

S-Acylation of Membrane Proteins by the zDHHC Family

by

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Abbreviations

2-BP	2-Bromopalmitate
5HTR2A	5-hydroxytryptamine receptor 2A
ABE	Acyl-biotin exchange
ABHD	α/β hydrolase fold domain
Acyl-CoA	Acyl-coenzyme A
Acyl-PEG	Acyl-polyethylene glycol
Acyl-RAC	Acyl resin-assisted capture
AEG1	Astrocyte elevated gene-1 protein
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANK	Ankyrin repeat
APS	Ammonium persulfate
APT	Acyl-protein thioesterases
ASC	Adaptor protein apoptosis-associated speck-like protein containing a caspase activation and recruitment domain
ATGL	Adipose triglyceride lipase
BRET	Bioluminescence resonance energy transfer
BSA	Bovine serum albumin
BST2	Bone marrow stromal antigen 2
cAMP	Cyclic AMP
CCL	Chemokine ligand
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
CFTR	Cystic fibrosis transmembrane conductance regulator
CHO	Chinese hamster ovary
CHX	Cycloheximide
CKAP4	Cytoskeleton-associated protein 4

CMA	Cyano-myracrylamide
COP	Coat protein complex
CRD	Cysteine-rich domain
CSP	Cysteine string protein
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C chemokine receptor type 4
DAG	Diacylglycerol
DISC	Death-inducing signalling complex
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECL	Extracellular loop
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FasR	Fas receptor
FBS	Foetal bovine serum
FRAP	Fluorescence recovery after photobleaching
GalNAc	N-Acetylgalactosamine
GALNT	N-Acetylgalactosaminyl-transferase
GAP-43	Growth-associated protein 43
GFP	Green fluorescent protein
GlcNAc	N-Acetylglucosamine
GNAI3	Guanine nucleotide-binding protein G(i) subunit alpha-3
GPCR	G protein-coupled receptor
GPI	Glycosylphosphatidylinositol
GPR37	G protein-coupled receptor 37

HD	Huntington's disease
HEK293T	Human embryonic kidney 293T
HIV-1	Human immunodeficiency virus 1
HTT	Huntingtin
ICL	Intracellular loop
IFITM3	Interferon-induced transmembrane protein 3
IP	Immunoprecipitation
JAK	Janus kinase
KSHV	Kaposi's sarcoma-associated herpesvirus
LB	Lysogeny broth
LF	Lipofectamine
LRP6	Low-density lipoprotein receptor-related protein 6
LTNP	Long-term non-progressor
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MIR2	Modulator of immune response 2
mPEG	Methoxy polyethylene glycol
NEK7	NIMA related kinase 7
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
OXTR	Oxytocin receptor
P2RY11	P2Y purinoceptor 11
PaCCT	Palmitoyltransferase conserved C-terminus
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline with tween
PD-L1	Programmed cell death ligand 1
PDZ	PSD-95/Discs-large/ZO-1
PECAM-1	Platelet endothelial cell adhesion molecule 1

PEI	Polyethylenimine
PK	Protein kinase
PPT	Palmitoyl-protein thioesterase
PSD-95	Postsynaptic density 95
RTK	Receptor tyrosine kinases
RUSH	Retention using selective hooks
SBP	Streptavidin binding protein
SCAP	Sterol regulatory element-binding protein cleavage-activating protein
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELENOK	Selenoprotein K
SEM	Standard error of mean
SH3	Src homology 3
SNAP25	Synaptosomal-associated protein 25
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SRP	Signal recognition particle
STAT	Signal transducer and activator of transcription
TBTA	Tris(benzyltriazolylmethyl)amine
TEMED	Tetramethylethylenediamine
TEMP	Type III endosome membrane protein
TfR	Transferrin receptor
TMPRSS2	Transmembrane serine protease 2
TPS	Total protein stain
TRC40	Transmembrane recognition complex 40
TSPAN4	Tetraspanin-4
V1aR	Vasopressin receptor 1A

V1bR	Vasopressin receptor 1B
VSV	Vesicular stomatitis virus
WT	Wild-type
zDABM	zDHHC ankyrin-binding motif
zDHHC	Zinc finger DHHC domain-containing proteins
β 1AR	β 1-adrenergic receptor
β 2AR	β 2-adrenergic receptor

Abstract

S-Acylation is a reversible lipid modification crucial for regulating membrane protein trafficking, stability, and signalling, and catalysed by the zDHHC family of protein acyltransferases. While its importance is well-established, the substrate specificity of individual zDHHC enzymes and the rules governing S-acylation across different transmembrane protein classes remain incompletely understood. Building on recent discoveries that the endoplasmic reticulum enzyme zDHHC6 exhibits broad activity towards single-pass transmembrane proteins with membrane-proximal cysteines, this thesis investigates the S-acylation of multi-transmembrane proteins, including GPCRs and tetraspanins, focusing on their recognition by zDHHC6 in comparison to Golgi-localised enzymes zDHHC3 and zDHHC15.

The first results chapter explores S-acylation of the GPCR CCR5, identifies new sites of S-acylation, shows that modification occurs after release from the endoplasmic reticulum, and demonstrates that zDHHC3 and zDHHC15, not zDHHC6, mediate this process. S-Acylation of CCR5 did not affect protein stability, whereas O-glycosylation did. The second results chapter analyses multi-transmembrane proteins, finding zDHHC6 activity against proteins with two and four transmembrane domains, but limited activity towards GPCRs, which are primarily substrates of zDHHC3 and zDHHC15. Mutational analysis rejected the idea that zDHHC6 specifically recognises membrane-proximal cysteines in transmembrane proteins, revealing a more complex enzyme-substrate interaction. S-Acylation of GPCRs on post-endoplasmic reticulum membranes was further supported by analysis of ER-trapped β 2AR. Fatty acid attachment to transmembrane proteins followed the selectivity of their modifying enzymes, regardless of whether target cysteines were located in the cytosol or membrane. The third results chapter reports the novel S-acylation of a single-pass protein, TEMP, and its modification by zDHHC6.

Overall, this work expands the catalogue of S-acylated transmembrane proteins and provides new insight into the determinants of zDHHC enzyme specificity, highlighting the importance of subcellular localisation, protein architecture, and lipid selectivity in regulating S-acylation.

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CHAPTER 1

INTRODUCTION

Chapter 1 - Introduction

1.1 Introduction to S-acylation

Lipid modifications play a central role in regulating protein localisation and function within cells. Among these, S-acylation is a reversible post-translational modification in which long-chain fatty acids are enzymatically attached to cysteine residues via a covalent thioester linkage between the carbonyl carbon of the acyl group and the thiol of the cysteine side chain (Chamberlain and Shipston, 2015, Zaballa and van der Goot, 2018). This lipidation can occur at multiple stages throughout the protein secretory pathway, and by increasing the hydrophobicity of target proteins, S-acylation often promotes the stable association of soluble proteins, or cytosolic domains of transmembrane proteins, with specific cellular membranes (Mesquita et al., 2024) (Malgapo and Linder, 2021). The modification was first described in 1979 by Schmidt and Schlesinger, who identified the palmitic acid esterification of the vesicular stomatitis virus (VSV) glycoprotein (Schmidt and Schlesinger, 1979). Secondary chick embryo fibroblasts were infected with VSV and labelled with ³⁵S-methionine, revealing bands corresponding to VSV glycoprotein, nucleoprotein, and matrix protein. Metabolic labelling with ³H-palmitate identified a band corresponding to the VSV glycoprotein, suggesting lipid incorporation on this protein. Pretreatment with organic solvents prior to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) did not remove this band, indicating that the lipid was covalently linked to the glycoprotein rather than being a contaminant. The fatty acid was subsequently released by mild alkali hydrolysis, which breaks the thioester bond through nucleophilic attack on the carbonyl carbon. The released fatty acid was then converted into a fatty acid methyl ester to allow analysis by radio-gas liquid chromatography. Comparison with known fatty acid methyl ester standards showed that approximately 80 % of the sample co-migrated with palmitic acid methyl ester, confirming the identity of the modification (Schmidt and Schlesinger, 1979). Although a range of fatty acids of varying chain lengths can be attached, palmitate appears to be the predominant lipid, and consequently, S-acylation is often referred to as palmitoylation (Muszbek et al., 1999, Chamberlain and Shipston, 2015). These fatty acids are attached to substrates by enzymes belonging to the zinc finger DHHC domain-containing (zDHHC) family and removed by members of the acyl-protein thioesterase (APT) and α/β hydrolase fold domain (ABHD) families.

It is now well established that S-acylation is a widespread post-translational modification across all eukaryotes. Proteomic analysis has identified thousands of cellular proteins that are modified by S-acylation, including both transmembrane and soluble proteins. Furthermore, S-acylation substrates belong to diverse protein families, including G protein-coupled receptors (GPCRs), receptor tyrosine kinases, transporters, ion channels, adhesion proteins, heterotrimeric and small G proteins, chaperones, scaffolds, and membrane traffic proteins (Mesquita et al., 2024, Li et al., 2025, Shang et al., 2025).

1.2 Targets of S-acylation

S-Acylation is now recognised as one of the most widespread lipid modifications in eukaryotic cells. It is estimated that approximately 20 % of the human proteome undergoes S-acylation, highlighting the scale at which this modification contributes to protein regulation (Mesquita et al., 2024). Since its initial discovery, technical advances have enabled the identification of thousands of S-acylated proteins across diverse eukaryotic systems (Zaballa and van der Goot, 2018).

The first large-scale S-acylation profile, published in 2006, identified 47 modified proteins in the yeast *Saccharomyces cerevisiae* (Roth et al., 2006). Since then, dozens of S-acylated proteomes across multiple species have been reported, collectively revealing thousands of candidate substrates and highlighting the widespread nature of this modification. However, the approaches used can generate false positives, and so it is important that the proteins identified in global S-acylation studies are confirmed using complementary validation strategies.

In 2015, SwissPalm was introduced as the first comprehensive database of protein S-acylation, containing information on proteins identified in both global and targeted studies (Blanc et al., 2015). The database compiled and cross-referenced experimental evidence from both literature and large-scale proteomic studies. Its initial release included approximately 5,000 candidate S-acylated proteins identified across 19 S-acylated proteomes and around 500 experimentally validated sites from seven species. By integrating data from validation studies, SwissPalm increased confidence in reported modifications and highlighted that more than 10 % of the proteome may undergo S-acylation. The updated version, SwissPalm2, expanded this resource by incorporating 38 large-scale proteomic datasets, a greater number of curated publications, and an increased set of validated sites (Blanc et al., 2019). It also uses evolutionary relationships and ortholog comparisons to strengthen confidence in proteomic hits. Proteins are described as S-acylated only when supported by direct experimental evidence such as metabolic labelling (including click chemistry or radioactive methods), mass spectrometry identification, or cysteine-targeting chemical approaches (acyl biotin exchange or acyl-resin assisted capture). Assignment of specific S-acylation sites requires data showing that cysteine substitution removes the modification. As of 2019, 671 articles providing direct evidence of S-acylation have been curated, resulting in 663 validated proteins and 787 unique sites. Additional information, such as putative sites identified by mass spectrometry, identified protein acyltransferases, and APTs has also been incorporated. Overall, SwissPalm2 encompasses 9,940 proteins from 17 species with evidence of S-acylation and 3,669 putative sites, offering an open-access and up-to-date platform for investigating this widespread protein modification (Blanc et al., 2019).

S-Acylated proteins can be broadly categorised into two major groups, peripheral membrane proteins and integral transmembrane proteins (Chamberlain and Shipston, 2015). For peripheral proteins, S-acylation often functions as a reversible membrane anchor, enabling transient or regulated membrane association. For example, small

GTPases such as H- and N-Ras undergo cycles of S-acylation that promote their recruitment to the plasma membrane and modulate their signalling activity; mutants lacking S-acylation show altered trafficking and impaired signalling functions (Rocks et al., 2005, Rubio et al., 2010). In many cases, S-acylation acts in combination with other lipid modifications. Proteins that are N-myristoylated or prenylated frequently require secondary S-acylation to achieve stable membrane attachment (Cadwallader et al., 1994, van't Hof and Resh, 1997). In integral membrane proteins, S-acylation typically occurs on cytosolic loops or C-terminal cysteine residues and can influence protein conformation, trafficking, and interactions with scaffolding or signalling complexes. In certain receptors, ion channels, and transporters, S-acylation modulates surface expression, endocytic recycling, or clustering within membrane microdomains, therefore shaping downstream signalling (Chamberlain and Shipston, 2015).

