The physiology of protein S-acylation

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Abstract

Protein S-acylation, the only fully reversible post-translational lipid modification of proteins, is emerging as a ubiquitous mechanism to control the properties and function of a diverse array of proteins and consequently physiological processes. S-acylation results from the enzymatic addition of long chain lipids, most typically palmitate, onto intracellular cysteine residues of soluble and transmembrane proteins via a labile thioester linkage. Addition of lipid results in increases in protein hydrophobicity that can impact on protein structure, assembly, maturation, trafficking and function. The recent explosion in global S-acylation (palmitoyl) proteomic profiling as a result of improved biochemical tools to assay S-acylation, in conjunction with the recent identification of enzymes that control protein S-acylation and de-acylation, has opened a new vista into the physiological function of S-acylation. This review introduces key features of S-acylation and tools to interrogate this process, and highlights the eclectic array of proteins regulated including: membrane receptors, ion channels and transporters, enzymes and kinases, signaling adapters and chaperones, cell adhesion and structural proteins. We highlight recent findings correlating disruption of S-acylation to pathophysiology and disease and discuss some of the major challenges and opportunities in this rapidly expanding field.

I. Introduction

Cellular proteins are regulated by a diverse range of chemical modifications on their amino acid sidechains (Figure 1). These various modifying groups can affect charge, hydrophobicity, and other aspects of protein chemistry, resulting in marked changes in the behaviour of protein molecules and hence in the control of physiological mechanisms. Lipidation is a general term encompassing the attachment of different lipids and lipid-like groups onto proteins (144, 208). These lipid modifications increase protein hydrophobicity and can impact on protein structure and the affinity of proteins for cellular membranes or membrane domains.

Glycosylphosphatidylinositol (GPI) groups anchor many cellular proteins to the outer surface of the plasma membrane, and cholesterol is attached to certain secreted proteins (60, 91, 208).. For proteins retained inside the cell, common lipidations are fatty acylation and isoprenylation (208). The latter modification involves the post-translational attachment of farnesyl and geranygeranyl chains onto C-terminal cysteine residues via an irreversible thioether linkage (208, 269).. The enzymes farnesyl transferase and geranylgeranyl transferase I recognise a strict "CAAX" (A is any aliphatic amino acid and X is any amino acid) consensus motif present at the extreme C-terminus of proteins. The amino acid occupying the X position in the CAAX motif determines if the protein is modified by farnesyl transferase or geranylgeranyl transferase I (134, 269). A different geranylgeranylation signal (non-CAAX) is recognised by the enzyme geranylgeranyl transferase II, which modifies Rab proteins (135).

The main types of fatty acylation characterised to-date are *N*-myristoylation and *S*-acylation. N-myristoylation most frequently involves the co-translational reaction of myristic acid with a glycine residue in the consensus sequence MGXXXS/T (where M is the initiating methionine and X is any amino acid) (8, 25, 208). As myristoyl chains are added to the amine group of the glycine, this modification requires the previous removal of the initiating methionine residue (65). *N*-myristoylation does not occur exclusively as a co-translational modification, and can also take place post-translationally when N-terminal glycine residues are exposed following protein cleavage, for example, during apoptosis (264).

S-acylation has proved more enigmatic than isoprenylation and *N*-myristoylation, however recent breakthroughs have shed light on the mechanisms and dynamics of this modification. *S*-acylation is exclusively post-translational, involving the attachment of fatty acids onto cysteine residues via a labile thioester linkage and thus, in contrast to other lipidations, S-acylation may be dynamic and reversible (Figure 2) (51, 144, 208, 215, 221, 232). This modification is frequently referred to as palmitoylation, reflecting the fact that palmitate is the predominant fatty acid attached to *S*-acylated proteins. Historically, *S*-acylation was relatively difficult to study due to the low sensitivity of available detection methods, the

absence of a defined consensus sequence and a dearth of information about the relevant enzymes responsible for this lipid modification (167). However recent breakthroughs have identified the *S*-acylation enzymes and developed highly sensitive techniques to study this process, leading to a marked increase in our knowledge of the mechanisms and outcomes of protein *S*-acylation.

In this review we first outline some general features of S-acylation including its ubiquity, reversibility and diversity of substrates before outlining some of the key regulatory effects S-acylation may have on protein function and subcellular localisation. We then review the enzymatic control of S-acylation and the implication of S-acylation in disease and outline methodologies exploited to assess and interrogate S-acylation. Finally, we discuss selected examples to highlight the diverse array of cellular proteins and physiological processes controlled by S-acylation – from control of gene transcription and cellular architecture to ion flux across membranes - before highlighting some of the key outstanding questions in the field.

II. General Features of *S*-Acylation

II.i S-acylation is a universal modification in eukaryotes

S-acylation is a highly conserved process occurring in all eukaryotic organisms that have been examined, and regulated by enzyme families (Figure 2) that are conserved from yeast to humans (74, 122, 167, 212). Proteomic profiling of *S*-acylated proteins in the yeast *Saccharomyces cerevisiae* identified 47 acylated proteins (212), whereas similar studies have detected several hundred *S*-acylated proteins in mammalian and other cell types (30, 83, 108, 118, 122, 139, 155-157, 162, 265, 266, 268, 276, 278, 282). The number of *S*-acylated proteins identified in these studies may well be a significant under-representation, as techniques employed to enrich the S-acylated proteome may be affected by the level and

dynamics of *S*-acylation on individual proteins. Whilst there is no evidence for *S*-acylation occurring in prokaryotes, many viral proteins undergo S-acylation catalysed by the host cell machinery, and indeed analysis of viral protein *S*-acylation has made a significant contribution to current knowledge about *S*-acylation in general e.g. (129).

II.ii S-acylation is a reversible modification

A key property of *S*-acylation that distinguishes it from other lipid modifications of cellular proteins is its reversibility. The labile nature of thioester bonds in the intracellular environment allows many (but not all) *S*-acylated proteins to undergo rapid cycles of *S*-acylation and deacylation (215). Classically, *S*-acylation dynamics have been studied using radiolabelling with [^{3}H] palmitic acid, which is converted into [^{3}H] palmitoyl-coA in cells and incorporated into *S*-acylated proteins. Early studies that employed [^{3}H] palmitate pulse-chase experiments highlighted the reversibility of *S*-acylation, for example the half-life of ^{3}H palmitate incorporated into N-Ras was shown to be around 20 minutes (154). These radiolabelling experiments have been instrumental in defining cellular *S*-acylation dynamics, however it is possible that this approach does not provide sufficient sensitivity to generate highly accurate turnover rates; for example, more recent work using an indirect reporter of *S*-acylation status suggested that deacylation of N-Ras may occur 10-20 times faster than calculated by pulse-chase analyses (209, 210). These apparent differences will require future clarification through the use of multiple approaches to determine palmitate turnover dynamics. The dynamic nature of *S*-acylation may therefore be more akin to other post-translational modifications that can have rapid turnover rates, such as phosphorylation, and cycles of *S*-acylation and deacylation are now known to play a fundamental role in regulating the intracellular localisation and function of many diverse proteins (215) (see section VI).

The reversibility of *S*-acylation sets this process apart from other lipid modifications and implies an important role for S-acylation in the dynamic regulation of cellular proteins. Nevertheless, it needs be emphasised that rapid cycling of acyl chains on *S*-acylated

proteins is not universal, and *S*-acylation turns over at a very low rate or not at all on other cellular proteins (157). Indeed marked differences can exist in the turnover rates of different S-acylation sites in the same protein (294). Thus, a major outstanding question in *S*-acylation research relates to how the dynamics of lipid turnover on *S*-acylated proteins is regulated and why different proteins display distinct turnover rates.

II.iii Diversity of Lipid Groups Attached to S-Acylated Proteins

The term 'palmitoylation' is often used synonymously with '*S*-acylation'. This nomenclature stems from the multitude of studies that have monitored *S*-acylation via incorporation of [^{3}H] palmitate into specific cellular proteins. Nonetheless, it is likely that palmitate (C16:0) is indeed the major lipid incorporated into endogenous *S*-acylated proteins, although other fatty acids such as stearate (C18:0) and oleate (C18:1) can also be added (253). One study used gas chromatography-mass spectrometry to quantify the different fatty acids that were released from total platelet proteins following hydroxylamine treatment (which cleaves thioester bonds between acyl groups and cysteine). This quantification found that of the fatty acids added to proteins via *S*-acylation, on average 74% were palmitate, 22% were stearate and 4% were oleate (174). This study provides a snap-shot of the lipid diversity on *S*-acylated proteins, but it is important to emphasise that there are likely to be other fatty acids incorporated at lower levels, and different cell types will likely exhibit distinct lipid profiles as will individual *S*-acylated proteins. Indeed the lipid profile of *S*-acylated proteins in platelets was markedly affected by the lipid composition of the extracellular environment, and there was also marked variability in the level of oleate in platelets from different subjects (at maximum, 100-fold) (174). Studies using cell lines have highlighted similar heterogeneity in fatty acids attached via *S*-acylation (140).

The challenge of dissecting the chemical identity of the lipid groups added to *S*-acylated proteins is widely recognised. The study described above employed gas chromatography-mass spectrometry to quantify the levels of individual lipids on platelet proteins, and this technique can also be applied to analyse the lipid profile of individual *S*-acylated proteins

(234); however this approach is not able to determine the exact cysteine residues that are modified by the detected fatty acids. However tandem mass spectrometry approaches have proved useful to profile the lipid species that are attached to specific cysteine residues in viral proteins. These analyses have revealed that the haemagluttinin protein of Influenza viruses can be modified by both palmitate and stearate, and intriguingly that stearate appears to be specifically added to cysteines located within the transmembrane domains of these proteins (128, 129). Whilst mass spectrometry approaches hold significant promise to dissect acyl chain heterogeneity in *S*-acylated proteins, technical hurdles have thus far prevented widespread use of this methodology, in particular for most low abundance mammalian membrane proteins.

II.iv. Diversity of S-Acylation Substrates

Proteomic profiling of *S*-acylated proteins from a variety of different cell types has revealed the diverse nature of the modified proteins (see section VI) (30, 83, 108, 118, 122, 139, 155-157, 162, 265, 266, 268, 276, 278, 282). *S*-acylated proteins can be broadly classified as transmembrane or peripheral membrane proteins, with the latter requiring acylation for stable membrane binding (Figure 3). Peripheral membrane *S*-acylated proteins often undergo 'dual' lipid modification, and thus can be further sub-classified accordingly as either: *N*-myristoylated/*S*-acylated, isoprenylated/*S*-acylated, or exclusively S-acylated (232). Exemplar proteins that are modified in these three ways include Src family kinases, Ras proteins, and the membrane fusion protein SNAP25, respectively (82, 98, 127). Transmembrane proteins that are modified by *S*-acylation are equally as diverse in nature as the *S*-acylated peripheral membrane proteins, and include ion channels, receptors and transporters (122).

III. Regulatory Effects of S-Acylation

S-acylation has been implicated in the control of multiple stages of the lifecycle of transmembrane and peripheral-membrane proteins (Figure 4): from protein assembly to trafficking and final degradation.

III.i Membrane binding of peripheral membrane proteins

For peripheral membrane proteins, the first and foremost function of *S*-acylation is to mediate stable membrane attachment (215). This requirement for *S*-acylation to promote stable membrane binding holds even for dual lipidated proteins, as single myristoyl or prenyl groups are not sufficient in this regard. Seminal work exploring the interaction of lipidated peptides with model membranes uncovered a fundamental principle, termed 'kinetic trapping' (222). These studies revealed that single myristoyl or prenyl groups only provide peptides with a weak membrane affinity, sufficient for transient membrane binding. In contrast, closely positioned dual lipid anchors, (either myristate/palmitate or farnesyl/palmitate) mediate strong, essentially irreversible, membrane-peptide interaction.

This kinetic trapping observed when peptides convert from having a single lipid group to being dual lipidated is highly relevant when one considers the intracellular distribution of lipidating enzymes. *N*-myristoyl and prenyl transferases, which mediate the co- and post-translational modification of soluble proteins, respectively, are localised in the cell cytosol (5, 26, 125, 254). In contrast, the enzymes that mediate *S*-acylation are exclusively membrane-associated (see section IV) and as a result this modification only occurs at cellular membranes. Thus, *N*-myristoylation or prenylation of proteins during or shortly after their synthesis in the cytosol bestows a weak membrane affinity that mediates transient interaction with intracellular membranes. These transient membrane interactions allow singly lipidated proteins to connect with membrane-bound *S*-acyl transferases, and subsequent *S*-acylation leads to stable membrane binding and kinetic trapping. The requirement of dual lipidation for stable membrane binding has been clearly shown for a variety of cellular proteins; for example, mutation of the *S*-acylation site(s) in Ras proteins leads to a weak

association with membranes, whereas mutation of the farnesylation signal leads to a loss of both *S*-acylation and membrane binding (81).

As mentioned previously, some proteins are exclusively *S*-acylated with no obvious primary membrane targeting signals. It has been suggested that such proteins rely upon a weak intrinsic membrane affinity to access intracellular membranes and undergo *S*-acylation and thus a similar kinetic trapping idea holds for these proteins. This mechanism of initial membrane interaction has been proposed to operate for SNAP25 and CSP (77, 78).

Although transmembrane proteins are irreversibly membrane-integrated, *S*-acylation of cysteine residues in the cytoplasmic domains of these proteins can dictate the membrane proximity of these domains (Figure 3). This can have a major effect on the overall structure/topology of the protein relative to the membrane.

III.ii Targeting to membrane micro-domains and sub-domains

The lipids within cellular membranes are diverse and have been proposed to undergo short-range ordering, as a result of packing preferences. This concept is readily observed in model membrane systems composed of ternary lipid mixtures, where cholesterol and saturated phospholipids phase separate from unsaturated phospholipids (20). This phase separation that occurs *in vitro* has been proposed to also occur in intact cellular membranes, leading to the formation of cholesterol- and saturated phospholipid-rich microdomains (229). It has been incredibly challenging to study these so-called lipid rafts in live cells due to the small size of these structures (<50 nm) (199) and several investigators have questioned whether such domains ever form or have meaningful functions in cellular membranes (173). The functions and composition of lipid rafts have largely been ascribed based upon results using cholesterol depletion experiments and detergent-insolubility as a descriptor for raft-associated proteins. Although there is intense debate about the extent to which detergent solubility characteristics of membrane proteins reflect association with cholesterol-rich lipid raft domains (141), it is clear that *S*-acylated proteins largely co-purify with cholesterol-enriched detergent insoluble membranes (161). As a result, *S*-acylation has been proposed

as an important signal for sequestration into lipid raft micro-domains, and thus as a regulator of the lateral distribution of proteins within membranes (136). *S*-acylation-dependent association of proteins with detergent-insoluble rafts has been proposed to regulate important cellular pathways such as exocytosis via the membrane fusion protein SNAP25 (214) and actin cytoskeleton remodelling and membrane organisation via Rac1 (176). Progress in this area of research is likely to be substantially enabled by the development of super-resolution imaging techniques that provide optical resolution on the order of raft size (49).

