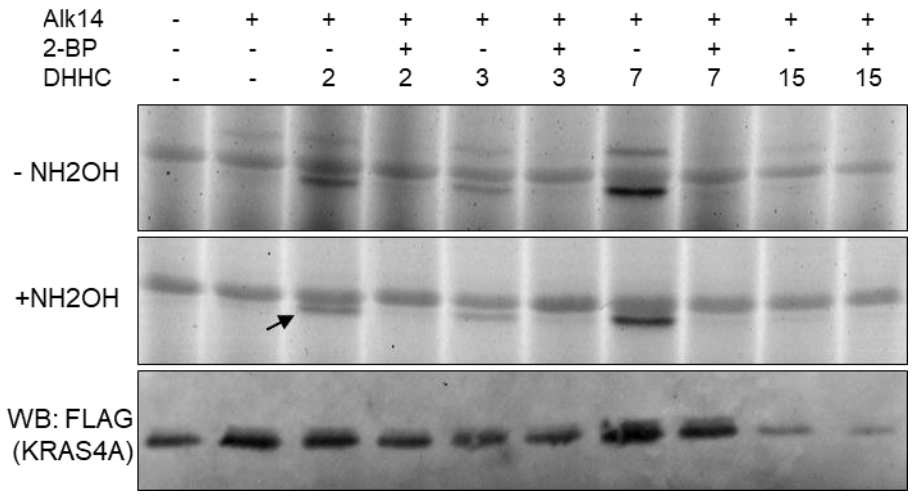


Figure 16. K-Ras4b DHHC dependent fatty acylation when treated with 2-BP.

K-Ras4b was overexpressed with the DHHC enzymes for 24 hours in HEK293T cells followed by one-hour pre-incubation with 100µM of 2-bromopalmitate and then six hours of combined 2-BP and 50µM Alk14 treatment. The cells were lysed and processed for in-gel fluorescence detection. Bottom membrane shows loading by western blot. 2-BP treatment can block the DHHC mediated fatty acylation of K-Ras4b, demonstrating the efficacy of 2-BP treatment in inhibiting DHHC mediated fatty acylation events. No 2-BP mediated novel fatty acylation is observed on K-Ras4b because it lacks a palmitoylated cysteine. Arrow denotes increased K-Ras4b fatty acylation due to DHHC overexpression.

CONCLUDING REMARKS

Our investigation has discovered a novel enzymatic activity by members of a family of enzymes thought only to catalyze a single enzymatic reaction. A mammalian lysine fatty acyltransferase has yet to be reported as of this publication, and our work demonstrates that members of the DHHC family are capable of directly transferring the modification on to the lysine residue of several members of the superfamily of GTPases. When we began our investigation, lysine fatty acylation was re-entering the scientific community's consciousness, and with our contributions, we hope to expand interest further, and our understanding of this poorly appreciated and understood modification as there are many questions to answer. Lysine fatty acylation has the potential to be a highly important modification such as that of phosphorylation and ubiquitination as we learn more about its functions. Our work has also expanded on our understanding of the DHHC family of enzymes. Not only does their cysteine fatty acyltransferase activity have biological consequences but so does their lysine fatty acyltransferase activity. A variety of pathologies have already been attributed to the cysteine transferase activity of the DHHCs, but the question is raised as to the importance of the DHHC's lysine fatty acyltransferase activity for these same pathologies. Differentiating the two function will be essential to understanding each activities' contribution to the pathologies associated with the DHHCs. Lastly, our findings demonstrate that one must remain open-minded when studying enzymes with a reported function; it may have an unknown activity.

APPENDIX

SUPPLEMENTAL DATA

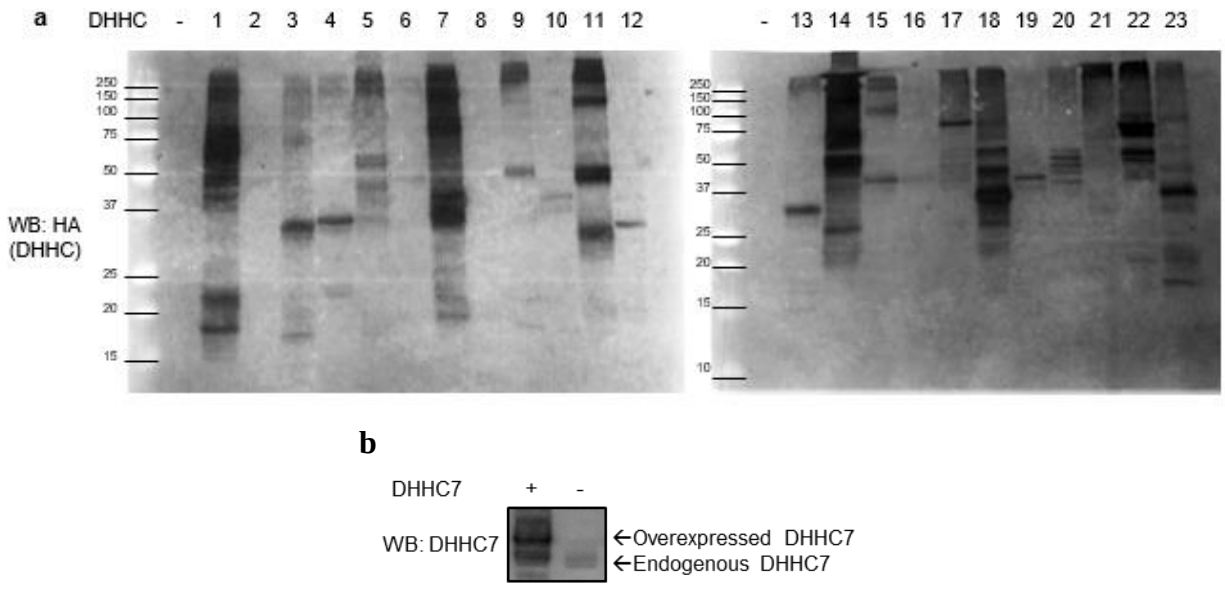


Figure S1. (**a**) Western showing expression level of the ectopically expressed DHHC enzymes in the K-Ras4a – DHHC screening. **(b)** Expression level of overexpressed DHHC7 compared to endogenous DHHC7.

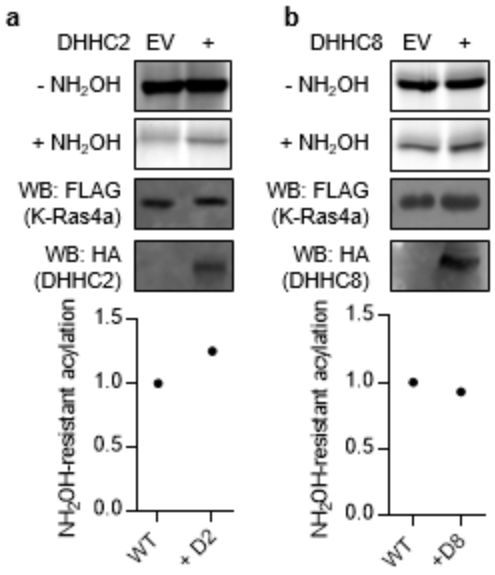


Figure S2. (**a**) K-Ras4a fatty acylation with over-expressed DHHC2 with quantification (n = 1). (EV: empty vector) (**b**) K-Ras4a fatty acylation with over-expressed DHHC8 with quantification (n = 1).

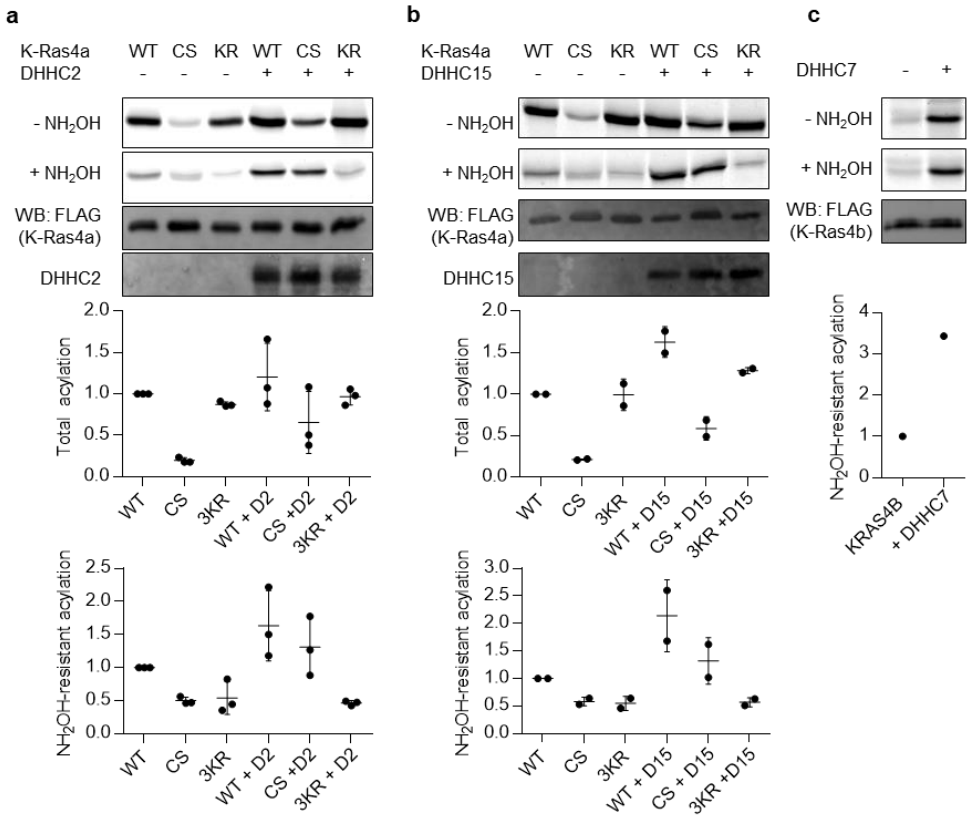
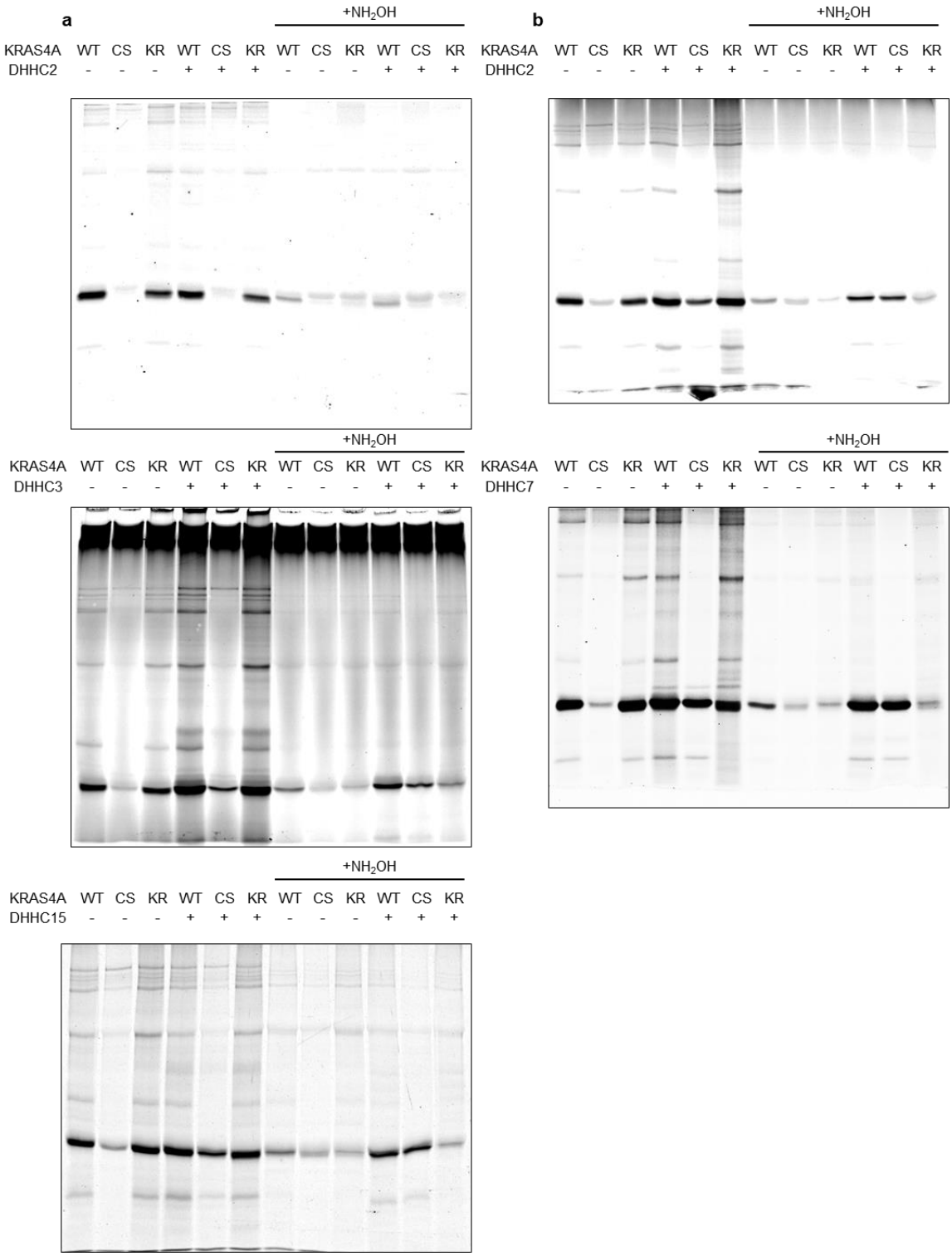
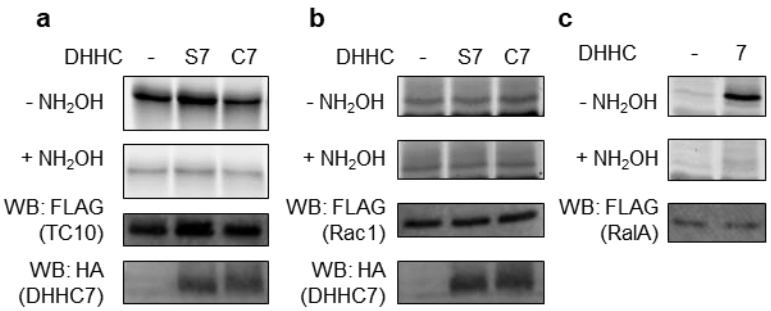