S-Acylated proteins are distributed across multiple cellular compartments, including the endoplasmic reticulum (ER), Golgi apparatus, endosomes, and plasma membrane (Zaballa and van der Goot, 2018). This distribution reflects both the subcellular localisation of distinct zDHHC enzymes and the dynamic trafficking routes of their substrates. Notably, many S-acylated proteins are enriched within cholesterol and glycosphingolipid-rich membrane microdomains, commonly referred to as lipid rafts (Levental et al., 2010). The addition of acyl chains promotes partitioning into these ordered membrane domains, which serve as platforms for signal transduction, vesicular trafficking, and cytoskeletal organisation. Lipid rafts facilitate protein clustering, enhance interactions with scaffold proteins, and coordinate downstream signalling pathways, vesicular trafficking, and cytoskeletal organisation (Levental, 2020).

Functionally, the S-acylated proteome encompasses a wide range of protein classes, including small GTPases, Src family kinases, soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, ion channels, and adhesion molecules (Hancock et al., 1990, Koegl et al., 1994, He and Linder, 2009, McClafferty et al., 2020, Yang et al., 2002). This diversity illustrates that S-acylation is not confined to a specific structural class of protein but instead represents a versatile regulatory mechanism which features across signalling networks, membrane trafficking pathways, and host-pathogen interactions. The broad substrate range, combined with the reversibility of the modification, enables S-acylation to function as a dynamic molecular switch.

1.3 Regulatory effects of S-acylation

1.3.1 Regulation of protein trafficking by S-acylation

S-Acylation plays a central role in regulating protein trafficking through the secretory and endocytic pathways. Precise protein localisation is essential for cellular function, as compartmentalisation enhances signalling efficiency while preventing inappropriate interactions or cellular damage (Krapf, 2018, Stalder and Gershlick, 2020). Increasing evidence identifies S-acylation as a key modulator of protein sorting, export, retention, and recycling.

In eukaryotic cells, proteins that are sorted by the secretory pathway are initially targeted to the ER either co-translationally or post-translationally. While both pathways depend on ER-targeting signals, only co-translational targeting involves ribosome-nascent chain complexes, whereas post-translational targeting occurs after synthesis via chaperone-assisted delivery to ER translocation machinery (Nyathi et al., 2013). Although no strict consensus sequence defines ER targeting, soluble proteins, type I transmembrane proteins (with luminal N-termini), and multi-pass transmembrane proteins containing extended luminal N-terminal regions typically possess an N-terminal cleavable signal peptide. This sequence usually contains a hydrophobic core and is flanked by positively-charged residues, and includes a signal peptidase cleavage site (Ng et al., 1996, Pool, 2022). In contrast, type II transmembrane proteins and multi-pass transmembrane proteins with a cytosolic N-terminus or a short luminal N-terminus usually contain internal signal sequences that are not cleaved (Pool, 2022). During co-translational translocation, signal sequences are recognised by the signal recognition particle (SRP), which directs the ribosome complex to the ER membrane (Nyathi et al., 2013). Proteins are translocated through the Sec61 translocon complex, and soluble proteins enter the ER lumen, whereas transmembrane proteins integrate into the lipid bilayer (Itskanov et al., 2021, Niesen et al., 2018, Sadlish et al., 2005, Ismail et al., 2008, Ojemalm et al., 2012). The transmembrane recognition complex 40 (TRC40) pathway is an example of a post-translational translocation pathway, which primarily targets tail-anchored proteins or shorter membrane proteins whose transmembrane domain emerges only after translation is complete (Schuldiner et al., 2008, Farkas and Bohnsack, 2021). In this process, cytosolic chaperone proteins bind the protein during translation before transferring the newly synthesised protein to the ATPase TRC40. This delivers proteins to the ER, where they are inserted into the membrane by a membrane insertase complex (Pool, 2022).

Within the ER, nascent proteins undergo folding and maturation. N-terminal signal sequences are cleaved by the signal peptidase complex (Liaci et al., 2021), chaperones assist folding and prevent aggregation (Adams et al., 2021), and further modifications, such as N-linked glycosylation, glycosylphosphatidylinositol (GPI) anchor addition, and disulphide bond formation, contribute to stability and activity (Barlowe and Miller, 2013). Export from the ER occurs via coat protein complex (COP) II-coated vesicles, assembled

following Sar1 activation and recruitment of Sec23/24 and Sec13/31 complexes (Sato and Nakano, 2007) (**Figure 1.1**). Cargo may be incorporated via bulk flow or selective capture mediated by cytosolic sorting motifs (Barlowe and Miller, 2013). ER-resident proteins are retained or retrieved through defined sequence motifs. Soluble luminal proteins bearing a KDEL (lysine-aspartate-glutamate-leucine) sequence are returned from the Golgi to the ER via COPI-coated vesicles after binding KDEL receptors (Cela et al., 2022), whereas transmembrane proteins containing C-terminal KKXX (L; lysine, X; any amino acid) motifs interact directly with COPI components to mediate retrograde transport (Letourneur et al., 1994, Cabrera et al., 2003). The Golgi is organised into cis, medial, and trans compartments, which have distinct resident enzyme profiles regulating additional modifications. Golgi processing includes O-linked glycosylation, glycan remodelling, and tyrosine sulphation, before proteins are released (Huang and Wang, 2017). Golgi-export to endosomes, lysosomes, or the plasma membrane is largely mediated by clathrin-coated vesicles (Huang and Wang, 2017, Stalder and Gershlick, 2020).

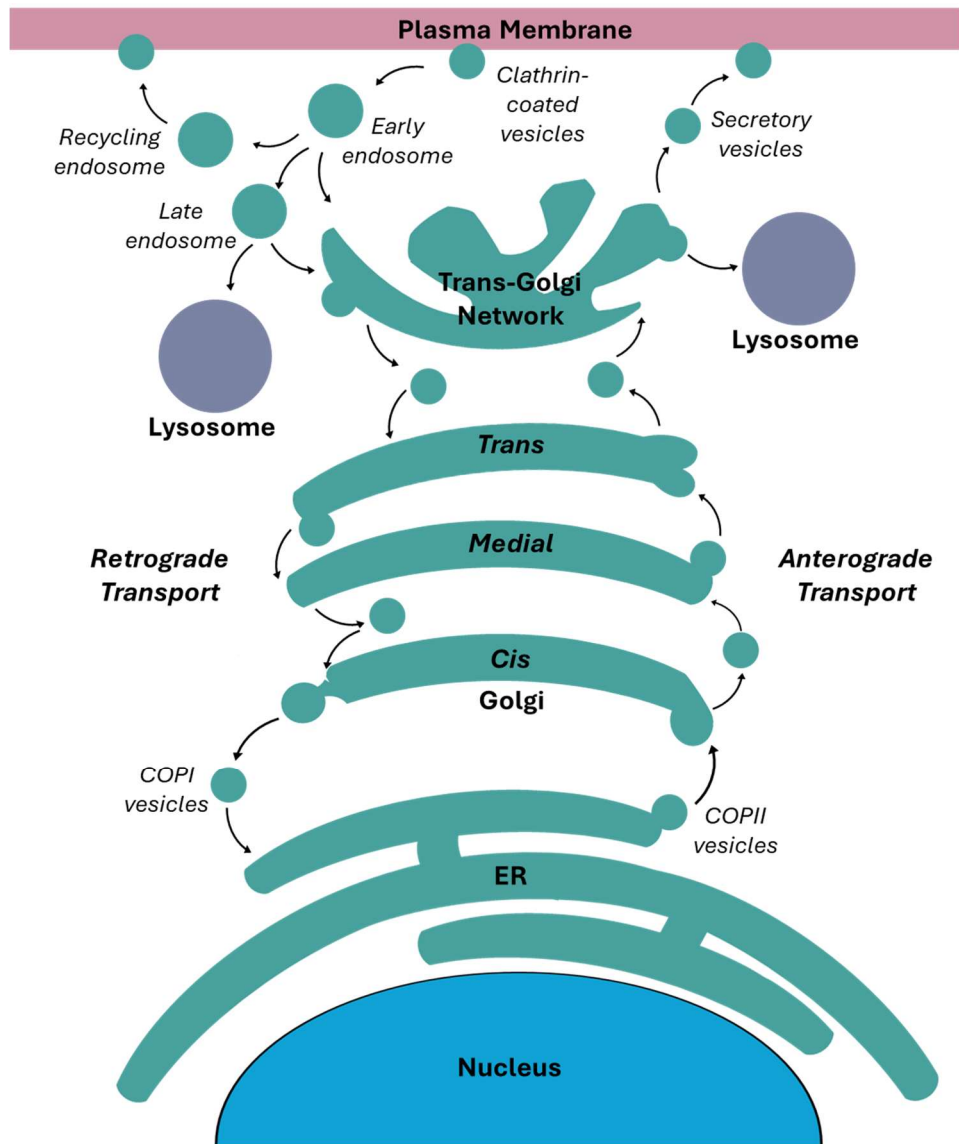


Figure 1.1. Schematic overview of protein sorting and trafficking within the endomembrane system. Newly synthesized soluble and transmembrane proteins in the endoplasmic reticulum (ER) are transported anterogradely via COPII-coated vesicles to the Golgi, where they pass through the cis-, medial-, and trans-Golgi and reach the trans-Golgi network (TGN) for sorting. From the TGN, proteins are delivered to the plasma membrane for secretion or membrane insertion, or can be delivered directly to lysosomes. In the endocytic pathway, proteins are internalised from the plasma membrane via clathrin-coated vesicles and delivered to early endosomes, where they are sorted for recycling back to the plasma membrane via recycling endosomes or for retrograde transport to the TGN, either directly from early endosomes or via late endosomal compartments. Late endosomes act as an additional sorting station, directing selected cargo toward lysosomal degradation. Retrograde transport also returns selected proteins and trafficking machinery from the Golgi to the ER via COPI-coated vesicles, maintaining organelle homeostasis and protein quality control.

S-Acylation intersects with these trafficking pathways at multiple stages, and affects proteins in a variety of different ways, for example, it can promote ER exit, stabilise membrane association and facilitate partitioning into specific membrane microdomains (**Figure 1.2**) (Daniotti et al., 2017). In other contexts, S-acylation regulates endocytic recycling or retention within specific compartments, therefore dynamically controlling steady-state localisation. Through these mechanisms, S-acylation integrates with the biosynthetic and vesicular trafficking machinery to fine-tune membrane protein distribution and cellular responsiveness (Mesquita et al., 2024).

The trafficking of soluble Ras proteins illustrates the functional importance of S-acylation. Following translation, Ras is modified at its C-terminal CAAX motif (C; cysteine, A; aliphatic amino acid, X; any amino acid), enabling initial membrane association (Casey et al., 1989, Hancock et al., 1990). The protein first undergoes farnesylation of the cysteine, followed by cleavage of the AAX residues, and the exposed prenylcysteine is then methylated to convert the originally hydrophilic C-terminus into a hydrophobic membrane-targeting domain (Wright and Philips, 2006). While it was found these modifications on the CAAX sequence were necessary to allow movement of Ras to the plasma membrane, an additional signal in this C-terminal region was required for both stable membrane attachment and subsequent trafficking from endomembrane compartments (Hancock et al., 1990). In H-Ras, S-acylation of Cys181 and Cys184, and in N-Ras of Cys181, is critical for efficient transport to the cell surface (Hancock et al., 1990). S-Acylation of Ras proteins has been shown to be mediated by the zDHHC9 sub-family (including -9, -14, -18 and -19), which form a functional complex with the accessory protein GCP16 (Swarthout et al., 2005, Nguyen et al., 2023, Yang et al., 2024). It was initially thought that this trafficking was direct to the plasma membrane, however, enzymes involved in the CAAX modification were found to be localised to the ER, while zDHHC9 is in Golgi membranes, suggesting H-Ras and N-Ras follow the classical secretory pathway (Dai et al., 1998, Schmidt et al., 1998, Swarthout et al., 2005). Indeed, imaging of both live and fixed cells confirmed the movement of Ras through the cytosolic face of the ER and Golgi, and this trafficking was also sensitive to Brefeldin A, which disrupts the secretory pathway (Choy et al., 1999, Apolloni et al., 2000).

