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STATE-OF-THE-ART REVIEW

Protein S-Palmitoylation: advances and challenges in studying a therapeutically important lipid modification

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The lipid post-translational modification S-palmitoylation is a vast developing field, with the modification itself and the enzymes that catalyse the reversible reaction implicated in a number of diseases. In this review, we discuss the past and recent advances in the experimental tools used in this field, including pharmacological tools, animal models and techniques to understand how palmitoylation controls protein localisation and function. Additionally, we discuss the obstacles to overcome in order to advance the field, particularly to the point at which modulating palmitoylation may be achieved as a therapeutic strategy.

Introduction

Lipid modifications

Post-translational modifications (PTMs) allow immense diversity in biological systems, often controlling substrate localisation, stability and function, and must be tightly regulated in order to maintain cell health. Over 400 chemically distinct covalent PTMs have been described to date, adding huge chemical complexity to the structure and function of the proteome [1]. Our understanding of the unique PTM profiles of individual proteins can improve knowledge of how to target them for therapeutic benefit. In the 1950s, the first evidence of covalent lipid transfer to a proteins in the brain was noted [2], but it was not until decades later that the theory of lipids as an essential component of protein trafficking gained traction. Now, several lipid modifications have been identified, many of which are involved in regulating integration and association of substrates with membranes through increasing hydrophobicity (Fig. 1) [3]. This is not done in a random fashion as initially thought, but utilising distinct membrane microdomains, known as lipid rafts, where the increased membrane fluidity may influence trafficking and protein–protein interactions, and therefore, lipid modified proteins have become a subject of great interest in recent years [4].

As such, it is estimated between 25 and 40% of substrates are modified by lipids in some way, making them one of the most common types of PTM and as such, a concerted effort has been made to classify and characterise them [5]. In mammalian cells, many types of fatty acylation (involving the addition of fatty acids between eight and twenty carbons in length) involve irreversible modifications of proteins. This includes N-

Abbreviations
17-ODYA, 17-octadecynoic acid; 2-BP, 2-bromopalmitate; ABE, acyl-biotin exchange; Acyl-RAC, resin-assisted capture of acylated proteins; APT, acyl protein thioesterase; DTT, 1,4-Dithiothreitol; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; MEND, massive endocytosis; MS, mass spectrometry; NCX, sodium/calcium exchanger; NKA, sodium/potassium ATPase; PLM, phospholemman; SILAC, Stable Isotope Labelling by Amino acids in Cell culture; TCEP, tris(2-carboxylethyl)phosphine; zDHHC-PAT, zDHHC-motif containing palmitoyl acyltransferase.

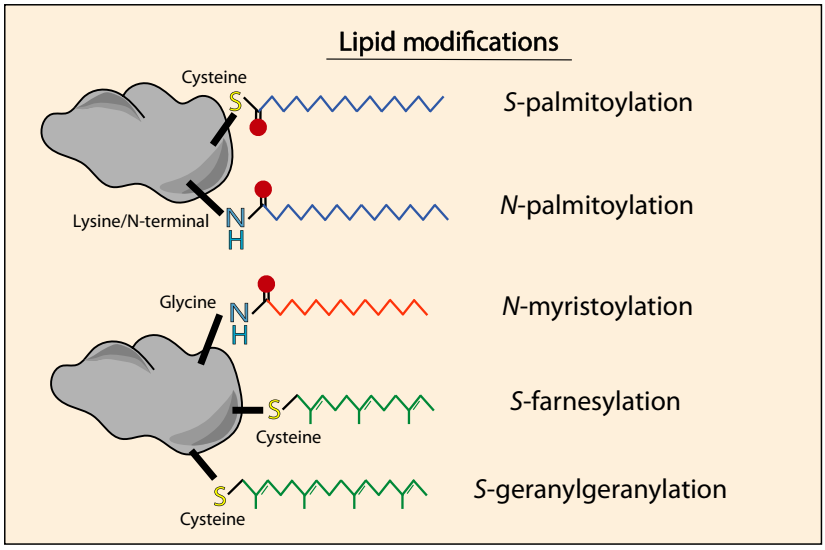

Fig. 1. Lipid post-translational modifications. Proteins can be subjected to multiple forms of lipid post-translational modification. These include cysteine modifications S-palmitoylation (C16:0), which forms a reversible thioester, and S-farnesylation (C15:0) and geranylgeranylation (C20:0), which form an irreversible thioether. Amide groups of lysines, glycines and N-terminal portions of proteins can be subjected to irreversible lipid modifications in the form of N-palmitoylation (C16:0; lysine/N-terminal) and N-myristoylation (C14:0; glycine). Adapted using information from (Chamberlain and Shipston, 2015). [29]

terminal myristoylation of a glycine by a 14-carbon (C14:0) myristoyl group, either co- or post-translationally, and has been implicated as a key step in many immune cell signalling cascades [6]. Similarly, prenylation provides a hydrophobic, membrane attracted C terminus through the enzymatic addition of farnesyl (C15:0) or geranylgeranyl (C20:0) to a cysteine residue, and the modification is now implicated as being dysregulated in several diseases including cancer [7]. Prenylation is not the only lipid cysteine modification, with their reactive sulfhydryl groups open to thioester linkage through S-acylation. These acyl additions can range in length from 16–20 carbons, with the C16:0, often palmitoyl derived from palmitoyl-CoA (S-palmitoylation), being the most common form. In fact, S-acylation and S-palmitoylation, in particular, have attracted much interest in the PTM field due to its reversible, enzyme-driven nature and wide ranging effects on substrate localisation and activity for both integral membrane and soluble proteins, with more than 10% of the proteome predicted to be targeted by S-palmitoylation [8].

S-acylation enzymology

Initially, studies into the G protein Giα1, the downstream effector of many important receptors, saw cysteine 3 (C3) auto-palmitoylated in the presence of palmitoyl-CoA. This was interpreted to mean that palmitoylation may be a spontaneous modification dependent on local fatty acid concentration, and therefore, modulation for therapeutic benefit would be challenging [9]. Progress in the field was therefore slow, with potential enzymes involved difficult to identify, even resulting in false positives [10]. Gradually evidence began emerging that palmitate turnover could be modulated, for example by isoproterenol stimulation of Gαs [11]. In 2002, a seminal study in yeast revealed that S-palmitoylation of Ras2 was catalysed by a zinc-finger motif containing enzyme, now known as a zDHHC-palmitoyl acyltransferase (zDHHC-PAT), named due to a conversed aspartate-histidine-histidine-cysteine motif in the enzyme active site [12,13]. These enzymes are multidomain integral membrane proteins, usually with intracellular N- and C-terminals, the latter of which is often long, poorly conserved and disordered, as well as a conserved ~ 55 amino acid cysteine-rich region between transmembrane domains 2 and 3 [14]. To date, 23 human isoforms have been identified and a study utilising GFP-tagged versions transfected and stained in HEK293 cells revealed distinct localisation patterns throughout the secretory pathway. Interestingly, some zDHHC-PATs show specific subcellular localisation, that is zDHHC5 to the plasma membrane, zDHHC7 and zDHHC8 to the Golgi and DHHC6 and DHHC13 to the endoplasmic reticulum (ER), while others appear in more than one location (Fig. 2) [15,16]. Together, these enzymes regulate the palmitoylation of substrates in every class of protein, in every tissue investigated to date, some of which, as will be discussed, having important physiological and pathophysiological consequences [17].

Palmitoylation can be reversed by slow hydrolysis of the thioester or catalytically by removal of the palmitoyl by soluble acyl thioesterases. Through investigation of H-Ras and Gα depalmitoylation in the 1990s, the first acyl protein thioesterase 1 (APT1) was

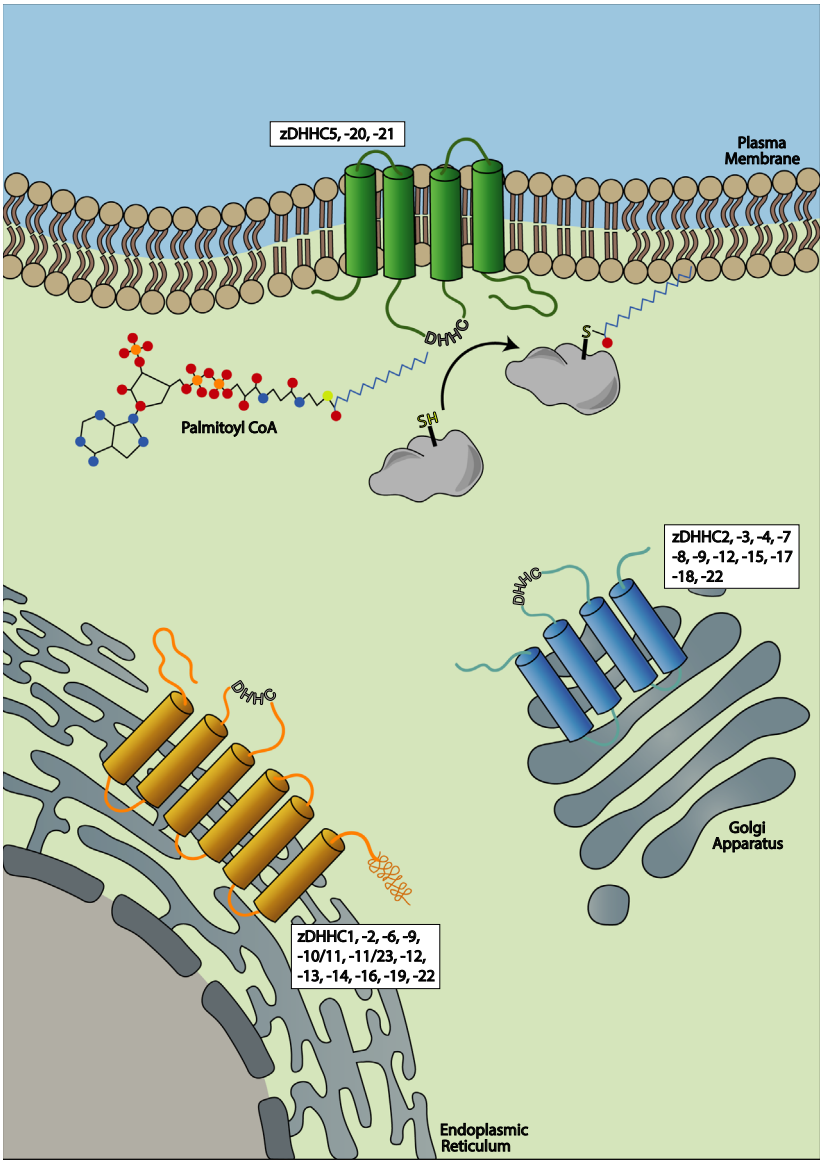

Fig. 2. Localisation of zDHHC-palmitoyl acyltransferases. zDHHC-palmitoyl acyltransferases (zDHHC-PATs) are located throughout the secretory pathway in the endoplasmic reticulum, Golgi apparatus and plasma membrane, with some solely located in one component and others found in multiple locations (for detailed information refer to Ohno, et al. 2006 [15] with updated information from Korycka, et al. 2012 [159]). zDHHC-PATs palmitoylate cysteine sulfhydryl groups (SH) by transferring palmitoyl (C16) from palmitoyl-CoA, often leading to targeting of the palmitoyl and attached target protein to intracellular membranes.

identified. Subsequent bioinformatics analysis revealed the existence of acyl protein thioesterase 2 (APT2), sharing ~ 68% homology to APT1 [18,19]. Both are members of the α/β serine hydrolase family, with active site serines crucial for their enzymatic activity, and are found localised in the cytosol as well as lysosomes [20]. Although thought to be the only method of enzymatic depalmitoylation, in recent years several studies have revealed other acyl thioesterases, such as a family of ABHD17 serine hydrolases that can remove palmitoyls from N-Ras where APT1 and APT2 could not [21], and more recently ABHD10, the first identified mitochondrial localised thioesterase, shedding light on the emerging field of mitochondrial palmitoylation [22].

Palmitoylation as a therapeutic target

The dynamic process of palmitoylating and depalmitoylating target proteins has led to great interest in understand how this PTM changes in disease states, and whether it may be tractable to target using small molecules. Until recently, the three main fields in which research has focussed were cancer, neurosciences and cardiovascular disease (for an overview see Fraser, et al. 2020 [23]).

Cancer

Numerous G proteins must be palmitoylated, both to anchor them to membranes and to segregate them

within microdomains [24]. The best-studied of these in the context of human disease are the small G proteins K-ras, H-ras and N-ras, which between them regulate a diverse array of intracellular signalling networks. A quarter of human cancers are driven by mis-sense mutations in ras isoforms [25], and since ras signalling is intimately linked to its membrane attachment the ras lipid anchors have generated considerable interest. K-ras4a and N-ras are palmitoylated at a single cysteine in their C-terminal hypervariable region, and H-ras at two cysteines in the same region. zDHHC9 has long been known to palmitoylate ras isoforms, and N-ras-mediated oncogenic transformation of cells is impeded in the absence of zDHHC9 [26]. However, promiscuity and redundancy in zDHHC-PAT relationships with their substrates is a common feature in this field: ras isoforms remain palmitoylated and oncogenic in the absence of zDHHC9, and indeed even following genetic deletion of numerous zDHHC-PATs [27]. Experimental strategies that target one individual zDHHC-PAT consequently have a limited impact on palmitoylation of a target protein, and the therapeutic tractability of zDHHC-PATs themselves is therefore questionable. On the other hand, as we develop our understanding of the 'substrate recognition rules' for zDHHC-PATs, small molecules targeting the feature(s) of a palmitoylated protein that are recognised by its zDHHC-PATs offer exciting potential to manipulate the palmitoylation of only that particular protein.