Figure S3. **(a)** Fatty acylation of K-Ras4a mutants with over-expressed DHHC2 and quantification (n = 3). **(b)** Fatty acylation of K-Ras4a mutants with over-expressed DHHC15 and quantification (n = 2). **(c)** Fatty acylation of K-Ras4b with overexpressed DHHC7 with quantification (n = 1). The individual plotted values are normalized to the WT control sample before and post 0.4M hydroxylamine treatment (error bars represent S.D.)



1370

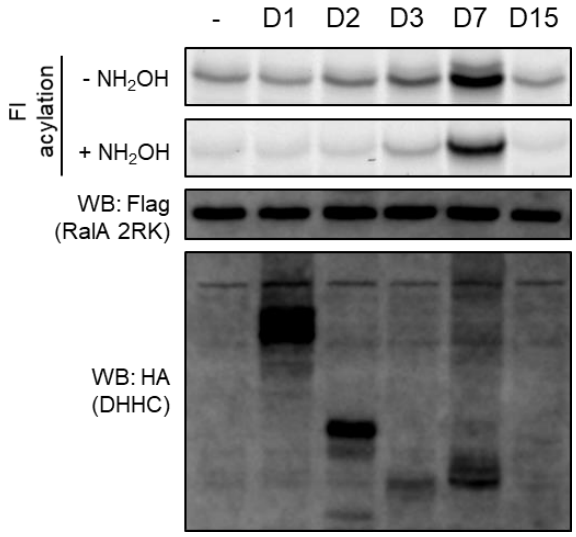
1371 Figure S4. Full fluorescence images of K-Ras4a mutants co-expressed with (a) DHHC1, (b)
1372 DHHC2, (c) DHHC3, (d) DHHC7, and (e) DHHC15



1373

1374 Figure S5**.** Fatty acylation of GTPases (a) TC10, (b) Rac1, (c) RalA) with overexpressed DHHC7.
1375 Here, we do not observe effect on either TC10 or Rac1 fatty acylation.

1376



1377

1378 Figure S6. RalA 2RK mutant gains hydroxylamine resistant fatty acylation when overexpressed
1379 with DHHC7. This gain of function acylation does not occur with DHHC2 or DHHC15 and
1380 weakly with DHHC3.

1381

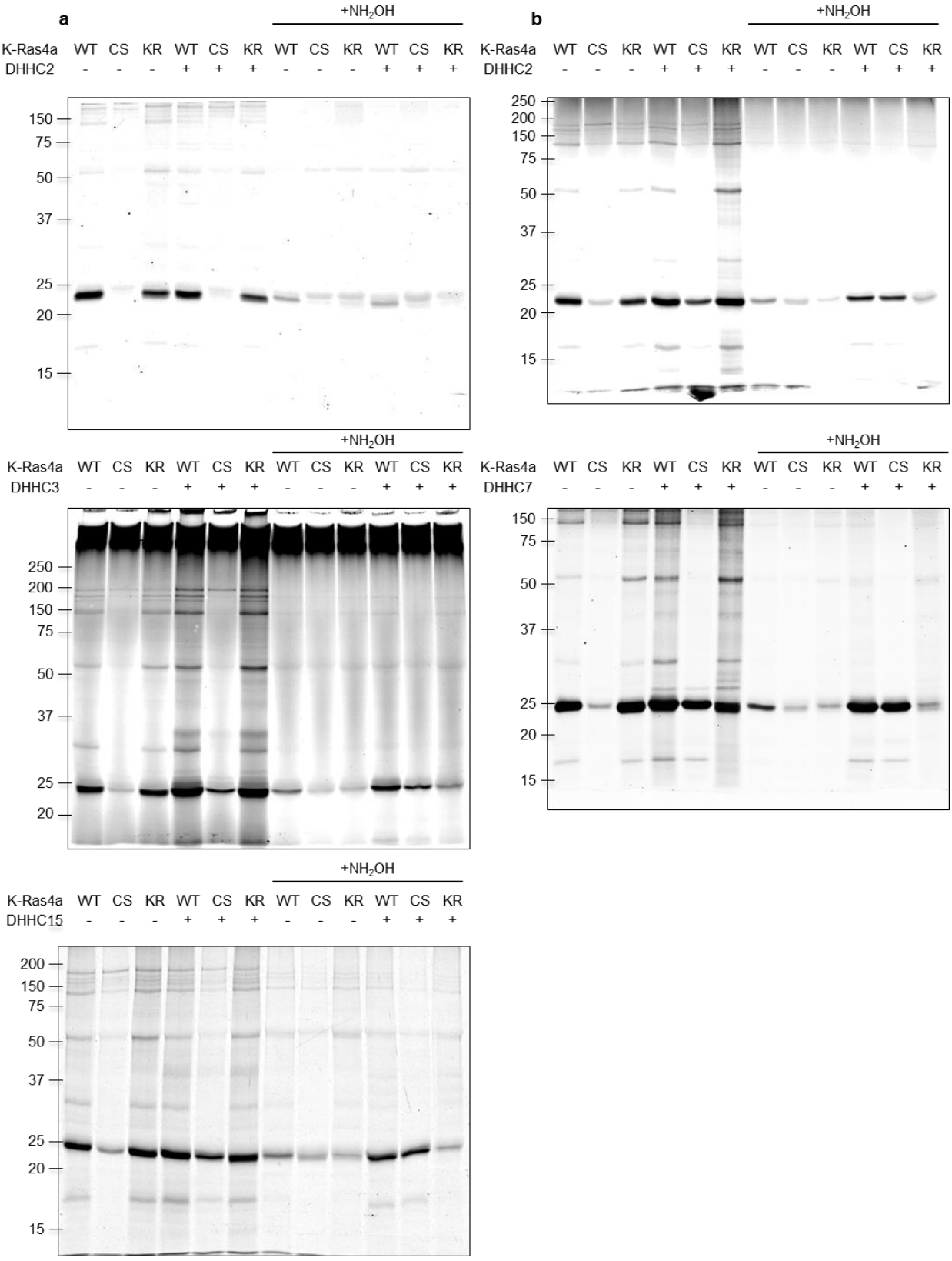


Figure S7. (**a**) Full fluorescence gel images of K-Ras4a coexpressed with DHHC1 – 23. Arrow

denotes K-Ras4a. (**b**) Full Coomassie blue stain gel of the same K-Ras4a coexpressed with DHHC1 – 23 fluorescence gel to show loading.

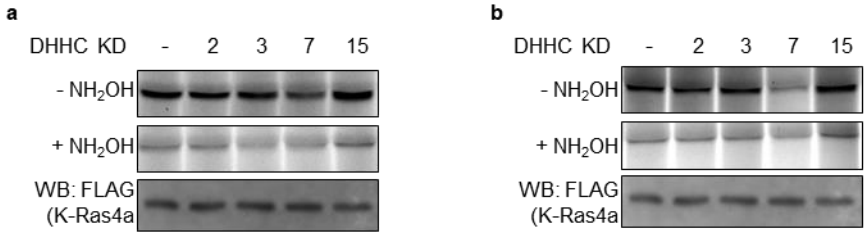


Figure S8. (a) (b) Additional K-Ras4a fatty acylation in DHHC2, 3, 7, 15 stable knockdown HEK293T cells. Accompanying data to Figure 7.

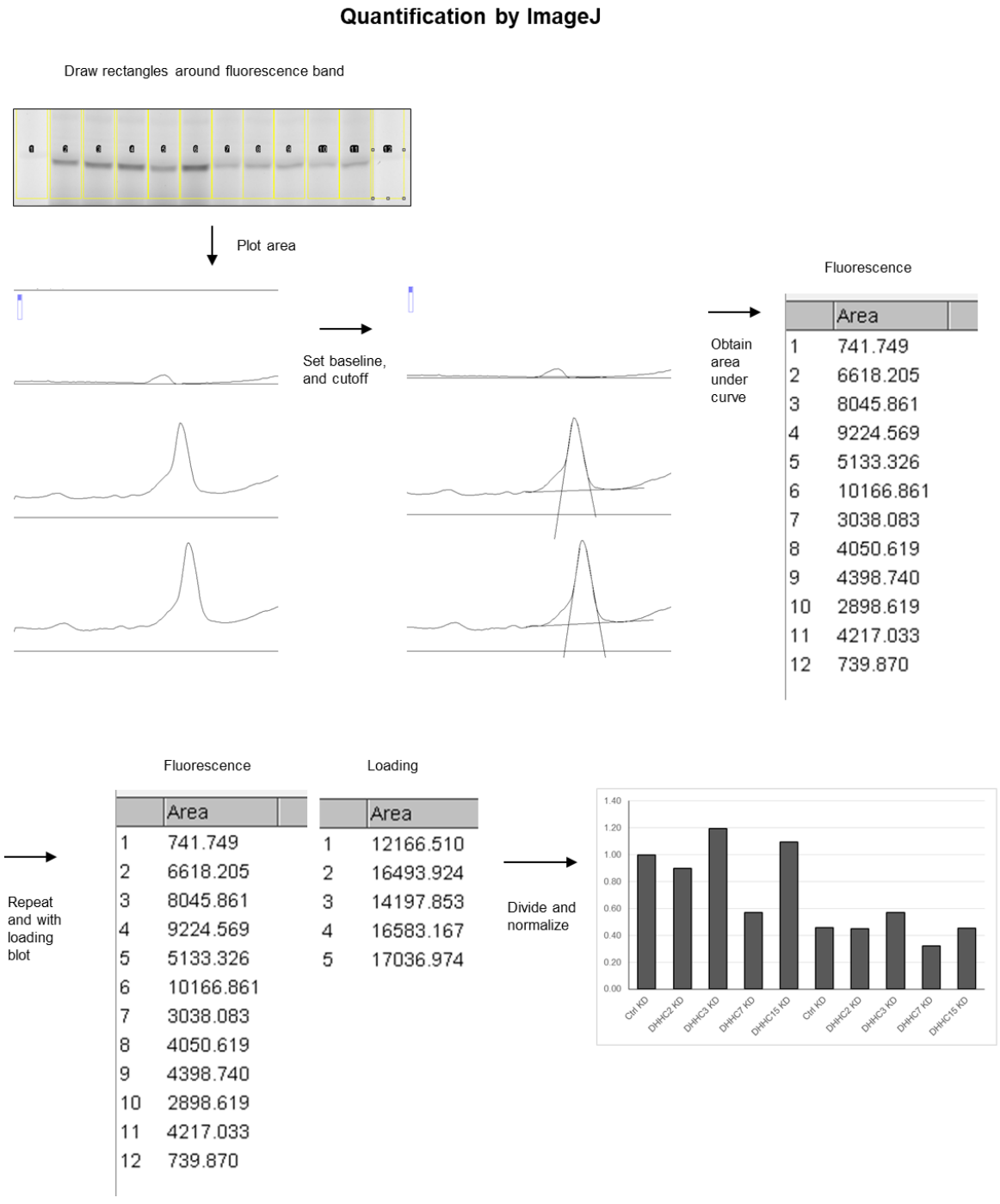


Figure S9. Quantification process by ImageJ. Shown fluorescence gel is the K-Ras4a fatty acylation in DHHC2, 3, 7, 15 stable knockdown in HEK293T cells.