N-Ras and H-Ras have half-lives longer than that of their S-acylation modifications, indicating that these lipid attachments are dynamically turned over (Lu and Hofmann, 1995, Baker et al., 2003, Magee et al., 1987). This led to the proposal of a reversible S-acylation cycle regulating Ras localisation. Golgi photobleaching experiments showed that, even when new protein synthesis was blocked, Golgi-associated N-Ras fluorescence recovered rapidly, consistent with retrograde movement from the plasma membrane (Goodwin et al., 2005). Non-acylated Ras mutants redistributed across Golgi and ER membranes with kinetics characteristic of cytosolic diffusion, suggesting that de-acylation enables membrane release and intracellular redistribution. Furthermore, N-Ras repopulated Golgi membranes more quickly than H-Ras, reflecting the requirement to remove only a single palmitoyl group rather than two (Rocks et al., 2005).

S-Acylation can influence more than just membrane binding of soluble proteins such as Ras; it can also mediate the membrane attachment of soluble loops of transmembrane proteins and affect the orientation of transmembrane domains within the lipid bilayer, particularly when the modification occurs on cysteines near the transmembrane domain. These changes in transmembrane domain orientation were suggested to help correct hydrophobic mismatch, which arises when the length of the transmembrane domain does not match the thickness of the surrounding membrane, potentially causing retention in the ER or misfolding (Chamberlain and Shipston, 2015). A clear example is the single-pass Wnt co-receptor low-density lipoprotein receptor-related protein 6 (LRP6), whose transmembrane helix is well-suited for the thicker plasma membrane but too long for the thinner ER bilayer (Abrami et al., 2008). S-Acylation of cysteines adjacent to the transmembrane domain was proposed to induce a tilt in the helix, effectively shortening the hydrophobic span relative to the ER membrane and alleviating the mismatch. In the absence of acylation, LRP6 is largely retained in the ER and targeted for degradation, whereas adjusting the transmembrane domain length to better fit the ER membrane allows ER exit even without acylation, indicating that the lipid modification primarily functions to optimise transmembrane domain orientation. By promoting correct helix tilt and reducing hydrophobic mismatch, S-acylation ensures proper folding, avoids ER quality control recognition, and enables efficient trafficking through the Golgi to the plasma membrane, where LRP6 carries out its signalling roles (Abrami et al., 2008, Chamberlain and Shipston, 2015).

α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA receptors) are tetrameric ion channels composed of four subunits, GluA 1-4, and provide a clear example of how S-acylation regulates the trafficking of multi-pass membrane proteins (Shang et al., 2025). AMPAR subunits undergo S-acylation at two major cysteine residues, one located in a cytosolic region adjacent to the second transmembrane domain and another in the C-terminal juxta-membrane region downstream of the fourth transmembrane domain (Hayashi et al., 2005). S-Acylation at the internal cysteine, mediated by zDHHC3, promotes retention within the Golgi apparatus, limiting forward trafficking to the plasma membrane (Yang et al., 2009). S-Acylation of the C-terminal cysteine modulates post-Golgi delivery by reducing protein kinase C (PKC)-dependent phosphorylation of GluA1, which promotes binding to the scaffold protein 4.1N and subsequent incorporation into the plasma membrane (Lin et al., 2009). At the cell surface, S-acylation plays a key role in regulating AMPAR turnover by controlling receptor stability. S-Acylated AMPARs can exhibit a reduced surface half-life, as they are more susceptible to activity-dependent internalisation following AMPA or N-methyl-D-aspartate stimulation (Corti and Duarte, 2023). In contrast, deacylation promotes interactions with protein 4.1N, spectrin, and actin, strengthening cytoskeletal anchoring and stabilising receptors at the plasma membrane, thereby prolonging their surface expression (Hayashi et al., 2005, Van Dolah et al., 2011). Therefore, S-acylation controls not only receptor trafficking from the Golgi but also the stability of receptors at the cell surface.

1.3.2 Regulation of protein-protein interactions, signalling, and stability by S-acylation

Beyond its role in membrane trafficking, S-acylation plays a central role in regulating protein-protein interactions, signalling, and protein stability (**Figure 1.2**). By increasing the hydrophobicity of modified cysteine residues and promoting membrane association, S-acylation influences the spatial positioning of proteins within the lipid bilayer. This spatial control shapes interaction networks by determining where signalling proteins localise, which partners they encounter, and the stability of the complexes they form.

S-Acylation can regulate signalling pathways by promoting the association of proteins with cholesterol- and glycosphingolipid-rich membrane microdomains, commonly referred to as lipid rafts. These regions are more ordered and tightly packed than the surrounding lipid bilayer due to their high concentration of saturated lipids and cholesterol, favouring the insertion of certain lipid-modified proteins (Simons and Ikonen, 1997). The saturated fatty acyl chains that are typically added during S-acylation increase the affinity of proteins for these ordered environments, promoting their segregation from more fluid, non-raft membrane regions (Levental et al., 2010, Zaballa and van der Goot, 2018). Lipid rafts function as dynamic signalling platforms that concentrate receptors, kinases, scaffold proteins, and adaptor molecules, facilitating efficient signal transduction by increasing the local concentration of signalling components and promoting the formation of multimeric protein complexes (Simons and Ikonen, 1997, Lingwood and Simons, 2010). By increasing the affinity of modified proteins for these raft domains, S-acylation contributes to the assembly and stabilisation of signalling networks at specific membrane sites, therefore enhancing the efficiency and specificity of signal propagation.

A clear demonstration of this mechanism is the death receptor Fas receptor or cluster of differentiation 95 (CD95), which initiates apoptosis upon ligand binding. S-Acylation of a cysteine residue within the cytoplasmic membrane proximal region of Fas receptor enhances its localisation to lipid rafts at the plasma membrane (Chakrabandhu et al., 2007). This localisation promotes receptor clustering and facilitates the formation of the death-inducing signalling complex (DISC), which recruits adaptor proteins such as Fas-associated protein with death domain, leading to the recruitment and activation of caspases, including caspase-8 (Aouad et al., 2004, Mollinedo and Gajate, 2022). Disruption of Fas S-acylation impairs receptor partitioning into lipid rafts, reduces DISC assembly, and diminishes apoptotic signalling, highlighting the importance of S-acylation in spatially organising receptors for efficient signal amplification (Chakrabandhu et al., 2007).

The targeting of S-acylated proteins to lipid rafts can coordinate protein interactions by mediating the spatial proximity with other raft-associated proteins. In addition, S-acylation can directly influence protein-protein interactions. The S-acylation of nucleotide-binding oligomerisation domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome illustrates the importance of S-acylation in regulating protein-protein interactions. NLRP3 is an intracellular innate immune receptor, mediating the

release of inflammatory cytokines and pyroptotic cell death via caspase-1 activation (Martin and Fairn, 2025). This process requires assembly of an inflammasome complex consisting of NLRP3, the adaptor protein apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), and the effector protein caspase-1 (He et al., 2016). NLRP3 is likely S-acylated at multiple cysteine residues, with distinct sites contributing to different functional outcomes, including subcellular localisation, stability, and trafficking. S-Acylation regulates protein-protein interactions in NLRP3 by modifying cysteine residues located within or adjacent to functional interaction domains, altering the ability of NLRP3 to engage binding partners required for inflammasome assembly. In the leucine-rich repeat domain, S-acylation at cysteine 837 and 838, mediated predominantly by zDHHC5, promotes NLRP3 activation and is essential for efficient recruitment of the kinase NIMA-related kinase 7 (NEK7) (Zheng et al., 2023, Williams and Peden, 2025). Mutations at either residue markedly reduce NEK7 binding, ASC speck formation, and downstream IL-1 β release, indicating that these modifications are required for productive inflammasome assembly. Mechanistically, these lipid modifications are proposed to enhance NEK7 engagement either by stabilising an interaction-competent conformation of NLRP3 or by enabling hydrophobic contacts between acyl chains and complementary non-polar regions on NEK7, although the latter remains to be fully validated. In addition, a second S-acylation site at cysteine 419, regulated by zDHHC17, similarly promotes NEK7 association and is required for full inflammasome activity, as mutation of this residue reduces NLRP3-NEK7 interaction, ASC oligomerisation, and IL-1 β secretion (Hu et al., 2024, Williams and Peden, 2025). Together, these findings indicate that site-specific S-acylation functions as a molecular switch that directly regulates NLRP3 protein-protein interactions, particularly through control of NEK7 binding, therefore licensing downstream inflammasome assembly.

S-Acylation can also modulate protein stability, in some cases protecting proteins from ubiquitination-dependent degradation, therefore prolonging their functional lifetime. Indeed, this is often linked to changes in raft association or protein-protein interactions. Sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP) is a chaperone protein that regulates cholesterol levels by transporting the transcription factor SREBP from the ER to the Golgi, where it is cleaved before entering the nucleus (Lee et al., 2020). zDHHC3-mediated S-acylation of SCAP at cysteine 264 at the Golgi is important for its interaction with SREBP and, therefore, for cholesterol synthesis (Wu et al., 2024). The expression of SCAP was decreased by either substitution of the modified cysteine or knockout of zDHHC3, whereas over-expression of zDHHC3 increased SCAP half-life, suggesting S-acylation is important for SCAP stability. This effect was identified to be due to S-acylation decreasing the ubiquitination of SCAP by the E3 ligase HACE1 (Wu et al., 2024).

Overall, S-acylation is increasingly recognised as a key modification controlling the localisation, micro-localisation, stability and protein-protein interactions of a diverse array of cellular proteins.