The majority of work on membrane microdomains has focussed on proteins associated with the plasma membrane, however intracellular compartments also exhibit heterogeneities in structure and composition. The endoplasmic reticulum (ER) is a case in point, and diversification of this compartment into several distinct sub-domains facilitates its role as a multi-functional organelle (152). Protein *S*-acylation appears to be an important factor in targeting proteins to specific ER sub-domains. Calnexin, which functions in the folding of glycoproteins in the ER, is *S*-acylated at the cytoplasmic side of its single transmembrane domain (132). Perturbing acylation of calnexin decreased the association of this protein with the ribosome-studded rough ER surrounding the nuclear envelope (132). Molecular modelling of the calnexin transmembrane domain and a short cytoplasmic region suggested that S-acylation may modify the orientation of the cytoplasmic tail with respect to the axis of the transmembrane helix (132), and these structural changes may facilitate targeting of calnexin, albeit by a presently unknown mechanism. A role for *S*-acylation has also emerged in targeting of proteins to the mitochondrial-associated membrane (MAM) another distinct ER sub-domain. The transmembrane thioredoxin family protein TMX and heme oxygenase-1 were found to require *S*-acylation for efficient targeting to the MAM (150). This study also suggested that S-acylation of calnexin was required for targeting to the MAM (150), and thus more work is clearly required in this area to understand the precise requirements and mechanisms whereby *S*-acylated proteins are segregated to defined domains in the ER.

III.iii Protein trafficking and intracellular localisation

S-acylation regulates the trafficking and localisation of a wide range of cellular proteins (72). Many effects on protein trafficking and localisation are likely to be attributable to the impact of *S*-acylation on membrane binding and micro-localisation discussed above. *S*-acylation will affect the localisation of peripheral membrane proteins simply by promoting accumulation of modified proteins on membranes containing the respective acylation enzymes. This *S*-acylation-dependent movement of proteins from the cytosol to membranes may allow other domains of the protein to interact with sorting adaptors, mediating trafficking to distinct membrane compartment(s). In this scenario, *S*-acylation is required for protein trafficking (by virtue of the requirement of this modification for membrane association) but it does not actively direct proteins to a specific intracellular compartment.

In addition to these 'passive' effects of *S*-acylation on protein localisation, *S*-acylation can also function as an active protein sorting signal. Short peptides from the lipidated C-terminus of H/N-Ras display a similar localisation to the full-length proteins, implying that lipid signals may be sufficient to specify movement of Ras proteins from endomembranes to the plasma membrane (80). At present it is not clear how S-acylation might regulate trafficking of these peptides but this may involve effects of *S*-acylation on protein micro-localisation. For example, cholesterol-rich domains at the Golgi have been proposed to act as platforms for the budding of transport vesicles delivering cargo to the plasma membrane (188). Thus, *S*-acylation might promote Golgi exit by driving protein association with such cholesterol-rich microdomains.

It can be challenging to tease apart the active effects of *S*-acylation on trafficking of peripheral membrane proteins from the requirement of this modification for stable membrane binding. However for multiply-acylated proteins such as the SNARE protein SNAP25, it is possible to examine effects of individual *S*-acylated cysteines on protein trafficking, without interfering with membrane binding. Indeed, recent work has suggested that the extent of *S*-acylation of SNAP25 (4 potential acylation sites) regulates the cycling of this protein between the plasma membrane and endosomes (73), which may impact endosomal fusion dynamics

(7). In this case, mutant SNAP25 proteins with 3 rather than 4 *S*-acylation acceptor sites exhibited a marked accumulation on endosomal membranes (73), likely highlighting a role for the extent of *S*-acylation in regulating endosome-to-plasma membrane trafficking of SNAP25. The mechanistic basis underlying the effects of multiple S-acylation on SNAP25 endosomal cycling are not known, however the number of acylation acceptor sites in SNAP25 has a marked effect on association of the protein with cholesterol-rich membranes (213), suggesting that lipid rafts might feature in the regulation of this cycling pathway.

As transmembrane proteins do not require *S*-acylation for stable membrane binding, the importance of this modification for trafficking is potentially easier to dissect. As a result, we currently have a clearer picture about the active effects of S-acylation on the trafficking of integral membrane proteins.

During protein translation, signal peptides and start/stop transfer sequences are employed to insert transmembrane proteins into the membrane of the endoplasmic reticulum (ER) and generate the required topological arrangement of the protein with respect to the membrane bilayer (223). Various chaperones and enzymes in the ER lumen facilitate protein folding, catalyse modifications such as glycosylation, and support oligomeric assembly of proteins into the necessary quaternary structure (237). *S*-acylation has been reported to regulate trafficking of a variety of transmembrane proteins from the ER (72, 215), including LRP6, a co-receptor for Wnt. LRP6 is a monotopic transmembrane protein that is *S*-acylated close to the cytoplasmic side of the membrane-spanning domain (1). LRP6 proteins with the S-acylation sites mutated were retained at the ER and failed to traffic to the plasma membrane. Intriguingly, this block in forward trafficking of *S*-acylation-deficient mutants was alleviated by reducing the length of the TMD of LRP6. This observation suggests that *S*-acylation may have the effect of 'reducing' the apparent length of the LRP6 TMD. Thus, the authors proposed that non-acylated LRP6 is trapped at the ER because hydrophobic mismatching occurs between the long TMD (23 amino acids) and the thin ER bilayer, and that *S*-acylation improves hydrophobic matching by inducing the TMD to tilt with respect to the membrane axis. Recent molecular dynamics simulations have supported the idea of interactions

between *S*-acyl chains and transmembrane helices, with the two *S*-acyl chains attached to the C-terminus of the G protein-coupled receptor rhodopsin reported to make frequent contacts with a transmembrane helix of the protein (183).

A similar role for *S*-acylation in regulating ER exit was described for the yeast chitin synthase, Chs3, a polytopic membrane protein. In this case, blocking *S*-acylation was found to promote aggregation and ER retention and a similar role for acylation in hydrophobic matching was proposed (133). Although the ER membrane has relatively low levels of cholesterol (or the yeast equivalent, ergosterol), *S*-acylation might also facilitate hydrophobic matching by targeting the modified protein into thicker cholesterol-rich domains on the ER membrane. A recent report also suggested that S-acylation may regulate the expression and localisation/maturation of both wild type and ΔF508 CFTR (159), highlighting a potential therapeutic role for drugs that modify S-acylation for the treatment of cystic fibrosis.

In addition to these effects of *S*-acylation on trafficking of newly-synthesised proteins, there are also numerous reports that this modification regulates movement of proteins between the plasma membrane and endosomal system. Again, it is difficult to find a consensus model to predict, for a specific protein, how S-acylation might modulate trafficking between the plasma membrane and endosomes. S-acylation in the C-terminus of AMPA receptor subunits regulates internalisation from the plasma membrane, and the mechanism involves the modulation of receptor interaction with the cytoskeletal protein 4.1N (86, 142). S-acylation of NMDA receptor subunits also regulates internalisation of this neurotransmitter receptor (87). However in this case, this results from downstream effects of S-acylation on phosphorylation of a tyrosine residue involved in receptor internalisation; S-acylation enhances phosphorylation, blocking internalisation and hence leading to receptor accumulation at the plasma membrane. In addition to effects of S-acylation on protein internalisation from the plasma membrane, there is also evidence that this modification can regulate recycling of proteins from endosomes back to the plasma membrane. This was observed for MUC1, a mucin-like protein present on the apical membrane of epithelia. MUC1 containing cysteine-to-alanine mutations at the transmembrane boundary that block S-

acylation exhibited an enhanced accumulation at recycling endosomes. In addition, the mutant protein displayed reduced co-immunoprecipitation with the adaptor protein AP1, which regulates vesicle formation at recycling endosomes, offering some mechanistic insight into how perturbation of S-acylation might impact MUC1 recycling (126). There is also evidence that S-acylation impacts the cycling of sortillin between endosomes and the Golgi apparatus. Sortillin is involved in the trafficking of soluble proteins from the Golgi to lysosomes, and cycles between the Golgi and endosomsal compartment. In this case, blocking palmitoylation interfered with trafficking of Sortillin back to the Golgi complex and led to enhanced degradation of the protein in lysosomes (160). S-acylation mutants of Sortillin appear to be more susceptible to ubquitination, explaining their increased targeting to lysosomes and degradation(47).

III.iv. Protein stability

Increased ubiquination and degradation is commonly observed when S-acylation of specific proteins is blocked. This interplay between S-acylation and ubiquitination was reported for the yeast SNARE protein Tlg1 (257). This protein is S-acylated in proximity to its single transmembrane domain and preventing this modification by deleting the relevant zDHHC enzyme (Swf1) leads to increased ubiquitination by the ubiquitin ligase Tul1 and vacuole targeting. In this case, S-acylation was suggested to fix the position of the transmembrane domain to prevent acidic residues coming into membrane contact, which can be a scenario leading to Tul1-mediated ubquitination. A similar interplay between S-acylation and ubiquitination has been identified for many other proteins including the anthrax toxin receptor TEM8 (2). S-acylation is thought to restrict TEM8 to non-raft domains of the plasma membrane, whereas S-acylation mutants associate with lipid rafts and undergo ubiquitination mediated by Cbl. The effect of S-acylation to restrict raft association of TEM8 is in contrast to the raft-targeting activity of this modification on many other proteins. These two examples given for Tlg1 and TEM8 emphasise how S-acylation can protect against premature degradation via distinct mechanisms: by preventing membrane interaction of

negatively charged phospholipids for Tlg1 and by regulating the lateral distribution in membranes for TEM8.

It is interesting to note that the limited number of proteomic analyses performed to-date in mouse lines with reduced expression of S-acyltransferase enzymes (zDHHC enzymes) have also noted that reduced S-acylation levels of specific proteins often correlates with an overall loss of expression. We anticipate that careful proteomic assessment of how S-acylation affects the expression levels of the cellular proteome will provide a detailed understanding of how widespread the link between S-acylation and protein stability is.

III.v. Functional effects of S-acylation from studies in mouse models and disease

Cell-based studies have highlighted the importance of *S*-acylation for the function of individual proteins in specific cellular pathways. For example, the extent of *S*-acylation dictates the lateral distribution of the membrane fusion protein SNAP25 in cholesterol-rich plasma membrane micro-domains, which affects the efficiency of regulated exocytosis in neuroendocrine cell (213, 214)**.** Perturbing the *S*-acylation of calnexin results in a loss of the protein from the perinuclear rough ER, which is enriched in ribosome-translocon complexes, with a corresponding defect in folding of cellular glycoproteins (132). More global physiological effects of *S*-acylation are reflected in the phenotypes of genetically modified mice with perturbations in specific *S*-acyl transferases (74) (see Table 1 and zDHHC enzymes, section IV.i.).

zDHHC5 genetrap mice with low expression levels of this enzyme have perturbed contextual fear conditioning, which indicates a deficit in learning and memory (138). The molecular basis for this behavioural change is not known but might be linked to interactions of zDHHC5 with post-synaptic proteins such as PSD95 (138) and GRIP1b (244). zDHHC8 knockout mice also exhibit behavioural abnormalities including changes in pre-pulse inhibition and in exploratory behaviour (172); these deficits may be caused by underlying changes in the formation/stability of excitatory synapses and perturbations in dendritic growth (171). zDHHC17 and zDHHC13 genetrap mice also exhibit behavioural deficits and changes in

neuronal function (230, 240). These highly-related enzymes *S*-acylate the huntingtin (HTT) protein (105), and furthermore zDHHC17 activity is positively modulated by HTT (106). Expansion of the poly-glutamine tract of HTT (as seen in Huntington's disease, HD) leads to a loss of *S*-acylation and also a reduced ability to regulate zDHHC17 *S*-acylation activity (106, 273), implying that changes in HTT S-acylation or its regulation of zDHHC enzymes might contribute to the deficits present in HD. The idea that loss of zDHHC17 activity might contribute to pathogenesis in HD is supported by recent work showing that zDHHC17 genetrap mice (insert at intron 5) display similar deficits as HD mouse models. The zDHHC17 mutant mice exhibit decreased striatal volume and a loss of medium spiny neurons, a reduction in the number of excitatory synapses, and a deficit in hippocampal long-term potentiation (164, 230). At the behavioural levels, the mice display deficits in motor co-ordination, pre-pulse inhibition, and hippocampal-dependent spatial learning tests (164, 230). Biochemically, zDHHC17 genetrap mice exhibit a partial loss of SNAP25 and PSD95 *S*-acylation (230), although it is not yet clear if these substrates contribute to the cellular and behavioural changes present in the mice. Given the strong homology between zDHHC17 and zDHHC13 it is likely that HTT can also regulate the activity of this latter enzyme. Indeed, zDHHC13 genetrap mice (insert in intron 1) exhibit a broadly similar neuropathology to zDHHC17 genetrap mice (240). Furthermore, the phenotypic changes seen in zDHHC13 mutant mice, such as a decreased striatal volume, occur later and are more progressive than in zDHHC17 mutant mice and as such mirror HD mouse model phenotypes better (240).

Other zDHHC mouse models that have been investigated include a different zDHHC13 loss-of-function mouse line, which expresses a truncated form of the zDHHC13 protein exhibits skin and hair defects (216). Furthermore, the mice also display a reduced life-span, systemic amyloidosis, osteoporosis and muscle wasting (216), with recent work linking the osteoporosis phenotype with a reduced S-acylation of membrane type 1- matrix metalloproteinase (233). Interestingly, no HD-like phenotype was seen in this zDHHC13 mouse model (233), unlike in the zDHHC13 genetrap model (240). A major difference

between these two zDHHC13 mouse lines is that one is hypomorphic whereas the other expresses a truncated (and therefore possible dominant-negative) form of the enzyme. Finally, a mouse line with a point mutation in the *zdhhc21* gene exhibits hair loss and skin abnormalities (163). The mutation in the *zdhhc21* gene leads to a single amino acid substitution in zDHHC21 and a resulting mis-localisation of the enzyme on ER rather than Golgi membranes (163). Future work on these and other zDHHC mutant mice will hopefully include an analysis of knock-in mutants in which the catalytic cysteine is mutated. This will be an important step to resolve effects arising due to a loss of S-acylation activity of the zDHHC enzymes from other non-canonical functions of the zDHHC proteins.

The reported phenotypes of zDHHC mutant mice clearly show the importance of individual enzymes for normal physiology. This is further emphasised by the reported links between human diseases and mutations in *ZDHHC* genes or changes in zDHHC expression levels (Table 2). Mutations in the *ZDHHC9* gene cause X-linked intellectual disability (ID) (202). Importantly, two point mutations linked with ID led to single amino acid changes in the DHHC-CR domain of zDHHC9, and these mutations have since been shown to affect enzyme autoacylation (a measure of activity) in *in vitro* assays (165). This provides strong evidence that intellectual disability can arise due to a deficit in the *S*-acylation of specific substrates of zDHHC9 rather than because of any other non-canonical functions of this enzyme. It will therefore be important in future work to identify proteins that have reduced *S*-acylation following inactivation of the zDHHC9 enzyme and to explore whether loss of these proteins leads to features seen in intellectual disability.

Despite earlier work reporting an association between an SNP in the *ZDHHC8* gene and an increased risk of developing schizophrenia (145), the majority of follow-up studies have failed to support such an association (270). However more recent work has suggested a possible link between zDHHC8 SNPs and smooth pursuit eye movement (which is perturbed in schizophrenia patients) (224) and cortical brain volume in schizophrenics (184).

Thioesterase enzymes, which catalyse protein deacylation, also have a critical role in brain function. This is exemplified by mutations in the *PPT1* gene, which cause infantile neuronal

ceroid lipofuscinosis (NCL), an early-onset neurodegenerative lysosomal-storage disease. PPT1 encodes a protein palmitoyl thioesterase that functions in the degradation of *S*-acylated proteins in lysosomes (262).