Neurosciences

The neurological phenotypes associated with the various zDHHC-PAT knockout models characterised to date (described below) highlight the importance of palmitoylation in the formation and maintenance of synapses. For example, the importance of the interaction between zDHHC5 and PSD-95 in the postsynaptic density is emphasised by the defects in hippocampal-dependent learning and memory observed in zDHHC5 knockout animals [28]. Palmitoylation influences synaptic plasticity, and importantly, its dysregulation has been associated with Huntington's disease, with zDHHC13 and zDHHC17 also implicated [28,29]. Interestingly, the finding that PSD-95 itself is palmitoylated by zDHHC2 [30] but not by zDHHC5 emphasises the multiplicity of zDHHC-PAT contributions to synapse assembly. Notably, this also highlights that colocalisation should not be assumed to be sufficient to infer an enzyme–substrate relationship between a palmitoylated protein and a zDHHC-PAT.

Given that 41% of synaptic proteins are palmitoylated [31], it is little wonder that mutations in a number of zDHHC-PATs ultimately lead to human neurological disorders [32]. Loss of function of zDHHC9, for example, is associated with X-linked intellectual disability [33] and epilepsy [34], while mutations in zDHHC15 [35] and zDHHC8 [36] are linked to X-linked mental retardation and schizophrenia, respectively. Recent work has established that the ability of zDHHC9 to palmitoylate the small G proteins N-ras and TC10 is a key step in promoting dendritic growth and synapse formation. [37] In the absence of zDHHC9, excitatory synapses dominate, which leads to seizure activity. Ultimately, then this zDHHC-PAT maintains the balance between excitatory and inhibitory neurotransmission in the hippocampus, and both zDHHC9 and its substrates represent candidates for therapeutic intervention in this group of patients.

Cardiovascular system

Palmitoylation in vascular health and disease

Palmitoylation of signalling molecules and ion transporters influences the physiology of both cardiac and vascular tissue. Although relatively few investigations have considered the role of palmitoylation in controlling the behaviour of vascular smooth muscle, our understanding of the importance of palmitoylation in the control of vascular tone has been aided by a number of studies focused on the endothelial cell layer that lines the vasculature, regulating vessel tone and therefore blood pressure. Central to the relationship between endothelial cells and vascular smooth muscle is endothelial nitric oxide synthase (eNOS), which must be palmitoylated in order to localise to caveolae in endothelial cells [38]. Palmitoylation-deficient eNOS produces less nitric oxide (NO) than the wild-type enzyme [39]. Interestingly, palmitoylation of eNOS *in vivo* is reported to be controlled in part by fatty acid availability. Genetic deletion of fatty acid synthase from endothelial cells impairs eNOS palmitoylation and NO production, while insulin resistance and fatty acid synthase downregulation lead to reduced eNOS palmitoylation, less NO production and endothelial dysfuncton [40]. This paradigm that fatty acid availability controls protein palmitoylation *in vivo* has not been widely explored. Given the relative paucity of knowledge regarding the control of zDHHC-PAT activity, it will be interesting to address in the future whether co-localisation of zDHHC-PATs, their substrates and fatty acid/fatty acyl-CoA synthesising

enzymes represents a general mechanism to control protein palmitoylation either globally, or in specific cellular microdomains.

The importance of protein palmitoylation in endothelial cell function was emphasised in a recent study that evaluated the impact of endothelial-specific deletion of the acylthioesterase APT1. Vascular remodelling after hindlimb ischaemia was blunted in the absence of endothelial APT1, a defect rescued by expressing mutant forms of R-ras, in which the hydrophobicity of the hypervariable region containing the palmitoylation sites was modified. Importantly, R-ras palmitoylation was also found to be enhanced by hyperglycaemia, which reduced APT-1 acetylation and impaired its enzymatic activity [41]. The impact of serum/extracellular glucose concentrations on palmitoylation of other APT-1 substrates remains to be determined, but it is an important consideration given most formulations of cell culture media contain supraphysiological glucose. This study also highlights an important aspect of palmitoylation in the regulation of protein function that is sometimes neglected by researchers: the importance of palmitate cycling on a substrate protein for its function [42]. In the case of R-ras, APT1 deficiency led to the accumulation of palmitoylated R-ras, but this did not enhance R-ras-mediated signalling. Other proteins also require palmitate cycling for function [43]. As will be discussed, experimental approaches that offer a snapshot of 'palmitoylation levels' cannot give sufficient insight into palmitoylation cycling on a protein of interest.

Alongside APT1, the PAT zDHHC21 has emerged as a key regulator of endothelial cell function. Although multiple zDHHC-PATs are capable of palmitoylating eNOS, only inhibition of zDHHC21 impairs eNOS-mediated NO production, placing this enzyme as a key nodal point in the regulation of blood pressure by the vascular endothelium [39]. Other endothelial substrates for zDHHC21 include phospholipase β1 and PECAM1, which regulate the integrity of the endothelial barrier and trans-endothelial migration of leucocytes, respectively. Transgenic mice lacking zDHHC21 are remarkably resistant to endothelial dysfunction caused by acute injury [44]. Ultimately then, zDHHC21 regulates inflammation, a process of central importance to a host of acute and chronic diseases, and is an attractive therapeutic target for anti-inflammatory agents.

Palmitoylation in cardiac function

The rhythmic release and removal of calcium from the cytoplasm of ventricular myocytes by a myriad of ion channels and transporters controls contraction and relaxation of the heart. The co-ordinated regulation of these channels and transporters is consequently essential for life, is established to be defective in numerous cardiac diseases and is regulated by dynamic palmitoylation (for an excellent review on this topic, see Essandoh, et al. 2020 [45]). In essence, all direct contributors to cardiac electrical excitability and calcium handling are palmitoylated (Fig. 3), from the voltage gated sodium channel Nav1.5 [46] that initiates the cardiac action potential, to L-type calcium channel subunits that mediate calcium influx into myocytes [47], to calcium reuptake (SERCA/phospholamban [48]) and removal (sodium calcium exchanger, NCX1 [49–55]) mechanisms and the ubiquitous Na/K ATPase [56–59], which maintains ion gradients across the sarcolemma. Palmitoylation of the cardiac ryanodine receptor RyR2, which is central to calcium-induced calcium release from intracellular stores, has not yet been reported, but it is notable that activity of the skeletal muscle isoform RyR1 is enhanced when it is palmitoylated [60].

The cell surface acyl transferase zDHHC5 is the best characterised of the cardiac zDHHC-PATs and is responsible for palmitoylation and regulation of at least two ion transporters in the cardiac sarcolemma. Inactivation of the cardiac sodium/calcium exchanger NCX1, responsible for cellular Ca efflux at the end of the contractile cycle, is controlled by zDHHC5 in cardiac muscle. The NCX1 inactivation process is mediated by a small polybasic region of its large regulatory loop, XIP, which is usually sequestered by the anionic phospholipid PIP_2. PIP_2 hydrolysis leading to the release of XIP leads to efficient NCX1 inactivation only when it is palmitoylated; in essence, palmitoylation sensitises NCX1 to PIP_2 [52,55]. Our understanding of the 'how, when and where' of NCX1 regulation by palmitoylation has expanded considerably recently. NCX1 palmitoylation is promoted by the presence of an amphipathic α-helix on the C-terminal side of the NCX1 palmitoylation site. Substitutions on the hydrophilic face of the helix reduce NCX1 palmitoylation, suggesting this face is recognised by the NCX1 palmitoylating enzymes [53,54]. Supporting this observation, insertion of this α-helix into a nonpalmitoylated protein is sufficient to direct its palmitoylation, but interestingly the same helix also affinity purifies the depalmitoylating enzyme APT1 from tissue lysates – suggesting it is also responsible for recruiting the NCX1 thioesterase [49]. Using FRET probes inserted into the regulatory intracellular loop of NCX1, we found that palmitoylation restructures existing NCX1 dimers to promote NCX1-NCX1 FRET. The structural

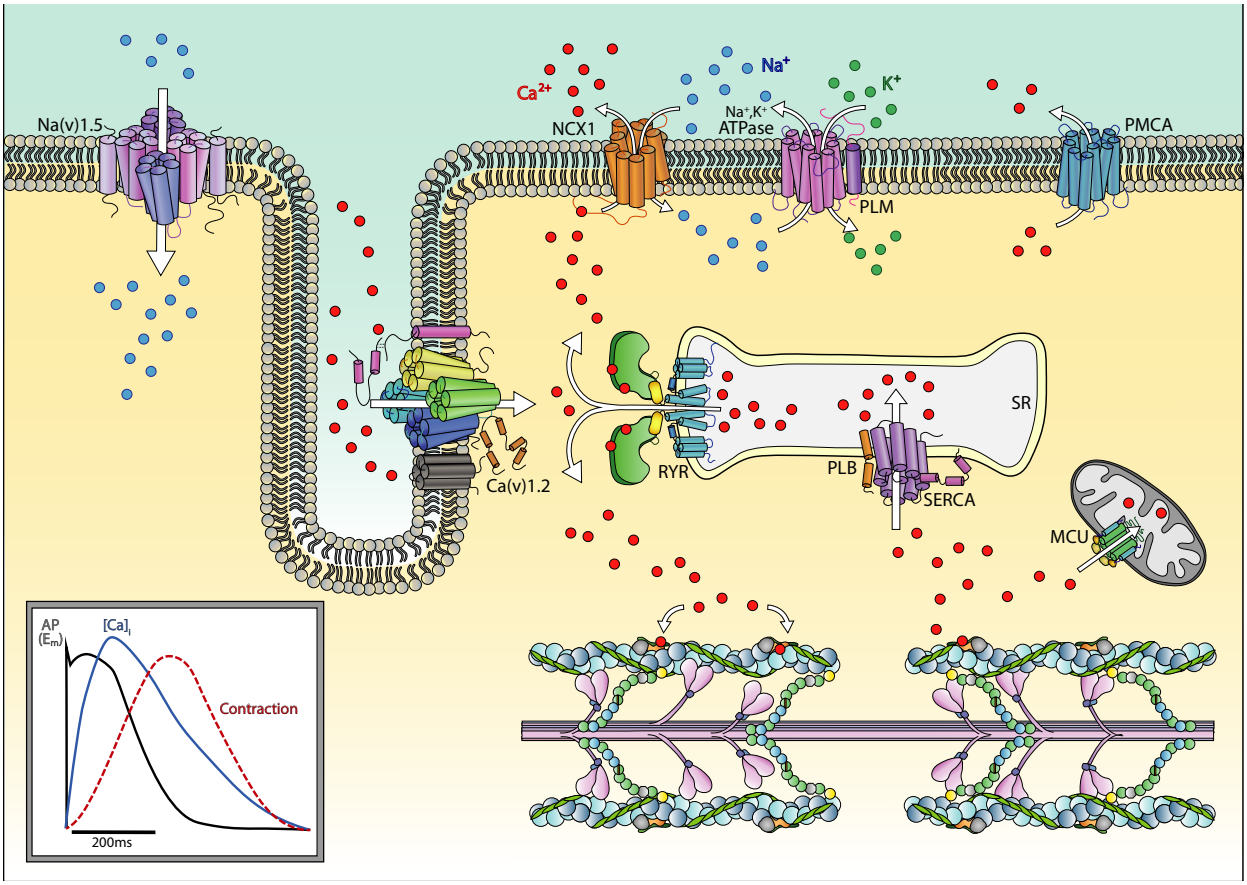

Fig. 3. Cardiac excitation contraction coupling. Arrival of an action potential (AP) at the sarcolemma results in activation of voltage gated sodium channels (Na(v)1.5) located there, allowing sodium to enter the cell. The subsequent increase in depolarisation activates L-type voltage gated calcium channels (Ca(v)1.2) located in the cardiomyocyte T-tubules, specialised invaginations that allow propagation of signal throughout the cell, allowing calcium ions to enter and bind to closely located ryanodine receptors (RYR) on the sarcoplasmic reticulum (SR), the internal calcium store of the cell. This triggers further calcium release from the SR which binds to the contractile machinery of the cell and triggers the sequence of events that will result in shortening of the cell and systole. At the end contraction, calcium is removed from the cell to return it to its resting state, mainly through the sarcoplasmic reticulum Ca^{2+}ATPase (SERCA), controlled by accessory protein phospholamban (PLB) back into the SR, and is also expelled from the cell via the Na^{+}/Ca^{2+} exchanger (NCX1) in exchange for sodium, the activity of which is closely coupled to the activity of the Na^{+},K^{+} ATPase and accessory protein phospholemman (PLM) (PLM). Calcium is also removed from the cell by the plasma membrane Ca^{2+} ATPase (PMCA) and the mitochondrial Ca^{2+} uniporter (MCU), to a lesser extent. Adapted from (Bers, 2002) [160].

rearrangement caused by NCX1 palmitoylation promotes XIP engagement with its binding site close to the NCX1 palmitoylation site, which explains the molecular basis by which palmitoylation promotes NCX1 inactivation [49]. While the physiological role of NCX1 inactivation has remained enigmatic, the finding that NCX1 sensitivity to inactivation is tuned by palmitoylation, and that this ultimately alters transmembrane Ca fluxes and modifies steady-state intracellular Ca [49,51], implies inactivation is a universal means to tune NCX1 mediated calcium fluxes in multiple tissues.

zDHHC5 recruitment and palmitoylation of NCX1 relies on a different amphipathic α-helix, positioned after the zDHHC5 fourth transmembrane domain, which is also the site of interaction of the cardiac Na/K ATPase (NKA) and the zDHHC5 accessory proteins Golga7b [61] and Golga7 [62]. The cardiac NKA is regulated by a small accessory subunit phospholemman (PLM), which is both palmitoylated and phosphorylated in cardiac muscle. Phosphorylation of PLM by protein kinases A and C at serines 63, 68 and 69 activates NKA, while it is inhibited by palmitoylation of PLM by zDHHC5, principally at cysteine 40 [56]. PLM recognition and palmitoylation by zDHHC5 rely on a protein–protein interaction between the NKA enzyme complex and the same amphipathic α-

helix after the fourth transmembrane domain of zDHHC5 that binds to NCX1. Recruitment of zDHHC5 to PLM is mediated by the catalytic NKA α-subunit, whose third intracellular loop interacts with zDHHC5 (raising the possibility that PLM homologues expressed in other tissues are also palmitoylated by zDHHC5 after it is recruited by the NKA α-subunit) [57,63,64]. A proximity biotinylation screen of zDHHC5 recently identified zDHHC20 and O-GlcNAc transferase as zDHHC5 interactors, and post-translational modifications of zDHHC5 by these interactors regulate NKA binding and PLM palmitoylation. zDHHC20 palmitoylates a dicysteine motif (zDHHC5 C236 & C237) immediately on the N-terminal side of the Na/K ATPase binding site to enhance zDHHC5 interaction with the Na/K ATPase and palmitoylation of PLM. O-GlcNAc transferase GlcNAcylates zDHHC5 S241 and also enhances PLM recruitment and palmitoylation [64].