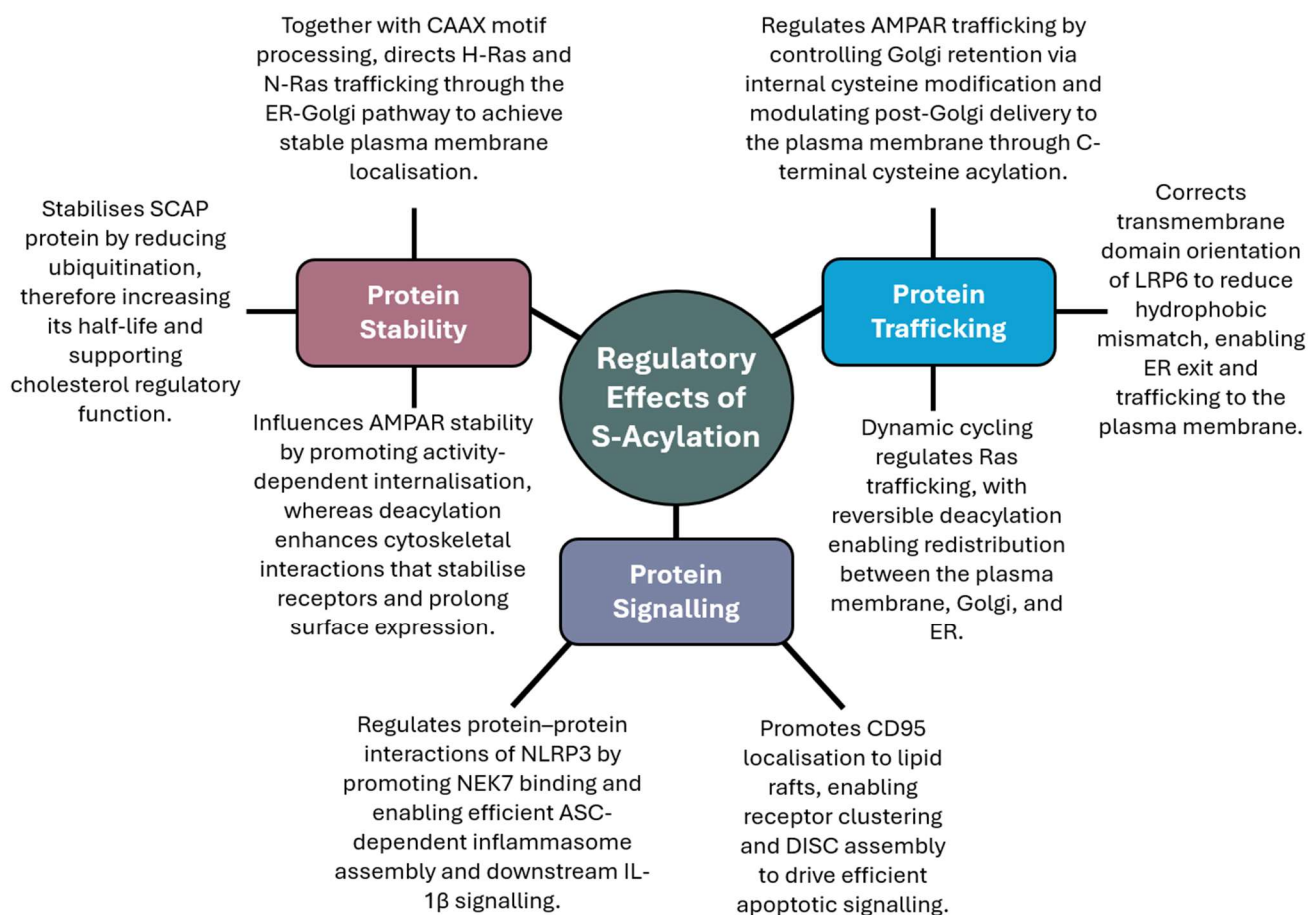


Figure 1.2. Regulatory effects of S-acylation. Diagram depicting examples of the effects of S-acylation on protein stability, trafficking, and signalling. Sterol regulatory element-binding protein cleavage-activating protein (SCAP), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), Low-density lipoprotein receptor-related protein 6 (LRP6), NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3), NIMA related kinase 7 (NEK7), apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), Fas receptor cluster of differentiation 95 (CD95), death-inducing signalling complex (DISC).

1.4 Approaches for studying S-acylation

Numerous methodologies have been developed to study protein S-acylation. One widely used approach is metabolic labelling, in which the incorporation of palmitate analogues into proteins is detected (Milligan et al., 1995). Traditionally, these analogues were radiolabelled. However, reagents such as [^3H] palmitate lack sensitivity, requiring large quantities of radioactive analogue and long exposure times. The speed and sensitivity of detection were substantially improved with the development of click chemistry-based

techniques, where non-radioactive, chemically modified fatty acid analogues containing azido or alkyne groups undergo cycloaddition reactions with tagged probes, enabling detection via fluorescent dyes or shifts in molecular weight (Charron et al., 2009, Salaun et al., 2020). Click chemistry methods have advanced the study of S-acylation of individual proteins, and also provided an approach for the large-scale purification of S-acylated proteins and their subsequent identification and analysis by mass spectrometry proteomics. Despite the advances that have been enabled by radiolabelled and click labelled fatty acids, these probes also have some limitations, including that they do not provide information on the endogenously incorporated fatty acids, they cannot be used for analysis of intact tissues, and issues around their incorporation into stably S-acylated proteins (Forrester et al., 2011). Additionally, it should be remembered that lipids used in click chemistry experiments are non-native, and the azido and alkyne groups may impart unwanted or unknown effects on modified proteins.

In addition to the use of fatty acid analogues, cysteine-centric approaches are also widely used to study S-acylation. One such approach is acyl-biotin exchange (ABE), in which free thiols on proteins are first blocked using an alkylating reagent such as N-ethylmaleimide, thereby preventing modification of non-acylated cysteine residues. Subsequently, hydroxylamine is applied to selectively cleave thioester bonds at S-acylated cysteines, removing the lipid modification and exposing these residues. The newly revealed thiols can then be labelled with a thiol-reactive reagent, such as biotin, permitting recovery on streptavidin beads and analysis by immunoblotting or mass spectrometry (Roth et al., 2006, Drisdell and Green, 2004). As an alternative to biotin-based enrichment, the free thiols exposed following hydroxylamine treatment can also be directly captured using sulfhydryl-reactive resins, an approach termed acyl resin-assisted capture (Acyl-RAC) (Forrester et al., 2011). Another related technique is the acyl-PEG exchange (APE) assay, in which polyethylene glycol (PEG) is used instead of biotin to label newly exposed thiols (Howie et al., 2014, Percher et al., 2016). PEGylation introduces a detectable mass shift, allowing analysis by SDS-PAGE and western blot and an estimation of the stoichiometry of S-acylation of a protein of interest. A similar PEGylation strategy was also introduced into click chemistry workflows to allow an estimation of the number of S-acylated cysteines and the total S-acylated pool of a protein of interest (Salaun et al., 2020).

The main advantage of acyl-group exchange techniques over the use of fatty acid analogues is that they are applicable to a broad range of sample types, including both cultured cells and intact tissues, for the detection of S-acylated proteins (Brigidi and Bamji, 2013, Chen et al., 2021). Furthermore, they can detect proteins carrying naturally occurring lipid modifications, rather than relying on synthetic analogues. Despite these strengths, there are notable drawbacks. These approaches do not provide information about the identity of the attached lipid, limiting insight into lipid-specific modification patterns. Careful experimental validation is essential, including the use of hydroxylamine-negative controls and cysteine mutagenesis to confirm true modification sites. In addition, thioester bonds are not unique to S-acylation and are found in other protein contexts, such as certain mitochondrial enzyme complexes, ubiquitin-

conjugating enzymes, and transient catalytic intermediates like glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which can result in false-positive detection (Roth et al., 2006, Wolfson-Stofko et al., 2013, Chen et al., 2021). Furthermore, these methods capture S-acylation at a single time point, although temporal studies can be performed to examine changes in modification over time (Chen et al., 2021, Fan et al., 2020).

These cysteine-centric and metabolic labelling approaches can be combined with proteomics. Once proteins have been captured and enriched using metabolic labelling, ABE, or acyl-RAC, they can be prepared for proteomic analysis by elution and digestion, followed by mass spectrometry. This allows the identification of S-acylated proteins, estimation of their relative abundance, and, when combined with site-specific digestion, the mapping of modified cysteine residues (Martin and Cravatt, 2009, Roth et al., 2006, Wang and Yang, 2021). In addition, these approaches can be applied in comparative studies to examine dynamic changes in S-acylation between different conditions, such as resting versus stimulated cells, providing insight into how this modification is regulated across the proteome (Colquhoun et al., 2015, Sobocinska et al., 2017, Chen et al., 2021).

Building on previous enrichment-based approaches, a new method has been developed to investigate S-acylation with enzyme-level specificity, referred to as the “bump-and-hole” system (Ocasio et al., 2024). In this approach, individual zDHHC enzymes are engineered with a modified lipid-binding pocket that can accommodate a complementary “bumped” fatty acid probe. The probe is selectively transferred only by the engineered zDHHC, allowing S-acylation of its direct substrates to be monitored without interference from other family members. Following incorporation, the tagged lipid can be ligated to a chemical reporter and analysed by proteomics, allowing identification of zDHHC-specific substrates and the precise sites of S-acylation across the proteome. This strategy provides a tool for linking specific zDHHC enzymes to their protein targets and exploring enzyme-specific S-acylation patterns in living cells.

1.5 Enzymology of S-acylation

1.5.1 zDHHC enzymes

S-Acylation is catalysed by a family of protein acyltransferases known as zDHHC enzymes, named after their conserved zinc-finger aspartate-histidine-histidine-cysteine (DHHC) motif that forms the catalytic active site. In humans, 23 genes encode zDHHC proteins, which differ in their subcellular localisation and substrate specificity (Chamberlain and Shipston, 2015, Fukata et al., 2004). These enzymes were first identified in *Saccharomyces cerevisiae* through the characterisation of Effect on Ras

Function 2 (Erf2), a protein containing a DHHC cysteine-rich domain (CRD). Yeast express two forms of Ras which, like their mammalian counterparts, require S-acylation for membrane targeting, as shown by experiments in which cysteine-to-serine substitutions caused Ras2 to lose its plasma membrane localisation (Bhattacharya et al., 1995). To identify genes required for Ras2 S-acylation, a genetic screen was performed in which an S-acylation-dependent Ras2 allele was expressed in *S. cerevisiae* (Bartels et al., 1999). This screen led to the discovery of the gene *ERF2*, and sequencing revealed that this gene encodes a 41 kDa integral membrane protein with four transmembrane domains and a DHHC cysteine-rich motif. Expression of Ras2 in *ERF2*-deficient cells resulted in a marked reduction of S-acylation, as determined by [³H]-palmitate metabolic labelling. Loss of *ERF2* also caused mis-localisation of Ras2 from the plasma membrane to internal membranes, as visualised through fluorescence microscopy. Subsequent work by Lobo et al. (2002) demonstrated that *ERF2*, in complex with the accessory protein *ERF4*, constitutes a functional S-acyltransferase responsible for Ras S-acylation (Lobo et al., 2002). At the same time, a study by Roth et al (2002) found that the enzyme *Akr1* was essential for S-acylation of yeast casein kinase (Roth et al., 2002). Importantly, *Akr1* contains a similar DHHC cysteine-rich domain as *ERF2*. Together, these results provided the first direct evidence that DHHC-containing proteins can function as acyltransferases.

These foundational studies in yeast were followed in 2004 by a report from the Fukata groups that described the cloning and initial analysis of a family of 23 zDHHC enzymes from mouse (Fukata et al., 2004). All human zDHHC enzymes are multi-pass transmembrane proteins predicted to contain between four and six transmembrane domains (Mesquita et al., 2024, Fukata et al., 2004, Rana et al., 2018). The majority possess four transmembrane domains; however, zDHHC4 and zDHHC24 are predicted to contain five, while zDHHC13, zDHHC17, and zDHHC23 contain six. In zDHHC enzymes with an even number of transmembrane domains, both the N- and C-termini are oriented towards the cytosol (**Figure 1.3**). The catalytic DHHC motif resides within a highly conserved 51-amino-acid CRD located in a cytosolic loop between transmembrane domain 2 and 3, positioned to access substrate cysteine residues (Politis et al., 2005, Zaballa and van der Goot, 2018).

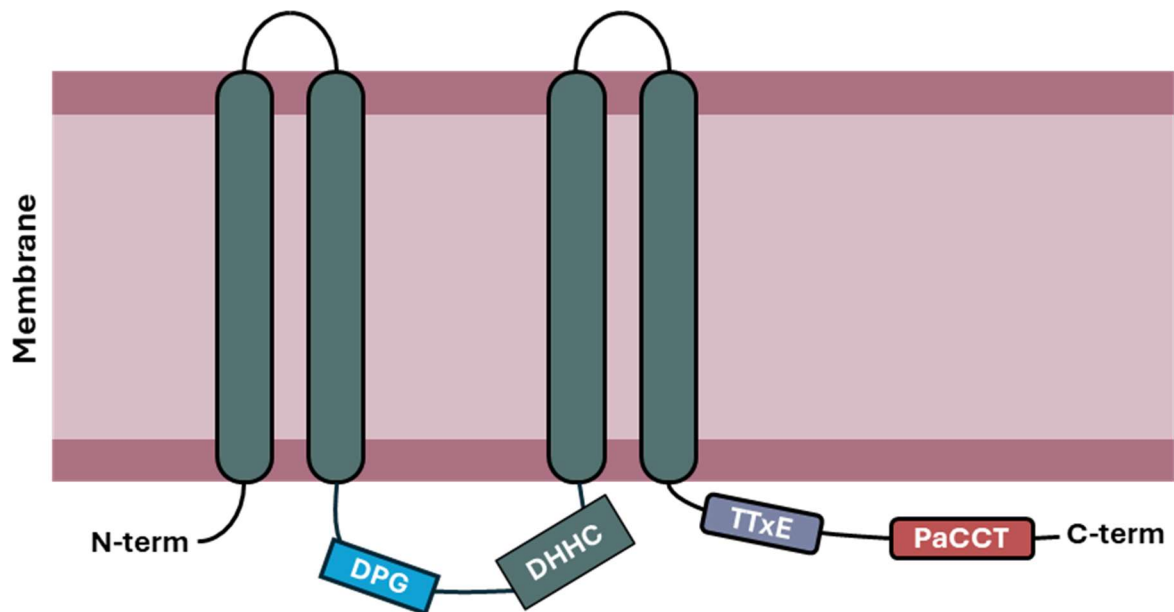


Figure 1.3. Schematic representation of a zDHHC enzyme. The enzyme is shown embedded in the membrane with four transmembrane domains (green) and both N- and C-termini located in the cytosol. The intracellular loop contains the DPG motif (blue) and the catalytic DHHC domain (green). The C-terminal region includes the TTxE motif (purple) and the PaCCT domain (red).