REFERENCES

(1) Jiang, H.; Khan, S.; Wang, Y.; Charron, G.; He, B.; Sebastian, C.; Du, J.; Kim, R.; Ge, E.; Mostoslavsky, R.et al. Sirt6 Regulates Tnf-Alpha Secretion through Hydrolysis of Long-Chain Fatty Acyl Lysine. *Nature* **2013,** *496*, 110-113.

(2) Jiang, H.; Zhang, X.; Lin, H. Lysine Fatty Acylation Promotes Lysosomal Targeting of Tnf-Alpha. *Sci. Rep.* **2016,** *6*, 24371.

(3) Resh, M. D. Covalent Lipid Modifications of Proteins. *Curr Biol* **2013,** *23*, R431-435.

(4) Stevenson, F. T.; Bursten, S. L.; Locksley, R. M.; Lovett, D. H. Myristyl Acylation of the Tumor Necrosis Factor Alpha Precursor on Specific Lysine Residues. *J. Exp. Med.* **1992,** *176*, 1053-1062.

(5) Stevenson, F. T.; Bursten, S. L.; Fanton, C.; Locksley, R. M.; Lovett, D. H. The 31-Kda Precursor of Interleukin 1 Alpha Is Myristoylated on Specific Lysines within the 16-Kda N-Terminal Propiece. *Proc. Natl. Acad. Sci. U.S.A.* **1993,** *90*, 7245-7249.

(6) Boyartchuk, V. L.; Ashby, M. N.; Rine, J. Modulation of Ras and a-Factor Function by Carboxyl-Terminal Proteolysis. *Science* **1997,** *275*, 1796-1800.

(7) Dai, Q.; Choy, E.; Chiu, V.; Romano, J.; Slivka, S. R.; Steitz, S. A.; Michaelis, S.; Philips, M. R. Mammalian Prenylcysteine Carboxyl Methyltransferase Is in the Endoplasmic Reticulum. *J. Biol. Chem.* **1998,** *273*, 15030-15034.

(8) Schafer, W. R.; Kim, R.; Sterne, R.; Thorner, J.; Kim, S. H.; Rine, J. Genetic and Pharmacological Suppression of Oncogenic Mutations in Ras Genes of Yeast and Humans. *Science* **1989,** *245*, 379-385.

(9) Taylor, J. S.; Reid, T. S.; Terry, K. L.; Casey, P. J.; Beese, L. S. Structure of Mammalian Protein Geranylgeranyltransferase Type-I. *EMBO J.* **2003,** *22*, 5963-5974.

(10) Long, S. B.; Casey, P. J.; Beese, L. S. Cocrystal Structure of Protein Farnesyltransferase Complexed with a Farnesyl Diphosphate Substrate. *Biochemistry* **1998,** *37*, 9612-9618.

(11) Sinclair, W. R.; Shrimp, J. H.; Zengeya, T. T.; Kulkarni, R. A.; Garlick, J. M.; Luecke, H.; Worth, A. J.; Blair, I. A.; Snyder, N. W.; Meier, J. L. Bioorthogonal Pro-Metabolites for Profiling Short Chain Fatty Acylation. *Chem Sci* **2018,** *9*, 1236-1241.

(12) Fujimoto, T.; Stroud, E.; Whatley, R. E.; Prescott, S. M.; Muszbek, L.; Laposata, M.; McEver, R. P. P-Selectin Is Acylated with Palmitic Acid and Stearic Acid at Cysteine 766 through a Thioester Linkage. *J. Biol. Chem.* **1993,** *268*, 11394-11400.

(13) Gutierrez, J. A.; Solenberg, P. J.; Perkins, D. R.; Willency, J. A.; Knierman, M. D.; Jin, Z.; Witcher, D. R.; Luo, S.; Onyia, J. E.; Hale, J. E. Ghrelin Octanoylation Mediated by an Orphan Lipid Transferase. *Proc. Natl. Acad. Sci. U.S.A.* **2008,** *105*, 6320-6325.

(14) Takada, R.; Satomi, Y.; Kurata, T.; Ueno, N.; Norioka, S.; Kondoh, H.; Takao, T.; Takada, S. Monounsaturated Fatty Acid Modification of Wnt Protein: Its Role in Wnt Secretion. *Dev. Cell* **2006,** *11*, 791-801.

(15) Burnaevskiy, N.; Fox, T. G.; Plymire, D. A.; Ertelt, J. M.; Weigele, B. A.; Selyunin, A. S.; Way, S. S.; Patrie, S. M.; Alto, N. M. Proteolytic Elimination of N-Myristoyl Modifications by the Shigella Virulence Factor Ipaj. *Nature* **2013,** *496*, 106-109.

(16) Johnson, D. R.; Bhatnagar, R. S.; Knoll, L. J.; Gordon, J. I. Genetic and Biochemical Studies of Protein N-Myristoylation. *Annu. Rev. Biochem.* **1994,** *63*, 869-914.

(17) Resh, M. D. Myristylation and Palmitylation of Src Family Members: The Fats of the Matter. *Cell* **1994,** *76*, 411-413.

(18) Patwardhan, P.; Resh, M. D. Myristoylation and Membrane Binding Regulate C-Src Stability and Kinase Activity. *Mol. Cell. Biol.* **2010,** *30*, 4094-4107.

(19) Hofmann, K. A Superfamily of Membrane-Bound O-Acyltransferases with Implications for Wnt Signaling. *Trends Biochem. Sci.* **2000,** *25*, 111-112.

(20) Kojima, M.; Hosoda, H.; Date, Y.; Nakazato, M.; Matsuo, H.; Kangawa, K. Ghrelin Is a Growth-Hormone-Releasing Acylated Peptide from Stomach. *Nature* **1999,** *402*, 656-660.

(21) Zou, C.; Ellis, B. M.; Smith, R. M.; Chen, B. B.; Zhao, Y.; Mallampalli, R. K. Acyl-Coa:Lysophosphatidylcholine Acyltransferase I (Lpcat1) Catalyzes Histone Protein O-Palmitoylation to Regulate Mrna Synthesis. *J. Biol. Chem.* **2011,** *286*, 28019-28025.

(22) Cases, S.; Smith, S. J.; Zheng, Y. W.; Myers, H. M.; Lear, S. R.; Sande, E.; Novak, S.; Collins, C.; Welch, C. B.; Lusis, A. J.et al. Identification of a Gene Encoding an Acyl Coa:Diacylglycerol Acyltransferase, a Key Enzyme in Triacylglycerol Synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **1998,** *95*, 13018-13023.

(23) Yen, C. L.; Monetti, M.; Burri, B. J.; Farese, R. V., Jr. The Triacylglycerol Synthesis Enzyme Dgat1 Also Catalyzes the Synthesis of Diacylglycerols, Waxes, and Retinyl Esters. *J. Lipid Res.* **2005,** *46*, 1502-1511.

(24) Hardy, R. Y.; Resh, M. D. Identification of N-Terminal Residues of Sonic Hedgehog Important for Palmitoylation by Hedgehog Acyltransferase. *J. Biol. Chem.* **2012,** *287*, 42881-42889.

(25) Abe, Y.; Kita, Y.; Niikura, T. Mammalian Gup1, a Homolog of Saccharomyces Cerevisiae Glycerol Uptake/Transporter 1, Acts as a Negative Regulator for N-Terminal Palmitoylation of Sonic Hedgehog. *FEBS J.* **2008,** *275*, 318-331.

(26) Yang, J.; Brown, M. S.; Liang, G.; Grishin, N. V.; Goldstein, J. L. Identification of the Acyltransferase That Octanoylates Ghrelin, an Appetite-Stimulating Peptide Hormone. *Cell* **2008,** *132*, 387-396.

(27) Shindou, H.; Hishikawa, D.; Harayama, T.; Yuki, K.; Shimizu, T. Recent Progress on Acyl Coa: Lysophospholipid Acyltransferase Research. *J. Lipid Res.* **2009,** *50 Suppl*, S46-51.

(28) Belenkaya, T. Y.; Wu, Y.; Tang, X.; Zhou, B.; Cheng, L.; Sharma, Y. V.; Yan, D.; Selva, E. M.; Lin, X. The Retromer Complex Influences Wnt Secretion by Recycling Wntless from Endosomes to the Trans-Golgi Network. *Dev. Cell* **2008,** *14*, 120-131.

(29) Herr, P.; Basler, K. Porcupine-Mediated Lipidation Is Required for Wnt Recognition by Wls. *Dev. Biol.* **2012,** *361*, 392-402.

(30) Jing, H.; Zhang, X.; Wisner, S. A.; Chen, X.; Spiegelman, N. A.; Linder, M. E.; Lin, H. Sirt2 and Lysine Fatty Acylation Regulate the Transforming Activity of K-Ras4a. *Elife* **2017,** *6*.

(31) Stanley, P.; Koronakis, V.; Hughes, C. Acylation of *Escherichia Coli* Hemolysin: A Unique Protein Lipidation Mechanism Underlying Toxin Function. *Microbiol. Mol. Biol. Rev.* **1998,** *62*, 309-333.

(32) Stanley, P.; Packman, L. C.; Koronakis, V.; Hughes, C. Fatty Acylation of Two Internal Lysine Residues Required for the Toxic Activity of Escherichia Coli Hemolysin. *Science* **1994,** *266*, 1992-1996.

(33) Zhou, Y.; Huang, C.; Yin, L.; Wan, M.; Wang, X.; Li, L.; Liu, Y.; Wang, Z.; Fu, P.; Zhang, N.et al. N(Epsilon)-Fatty Acylation of Rho Gtpases by a Martx Toxin Effector. *Science* **2017,** *358*, 528-531.

(34) Braun, P. E.; Radin, N. S. Interactions of Lipids with a Membrane Structural Protein from Myelin. *Biochemistry* **1969,** *8*, 4310-4318.

(35) Stoffyn, P.; Folch-Pi, J. On the Type of Linkage Binding Fatty Acids Present in Brain White Matter Proteolipid Apoprotein. *Biochem. Biophys. Res. Commun.* **1971,** *44*, 157-161.

(36) Quesnel, S.; Silvius, J. R. Cysteine-Containing Peptide Sequences Exhibit Facile Uncatalyzed Transacylation and Acyl-Coa-Dependent Acylation at the Lipid Bilayer Interface. *Biochemistry* **1994,** *33*, 13340-13348.

(37) O'Brien, P. J.; St Jules, R. S.; Reddy, T. S.; Bazan, N. G.; Zatz, M. Acylation of Disc Membrane Rhodopsin May Be Nonenzymatic. *J. Biol. Chem.* **1987,** *262*, 5210-5215.

(38) Duncan, J. A.; Gilman, A. G. Autoacylation of G Protein Alpha Subunits. *J. Biol. Chem.* **1996,** *271*, 23594-23600.

(39) Knudsen, J.; Neergaard, T. B.; Gaigg, B.; Jensen, M. V.; Hansen, J. K. Role of Acyl-Coa Binding Protein in Acyl-Coa Metabolism and Acyl-Coa-Mediated Cell Signaling. *J Nutr* **2000,** *130*, 294S-298S.

(40) Blachnio-Zabielska, A. U.; Koutsari, C.; Jensen, M. D. Measuring Long-Chain Acyl-Coenzyme a Concentrations and Enrichment Using Liquid Chromatography/Tandem Mass Spectrometry with Selected Reaction Monitoring. *Rapid Commun. Mass Spectrom.* **2011,** *25*, 2223-2230.

(41) Keller, C. A.; Yuan, X.; Panzanelli, P.; Martin, M. L.; Alldred, M.; Sassoè-Pognetto, M.; Lüscher, B. The Γ2 Subunit of Gabaa Receptors Is a Substrate for Palmitoylation by Godz. *J. Neurosci.* **2004,** *24*, 5881-5891.

(42) Fukata, M.; Fukata, Y.; Adesnik, H.; Nicoll, R. A.; Bredt, D. S. Identification of Psd-95 Palmitoylating Enzymes. *Neuron* **2004,** *44*, 987-996.

(43) Putilina, T.; Wong, P.; Gentleman, S. The Dhhc Domain: A New Highly Conserved Cysteine-Rich Motif. *Mol. Cell. Biochem.* **1999,** *195*, 219-226.