Importantly, zDHHC5 palmitoylation of sarcolemmal substrates such as PLM has been implicated in the pathophysiology of myocardial infarction, which is caused by reperfusion of cardiac tissue after a period of oxygen deprivation. In multiple cell types, including fibroblasts and cardiac myocytes, calcium overload during the reperfusion phase when oxygen is reintroduced leads to mitochondrial redox stress, causing mitochondrial permeability transition pore opening and release of Coenzyme A into the cytoplasm, which is immediately acylated into a substrate for zDHHC5. zDHHC5 palmitoylates plasma membrane proteins, including PLM, causing their clustering in the surface membrane. This triggers the process of massive endocytosis (MEND), in which up to 70% of the cell surface membrane is internalised and is crucial to the damage caused during reperfusion [65]. Proteins preferentially palmitoylated and internalised in this process include ion transporters such as NKA and NCX1, which typically have bulky intracellular regions that can be accommodated in curved membrane domains [52,55] (for which palmitoylated proteins display a high affinity [66]). zDHHC5 and PLM knockout mouse hearts and myocytes show reduced MEND and enhanced functional recovery following anoxia-reperfusion, strongly implicating zDHHC5 as a key mediator in this process [67]. The dependence of this extensive plasma membrane remodelling on acyl-CoA synthesis suggests that fatty acyl-CoA availability is one factor that limits (and therefore controls) zDHHC5-mediated palmitoylation of substrates.

Evidently then, the positioning and post-translational modifications of the NKA and NCX1-interacting 'substrate recognition helix' in zDHHC5 are important regulators of substrate recruitment, and ultimately therefore substrate palmitoylation. A recent study has also established that the enzymatic activity of zDHHC5 can be directly inhibited by phosphorylation of a conserved tyrosine in the same intracellular loop as the zDHHC5 active site [68]. With zDHHC5 subcellular localisation [69,70], acyl-CoA availability [67] and adaptor protein interactions [61,62] also established to regulate substrate recruitment, the complexity of the zDHHC5 upstream regulatory milieu grows ever more convoluted.

Palmitoylation of pathogen and parasite proteins

Understanding the role of palmitoylation in toxins, parasites and infectious diseases is an area of increasing interest, particular due to the recent SARS-CoV-2-mediated pandemic. In terms of toxins, recent evidence implicated zDHHC5 as a central factor in the mechanism of action of anthrax toxin through microdomain partitioning [71], while discovery of a large portion of the *Plasmodium falciparum* palmitome suggests palmitoylation may play a significant role in regulation of its parasitic function [72].

Viruses hijack host machinery to modify their own proteins for the benefit of viral replication, and their ability to use palmitoylation machinery to modify their own viral proteins was first discovered in 1979. Since then, the majority of research has focussed on the role of palmitoylation in enveloped viruses (reviewed by Sobocińska, et al. 2018 [73]), viruses surrounded by a glycoprotein rich lipid bilayer, of which the SARS-CoV-2 virus is classified. Several SARS-CoV-2 proteins are palmitoylated and have been found to associate with zDHHC-PATs using proximity biotinylation, with zDHHC3 and zDHHC13 the most commonly identified in the vicinity of viral proteins [74]. The SARS-CoV-2 spike protein uses its receptor binding domain to bind to angiotensin-converting enzyme 2 receptors and invade host respiratory cells, and therefore, mechanisms to prevent spike function or interactions may be therapeutically beneficial [75]. Interestingly, research several years ago identified spike protein S-palmitoylation in SARS-CoV-1 and investigated four cysteine clusters, some of which were important for spike expression and viral fusion [76,77].

Experimental tools

Techniques to measure protein palmitoylation

Multiple techniques have been developed to investigate protein palmitoylation. Some are capable of providing

a snapshot of steady-state palmitoylation levels in the cell, whereas others can report on palmitoylation dynamics. It is important to be aware of the limitations of each technique and in particular, the questions that cannot be addressed using the techniques available.

Radiolabelling

The palmitoylation field was limited for several years due to a lack of experimental tools. Developed in the 1980s [78], radioactive assays labelling the cellular pool of fatty acids with [^{3}H]-palmitate, followed by purification, electrophoresis and autoradiography, were commonly used in live cells. Although beneficial in allowing pulse-chase identification of palmitoylation turnover, including demonstrating the rapid activation-driven depalmitoylation of H-ras [79], these techniques were limited in the information they could give on palmitoylation stoichiometry, and were often laborious and time consuming. In particular, these experiments typically employed mCurie quantities of [^{3}H]-palmitate and required exposure of dried gels to X-ray films for many weeks. For researchers more accustomed to the rapid turnaround of experiments, for example employing ^{32}P labelling of phosphoproteins, it was common for autoradiographs to come out frustratingly blank unless they lay forgotten for many months in the −80°C freezer. As such, [^{3}H]-palmitate is no longer widely used in the field, but remains the 'gold standard' technique to demonstrate lipidation modifications in general [80]. However, there is potential for incorporation of labelled palmitate into serine/threonine residues via oxyester linkage [81]. Additionally, given the preference of some zDHHC-PATs for fatty acid chain lengths other than 16-carbon and the fact that extended incubation periods with [^{3}H]-palmitate can lead to the labelled palmitate being metabolised and entering different pools of cellular fatty acids, there are weaknesses to this approach that must always be considered [14].

Affinity purification

The chemically inert nature of palmitate and fatty acids in general limited efforts to affinity purify lipidated proteins for many years. The development of the biotin-switch technique, now routinely used to identify numerous cysteine PTMs in both cells and tissues, enabled affinity purification and proteome-level characterisation of palmitoylated proteins using an approach referred to as acyl-biotin exchange (ABE) [82]. This approach relies on alkylation of free cysteines (most commonly with N-ethylmaleimide) under denaturing conditions, specific cleavage of the thioester bond between palmitate and the palmitoylated cysteine (most commonly with neutral hydroxylamine), reaction of the newly revealed cysteines with a sulfhydryl-reactive biotinylation reagent and finally affinity purification of biotinylated proteins using streptavidin beads (Fig. 4).

In a similar vein, the technique of acyl-resin-assisted capture (Acyl-RAC), a refinement of ABE, dispensed with the biotinylation step and instead combined the thioester cleavage and capture steps utilising 2-thiopyridyl disulphide-containing thiopropyl Sepharose beads (Fig. 4) [83]. Both Acyl-RAC and ABE are widely used as they allow direct assessment of the fraction of a particular protein in a cell or tissue that is palmitoylated – by comparing the degree of enrichment of that protein compared to the initial unfractionated cell lysate. Both techniques are labour-intensive however, with clean-up steps (either precipitation or desalting) required after cysteine alkylation and cysteine biotinylation. A refinement, in which cysteine alkylation is followed by chemical scavenging of N-ethylmaleimide using 2,3-dimethyl-1,3-butadiene in an aqueous Diels-Alder 4 + 2 cycloaddition reaction, removed the need for any clean-up steps but has not yet been widely adopted [84].

All these techniques efficiently purify all palmitoylated proteins from a cell lysate providing an accurate 'snapshot' of steady-state palmitoylation levels in the cell, but are limited in that they cannot provide information on palmitoylation/depalmitoylation dynamics, nor are they capable of detecting changes from singly to multiply palmitoylated states. Two factors, achieving complete alkylation of free cysteines and the specificity of the thioester cleavage step, are central to the success of this approach. To avoid false positives, it is important to consider the nature of the protein of interest. Proteins containing multiple disulphides evade initial alkylation, leaving them free to disulphide exchange with the thiopyridyl disulphide group, and therefore, an initial reducing step utilising 1,4-Dithiothreitol (DTT) or tris(2-carboxylethyl)phosphine (TCEP) can prevent this. In terms of hydroxylamine specific cleavage, conducting duplicate assays in which hydroxylamine is omitted from one reaction can confirm specificity. It is also important to be aware that hydroxylamine-dependent affinity purification of a protein is evidence that a thioester bond exists in that particular protein, which is only indirect evidence that it is palmitoylated. Thioesters are common in active site cysteines, for example GAPDH [85], and universal to E3 ubiquitin ligase enzymes [86], and these may be

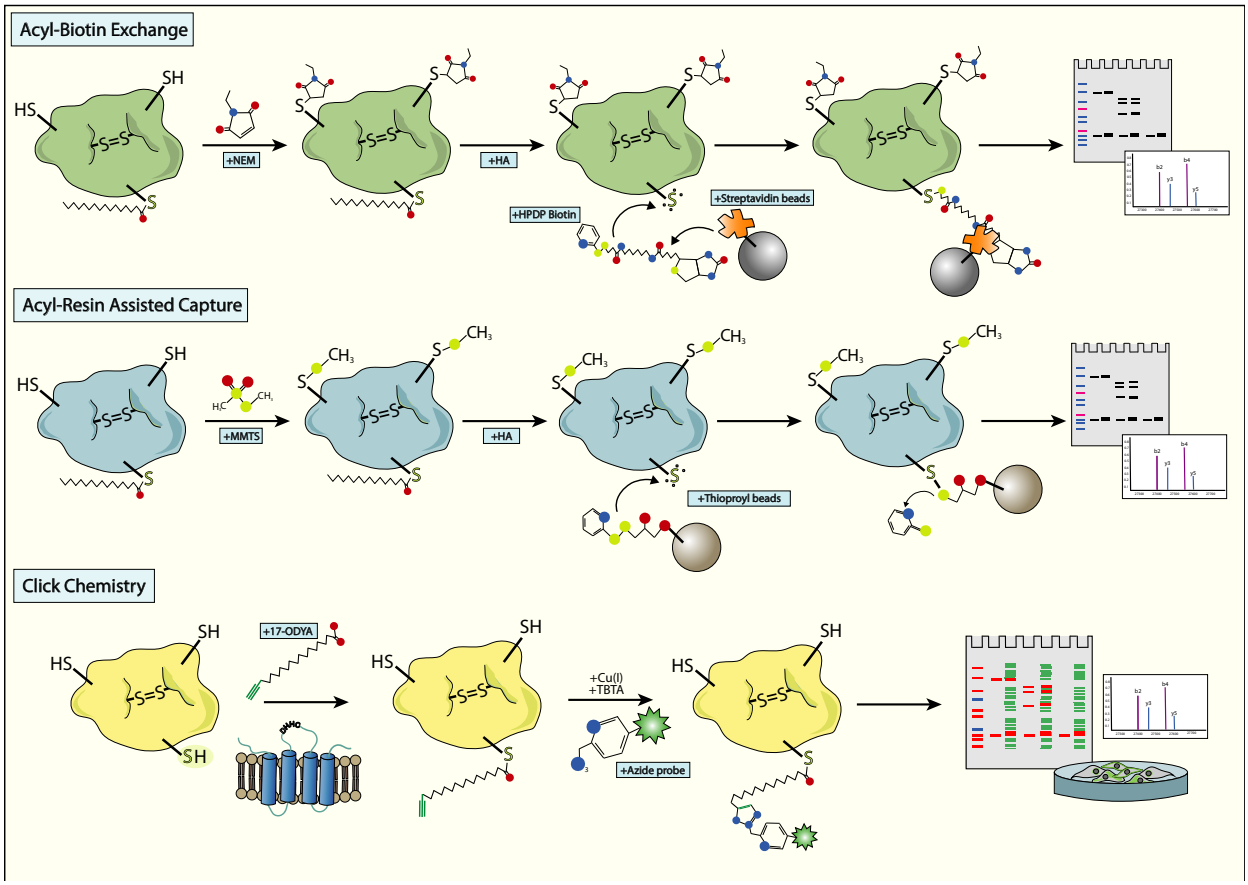

Fig. 4. Nonradiolabelled methods of detecting protein palmitoylation. In an effort to provide safer alternatives to radioactive palmitoylation assays, purification methods such as acyl-biotin exchange (ABE) and Acyl-RAC-assisted capture (Acyl-RAC) and click chemistry-based detection techniques have been developed. ABE and Acyl-RAC involve alkylation of free cysteines with either N-ethylmaleimide (NEM) or methylation with S-methyl methanethiosulfonate (MMTS), followed by cleavage of thioesters at acylation sites with hydroxylamine (HA). For ABE, this is followed by incubation and conjugation of a HPDP-biotin molecule to the newly freed cysteine, and purification with streptavidin containing beads of agarose, whereas for Acyl-RAC, the freed cysteine can be immediately conjugated to thiopropyl sepharose containing beads. By comparing initial lysate (unfractionated), percentage palmitoylation of any proteins in the sample can be determined by SDS-PAGE. Click chemistry utilises the cells palmitoylation machinery to conjugate an alkyne-containing fatty acid (commonly 17-ODYA to detect palmitoylation), followed by incubation with an azide-containing click chemistry probe, with a suitable detection system attached (biotin or fluorescence), which can then be analysed via SDS-PAGE or fluorescence microscopy. All three techniques can be combined with mass spectrometry to give unbiased, comprehensive indication of palmitoylated proteins.

purified in an Acyl-RAC reaction even if no palmitoylation occurs.