Several additional conserved motifs are present in zDHHC enzymes beyond the catalytic DHHC domain. These include an aspartate-proline-glycine (DPG) motif located N-terminal to the DHHC sequence, a threonine-threonine-x-glutamate (TTxE) motif (where x represents any amino acid) positioned in the C-terminus, and a palmitoyltransferase conserved C-terminus (PaCCT) motif at the extreme C-terminus. The strong evolutionary conservation of these elements suggests functional importance; however, their precise mechanistic contributions remain incompletely defined (Chamberlain and Shipston, 2015, Zaballa and van der Goot, 2018). Structural analysis of human zDHHC20 has provided insight into the TTxE motif. In the crystal structure, threonine 241 within the TTxE sequence forms a hydrogen bond with the conserved aspartate residue of the DHHC domain, linking the C-terminal region to the catalytic core (Rana et al., 2018). Substitution of the two threonine residues to alanine (AAxE) significantly reduced enzymatic activity, indicating that the motif contributes to maintenance of catalytic ability, likely through stabilisation of the structure rather than direct participation in acyl transfer. The PaCCT motif was first identified in yeast acyltransferases Swf1 and Pfa3, where it was shown to be important for *in vivo* acyltransferase function (Gonzalez Montoro et al., 2009). In zDHHC20, substitution of a conserved residue within this motif, asparagine 266, to alanine led to reduced enzymatic activity, supporting a role in structural stabilisation of the catalytic domain (Rana et al., 2018). Collectively, the available data suggest that the DPG, TTxE, and PaCCT motifs contribute to the structural

organisation and enzymatic efficiency of zDHHC enzymes, although none have been demonstrated to participate directly in the chemical steps or acyl transfer.

Despite these shared structural features, considerable diversity exists across the zDHHC enzyme family. One notable variant is zDHHC13, which contains a closely related aspartate-glutamine-histidine-cysteine (DQHC) motif in place of the canonical DHHC sequence. Although this substitution alters the conserved histidine residue within the catalytic core, zDHHC13 retains its acyltransferase activity, indicating that some flexibility exists within the catalytic motif provided the essential cysteine and supporting residues are preserved (Zaballa and van der Goot, 2018, Lemonidis et al., 2014). In contrast, replacing the DHHC domain of zDHHC17, a closely related family member, with a DQHC motif abolishes its ability to S-acylate substrates such as cysteine string protein (CSP) and synaptosomal-associated protein 25 (SNAP25). These findings indicate that the functional competence of the DQHC motif in zDHHC13 likely depends on additional structural or contextual features that are not transferrable to zDHHC17 (Lemonidis et al., 2014). In addition to variation within the catalytic motif, zDHHC enzymes display a substantial heterogeneity in overall size. Protein length ranges from approximately 263 amino acids in zDHHC22 to 768 amino acids in zDHHC8, with most divergence occurring in the cytosolic N- and C-terminal regions (Malgapo and Linder, 2021). These terminal domains are not as conserved between family members and frequently contain protein-protein interaction motifs, trafficking signals, and regulatory elements. Such variable domains are thought to contribute to substrate recognition, subcellular localisation, and regulation therefore enabling distinct zDHHC enzymes to modify discrete subsets of protein substrates despite containing a conserved catalytic core.

Mechanistic understanding of zDHHC-mediated S-acylation was significantly advanced by the first X-ray crystal structure of a mammalian zDHHC enzyme, zDHHC20, reported by Rana *et al.* in 2018 (Rana et al., 2018). This structure revealed that the four transmembrane helices assemble into a hydrophobic, “tepee”-shaped pocket within the membrane, forming a cavity specifically adapted to accommodate the acyl chain during catalysis. The DHHC catalytic motif is positioned at the cytosolic-membrane interface, directly at the entrance of this pocket, allowing the catalytic cysteine to engage the acyl chain while maintaining access to the cytosolic environment (Mesquita et al., 2024, Rana et al., 2018). Several residues lining this cavity, including tryptophan 158, phenylalanine 171, phenylalanine 174, and leucine 227, form hydrophobic contacts with the acyl chain, and some of these residues are conserved across the zDHHC family. Together, these observations suggest that the hydrophobic cavity contributes to positioning and stabilisation of the acyl group during formation of the acyl-enzyme intermediate, although the precise contributions of individual residues to catalysis and substrate specificity remain to be fully established (Rana et al., 2018).

1.5.2 zDHHC mechanism of action

S-Acylation by zDHHC enzymes is widely proposed to proceed via a two-step “ping-pong” reaction mechanism (**Figure 1.4**), using a fatty acyl-coenzyme A (acyl-CoA) as a lipid donor. In the first step, the catalytic cysteine within the DHHC motif undergoes auto-acylation, forming a transient thioester linkage between the enzyme and the acyl chain (Jennings and Linder, 2012, Mitchell et al., 2010). This reaction is initiated by nucleophilic attack of the deprotonated cysteine thiolate on the thioester bond of acyl-CoA, resulting in the release of coenzyme A and the generation of the acyl-enzyme intermediate. In the second step, the acyl chain is transferred from the enzyme to a cysteine residue on the substrate protein, regenerating the free enzyme and enabling subsequent catalytic cycles (Jennings and Linder, 2012, Stix et al., 2020, Gonzalez Montoro et al., 2015). This transfer is predicted to occur via nucleophilic attack by the substrate cysteine thiol on the acyl carbonyl carbon of the acyl-enzyme thioester, thereby forming a new thioester bond between the lipid and the substrate (Stix et al., 2020, Mitchell et al., 2006, Jennings and Linder, 2012).

Structural and biochemical studies have provided important insight into the catalytic mechanism underlying this process. Analysis of the zDHHC20 crystal structure suggests that the first histidine residue within the DHHC motif plays a key role in activating the catalytic cysteine and stabilising the acyl-enzyme intermediate (Rana et al., 2018). In this model, polarisation of histidine 154 by the adjacent aspartate residue (D153) promotes deprotonation of the catalytic cysteine (C156), generating a reactive thiolate capable of attacking the acyl-CoA thioester bond. The special positioning of these residues within the conserved CRD supports their coordinated involvement in catalysis. Following acyl transfer to the substrate, the regenerated cysteine is available for further rounds of auto-acylation.

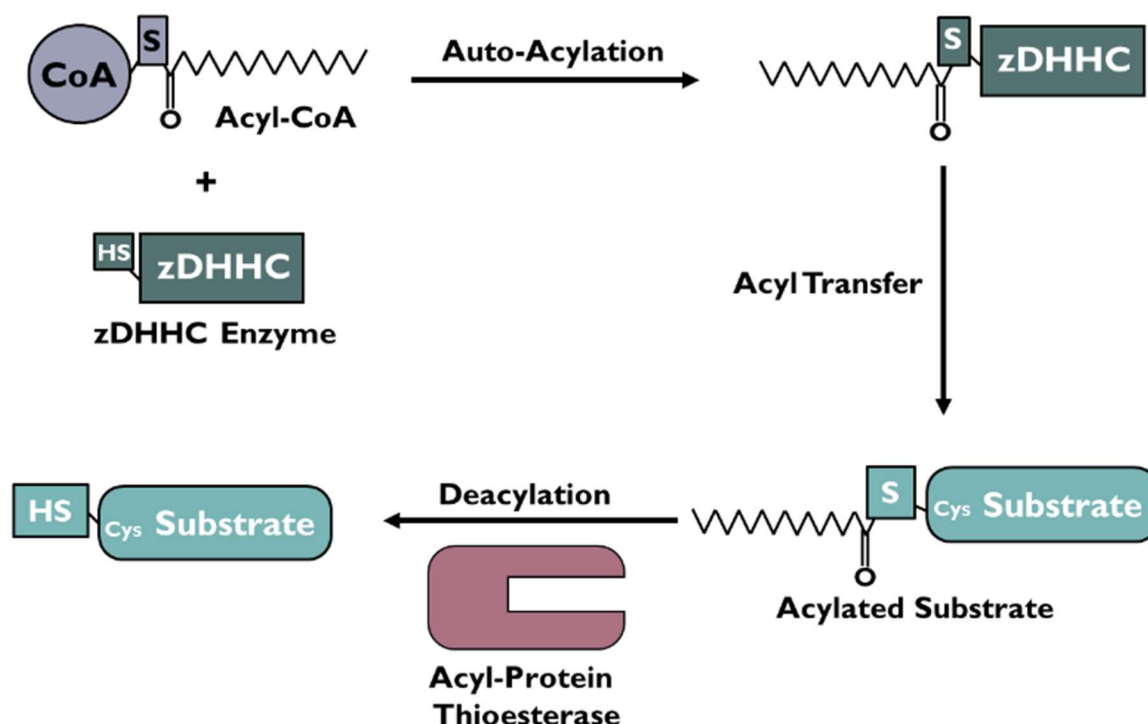


Figure 1.4. Schematic depicting the mechanism of zDHHC-mediated S-acylation. Initially, the zDHHC enzyme (green) is auto-acylated on the cysteine residue of the DHHC motif using an acyl group from a donor acyl-CoA (purple). This acyl group is then transferred from the zDHHC enzyme onto the substrate protein (blue) and this can be reversed by acyl-protein thioesterases (pink).

Although the two-step catalytic mechanism is widely accepted for zDHHC enzymes, the requirement for a stable auto-acylated intermediate remains a subject of discussion. In particular, acyl-enzyme intermediates have not been readily detected for zDHHC13, zDHHC17, zDHHC19, or zDHHC22 in [^3H]-palmitate metabolic labelling experiments (Lemonidis et al., 2014, Ohno et al., 2012). Consistent with this, auto-acylation of zDHHC17 was not observed using metabolic labelling with fatty acid alkyne followed by click chemistry to an azide-conjugated dye (Verardi et al., 2017). Moreover, incubation of purified zDHHC17 with a fluorescent analogue of palmitoyl-CoA yielded only a weak signal compared with zDHHC15, despite zDHHC17 retaining the ability to S-acylate SNAP25b. These findings have prompted suggestions that certain zDHHC enzymes may operate via alternative catalytic strategies or form highly transient acyl-enzyme intermediates that evade detection. However, the apparent absence of detectable intermediates may instead reflect differences in intermediate stability, catalytic turnover, or experimental sensitivity rather than a fundamentally distinct mechanism (Greaves et al., 2017). It is also possible that, for enzymes such as zDHHC17, substrate binding precedes acyl-enzyme formation, thereby shortening the lifetime of any free auto-acylated intermediate. Therefore, while mechanistic nuances may exist among individual

zDHHC family members, current structural and biochemical evidence broadly supports a conserved acyl-enzyme intermediate-based model for S-acyl transfer.