In addition to brain disorders such as intellectual disability, schizophrenia and NCL, there is also evidence linking changes in zDHHC expression, both up- and down-regulation, with various cancers. For example, the *ZDHHC11* gene is present within a region of chromosome 5 that has an increased copy number in lung and bladder cancers (120, 271) and zDHHC9 expression is increased in colorectal tumours (16). In contrast, zDHHC2 has been reported to be down-regulated in colorectal cancers (186) and also in gastric adenocarcinoma (272), and zDHHC14 expression is down-regulated in testicular germ cell tumours and prostate cancer (280). These findings may suggest a potential tumour suppressor function of zDHHC2 and zDHHC14, however it is worth noting that increased zDHHC14 expression was seen in gastric cancer and leukaemia (11, 283), suggesting that this protein actually might be oncogenic in certain settings.

A major area of investigation should now centre on delineating the substrate(s) of zDHHC enzymes that are linked with specific disease states. This would lead to a greater understanding of the specific proteins and physiological pathways that are likely to be affected by changes in zDHHC function. It should also be remembered that in come cases, diseases may be linked with up- or down-regulation of non-canonical activities of zDHHC enzymes.

IV. Acylation Enzymes and the Control of Acylation

IV.i. S-acyl transferases: zDHHCs

The biggest breakthrough in the *S*-acylation field in recent years was the discovery of the enzymes that control this process. Independent studies in *Saccharomyces cerevisiae* identified proteins that were required for *S*-acylation of Ras2p and Yck1p (14, 147, 211,

287). Analysis of the sequences of these *S*-acyl transferases, Erf2p and Akr1p, revealed a common 51 amino acid zinc finger domain with a conserved 'DHHC' motif (note this motif is actually DYHC in Akr1p) (167). This pioneering work in *S. cerevisiae* paved the way for the subsequent discovery of a large family (24 in human genome) of mammalian genes encoding proteins containing this DHHC domain (61, 107, 124). It is now established that the large majority of these mammalian zDHHC proteins are bona fide *S*-acyl transferases (74, 109). However it is important to note that other acylation-independent functions have additionally been ascribed to some members of the family, including in membrane transport, intracellular signalling, and cytoskeletal regulation (42, 69, 101, 274). Experiments using purified zDHHC proteins are consistent with the idea that *S*-acylation by these enzymes is a two-step mechanism involving autoacylation of the DHHC motif cysteine and subsequent acyl transfer to the substrate protein (112, 166).

All zDHHC family members characterised to-date are predicted polytopic membrane proteins with the catalytic DHHC domain facing the cytosol (Figure 2C) (196). The majority of zDHHC proteins are localised to ER and Golgi membranes, with a small number present on post-Golgi compartments (182). At present there is little information available on the intramolecular signals that specify intracellular localisation, although lysine-based sorting signals in the extreme C-terminal tails of zDHHC4 and zDHHC6 restrict these specific isoforms to the ER (68).

Whilst zDHHC enzymes are likely to display a certain degree of redundancy and have partially over-lapping substrates, it is also true that specific substrates are dependent upon individual zDHHC enzymes for their efficient modification. Proteomic analyses following knockdown of individual zDHHC enzymes in *S. cerevisiae* clearly highlighted that efficient *S*-acylation of specific substrates requires individual zDHHC enzymes (212). In concurrence, other focussed studies showed that deletion of Pfa4 led to an ablation of S-acylation of the chitin synthase Chs3 (133) and Swf1p deletion blocked S-acylation of the SNARE protein Tlg1p (257). Furthermore, *S*-acylation of Tlg1 following depletion of Swf1 was not rescued by high level expression of three other yeast zDHHC proteins, although Pfa3 had a modest

effect on S-acylation of this SNARE protein (67). Similarly, S-acylation of Chs3p was not rescued by either Swf1, Pfa3 or Erf2 (67). Indeed, specific zDHHC enzymes are required for the S-acylation of different sites on the same protein, for example this is true for the STREX variant of BK potassium channels (248, 249). However superimposed upon this zDHHC-substrate specificity is an element of zDHHC enzymes acting on over-lapping substrates (74). Thus, VAC8 *S*-acylation in an *S. cerevisiae* strain depleted of the zDHHC protein Pfa3 could be rescued by over-expression of other yeast zDHHC enzymes (104), and analysis of semi-synthetic Ras constructs suggested that multiple zDHHC enzymes could regulate *S*-acylation of these proteins (209). However a key point to note is that although several zDHHC enzymes might be able to *S*-acylate a specific substrate, the enzymes that are important *in vivo* will depend upon their relative expression profiles; thus distinct zDHHC enzymes might be important for the S-acylation of a specific substrate in different cell types.

Despite the debate around the level of specificity in zDHHC-substrate interactions, several studies have identified domains and residues in both substrates and enzymes that are important for interaction specificity. The catalytic DHHC-CR domain does not appear to be sufficient for substrate S-acylation or to specificity substrate selectivity, This point is emphasised by a study showing that although zDHHC3 is highly active against SNAP23, transplanting the DHHC domain from this protein into an inactive enzyme (zDHHC15) did not allow zDHHC15 to acylate SNAP23 (75). Instead, zDHHC enzyme protein activity likely requires the coordinated activities of the DHHC domain and other regions of the enzyme. zDHHC17 and zDHHC13 (also known as HIP14 and HIP14L, respectively) are unique in containing N-terminal ankyrin repeat domains, which appear to specify substrate binding (281). Indeed ligating the ankyrin-repeat domain of zDHHC17 onto the N-terminus of zDHHC3 led to the S-acylation of zDHHC17 substrates by zDHHC3, highlighting the importance of this protein interaction domain for substrate recognition and subsequent S-acylation (105). The *S. cerevisiae* orthologue of zDHHC17, Akr1p, also contains an ankyrin-repeat extension, and although mutants lacking the ankyrin repeat domain were able to *S*-acylate Yck2p, the extent of this acylation was greatly reduced compared with full-length

Akr1p (94). More recent work showed that a PDZ ligand in the C-terminus of zDHHC5/zDHHC8 regulates binding to and *S*-acylation of GRIP1b, an adaptor protein containing multiple PDZ domains (244). Combined depletion of these zDHHC5 and zDHHC8 led to an almost complete loss of GRIP1b acylation in hippocampal neurons (244). Thus, at least some zDHHC enzymes recognise their substrates via defined domains, which then couple with the DHHC-CR domain to mediate substrate S-acylation.

There has also been some progress in identifying elements in *S*-acylated substrate proteins that are required for their recognition by specific zDHHC enzymes. A proline residue 25 amino acids downstream of the acylated cysteine-rich domain of SNAP25 is required for modification of this protein by zDHHC17 but not by zDHHC3 (77). In addition, a phenylalanine residue immediately upstream of the cysteine cluster is required for S-acylation of the SNAP25b isoform by DHHC15 (75). Other work has shown that domains downstream from modified cysteines can also function to prevent promiscuous zDHHC-substrate interactions. Full-length Vac8 was specifically S-acylated by Pfa3 *in vitro* but the isolated S-acylation domain was also modified by 4 other yeast zDHHC proteins (175). This observation emphasises the importance of considering changes in zDHHC-substrate specificity when analysing isolated protein domains or truncated proteins.

Although a comprehensive analysis of zDHHC-dependent *S*-acylation has been undertaken in *S. cerevisiae* (212), we currently lack detailed information on the effects of knockdown of specific mammalian zDHHC enzymes on global S-acylation. One study examining cultured neural stem cells from the forebrains of zDHHC5 gene-trapped mice (which express zDHHC5 at around 5% of the levels present in cells from control mice) reported that *S*-acylation of flotillin-2 was reduced by around 10-fold (139). In addition to flotillin-2, a group of approximately 20 other *S*-acylated proteins displayed decreased expression levels in zDHHC5 knockdown cells (139), possibly reflecting a requirement for *S*-acylation to stabilise these proteins. Interestingly, analysis of the brain S-acylated proteome from zDHHC17 genetrap mice also showed that S-acylation of flotillin-1 and -2 was reduced by 36%, with a corresponding decrease in expression levels. A further 15 proteins were identified that had a

>10% loss in S-acylation (266). Further analysis of global *S*-acylation in zDHHC knock-out mice will provide invaluable data on the substrates of individual DHHC proteins.

Although knowledge of how zDHHC-substrate specificity is encoded is rapidly evolving, there has been little work examining how specific zDHHC enzymes contribute to the lipid profile of *S*-acylated proteins (section II.iii). A recent study has shed some light on this issue by highlighting that different zDHHC enzymes incorporate and transfer different lipids with distinct efficiencies. The ability of different 'competing' lipids to block ^{3}H palmitate incorporation into zDHHC2 and zDHHC3, and subsequent transfer to substrate proteins was compared (112). Intriguingly, whereas only C14 and C16 lipids were efficient inhibitors of zDHHC3 autoacylation and substrate S-acylation, C14-C20 lipids all inhibited zDHHC2 to a similar extent. Further analysis confirmed that zDHHC2 transferred C18:0, C18:1 and C20:4 lipids to a model substrate protein with broadly similar efficiencies, whereas zDHHC3 strongly favoured C14/C16 lipids over C18/C20 lipids (112). It will be of particular interest in further work to delineate the sequence/structural basis for these differences in fatty acid specificity, and also to test whether zDHHC2 is responsible for the incorporation of stearic acid into the TMD of haemmaglutinin (129).

IV.ii. S-acyl thioesterases

In contrast to central role played by zDHHC enzymes in cellular *S*-acylation, far less is known about the enzymes and mechanisms that mediate protein deacylation. In fact, candidate thioesterases were identified many years prior to the realisation that zDHHC enzymes function as *S*-acyl transferases: Protein palmitoyl thioesterase 1 (PPT1) and PPT2 are targeted to lysosomes and catalyse deacylation during protein degradation (23, 24, 93, 235, 261), whereas acyl protein thioesterase 1 (APTI) is a cytoplasmic enzyme, implicated in dynamic *S*-acylation cycling of proteins (48). APT1 is active against proteins including eNOS, H/N-Ras, Gα subunits, and BK potassium channels (48, 249, 279). A related protein, APT2 (~ 64% identical at amino acid level to APT1), has been reported to function as a

thioesterase against GAP43 and H-Ras but not BK potassium channels (249, 251). An additional protein, APT1-like thioesterase has activity against BK potassium channels (249). Research into the role of APT proteins in dynamic S-acylation has been supported by the recent development of thioesterase inhibitors. Palmostatin B, which is a broad spectrum inhibitor of APT proteins and other serine hydrolases, was employed to highlight the role of thioesterase enzymes (and specifically APT1) in dynamic *S*-acylation of Ras proteins (40). However, our knowledge of thioesterase regulation of dynamic S-acylation is still very much in its infancy. Indeed, recent studies using broad spectrum serine lipase inhibitors have revealed a significant subset of the large serine hydrolase family (that include the APTs and PPTs (12, 284)) are responsible for cellular depalmitoylation (157). This point is highlighted by a recent study that reported the blockade of deacylation of R7BP (a regulator of G protein signalling) by the general serine hydrolase inhibitors Palmostatin B and HDFP (115). This led to a redistribution of the protein from the plasma membrane onto endomembrane compartments (115). Intriguingly, these effects on R7BP deacylation and localisation were not replicated by either RNAi knockdown of APT1/APT2 or by specific inhibitors of these enzymes. Indeed, a combination of RNAi knockdown of APT1 and APT2 and treatment with specific inhibitors of both of these enzymes also failed to affect R7BP deacylation (115). These results thus suggest that there are likely to be many other serine hydrolase enzymes that function as deacylation enzymes, and which presumably add an additional layer of specificity into dynamic S-acylation of cellular proteins.

IV.iii. MBOATs and Acylation of Secreted Proteins

Although the major focus of this review is *S*-acylation, it is worthwhile noting that several secreted proteins are modified by different types of acylation that are critical for their signalling functions. Wnt, hedgehog and ghrelin are all acylated but, in contrast to substrates of the DHHC family, acylation occurs on the luminal side of intracellular membranes. The enzymes that mediate acylation of these secreted proteins belong to the MBOAT family of membrane-bound *O*-acyl transferases (21, 28). Wnt undergoes modification with palmitate

on a conserved cysteine residue, whereas the monounsaturated palmitoleate is added to a conserved serine residue. Porcupine mediates acylation of Wnt, at least with respect to palmitoleate attachment to serine (242). Palmitate is attached via an amide linkage to the N-terminal cysteine of hedgehog (following signal peptide cleavage), and this modification is catalysed by hedgehog acyltransferase (193). Finally ghrelin *O*-acyltransferase transfers an *O*-octanoyl group onto a conserved serine residue of the peptide hormone ghrelin (275). The importance of these acyl modifications for the physiology of a diverse group of essential secreted proteins has led to an increasing interest in MBOATs as novel drug targets. In particular, there is a major interest in targeting the abnormal hedgehog signalling that is present in a high percentage of pancreatic and other cancer types via chemical inhibitors of Hhat (243). Palmitoylation and cholesterylation are essential for the biological activity of hedgehog, and recent work has reported the identification of novel small molecule inhibitors Hhat (195), which successfully block proliferation of pancreatic cancer cells (194). This example highlights the emerging clinical significance of MBOAT proteins and the potential to target these enzymes as novel drug targets in different disease states.

IV.iv. Regulation of S-acylation

Although *S*-acylation is a major regulator of cellular proteins and pathways, there is little information about how the dynamics of this process are regulated. *S*-acylation clearly is regulated as the acylation status of many proteins is modified in response to specific cues, such as PSD95, which exhibits changes in acylation that correlate with synaptic activity (38, 52). Regulation of *S*-acylation might occur via effects on zDHHC enzymes, thioesterases, or direct effects on specific substrates. There is certainly constitutive regulation of specific zDHHC enzymes, for example, zDHHC9 function as an acyltransferase requires an obligatory co-factor, GCP16 (241). On the other hand, zDHHC17 has an intrinsic S-acyltransferase activity that was recently suggested to be positively modulated by Huntingtin (106). A HTT-zDHHC17 complex immunoprecipitated from rat brain displayed acyltransferase activity towards recombinant SNAP25 (106). Depletion of HTT led to a

reduction of zDHHC17 *S*-acylation and a corresponding reduction in S-acylation of SNAP25 and GluR1 (106). Interestingly, whereas a COS cell lysate containing wild-type HTT enhanced S-acylation of recombinant SNAP25 and PSD95 by zDHHC17, mutant HTT with a polyglutamine tract expansion did not stimulate S-acylation.

There is also evidence that zDHHC enzymes are regulated by dynamic changes in their localisation. zDHHC2, which mediates activity-dependent *S*-acylation of PSD95, is regulated by dynamic changes in its localisation. This zDHHC enzymes enters a dynamic cycling pathway that traffics it between the plasma membrane and recycling endosomes (71). Blocking synaptic activity, which leads to enhanced *S*-acylation of PSD95 also modulates zDHHC2 trafficking (179), promoting its accumulation at the plasma membrane from where it can effectively modify PSD95 (62).

Another potential mode of zDHHC2 regulation is via changes in its oligomeric state. Both zDHHC2 and zDHHC3 were shown to exhibit dimerization/oligomerisation, with the higher molecular weight forms potentially linked to a less active state of the enzymes (131). Future work should reveal if regulation of S-acyltransferase activity by self-association (and perhaps also hetero-multimerisation of zDHHC enzymes) might represent a mode of zDHHC regulation that is widespread through this enzyme family.

An additional mode of regulation has been suggested to exist for zDHHC5. Growth factor withdrawal from neural stem cells led to a rapid proteasome-dependent degradation of zDHHC5 (139), which was inhibited by EGF and FGF. Thus, zDHHC5 activity is likely to be regulated via expression changes in response to specific extracellular cues (139),.