(44) Bohm, S.; Frishman, D.; Mewes, H. W. Variations of the C2h2 Zinc Finger Motif in the Yeast Genome and Classification of Yeast Zinc Finger Proteins. *Nucleic Acids Res.* **1997,** *25*, 2464-2469.

(45) Gonzalez Montoro, A.; Quiroga, R.; Valdez Taubas, J. Zinc Co-Ordination by the Dhhc Cysteine-Rich Domain of the Palmitoyltransferase Swf1. *Biochem. J.* **2013,** *454*, 427-435.

(46) Gottlieb, C. D.; Zhang, S.; Linder, M. E. The Cysteine-Rich Domain of the Dhhc3 Palmitoyltransferase Is Palmitoylated and Contains Tightly Bound Zinc. *J. Biol. Chem.* **2015,** *290*, 29259-29269.

(47) Reprinted with permission from "Jiang, H.; Zhang, X.; Chen, X.; Aramsangtienchai, P.; Tong, Z.; Lin, H. Protein Lipidation: Occurrence, Mechanisms, Biological Functions, and Enabling Technologies. Chem. Rev. 2018, 118, 919-988." Copyright (2018) American Chemical Society

(48) Baumgart, F.; Corral-Escariz, M.; Pérez-Gil, J.; Rodríguez-Crespo, I. Palmitoylation of R-Ras by Human Dhhc19, a Palmitoyl Transferase with a Caax Box. *Biochim. Biophys. Acta* **2010,** *1798*, 592-604.

(49) Swarthout, J. T.; Lobo, S.; Farh, L.; Croke, M. R.; Greentree, W. K.; Deschenes, R. J.; Linder, M. E. Dhhc9 and Gcp16 Constitute a Human Protein Fatty Acyltransferase with Specificity for H- and N-Ras. *J. Biol. Chem.* **2005,** *280*, 31141-31148.

(50) Lakkaraju, A. K.; Abrami, L.; Lemmin, T.; Blaskovic, S.; Kunz, B.; Kihara, A.; Dal Peraro, M.; van der Goot, F. G. Palmitoylated Calnexin Is a Key Component of the Ribosome–Translocon Complex. *EMBO J.* **2012,** *31*, 1823-1835.

(51) Hou, H.; John Peter, A. T.; Meiringer, C.; Subramanian, K.; Ungermann, C. Analysis of Dhhc Acyltransferases Implies Overlapping Substrate Specificity and a Two-Step Reaction Mechanism. *Traffic* **2009,** *10*, 1061-1073.

(52) Roth, A. F.; Wan, J.; Bailey, A. O.; Sun, B.; Kuchar, J. A.; Green, W. N.; Phinney, B. S.; Yates, J. R.; Davis, N. G. Global Analysis of Protein Palmitoylation in Yeast. *Cell* **2006,** *125*, 1003-1013.

(53) Rocks, O.; Gerauer, M.; Vartak, N.; Koch, S.; Huang, Z.-P.; Pechlivanis, M.; Kuhlmann, J.; Brunsveld, L.; Chandra, A.; Ellinger, B.et al. The Palmitoylation Machinery Is a Spatially Organizing System for Peripheral Membrane Proteins. *Cell* **2010,** *141*, 458-471.

(54) Gorleku, O. A.; Barns, A. M.; Prescott, G. R.; Greaves, J.; Chamberlain, L. H. Endoplasmic Reticulum Localization of Dhhc Palmitoyltransferases Mediated by Lysine-Based Sorting Signals. *J. Biol. Chem.* **2011,** *286*, 39573-39584.

(55) Stornaiuolo, M.; Lotti, L. V.; Borgese, N.; Torrisi, M. R.; Mottola, G.; Martire, G.; Bonatti, S. Kdel and Kkxx Retrieval Signals Appended to the Same Reporter Protein Determine Different Trafficking between Endoplasmic Reticulum, Intermediate Compartment, and Golgi Complex. *Mol Biol Cell* **2003,** *14*, 889-902.

(56) Greaves, J.; Carmichael, J. A.; Chamberlain, L. H. The Palmitoyl Transferase Dhhc2 Targets a Dynamic Membrane Cycling Pathway: Regulation by a C-Terminal Domain. *Mol. Biol. Cell* **2011,** *22*, 1887-1895.

(57) Noritake, J.; Fukata, Y.; Iwanaga, T.; Hosomi, N.; Tsutsumi, R.; Matsuda, N.; Tani, H.; Iwanari, H.; Mochizuki, Y.; Kodama, T.et al. Mobile Dhhc Palmitoylating Enzyme Mediates Activity-Sensitive Synaptic Targeting of Psd-95. *J. Cell Biol.* **2009,** *186*, 147-160.

(58) Ohno, Y.; Kihara, A.; Sano, T.; Igarashi, Y. Intracellular Localization and Tissue-Specific Distribution of Human and Yeast Dhhc Cysteine-Rich Domain-Containing Proteins. *Biochim. Biophys. Acta* **2006,** *1761*, 474-483.

(59) Kilpatrick, C. L.; Murakami, S.; Feng, M.; Wu, X.; Lal, R.; Chen, G.; Du, K.; Luscher, B. Dissociation of Golgi-Associated Dhhc-Type Zinc Finger Protein (Godz)- and Sertoli Cell Gene with a Zinc Finger Domain-Beta (Serz-Beta)-Mediated Palmitoylation by Loss of Function Analyses in Knock-out Mice. *J. Biol. Chem.* **2016,** *291*, 27371-27386.

(60) Fang, C.; Deng, L.; Keller, C. A.; Fukata, M.; Fukata, Y.; Chen, G.; Luscher, B. Godz-Mediated Palmitoylation of Gaba(a) Receptors Is Required for Normal Assembly and Function of Gabaergic Inhibitory Synapses. *J. Neurosci.* **2006,** *26*, 12758-12768.

(61) Greaves, J.; Gorleku, O. A.; Salaun, C.; Chamberlain, L. H. Palmitoylation of the Snap25 Protein Family Specificity and Regulation by Dhhc Palmitoyl Transferases. *J. Biol. Chem.* **2010,** *285*, 24629-24638.

(62) Singaraja, R. R.; Hadano, S.; Metzler, M.; Givan, S.; Wellington, C. L.; Warby, S.; Yanai, A.; Gutekunst, C.-A.; Leavitt, B. R.; Yi, H.et al. Hip14, a Novel Ankyrin Domain-Containing Protein, Links Huntingtin to Intracellular Trafficking and Endocytosis. *Hum. Mol. Genet.* **2002,** *11*, 2815-2828.

(63) Huang, K.; Sanders, S.; Singaraja, R.; Orban, P.; Cijsouw, T.; Arstikaitis, P.; Yanai, A.; Hayden, M. R.; El-Husseini, A. Neuronal Palmitoyl Acyl Transferases Exhibit Distinct Substrate Specificity. *FASEB J.* **2009,** *23*, 2605-2615.

(64) Li, Y.; Hu, J.; Hofer, K.; Wong, A. M.; Cooper, J. D.; Birnbaum, S. G.; Hammer, R. E.; Hofmann, S. L. Dhhc5 Interacts with Pdz Domain 3 of Post-Synaptic Density-95 (Psd-95) Protein and Plays a Role in Learning and Memory. *J. Biol. Chem.* **2010,** *285*, 13022-13031.

(65) Thomas, G. M.; Hayashi, T.; Chiu, S. L.; Chen, C. M.; Huganir, R. L. Palmitoylation by Dhhc5/8 Targets Grip1 to Dendritic Endosomes to Regulate Ampa-R Trafficking. *Neuron* **2012,** *73*, 482-496.

(66) Verardi, R.; Kim, J. S.; Ghirlando, R.; Banerjee, A. Structural Basis for Substrate Recognition by the Ankyrin Repeat Domain of Human Dhhc17 Palmitoyltransferase. *Structure* **2017**, DOI:10.1016/j.str.2017.06.018 10.1016/j.str.2017.06.018.

(67) Lemonidis, K.; Sanchez-Perez, M. C.; Chamberlain, L. H. Identification of a Novel Sequence Motif Recognized by the Ankyrin Repeat Domain of Zdhhc17/13 S-Acyltransferases. *J. Biol. Chem.* **2015,** *290*, 21939-21950.

(68) Gonzalez Montoro, A.; Chumpen Ramirez, S.; Quiroga, R.; Valdez Taubas, J. Specificity of Transmembrane Protein Palmitoylation in Yeast. *PLoS One* **2011,** *6*, e16969.

(69) El-Husseini, A. E.; Craven, S. E.; Chetkovich, D. M.; Firestein, B. L.; Schnell, E.; Aoki, C.; Bredt, D. S. Dual Palmitoylation of Psd-95 Mediates Its Vesiculotubular Sorting, Postsynaptic Targeting, and Ion Channel Clustering. *J. Cell Biol.* **2000,** *148*, 159-172.

(70) Plain, F.; Congreve, S. D.; Yee, R. S. Z.; Kennedy, J.; Howie, J.; Kuo, C. W.; Fraser, N. J.; Fuller, W. An Amphipathic Alpha-Helix Directs Palmitoylation of the Large Intracellular Loop of the Sodium/Calcium Exchanger. *J. Biol. Chem.* **2017,** *292*, 10745-10752.

(71) Schweizer, A.; Rohrer, J.; Kornfeld, S. Determination of the Structural Requirements for Palmitoylation of P63. *J. Biol. Chem.* **1995,** *270*, 9638-9644.

(72) Yik, J. H.; Weigel, P. H. The Position of Cysteine Relative to the Transmembrane Domain Is Critical for Palmitoylation of H1, the Major Subunit of the Human Asialoglycoprotein Receptor. *J. Biol. Chem.* **2002,** *277*, 47305-47312.

(73) Jennings, B. C.; Linder, M. E. Dhhc Protein S-Acyltransferases Use Similar Ping-Pong Kinetic Mechanisms but Display Different Acyl-Coa Specificities. *J. Biol. Chem.* **2012,** *287*, 7236-7245.

(74) Greaves, J.; Munro, K. R.; Davidson, S. C.; Riviere, M.; Wojno, J.; Smith, T. K.; Tomkinson, N. C. O.; Chamberlain, L. H. Molecular Basis of Fatty Acid Selectivity in the Zdhhc Family of S-Acyltransferases Revealed by Click Chemistry. *Proc. Natl. Acad. Sci. U. S. A.* **2017,** *114*, E1365-E1374.

(75) Rana, M. S.; Kumar, P.; Lee, C. J.; Verardi, R.; Rajashankar, K. R.; Banerjee, A. Fatty Acyl Recognition and Transfer by an Integral Membrane S-Acyltransferase. *Science* **2018,** *359*.

(76) Casey, W. M.; Gibson, K. J.; Parks, L. W. Covalent Attachment of Palmitoleic Acid (C16:1 Delta 9) to Proteins in Saccharomyces Cerevisiae. Evidence for a Third Class of Acylated Proteins. *J. Biol. Chem.* **1994,** *269*, 2082-2085.

(77) Hallak, H.; Muszbek, L.; Laposata, M.; Belmonte, E.; Brass, L. F.; Manning, D. R. Covalent Binding of Arachidonate to G Protein Alpha Subunits of Human Platelets. *J. Biol. Chem.* **1994,** *269*, 4713-4716.

(78) Muszbek, L.; Laposata, M. Covalent Modification of Proteins by Arachidonate and Eicosapentaenoate in Platelets. *J. Biol. Chem.* **1993,** *268*, 18243-18248.

(79) DeMar, J. C., Jr.; Anderson, R. E. Identification and Quantitation of the Fatty Acids Composing the Coa Ester Pool of Bovine Retina, Heart, and Liver. *J. Biol. Chem.* **1997,** *272*, 31362-31368.

(80) Bizzozero, O. A.; McGarry, J. F.; Lees, M. B. Acylation of Endogenous Myelin Proteolipid Protein with Different Acyl-Coas. *J. Biol. Chem.* **1987,** *262*, 2138-2145.

(81) Zeng, F. Y.; Kaphalia, B. S.; Ansari, G. A.; Weigel, P. H. Fatty Acylation of the Rat Asialoglycoprotein Receptor. The Three Subunits from Active Receptors Contain Covalently Bound Palmitate and Stearate. *J. Biol. Chem.* **1995,** *270*, 21382-21387.

(82) Okubo, K.; Hamasaki, N.; Hara, K.; Kageura, M. Palmitoylation of Cysteine 69 from the Cooh-Terminal of Band 3 Protein in the Human Erythrocyte Membrane. Acylation Occurs in the Middle of the Consensus Sequence of F--I-Iiclavl Found in Band 3 Protein and G2 Protein of Rift Valley Fever Virus. *J. Biol. Chem.* **1991,** *266*, 16420-16424.