1.5.3 zDHHC subcellular localisation

zDHHC enzymes are polytopic membrane proteins that reside at distinct intracellular membranes, spatially restricting their access to potential substrates. Early overexpression studies in HEK293T cells using immunofluorescence microscopy showed that zDHHC enzymes are predominantly localised to the ER and Golgi apparatus, with a subset present at the plasma membrane (Ohno et al., 2006). More recent studies implemented super-resolution microscopy to generate a higher-resolution understanding of zDHHC localisation (Ernst et al., 2018). They reported that zDHHC-1, -4, -6 and -20 are localised at the ER, and zDHHC-2 and -5 are localised at the plasma membrane, with the remainder of the enzymes at the Golgi (zDHHC20 was reported to be at both the ER and Golgi). The majority of Golgi enzymes were localised at the cis-Golgi, whereas zDHHC9 and zDHHC11 were more oriented towards the trans-Golgi compartment.

zDHHC localisation is not static and can vary between cell types or in response to cellular conditions. For example, zDHHC2, initially described as an ER/Golgi enzyme by Ohno *et al.* (2006), was subsequently reported to be localised at the plasma membrane, endosomes and vesicles in HEK293T and PC12 cells and in primary hippocampal neurons (Ohno et al., 2006, Greaves et al., 2010, Salaun et al., 2017), and this post-Golgi localisation was confirmed in the study of Ernst et al. (2018). Furthermore, fluorescence recovery after photobleaching (FRAP) experiments showed that the localisation of zDHHC2 was dynamic in PC12 cells and that the enzyme cycles between the plasma membrane and endosomes (Greaves et al., 2011). A dynamic localisation was also reported in hippocampal neurons, where activity blockade was associated with the movement of zDHHC2 vesicles from dendritic shafts to spines (Noritake et al., 2009). In neurons, zDHHC5 also displays a dynamic localisation. It resides at synaptic membranes but undergoes activity-dependent endocytosis to dendritic shafts, enabling interaction with its substrate δ -catenin (Brigidi et al., 2015).

The mechanisms underlying the distinct localisation of zDHHC enzymes have been identified for some isoforms. The entry of zDHHC2 into the dynamic endosome-plasma membrane cycling pathway was shown to depend on a C-terminal sequence, and swapping the corresponding region from the related Golgi enzyme zDHHC15, led to the movement of zDHHC2 away from the plasma membrane (Greaves et al., 2010). Subsequent work showed that the C-terminal region of zDHHC2 contains phosphorylation-dependent sorting signals in this region of the enzyme that are important for determining the localisation of the enzyme (Salaun et al., 2017). Interestingly, the activity-dependent internalisation of zDHHC5 in hippocampal neurons is also regulated by phosphorylation. In this case, phosphorylation of a tyrosine-based sorting signal by Fyn kinase was shown to block internalisation (Brigidi et al., 2015).

Sorting motifs have also been identified on the ER enzymes zDHH4 and zDHH6. These enzymes rely on lysine-based motifs present at the extreme C-termini for ER retention. For zDHH6, the minimal motif was KKXX, whereas for zDHH4, a KXX motif mediates ER retention (Gorleku et al., 2011). Removal of these motifs led to the rerouting of zDHH4 to the Golgi, and the movement of zDHH6 to various membrane compartments, including the plasma membrane. Furthermore, appending the KKXX/ KXX sequences to the Golgi enzyme zDHH3 led to its redistribution to ER membranes. Finally, the Golgi enzyme zDHH21 fails to reach the Golgi and is instead retained in the ER upon deletion of a single amino acid, phenylalanine 233, in the C-terminus (Mill et al., 2009). However, the mechanism underlying the effect of phenylalanine 233 on localisation is not currently clear.

1.5.4 Thioesterase enzymes mediate protein deacylation

A defining feature of S-acylation is its reversibility, distinguishing it from many other lipid modifications and enabling dynamic regulation analogous to that observed with phosphorylation and ubiquitination (Chamberlain and Shipston, 2015). This reversibility permits proteins to undergo repeated cycles of S-acylation, therefore allowing precise control over protein localisation, stability, and signalling.

Proteomic analyses indicate that although S-acylation of many proteins is relatively stable over several hours, exhibiting low turnover rates (Martin et al., 2011), this stability is not universal. Turnover rates vary considerably between proteins and even between individual cysteine residues within the same protein. Certain subsets of signalling proteins undergo rapid cycles of acylation and deacylation, with direct consequences for their localisation and activity (Chamberlain and Shipston, 2015, Chen et al., 2021). For example, plasma membrane localisation of the Src kinase family protein Fyn depends on S-acylation, which exhibits an approximate turnover of 1.5-to-2 hours, therefore directly influencing the signalling role of this protein (Wolven et al., 1997, Zeidman et al., 2009). In other cases, turnover is regulated by cellular signalling events such as change in activation state. The small GTPase H-Ras exhibits activation-state-dependent acylation dynamics, with a S-acylation turnover of approximately 150 minutes in its inactive GDP-bound state that decreases to approximately 10 minutes in the active GTP-bound state (Baker et al., 2003). These observations highlight the importance of tightly regulated deacylation mechanisms, which allow cells to fine-tune membrane association and downstream signalling.

Deacylation is mediated by different classes of thioesterase enzymes that differ in subcellular localisation and substrate preference. The most extensively characterised are the APTs (Duncan and Gilman, 1998). In vertebrates, two conserved APT enzymes, APT1 and APT2, have been characterised, both of which require S-acylation for their activity. These soluble proteins, around 200 amino acids in size, belong to the serine hydrolase family and adopt a canonical α/β -hydrolase fold. Although predominantly cytosolic, both enzymes associate with membranes following S-acylation of an N-

terminal cysteine residue (C2). The S-acylation-dependent membrane association allows interaction of APT1 and APT2 with their substrates (Kong et al., 2013, Vartak et al., 2014). In both APT1 and APT2, substitution of C2 results in redistribution from membranes to the cytosol in both fluorescence microscopy and biochemical fractionation assays (Abrami et al., 2021, Kong et al., 2013).

S-Acylation also influences the stability of the APT enzymes. Preventing APT2 S-acylation reduces its half-life from approximately 5 hours to 3 hours, whereas treatment with the de-acylation inhibitor palmostatin B extends the half-life of wild-type APT2 to around 20 hours. Structural analysis using X-ray crystallography and molecular dynamics simulations identified a membrane-interacting region in APT2 termed the β tongue, composed of two antiparallel β -strands and a predominantly hydrophobic helix (Abrami et al., 2021). Mutations within this region abolish APT2 S-acylation in acyl-RAC assays and result in redistribution to soluble fractions, similar to the C2 mutant. These findings support a mechanism in which the β tongue mediates temporary membrane association, enabling subsequent S-acylation of C2 and stabilised membrane association. The zDHC enzymes zDHC3 and zDHC7 were identified as the enzymes responsible for APT2 S-acylation through siRNA knockdown and metabolic labelling experiments (Abrami et al., 2021).

Membrane association of the APT enzymes allows deacylation of their substrates. Catalysis by APT enzymes requires a conserved serine-histidine-aspartate triad. Substitution of the catalytic serine (S119 in APT1 or S122 in APT2) abolishes enzymatic activity (Won et al., 2016). Structural studies revealed a hydrophobic cleft within the catalytic domain that accommodates the acyl chain during hydrolysis, positioning the thioester bond for nucleophilic attack by the active-site serine (Devedjiev et al., 2000, Chen et al., 2021, Abrami et al., 2021). Mutation of hydrophobic residues within this pocket to arginine impairs APT2-mediated deacylation of zDHC6, demonstrating the functional importance of this region (Abrami et al., 2021).

Importantly, both APT1 and APT2 undergo auto-deacylation, enabling them to cycle between membrane-bound and cytosolic states and, therefore, dynamically regulate substrate access (Vartak et al., 2014). In astrocytes, palmostatin B treatment increases membrane localisation of APT1 and APT2 in a dose-dependent manner and elevates levels of their S-acylated forms, consistent with self- or cross-deacylation (Kong et al., 2013). Knockdown of APT1 increases membrane-associated APT2, suggesting that APT1 deacylates APT2. In contrast, APT2 knockdown does not significantly alter APT1 membrane levels, indicating limited reciprocal regulation. Furthermore, mutation of the catalytic serine to alanine results in strong membrane localisation rather than the mixed cytosolic/membrane distribution of wild-type protein, further supporting a role for auto-deacylation in regulating localisation (Kong et al., 2013).

Despite substantial sequence and structural similarity and some overlapping substrate profiles, APT1 and APT2 exhibit substrate selectivity (Won et al., 2018). APT1 has been implicated in deacylation of Gai proteins, certain small GTPases, and nitric oxide

synthase, whereas APT2 targets include growth-associated protein 43 (GAP-43) and the acyltransferase zDHHC6 (Amara et al., 2019, Chen et al., 2021).

Beyond the APTs, additional thioesterases contribute to S-acylation dynamics. Palmitoyl-protein thioesterase 1 (PPT1) is a lysosomal hydrolase initially identified through its ability to deacylate H-Ras (Camp and Hofmann, 1993, Verkruyse and Hofmann, 1996). Like the APTs, PPT1 contains a serine-histidine-aspartate catalytic triad and it has over 100 reported substrates with diverse functions (Gorenberg et al., 2022). PPT2, a lysosomal homologue of PPT1, also displays acyl thioesterase activity but appears to have a distinct substrate profile (Soyombo and Hofmann, 1997). The identification of the ABHD17 subfamily, which deacylates postsynaptic density 95 (PSD-95) and N-Ras, further expanded knowledge of the enzymatic network regulating S-acylation cycles (Lin and Conibear, 2015, Yokoi et al., 2016). Collectively, these thioesterases ensure that S-acylation remains tightly controlled and reversible modification, enabling dynamic interaction with signalling pathways.

1.5.5 Development of inhibitors of S-acylation enzymes

Despite the central role of S-acylation in regulating protein trafficking, signalling, and stability, research into zDHHC enzymes has been hindered by a lack of specific experimental tools. In particular, the scarcity of high-quality antibodies against endogenous zDHHC enzymes, combined with their low cellular expression levels (Mesquita et al., 2024), has limited the ability to study their physiological roles and interactions with substrates (Lan et al., 2021). As a result, many studies have relied on ectopically expressed, tagged zDHHC constructs, which may not fully recapitulate native enzyme behaviour (Malgapo and Linder, 2021).

Chemical inhibitors of S-acylation could provide a complementary approach to genetic knockouts or knockdowns, offering a rapid, reversible, and less complex means of probing zDHHC function and assessing their potential as therapeutic targets (Elliot Murphy and Banerjee, 2022, Zhang and Thomas, 2024, Lan et al., 2021, Zheng et al., 2013). Such tools would allow researchers to interrogate specific enzyme-substrate interactions and downstream biochemical effects without perturbing developmental processes or leading to compensatory changes in enzyme expression, as can occur with genetic manipulation.

Historically, 2-bromopalmitate (2-BP) has been the most widely used inhibitor of S-acylation. While previously identified as an inhibitor of lipid metabolism, 2-BP was shown to inhibit S-acylation and myristoylation of the Src-family kinase Fyn (Burges et al., 1968, Webb et al., 2000). Mechanistically, 2-BP enters the acyl-binding pocket of zDHHC enzymes and covalently modifies the catalytic cysteine within the DHHC motif, irreversibly preventing auto-acylation and subsequent substrate modification (Webb et al., 2000, Zheng et al., 2013, Lan et al., 2021).

However, the utility of 2-BP is limited by poor specificity and significant cytotoxicity. In addition to being non-selective towards different zDHHC enzyme isoforms, it also inhibits multiple enzymes beyond zDHHC family members, and its inhibitory effects often depend on high concentrations rather than selective binding (Coleman et al., 1992). Moreover, 2-BP has been shown to inhibit APT1 and APT2, the enzymes responsible for reversing S-acylation, paradoxically opposing its intended effect on protein acylation (Pedro et al., 2013). In HEK293T cells, concentrations between 20-80 μ M caused cytotoxicity after 6-24 hours of exposure (Lan et al., 2021). More recently, cyanomylacrylamide (CMA) was developed as a next-generation inhibitor. CMA demonstrates improved specificity for zDHHC enzymes compared with 2-BP, but it still lacks the potency and selectivity required for detailed mechanistic studies or therapeutic applications (Azizi et al., 2021, Pei and Piao, 2024).