A recent study also highlighted regulatory mechanisms occurring at the mRNA level. Micro-RNAs have been shown to regulate the expression of both zDHHC enzymes and thioesterases. Whereas miR-138 regulates expression of APT1, leading to activation of $G_{\alpha 13}$ and spine shrinkage (228), miR-134 was shown to target zDHHC9 leading to a loss of plasma membrane targeting of H-Ras in GABAergic cortical interneurons (27). Interestingly, miR-138 and miR-134 appear to respond differently to neuronal activity, with miR-138 being repressed and miR-134 being activated by neuronal activation (27, 228).

Regulation of substrate *S*-acylation can also occur independently of direct effects on zDHHC or APT enzymes. H-Ras typically displays an intracellular distribution across the Golgi and plasma membrane, with Golgi accumulation reflecting the continuous return of the protein to this compartment following depalmitoylation. Although the final 10 amino acids of H-Ras (containing both the farnesylated CAAX motif and palmitoylated cysteines) are sufficient for plasma membrane targeting (80), it was noted that this amino acid sequence displayed less Golgi localisation than the final 19 amino acids of H-Ras, when both sequences were fused to EGFP (6). This difference reflected a higher level of *S*-acylation of the 10 amino acid construct compared with the 19 amino acid domain, which was proposed to be due to a slower rate of deacylation (6). Pharmacological and mRNA knockdown experiments combined with site-directed mutagenesis of H-Ras suggested that binding of the *cis-trans* prolyl isomerase FKBP12 and *cis-trans* isomerisation of the glycine178-proline179 peptide bond (present in the 19 amino acid but not the 10 amino acid construct) was responsible for the enhanced deacylation rate of the longer H-Ras construct (6). A similar role for prolyl isomerases has been suggested in the regulation of Rac1 *S*-acylation, where pharmacological inhibition of prolyl isomerase activity (FK508) or mutation of proline residues adjacent to the acylation site increased incorporation of ^{3}H palmitate into Rac1 (176). One possibility to explain these effects is that prolyl isomerase action leads to an increased accessibility of the *S*-acylated cysteines to thioesterase enzymes; more work to explore how widespread the role of prolyl isomerisation is in the regulation of protein de-acylation is clearly warranted.

The *S*-acylation status of some proteins is also subject to regulation by different post-translational modifications. In particular, there appears to be bidirectional interplay between *S*-acylation and phosphorylation for many proteins. In most cases the presence of one of these modifications is mutually exclusive or at least inhibitory to the other. PKA phosphorylation of the STREX variant of BK potassium channels interfered with S-acylation of adjacent cysteine residues, preventing membrane association of the C-terminal channel tail, and these effects were linked with PKA regulation of channel activity (111, 247).

Similarly, PKA phosphorylation of PDE10A2 inhibited S-acylation and membrane binding of this peripheral membrane protein (32).

In addition to phosphorylation-acylation interplay, there is also 'competition' by different cysteine modifications. Several studies have shown changes in protein *S*-acylation following cell treatment with nitric oxide donors (10, 13, 97). However as intracellular diffusion of NO is limited, physiologically-relevant protein nitrosylation (which occurs on cysteine residues) might be limited to substrates that are in close proximity to nitric oxide synthase. In this regard, a recent study highlighted mutually competitive effects of *S*-acylation and S-nitrosylation on Cys-3 and Cys-5 of PSD95, a protein that binds directly to neuronal nitric oxide synthase (nNOS) (102). *S*-acylation of PSD95 on Cys3/Cys5 is essential for synaptic targeting of this protein and neurotransmitter receptor clustering (52). PSD-95 acts as a scaffold to couple Ca^{2+} influx through NMDA channels to activation of nNOS activity (18, 218). Physiological production of NO was shown to lead to S-nitrosylation of Cys3/Cys5 and a decrease in PSD95 S-acylation and subsequent synaptic clustering. These effects were blocked by inhibition of nNOS (102). Conversely, depletion of zDHHC8, which was reported to *S*-acylate PSD95 (171), led to an increase in PSD95 nitrosylation (102). This study thus highlights an intriguing reciprocal regulation of S-acylation and S-nitrosylation that is relevant to synaptic strength and plasticity.

A recent study also highlighted a role for metabolic stress in the regulation of protein *S*-acylation (22). Cardiac tissue from high fat and high sucrose fed mice or cultured arterial endothelial cells treated with high palmitate and high glucose were found to contain significantly reduced levels of *S*-acylated H-Ras and eNOS, and a corresponding reduction in plasma membrane targeting of these proteins. These effects on S-acylation were suggested to be caused by reactive oxygen species generated in endothelial cells in response to metabolic stress. Mass spectrometry analysis revealed that H-Ras from treated endothelial cells was *S*-glutathiolated on either Cys181 or Cys184 following metabolic stress, presumably competing or displacing *S*-acylation at these residues.

V. Tools to analyse S-acylation

With the discovery of the zDHHC family of acyltransferases and recent advances in proteomic techniques to assay S-acylation, progress in the field has begun to accelerate. However, relative to other post-translational modifications, such as phosphorylation, the range of pharmacological, proteomic and genetic tools to investigate the functional role of S-acylation remains somewhat limited. Thus there is a real need, and opportunity, to develop improved tools to interrogate and manipulate S-acylation to develop our understanding of the physiological function of this PTM in health and disease.

V.i. Assays of S-acylation

Although no 'consensus' sequence for S-acylation exists a number of freely available prediction algorithms, such as CSS-palm v4.0 (203), provide an *in silico* platform to inform experimental approaches for candidate targets.

Until relatively recently, radiolabelled palmitate (typically ^{3}H but also ^{14}C and ^{125}I-labelled) incorporation (metabolic labelling) has been the mainstay for experimental validation of protein *S*-acylation over the last four decades. However, while this tool has been instrumental in studying the *S*-acylation of individual proteins it is not suitable for studying protein acylation in intact tissues, suffers from an inherent lack of sensitivity (detection can take months) and is not readily amenable to higher throughput proteomic analysis. To overcome many of these issues a range of alternative metabolic (lipid centric) labelling approaches and indirect assays of cysteine accessibility (cysteine centric) assays have been developed that are complimentary approaches.

Lipid centric (metabolic) labelling assays: Metabolic labelling approaches are most suited to analysis of isolated cells, rather than tissues, but provide information on dynamic palmitoylation of proteins during the relatively short (~4h) labelling period and insight into the species of lipid bound to cysteine residues. Alternative labels have now been developed

including a variety of biorthagonal lipid probes (for reviews see (30, 83-85, 157, 250) such as the commercially available 17-octadecynoic acid (17-ODYA), that can be used in both fluoresecent imaging and protein purification assays. These labels are modified fatty acids, with chemical reactive groups, such as an alkyne or azide group, that are incorporated into S-acylated proteins by the zDHHC enzymes. Using Staudinger ligation or 'click' chemistry a range of fluorophores or protein capture reagents can then be conjugated to the reactive group. In particular, development of a family of ω-alkynyl fatty acid probes of different chain lengths (such as Alk-C16 and Alk-C18) have been exploited for proteomic profiling as well as single cell imaging with improved sensitivity (63) and have been used for proteomic profiling of S-acylated proteins in a variety of cell lines (see (31, 83, 156, 157, 278, 282). In all these metabolic labelling assays it is essential to discriminate incorporation via S-acylation, through a labile thioester linkage to cysteine residues, rather than lipid incorporation via other mechanisms including: onto free N-terminal cysteines of proteins via an amide linkage (N-palmitoylation), addition via an oxyester linkage to a serine residue (O-palmitoylation and oleoylation) as well as myristate via amide linkages on glycine residues (84, 144, 219). The most common method to discriminate S-acylation is to use hydroxylamine cleavage (at neutral pH) of the lipid: only lipids attached via a thioester bond (s-acylation) are cleaved.

Cysteine centric (cysteine accessibility) assays: Acyl-biotin exchange (ABE) and resin-assisted capture (Acyl-RAC): To circumvent the limitations of the labelling approach, which requires metabolic incorporation of lipid typically to isolated cells, a number of related approaches have been developed that exploit the exposure of a reactive cysteine following hydroxylamine cleavage (at neutral pH) of the cysteine-acyl thioester linkage. The newly exposed cysteine thiol can then react with cysteine-reactive groups (such as biotin-BMCC or biotin-HPDP used in the ABE approach (43-45, 265)) or thiopropyl sepharose (used in Acyl-RAC (56)) typically to allow purification of S-acylated proteins. While it must be remembered that these assays do not determine the nature of the bound lipid, rather the presence of a cysteine with a cleavable thioester linkage, these approaches have been exploited to

determine the 'S-acylated proteome' in a number of species and tissues (118, 122, 156, 162, 265, 266, 268, 276, 282). These methods have also been adapted to allow more quantitative labelling *in vivo* (266) and have been reported to improve detection of high molecular weight proteins. Cysteine accessibility approaches determine the net amount of pre-existing S-acylated proteins; however, caution is required to eliminate false positives, in particular it is necessary to fully block all reactive cysteines prior to hydroxylamine cleavage.

In conjunction with site–directed mutagenesis of candidate S-acylated cysteine residues, the lipid- and cysteine- have provided substantial insight into the diversity of proteins that are S-acylated. These approaches can be refined to determine peptides that encompass the S-acylated cysteine. However, mass spectrometry–based approaches to identify the native lipid specifically bound to S-acylated cysteines remain a significant challenge, in particularly for low abundance proteins such as membrane proteins (114, 128, 129, 158, 234). Similarly, tools widely available for other PTMs such as phospho-specific antibodies, to allow assay in tissue sections for example, are not available in the S-acylation field. A recent novel approach has been the exploitation of an intrabody that recognises the S-acylation induced conformational changes in the scaffolding protein PSD-95, rather than S-acylation *per se* and this can be used as an indirect measure of S-acylation in cells (62).

V.ii. Pharmacological manipulation of S-acylation

Although significant advances have been made with respect to assays for detecting S-acylated proteins, the pharmacological toolkit to manipulate S-acylation in vitro and in vivo remains very limited, compared to many other PTMs. For example, the palmitate analogue 2- bromopalmitate (2-BP), that is very widely used for functional analysis of S-acylation, should be used with caution, even though it remains our best pharmacological inhibitor of zDHHCs (207, 289). Unfortunately, 2-BP does not show selectivity toward specific zDHHC proteins (113); also inhibits acylthioesterases (192); is a non-selective inhibitor of lipid

metabolism and many proteins with a membrane accessible cysteine residue (see for example, (39)) and; at high concentrations has many pleiotropic effects on cells, including cytotoxicity (207). Other, less widely used, lipid inhibitors include cerulenin, which affects many aspects of lipid metabolism, and tunicamycin that also inhibits N-linked glycosylation (207). Although some non-lipid inhibitors have been developed, these are not widely used (46, 113) and there are currently no known activators of zDHHCs or compounds that inhibit specific zDHHCs. In the last few years, a number of inhibitors for the acylthioesterases APT1 and APT2 have been developed (3, 12, 40). However, several of these compounds, such as palmostatin B, are active against several members of the larger serine hydrolase family. Clearly, the development of novel S-acylation inhibitors and activators that display both specificity and zDHHC selectivity would represent a substantial advance for investigation of S-acylation.

V.iii. Genetic tools and models

Since the seminal discovery of the mammalian enzymes that control S-acylation (61) both overexpression and knockdown strategies have been developed to interrogate S-acylation. Overexpression studies have predominantly exploited expression of candidate zDHHCs,and their catalytically 'dead' DHHS mutants, in heterologous expression or native systems and analyzed increases in ^{3}H-palmitate incorporation to define zDHHCs that may S-acylate specific proteins. Although this is a powerful approach, caution is required as overexpression can result in S-acylation of cysteine residue(s) that are not endogenously S-acylated in native cells (248). Increasingly, knockdown of endogenous zDHHCs using siRNA, and related approaches, is beginning to reveal the identity of zDHHCs that S-acylate native proteins. However, relatively few studies have taken a systematic knockdown approach to identify zDHHCs (for examples see (132, 248, 249)). Furthermore, as some zDHHCs and APTs are themselves palmitoylated, the functional effect of overexpressing or knocking down individual zDHHCs on the localization and activity of other zDHHCs, and APTs, must also be carefully determined. For example, siRNA-mediated knockdown of zDHHC 5, 7 or 17

in HEK293 cells paradoxically results in an upregulation of zDHHC23 mRNA expression (249). An important, caveat of many of these approaches is the lack of tools to analyse native zDHHC protein expression due to the lack of high quality antibodies for western blotting and immunocytochemistry for most zDHHCs. Increasingly, a variety of mouse Gene-trap and global as well as conditional knockout models for some zDHHCs are becoming available although full phenotypic analysis is limited in most cases (see section III.iv). Similar overexpression and knockdown approaches have also started to be exploited to interrogate APT function.

VI. Diversity of proteins regulated by S-acylation

As highlighted in the previous sections, an eclectic array of proteins from viruses to man (Figure 5), have been reported to be S-acylated and the 'catalogue' is ever expanding with the increasing number of S-acylation (palmitoyl) proteomic screens (30, 83, 108, 118, 122, 139, 155-157, 162, 265, 266, 268, 276, 278, 282). Furthermore, available predictive tools suggest a large proportion of the cellular proteome may be S-acylated.

In this section we highlight a number of cellular pathways and protein families in eukaryotes that have been shown to be S-acylated. While not exhaustive, we attempt to emphasise the diverse array of physiological processes and mechanisms that may be regulated by S-acylation to reveal both the opportunities and challenges for investigators entering the field.

VI.i Membrane receptors and signalling

Multiple types of cellular signalling receptors are now known to be regulated by S-acylation, from classical G-protein coupled receptors to the more recent insights into membrane receptors for steroid hormones.

VI.i.a G-protein coupled receptor (GPCR) signalling

Since the 'prototypical' G-protein coupled receptor, rhodopsin, was shown to be S-acylated more than 30 years ago (180, 185) the large majority of GPCRs examined have been shown to be S-acylated at typically one, two or three residues in the C-terminal cytoplasmic tail following the last transmembrane domain (for reviews see (37, 201). Notable exceptions include the GnRH receptor and a Thromboxane A2 splice variant that lack the conserved cysteine residues. However, although the S-acylated cysteines are conserved, and S-acylation has been proposed to generate additional 'loops' in the C-terminus for many GPCRs, the effect of S-acylation on particular GPCRs cannot be easily predicted and the reported functional effects are as diverse as the family of GPCRs and their agonists. Indeed. In the β1-adrenoreceptor different sites of S-acylation in the C-terminal tail turn over at different rates and control distinct functions (294).