Antibody-based approaches

Although both affinity purification techniques allow evaluation of the acylated fraction of a protein, they cannot distinguish between fatty acid chain length and detect palmitoylation exclusively. As such, there has been attempts to generate specific antibodies that detect protein palmitoylation, but these have been largely unsuccessful due to the difficultly in raising an antibody against the hydrophobic modification. Indeed, there are very few examples of antibodies specific to cysteine post-translational modifications being successfully produced (although see [87]), and despite extensive efforts we have failed to develop such tools. A recent study reports a pan-palmitoylation antibody for the detection of the palmitoylated fraction of a protein, but this has yet to be widely trialled or adopted [88].

Cysteine PEGylation

A useful adaptation of the ABE/Acyl-RAC assay is to use cysteine-reactive molecules to induce a molecular

weight shift after the hydroxylamine cleavage step. Rather than allowing capture of palmitoylated proteins, this causes a 'laddering' of the protein of interest, detected using SDS-PAGE and western blot [63,64]. The extent of the laddering is dependent on the number of palmitoylation sites occupied in the protein, and the stoichiometry of palmitoylation. Commercially available cysteine-reactive PEG maleimides inducing a 5 kDa or 10 kDa molecular mass shift are commonly used. In our hands, application of this technique is limited to proteins that migrate on SDS-PAGE as single discrete bands smaller than 100 kDa in size. Despite this limitation, PEGylation is the only reliable method to count palmitoylation sites in proteins, and to detect transitions between singly and multiply palmitoylated states.

Click chemistry

Driven by an effort to steer away from radioactivity methods, the development of chemical probes for the detection and characterisation of palmitoylated proteins using click chemistry was a significant advancement in the field, and remains a widely used method (reviewed in Gao and Hannoush, 2018 [89]). The first use of this technique involved an array of azido-containing fatty acids of different carbon lengths, which can metabolically incorporate into appropriate acylation sites (commonly 17-octadecyonic acid (17-ODYA) for palmitoylation [90]). This is followed by Cu(I)-catalysed [3 + 2] Huisgen cycloaddition or 'clicking' to a suitable alkyne-containing detection system (biotin or a fluorophore) and analysis using electrophoresis or fluorescence microscopy (Fig. 4) [91]. Both azido and alkyne versions of the probes exist, representing a host of lipid modifications, and in terms of palmitoylation have been crucial in identifying novel palmitoylated proteins, confirming sites of modification and aiding in the understanding of fatty acid selectivity, where probes of carbon length 14–18 and zDHHC-PAT preference were characterised and manipulated through mutagenesis [92]. Indeed, adoption of click chemistry has led to significant developments across many fields. For example, the azido-palmitic acid probes revealed the functional role of palmitoylation in tumour necrosis factor alpha [93] and epidermal growth factor receptor [94] signalling (facilitated through zDHHC20) [95], which may lead to new therapeutic approaches for K-ras-positive lung cancer.

However, there are limitations to this technique that must be considered, including that electrophoresis of whole-cell click-chemistry experiments may limit the detection of proteins palmitoylated with low stoichiometry, and therefore may require additional purification steps or coupling with more sensitive, yet costly, proteomic analysis, to yield clear results. Our own unpublished experience with these probes is that certain cell types, cardiac myocytes in particular, do not consistently incorporate them into the palmitoyl proteome. The existence of a rigid cell wall in plant cells also impairs probe uptake and incorporation. Additionally, the commonly used 17-ODYA presents several experimental caveats which may limit use, as it has been reported to nonspecifically label N-myristoylation sites (likely involving the incorporation of metabolic products from 17-ODYA degradation into these sites [90]), as well be incorporated into glycosylphosphatidylinositol moieties of proteins [96]. With an ever-growing array of probes available, it is important to also consider the functional consequences of the azido/alkyne addition to the hydrophobicity of the fatty acid and therefore its affinity for the membrane and representation of endogenous acylation. Fortunately, these probes are extensively characterised (reviewed by Hannoush and Sun, 2010 [97]) with study suggesting that alkyne labelled fatty acids may incorporate more effectively into proteins [98], and then, azide detection systems may have a greater efficiency [99]. As with affinity purification, precautions such as confirming the specificity of the reaction with neutral hydroxylamine should be considered.

Nevertheless, click chemistry is a valuable tool in a palmitoylation researcher's arsenal, as where affinity purification techniques cannot provide information on palmitoylation turnover, click chemistry can be utilised through control of probe incubation [100], and is reported to produce a much lower proportion of false positive compared to ABE [90]. The combined use of these techniques when investigating any novel palmitoylated protein appears crucial, as recent evidence suggests the estimated stoichiometry of protein palmitoylation can vary between techniques [101]. Currently, click chemistry is limited to analysis of lysed or fixed cells and its development in the field of live cell and *in vivo* imaging would be invaluable, allowing spatiotemporal tracking of palmitoylated proteins, significantly enhancing our understanding of this dynamic modification. Nonetheless, recent advancements in coupling click chemistry with high and even superresolution microscopy [102] and its use in the generation of a high throughput small molecule screen for palmitoylation modulators [103] is promising. Additionally, click chemistry, as well as affinity purification techniques, are becoming more accessible and applicable

to scientific projects due to the researcher-led development of commercial assay kits [104].

Advances in quantitative proteomics

The above techniques can be successfully used to confirm selected, candidate S-acylated proteins using western blotting as an endpoint. However, by combining with mass spectrometry (MS)-based proteomics techniques, an unbiased, comprehensive list of acylated proteins can be elucidated from a number of cell and tissue types. This technique aided in the first substantial global profiling of palmitoylated proteins in yeast [27], whereby tandem MS analysis after purification in the presence and absence of hydroxylamine identified a host of novel S-acylated proteins. Additionally, adapting a quantitative approach for MS analysis allowed consideration of the relative abundance of detected peptides between duplicate purifications conducted in the absence and presence of hydroxylamine. This is an important refinement as it limits the number of false positives and negatives (proteins / peptides purified in the absence of hydroxylamine) [105]. Given the exceptional sensitivity of MS-based approaches, we caution researchers to always consider whether a biologically meaningful fraction of their protein of interest is palmitoylated. The presence of a particular protein in a list of several thousand proteins identified by MS is no guarantee that palmitoylation of the protein in question merits investigation. Spurious palmitoylation sites and false positives are often observed [106], less frequently reported.

A significant advantage afforded by the MS-based approach is the opportunity to gain information about palmitoylation sites. Broadly speaking, there are two ways to achieve this. Tryptic digestion immediately before or immediately after affinity purification using acyl-RAC or ABE will capture only palmitoylated peptides for MS detection. Using this approach, all peptides detected will contain cysteines, so for those peptides containing a single cysteine the palmitoylation site is identified. Using acyl-RAC and ABE this technique identified 84 and 168 palmitoylation sites, respectively, in affinity-purified peptides from mammalian cells [83,107]. Alternatively, a differential alkylation approach can be used, in which the initial cysteine alkylation/block step (Fig. 4) employs one alkylating agent, and previously palmitoylated cysteines are alkylated with a different reagent (or left in the reduced state) after capturing intact proteins by acyl-RAC or ABE. To successfully identify palmitoylation sites, the differential alkylation approach requires good protein coverage in the MS analysis. The most comprehensive example of this, which identified 906 palmitoylation sites in mouse forebrain [108], represents an important resource for the field.

Quantitative MS was first used to investigate the extent of palmitoylated substrates in neurons, where seminal work demonstrated dramatic remodelling of the synaptic palmitome associated with neuronal activity, [109] and opened the door to a renaissance for the field of dynamic palmitoylation. Acyl-RAC and ABE have now catalogued tissue and cell palmitomes in multiple tissues (curated in the SwissPalm database [8]), as well as assisting in the identification of enzyme/substrate pairs. For example, label-free MS analysis of palmitoylated proteins prepared using acyl-RAC from the liver in the presence and absence of zDHHC13 (which is associated with abnormal liver function) identified substantial remodelling of the palmitome caused by the absence of a single PAT [110]. In fact, as well as identifying novel palmitoylated proteins, this technique has been applied to identify substrates of numerous zDHHC-PATs in the setting of cancer [111] and neuronal development [112].

Quantitative proteomics are not just been restricted to acyl-RAC and ABE, with several click chemistry probes available to combine pulse-chase methods with MS techniques [89]. The first such study used Stable Isotope Labelling by Amino acids in Cell culture (SILAC) in combination with 17-ODYA labelling to reveal the global palmitoylation dynamics in mouse T-cell hybridoma cells [113]. Not only did this investigation aid in our understanding of palmitate cycling on heterotrimeric and small G proteins such as H-ras and N-ras, it also identified chemical tools to probe the behaviour of depalmitoylating enzymes. The insights into palmitoylation dynamics afforded by applying quantitative MS in combination clickable palmitate analogues are consequently formidable. For those interested in cellular dynamics and signalling events, being able to visualise the global turnover the palmitome supports easy identification of proteins of interest.

Tools to pharmacologically manipulate protein palmitoylation

Once palmitoylation of a target protein is established, altering its palmitoylation pharmacologically in order to understand elucidate the functional consequences is beneficial. Similarly to the development of detection methods, this field was limited for many years due to lack of suitable tools and only now are more robust methods beginning to replace more crude techniques once employed.

Inhibiting zDHHC-palmitoyl acyltransferases

As will be discussed, zDHHC-PATs can show some form of redundancy, limiting the information gained from individual knockout models on their function and any subsequent change in substrate behaviour. Pharmacologically inhibiting a select zDHHC-PAT for shorter periods of time would be informative; however, attempts to make zDHHC-PAT specific inhibitors, targeting the active site, have not been fruitful thus far. This is due to the lack of structural understanding of these integral membrane proteins, including difficultly in resolving high resolution structures. However, this was recently achieved for both zDHHC15 and zDHHC20 [114], in part through utilisation of the general zDHHC-PAT inhibitor 2-bromopalmitate (2-BP). Often referred to as a 'suicide inhibitor', 2-BP, which is thought to irreversibly alter the zDHHC active site cysteine through nucleophilic displacement and alkylation, has been widely used to determine the effect of reducing palmitoylation on proteins of interest [115]. However, there are several caveats to its use including poor potency and bioavailability, which limits translational studies, and several groups have now documented its extensive off-target effects, suggesting up to 99% of its targets are not zDHHC-PATs [116]. Additionally, its exact mechanism of action is still unknown. Some of its cellular effects are caused by altering lipid metabolism, and it has long been known to inhibit mitochondrial fatty acid oxidation independent of any influence on zDHHC-PATs [117]. This is supported by mass spectrometry suggesting it does not favour zDHHC-PATs over other proteins [116,118,119]. Interestingly, it may also paradoxically inhibit depalmitoylation through APT1 [120]. Despite this, it is still widely used due to lack of suitable alternatives, frequently at concentrations above its IC_{50} of 10μM [115], and therefore, caution must be applied in concluding any functional effects from its use.

Additionally, lipid-based alternatives such as antibiotics tunicamycin, which has been shown to inhibit the palmitoylation of calcium channels [121] and presynaptic plasticity protein GAP-43 [122], and cerulenin, which has been reported to inhibit palmitoylation of fatty acid uptake channel CD36 [123], can be used. However, again these remaining relatively uncharacterised in terms of mechanism of action and off-target effects. As will be discussed, more recent attempts to identify inhibitors have utilised high throughput screening, including pharmacological control over DHHC-PAT active site autopalmitoylation although their broader effectiveness still remains to be determined [124].

Inhibiting acylthioesterases

Another route through which palmitoylation can be influenced is through inhibiting depalmitoylation. As the first described acylthioesterase, efforts to find an inhibitor of APT1 lead to the development of palmostatin-B, through comparing structural similarities of APT1 to gastric lipase and basing a compound screen on features of an inhibitor of the latter [125]. Palmostatin-B reversibly inhibits and inactivates APT1, and inhibited depalmitoylation of H-ras and N-ras leading to loss of membrane and Golgi localisation [125]. The specificity of this particular tool compound for APT1 is questionable, given the finding that palmostatin B but not ML-348 inhibits depalmitoylation of N-ras [21]. It is now generally accepted that palmostatin B acts as a rather broad spectrum thioesterase inhibitor, targeting APT1 and APT2 as well as serine hydrolases in the ABHD family [21]. ML-348 specifically targets APT1, and ML-349 inhibits APT2 [126]. Given the *in vivo* bioavailability of these compounds it is perhaps surprising that to date relatively few studies have used them to specifically target depalmitoylation in animal models. For palmostatin-B, this again could be largely due to reported off-target effects [21,127] and poor drug-like properties resulting in lack of stability [18]. ML348 and ML349 often report more specific inhibiting activity evidenced by results from siRNA experiments [128] and show more promise in their use *in vivo* [126,129].