A study by Salaun *et al.* (2022) undertook a high-throughput screen of 350,000 compounds to identify inhibitors of zDHHC2 auto-acylation (Salaun et al., 2022). This led to the discovery of two compounds, TTZ-1 and TTZ-2, that showed partial selectivity towards zDHHC enzymes expressed in HEK293T cells. Specifically, both compounds were effective at blocking the S-acylation of the substrate protein SNAP25 by both zDHHC2 and the related isoform zDHHC15. They also displayed reduced inhibitory activity towards zDHHC17. Interestingly however, the compounds did not block the S-acylation of SNAP25 by zDHHC3 or zDHHC7. As TTZ-1 and TTZ-2 were identified as inhibitors of auto-acylation, this suggests that partially selective inhibitors might be developed by targeting the DHHC active site. Overall, there remains a pressing need for high-quality chemical tools to dissect zDHHC function. There may be potential to develop TTZ-1 and TTZ-2 further to improve selectivity and potency but targeting enzyme-substrate interactions rather than the enzyme active site might provide a better route to inhibitor selectivity (Lemonidis et al., 2017a, Plain et al., 2020).

In contrast to the current lack of potent and selective inhibitors of zDHHC enzymes, there has been significant success in developing selective inhibitors of thioesterase enzymes. This is highlighted by the development of ML348 and ML349 as selective inhibitors of APT1 and APT2, respectively (Adibekian et al., 2010a, Adibekian et al., 2010b). These compounds were identified through high-throughput screening using fluorescence polarization-activity-based protein profiling (fluopol-ABPP). In this assay, a fluorescent probe bound to the enzyme rotates slowly due to the increased size, producing polarized light. When a competitive inhibitor displaces the probe, the free probe rotates more rapidly, leading to depolarization of the emitted light (Porta and Steel, 2023). The co-crystal structure of ML348 and ML349 with their respective APT enzymes revealed that both compounds occupy the acyl-binding pocket, but adopt distinct orientations and interactions. These differences explain their isoform specificity (Won et al., 2016).

A particular challenge in relation to the inhibition of S-acylation is how to generate specificity towards a specific S-acylated protein. Thus, even the highly selective ML348 and ML349 compounds will disrupt S-acylation of multiple proteins (i.e. all the targets of APT1 and APT2). An interesting strategy towards inhibiting the S-acylation of specific

substrate proteins was recently developed by Kuo *et al.* (2025) (Kuo *et al.*, 2025). Here, they showed that fusing APT enzymes to nanobodies could be used to mediate the selective deacylation of the nanobody target. Temporal control is provided by a chemogenetic switch built into the nanobody where a conformationally switchable protein domain is incorporated so that, in the absence of its ligand, the nanobody can bind the target and recruit the thioesterase, while addition of the ligand triggers a structural change that disrupts this interaction, effectively halting deacylation. This design provides a way to interfere in a regulated manner with the S-acylation of specific substrates of interest.

1.6 zDHHC enzymes and disease

Given its central role in regulating protein localisation, signalling, and stability, it is unsurprising that dysregulation of S-acylation and deacylation has been linked to a wide spectrum of human diseases, including cancer, metabolic disorders such as diabetes, and numerous neurological and neurodegenerative conditions (Mesquita *et al.*, 2024) (Chamberlain *et al.*, 2021, Malgapo and Linder, 2021). Perturbations in S-acylation can arise through altered expression or mutation of zDHHC enzymes, changes in thioesterase activity, or disruption of substrate recognition, collectively leading to aberrant signalling and cellular dysfunction.

1.6.1 S-Acylation in cancer

Multiple zDHHC enzymes and their substrates have been implicated in oncogenesis, tumour progression, and immune evasion (**Figure 1.5**) (Chen *et al.*, 2021, Zhou *et al.*, 2023). S-Acylation can modulate the stability or signalling capacity of proteins that promote proliferation, migration, or survival. For instance, zDHHC3 was shown to promote breast cancer cell migration through S-acylation of integrin $\beta 4$, a transmembrane adhesion receptor that cooperates with growth factor receptors to enhance cell motility and invasion, at multiple cysteine residues within its cytoplasmic juxta-membrane region (Gagnoux-Palacios *et al.*, 2003). S-acylation of integrin $\beta 4$ is required for its efficient localisation into lipid rafts and for downstream signalling activity (Sharma *et al.*, 2012, Soung and Chung, 2011). Inhibition of zDHHC3 auto-acylation using the polyphenol curcumin reduced integrin $\beta 4$ S-acylation and breast cancer cell migration, highlighting the therapeutic potential of targeting the S-acylation machinery (Coleman *et al.*, 2015).

In addition to promoting invasion, S-acylation can also enhance immune evasion in the tumour microenvironment. The immune checkpoint protein programmed cell death ligand 1 (PD-L1), which suppresses T-cell activation by engaging PD-1 on T cells, is a single-pass transmembrane protein (Lin *et al.*, 2024). zDHHC9-mediated S-acylation of cysteine 272 increases PD-L1 stability by reducing ubiquitination (Yang *et al.*, 2019).

PD-L1 S-acylation has been detected in primary human breast tumour tissues and breast cancer cell lines, and mutating the key S-acylation site (C272) reduces PD-L1 stability, enhances T-cell-mediated tumour cell killing, and reduces tumour growth *in vivo*. These findings highlight aberrant S-acylation as a mechanism that allows tumours to evade immune surveillance, demonstrating its impact on both cancer cell-driven signalling and tumour-immune interactions during disease progression.

Epidermal growth factor receptor (EGFR) signalling, frequently dysregulated in cancer, is also modulated by S-acylation (Liu et al., 2022). The effects of S-acylation on EGFR are highly context-dependent. In lung cancer, S-acylation of cysteine 797 enhances EGFR stability and promotes tumour cell growth (Bollu et al., 2015). Inhibition of S-acylation reduces tumour growth and increases sensitivity to tyrosine kinase inhibitors, suggesting that S-acylation contributes to therapeutic resistance (Ali et al., 2018). Conversely, in breast cancer, inhibition of zDHHC20-mediated S-acylation at cysteine 1025 increases EGFR stability and signalling, therefore promoting tumour cell survival (Runkle et al., 2016). These contrasting findings underscore that the functional consequences of S-acylation are highly substrate and context-specific, reflecting the complex integration of this modification within broader signalling networks.

1.6.2 S-Acylation in neurological disorders

Disruption of S-acylation has been strongly associated with neurological disorders (**Figure 1.5**). Proper neuronal development and synaptic function rely heavily on tightly regulated membrane targeting and scaffolding of receptors and signalling proteins, processes in which S-acylation plays a pivotal role (Peng et al., 2024).

Mutations in *ZDHHC9* have been shown to cause X-linked intellectual disability associated with Rolandic epilepsy, providing direct genetic evidence that impaired S-acylation contributes to neurodevelopmental pathology (Baker et al., 2015). Furthermore, genome-wide association studies have linked variants in *zDHHC5* and *zDHHC18* to schizophrenia risk (Zhao et al., 2018, Mueller and Meador-Woodruff, 2020), and single nucleotide polymorphisms (SNPs) in *ZDHHC8*, particularly rs175174, have been proposed to associate with schizophrenia susceptibility, although findings have been inconsistent across populations, highlighting the complexity of genetic contributions (Xu et al., 2010).

There is also evidence linking S-acylation to neurodegenerative diseases, such as Huntington's disease (HD), a neurodegenerative disorder caused by a CAG repeat expansion in the huntingtin (*HTT*) gene (The Huntington's Disease Collaborative Research Group, 1993). *HTT* is S-acylated by *zDHHC17* and *zDHHC13* at conserved cysteine residues, which regulates its subcellular trafficking, stability, and functional interactions (Lemarie et al., 2023, Sanders and Hayden, 2015). In HD, mutant huntingtin contains an extended polyglutamine tract that impairs its interaction with *zDHHC17*, resulting in reduced S-acylation (Yanai et al., 2006). This loss of modification is associated with altered localisation of *HTT* and toxicity. Restoring S-acylation in cellular models using APT

inhibitors reduces mutant HTT aggregation and cytotoxicity, providing proof-of-principle that modulating the S-acylation cycle has therapeutic potential (Lemarie et al., 2021, Lemarie et al., 2023). In addition to being a substrate of zDHHC17, it was further shown that HTT can positively modulate the activity of zDHHC17, and that this regulation is lost with mutant HTT (Huang et al., 2011). Indeed, mice lacking zDHHC17 display neuropathological and behavioural abnormalities similar to key features of HD, including motor deficits and synaptic dysfunction, demonstrating that loss of zDHHC17-mediated acylation can phenocopy aspects of the human disease (Singaraja et al., 2011, Young et al., 2012). However, HTT S-acylation was not affected in this model, suggesting that other zDHHC enzymes (such as zDHHC13) may compensate. In contrast, the S-acylation of other neuronal substrates involved in synaptic transmission, including PSD-95, was affected, suggesting that altered S-acylation networks, perhaps linked to loss of zDHHC17 regulation by mutant HTT, might broadly contribute to HD-like pathology (Singaraja et al., 2011).

1.6.3 S-Acylation in metabolic disorders

In addition to its links with cancer and neurological disease, dysregulation of S-acylation has emerged as a key contributor in several metabolic disorders, including type 2 diabetes and fatty liver disease (**Figure 1.5**). In type 2 diabetes, altered cardiac metabolism can lead to organ dysfunction and increased patient mortality (Ritchie and Abel, 2020). S-Acylation can impact the function of a receptor involved in cardiac fatty acid metabolism, the fatty acid transporter cluster of differentiation 36 (CD36), a multi-pass membrane protein critical for long-chain fatty acid uptake in cardiomyocytes and peripheral tissues. In diabetes, CD36 becomes abnormally localised to the sarcolemma due to increased zDHHC4-mediated S-acylation. This correlates with enhanced fatty acid oxidation, elevated triglyceride storage, and impaired cardiac function in diabetic animal models and insulin-resistant human iPSC-derived cardiomyocytes. Inhibition of zDHHC4 by CMA decreases CD36 S-acylation, normalises CD36 trafficking and improves functional outcomes, demonstrating that dysregulated S-acylation directly contributes to metabolic pathology (Dennis et al., 2025).

Another example is adipose triglyceride lipase (ATGL), a key enzyme in hepatic lipid metabolism. ATGL catalyses the initial step of triglyceride hydrolysis in hepatocytes, and its proper function is essential for lipid droplet catabolism and prevention of lipid overload. S-Acylation on multiple cysteine residues, particularly cysteine 15, mediated by zDHHC11, is required for its catalytic activity in human liver cells (Zheng et al., 2024). When the S-acylation site on ATGL is mutated or zDHHC11 is absent, ATGL becomes catalytically inactive despite correct localisation, leading to the accumulation of lipid droplets and defective lipolysis in hepatocytes. This disruption promotes hepatocyte lipid retention, a hallmark of non-alcoholic fatty liver disease (NAFLD), highlighting the importance of S-acylation in regulating lipid homeostasis in human liver (Carr et al., 2016, Zheng et al., 2024).