S-acylation has been reported to control tertiary structure including by controlling receptor dimerization in β2-adrenergic and μ-opioid GPCRs (36, 290). Furthermore, recent insights from mice lacking S-acylated rhodopsin, via knock-in mutations of the S-acylated cysteines, reveals an important structural role for S-acylation by controlling the stability of rhodopsin, rather than its signalling via G-proteins, *in vivo* (153). S-acylation is required for correct maturation in the ER, subsequent trafficking to the plasma membrane and targeting to 'lipid raft' domains for several, but not all, GPCRs. Moreover, efficient coupling between agonist binding and G-protein activation is reported for several GPCRs, including adrenalin stimulation of the β2-adrenoreceptor through Gs proteins to activate adenylate cyclase. Here, the S-acylation deficient β2-adrenergic receptor C341G mutant is less efficient at stimulating adenylate cyclase activity than the wild type protein (181), an effect due to the C341G mutant being hyperphosphorylated by PKA and thus resembling a desensitised receptor, again demonstrating important cross-talk between distinct signalling pathways (169, 170). Moreover, for some GPCRs S-acylation can act as a switch to allow differential coupling with different effectors. In the V2 vasopressin receptor, S-acylation is required for stimulation of the MAPK pathway following receptor internalisation (29) and in the endothelin

A receptor is required for coupling through Gq to regulate phospholipase C (103). In both cases, S-acylation did not affect coupling to adenylate cyclase through Gs. In contrast, de-acylation of the proteinase-activated receptor 2 promotes activation of the ERK pathway highlight diverse effects of S-acylation of GPCR coupling (17). Agonists can also modify the S-acylation status of GPCRs (148) and S-acylation controls receptor desensitization and internalisation.

Recent studies reveal how S-acylation-dependent control of GPCR signalling is potentially even more complex as both G-proteins, multiple effectors as well as a range of molecules that regulate GPCR signalling may also be S-acylated.

The Galpha subunits of heterotrimeric G-proteins themselves are S-acylated and S-acylation is required for plasma membrane localisation (37, 201). For example, zDHHC3 & 7 overexpression robustly S-acylated Gq, Gs and Gi subunits expressed in HEK293 cells. S-acylation controls Gq shuttling between the Golgi, where Gq is S-acylated, and the plasma membrane with inhibition of Gq S-acylation blocking α1-adrenergic signalling through Gq (255).

Several members of two important protein families that control GPCR function are also S-acylated: members of the GPCR kinase (GRK) family important in the control of GPCR desensitization and the regulator of G-protein signalling (RGS) proteins that regulate the activity of G-proteins themselves, by accelerating GTP hydrolysis, and terminating Gi/o signalling by speeding Gi/o deactivation at the plasma membrane (37, 201).. As examples, GRK6 is S-acylated at a cluster of three cysteine residues in the C-terminus that promotes its targeting to the plasma membrane and stimulates kinase activity thus enhancing GPCR desensitization (117, 238). Several members of the RGS family are S-acylated at the N-terminus however the role of S-acylation differs between family members with both positive and negative effects on GTPase activity reported. However, S-acylation also indirectly regulates some members of the RGS family. For example, membrane targeting of RGS-R7

is controlled through assembly with an S-acylated R7 binding protein (R7-BP), that also acts as an allosteric activator, to control G-protein coupled potassium channel activity (115). In neurones, R7-BP S-acylation is dynamically controlled by zDHHC2, furthermore R7-BP S-acylation is increased upon activation of Gi-linked GPCRs through slowing of de-acylation R7-BP revealing an intimate interplay between G-protein signalling and control of G-protein activity via S-acylation of R7-BP (116). In addition, several downstream effectors of G-proteins, including some members of the Rho guanine nucleotide exchange factor (RhoGEF) family, are also S-acylated. For p63RhoGEF, a Gq regulated RhoGEF, S-acylation of a conserved cluster of cysteine residues (C23, 25 & 26) in the N-terminal domain target p63RhoGEF to the plasma membrane. This constitutive targeting (unlike most other RhoGEFs that are sequestered in the cytosol) to the plasma membrane promotes basal activity of p63RhoGEF as mutation of the S-acylated cysteines reduces activity and re-localises it to the cytoplasm (9).

Clearly defining the network of S-acylation within the GPCR-signalling complex will remain a significant, yet important challenge, to identify key principles and nodes that are critical for signalling by this very important class of cell-surface receptors.

VI.i.ii Membrane receptors for steroid hormones

The 'classical' steroid receptors exist predominantly in the nucleus where they mediate both ligand-dependent and independent control of gene transcription. However, for many steroid receptors, including the oestrogen (ERα), progesterone PR) and androgen (AR) receptors, a significant fraction of the receptor may be localised at the plasma membrane where they can mediate non-genomic steroid signalling cascades. The membrane targeted fraction of these receptors are S-acylated, at a single cysteine residue, in the ligand binding domain of the receptor (191) by zDHHCs 7 and 21 (189). S-acylation is required for both membrane targeting as well as the rapid, non-genomic effects of these steroids. For example, site

directed mutation of the S-acylated cysteine to alanine in the ERα receptor, prevent ERα□□□□□ing □□□□□plasma membrane associated □□□□□□□□□□coupled receptor that controls non-genomic regulation of the MAPK/ERK and PI3Kinase/AKT signalling pathways. In contrast, de-acylation of ERα promotes□□□□□□□□□□□□□□□□□□□□ ERα degradation and abrogates oestrogen induced gene transcription revealing cross-talk between the non-genomic and genomic mechanism of action of oestrogen (130). Recent analysis of mice with knock-in mutations of the S-acylated cysteine in ERα, to alanine, reveal discrete developmental and physiological functions controlled via the membrane (S-acylated) ERα receptors including in the female reproductive, endocrine and vascular systems (4, 190). The *in vivo* roles of PR and AR S-acylation remain to be fully explored. Furthermore, whether other steroid receptors, that also have the conserved cysteine in the ligand binding domain, and have been reported to have membrane mediated effects (e.g receptors for cortisol) are also regulated by S-acylation remains to be examined.

S-acylation is also critical for the rapid non-genomic effects of thyroid hormone (TH) signalling via a novel membrane TH receptor, that lacks the conserved cysteine residue seen in the ER and other steroid receptors above (119). In this case, the membrane receptor is generated by translational initiation from an internal methionine (M150) of the ligand-activated transcription factor receptor TRα. This produces a receptor (p30 TRα1) that is unable to stimulate transcriptional but is instead targeted to caveolae through S-acylation of two cysteine residues C254 and C255 in osteoblasts. S-acylation of p30 TRα1 is essential for the non-genomic actions of thyroid hormone as signalling through the NO/cGMP signalling cascade to ultimately control extracellular signal related kinase (ERK) and Akt was abolished in mutants deficient for S-acylation. Thus this novel receptor is required for the rapid non-genomic signalling of thyroid hormone and the control of cell survival and proliferation.

VI.ii Membrane and vesicle trafficking

Vesicle trafficking, exocytosis and endocytosis are fundamental mechanisms for controlling bulk trafficking of proteins in cells as well as the control of intercellular communication through the release of hormones and neurotransmitters. S-acylated proteins are involved in both vesicle translocation as well as fusion/retrieval from membranes.

VI.ii.a Vesicle trafficking and exocytosis

The fusion of vesicles to their target membrane requires assembly of SNARE complex proteins to promote membrane fusion. In neurones multiple components of the vesicle fusion machinery are S-acylated including both the pre-synaptic membrane SNARE proteins syntaxin and SNAP25, the vesicle protein synaptobrevin (VAMP2) as well as the calcium sensor for fusion Synaptotagmin 1 (see (122, 200). S-acylation controls multiple aspects of protein sorting and localisation to membrane microdomains for these proteins.

Multiple members of the syntaxin family, including both neuronal (e.g syntaxin 1) and non-neuronal (e.g syntaxin 11) expressed syntaxins, have been reported to be S-acylated although in most cases the functional role is very poorly understood. The endosomal syntaxin 7, S-acylated at a cysteine residue (C239) juxtaposed to its single transmembrane domain is important for trafficking between endosomal and plasma membranes as its S-acylation deficient mutant is trapped on the plasma membrane (90). Recent analysis of the atypical syntaxin, syntaxin 11, that lacks a transmembrane domain, reveals an important role for S-acylation in membrane targeting, and localisation of syntaxin11 to the immunological synapse in natural killer (NK) cells and association of loss of S-acylation dependent targeting in familial hemophagocytic lymphohistiocytosis type 4 (FHL-4) (92). Syntaxin 11 is widely expressed in immune cells and in NK cells is required for secretory lysosome exocytosis, required for elimination of pathogens or tumor cells a function severely abrogated in FHL-4. In NK cells, Syntaxin 11 is S-acylated at a cluster of cysteine residues at the C-terminus of the protein, a region that is lost due to frameshift mutations (e.g Q286X) in patients with

FHL-4. Syntaxin that is not S-acylated does not bind membranes resulting in disruption of its interaction with the Sec-1/munc18-like protein Munc18-2 at the immunological synapse in activated NK cells.

SNAP25, and the related non-neuronal protein SNAP23, are both peripheral membrane proteins that interact with membranes through S-acylation of multiple cysteines in cysteine rich domains of the proteins. SNAP25, plays a role in both synaptic and endosomal membrane fusion events and its targeting to membranes is controlled by S-acylation of a cluster of four cysteine residues C85, C88, C90 & C92, predominantly by zDHHCs 3, 7 and 17 in the Golgi network (75). Importantly, the number of cysteine residues of SNAP25 that are S-acylated appears to act as a code to specify the precise sub cellular distribution in the recycling pathway between recycling endosomes, the trans Golgi network (TGN) and the plasma membrane (73). For example, in neuroendocrine PC12 cells mutation of any individual S-acylated cysteine to leucine enhanced SNAP25 localisation at recycling endosomes and the TGN although mutation of either C88 or C90. had the greatest effect. As SNAP25 S-acylation is dynamic this suggests that controlling the extent of SNAP-25 S-acylation is a key determinant in defining its localisation and supports multiple lines of evidence that S-acylation is not a simple membrane anchor. Moreover, the number of S-acylated cysteines in both SNAP25 and SNAP23 are important determinants in the role of these proteins to support secretion in neuroendocrine cells. For example, engineering an additional cysteine into SNAP25 (to have 5 rather than 4 S-acylated cysteines) results in reduced stimulated secretion compared to wild-type whereas in a converse experiment deleting a cysteine in SNAP 23 (so it has 4 rather than the normal 5) results in enhanced secretion (214).

Cysteine string protein α (CSPα) is a ubiquitously expressed DnaJ chaperone protein that regulates secretory vesicle dynamics likely by stabilising multiple synaptic proteins including SNAP25, and represents one of the most heavily S-acylated proteins per mole. S-acylation of CSP occurs on multiple residues in a core cysteine-rich domain. Mutations within this

cysteine rich domain (L115R or ΔL116), recently shown to cause the neurodegenerative disorder adult-onset neuronal ceroid lipofuscinosis, result in aggregation of CSPα (76). The mutations mistarget CSPα resulting in aggregates of mutant CSPα that are membrane bound with incorporated palmitate. Although oligomerization of mutant CSPα can also occur in the absence of S-acylation (286) chemical de-acylation solubilises the aggregates in cell lines and in post-mortem brains of patients suggesting that CSPα S-acylation of these mutants is a major driver of the onset of this disease and aggregate formation (76).

Synaptobrevin is S-acylated at a single cysteine residue within its transmembrane domain and although the functional role of S-acylation is not known S-acylation of synaptobrevin may be developmentally regulated as synaptic vesicles prepared from adult rat brain efficiently incorporated ^{3}H-palmitate whereas vesicles isolated from embryonic brains did not (259).

Synaptotagmins represent a large family of calcium-sensors for vesicle fusion with a single transmembrane domain. The major neuronal isoform synaptotagmin 1 is S-acylated at multiple cysteine residues at the boundary between the transmembrane domain and cytosolic domain. In neurones, synaptotagmin1 S-acylation is enhanced by inhibiting synaptic activity and mutation of the S-acylated cysteines results in enhanced surface expression and diffuse localisation rather than punctate localisation to presynaptic membranes (121) suggesting a role of S-acylation in clustering and stability at the plasma membrane. Synaptotagmin VII (Syt VII) is an example of a more broadly expressed synaptotagmin that is localised to lysozomes, as well as other compartments, in a variety of cells including macrophages. In bone marrow macrophages from SytVII knockout mice, phagocytosis, which is dependent upon lysosomal membrane delivery to the cell surface, is disrupted and this could be restored by overexpression of wild-type but not S-acylation deficient mutants (55). Wild-type SytVII was correctly targeted to lysozomes where it is assembled in clusters with the tetraspanin CD63 whereas S-acylation deficient SytVII

mutants localised at the Golgi and did not assemble with CD63. S-acylation dependent assembly of SytVII with CD63 is required at the level of the Golgi to allow SytVII to traffic to lysozomes directed by the lysosomal targeting motifs of CD63.

VI.ii.b Microtubule-mediated trafficking

S-acylation has also been implicated in the control of dynein-dependent motor protein function that controls multiple aspects of cellular function: from long range transport of cargo vesicles to mitosis. The anterograde motor protein dynein is an important microtubule associated motor protein and is regulated by a protein complex composed of the proteins LIS1 and the evolutionary conserved NUDE proteins Ndel1 and Nde1. Both Ndel1 and Nde1 are S-acylated, with zDHHCs 2, 3 and 7 the major candidates, most likely at multiple sites although in both proteins conserved C273 in the Ndel1 dynein binding domain appears to be the major residue (227). However the effect of S-acylation is divergent between Ndel1 and its paralog Nde1. For Ndel1, S-acylation reduces the ability of Ndel1 to interact with cytoplasmic dynein that results in reduced dynein activity and hence disruption of a number of key dynein–dependent processes including maintenance of the Golgi apparatus, trafficking from ER to Golgi and long range vesicular trafficking in neurites. In contrast, S-acylation of Nde1 does not significantly affect dynein activity or function. Importantly, S-acylation of Ndel1 is involved in neuronal migration both *in vivo* and *in vitro*. *In vivo* (using *in utero* electroporation), expression of a dominant negative mutant of zDHHC7, or the C273S Ndel1 mutant, resulted in reduced neuronal migration in the developing cortex (227).

VI.ii.c Massive endocytosis

Endocytosis of transmembrane proteins may occur through multiple mechanisms including the ‘classical’ clathrin-dependent pathway as well as clathrin-independent pathway. For a variety of proteins, clathrin-independent endocytosis may occur through S-acylation dependent partitioning of the protein into cholesterol and sphingolipid rich lipid raft microdomains. Recently, a mechanism (massive endocytosis, MEND) that promotes large scale (e.g up to 50%) endocytosis of such ordered plasma membranes has been described

to be, in part, triggered by S-acylation in cardiac myocytes and other cells (100, 143). MEND is activated in cardiac cells upon calcium influx or insults that that result in elevations in acyl-CoA in the cytoplasm, along with activation of PKC. These conditions are manifest following re-oxygenation of cardiac cells following acute ischaemic events, for example during heart attacks. Thus reoxygenation of anoxic cardiac myocytes initates MEND, a process that is proposed to result from formation of permeability transition pores in the mitochondrial membrane resulting in release of Coenzyme A into the cytoplasm. The release of Coenzyme A results in a rapid increase in acyl-CoA providing an unlimited supply of acyl-CoA for zDHHC enzymes with consequent enhanced S-acylation of multiple membrane proteins including phospholemman and flotillin. The increased S-acylation of such proteins is then hypothesised to result in clustering and association of these proteins in ordered lipid raft domains and thus promoting endocytosis. Although the precise mechanisms that result in such massive membrane internalisation remain to be established, knockdown of zDHHC5 in cardiac myocytes (via siRNA or using myocytes from a mouse with a hypomorphic allele of zDHHC5 largely prevented MEND (143). Furthermore, hypomorphic zDHHC5 mice retained ventricular function following ischaemia suggesting that zDHHC5 mediated S-acylation is a critical check-point in MEND and reperfusion injury in the heart.