Novel approaches

Although inhibitors targeting the active site of zDHHC-PATs and acylthioesterases would no doubt provide valuable research tools, each individual enzyme can have several varied targets in a cell or tissue and specifically targeting the interaction between zDHHC-PAT/acylthioesterase and substrate may be more therapeutically relevant. Although, as will be discussed, the recognition rules for zDHHC-PAT and substrate are still undetermined, evidence suggests unique features such as SH3 regulatory domains or ankyrin repeats may play a role. This was elegantly demonstrated over a decade ago with Golgi-localised zDHHC17 whereby fusion of its ankyrin repeat domain to zDHHC3 enabled it to interact and palmitoylate zDHHC17 substrates [130]. Additionally, we have shown recently that it is possible to selectively disrupt the zDHHC5-mediated palmitoylation of PLM through blocking the aforementioned direct interaction of the PAT with NKA. Using peptide array technology, we identified the key residues of zDHHC5

involved in interaction with the NKA and designed a stearate-tagged cell-penetrating peptide disruptor which reduced PLM palmitoylation in both HEK293 and cardiac myocytes [64]. Being able to selectively disrupt palmitoylation of PLM to increase NKA activity may represent a novel treatment strategy for heart failure and cardiac hypertrophy [57]. Of course, peptide disruptors are limited therapeutically due to their often poor bioavailability and pharmacokinetic properties, but may pave the way to understanding how to produce small molecule drugs to target these interactions specifically, and provide powerful tools for understanding palmitoylation mechanisms.

Additionally, several groups have investigated the use of classic and novel compounds to drive an increase or decrease in palmitoylation. The simplest possible example of this is palmitate supplementation, which enhances the palmitoylation status of some proteins in cell culture [46,49]. However, the broad applicability of this approach to all cell types and *in vivo* is questionable. Fatty acid excess, for example following high fat diet, has been found to drive reduced palmitoylation of H-ras as a result of mitochondrial redox stress [131]. In the presence of reactive oxygen species cysteines that are depalmitoylated can be reversibly or irreversibly oxidised, reducing the palmitoylation of the protein of interest in the presence of excess palmitate.

A well-established technique to determine the effect of loss of palmitoylation of individual substrate function is through site-directed mutagenesis, most commonly of the palmitoylated cysteine to an unpalmitoylatable alanine. However recently, this approach has been used to produce 'gain of function' palmitoylation mutants, including a novel 'lipidation mimic' strategy which replaces the cysteine with the most hydrophobic amino acid, tryptophan, in order to mimic fatty acid attachment, which was able to partially rescue the phenotype of an alanine mutant [132]. A recently published refinement of this approach used unnatural amino acids (i.e. those formed from a codon sequence not commonly used by cells, e.g. TAG) whereby the amino acid is genetically introduced, with either a strained alkyne/acyl-modified azide attached, in replace of the cysteine. This amino acid can then be conjugated in an inverse-electron-demand Diels-Adler cycloaddition reaction to a fluorescent detection system in live cells, providing a very powerful tool to understand the functional effect of increased palmitoylation on individual proteins [133] Although this is a significant advance, especially due to lack of pharmacological compounds, mutation of palmitoylated cysteines may have unintentional consequences on other cysteine modifications such a redox modifications S-glutathionylation and S-nitrosylation, as it is typically solvent exposed cysteines that participate in palmitoylation and are thus open to additional post-translational modifications [134].

On the other hand, methods to enhance depalmitoylation are also emerging. Using a cell penetrating, amphiphillic compound, in order to optimise targeting to the membrane, Rudd, et al. were able to show depalmitoylation of H-ras *in vitro*. Importantly, use of the compound in an *in vivo* model of infantile neuronal ceroid lipofuscinosis (a degenerative disease caused by mutations in the gene encoding depalmitoylation enzyme palmitoyl-protein thioesterase 1 (PPT1)) showed that after treatment, once mislocalised proteins, including GAP-43, were reduced. In theory, this approach allows an understanding of global depalmitoylation without altering other functional aspects of these proteins or the palmitoylation machinery [135].

The usefulness of *in-vivo* transgenic models to study protein palmitoylation

Numerous transgenic animal models are now available to investigate the importance of protein palmitoylation in physiology and pathology. Perhaps reflecting the redundancy of the zDHHC-PAT family, zDHHC knockout animals reported to date are generally viable and display relatively modest phenotypes (with the notable exceptions of zDHHC8 [136] and zDHHC16 [48]). Several of these models exhibit behavioural defects, reflecting the functional diversity and importance of synaptic zDHHC-PAT substrates. For example, zDHHC17 knockout impairs glutaminergic synapse function, leading to defects in hippocampal memory and synaptic plasticity [137], and knockout of zDHHCs 3, 5, 11, 23 and 24 all cause behavioural and/or neurological defects, according to the International Mouse Phenotyping Consortium Database.

The Golgi-localised zDHHC-PATs zDHHC3 and zDHHC7 are widely acknowledged to act as 'high capacity, low specificity' palmitoylating enzymes in the Golgi apparatus [138], where they share overlapping substrates, for example heterotrimeric G protein alpha subunits [139]. Interestingly, palmitoylation of the zDHHC3 substrates GABA-A receptor γ2 subunit and GAP43 was modestly reduced but not abolished in zDHHC3 knockout animals [140], suggesting that other zDHHC-PATs are not capable of fully substituting for zDHHC3. This emphasises that knockout models have merit to identify enzyme/substrate relationships between zDHHC-PATs and palmitoylated proteins, albeit not offering a 'black and white'

picture as a result of the functional redundancy between zDHHC-PATs. Importantly, dual knockout of zDHHC3 and zDHHC7 causes perinatal lethality [140] in the majority of animals, confirming the concept of overlapping substrate specificities for these enzymes.

The functional redundancy of zDHHC-PATs remains a theme in other investigations. For example, acute siRNA-mediated knockdown of multiple zDHHC-PATs reduces large conductance calcium and voltage-activated potassium channel palmitoylation [141], but no single zDHHC-PAT is solely responsible for palmitoylating this channel. Similarly, CRISPR-mediated zDHHC5 knockout reduces but does not abolish palmitoylation of NCX1 [49], while thirteen different zDHHC-PATs were found to be capable of palmitoylating Gαi3 in an overexpression study [142]. Evidently, substrates are promiscuous in their choice of zDHHC-PATs, and there remains much work to be done to understand the 'recognition rules' that apply within these relationships. Promiscuity appears to be the rule whether a protein is a peripheral or integral membrane protein. However, concept of 'local palmitoylation' for integral membrane proteins has recently been proposed in which an important governing factor in the enzyme/substrate relationship is co-localisation of zDHHC-PAT and substrate [143]. It is certainly conceivable that palmitoylated integral membrane proteins that are generally confined to one subcellular compartment during their functional lifetime may be palmitoylated by a small number of zDHHC-PATs in their 'home' compartment (e.g. NCX1 by zDHHC5), while being palmitoylated by other enzymes during their passage through the secretory pathway.

Future challenges

Substrate recognition rules

SwissPalm, a curated database of palmitoylated proteins, which includes known palmitoylated proteins, known palmitoylation sites, and enzyme/substrate relationships for palmitoylating and depalmitoylating enzymes is a key resource for this field [8]. As enzyme/substrate pairs are established and this database matures, the opportunity to improve our understanding of zDHHC-PAT relationships with their substrates will expand. To date, palmitoylation site prediction algorithms based on palmitoylation sites identified using proteomics have failed to offer significant insight into the rules underlying substrate recognition. Substrate recognition rules for protein kinases based on whole phosphoproteomic data would yield little value, so it is little wonder that whole palmitome analyses has failed here. We also suggest that the use of scoring matrices based on the position of a particular palmitoylated cysteine relative to nearby amino acids is not a sensible approach. Palmitoylation of NCX1, for example, is completely unaffected when a single amino acid is inserted between the palmitoylated cysteine and the amphipathic α-helix recognised by the NCX1 zDHHC-PAT [54]. Claudin-3 palmitoylation can occur at multiple positions within its transmembrane region [144]. In other words, proximity but not absolute position relative to a 'palmitoylation signal' in a protein dictates cysteine palmitoylation: the positional scoring matrices that served kinase biology so well are no use for palmitoylation.

Where the palmitoylation field could learn some tricks from the protein phosphorylation field is in the rational design of derivatised substrates for engineered zDHHC-PATs – the so-called 'bump-hole' strategy. Pioneered by Kevan Shokat, this approach engineers the kinase active site to accept an ATP derivative that cannot be accommodated by wild-type kinases [145]. Use of this modified ATP by the kinase tags its substrates for affinity purification and identification. Our understanding of the structure of zDHHC-PATs [14], and the way that the fatty acid substrate is accommodated within the transmembrane domains [92] means that engineering zDHHC-PATs that can accept a modified substrate is feasible. This would allow us to definitively identify zDHHC-PAT substrates in multiple cell types, paving the way for a deeper understanding of the enzyme/substrate relationships, and offering the potential to pharmacologically target these relationships.

Accessory Proteins and zDHHC-PAT regulatory pathways

As we strive to further characterise zDHHC-PATs, it is increasingly important to consider the influence of accessory proteins, as they may provide novel routes of targeting [146]. This has long been known from study of zDHHC9, where co expression with accessory protein GCP16 is essential to its activity [147]. Targeting this relationship may therefore offer an opportunity to impair palmitoylation of ras isoforms. The example of zDHHC5 is also relevant here. In some model systems, this PAT relies on the accessory protein Golga7 [62] (the same protein as GCP16); in some model systems, Golga7b [61] is present, and in other systems substrates bind directly to the same region of zDHHC5 as these accessory proteins [49,64]. The presence and absence of these proteins, their homologues

and the activity of enzymes that post-translationally modify substrate binding sites all contribute to zDHHC5 regulation. We suggest that proximity biotinylation, which successfully identified zDHHC20 and O-GlcNAc transferase upstream of zDHHC5, has significant potential for unpicking zDHHC-PAT regulatory pathways, as well as identifying substrates [64]. In the case of several PATs, it is impossible to detect a physical interaction with substrate using either affinity purification or genetic screens [138]. We therefore propose that BioID and APEX not only offer the best way forward to identify zDHHC-PAT interaction networks, but also to determine how these interaction networks remodel during signalling and disease, although it is important to consider that co-localisation or direct interaction of zDHHC-PAT and substrate does not always infer substrate modification (e.g. PSD-95 colocalises with zDHHC5 [28], but is not palmitoylated by this enzyme [148]).

Importantly, zDHHC-PATs themselves are subject to a host of PTMs, which adds to the complexity of S-acylation as a modification. Although auto-acylation of the active site cysteine is generally thought to be an intermediate step in zDHHC-PAT catalysis, [149] additional S-palmitoylation has been reported to regulate the activity of several zDHHC-PATs. S-palmitoylation of zDHHC13 is reduced in a model of Huntington's disease and limiting the presence of its substrate, the huntingtin protein, decreases its enzymatic activity *in vivo* [150]. As mentioned, proximity biotinylation revealed palmitoylation of zDHHC5 by zDHHC20, which has knock-on effects on sodium pump recruitment [64]. Additionally, several studies have shown the interplay between S-palmitoylation and phosphorylation of transporters [151], ion channels [152] and regulatory proteins [56], and there is now increasing evidence of phosphorylation-mediated control of zDHHC-PAT function. Of note, LYN kinase-mediated phosphorylation in the intracellular loop of zDHHC5 that contains the active site leads to its inactivation, ultimately regulating CD36 palmitoylation in a feedback loop mechanism [68]. Phosphorylation of the zDHHC5 C tail by the tyrosine kinase Fyn regulates its subcellular localisation (and consequently substrate interactions) in synapses [153]. Similarly, limiting zDHHC3 tyrosine phosphorylation leads to increased auto-acylation and substrate palmitoylation, with implications for neuronal development [154].

Aside from PTMs, a few studies are beginning to highlight the influence of microRNAs on zDHHC-PAT activity. Expression zDHHC21, of which the serotonin-1A receptor is a substrate, is negatively regulated by miR-30e, levels of which are increased in the postmortem brains of patients with major depressive disorder with corresponding loss of serotonin-1A palmitoylation [155]. Similarly, miR-134, a key modifier in synaptic plasticity, was shown to regulate zDHHC9 with potential knock-on effects for its substrate H-Ras [156]. Clearly then, transcriptional control of zDHHC-PAT abundance, as well as post-transcriptional and post-translational mechanisms all require further investigation.

Better experimental tools

As mentioned above, the reliance of the palmitoylation field on the compound 2-bromopalmitate (2-BP) remains a significant barrier. Ultimately, we suggest that the lack of specificity of this reagent risks undermining progress. While we acknowledge that pharmacological inhibitors of zDHHC-PATs are lacking, we urge caution using 2-BP. This reagent is only suitable to use alongside more specific genetic approaches to impair palmitoylation of a protein of interest (e.g. zDHHC-PAT silencing, palmitoylation site mutagenesis). Even if 2-BP were to be performing precisely as intended (as a global palmitoylation inhibitor), the value of experiments in which the entire cellular palmitome is remodelled is questionable. Given the well-established off-target effects, alternative reagents are urgently required. Two recent high-throughput screens measuring palmitoylation of dual leucine-zipper kinase [157] and ras [103] as endpoints have highlighted that zDHHC-PATs may be tractable to inhibition by small molecules in compound libraries. Similarly, a high-throughput screen for inhibitors of yeast zDHHC Erf2 revealed compounds that could inhibit PAT autopalmitoylation and Ras-dependent growth [158]. While the conservation of the zDHHC-PATs makes it unlikely that isoform-specific zDHHC-PAT inhibitors targeting the active site can be found, alternative approaches may bear fruit. The recent report that substrate recruitment can be manipulated offers hope that individual substrates could be targeted. If there are isoform-specific differences in palmitoyl-CoA recruitment by the different zDHHC-PATs, this may offer another route to pharmacologically target different PAT families.