(83) Lobo, S.; Greentree, W. K.; Linder, M. E.; Deschenes, R. J. Identification of a Ras Palmitoyltransferase in Saccharomyces Cerevisiae. *J. Biol. Chem.* **2002,** *277*, 41268-41273.

(84) Rana, M. S.; Kumar, P.; Lee, C.-J.; Verardi, R.; Rajashankar, K. R.; Banerjee, A. Fatty Acyl Recognition and Transfer by an Integral Membrane <Em>S</Em>-Acyltransferase. *Science* **2018,** *359*.

(85) Ohno, Y.; Kashio, A.; Ogata, R.; Ishitomi, A.; Yamazaki, Y.; Kihara, A. Analysis of Substrate Specificity of Human Dhhc Protein Acyltransferases Using a Yeast Expression System. *Mol Biol Cell* **2012,** *23*, 4543-4551.

(86) Huang, K.; Sanders, S. S.; Kang, R.; Carroll, J. B.; Sutton, L.; Wan, J.; Singaraja, R.; Young, F. B.; Liu, L.; El-Husseini, A.et al. Wild-Type Htt Modulates the Enzymatic Activity of the Neuronal Palmitoyl Transferase Hip14. *Hum. Mol. Genet.* **2011,** *20*, 3356-3365.

(87) Yang, Z.; Champoux, J. J. The Role of Histidine 632 in Catalysis by Human Topoisomerase I. *J. Biol. Chem.* **2001,** *276*, 677-685.

(88) Mitchell, D. A.; Vasudevan, A.; Linder, M. E.; Deschenes, R. J. Protein Palmitoylation by a Family of Dhhc Protein S-Acyltransferases. *J. Lipid Res.* **2006,** *47*, 1118-1127.

(89) Gonzalez Montoro, A.; Chumpen Ramirez, S.; Valdez Taubas, J. The Canonical Dhhc Motif Is Not Absolutely Required for the Activity of the Yeast S-Acyltransferases Swf1 and Pfa4. *J. Biol. Chem.* **2015,** *290*, 22448-22459.

(90) Marino, S. M.; Gladyshev, V. N. Analysis and Functional Prediction of Reactive Cysteine Residues. *J. Biol. Chem.* **2012,** *287*, 4419-4425.

(91) Poole, L. B. The Basics of Thiols and Cysteines in Redox Biology and Chemistry. *Free Radic Biol Med* **2015,** *80*, 148-157.

(92) Dietrich, L. E.; Ungermann, C. On the Mechanism of Protein Palmitoylation. *EMBO Rep* **2004,** *5*, 1053-1057.

(93) Reprinted with permission from "Jiang, H.; Zhang, X.; Chen, X.; Aramsangtienchai, P.; Tong, Z.; Lin, H. Protein Lipidation: Occurrence, Mechanisms, Biological Functions, and Enabling Technologies. Chem. Rev. 2018, 118, 919-988." Copyright (2018) American Chemical Society

(94) Reprinted with permission from "Rana, M. S.; Kumar, P.; Lee, C.-J.; Verardi, R.; Rajashankar, K. R.; Banerjee, A. Fatty acyl recognition and transfer by an integral membrane S-acyltransferase. Science 2018, 359." Copyright (2018) Science

(95) Abrami, L.; Dallavilla, T.; Sandoz, P. A.; Demir, M.; Kunz, B.; Savoglidis, G.; Hatzimanikatis, V.; van der Goot, F. G. Identification and Dynamics of the Human Zdhhc16-Zdhhc6 Palmitoylation Cascade. *Elife* **2017,** *6*.

(96) Lievens, P. M.; Kuznetsova, T.; Kochlamazashvili, G.; Cesca, F.; Gorinski, N.; Galil, D. A.; Cherkas, V.; Ronkina, N.; Lafera, J.; Gaestel, M.et al. Zdhhc3 Tyrosine Phosphorylation Regulates Neural Cell Adhesion Molecule Palmitoylation. *Mol. Cell. Biol.* **2016,** *36*, 2208-2225.

(97) Lai, J.; Linder, M. E. Oligomerization of Dhhc Protein S-Acyltransferases. *J. Biol. Chem.* **2013,** *288*, 22862-22870.

(98) Mitchell, D. A.; Hamel, L. D.; Ishizuka, K.; Mitchell, G.; Schaefer, L. M.; Deschenes, R. J. The Erf4 Subunit of the Yeast Ras Palmitoyl Acyltransferase Is Required for Stability of the Acyl-Erf2 Intermediate and Palmitoyl Transfer to a Ras2 Substrate. *J. Biol. Chem.* **2012,** *287*, 34337-34348.

(99) Clapham, D. E. Calcium Signaling. *Cell* **2007,** *131*, 1047-1058.

(100) Silvius, J. R.; l'Heureux, F. Fluorimetric Evaluation of the Affinities of Isoprenylated Peptides for Lipid Bilayers. *Biochemistry* **1994,** *33*, 3014-3022.

(101) Shahinian, S.; Silvius, J. R. Doubly-Lipid-Modified Protein Sequence Motifs Exhibit Long-Lived Anchorage to Lipid Bilayer Membranes. *Biochemistry* **1995,** *34*, 3813-3822.

(102) Chandra, A.; Grecco, H. E.; Pisupati, V.; Perera, D.; Cassidy, L.; Skoulidis, F.; Ismail, S. A.; Hedberg, C.; Hanzal-Bayer, M.; Venkitaraman, A. R.et al. The Gdi-Like Solubilizing Factor Pdedelta Sustains the Spatial Organization and Signalling of Ras Family Proteins. *Nat. Cell Biol.* **2012,** *14*, 148-158.

(103) Rocks, O.; Peyker, A.; Kahms, M.; Verveer, P. J.; Koerner, C.; Lumbierres, M.; Kuhlmann, J.; Waldmann, H.; Wittinghofer, A.; Bastiaens, P. I. An Acylation Cycle Regulates Localization and Activity of Palmitoylated Ras Isoforms. *Science* **2005,** *307*, 1746-1752.

(104) Hancock, J. F.; Paterson, H.; Marshall, C. J. A Polybasic Domain or Palmitoylation Is Required in Addition to the Caax Motif to Localize P21ras to the Plasma Membrane. *Cell* **1990,** *63*, 133-139.

(105) Landles, C.; Bates, G. P. Huntingtin and the Molecular Pathogenesis of Huntington's Disease. Fourth in Molecular Medicine Review Series. *EMBO Rep.* **2004,** *5*, 958-963.

(106) Yanai, A.; Huang, K.; Kang, R.; Singaraja, R. R.; Arstikaitis, P.; Gan, L.; Orban, P. C.; Mullard, A.; Cowan, C. M.; Raymond, L. A.et al. Palmitoylation of Huntingtin by Hip14 Is Essential for Its Trafficking and Function. *Nat. Neurosci.* **2006,** *9*, 824-831.

(107) Coe, J. G.; Lim, A. C.; Xu, J.; Hong, W. A Role for Tlg1p in the Transport of Proteins within the Golgi Apparatus of Saccharomyces Cerevisiae. *Mol. Biol. Cell* **1999,** *10*, 2407-2423.

(108) Siniossoglou, S.; Pelham, H. R. An Effector of Ypt6p Binds the Snare Tlg1p and Mediates Selective Fusion of Vesicles with Late Golgi Membranes. *EMBO J.* **2001,** *20*, 5991-5998.

(109) Valdez-Taubas, J.; Pelham, H. Swf1-Dependent Palmitoylation of the Snare Tlg1 Prevents Its Ubiquitination and Degradation. *EMBO J.* **2005,** *24*, 2524-2532.

(110) Percherancier, Y.; Planchenault, T.; Valenzuela-Fernandez, A.; Virelizier, J. L.; Arenzana-Seisdedos, F.; Bachelerie, F. Palmitoylation-Dependent Control of Degradation, Life Span, and Membrane Expression of the Ccr5 Receptor. *J. Biol. Chem.* **2001,** *276*, 31936-31944.

(111) La Rosa, P.; Pesiri, V.; Leclercq, G.; Marino, M.; Acconcia, F. Palmitoylation Regulates 17beta-Estradiol-Induced Estrogen Receptor-Alpha Degradation and Transcriptional Activity. *Mol. Endocrinol.* **2012,** *26*, 762-774.

(112) Wang, J.; Xie, Y.; Wolff, D. W.; Abel, P. W.; Tu, Y. Dhhc Protein-Dependent Palmitoylation Protects Regulator of G-Protein Signaling 4 from Proteasome Degradation. *FEBS Lett.* **2010,** *584*, 4570-4574.

(113) Lam, K. K.; Davey, M.; Sun, B.; Roth, A. F.; Davis, N. G.; Conibear, E. Palmitoylation by the Dhhc Protein Pfa4 Regulates the Er Exit of Chs3. *J. Cell Biol.* **2006,** *174*, 19-25.

(114) Bhattacharyya, R.; Barren, C.; Kovacs, D. M. Palmitoylation of Amyloid Precursor Protein Regulates Amyloidogenic Processing in Lipid Rafts. *J. Neurosci.* **2013,** *33*, 11169-11183.

(115) Hayashi, T.; Rumbaugh, G.; Huganir, R. L. Differential Regulation of Ampa Receptor Subunit Trafficking by Palmitoylation of Two Distinct Sites. *Neuron* **2005,** *47*, 709-723.

(116) Dharmaiah, S.; Bindu, L.; Tran, T. H.; Gillette, W. K.; Frank, P. H.; Ghirlando, R.; Nissley, D. V.; Esposito, D.; McCormick, F.; Stephen, A. G.et al. Structural Basis of Recognition of Farnesylated and Methylated Kras4b by Pdeδ. *Proc. Natl. Acad. Sci. U.S.A.* **2016,** *113*, E6766-E6775.

(117) Sutton, L. M.; Sanders, S. S.; Butland, S. L.; Singaraja, R. R.; Franciosi, S.; Southwell, A. L.; Doty, C. N.; Schmidt, M. E.; Mui, K. K. N.; Kovalik, V.et al. Hip14l-Deficient Mice Develop Neuropathological and Behavioural Features of Huntington Disease. *Hum. Mol. Genet.* **2012,** *22*, 452-465.

(118) Sanders, S. S.; Hou, J.; Sutton, L. M.; Garside, V. C.; Mui, K. K.; Singaraja, R. R.; Hayden, M. R.; Hoodless, P. A. Huntingtin Interacting Proteins 14 and 14-Like Are Required for Chorioallantoic Fusion During Early Placental Development. *Dev. Biol.* **2015,** *397*, 257-266.

(119) Skotte, N. H.; Sanders, S. S.; Singaraja, R. R.; Ehrnhoefer, D. E.; Vaid, K.; Qiu, X.; Kannan, S.; Verma, C.; Hayden, M. R. Palmitoylation of Caspase-6 by Hip14 Regulates Its Activation. *Cell Death Differ.* **2017,** *24*, 433-444.

(120) Mukai, J.; Liu, H.; Burt, R. A.; Swor, D. E.; Lai, W.-S.; Karayiorgou, M.; Gogos, J. A. Evidence That the Gene Encoding Zdhhc8 Contributes to the Risk of Schizophrenia. *Nat. Genet.* **2004,** *36*, 725-731.

(121) Moutin, E.; Nikonenko, I.; Stefanelli, T.; Wirth, A.; Ponimaskin, E.; De Roo, M.; Muller, D. Palmitoylation of Cdc42 Promotes Spine Stabilization and Rescues Spine Density Deficit in a Mouse Model of 22q11.2 Deletion Syndrome. *Cereb. Cortex* **2016**, DOI:10.1093/cercor/bhw183 10.1093/cercor/bhw183.

(122) Glaser, B.; Schumacher, J.; Williams, H. J.; Jamra, R. A.; Ianakiev, N.; Milev, R.; Ohlraun, S.; Schulze, T. G.; Czerski, P. M.; Hauser, J.et al. No Association between the Putative Functional Zdhhc8 Single Nucleotide Polymorphism Rs175174 and Schizophrenia in Large European Samples. *Biol Psychiatry* **2005,** *58*, 78-80.

(123) Demily, C.; Legallic, S.; Bou, J.; Houy-Durand, E.; Van Amelsvoort, T.; Zinkstok, J.; Manouvrier-Hanue, S.; Vogels, A.; Drouin-Garraud, V.; Philip, N.et al. Zdhhc8 Single Nucleotide Polymorphism Rs175174 Is Not Associated with Psychiatric Features of the 22q11 Deletion Syndrome or Schizophrenia. *Psychiatr Genet* **2007,** *17*, 311-312.