VI.iii Ion channels and Transporters

S-acylation has been reported to control a diverse array of ion channels as well as transporters that move ions, lipids or other solutes across membranes. S-acylation is important for controlling both channel/transporter activity as well as trafficking to target membranes, effects that are mediated by S-acylation of the pore/transporter subunits or associated regulatory/accessory subunits. Recent extensive reviews have highlighted the diversity of ion channels and mechanisms/properties regulated by S-acylation and thus here we focus on a few select examples to illustrate key points (225, 226, 246).

VI.iii.a Ion channels

Since the discovery of the first S-acylated ion channels more than 25 years ago (220, 239) more than 50 different ion channel subunits have been reported to be S-acylated (for reviews see (225, 226, 246). S-acylation has been reported to control all aspects of the ion channel life cycle from assembly, trafficking, kinetics and regulation by other PTMs at the destination membrane to degradation and sensitivity to toxins and pharmacological agents. Importantly, ion channels have been shown to be regulated by S-acylation of pore forming subunits as well as an array of regulatory subunits that control channel trafficking and function. To illustrate some of the key features and diverse roles S-acylation plays in ion channel function two exemplars from the ligand-gated and voltage gated ion channel families will be discussed: the glutamate activated α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors and large conductance calcium- and voltage activated potassium (BK) channels, respectively.

AMPA receptors (AMPAR) are critical determinants of glutamatergic signalling in central synapses and the control of AMPAR surface expression and function key to mechanisms of synaptic plasticity. The pore forming subunits, encoded by four genes Glu1-4, are S-acylated at two distinct sites. S-acylation of a single cysteine residue in Glu1, that is located adjacent to a cytosolic hydrophobic segment between two transmembrane domains, promotes retention of AMPAR at the Golgi apparatus (86). In contrast, S-acylation of a cluster of cysteine residues in the cytosolic C-terminus of GluA1, just downstream of the last transmembrane domain, blocks PKC-mediated phosphorylation of GluA1 likely through a mechanism of steric hindrance (142). This S-acylation-dependent regulation of PKC phosphorylation prevents GluA1 binding to its cytoskeletal adapter protein 4.1N and reducing AMPAR insertion into the plasma membrane. The functional diverse effects of S-acylation at two distinct site within the same pore-forming subunit are also observed for BK

channels. The pore forming subunit of BK channels encoded by the single gene KCNMA1, is again S-acylated at two sites. Firstly, S-acylation of a cluster of cysteine residues (C53 & 56) in a cytosolic loop between the first two transmembrane domains is required for efficient trafficking of the BK channel from the ER to Golgi and subsequently the cell surface (110, 249). In contrast, S-acylation of a BK channel C-terminal splice variant (STREX) in a cysteine-rich cytosolic domain acts as a switch to specify STREX BK channel regulation by AGC-family protein kinases (247). Channels S-acylated in the STREX insert are inhibited by PKA-dependent protein phosphorylation but are insensitive to PKC regulation. In contrast, de-acylation of the STREX insert switches the channel from being inhibited by PKA to being inhibited by PKC (292), a mechanism proposed to involve accessibility of the PKC phosphorylation site only when the STREX domain is not S-acylated and hence dissociated from the plasma membrane.

Importantly, in both AMPAR and BK channels the distinct sites of S-acylation appear to be under control of distinct members of the zDHHC family. How, zDHHCs discriminate between these distinct sites is as yet unknown.

While S-acylation of the pore-forming subunits can have differential effects, the diversity of effects of S-acylation on channel function is also illustrated in these two channel types through S-acylation of distinct regulatory subunits and adapter proteins. For example, S-acylation of the AMPAR interacting protein PICK1, retains endocytosed AMPAR in intracellular compartments and thus contributes to long-term depression of glutamatergic synapses (245). Another AMPAR interacting protein Grip1b, that is also S-acylated by zDHHC 5 & 8 as for PICK1, accelerates local recycling of AMPAR to the plasma membrane in dendritic spines through interaction of S-acylated Grip1b with dendritic trafficking vesicles (244). Moreover, S-acylation of another AMPAR interacting adapter protein, PSD-95, in dendritic spines controls AMPAR clustering at the postsynaptic membrane (62). The role of regulatory subunits in BK channels is likely to be as complex. For example, S-acylation of the regulatory β4-subunit, at a single juxtatransmebrane cysteine residue C193, is required

for the up-regulation of BK channel surface expression when β4-subunits are expressed with pore-forming subunits in neurones through control of ER exit. Intriguingly, this S-acylation dependent mechanism is only manifest with specific C-terminal splice variants of the pore-forming subunit illustrating how surface expression may be finely and differentially tuned in different cell types (33). Such mechanisms likely contribute to the diverse roles that BK channels play in an eclectic range of physiological process: from control of neuronal excitability to endocrine, vascular and renal function.

A recent intriguing study has also revealed a role for S-acylation in controlling trafficking of the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a chloride and bicarbonate channel that is central to the pathogenesis of the enhanced mucus lining the lung in cystic fibrosis (159). Several disease causing mutations of CFTR, including F508del, result in reduced, but not abolished, trafficking to the cell surface as deletion of Phe^{508} results in misfolded CFTR that is recognised for degradation by ER-associated degradation (ERAD). Rescue of F508del surface expression by small molecule correctors allows functional rescue as the F508del mutant retains partial transport activity at the plasma membrane. Both wild-type CFTR and the F508del mutant are S-acylated, with zDHHCs 3 and 7 likely candidates, resulting in increased steady-state levels of the protein. S-acylation promotes biogenesis of core-glycosylated CFTR promoting trafficking of both wild-type and F508del mutant channels from the ER to the Golgi where they are sequestered. It is likely that additional de-acylation steps are required for efficient transport to the cell surface from the Golgi. However, manipulation of mutant CFTR S-acylation, in conjunction with established small molecule enhancers of trafficking/function may provide a new strategy for treatment of this disabling disorder.

VI.iii.b Ion, solute and lipid transporters

A wide variety of neurotransmitter, ion, solute and lipid transporters have also now been revealed to be regulated by S-acylation and below we highlight some key examples.

Dopamine transport is essential in controlling synaptic levels of dopamine and consequent regulation of a wide range of nervous system functions including in emotion, reward and motor pathways. The efficiency of active reuptake of dopamine from the extracellular space, back into presynaptic nerve terminals in central synapses, by the dopamine transporter (DAT) is enhanced by S-acylation of the transporter at at least two sites, including a cysteine (C580) at the intracellular end of transmembrane 12 (57). Acute pharmacological inhibition of S-acylation, using 2-BP, reduced transporter V0.5max in synaptosomes without affecting transporter expression or surface membrane expression. However chronic inhibition, or site-directed mutagenesis of C580 to serine, resulted in enhanced receptor degradation and enhanced protein kinase C-dependent down-regulation of transporter function (57). Dopamine transporters are targets for both therapeutic drugs and drugs of abuse, and thus both short and long term effects of S-acylation may have major impacts on dopamine neurotransmission and dopaminergic disorders. However, whether DAT S-acylation is compromised in disease states, such as Parkinson;s and schizophrenia, or can be targeted to enhance dopamine transport remains to be examined.

The ATP-binding cassette transporters ABCA1 and ABCG1 are both found at the plasma membrane, where they transport cholesterol out of the cell to apoliporotein A1 and subsequent formation of high density lipoprotein (LDL) (79, 231). In both cases S-acylation is required for efficient trafficking of the transporter from the ER to plasma membrane thus transporter deacylation results in reduced cholesterol export in a variety of cell types. ABCA1 is S-acylated on 5 cysteine residues, with two residues within the intracellular N-terminus (C3 and C23) and a dicysteine motif (C1110 and C1111) in the large intracellular linker between transmembrane domains 6 and 7. Mutation of any cysteine has similar effects on ABCA1 surface expression and cholesterol efflux suggesting the cysteine residues play similar functions (231). ABCA1 is S-acylated by multiple zDHHCs although zDHHC8 shows the most robust S-acylation in overexpression assays. Moreover, zDHHC8 overexpression up regulates ABCA1 transporter function (231). In contrast, ABCG1 is S-acylated at 5

residues although residue C311 appears to be primarily responsible for controlling trafficking and cholesterol efflux (79). This again highlights that cysteines at distinct locations with proteins can display similar or distinct functions. The apolipiporoteins are also S-acylated and S-acylation of ApoB at C1085 is important for its intracellular sorting and formation of LDL (288). Whether dysregulation of ABC transporter S-acylation is associated with disruption of cholesterol transport in a range of disorders including artherosclerosis, Macula degeneration and some cancers remains to be determined.

The glucose transporters Glut1 and Glut4 are both reported to be S-acylated and the level of S-acylation changes in diet-induced models of hyperglycaemia or obesity respectively (198, 205). However, although the functional role of Glut1 or 4 S-acylation is not fully understood, S-acylation plays an important role in controlling insulin dependent Glut4 translocation to the plasma membrane via indirect mechanisms. Several components important for Glut4 vesicle trafficking, including cytoplasmic linker protein 170-related 59 kDa (ClipR59), involved in regulating glucose homeostasis and peripheral insulin sensitivity, are S-acylated in adipocytes. ClipR-59, acts as a scaffold to drive phosphorylation of the Rab GTPase activating protein AS160 (that is also S-acylated Ren:2013fa) by the protein kinase Akt to drive insulin-dependent recruitment of Glut4 to the plasma membrane. S-acylation of two cysteine residues (C534 and C535) of ClipR-59 promotes membrane interaction and allows ClipR-59 to recruit the kinase Akt to the plasma membrane (206). ClipR-59 assembles with, and is predominantly S-acylated by, zDHHC17. Knockdown of zDHHC17 prevents ClipR-59 and Akt recruitment to the plasma membrane and impairs insulin-induced Glut4 translocation to the plasma membrane. As multiple components required for Glut 4 trafficking, including Glut 4 itself, are S-acylated this PTM may play a complex role in Glut4 translocation and insulin signalling.

Phospholemman (FXYD1) is a transmembrane protein that is a major regulatory subunit of the sodium pump (Na/K ATPase) function in cardiac cells and a major target for PKA and PKC mediated regulation of the pump. FXYD1 may be S-acylated at two cysteines (C40

and C42) in its short intracellular N-terminus (256). S-acylation has neo effect on FXYD1 membrane expression but S-acylation increases the half-life of FXYD1 expression. Importantly, S-acylation was essential for FXYD1 to mediate inhibition of pump activity: non-S-acylated FXYD1 was unable to inhibit pump activity. Furthermore, PKA dependent phosphorylation of FXYD1 downstream of the sites of S-acylation promoted FXYD1 S-acylation. Other members of the FXYD family are also predicted to be S-acylated and thus may control pump activity in other tissues via a S-acylation dependent mechanism.

An intriguing function of S-acylation in transporter function is to control distinct functions of phospholipid scramblases (PLSCR) 1 & 3. PLSCR1 is a calcium-binding protein that controls phospholipid transfer across the plasma membrane. PLSCR1 contributes to the reorganisation of lipids in a number of cell types including platelets and red blood cells in particular in response to elevated intracellular calcium in response to cell injury or apoptosis. PLSCR1 is multiply S-acylated in a core –^{184}CCCPCC- motif that targets it to lipid raft domains and is essential for trafficking to the plasma membrane (267)**.** In contrast, de-acylated PLSRC1 is targeted to the nucleus where it can bind DNA and activate transcriptional cascades. Under normal conditions the large majority of PLSCR1 is located in the plasma membrane or endosomal membranes. The ability of S-acylation to act as a switch of PLSCR1 function is important following the rapid upregulation of PLSCR1 expression following exposure to cytokines when newly synthesised PLSCR1 rapidly accumulates in the nucleus. Intriguingly S-acylation also appears to play a role in differential targeting of the scramblase PLSCR3: in this case to the mitochondrion, rather than the nucleus. S-acylation of a cluster of five cysteine residues (-^{159}CGCSCCPC-) in PLSCR3 is required for mitochondrial localisation in macrophages. At the mitochondrion PLSCR3 transports cardiolipin from the inner to outer mitochondrial membrane that ultimately results in release of cytochrome C into the cytosol and induction of apoptosis. The removal of PLSCR3 from the mitochondrion upon de-acylation reduces apoptosis supporting that S-

acylation of PLSCR3 contributes to the pro-apoptotic function of PSCLR3 in macrophages (162).

VI.iv Signalling adapters and chaperones

The spatial and temporal coordination of cellular signalling cascades is, in large part, orchestrated by a diverse array of signalling adapter and chaperone proteins that reulate the organisation of macromolecular signalling complexes. A large number of such adapter proteins are S-acylated and we are beginning to understand some of the roles of S-acylation in controlling information flow through these signalling networks.

PSD-95 is the most abundant abundant scaffolding protein in the post-synaptic density of glutamatergic chemical synapses where it coordinates the assembly of a variety of ligand-gated ion channels (including AMPA and NMDAR) and other signalling proteins and transmembrane adhesion molecules essential for the correct assembly and function of glutamatergic chemical synapses. PSD-95 is S-acylated at its N-terminus on two residues by multiple zDHHC enzymes including zDHHC2, and is essential for its post-synaptic targeting (61, 252). PSD-95 S-acylation appears to play a primary role in assembly and maturation of dendritic spines through establishment of domains of PSD-95 and its interacting molecules. Recent evidence, exploiting a conformation specific antibody that recognises the S-acylated conformation of PSD-95, revealed local acylation-deacylation cycles of PSD-95, driven by zDHHC2, serve to create nanodomains of clustered PSD-95 that are maintained in the postsynaptic membrane of dendritic spines (62). Importantly, activity-dependent remodelling and expansion of the PSD involves establishment of new PSD-95 nanodomains driven by increases in plasma membrane inserted zDHHC2 (62) that allow recruitment and stabilisation of PSD-95 into the nanodomains. Such PSD remodelling is thought to be central to the mechanisms underlying synaptic plasticity at synapses.

Ankyrin-G is another adapter protein that interacts with multiple transmembrane proteins anchoring them to the sub-membrane network of βII-spectrin and actin. Ankyrin G is S-acylated towards its N-terminus, via zDHHC5 and 8, allowing Ankyrin-G to localise to the lateral membrane of polarized columnar epithelial MDCK cells. (88, 89) Knockdown of zDHHC5 and 8, or site directed mutagenesis of the Ankyrin–G S-acylated cysteine mimics the effect of Ankyrin-G knockout itself: preventing lateral membrane assembly and thus significantly reducing epithelial cell height. Intriguingly, this mechanism is also dependent upon phospholipid interactions with βII-spectrin, that binds to Ankyrin-G, in micron-sized domains in the lateral membrane in which zDHHC5 and 8 are also co-localised (88). βII-spectrin mutants that cannot bind Ankyrin-G or phospholipids also disrupt lateral membrane assembly. This highlights that an important interaction between two distinct lipid–dependent mechanisms is required for lateral membrane assembly: S-acylation of the adapter protein Ankyrin-G and phospholipid binding to its binding partner βII-spectrin. Whether such a mechanism also controls assembly of other Ankyrin-G dependent complexes, such as at the axon initial segment, and how assembly controls lateral membrane assembly, perhaps through control of adhesion molecules, that control intercellular contacts, remains to be explored.