Overall, although there are still many areas to address, there is no doubt that the study of palmitoylation has advanced significantly in recent years. With constantly improving pharmacological options, experimental models and understanding of palmitoylation enzymology, our knowledge of the therapeutic relevance of the modification, and the novel ways to by which it can be modulated, will only increase.

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Author contributions

Alice Main wrote original draft. William Fuller reviewed and edited the manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

References

1 Walsh CT, Garneau-Tsodikova S & Gatto GJ (2005) Protein posttranslational modifications: The chemistry of proteome diversifications. *Angewandte Chemie – International Edition* **44**, 7342–7372.
2 Folch J & Lees M (1951) Proteolipides, a new type of tissue lipoproteins; their isolation from brain. *J Biol Chem* **191**, 807–817.
3 Haucke V & Di Paolo G (2007) Lipids and lipid modifications in the regulation of membrane traffic. *Curr Opin Cell Biol* **19**, 426–435.
4 Sezgin E, Levental I, Mayor S & Eggeling C (2017) The mystery of membrane organization: Composition, regulation and roles of lipid rafts. *Nat Rev Mol Cell Biol* **18**, 361–374.
5 Levental I, Grzybek M & Simons K (2010) Greasing their way: Lipid modifications determine protein association with membrane rafts. *Biochemistry* **49**, 6305–6316.
6 Udenwobele DI, Su RC, Good SV, Ball TB, Shrivastav SV & Shrivastav A (2017) Myristoylation: An important protein modification in the immune response. *Front Immunol* **8**, 751.
7 Wang M & Casey PJ (2016) Protein prenylation: Unique fats make their mark on biology. *Nat Rev Mol Cell Biol* **17**, 110–122.
8 Blanc M, David F, Abrami L, Migliozzi D, Armand F, Bürgi J, van der Goot FG (2015) SwissPalm: Protein Palmitoylation database. *F1000Res* **4**, 261.
9 Duncan JA & Gilman AG (1996) Autoacylation of G protein α subunits. *J Biol Chem* **271**, 23594–23600.
10 Liu L, Dudler T & Gelb MH (1996) Purification of a protein palmitoyltransferase that acts on H-Ras protein and on a C-terminal N-Ras peptide. *J Biol Chem* **271**, 23269–23276.
11 Wedegaertner PB & Bourne HR (1994) Activation and depalmitoylation of Gsα. *Cell* **77**, 1063–1070.
12 Mitchell DA, Vasudevan A, Linder ME & Deschenes RJ (2006) Protein palmitoylation by a family of DHHC protein S-acyltransferases. *J Lipid Res* **47**, 1118–1127.
13 Lobo S, Greentree WK, Linder ME & Deschenes RJ (2002) Identification of a Ras palmitoyltransferase in Saccharomyces cerevisiae. *J Biol Chem* **277**, 41268–41273.
14 Rana MS, Lee CJ & Banerjee A (2018) The molecular mechanism of DHHC protein acyltransferases. *Biochem Soc Trans* **47**, 157–167.
15 Ohno Y, Kihara A, Sano T & Igarashi Y (2006) Intracellular localization and tissue-specific distribution of human and yeast DHHC cysteine-rich domain-containing proteins. *Biochim Biophys Acta - Mol Cell Biol Lipids* **1761**, 474–483.
16 Aicart-Ramos C, Valero RA & Rodriguez-Crespo I (2011) Protein palmitoylation and subcellular trafficking. *Biochim Biophys Acta - Biomembr* **1808**, 2981–2994.
17 Greaves J & Chamberlain LH (2011) DHHC palmitoyl transferases: Substrate interactions and (patho)physiology. *Trends Biochem. Sci* **36**, 245–253.
18 Davda D & Martin BR (2014) Acyl protein thioesterase inhibitors as probes of dynamic S-palmitoylation. *MedChemComm* **5**, 268–276.
19 Duncan JA & Gilman AG (1998) A cytoplasmic acyl-protein thioesterase that removes palmitate from G protein α subunits and p21(RAS). *J Biol Chem* **273**, 15830–15837.
20 Lin DTS & Conibear E (2015) Enzymatic protein depalmitoylation by acyl protein thioesterases. *Biochem Soc Trans* **43**, 193–198.
21 Lin DTS & Conibear E (2015) ABHD17 proteins are novel protein depalmitoylases that regulate N-Ras palmitate turnover and subcellular localization. *Elife* **4**.
22 Cao Y, Qiu T, Kathayat RS, Azizi SA, Thorne AK, Ahn D, Fukata Y, Fukata M, Rice PA & Dickinson BC (2019) ABHD10 is an S-depalmitoylase affecting redox homeostasis through peroxiredoxin-5. *Nat Chem Biol* **15**, 1232–1240.
23 Fraser NJ, Howie J, Wypijewski KJ & Fuller W (2020) Therapeutic targeting of protein S-acylation for the treatment of disease. *Biochem Soc Trans* **48**, 281–290.
24 Zhou Y & Hancock JF (2015) Ras nanoclusters: Versatile lipid-based signaling platforms. *Biochimica et Biophysica Acta - Molecular Cell Research* **1853**, 841–849.
25 Hobbs GA, Der CJ & Rossman KL (2016) RAS isoforms and mutations in cancer at a glance. *J. Cell Sci* **129**, 1287–1292.
26 Cuiffo B & Ren R (2010) Palmitoylation of oncogenic NRAS is essential for leukemogenesis. *Blood* **115**, 3598–3605.

27 Roth AF, Wan J, Bailey AO, Sun B, Kuchar JA, Green WN, Phinney BS, Yates JR & Davis NG (2006) Global Analysis of Protein Palmitoylation in Yeast. *Cell* **125**, 1003–1013.
28 Li Y, Hu J, Höfer K, Wong AMS, Cooper JD, Birnbaum SG, Hammer RE & Hofmann SL (2010) 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* **285**, 13022–13031.
29 Chamberlain LH & Shipston MJ (2015) The physiology of protein s-acylation. *Physiol Rev* **95**, 341–376.
30 Noritake J, Fukata Y, Iwanaga T, Hosomi N, Tsutsumi R, Matsuda N, Tani H, Iwanari H, Mochizuki Y, Kodama T *et al.* (2009) Mobile DHHC palmitoylating enzyme mediates activity-sensitive synaptic targeting of PSD-95. *J. Cell Biol* **186**, 147–160.
31 Sanders SS, Martin DDO, Butland SL, Lavallée-Adam M, Calzolari D, Kay C, Yates JR & Hayden MR (2015) Curation of the Mammalian Palmitoylome Indicates a Pivotal Role for Palmitoylation in Diseases and Disorders of the Nervous System and Cancers. *PLoS Comput. Biol* **11**.
32 Cho E & Park M (2016) Palmitoylation in Alzheimer's disease and other neurodegenerative diseases. *Pharmacol Res* **111**, 133–151.
33 Raymond FL, Tarpey PS, Edkins S, Tofts C, O'Meara S, Teague J, Butler A, Stevens C, Barthorpe S, Buck G *et al.* (2007) 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* **80**, 982–987.
34 Baker K, Astle DE, Scerif G, Barnes J, Smith J, Moffat G, Gillard J, Baldeweg T & Raymond FL (2015) Epilepsy, cognitive deficits and neuroanatomy in males with ZDHHC 9 mutations. *Ann. Clin. Transl. Neurol* **2**, 559–569.
35 Mansouri MR, Marklund L, Gustavsson P, Davey E, Carlsson B, Larsson C, White I, Gustavson KH & Dahl N (2005) Loss of ZDHHC15 expression in a woman with a balanced translocation t(X;15)(q13.3; cen) and severe mental retardation. *Eur. J. Hum. Genet* **13**, 970–977.
36 Mukai J, Liu H, Burt RA, Swor DE, Lai WS, Karayiorgou M & Gogos JA (2004) Evidence that the gene encoding ZDHHC8 contributes to the risk of schizophrenia. *Nat. Genet* **36**, 725–731.
37 Shimell JJ, Shah BS, Cain SM, Thouta S, Kuhlmann N, Tatarnikov I, Jovellar DB, Brigidi GS, Kass J, Milnerwood AJ *et al.* (2019) The X-Linked Intellectual Disability Gene Zdhhc9 Is Essential for Dendrite Outgrowth and Inhibitory Synapse Formation. *Cell Rep* **29**, 2422–2437.e8.
38 Liu J, García-Cardeña G & Sessa WC (1996) Palmitoylation of endothelial nitric oxide synthase is necessary for optimal stimulated release of nitric oxide: Implications for caveolae localization. *Biochemistry* **35**, 13277–13281.
39 Fernández-Hernando C, Fukata M, Bernatchez PN, Fukata Y, Lin MI, Bredt DS & Sessa WC (2006) Identification of Golgi-localized acyl transferases that palmitoylate and regulate endothelial nitric oxide synthase. *J. Cell Biol* **174**, 369–377.
40 Wei X, Schneider JG, Shenouda SM, Lee A, Towler DA, Chakravarthy MV, Vita JA & De Semenkovich CF (2011) Novo lipogenesis maintains vascular homeostasis through endothelial nitric-oxide synthase (eNOS) palmitoylation. *J Biol Chem* **286**, 2933–2945.
41 Wei X, Adak S, Zayed M, Yin L, Feng C, Speck SL, Kathayat RS, Zhang Q, Dickinson BC & Semenkovich CF (2020) Endothelial Palmitoylation Cycling Coordinates Vessel Remodeling in Peripheral Artery Disease. *Circ. Res* **127**, 249–265.
42 Baekkeskov S & Kanaani J (2009) Palmitoylation cycles and regulation of protein function (Review). *Mol Membr Biol* **26**, 42–54.
43 Jia L, Chisari M, Maktabi MH, Sobieski C, Zhou H, Konopko AM, Martin BR, Mennerick SJ & Blumer KJ (2014) A mechanism regulating g protein-coupled receptor signaling that requires cycles of protein palmitoylation and depalmitoylation. *J Biol Chem* **289**, 6249–6257.
44 Beard RS, Yang X, Meegan JE, Overstreet JW, Yang CGY, Elliott JA, Reynolds JJ, Cha BJ, Pivetti CD, Mitchell DA *et al.* (2016) Palmitoyl acyltransferase DHHC21 mediates endothelial dysfunction in systemic inflammatory response syndrome. *Nat Commun* **7**, 12823.
45 Essandoh K, Philippe JM, Jenkins PM & Brody MJ (2020) Palmitoylation: A Fatty Regulator of Myocardial Electrophysiology. *Front Physiol* **11**, 108.
46 Pei Z, Xiao Y, Meng J, Hudmon A & Cummins TR (2016) Cardiac sodium channel palmitoylation regulates channel availability and myocyte excitability with implications for arrhythmia generation. *Nat Commun* **7**, 12035.
47 Chien AJ, Gao T, Perez-Reyes E & Hosey MM (1998) Membrane targeting of L-type calcium channels. Role of palmitoylation in the subcellular localization of the β(2a) subunit. *J Biol Chem* **273**, 23590–23597.
48 Zhou T, Li J, Zhao P, Liu H, Jia D, Jia H, He L, Cang Y, Boast S, Chen YH *et al.* (2015) Palmitoyl acyltransferase Aph2 in cardiac function and the development of cardiomyopathy. *Proc Natl Acad Sci U S A* **112**, 15666–15671.
49 Gök C, Plain F, Robertson AD, Howie J, Baillie GS, Fraser NJ & Fuller W (2020) Dynamic Palmitoylation of the Sodium-Calcium Exchanger Modulates Its Structure, Affinity for Lipid-Ordered Domains, and Inhibition by XIP. *Cell Rep* **31**, 107697.