(124) Moraes, L. S.; Santos, A. C. C.; Ferreira-Fernandes, H.; Yoshioka, F. K. N.; Teixeira, S. S.; Guimaraes, A. C.; da Rocha, C. A. M.; Rey, J. A.; Pinto, G. R.; Burbano, R. R. Lack of Association between Comt Val158met and Zdhhc8 Rs175174 Polymorphisms and Susceptibility to Schizophrenia in a Brazilian Population. *Psychiatr Genet* **2017,** *27*, 197-198.

(125) Xu, M.; St Clair, D.; He, L. Testing for Genetic Association between the Zdhhc8 Gene Locus and Susceptibility to Schizophrenia: An Integrated Analysis of Multiple Datasets. *Am J Med Genet B Neuropsychiatr Genet* **2010,** *153B*, 1266-1275.

(126) Raymond, F. L.; Tarpey, P. S.; Edkins, S.; Tofts, C.; O'Meara, S.; Teague, J.; Butler, A.; Stevens, C.; Barthorpe, S.; Buck, G.et al. Mutations in Zdhhc9, Which Encodes a Palmitoyltransferase of Nras and Hras, Cause X-Linked Mental Retardation Associated with a Marfanoid Habitus. *Am. J. Hum. Genet.* **2007,** *80*, 982-987.

(127) Mansouri, M. R.; Marklund, L.; Gustavsson, P.; Davey, E.; Carlsson, B.; Larsson, C.; White, I.; Gustavson, K.-H.; Dahl, N. Loss of Zdhhc15 Expression in a Woman with a Balanced Translocation T(X;15)(Q13.3;Cen) and Severe Mental Retardation. *Eur. J. Hum. Gen.* **2005,** *13*, 970-977.

(128) Kokkola, T.; Kruse, C.; Roy-Pogodzik, E. M.; Pekkinen, J.; Bauch, C.; Honck, H. H.; Hennemann, H.; Kreienkamp, H. J. Somatostatin Receptor 5 Is Palmitoylated by the Interacting Zdhhc5 Palmitoyltransferase. *FEBS Lett.* **2011,** *585*, 2665-2670.

(129) Rasmussen, S. G.; Choi, H. J.; Rosenbaum, D. M.; Kobilka, T. S.; Thian, F. S.; Edwards, P. C.; Burghammer, M.; Ratnala, V. R.; Sanishvili, R.; Fischetti, R. F.et al. Crystal

Structure of the Human Beta2 Adrenergic G-Protein-Coupled Receptor. *Nature* **2007,** *450*, 383-387.

(130) Palczewski, K.; Kumasaka, T.; Hori, T.; Behnke, C. A.; Motoshima, H.; Fox, B. A.; Trong, I. L.; Teller, D. C.; Okada, T.; Stenkamp, R. E.et al. Crystal Structure of Rhodopsin: A G Protein-Coupled Receptor. *Science* **2000,** *289*, 739-745.

(131) Yan, S. M.; Tang, J. J.; Huang, C. Y.; Xi, S. Y.; Huang, M. Y.; Liang, J. Z.; Jiang, Y. X.; Li, Y. H.; Zhou, Z. W.; Ernberg, I.et al. Reduced Expression of Zdhhc2 Is Associated with Lymph Node Metastasis and Poor Prognosis in Gastric Adenocarcinoma. *PLOS ONE* **2013,** *8*, e56366.

(132) Planey, S. L.; Keay, S. K.; Zhang, C. O.; Zacharias, D. A. Palmitoylation of Cytoskeleton Associated Protein 4 by Dhhc2 Regulates Antiproliferative Factor-Mediated Signaling. *Mol. Biol. Cell* **2009,** *20*, 1454-1463.

(133) Oo, H. Z.; Sentani, K.; Sakamoto, N.; Anami, K.; Naito, Y.; Uraoka, N.; Oshima, T.; Yanagihara, K.; Oue, N.; Yasui, W. Overexpression of Zdhhc14 Promotes Migration and Invasion of Scirrhous Type Gastric Cancer. *Oncol. Rep.* **2014,** *32*, 403-410.

(134) Tian, H.; Lu, J. Y.; Shao, C.; Huffman, K. E.; Carstens, R. M.; Larsen, J. E.; Girard, L.; Liu, H.; Rodriguez-Canales, J.; Frenkel, E. P.et al. Systematic Sirna Screen Unmasks Nsclc Growth Dependence by Palmitoyltransferase Dhhc5. *Mol. Cancer. Res.* **2015,** *13*, 784-794.

(135) Mill, P.; Lee, A. W. S.; Fukata, Y.; Tsutsumi, R.; Fukata, M.; Keighren, M.; Porter, R. M.; McKie, L.; Smyth, I.; Jackson, I. J. Palmitoylation Regulates Epidermal Homeostasis and Hair Follicle Differentiation. *PLoS Genet.* **2009,** *5*, e1000748.

(136) Chen, L. Y.; Yang-Yen, H. F.; Tsai, C. C.; Thio, C. L.; Chuang, H. L.; Yang, L. T.; Shen, L. F.; Song, I. W.; Liu, K. M.; Huang, Y. T.et al. Protein Palmitoylation by Zdhhc13 Protects Skin against Microbial-Driven Dermatitis. *J. Invest. Dermatol.* **2017,** *137*, 894-904.

(137) Marin, E. P.; Jozsef, L.; Di Lorenzo, A.; Held, K. F.; Luciano, A. K.; Melendez, J.; Milstone, L. M.; Velazquez, H.; Sessa, W. C. The Protein Acyl Transferase Zdhhc21 Modulates Alpha1 Adrenergic Receptor Function and Regulates Hemodynamics. *Arterioscler Thromb Vasc Biol* **2016,** *36*, 370-379.

(138) Saleem, A. N.; Chen, Y.-H.; Baek, H. J.; Hsiao, Y.-W.; Huang, H.-W.; Kao, H.-J.; Liu, K.-M.; Shen, L.-F.; Song, I.-W.; Tu, C.-P. D.et al. Mice with Alopecia, Osteoporosis, and Systemic Amyloidosis Due to Mutation in Zdhhc13, a Gene Coding for Palmitoyl Acyltransferase. *PLoS Genet.* **2010,** *6*, e1000985.

(139) Liu, K. M.; Chen, Y. J.; Shen, L. F.; Haddad, A. N.; Song, I. W.; Chen, L. Y.; Chen, Y. J.; Wu, J. Y.; Yen, J. J.; Chen, Y. T. Cyclic Alopecia and Abnormal Epidermal Cornification in Zdhhc13-Deficient Mice Reveal the Importance of Palmitoylation in Hair and Skin Differentiation. *J. Invest. Dermatol.* **2015,** *135*, 2603-2610.

(140) Shen, L. F.; Chen, Y. J.; Liu, K. M.; Haddad, A. N. S.; Song, I. W.; Roan, H. Y.; Chen, L. Y.; Yen, J. J. Y.; Chen, Y. J.; Wu, J. Y.et al. Role of S-Palmitoylation by Zdhhc13 in Mitochondrial Function and Metabolism in Liver. *Sci. Rep.* **2017,** *7*, 2182.

(141) Du, K.; Murakami, S.; Sun, Y.; Kilpatrick, C. L.; Luscher, B. Dhhc7 Palmitoylates Glucose Transporter 4 (Glut4) and Regulates Glut4 Membrane Translocation. *J. Biol. Chem.*

2017, *292*, 2979-2991.

(142) Chen, B.; Zheng, B.; DeRan, M.; Jarugumilli, G. K.; Fu, J.; Brooks, Y. S.; Wu, X. Zdhhc7-Mediated S-Palmitoylation of Scribble Regulates Cell Polarity. *Nat. Chem. Biol.* **2016,** *12*, 686-693.

(143) Aramsangtienchai, P.; Spiegelman, N. A.; Cao, J.; Lin, H. S-Palmitoylation of Junctional Adhesion Molecule C Regulates Its Tight Junction Localization and Cell Migration. *J. Biol. Chem.* **2017,** *292*, 5325-5334.

(144) Veit, M.; Siche, S. S-Acylation of Influenza Virus Proteins: Are Enzymes for Fatty Acid Attachment Promising Drug Targets? *Vaccine* **2015,** *33*, 7002-7007.

(145) Wang, S.; Mott, K. R.; Wawrowsky, K.; Kousoulas, K. G.; Luscher, B.; Ghiasi, H. Binding of Hsv-1 Ul20 to Godz Affects Its Palmitoylation and Is Essential for Infectivity and Proper Targeting and Localization of Ul20 and Gk. *J. Virol.* **2017**, DOI:10.1128/JVI.00945-17 10.1128/JVI.00945-17.

(146) Lin, Y. H.; Doms, A. G.; Cheng, E.; Kim, B.; Evans, T. R.; Machner, M. P. Host Cell-Catalyzed S-Palmitoylation Mediates Golgi Targeting of the Legionella Ubiquitin Ligase Gobx. *J. Biol. Chem.*

2015, *290*, 25766-25781.

(147) Reinicke, A. T.; Hutchinson, J. L.; Magee, A. I.; Mastroeni, P.; Trowsdale, J.; Kelly, A. P. A Salmonella Typhimurium Effector Protein Sifa Is Modified by Host Cell Prenylation and S-Acylation Machinery. *J. Biol. Chem.* **2005,** *280*, 14620-14627.

(148) Camp, L. A.; Hofmann, S. L. Purification and Properties of a Palmitoyl-Protein Thioesterase That Cleaves Palmitate from H-Ras. *J. Biol. Chem.* **1993,** *268*, 22566-22574.

(149) Verkruyse, L. A.; Hofmann, S. L. Lysosomal Targeting of Palmitoyl-Protein Thioesterase. *J. Biol. Chem.* **1996,** *271*, 15831-15836.

(150) Camp, L. A.; Verkruyse, L. A.; Afendis, S. J.; Slaughter, C. A.; Hofmann, S. L. Molecular Cloning and Expression of Palmitoyl-Protein Thioesterase. *J. Biol. Chem.* **1994,** *269*, 23212-23219.

(151) Duncan, J. A.; Gilman, A. G. A Cytoplasmic Acyl-Protein Thioesterase That Removes Palmitate from G Protein A Subunits and P21ras. *J. Biol. Chem.* **1998,** *273*, 15830-15837.

(152) Flaumenhaft, R.; Rozenvayn, N.; Feng, D.; Dvorak, A. M. Snap-23 and Syntaxin-2 Localize to the Extracellular Surface of the Platelet Plasma Membrane. *Blood* **2007,** *110*, 1492-1501.

(153) Duncan, J. A.; Gilman, A. G. Characterization of Saccharomyces Cerevisiae Acyl-Protein Thioesterase 1, the Enzyme Responsible for G Protein Alpha Subunit Deacylation in Vivo. *J. Biol. Chem.* **2002,** *277*, 31740-31752.

(154) Yeh, D. C.; Duncan, J. A.; Yamashita, S.; Michel, T. Depalmitoylation of Endothelial Nitric-Oxide Synthase by Acyl-Protein Thioesterase 1 Is Potentiated by Ca2+-Calmodulin. *J. Biol. Chem.* **1999,** *274*, 33148-33154.

(155) Devedjiev, Y.; Dauter, Z.; Kuznetsov, S. R.; Jones, T. L.; Derewenda, Z. S. Crystal Structure of the Human Acyl Protein Thioesterase I from a Single X-Ray Data Set to 1.5 A. *Structure* **2000,** *8*, 1137-1146.

(156) Tomatis, V. M.; Trenchi, A.; Gomez, G. A.; Daniotti, J. L. Acyl-Protein Thioesterase 2 Catalyzes the Deacylation of Peripheral Membrane-Associated Gap-43. *PLoS One* **2010,** *5*, e15045.

(157) Kong, E.; Peng, S.; Chandra, G.; Sarkar, C.; Zhang, Z.; Bagh, M. B.; Mukherjee, A. B. Dynamic Palmitoylation Links Cytosol-Membrane Shuttling of Acyl-Protein Thioesterase-1 and Acyl-Protein Thioesterase-2 with That of Proto-Oncogene H-Ras Product and Growth-Associated Protein-43. *J. Biol. Chem.* **2013,** *288*, 9112-9125.

(158) Vartak, N.; Papke, B.; Grecco, Hernan E.; Rossmannek, L.; Waldmann, H.; Hedberg, C.; Bastiaens, Philippe I. H. The Autodepalmitoylating Activity of Apt Maintains the Spatial Organization of Palmitoylated Membrane Proteins. *Biophys. J.* **2014,** *106*, 93-105.