The large family of cAMP-dependent protein kinase A (PKA) anchoring proteins (AKAPs) allow efficient signalling through PKA signalling pathways. Several members of the AKAP family are S-acylated and target PKA (via binding to the regulatory R subunits) to multiple effectors as well as other components of the signalling machinery. For example, the small AKAP15/18 targets PKA to the plasma membrane to promote PKA-dependent regulation of L-type calcium channels in neurones, endocrine and cardiac cells (58, 70). In hippocampal neurones, activity-dependent S-acylation of the multi-functional AKAP79/150 at two sites (C36 and C129) within its N-terminal polybasic domain targets AKAP79/150 to lipid rafts and recycling endosomal membranes in dendrites (123). This recruitment was important for AMPAR recruitment and synaptic plasticity, in part likely due to the role of AKAP79/150 in

coordinating AMPAR phosphorylation, as AKAP79/150 mutants that lack the S-acylation site do not support AMPAR trafficking and synaptic potentiation in vitro. In other systems, this S-acylation dependent recruitment of AKAP79/150 to lipid rafts, from non-raft domains, promotes assembly with the calcium-dependent adenylate cyclase, AC8 (41) to coordinate PKA-dependent phosphorylation of multiple raft proteins upon store-operated calcium entry.

Transmembrane adapter proteins (TRAPs) represent a large and diverse family of proteins that can coordinate signal transduction modules at the plasma membrane (236). A significant number of TRAPs have been reported to be S-acylated, with linker of T-cell activation (LAT) representing a protypical TRAP expressed in T-cells, as well as other cells of the immune system, and important for T-cell receptor signalling. S-acylation of two juxtamembrane cysteine residues (C26 & C29) in LAT are required for its partitioning into lipid rafts with either mutation of the cysteines or pharmacological inhibition of S-acylation disrupting raft partitioning (137). S-acylation of C26,that is closest to the plasma membrane appears to play the dominant role in raft localisation in model systems again pointing to the importance of cysteine positioning for function. Moreover, mutation of C26 increaseed the localisation of LAT on intracellular membranes. Correct raft partitioning is required for assembly of LAT with a range of proteins and assembly of a multimolecular signalling complex. It is likely that S-acylation of other TRAPs plays a similar role.

Calnexin is a transmembrane chaperone localised in the endoplasmic reticulum where it facilitates the folding of nascent chains of glycosylated proteins and preventing their aggregation (see Section III.ii) (132). In addition calnexin plays a role in ER calcium content and the regulation of calcium signalling between the ER and mitochondria required for control of mitochondrial membrane potential (150, 151). Recent studies have implicated S-acylation in controlling these distinct properties, at least in part, by controlling the spatial organisation of calnexin in the ER and the assembly of calnexin with distinct protein complexes. Calnexin is S-acylated at two juxtamembrane cysteine residues, predominantly C502, and appears to be largely catalysed by zDHHC6 (132, 150). S-acylation of calnexin

promotes calnexin localisation to two distinct regions of the ER: i) the perinuclear rough ER (RER) that is the site of the ribosome translocon complex (RTC) and ii) the mitochondria-associated membrane (MAM) that provides communication between the ER and mitochondria. In contrast, de-acylation redistributes calnexin to the tubular ER. At the RER S-acylated calnexin assembles with core components of the RTC leading to recruitment of the actin cytoskeleton and stabilisation of the super-complex (132). S-acylation of calnexin at the RTC is thus essential for the ability of calnexin to perform its chaperone function and capture nascent polypeptides and promote their folding as they emerge from the translocon. Inhibition of zDHHC6 or mutation of the calnexin S-acylatied cysteines disrupts glycoprotein folding. In contrast, at the MAM, S-acylated calnexin assembles with the sarcoplasmic reticulum ca2+-transport ATPase 2b (SERCA2b) and reduces the ability of mitochondria to uptake Ca2+ from the ER (151). De-acylation of calnexin prevents its association with SERCA2b with subsequent loss of the inhibitory effect of calnexin on mitochondrial Ca^{2+} uptake from the ER. Importantly, following a short term ER stress calnexin becomes de-acylated thus shifting its function away from Ca^{2+} homeostasis and promotes its ER quality control functions, for example by interaction of de-acylated calnexin with the oxidoreductase ERp57. Taken together these data indicate S-acylation as an important switch to allow differential localisation and functions of calnexin in the ER with impacts on both protein folding and mitochondrial homeostasis.

VI.v Transcriptional regulation

Increasing evidence suggests that S-acylation may control transcription through a variety of mechanisms. As outlined above, S-acylation of steroid receptors can determine whether the receptor acts as a receptor at the plasma membrane (S-acylated receptor) or conversely controls transcription via DNA binding in the de-acylated state (130, 189-191). Recent evidence also points to S-acylation controlling translocation of another transcription factor, nuclear factor of activated T-cells 5a, NFAT5a, from the plasma membrane to the nucleus to

control gene transcription (50). NFAT5a is myristoylated and S-acylated at its N-terminus with S-acylation being required for the localisation of NFAT5a at the plasma membrane under conditions of cells in the isotonic state. However, upon osmotic stress NFAT5a is released from the plasma membrane and translocates to the nucleus, a process that likely requires de-acylation as site-directed mutants that lack the S-acylated cysteine, but not myristoylated residue, or acute inhibition of S-acylation with 2-BP, result in NFAT5a being located in the nucleus.

Transcription requires remodelling of chromatin structure in the nucleus, a process intricately controlled by the post-translational regulation of histones, including by methylation and acetylation. Recent proteomic screens of S-acylation substrates in Jurkat cells revealed that a number of histone H3 variants, including H3.2, are S-acylated with the conserved cysteine C110 being a major site of S-acylation (268). The functional impact of H3 S-acylation has yet to be fully established although the localisation of H3 to the nuclear shell fraction in HeLa cells may support a role in perinuclear tethering of chromatin, a process associated with inactive heterochromatin. Intriguingly, 2-BP treatment of P19 mouse embryonic carcinoma cells impaired neuronal differentiation and cell cycle exit with associated changes in H3, and Histone 4 (H4), acetylation (34). This suggests that S-acylation may, directly or indirectly control histone acetylation and hence chromatin remodelling. Of note, H4 has also been reported to be O-acylated on serine 47 (S47) by nuclear localised acyl-CoA: lysophosphatidylcholine acyltransferase (Lpcat1) via a calcium-dependent mechanism (293). Knockdown of Lpcat1, the cytosolic form of which also catalyzes an acyltransferase reaction by adding a palmitate to the sn-2 position of lysophospholipids, or the H4 S47A mutant decreased global mRNA synthesis.

VI.vi Enzymes and kinases

S-acylation is now implicated in controlling a wide diversity of protein and lipid kinases as well as an eclectic array of other enzymes and signalling proteins.

VI.vi.a Tyrosine kinases

Perhaps the best studied examples of S-acylated protein kinases are members of the Src-family of non-receptor tyrosine kinases (SFK) implicated in a wide diversity of signalling responses including cell proliferation, changes in cell-morphology and migration as well as differentiation. Members of the family are co-translationally myristoylated at glycine residue 2 but are differentially S-acylated at cysteine residues immediately downstream of the myristoylated glycine (127). For example, Src-kinase is not S-acylated, whereas Lyn and Yes kinases are mono-acylated at C3 whereas Fyn is S-acylated at both C3 and C6 cysteine residues. The subcellular organisation of these kinases is important for their functional role and the degree of S-acylation has been proposed to control the mode of SFK trafficking and predominant subcellular localisation. In an elegant series of studies, exploiting both site-directed mutants and cysteine 'knock-in' experiments Sato and co-workers revealed three distinct modes of SFK trafficking (217). Non S-acylated SFKs, such as Src, enter a rapidly recycling pathway between endosome and the plasma membrane that is controlled by myristoylation. In contrast, SFKs that may be singly S-acylated, such as Lyn and Yes are S-acylated in the Golgi apparatus where they enter the secretory pathway for delivery to the cell surface. In this case, if Lyn or Yes are not S-acylated they remain largely in the Golgi apparatus due to their myristoylation. In contrast, dually S-acylated Fyn is targeted directly to the plasma membrane. Introduction of an addition S-acylated cysteine in Yes, or mutation of just C6 in Fyn, resulted in the respective mutant SFKs entering the dually- or mono- S-acylation pathways respectively. Taken together, at least for these peripheral membrane proteins, the extent of S-acylation acts as a postcode to direct the kinases to distinct compartments. For most SFKs the repertoire of zDHHCs that control S-acylation are not well established. For Fyn, zDHHC21 has been identified as a candidate and in mice with a

loss of function mutation in zDHHC21 Fyn is mislocalised in hair follicles and mice have significant hair loss (163)

VI.vi.b *Lipid kinases*

Phosphatidylinositol 4-phosphate (PI4P) is an important phospholipid required for Golgi membrane trafficking and control of phosphoinositide biosynthesis and signalling at the plasma membrane. At the Golgi, PI4P is predominantly synthesised by the lipid kinase phosphatidinylinositol 4-kinase IIα (PI4KIIα) that is very tightly associated with the membrane even though it lacks any transmembrane domains. PI4P assembles with zDHHC3 and 7 in lipid raft domains in the Trans-Golgi network in a cholesterol dependent mechanism (149). zDHHC3 and 7 S-acylate multiple cysteines in the ^{173}CCPCC motif within the catalytic core of PI4KIIα that is essential for both assembly with the zDHHCs and localisation in lipid raft domains (15, 149). Mutation of these cysteines, or knockdown of the cognate zDHHCs also significantly reduces PI4P kinase activity and consequently Golgi PI4P levels.

VI.vi.c RAS superfamily of small GTPases

The rat sarcoma (Ras) superfamily of small GTPases, comprising Rac, Rho, Rab, Arf and Ran subfamilies are important signal transducers that act as switches to control a diverse array of downstream effectors and regulate a variety of physiological mechanisms including cell growth, movement, differentiation and survival.

The importance of S-acylation of H-Ras and N-Ras, in conjunction with prenylation, in controlling their trafficking and localisation at the plasma membrane to mediate effective signalling has long been recognised (for review see (144). Importantly, H- and N-Ras plasma membrane targeting is intricately controlled by cycles of S-acylation and de-acylation coordinated through the activity of predominantly Golgi-localised zDHHCs and

palmitoylthioesterases at other internal membranes to promote accumulation of these GTPases at the plasma membrane (40, 209, 258).

The Rho GTPase Rac1 is also S-acylated but at a cysteine residue (C178) in the C-terminus that is more proximal than the cysteine residues of H- and N-RAS that are located adjacent to the prenylated –CAAX box (176). S-acylation is required for correct localisation of Rac1 in lipid rafts at the plasma membrane and de-acylated Rac1 shows reduced GTP loading and increased oligomerization, with reduced activation of its downstream effector PAK kinase leading to defects in cell spreading and migration. Another intriguing variation on this theme, that also highlights the importance of alternative splicing in determining S-acylation of many proteins, is the example of the Rho GTPase Cdc42 (122).. The 'canonical' Cdc42 splice form is ubiquitously expressed and membrane association is determined by prenylation of the C-terminus and differs from H-Ras and N-Ras in lacking the C-terminal S-acylation sites. However, a brain specific C-terminal splice variant replaces the C-terminal prenylation motif to a sequence with a di-cysteine motif (-CCAX) just upstream of the C-terminus. This brain specific isoform (bCdc42) was reported to be S-acylated and preferentially targeted to dendritic spines, compared to the canonical variant that locates in both spines and dendritic shaft. The S-acylated bCdc42 variant being more efficient at promoting spine remodelling in cultured neurones based on studies using mutants of both cysteines in the –CCAX terminus. However, functional diversity of the bCdc42 variant is expanded further as bCdc42 can exist in two forms via a novel form of –CAAX processing (178): a form that is tandemly prenylated and S-acylated, as it bypasses the canonical post-prenylation steps of proteolysis and carboxymethylation, with S-acylation occurring on the second cysteine of the –CCAX terminus; and a 'canonical –CAAX' processed form of bCdc42 that is not S-acylated. The dually prenylated and S-acylated form of bCdc42 does not interact with RhoGDIα thus promoting the amount of bCdc42 at the plasma membrane and potentiating bCdc42 signalling.

VI.vi.d Other enzymes

Superoxide dismutase 1 (SOD1) is a ubiquitously expressed protein that protects cells from oxidative damage and mutations are associated with the familial variant of Amyotrophic lateral sclerosis (Lou Gehrig disease) as well as other disease of oxidative damage including artherosclerosis and diabetes. In endothelial cells, SOD1 is S-acylated on an N-terminal (C6) cysteine residue that, based on crystal structures of the mature protein is normally buried within the protein; this cysteine is also associated with other cysteine-dependent interactions, including disulphide bond formation (155). Whether C6 is S-acylated in the mature protein or in the apo-protein, in which C6 is thought to be exposed, remains to be determined. Several mutations of C6 are found in ALS patients with increased aggregation of SOD1, and thus S-acylation of C6 has been proposed as a potential protective mechanism especially in the aggregate prone apoprotein (155). In endothelial cells, S-acylation of SOD1 is mediated predominantly via zDHHC21 and zDHHC21 knockdown, or C6S mutation, reduces SOD nuclear localisation and enzyme activity (155).

S-acylation is also an important determinant of the function of several enzymes that produce gaseous transmitters such as nitric oxide (NO) and carbon monoxide (CO). For example, in endothelial cells endothelial nitric oxide, eNOS is the major enzyme controlling nitric oxide production that is essential for vascular function. eNOS is dually myristoylated and S-acylated (C15 and C26), largely through zDHHC21 in endothelial cells, although several other enzymes can S-acylate eNOS in overexpression assays (54, 64, 146). S-acylation is crucial for eNOS to be targeted to the plasma membrane and caveolae as zDHHC21 knockdown or site directed mutation of C15 and C26 results in a more perinuclear and Golgi localisation of the enzyme (54). Furthermore, inhibition of eNOS S-acylation abrogates both basal and stimulated NO synthesis in endothelial cells. S-acylation is also important for targeting haem oxygenase 1 to the mitochondrial associated membrane region of the ER as disruption of HMOX1 S-acylation redistributed the enzyme to other ER localisations (150). HMOX1 degrades haem to produce the anti-apoptotic gas carbon monoxide that is also an important determinant of mitochondrial and ion channel function. However, whether S-

acylation also controls HMOX1 activity *per se*, in addition to its role in HMOX1 localisation, is currently not known.

Enzymes involved in termination of signalling cascades are also regulated by S-acylation with the phosphodiesterase 10A (PDE10A) being another remarkable example of how S-acylation works with other PTMs to control function (32). PDE10A is a dual (cAMP and cGMP) cyclic nucleotide PDE that is highly enriched in the striatum. PDE10A inhibitors and mouse models have implicated this PDE as a potential ant-psychotic strategy for disorders such as schizophrenia through compartmentalisation of cyclic nucleotide signalling cascades. PDE10A exists as two N-terminal splice variants: the longer PDE10A2 variant is predominantly membrane localised, including at postsynaptic membranes, that is controlled by S-acylation of C11. S-acylation of PDE10A2 is largely mediated via zDHHCs 7 & 19 in overexpression assays and mutation of C11 in PDE10A2, or the shorter PDE10A1 variant that lacks C11, has a cytosolic localisation and reduced trafficking to distal dendrites (32). Importantly, S-acylation of PDE10A2 is inhibited by PKA, or PKG, mediated phosphorylation of a Threonine residue (T16) immediately downstream of the S-acylated cysteine. Thus elevations of cAMP, or cGMP, prevent PDE10A2 S-acylation and trafficking to the membrane although acute stimulation of these pathways does not remove PDE10A2 already resident at the plasma membrane (32).