50 Gök C & Fuller W (2020) Regulation of NCX1 by palmitoylation. *Cell Calcium* **86**, 102158.
51 Gök C & Fuller W (2020) Topical review: Shedding light on molecular and cellular consequences of NCX1 palmitoylation. *Cell Signal* **76**, 109791.
52 Reilly L, Howie J, Wypijewski K, Ashford MLJ, Hilgemann DW & Fuller W (2015) Palmitoylation of the Na/Ca exchanger cytoplasmic loop controls its inactivation and internalization during stress signaling. *FASEB J* **29**, 4532–4543.
53 Plain F, Turnbull D, Fraser NJ & Fuller W (2017) Understanding the rules governing NCX1 palmitoylation. *Channels (Austin)* **11**, 377–379.
54 Plain F, Congreve SD, Yee RSZ, Kennedy J, Howie J, Kuo CW, Fraser NJ & Fuller W (2017) An amphipathic -helix directs palmitoylation of the large intracellular loop of the sodium/calcium exchanger. *J Biol Chem* **292**, 10745–10752.
55 Fuller W, Reilly L & Hilgemann DW (2016) S-palmitoylation and the regulation of NCX1. *Channels* **10**, 75–77.
56 Tulloch LB, Howie J, Wypijewski KJ, Wilson CR, Bernard WG, Shattock MJ & Fuller W (2011) The inhibitory effect of phospholemman on the sodium pump requires its palmitoylation. *J Biol Chem* **286**, 36020–36031.
57 Howie J, Wypijewski KJ, Plain F, Tulloch LB, Fraser NJ & Fuller W (2018) Greasing the wheels or a spanner in the works? Regulation of the cardiac sodium pump by palmitoylation. *Crit Rev Biochem Mol Biol* **53**, 175–191.
58 Fuller W, Tulloch LB, Shattock MJ, Calaghan SC, Howie J & Wypijewski KJ (2013) Regulation of the cardiac sodium pump. *Cell Mol Life Sci* **70**, 1357–1380.
59 Howie J, Tulloch LB, Shattock MJ & Fuller W (2013) Regulation of the cardiac Na(+) pump by palmitoylation of its catalytic and regulatory subunits. *Biochem Soc Trans* **41**, 95–100.
60 Chaube R, Hess DT, Wang YJ, Plummer B, Sun QA, Laurita K & Stamler JS (2014) Regulation of the skeletal muscle ryanodine receptor/Ca2+- release channel RyR1 by S-palmitoylation. *J Biol Chem* **289**, 8612–8619.
61 Woodley KT & Collins MO (2019) S-acylated Golga7b stabilises DHHC 5 at the plasma membrane to regulate cell adhesion. *EMBO Rep* **20**, e47472.
62 Ko PJ, Woodrow C, Dubreuil MM, Martin BR, Skouta R, Bassik MC & Dixon SJ (2019) A ZDHHC5-GOLGA7 protein acyltransferase complex promotes nonapoptotic cell death. *Cell Chem Biol* **26**, 1716–1724.
63 Howie J, Reilly L, Fraser NJ, Walker JMV, Wypijewski KJ, Ashford MLJ, Calaghan SC, McClafferty H, Tian L, Shipston MJ *et al.* (2014) Substrate recognition by the cell surface palmitoyl transferase DHHC5. *Proc Natl Acad Sci U S A* **111**, 17534–17539.
64 Plain F, Howie J, Kennedy J, Brown E, Shattock MJ, Fraser NJ & Fuller W (2020) Control of protein palmitoylation by regulating substrate recruitment to a zDHHC-protein acyltransferase. *Commun Biol* **3**.411
65 Hilgemann DW, Fine M, Linder ME, Jennings BC & Lin MJ (2013) Massive endocytosis triggered by surface membrane palmitoylation under mitochondrial control in BHK fibroblasts. *Elife* **2**, e01293.
66 Larsen JB, Jensen MB, Bhatia VK, Pedersen SL, Bjørnholm T, Iversen L, Uline M, Szleifer I, Jensen KJ, Hatzakis NS *et al.* (2015) Membrane curvature enables N-Ras lipid anchor sorting to liquid-ordered membrane phases. *Nat Chem Biol* **11**, 192–194.
67 Lin MJ, Fine M, Lu JY, Hofmann SL, Frazier G & Hilgemann DW (2013) Massive palmitoylation-dependent endocytosis during reoxygenation of anoxic cardiac muscle. *Elife* **2**, e01295.
68 Hao JW, Wang J, Guo H, Zhao YY, Sun HH, Li YF, Lai XY, Zhao N, Wang X, Xie C *et al.* (2020) CD36 facilitates fatty acid uptake by dynamic palmitoylation-regulated endocytosis. *Nat Commun* **11**, 4765.
69 Brigidi GS, Santyr B, Shimell J, Jovellar B & Bamji SX (2015) Activity-regulated trafficking of the palmitoyl-acyl transferase DHHC5. *Nat Commun* **6**, 8200.
70 Brigidi GS, Sun Y, Beccano-Kelly D, Pitman K, Mobasser M, Borgland SL, Milnerwood AJ & Bamji SX (2014) Palmitoylation of δ-catenin by DHHC5 mediates activity-induced synapse plasticity. *Nat Neurosci* **17**, 522–532.
71 Sergeeva OA & van der Goot FG (2019) Anthrax toxin requires ZDHHC5-mediated palmitoylation of its surface-processing host enzymes. *Proc Natl Acad Sci U S A* **116**, 1279–1288.
72 Jones ML, Collins MO, Goulding D, Choudhary JS & Rayner JC (2012) Analysis of protein palmitoylation reveals a pervasive role in Plasmodium development and pathogenesis. *Cell Host Microbe* **12**, 246–258.
73 Sobocińska J, Roszczenko-Jasińska P, Ciesielska A & Kwiatkowska K (2018) Protein Palmitoylation and Its Role in Bacterial and Viral Infections. *Front Immunol* **8**, 2003.
74 Samavarchi-Tehrani P, Abdouni H, Knight JD, Astori A, Samson R, Lin Z-Y, Kim D-K, Knapp JJ, St-Germain J, Go CD, Larsen B, Wong CJ, Cassonnet P, Demeret C, Jacob Y, Roth FP, Raught B & Gingras A-C. A SARS-CoV-2 – host proximity interactome. *bioRxiv* [PREPRINT] 2020.09.03.282103 (2020) doi:10.1101/2020.09.03.282103.
75 Yang J, Petitjean SJL, Koehler M, Zhang Q, Dumitru AC, Chen W, Derclaye S, Vincent SP, Soumillion P &

Alsteens D (2020) Molecular interaction and inhibition of SARS-CoV-2 binding to the ACE2 receptor. *Nat Commun* **11**, 4541.
76 Petit CM, Chouljenko VN, Iyer A, Colgrove R, Farzan M, Knipe DM & Kousoulas KG (2007) Palmitoylation of the cysteine-rich endodomain of the SARS-coronavirus spike glycoprotein is important for spike-mediated cell fusion. *Virology* **360**, 264–274.
77 Thorp EB, Boscarino JA, Logan HL, Goletz JT & Gallagher TM (2006) Palmitoylations on Murine Coronavirus Spike Proteins Are Essential for Virion Assembly and Infectivity. *J Virol* **80**, 1280–1289.
78 Schlesinger MJ, Magee AI & Schmidt MF (1980) Fatty acid acylation of proteins in cultured cells. *J Biol Chem* **21**, 10021–10024.
79 Baker TL, Zheng H, Walker J, Coloff JL & Buss JE (2003) Distinct rates of palmitate turnover on membrane-bound cellular and oncogenic H-Ras. *J Biol Chem* **278**, 19292–19300.
80 Martin DDO, Vilas GL, Prescher JA, Rajaiah G, Falck JR, Bertozzi CR & Berthiaume LG (2008) Rapid detection, discovery, and identification of post-translationally myristoylated proteins during apoptosis using a bio-orthogonal azidomyristate analog. *FASEB J* **22**, 797–806.
81 Pedone KH, Bernstein LS, Linder ME & Hepler JR (2009) Analysis of Protein Palmitoylation by Metabolic Radiolabeling Methods. Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-59745-198-7_166. in 1623–1636.
82 Drisdel RC & Green WN (2004) Labeling and quantifying sites of protein palmitoylation. *Biotechniques* **36**, 276–285.
83 Forrester MT, Hess DT, Thompson JW, Hultman R, Moseley MA, Stamler JS & Casey PJ (2011) Site-specific analysis of protein S-acylation by resin-assisted capture. *J Lipid Res* **52**, 393–398.
84 Hurst CH, Turnbull D, Plain F, Fuller W & Hemsley PA (2017) Maleimide scavenging enhances determination of protein S-palmitoylation state in acyl-exchange methods. *Biotechniques* **62**, 69–75.
85 Alberts B, Johnson A, Lewis J, Raff M, Roberts K & Walter P (2002) How Cells Obtain Energy from Food. In Molecular Biology of the Cell, 4th edn. Garland Science, New York.
86 Passmore LA & Barford D (2004) Getting into position: the catalytic mechanisms of protein ubiquitylation. *Biochem J* **379**, 513–525.
87 Maller C, Schröder E & Eaton P (2011) Glyceraldehyde 3-phosphate dehydrogenase is unlikely to mediate hydrogen peroxide signaling: Studies with a novel anti-dimedone sulfenic acid antibody. *Antioxidants Redox Signal* **14**, 49–60.
88 Fang C, Zhang X, Zhang L, Gao X, Yang P & Lu H (2016) Identification of Palmitoylated Transitional Endoplasmic Reticulum ATPase by Proteomic Technique and Pan Antipalmitoylation Antibody. *J Proteome Res* **15**, 956–962.
89 Gao X & Hannoush RN (2018) A Decade of Click Chemistry in Protein Palmitoylation: Impact on Discovery and New Biology. *Cell Chemical Biology* **25**, 236–246.
90 Martin BR & Cravatt BF (2009) Large-scale profiling of protein palmitoylation in mammalian cells. *Nat Methods* **6**, 135–138.
91 Kostiuk MA, Corvi MM, Keller BO, Plummer G, Prescher JA, Hangauer MJ, Bertozzi CR, Rajaiah G, Falck JR & Berthiaume LG (2008) Identification of palmitoylated mitochondrial proteins using a bio-orthogonal azido-palmitate analogue. *FASEB J* **22**, 721–732.
92 Greaves J, Munro KR, Davidson SC, Riviere M, Wojno J, Smith TK, Tomkinson NCO & Chamberlain LH (2017) Molecular basis of fatty acid selectivity in the zDHHC family of S-acyltransferases revealed by click chemistry. *Proc Natl Acad Sci U S A* **114**, E1365–E1374.
93 Poggi M, Kara I, Brunel JM, Landrier JF, Govers R, Bonardo B, Fluhrer R, Haass C, Alessi MC & Peiretti F (2013) Palmitoylation of TNF alpha is involved in the regulation of TNF receptor 1 signalling. *Biochim Biophys Acta - Mol Cell Res* 1833, 602–612.
94 Kharbanda A, Walter DM, Gudiel AA, Schek N, Feldser DM & Witze ES (2020) Blocking EGFR palmitoylation suppresses PI3K signaling and mutant KRAS lung tumorigenesis. *Sci Signal* **13**, 2364.
95 Runkle KB, Kharbanda A, Stypulkowski E, Cao XJ, Wang W, Garcia BA & Witze ES (2016) Inhibition of DHHC20-Mediated EGFR Palmitoylation Creates a Dependence on EGFR Signaling. *Mol Cell* **62**, 385–396.
96 Sobocinska J, Roszczenko-Jasinska P, Zareba-Kozio M, Hromada-Judycka A, Matveichuk OV, Traczyk G, Ukasiuk K & Kwiatkowska K (2018) Lipopolysaccharide Upregulates Palmitoylated Enzymes of the Phosphatidylinositol Cycle: An Insight from Proteomic Studies. *Mol Cell Proteomics* **17**, 233–254.
97 Hannoush RN & Sun J (2010) The chemical toolbox for monitoring protein fatty acylation and prenylation. *Nat Chem Biol* **6**, 498–506.
98 Heal WP, Wickramasinghe SR, Leatherbarrow RJ & Tate EW (2008) N-Myristoyl transferase-mediated protein labelling in vivo. *Org Biomol Chem* **6**, 2308–2315.
99 Charron G, Zhang MM, Yount JS, Wilson J, Raghavan AS, Shamir E & Hang HC (2009) Robust fluorescent detection of protein fatty-acylation with chemical reporters. *J Am Chem Soc* **131**, 4967–4975.
100 Hannoush RN & Arenas-Ramirez N (2009) Imaging the lipidome: ω-alkynyl fatty acids for detection and