(159) Lin, D. T.; Conibear, E. Abhd17 Proteins Are Novel Protein Depalmitoylases That Regulate N-Ras Palmitate Turnover and Subcellular Localization. *Elife* **2015,** *4*, e11306.

(160) Yokoi, N.; Fukata, Y.; Sekiya, A.; Murakami, T.; Kobayashi, K.; Fukata, M. Identification of Psd-95 Depalmitoylating Enzymes. *J. Neurosci.* **2016,** *36*, 6431-6444.

(161) Peseckis, S. M.; Deichaite, I.; Resh, M. D. Iodinated Fatty Acids as Probes for Myristate Processing and Function. Incorporation into Pp60v-Src. *J. Biol. Chem.* **1993,** *268*, 5107-5114.

(162) Schlesinger, M. J.; Magee, A. I.; Schmidt, M. F. Fatty Acid Acylation of Proteins in Cultured Cells. *J. Biol. Chem.* **1980,** *255*, 10021-10024.

(163) Drisdel, R. C.; Green, W. N. Labeling and Quantifying Sites of Protein Palmitoylation. *Biotechniques* **2004,** *36*, 276-285.

(164) Wan, J.; Roth, A. F.; Bailey, A. O.; Davis, N. G. Palmitoylated Proteins: Purification and Identification. *Nat. Protoc.* **2007,** *2*, 1573-1584.

(165) Kang, R.; Wan, J.; Arstikaitis, P.; Takahashi, H.; Huang, K.; Bailey, A. O.; Thompson, J. X.; Roth, A. F.; Drisdel, R. C.; Mastro, R.et al. Neural Palmitoyl-Proteomics Reveals Dynamic Synaptic Palmitoylation. *Nature* **2008,** *456*, 904-909.

(166) Forrester, M. T.; Thompson, J. W.; Foster, M. W.; Nogueira, L.; Moseley, M. A.; Stamler, J. S. Proteomic Analysis of S-Nitrosylation and Denitrosylation by Resin-Assisted Capture. *Nat. Biotechnol.* **2009,** *27*, 557-559.

(167) Percher, A.; Ramakrishnan, S.; Thinon, E.; Yuan, X.; Yount, J. S.; Hang, H. C. Mass-Tag Labeling Reveals Site-Specific and Endogenous Levels of Protein S-Fatty Acylation. *Proc. Natl. Acad. Sci. U. S. A.* **2016,** *113*, 4302-4307.

(168) Wang, Q.; Chan, T. R.; Hilgraf, R.; Fokin, V. V.; Sharpless, K. B.; Finn, M. G. Bioconjugation by Copper(I)-Catalyzed Azide-Alkyne [3 + 2] Cycloaddition. *J. Am. Chem. Soc.* **2003,** *125*, 3192-3193.

(169) Speers, A. E.; Cravatt, B. F. Profiling Enzyme Activities in Vivo Using Click Chemistry Methods. *Chem. Biol.* **2004,** *11*, 535-546.

(170) Ji, Y.; Leymarie, N.; Haeussler, D. J.; Bachschmid, M. M.; Costello, C. E.; Lin, C. Direct Detection of S-Palmitoylation by Mass Spectrometry. *Anal. Chem.* **2013,** *85*, 11952-11959.

(171) Sorek, N.; Yalovsky, S. Analysis of Protein S-Acylation by Gas Chromatography-Coupled Mass Spectrometry Using Purified Proteins. *Nat. Protoc.* **2010,** *5*, 834-840.

(172) McClure, M.; DeLucas, L. J.; Wilson, L.; Ray, M.; Rowe, S. M.; Wu, X.; Dai, Q.; Hong, J. S.; Sorscher, E. J.; Kappes, J. C.et al. Purification of Cftr for Mass Spectrometry Analysis: Identification of Palmitoylation and Other Post-Translational Modifications. *Protein Eng. Des. Sel.* **2012,** *25*, 7-14.

(173) Ren, J.; Wen, L.; Gao, X.; Jin, C.; Xue, Y.; Yao, X. Css-Palm 2.0: An Updated Software for Palmitoylation Sites Prediction. *Protein Eng. Des. Sel.* **2008,** *21*, 639-644.

(174) Xue, Y.; Chen, H.; Jin, C.; Sun, Z.; Yao, X. Nba-Palm: Prediction of Palmitoylation Site Implemented in Naive Bayes Algorithm. *BMC Bioinform.* **2006,** *7*, 458.

(175) Wang, X. B.; Wu, L. Y.; Wang, Y. C.; Deng, N. Y. Prediction of Palmitoylation Sites Using the Composition of K-Spaced Amino Acid Pairs. *Protein Eng. Des. Sel.* **2009,** *22*, 707-712.

(176) Wang, J. Y.; Yao, X.; Huang, J. New Tricks for Human Farnesyltransferase Inhibitor: Cancer and Beyond. *Medchemcomm* **2017,** *8*, 841-854.

(177) "Reprinted from European Journal of Cell Biology, Indranil De, Sushabhan Sadhukhan, Emerging Roles of DHHC-mediated Protein S-palmitoylation in Physiological and Pathophysiological Context, 2018, https://doi.org/10.1016/j.ejcb.2018.03.005, with permission from Elsevier"

(178) Adapted with permission from "Jiang, H.; Zhang, X.; Chen, X.; Aramsangtienchai, P.; Tong, Z.; Lin, H. Protein Lipidation: Occurrence, Mechanisms, Biological Functions, and

Enabling Technologies. Chem. Rev. 2018, 118, 919-988." Copyright (2018) American Chemical Society

(179) Oku, S.; Takahashi, N.; Fukata, Y.; Fukata, M. In Silico Screening for Palmitoyl Substrates Reveals a Role for Dhhc1/3/10 (Zdhhc1/3/11)-Mediated Neurochondrin Palmitoylation in Its Targeting to Rab5-Positive Endosomes. *J. Biol. Chem.* **2013,** *288*, 19816-19829.

(180) Oyama, T.; Miyoshi, Y.; Koyama, K.; Nakagawa, H.; Yamori, T.; Ito, T.; Matsuda, H.; Arakawa, H.; Nakamura, Y. Isolation of a Novel Gene on 8p21.3-22 Whose Expression Is Reduced Significantly in Human Colorectal Cancers with Liver Metastasis. *Genes Chromosomes Cancer* **2000,** *29*, 9-15.

(181) Zeidman, R.; Buckland, G.; Cebecauer, M.; Eissmann, P.; Davis, D. M.; Magee, A. I. Dhhc2 Is a Protein *S* -Acyltransferase for Lck. *Mol. Membr. Biol.* **2011,** *28*, 473-486.

(182) Jia, L.; Linder, M. E.; Blumer, K. J. Gi/O Signaling and the Palmitoyltransferase Dhhc2 Regulate Palmitate Cycling and Shuttling of Rgs7 Family-Binding Protein. *J. Biol. Chem.* **2011,** *286*, 13695-13703.

(183) Zhang, J.; Planey, S. L.; Ceballos, C.; Stevens, S. M.; Keay, S. K.; Zacharias, D. A. Identification of Ckap4/P63 as a Major Substrate of the Palmitoyl Acyltransferase Dhhc2, a Putative Tumor Suppressor, Using a Novel Proteomics Method. *Mol. Cell. Proteomics* **2008,** *7*, 1378-1388.

(184) Sharma, C.; Yang, X. H.; Hemler, M. E. Dhhc2 Affects Palmitoylation, Stability, and Functions of Tetraspanins Cd9 and Cd151. *Mol. Biol. Cell* **2008,** *19*, 3415-3425.

(185) Fernández-Hernando, C.; Fukata, M.; Bernatchez, P. N.; Fukata, Y.; Lin, M. I.; Bredt, D. S.; Sessa, W. C. Identification of Golgi-Localized Acyl Transferases That Palmitoylate and Regulate Endothelial Nitric Oxide Synthase. *J. Cell Biol.* **2006,** *174*, 369-377.

(186) Fairbank, M.; Huang, K.; El-Husseini, A.; Nabi, I. R. Ring Finger Palmitoylation of the Endoplasmic Reticulum Gp78 E3 Ubiquitin Ligase. *FEBS Lett.* **2012,** *586*, 2488-2493.

(187) Woolfrey, K. M.; Sanderson, J. L.; Dell'Acqua, M. L. The Palmitoyl Acyltransferase Dhhc2 Regulates Recycling Endosome Exocytosis and Synaptic Potentiation through Palmitoylation of Akap79/150. *J. Neurosci.* **2015,** *35*, 442-456.

(188) Sharma, C.; Rabinovitz, I.; Hemler, M. E. Palmitoylation by Dhhc3 Is Critical for the Function, Expression, and Stability of Integrin Alpha6beta4. *Cell. Mol. Life Sci.* **2012,** *69*, 2233-2244.

(189) Oh, Y.; Jeon, Y. J.; Hong, G. S.; Kim, I.; Woo, H. N.; Jung, Y. K. Regulation in the Targeting of Trail Receptor 1 to Cell Surface Via Godz for Trail Sensitivity in Tumor Cells. *Cell Death. Differ.* **2012,** *19*, 1196-1207.

(190) Tsutsumi, R.; Fukata, Y.; Noritake, J.; Iwanaga, T.; Perez, F.; Fukata, M. Identification of G Protein Alpha Subunit-Palmitoylating Enzyme. *Mol. Cell. Biol.* **2009,** *29*, 435-447.

(191) Greaves, J.; Salaun, C.; Fukata, Y.; Fukata, M.; Chamberlain, L. H. Palmitoylation and Membrane Interactions of the Neuroprotective Chaperone Cysteine-String Protein. *J. Biol. Chem.* **2008,** *283*, 25014-25026.

(192) Lu, D.; Sun, H. Q.; Wang, H.; Barylko, B.; Fukata, Y.; Fukata, M.; Albanesi, J. P.; Yin, H. L. Phosphatidylinositol 4-Kinase Iialpha Is Palmitoylated by Golgi-Localized Palmitoyltransferases in Cholesterol-Dependent Manner. *J. Biol. Chem.* **2012,** *287*, 21856-21865.

(193) Tian, L.; McClafferty, H.; Jeffries, O.; Shipston, M. J. Multiple Palmitoyltransferases Are Required for Palmitoylation-Dependent Regulation of Large Conductance Calcium- and Voltage-Activated Potassium Channels. *J. Biol. Chem.* **2010,** *285*, 23954-23962.

(194) Thomas, G. M.; Hayashi, T.; Huganir, R. L.; Linden, D. J. Dhhc8-Dependent Pick1 Palmitoylation Is Required for Induction of Cerebellar Long-Term Synaptic Depression. *J. Neurosci.* **2013,** *33*, 15401-15407.

(195) Taruno, A.; Sun, H.; Nakajo, K.; Murakami, T.; Ohsaki, Y.; Kido, M. A.; Ono, F.; Marunaka, Y. Post-Translational Palmitoylation Controls the Voltage Gating and Lipid Raft Association of Calhm1 Channel. *J. Physiol.* **2017**, DOI:10.1113/JP274164 10.1113/JP274164.

(196) Segal-Salto, M.; Sapir, T.; Reiner, O. Reversible Cysteine Acylation Regulates the Activity of Human Palmitoyl-Protein Thioesterase 1 (Ppt1). *PLOS ONE* **2016,** *11*, e0146466.

(197) Brigidi, G. S.; Sun, Y.; Beccano-Kelly, D.; Pitman, K.; Mobasser, M.; Borgland, S. L.; Milnerwood, A. J.; Bamji, S. X. Palmitoylation of Delta-Catenin by Dhhc5 Mediates Activity-Induced Synapse Plasticity. *Nat. Neurosci.* **2014,** *17*, 522-532.

(198) Li, Y.; Martin, B. R.; Cravatt, B. F.; Hofmann, S. L. Dhhc5 Protein Palmitoylates Flotillin-2 and Is Rapidly Degraded on Induction of Neuronal Differentiation in Cultured Cells. *J. Biol. Chem.* **2012,** *287*, 523-530.

(199) Howie, J.; Reilly, L.; Fraser, N. J.; Vlachaki Walker, J. M.; Wypijewski, K. J.; Ashford, M. L.; Calaghan, S. C.; McClafferty, H.; Tian, L.; Shipston, M. J.et al. Substrate Recognition by the Cell Surface Palmitoyl Transferase Dhhc5. *Proc. Natl. Acad. Sci. U.S.A.* **2014,** *111*, 17534-17539.

(200) Fredericks, G. J.; Hoffmann, F. W.; Rose, A. H.; Osterheld, H. J.; Hess, F. M.; Mercier, F.; Hoffmann, P. R. Stable Expression and Function of the Inositol 1,4,5-Triphosphate Receptor Requires Palmitoylation by a Dhhc6/Selenoprotein K Complex. *Proc. Natl. Acad. Sci. U.S.A.* **2014,** *111*, 16478-16483.