S-acylation is also implicated in the control of enzymes involved in proteolytic processing as evidenced by the amyloid precursor protein (APP) processing enzymes aspartyl protease β-site APP-cleaving enzyme 1 (BACE1) and the multi-protein complex γ-secretase. APP processing involves sequential proteolysis by BACE1 and γ-secretase to generate Aβ peptides that accumulate in the brains of patients with Alzheimer's disease. BACE1 is S-acylated at four cysteine resides at the junction of transmembrane and cytosolic domains and controls BACE1 localisation to 'lipid rafts' as site directed mutagenesis of these residues localised BACE1 to non-raft domains (263). However, although S-acylation controlled raft localisation, it had no effect on APP processing suggesting that BACE1 activity is similar in

both raft and non-raft domains. A similar mechanism, to control lipid raft localisation, with little if no effect on processing, is also observed with the effects of S-acylation of γ-secretase (35). Two subunits of γ-secretase, Nicastrin and APH-1, are S-acylated with Nicastrin S-acylated at a single site (C689) whereas APH-1 is S-acylated at two sites (C182 & C245). S-acylation increased stability of Nicastrin and APH-1 and localised γ-secretase to detergent resistant 'lipid rafts' without affecting complex assembly (35). However, S-acylation of the subunits was not required for processing of APP or other substrates, at least in cultured cells. This suggests the role of S-acylation is to protect the two nascent subunits from degradation until assembly into the complex (35). APP processing has been reported to be controlled indirectly by zDHHC12 by tethering APP at the Golgi membrane and inhibition of the generation of APP-containing vesicle and subsequent trafficking to the plasma membrane (168). However, the targets for zDHHC12 are unknown, APP itself is unlikely as it lacks a cytosolic cysteine residue, moreover, similar effects were seen with a catalytically-dead zDHHC12 in overexpression assays suggesting the effect of APP trafficking may be independent of the acyltransferase activity of zDHHC12. Moreover, the mutant zDHHC12 also increased non-amyloidogenic α-cleavage of APP. Thus while this highlights another possible non-PAT function for zDHHC12, the role of S-acylation in APP processing remains elusive.

VI.vii. Cell adhesion molecules:

An increasing number of cell adhesion molecules (122) are reported to be S-acylated with S-acylation controlling both membrane localisation and signalling including neurofascin, integrin-tetraspanin complexes and neural cell adhesion molecules.

Neurofascin, a member of the L1 family of cell adhesion proteins, is S-acylated at a conserved cysteine residue (C1213) in the predicted membrane spanning domains. S-acylation did not affect neurofascin membrane localisation, targeting to axons nor its ability

to interact with the adapter Ankyin-G or cell-adhesion activity in neuroblastoma cells. However, S-acylation targeted neurofascin to low density membrane compartments resembling caveolae (204).

S-acylation also plays a complex role in controlling the formation of membrane microdomains enriched for tetraspanins and a variety of integrins that control cell morphology, migration and signalling. Tetraspanins and integrins can assemble into complex signalling networks dependent upon both S-acylation of individual tetraspanins and integrins. For example, S-acylated integrins (α3, α6 and β4) can coexist with a number of tetraspanins such as CD9, CD63 and CD81. β4 integrins are S-acylated at multiple cysteine residues and that β4 integrins cannot be S-acylated result in disruption of both integrin and tetraspanin interactions, clustering of tetraspanins, as well as integrin signalling and control of cell morphology (277).

The S-acylation of NCAMs themselves can also be dynamically regulated to control axonal outgrowth and targeting. In neuroblastoma cells, the two major NCAM isoforms NCAM140 and NCAM180 are S-acylated and this can be stimulated by FGF2-mediated signalling through the FGF receptor (197). This mechanism appears to involve elevation of zDHHC7 activity that is the major zDHHC S-acylating these NCAMs. The NCAMs are S-acylated at multiple cysteine residues that targets the NCAM to lipid rafts and subsequent downstream signalling through tyrosine focal adhesion kinase and extracellular signal regulated 1/2 kinase to stimulate neurite outgrowth (177). Deleted in colorectal cancer (DCC) is homologous to neuronal cell adhesion molecules (NCAM) and is a receptor for the guidance molecule netrin-1 that controls commissural axons in the developing nervous system. DCC is S-acylated at a juxtamembrane cysteine that is important for targeting DCC to lipid rafts and this S-acylation dependent targeting is required for netrin-1 signalling to ERK and neurite outgrowth (99).

S-acylation control of other cell adhesion molecules also plays and important role in non-neuronal systems. In endothelial cells, the junctional adhesion molecule platelet endothelial cell adhesion molecule-1 (PECAM1, CD31), is S- acylated by zDHHC21 on C595 although zDHHC3 may play a role in other cell types such as HEK293 cells. Knockdown of zDHHC21 leads to a significant reduction in PECAM1 surface expression although this is associated with a decrease in total PECAM1 expression suggesting S-acylation controls PECAM1 stability (155). As PECAM1 is implicated in a diverse array of endothelial function from angiogenesis to flow sensing and transepithelial cell migration change sin S-acylation may control these important functions of endothelia.

The examples above illustrate where the cell adhesion molecule itself is S-acylated however, control of cell adhesion molecule function may also be controlled by other S-acylated proteins they interact with. A recent example, is the activity dependent control of synaptic cadherin complexes by its S-acylated binding partner δ-catenin and the control of synaptic structure and organisation involved in memory formation (19). In hippocampal neurones, increases in synaptic activity, for example as observed following protocols that induce long-term potentiation in vitro, or in vivo such as context-dependent fear conditioning, results in a transient increase in δ-catenin S-acylation mediated via zDHHC5. S-acylation of δ-catenin, predominantly at a di-cysteine motif (C960 and C961) in its C-terminus, targets δ-catenin to the plasma membrane facilitating its recruitment to synaptic clusters of cadherin. The functional effect is to stabilise cadherin at the synapse and promote activity dependent remodelling of postsynaptic spines and recruitment of synaptic AMPARs resulting in increased efficacy of neurotransmission.

VI.viii S-acylated proteins of viruses, protozoa and plants

The discussion above has focussed on the role of S-acylation in predominantly mammalian systems. However, S-acylation plays a critical role in a diverse array of species from viruses to protozoans and plants, although bacteria lack the machinery for enzymatic S-acylation.

Viral proteins were the first S-acylated proteins identified (221). Since then several classes of viral protein have been shown to be S-acylated including: spike proteins involved in membrane fusion (such as hemagluttinin (HA)) from influenza virus Viruses; viroporins such as the M2 ion channel protein of Influenza and; a variety of peripheral membrane proteins (for review see (260)). Although the role of S-acylation is not fully understood in many cases S-acylation plays a role in targeting viral proteins to lipid raft domains and controlling aspects of virus entry into host cells by controlling spike protein mediated membrane fusion events as well as subsequent virus assembly and release from cells. In contrast to many cellular S-acylated proteins, most viral proteins do not appear to undergo cycles of S-acylation and de-acylation, most likely reflecting that viral proteins are used only once in the viral lifecycle. Furthermore, the cellular enzymes that control viral protein S-acylation are ill defined, although as viral protein S-acylation occurs at the ER and Golgi it is likely to be a subset of the zDHHCs localised to these sites. Intriguingly, S-acylation is also important for host resistance to influenza viral infections. For example, S-acylation of interferon-induced transmembrane protein 3 (IFITM3) is required for its membrane clustering and antiviral activity (282).

S-acylation plays a major role in all aspects of the lifecycle and function of the trypanosomatids, eukaryotyic single-celled flagellated protozoans, including the major species involved in human diseases such as the Leishmania's, Chagas disease and African sleeping sickness (for review see (66)). For example, proteomic screens have identified > 100 S-acylated proteins in *Trypanosoma brucei* (53) and global inhibition of S-acylation is lethal to these organisms.

The major cause of mortality in malaria infection, *Plasmodium falciparum*, also expresses a large (>400) compliment of S-acylated proteins several of which are involved in controlling the actin-myosin machinery required for these organisms to invade red blood cells of infected individuals and for asexual development (118). S-acylation has also been shown to play multiple roles in the lytic cycle of another member of the same phylum, *Toxoplasma gondii*, the causative agent of toxoplasmosis (see (59)).

A large number of S-acylated proteins have been identified in Yeast (*Saccharomyces cerevisiae*) including many proteins involved in vesicle fusion/trafficking and amino acid transport (212). In *S. pombe* the level of Erf2 DHHC activity controls meiotic entry with high Erf2 activity promoting meiosis, in part through S-acylation of the small GTPase Rho3 whereas low Erf2 activity delays entry into meiosis (285). S-acylation also controls telomere dynamics in yeast as S-acylation, via the DHHC Pfa, targets the telomere interacting protein Rif-1 to discrete foci at the periphery of the inner nuclear membrane allowing assembly of 'telomere clusters' and subsequent regulation of telomere dynamics and silencing (187).

More than 500 S-acylated proteins have been identified in Arabidopsis root cells (95) and S-acylation is emerging as a major signalling pathway controlling multiple aspects of plant development and growth (96) including development of salt tolerance in Arabdopsis (291)

VII. Conclusions and perspectives

In this review we highlighted the key features of protein S-acylation, highlight the diversity of proteins controlled by this essential post-translational modification and the physiological function of S-acylation in health and disease. As we outline, major goals, opportunities and challenges are to understand:

i) the mechanisms by which S-acylation is controlled, how covalent addition of lipid can control diverse properties of proteins and how this is spatiotemporally coordinated.

ii) the physiological relevance of S-acylation at a systems and whole organism level.

To address these issues it is clear that new tools are urgently required. However, the recent development of new proteomic, chemical biology, imaging and genetic tools are enabling studies to understand and interrogate S-acylation at multiple levels of analysis.

Key questions for the future that need to be addressed include:

i) How is zDHHC substrate specificity and activity controlled? A key challenge is to understand how zDHHCs themselves are regulated by post-transcriptional (e.g. mRNA splicing) and post-translational regulation, including by S-acylation itself. How are substrates recognised and how is this spatiotemporally coordinated?

ii) Can we pharmacologically control distinct zDHHC and APTs? No specific inhibitors or activators of zDHHCs exist although a limited number of acylthioesterase inhibitors are now being explored. Targeting zDHHC will be a major challenge, in particular as we have no structural determination of any zDHHC to guide rationale drug design.

iii) How is de-acylation controlled? The acyl thioesterases that control de-acylation and the spatiotemporal dynamics of de-acylation of most proteins is unknown. It is likely that several members of the larger serine hydrolase superfamily also function as deacylating enzymes for a range of proteins.

iv) When and where does S-acylation occur? Recombinant zDHHCs may be localised in multiple compartments including the ER, Golgi and plasma membrane but for most zDHHCs we do not know the subcellular distribution of zDHHCs in native cells. The lack of high quality antibodies against zDHHCs remains a major bottle neck. Furthermore, determining the cellular compartments

where specific proteins are S-acylated/de-acylated remains a significant challenge. The spatiotemporal dynamics of S-acylation for most proteins is very poorly understood and improved tools to quantify the spatiotemporal dynamics are required.

v) How does S-acylation integrate with other post-transcriptional and post-translational modifications to control cellular function? It is clear that significant cross-talk between S-acylation and other signalling pathways may occur. Insight into the rules, mechanisms and cross-talk of S-acylation with these modifications has broad implications for cellular signalling.

vi) What is the functional impact of S-acylation at the systems/whole organism level? With recent developments of genome editing tools we now have the opportunity to interrogate the functional impact of S-acylation, and its disruption, on physiological homeostasis. For example, few studies have exploited 'target-protein-centric' (e.g. site-directed mutagenesis of S-acylated cysteines) as well as 'S-acylation centric' (e.g. knockout of specific zDHHC activity) approaches to understand how S-acylation controls the physiological function of key S-acylated proteins. Furthermore, more rigorous development and analysis of global and conditional zDHHC knockout animals should provide insight into both the array of proteins controlled by distinct subsets of zDHHCs as well as the physiological processes controlled by these enzymes.

vii) Is S-acylation disrupted in disease? While disruption of S-acylation has been associated with a variety of major disorders our understanding of how S-acylation may be dynamically controlled during normal ageing, in response to homeostatic challenge, or how it is disrupted in disease states remains rudimentary. A clearer understanding of loss of S-acylation regulation will be key to defining potential therapeutic opportunities to manipulate S-acylation and its associated pathways in disease.

We hope this review provides further impetus for both established S-acylation investigators to address these issues as well as to provide a platform for investigators new to the field to explore the scope and opportunities to understand the physiology of S-acylation. The development of new tools has the potential to provide a paradigm shift in our understanding of the physiology of S-acylation and the promise of potential new therapeutic avenues for a diverse array of major human disorders.

Acknowledgements

Work in the authors laboratories is generously supported by the Wellcome Trust, Medical Research Council, Biotechnology and Biological Sciences Research Council, British Heart Foundation and Diabetes UK. The author declares no competing financial interests.

Figure legends

Figure 1: Major lipid modifications of proteins.

S-acylation is reversible due to the labile thioester bond between the lipid (typically, but not exclusively, palmitate) and a cysteine amino acid of a protein. The zDHHC family of palmitoyl acyltransferases mediate S-acylation. Other major lipid modifications result from stable bond formation between either the N-terminal amino acid (amide) or the amino acid side chains in the protein (thioether, and oxyester). Distinct enzyme families control these lipid modifications: N-myristoyltransferase (NMT) controls myristoylation of many proteins such as the src-family kinase, Fyn kinase; amide-linked palmitoylation of the secreted sonic hedgehog protein is mediated by Hedgehog acyltransferase (Hhat), a member of the membrane bound O-acyl transferase (MBOAT) family. Prenyl transferases catalyse farnesylation (farnesyltransferase, FTase) or geranylgeranylation (geranylgeranyl transferase I and II, GGTase I and II) of small GTPase proteins such as RAS and the Rab proteins respectively. Porcupine (Porcn) a member of the MBOAT family acylates secreted proteins such as Wnt.

Figure 2: Protein S-acylation: a *reversible* lipid post-translational modification controlled by the zDHHC family of acyltransferases

a) zDHHC enzymes typically utilise co-enzyme A (CoA)-palmitate however, other long chain fatty acids (either saturated or unsaturated) can also be used. Deacylation is mediated by a number of acyl thioesterases of the serine hydrolase family. **b)** Phylogenetic tree showing the relationships of the DHHC-CR domain of the 23 human zDHHC acyltransferases that are **c**) predicted transmembrane proteins (typically with 4, or 6, transmembrane domains) with the catalytic DHHC domain located in a cytosolic loop.

Figure 3: Location of sites of S-acylation in transmembrane and peripheral-membrane proteins.

Schematic illustrating different locations of cysteine S-acylation in transmembrane and peripheral membrane proteins. In many cases S-acylation allows a terminus or loop of a protein to associate with the membrane interface. S-acylation can also confer structural constraints in particular when located close to transmembrane domains where S-acylation has been proposed to control transmembrane orientation that may be important for controlling hydrophobic mismatch in different sub-cellular membrane compartments.

Figure 4: S-acylation and regulation of the protein lifecycle

Reversible S-acylation regulates multiple steps in the lifecycle of membrane and peripheral-membrane proteins including: assembly and (a) ER exit; (b) maturation and Golgi exit; (c) sorting and trafficking to target membranes; (d) recycling and internalisation; (e) clustering and localization in membrane microdomains; (f) control of properties and regulation by other signalling pathways; (g) partitioning of peripheral membrane proteins between the cytosol and membranes and; (h) recycling and final degradation.

Figure 5: Diversity of S-acylated proteins

Schematic illustrating the diversity of proteins demonstrated to be S-acylated. A large number of proteins identified to date are involved in cellular transport and signalling although structural, chaperone, cell adhesion and proteins required for translational/transcription are also S-acylated.

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