cellular visualization of lipid-modified proteins. *ACS Chem Biol* **4**, 581–587.
101 Thinon E, Fernandez JP, Molina H & Hang HC (2018) Selective Enrichment and Direct Analysis of Protein S-Palmitoylation Sites. *J Proteome Res* **17**, 1907–1922.
102 Gao X & Hannoush RN (2014) Single-cell in situ imaging of palmitoylation in fatty-acylated proteins. *Nat Protoc* **9**, 2607–2623.
103 Ganesan L, Shieh P, Bertozzi CR & Levental I (2017) Click-Chemistry Based High Throughput Screening Platform for Modulators of Ras Palmitoylation. *Sci Rep* **7**, 41147.
104 Introduction to CAPTUREome S-palmitoylated protein kit. https://badrilla.com/captureome-s-palmitoylated-protein-kit.
105 Roth AF, Wan J, Green WN, Yates JR & Davis NG (2006) Proteomic identification of palmitoylated proteins. *Methods* **40**, 135–142.
106 Gottlieb CD, Zhang S & Linder ME (2015) The cysteine-rich domain of the DHHC3 palmitoyltransferase is palmitoylated and contains tightly bound zinc. *J Biol Chem* **290**, 29259–29269.
107 Yang W, Di Vizio D, Kirchner M, Steen H & Freeman MR (2010) Proteome scale characterization of human S-acylated proteins in lipid raft-enriched and non-raft membranes. *Mol Cell Proteomics* **9**, 54–70.
108 Collins MO, Woodley KT & Choudhary JS (2017) Global, site-specific analysis of neuronal protein S-acylation. *Sci Rep* **7**, 4683.
109 Kang R, Wan J, Arstikaitis P, Takahashi H, Huang K, Bailey AO, Thompson JX, Roth AF, Drisdel RC, Mastro R *et al.* (2008) Neural palmitoyl-proteomics reveals dynamic synaptic palmitoylation. *Nature* **456**, 904–909.
110 Shen LF, Chen YJ, Liu KM, Haddad ANS, Song IW, Roan HY, Chen LY, Yen JJY, Chen YJ, Wu JY *et al.* (2017) Role of S-Palmitoylation by ZDHHC13 in Mitochondrial function and Metabolism in Liver. *Sci Rep* **7**, 2182.
111 Zhang J, Planey SL, Ceballos C, Stevens SM, Keay SK & Zacharias DA (2008) 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* **7**, 1378–1388.
112 Li Y, Martin BR, Cravatt BF & Hofmann SL (2012) DHHC5 protein palmitoylates flotillin-2 and is rapidly degraded on induction of neuronal differentiation in cultured cells. *J Biol Chem* **287**, 523–530.
113 Martin BR, Wang C, Adibekian A, Tully SE & Cravatt BF (2012) Global profiling of dynamic protein palmitoylation. *Nat Methods* **9**, 84–89.
114 Rana MS, Kumar P, Lee CJ, Verardi R, Rajashankar KR & Banerjee A (2018) Fatty acyl recognition and transfer by an integral membrane S-acyltransferase. *Science* **359**, eaao6326.
115 Jennings BC, Nadolski MJ, Ling Y, Baker MB, Harrison ML, Deschenes RJ & Linder ME (2009) 2-bromopalmitate and 2-(2-hydroxy-5-nitro-benzylidene)-benzo[b] thiophen-3-one inhibit DHHC-mediated palmitoylation in vitro. *J Lipid Res* **50**, 233–242.
116 Davda D, El Azzouny MA, Tom CTMB, Hernandez JL, Majmudar JD, Kennedy RT & Martin BR (2013) Profiling targets of the irreversible palmitoylation inhibitor 2-bromopalmitate. *ACS Chem Biol* **8**, 1912–1917.
117 Chase JF & Tubbs PK (1972) Specific inhibition of mitochondrial fatty acid oxidation by 2-bromopalmitate and its coenzyme A and carnitine esters. *Biochem J* **129**, 55–65.
118 Draper JM & Smith CD (2009) Palmitoyl acyltransferase assays and inhibitors (Review). *Mol Membr Biol* **26**, 5–13.
119 Zheng B, Deran M, Li X, Liao X, Fukata M & Wu X (2013) 2-Bromopalmitate analogues as activity-based probes to explore palmitoyl acyltransferases. *J Am Chem Soc* **135**, 7082–7085.
120 Pedro MP, Vilcaes AA, Tomatis VM, Oliveira RG, Gomez GA & Daniotti JL (2013) 2-Bromopalmitate Reduces Protein Deacylation by Inhibition of Acyl-Protein Thioesterase Enzymatic Activities. *PLoS One* **8**, e75232.
121 Hurley JH, Cahill AL, Currie KPM & Fox AP (2000) The role of dynamic palmitoylation in Ca2+ channel inactivation. *Proc Natl Acad Sci U S A* **97**, 9293–9298.
122 Trenchi A, Gomez GA & Daniotti JL (2009) Dual acylation is required for trafficking of growth-associated protein-43 (GAP-43) to endosomal recycling compartment via an Arf6-associated endocytic vesicular pathway. *Biochem J* **421**, 357–369.
123 Jochen AL, Hays J & Mick G (1995) Inhibitory effects of cerulenin on protein palmitoylation and insulin internalization in rat adipocytes. *Biochim Biophys Acta (BBA)/Lipids Lipid Metab* **1259**, 65–72.
124 Hamel D & L., J. Lenhart, B., A. Mitchell, D., G. Santos, R., A. Giulianotti, M. & J. Deschenes, R. (2016) Identification of Protein Palmitoylation Inhibitors from a Scaffold Ranking Library. *Comb Chem High Throughput Screen* **19**, 262–274.
125 Dekker FJ, Rocks O, Vartak N, Menninger S, Hedberg C, Balamurugan R, Wetzel S, Renner S, Gerauer M, Schölermann B *et al.* (2010) Small-molecule inhibition of APT1 affects Ras localization and signaling. *Nat Chem Biol* **6**, 449–456.
126 Adibekian A, Martin BR, Chang JW, Hsu KL, Tsuboi K, Bachovchin DA, Speers AE, Brown SJ, Spicer T, Fernandez-Vega V *et al.* (2012) Confirming target engagement for reversible inhibitors in vivo by

kinetically tuned activity-based probes. *J Am Chem Soc* **134**, 10345–10348.
127 Vujic I, Sanlorenzo M, Esteve-Puig R, Vujic M, Kwong A, Tsumura A, Murphy R, Moy A, Posch C, Monshi B *et al.* (2016) Acyl protein thioesterase 1 and 2 (APT-1, APT-2) inhibitors palmostatin B, ML348 and ML349 have different effects on NRAS mutant melanoma cells. *Oncotarget* **7**, 7297–7306.
128 Hernandez JL, Davda D, Kit CS & M., Majmudar, J. D., Won, S. J., Gang, M., Pasupuleti, S. C., Choi, A. I., Bartkowiak, C. M. & Martin, B. R. (2017) APT2 inhibition restores scribble localization and s-palmitoylation in snail-transformed cells. *Cell Chem Biol* **24**, 87–97.
129 Chen S, Han C, Miao X, Li X, Yin C, Zou J, Liu M, Li S, Stawski L, Zhu B *et al.* (2019) Targeting MC1R depalmitoylation to prevent melanomagenesis in redheads. *Nat Commun* **10**, 877.
130 Huang K, Sanders S, Singaraja R, Orban P, Cijsouw T, Arstikaitis P, Yanai A, Hayden MR & El-Husseini A (2009) Neuronal palmitoyl acyl transferases exhibit distinct substrate specificity. *FASEB J* **23**, 2605–2615.
131 Burgoyne JR, Haeussler DJ, Kumar V, Ji Y, Pimental DR, Zee RS, Costello CE, Lin C, McComb ME, Cohen RA *et al.* (2012) Oxidation of HRas cysteine thiols by metabolic stress prevents palmitoylation *in vivo* and contributes to endothelial cell apoptosis. *FASEB J* **26**, 832–841.
132 Neviani V, Deventer S, Wörner TP, Xenaki KT, Waterbeemd M, Rodenburg RNP, Wortel IMN, Kuiper JK, Huisman S, Granneman J *et al.* (2020) Site-specific functionality and tryptophan mimicry of lipidation in tetraspanin CD9. *The FEBS Journal* **287**, 5323–5344.
133 Li Y, Wang S, Chen Y, Li M, Dong X, Hang HC & Peng T (2020) Site-specific chemical fatty-acylation for gain-of-function analysis of protein: S -palmitoylation in live cells. *Chem Commun* **56**, 13880–13883.
134 Main A, Robertson-Gray O & Fuller W (2020) Cyclophilin D palmitoylation and permeability transition: a new twist in the tale of myocardial ischaemia–reperfusion injury. *Cardiovasc Res* **117**, 15–17. https://doi.org/10.1093/cvr/cvaa149.
135 Rudd AK, Brea RJ & Devaraj NK (2018) Amphiphile-Mediated Depalmitoylation of Proteins in Living Cells. *J Am Chem Soc* **140**, 17374–17378.
136 Strassburger K, Kang E & Teleman AA (2019) Drosophila ZDHHC8 palmitoylates scribble and Ras64B and controls growth and viability. *PLoS One* **14**, e0198149.
137 Milnerwood AJ, Parsons MP, Young FB, Singaraja RR, Franciosi S, Volta M, Bergeron S, Hayden MR & Raymond LA (2013) Memory and synaptic deficits in Hip14/DHHC17 knockout mice. *Proc Natl Acad Sci U S A* **110**, 20296–20301.
138 Lemonidis K, Gorleku OA, Sanchez-Perez MC, Grefen C & Chamberlain LH (2014) The Golgi S-acylation machinery comprises zDHHC enzymes with major differences in substrate affinity and S-acylation activity. *Mol Biol Cell* **25**, 3870–3883.
139 Tsutsumi R, Fukata Y, Noritake J, Iwanaga T, Perez F & Fukata M (2009) Identification of G Protein α Subunit-Palmitoylating Enzyme. *Mol Cell Biol* **29**, 435–447.
140 Kilpatrick CL, Murakami S, Feng M, Wu X, Lal R, Chen G, Du K & Luscher B (2016) Dissociation of Golgi-associated DHHC-type zinc finger protein (GODZ)- and Sertoli cell gene with a zinc finger domain-β (SERZ-β)-mediated palmitoylation by loss of function analyses in knock-out mice. *J Biol Chem* **291**, 27371–27386.
141 Tian L, McClafferty H, Jeffries O & Shipston MJ (2010) Multiple palmitoyltransferases are required for palmitoylation-dependent regulation of large conductance calcium- and voltage-activated potassium channels. *J Biol Chem* **285**, 23954–23962.
142 Ohno Y, Kashio A, Ogata R, Ishitomi A, Yamazaki Y & Kihara A (2012) Analysis of substrate specificity of human DHHC protein acyltransferases using a yeast expression system. *Mol Biol Cell* **23**, 4543–4551.
143 Philippe JM & Jenkins PM (2019) Spatial organization of palmitoyl acyl transferases governs substrate localization and function. *Mol Membr Biol* **35**, 60–75.
144 Rodenburg RNP, Snijder J, Van De Waterbeemd M, Schouten A, Granneman J, Heck AJR & Gros P (2017) Stochastic palmitoylation of accessible cysteines in membrane proteins revealed by native mass spectrometry. *Nat Commun* **8**, 1280.
145 Allen JJ, Li M, Brinkworth CS, Paulson JL, Wang D, Hübner A, Chou WH, Davis RJ, Burlingame AL, Messing RO *et al.* (2007) A semisynthetic epitope for kinase substrates. *Nat Methods* **4**, 511–516.
146 Salaun C, Locatelli C, Zmuda F, Cabrera Gonzaíez J & Chamberlain LH (2020) Accessory proteins of the zDHHC family of S-acylation enzymes. *J Cell Sci* **133**, jcs251819.
147 Swarthout JT, Lobo S, Farh L, Croke MR, Greentree WK, Deschenes RJ & Linder ME (2005) DHHC9 and GCP16 constitute a human protein fatty acyltransferase with specificity for H- and N-Ras. *J Biol Chem* **280**, 31141–31148.
148 Fukata M, Fukata Y, Adesnik H, Nicoll RA & Bredt DS (2004) Identification of PSD-95 palmitoylating enzymes. *Neuron* **44**, 987–996.
149 Stix R, Lee CJ, Faraldo-Gómez JD & Banerjee A (2020) Structure and mechanism of DHHC protein acyltransferases. *J Mol Biol* **432**, 4983–4998.
150 Huang K, Sanders SS, Kang R, Carroll JB, Sutton L, Wan J, Singaraja R, Young FB, Liu L, El-Husseini A *et al.* (2011) Wild-type HTT modulates the enzymatic

activity of the neuronal palmitoyl transferase HIP14. *Hum Mol Genet* **20**, 3356–3365.
151 Moritz AE, Rastedt DE, Stanislowski DJ, Shetty M, Smith MA, Vaughan RA & Foster JD (2015) Reciprocal phosphorylation and palmitoylation control dopamine transporter kinetics. *J Biol Chem* **290**, 29095–29105.
152 Tian L, Jeffries O, McClafferty H, Molyvdas A, Rowe ICM, Saleem F, Chen L, Greaves J, Chamberlain LH, Knaus HG *et al*. (2008) Palmitoylation gates phosphorylation-dependent regulation of BK potassium channels. *Proc Natl Acad Sci U S A* **105**, 21006–21011.
153 Chen JJ, Marsden AN, Scott CA, Akimzhanov AM & Boehning D (2020) DHHC5 Mediates β-Adrenergic Signaling in Cardiomyocytes by Targeting Gα Proteins. *Biophys J* **118**, 826–835.
154 Lievens PM-J, Kuznetsova T, Kochlamazashvili G, Cesca F, Gorinski N, Galil DA, Cherkas V, Ronkina N, Lafera J, Gaestel M *et al*. (2016) ZDHHC3 Tyrosine Phosphorylation Regulates Neural Cell Adhesion Molecule Palmitoylation. *Mol Cell Biol* **36**, 2208–2225.
155 Gorinski N, Bijata M, Prasad S, Wirth A, Abdel Galil D, Zeug A, Bazovkina D, Kondaurova E, Kulikova E, Ilchibaeva T *et al*. (2019) Attenuated palmitoylation of serotonin receptor 5-HT1A affects receptor function and contributes to depression-like behaviors. *Nat Commun* **10**, 3924.
156 Chai S, Cambronne XA, Eichhorn SW & Goodman RH (2013) MicroRNA-134 activity in somatostatin interneurons regulates H-Ras localization by repressing the palmitoylation enzyme, DHHC9. *Proc Natl Acad Sci U S A* **110**, 17898–17903.
157 Martin DDO, Kanuparthi PS, Holland SM, Sanders SS, Jeong HK, Einarson MB, Jacobson MA & Thomas GM (2019) Identification of novel inhibitors of DLK palmitoylation and signaling by high content screening. *Sci Rep* **9**, 3632.
158 Hamel LD, Lenhart BJ, Mitchell DA, Santos RG, Giulianotti MA & Deschenes RJ (2016) Identification of protein palmitoylation inhibitors from a scaffold ranking library. *Comb Chem High Throughput Screen* **19**, 262–274.
159 Korycka J, Łach A, Heger E, Boguslawska DM, Wolny M, Toporkiewicz M, Augoff K, Korzeniewski J & Sikorski AF (2012) Human DHHC proteins: A spotlight on the hidden player of palmitoylation. *Eur J Cell Biol* **91**, 107–117.
160 Bers DM (2002) Cardiac excitation–contraction coupling. *Nat* **415**, 198–205.