(201) Pedram, A.; Razandi, M.; Deschenes, R. J.; Levin, E. R. Dhhc-7 and -21 Are Palmitoylacyltransferases for Sex Steroid Receptors. *Mol. Biol. Cell* **2012,** *23*, 188-199.

(202) Rossin, A.; Durivault, J.; Chakhtoura-Feghali, T.; Lounnas, N.; Gagnoux-Palacios, L.; Hueber, A. O. Fas Palmitoylation by the Palmitoyl Acyltransferase Dhhc7 Regulates Fas Stability. *Cell Death Differ.* **2015,** *22*, 643-653.

(203) Milde, S.; Coleman, M. P. Identification of Palmitoyltransferase and Thioesterase Enzymes That Control the Subcellular Localization of Axon Survival Factor Nicotinamide Mononucleotide Adenylyltransferase 2 (Nmnat2). *J. Biol. Chem.* **2014,** *289*, 32858-32870.

(204) Singaraja, R. R.; Kang, M. H.; Vaid, K.; Sanders, S. S.; Vilas, G. L.; Arstikaitis, P.; Coutinho, J.; Drisdel, R. C.; El-Husseini Ael, D.; Green, W. N.et al. Palmitoylation of Atp-Binding Cassette Transporter A1 Is Essential for Its Trafficking and Function. *Circ. Res.* **2009,** *105*, 138-147.

(205) Tian, L.; McClafferty, H.; Knaus, H.-G.; Ruth, P.; Shipston, M. J. Distinct Acyl Protein Transferases and Thioesterases Control Surface Expression of Calcium-Activated Potassium Channels. *J. Biol. Chem.* **2012,** *287*, 14718-14725.

(206) Mizumaru, C.; Saito, Y.; Ishikawa, T.; Yoshida, T.; Yamamoto, T.; Nakaya, T.; Suzuki, T. Suppression of App-Containing Vesicle Trafficking and Production of Beta-Amyloid by Aid/Dhhc-12 Protein. *J. Neurochem.* **2009,** *111*, 1213-1224.

(207) Michelson, M.; Ben-Sasson, A.; Vinkler, C.; Leshinsky-Silver, E.; Netzer, I.; Frumkin, A.; Kivity, S.; Lerman-Sagie, T.; Lev, D. Delineation of the Interstitial 6q25 Microdeletion Syndrome: Refinement of the Critical Causative Region. *Am. J. Hum. Genet.* **2012,** *158A*, 1395-1399.

(208) Yu, L.; Reader, J. C.; Chen, C.; Zhao, X. F.; Ha, J. S.; Lee, C.; York, T.; Gojo, I.; Baer, M. R.; Ning, Y. Activation of a Novel Palmitoyltransferase Zdhhc14 in Acute Biphenotypic Leukemia and Subsets of Acute Myeloid. *Leukemia* **2011,** *25*, 367-371.

(209) Yang, G.; Cynader, M. S. Regulation of Protein Trafficking: Jnk3 at the Golgi Complex. *Cell Cycle* **2014,** *13*, 5-6.

(210) Zhang, F.; Di, Y.; Li, J.; Shi, Y.; Zhang, L.; Wang, C.; He, X.; Liu, Y.; Wan, D.; Huo, K.et al. Molecular Cloning and Characterization of Human Aph2 Gene, Involved in Ap-1 Regulation by Interaction with Jab1. *Biochim. Biophys. Acta* **2006,** *1759*, 514-525.

(211) Zhou, T.; Li, J.; Zhao, P.; Liu, H.; Jia, D.; Jia, H.; He, L.; Cang, Y.; Boast, S.; Chen, Y. H.et al. Palmitoyl Acyltransferase Aph2 in Cardiac Function and the Development of Cardiomyopathy. *Proc. Natl. Acad. Sci. U.S.A.* **2015,** *112*, 15666-15671.

(212) Milnerwood, A. J.; Parsons, M. P.; Young, F. B.; Singaraja, R. R.; Franciosi, S.; Volta, M.; Bergeron, S.; Hayden, M. R.; Raymond, L. A. Memory and Synaptic Deficits in Hip14/Dhhc17 Knockout Mice. *Proc. Natl. Acad. Sci. U.S.A.* **2013,** *110*, 20296-20301.

(213) Ren, W.; Sun, Y.; Du, K. Dhhc17 Palmitoylates Clipr-59 and Modulates Clipr-59 Association with the Plasma Membrane. *Mol. Cell. Biol.* **2013,** *33*, 4255-4265.

(214) Huang, K.; Yanai, A.; Kang, R.; Arstikaitis, P.; Singaraja, R. R.; Metzler, M.; Mullard, A.; Haigh, B.; Gauthier-Campbell, C.; Gutekunst, C.-A.et al. Huntingtin-Interacting Protein Hip14 Is a Palmitoyl Transferase Involved in Palmitoylation and Trafficking of Multiple Neuronal Proteins. *Neuron* **2004,** *44*, 977-986.

(215) Łach, A.; Grzybek, M.; Heger, E.; Korycka, J.; Wolny, M.; Kubiak, J.; Kolondra, A.; Bogusławska, D. M.; Augoff, K.; Majkowski, M.et al. Palmitoylation of Mpp1 (Membrane-Palmitoylated Protein 1)/P55 Is Crucial for Lateral Membrane Organization in Erythroid Cells. *J. Biol. Chem.* **2012,** *287*, 18974-18984.

(216) Yang, G.; Zhou, X.; Zhu, J.; Liu, R.; Zhang, S.; Coquinco, A.; Chen, Y.; Wen, Y.; Kojic, L.; Jia, W.et al. Jnk3 Couples the Neuronal Stress Response to Inhibition of Secretory Trafficking. *Sci. Signal.* **2013,** *6*, ra57-.

(217) Runkle, K. B.; Kharbanda, A.; Stypulkowski, E.; Cao, X. J.; Wang, W.; Garcia, B. A.; Witze, E. S. Inhibition of Dhhc20-Mediated Egfr Palmitoylation Creates a Dependence on Egfr Signaling. *Mol. Cell* **2016,** *62*, 385-396.

(218) Draper, J. M.; Smith, C. D. Dhhc20: A Human Palmitoyl Acyltransferase That Causes Cellular Transformation. *Molec. Membrane Biol.* **2010,** *27*, 123-136.

(219) Marin, E. P.; Derakhshan, B.; Lam, T. T.; Davalos, A.; Sessa, W. C. Endothelial Cell Palmitoylproteomic Identifies Novel Lipid-Modified Targets and Potential Substrates for Protein Acyl Transferases. *Circ. Res.* **2012,** *110*, 1336-1344.

(220) Goytain, A.; Hines, R. M.; Quamme, G. A. Huntingtin-Interacting Proteins, Hip14 and Hip14l, Mediate Dual Functions, Palmitoyl Acyltransferase and Mg2+ Transport. *J. Biol. Chem.* **2008,** *283*, 33365-33374.

(221) Song, I.-W.; Li, W.-R.; Chen, L.-Y.; Shen, L.-F.; Liu, K.-M.; Yen, J. J. Y.; Chen, Y.-J.; Chen, Y.-J.; Kraus, V. B.; Wu, J.-Y.et al. Palmitoyl Acyltransferase, Zdhhc13, Facilitates Bone Mass Acquisition by Regulating Postnatal Epiphyseal Development and Endochondral Ossification: A Mouse Model. *PLOS ONE* **2014,** *9*, e92194.

(222) Chalkiadaki, A.; Guarente, L. Sirtuins Mediate Mammalian Metabolic Responses to Nutrient Availability. *Nat. Rev. Endocrinol.* **2012,** *advance online publication*.

(223) Houtkooper, R. H.; Pirinen, E.; Auwerx, J. Sirtuins as Regulators of Metabolism and Healthspan. *Nat. Rev. Mol. Cell Biol.* **2012,** *13*, 225-238.

(224) Haigis, M. C.; Sinclair, D. A. Mammalian Sirtuins: Biological Insights and Disease Relevance. *Annu. Rev. Pathol.* **2010,** *5*, 253-295.

(225) Imai, S.-i.; Armstrong, C. M.; Kaeberlein, M.; Guarente, L. Transcriptional Silencing and Longevity Protein Sir2 Is an Nad-Dependent Histone Deacetylase. *Nature* **2000,** *403*, 795-800.

(226) Du, J.; Zhou, Y.; Su, X.; Yu, J.; Khan, S.; Jiang, H.; Kim, J.; Woo, J.; Kim, J. H.; Choi, B. H.et al. Sirt5 Is an Nad-Dependent Protein Lysine Demalonylase and Desuccinylase. *Science* **2011,** *334*, 806-809.

(227) Zhang, X.; Spiegelman, N. A.; Nelson, O. D.; Jing, H.; Lin, H. Sirt6 Regulates Ras-Related Protein R-Ras2 by Lysine Defatty-Acylation. *Elife* **2017,** *6*.

(228) Cao, J.; Sun, L.; Aramsangtienchai, P.; Spiegelman, N. A.; Zhang, X.; Seto, E.; Lin, H. Hdac11 Regulates Type I Interferon Signaling through Defatty-Acylation of Shmt2. *bioRxiv* **2017**, DOI:10.1101/211706 10.1101/211706.

(229) Aramsangtienchai, P.; Spiegelman, N. A.; He, B.; Miller, S. P.; Dai, L.; Zhao, Y.; Lin, H. Hdac8 Catalyzes the Hydrolysis of Long Chain Fatty Acyl Lysine. *ACS Chem. Biol.* **2016,** *11*, 2685-2692.

(230) Korycka, J.; Lach, A.; Heger, E.; Boguslawska, D. M.; Wolny, M.; Toporkiewicz, M.; Augoff, K.; Korzeniewski, J.; Sikorski, A. F. Human Dhhc Proteins: A Spotlight on the Hidden Player of Palmitoylation. *Eur J Cell Biol* **2012,** *91*, 107-117.

(231) Stults, J. T.; Griffin, P. R.; Lesikar, D. D.; Naidu, A.; Moffat, B.; Benson, B. J. Lung Surfactant Protein Sp-C from Human, Bovine, and Canine Sources Contains Palmityl Cysteine Thioester Linkages. *Am J Physiol* **1991,** *261*, L118-125.

(232) Tapon, N.; Hall, A. Rho, Rac and Cdc42 Gtpases Regulate the Organization of the Actin Cytoskeleton. *Curr Opin Cell Biol* **1997,** *9*, 86-92.

(233) Monypenny, J.; Zicha, D.; Higashida, C.; Oceguera-Yanez, F.; Narumiya, S.; Watanabe, N. Cdc42 and Rac Family Gtpases Regulate Mode and Speed but Not Direction of Primary Fibroblast Migration During Platelet-Derived Growth Factor-Dependent Chemotaxis. *Mol Cell Biol* **2009,** *29*, 2730-2747.

(234) Swart-Mataraza, J. M.; Li, Z.; Sacks, D. B. Iqgap1 Is a Component of Cdc42 Signaling to the Cytoskeleton. *J. Biol. Chem.* **2002,** *277*, 24753-24763.

(235) Davda, D.; El Azzouny, M. A.; Tom, C. T.; Hernandez, J. L.; Majmudar, J. D.; Kennedy, R. T.; Martin, B. R. Profiling Targets of the Irreversible Palmitoylation Inhibitor 2-Bromopalmitate. *ACS Chem. Biol.* **2013,** *8*, 1912-1917.

(236) Charron, G.; Zhang, M. M.; Yount, J. S.; Wilson, J.; Raghavan, A. S.; Shamir, E.; Hang, H. C. Robust Fluorescent Detection of Protein Fatty-Acylation with Chemical Reporters. *J Am Chem Soc* **2009,** *131*, 4967-4975.

(237) Liu, H.; Naismith, J. H. An Efficient One-Step Site-Directed Deletion, Insertion, Single and Multiple-Site Plasmid Mutagenesis Protocol. *BMC Biotechnol* **2008,** *8*, 91.

(238) Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W. Nih Image to Imagej: 25 Years of Image Analysis. *Nat Methods* **2012,** *9*, 671-675.

(239) Wang, X. Y.; Gan, M. X.; Li, Y.; Zhan, W. H.; Han, T. Y.; Han, X. J.; Cheng, J. Q.; Wang, J. B. Cdc42 Induces Egf Receptor Protein Accumulation and Promotes Egf Receptor Nuclear Transport and Cellular Transformation. *FEBS Lett.* **2015,** *589*, 255-262.