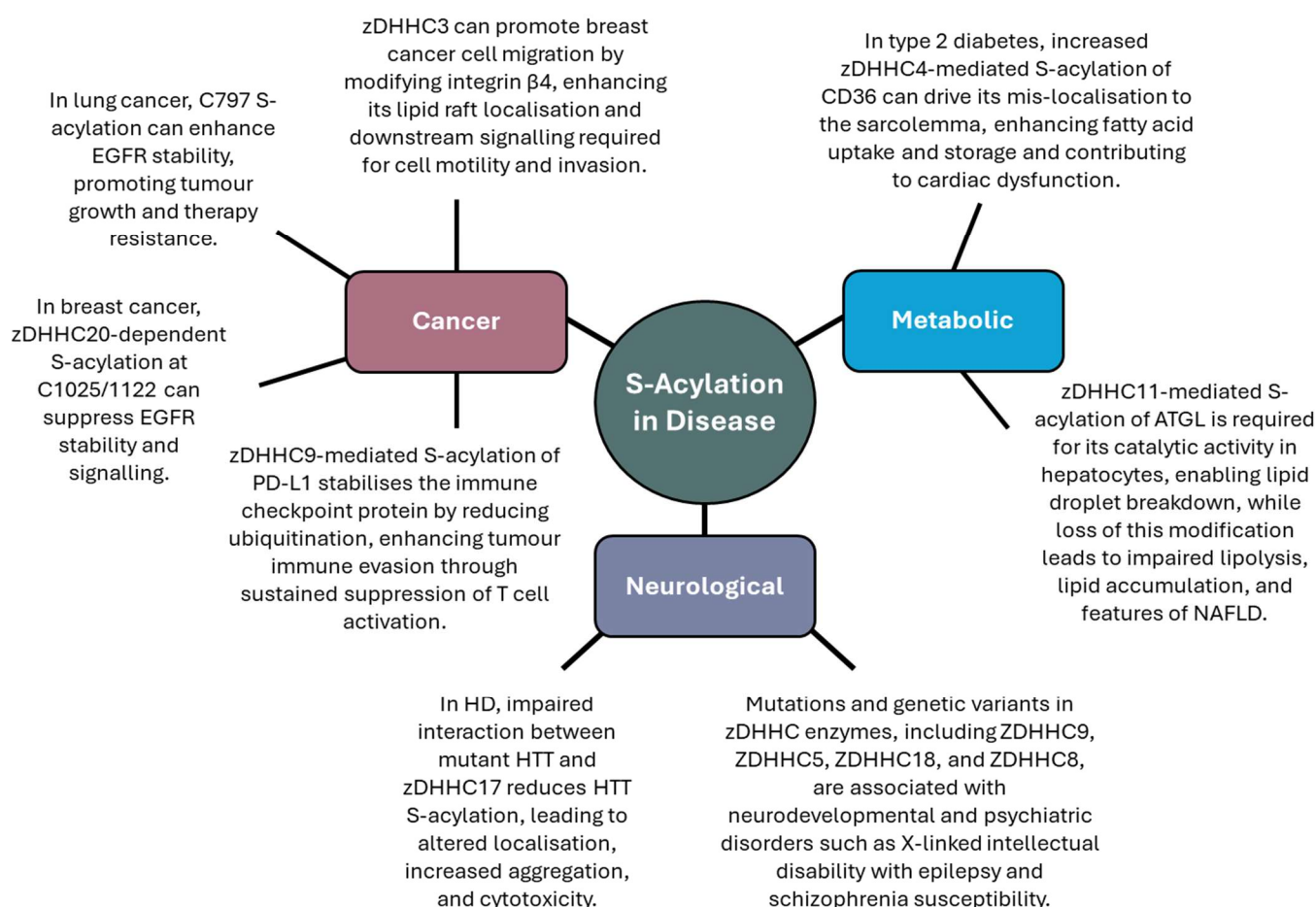


Figure 1.5. Functional roles of S-acylation in disease. S-Acylation regulates protein trafficking, stability, membrane organisation, and protein-protein interactions, therefore shaping key signalling pathways in cancer, neurological disease, and metabolic disease. Programmed cell death ligand 1 (PD-L1), epidermal growth factor receptor (EGFR), huntingtin (HTT), Huntington's disease (HD), cluster of differentiation 36 (CD36), adipose triglyceride lipase (ATGL), non-alcoholic fatty liver disease (NAFLD).

Together, this body of evidence establishes S-acylation as a central regulator of cellular homeostasis rather than a merely modulatory post-translational modification. By controlling protein localisation, interaction networks, signalling efficiency, and stability, S-acylation integrates into virtually every major cellular pathway, from immune regulation and growth factor signalling to neuronal and metabolic processes. Disruption of the delicate balance between acylation and de-acylation can rewire signalling networks, destabilise membrane proteins, and drive pathological processes across cancer, neurological, and metabolic disorders. Given the pervasive involvement of zDHHC enzymes in human disease, a detailed understanding of their regulation, substrate specificity, and context-dependent functions is critical.

1.7 Fatty acid profiles in S-acylated proteins and the role of zDHHC lipid selectivity

Palmitate (C16:0) is widely considered the predominant lipid added to proteins during S-acylation, to such an extent that the modification is often colloquially referred to as palmitoylation. Often, the most abundant lipid donor in cells is palmitoyl-CoA, which likely explains why palmitate is the most frequently incorporated fatty acid (Malgapo and Linder, 2021). Despite this dominance, it is now well recognised that multiple lipid species can be attached to proteins through S-acylation (Busquets Hernandez et al., 2025).

The identity and prevalence of fatty acids attached to S-acylated proteins have been examined in human platelets. Hydroxylamine selectively cleaves thioester-linked fatty acids from cysteine residues, generating fatty acid hydroxamates that can subsequently undergo acid methanolysis to form fatty acid methyl esters for analysis by gas chromatography-mass spectrometry (Muszbek et al., 1999). Analysis of proteins from 14 platelet samples revealed that approximately 74 % of S-acylation occurred with palmitate, with the remaining fatty acids consisting primarily of stearate (C18:0) and a small proportion of oleate (C18:1). Importantly, lipid utilisation was shown to depend on cellular availability: incubation of platelets with exogenous fatty acids resulted in a 26 % increase in palmitoylation and a 30 % increase in stearylation, respectively, reinforcing the role of fatty acid availability as a determinant of S-acylation patterns (Muszbek et al., 1999). More recent studies have used mass spectrometry to study the fatty acids released from S-acylated proteins in different tissues and cell types. Here, the amount of C18 fatty acyl chains (C18:0, C18:1, C18:2) released from S-acylated proteins exceeded the amount of C16:0 in all tissues tested (Busquets-Hernandez et al., 2024).

The fatty acids attached to individual proteins have not been extensively reported. The calmodulin-binding protein GAP-43 is S-acylated at cysteine residues 3 and 4 within its N-terminus, a modification essential for its membrane association (Liu et al., 1993). Analysis of the lipids attached to GAP-43 revealed that approximately 35 % of analysed peptides were S-acylated on one or both cysteines by either palmitate or stearate, indicating heterogeneous S-acylation (Liang et al., 2002). Notably, even a single cysteine residue can be modified by different fatty acids. The membrane-associated protein guanine nucleotide-binding protein G(i) subunit alpha-3 (GNAI3) is S-acylated at a single site, cysteine 3, and has been shown to incorporate analogues of both palmitate and stearate. Mass spectrometry analysis demonstrated endogenous acylation by palmitate and oleate, with stearate found to be predominantly desaturated prior to attachment (Nuskova et al., 2021). Moreover, the balance between palmitoylation and stearylation/oleoylation could be shifted by increasing exposure to the corresponding fatty acids, further confirming that lipid availability strongly influences the modification.

The incorporation of saturated versus unsaturated fatty acids can significantly affect protein localisation and function. The Src family kinase Fyn was shown to incorporate a range of saturated and unsaturated fatty acids when expressed in CHO-1 cells supplemented with isotope-labelled fatty acid analogues (Liang et al., 2001). All tested analogues (C14:0, C16:0, C18:0, and C18:1) were successfully incorporated, while

substitution of cysteine residues 3 and 6 (previously identified as the S-acylation sites of Fyn) abolished incorporation (Shenoy-Scaria et al., 1994, Alland et al., 1994, Liang et al., 2001). Fractionation experiments in COS-1 cells demonstrated that Fyn localised predominantly to membranes regardless of the fatty acid incorporated. However, localisation to membrane rafts differed markedly: approximately 70 % of Fyn modified with saturated fatty acids (palmitate or stearate) partitioned into purified raft fractions, compared with only ~40 % of Fyn modified with unsaturated oleate (C18:1) (Liang et al., 2001). Similar results were observed in Jurkat T cells, where ~75 % of Fyn was associated with raft fractions following stearic acid treatment, compared with ~40 % following treatment with arachidonic acid (C20:4) or eicosapentaenoic acid (C20:5). In line with these findings, treatment of T cells with polyunsaturated fatty acids reduced mitogen-activated protein kinase (MAPK) pathway activation by approximately 70 % relative to stearic acid, which had no effect (Liang et al., 2001). Comparable effects were observed for GNAI3, where treatment with C18:0 reduced association with detergent-resistant raft membranes and diminished PKB activation by impairing recruitment of the adaptor protein Grb2-associated binding protein 1 to the epidermal growth factor receptor (Nuskova et al., 2021).

Fatty acid preferences have also been documented for single-transmembrane viral glycoproteins. The Newcastle disease virus fusion protein F contains a cysteine residue (C523) near the cytoplasmic end of its transmembrane domain, which was shown by mass spectrometry to be modified with stearate (Kordyukova et al., 2010). In contrast, the VSV G protein contains a single S-acylated cysteine located six residues from the transmembrane domain in its C-terminal region, which is modified by palmitate. The Semliki Forest virus glycoprotein E1 is also S-acylated at a single cysteine within its transmembrane domain near the C-terminus and is primarily modified by stearate. The E2 glycoprotein contains four cysteine residues: one within the transmembrane domain, one in the proximal C-terminus, and two further into the C-terminal region. Evidence suggests that the transmembrane cysteine is modified by stearate, with possible minor modification of the adjacent C-terminal cysteine, whereas the two more distal C-terminal cysteines are likely modified exclusively by palmitate (Kordyukova et al., 2010). A similar pattern has been reported for influenza virus hemagglutinin, which contains three S-acylated cysteine residues: one within the transmembrane domain and two in the C-terminal tail (Veit et al., 1991). The transmembrane cysteine was found to be modified by stearate, while the C-terminal cysteines were palmitoylated (Kordyukova et al., 2008). Relocation of the transmembrane cysteine into the cytoplasm prevented stearate incorporation and resulted in palmitoylation at all three sites (Brett et al., 2014). Together, these findings suggest that, for these viral proteins, cysteine proximity to the transmembrane domain influences fatty acid selection, with stearate favoured at transmembrane or juxta-membrane positions and palmitate favoured at more distal cytoplasmic sites.

In addition to substrate-specific preferences, zDHHC enzymes themselves can exhibit biases toward particular fatty acids. The first X-ray crystal structure of a human zDHHC enzyme, zDHHC20, revealed the presence of a hydrophobic pocket formed by the

organisation of its transmembrane domains within the membrane. The geometry of this pocket has been proposed to determine the length of the acyl chain that can be accommodated and transferred to substrates, therefore contributing to enzyme-specific acyl chain selectivity (Rana et al., 2018). Early evidence for differential lipid preference was observed between zDHHC2 and zDHHC3: zDHHC2 efficiently transferred acyl chains of 16, 18, or 20 carbons, whereas zDHHC3 showed reduced efficiency for chains longer than 16 carbons (Jennings and Linder, 2012). The S-acylation of SNAP25 by several zDHHC enzymes (zDHHC2, -3, -7, -15, and -17) was subsequently examined using fatty acid probes of varying lengths (C14:0, C16:0, and C18:0). zDHHC3, zDHHC7, and zDHHC15 preferentially utilised C14:0 and C16:0 over C18:0, while zDHHC2 showed no clear preference and zDHHC17 favoured C16:0 and C18:0 over C14:0 (Greaves et al., 2017). Analysis of enzyme auto-acylation revealed a similar pattern of fatty acid preference, with the exception of zDHHC17, for which auto-acylation could not be detected. Expansion of this auto-acylation analysis to additional zDHHC enzymes demonstrated that zDHHC4 showed no detectable preference, zDHHC5 and zDHHC11 favoured C14:0 and C16:0, and zDHHC23 preferentially utilised C18:0 (Greaves et al., 2017).

This demonstrates that S-acylation is a far more diverse and regulated modification than the term palmitoylation implies. Multiple saturated and unsaturated lipids can be attached in a context-dependent manner, influenced by fatty acid availability, cysteine positioning relative to transmembrane domains, and the intrinsic acyl chain preferences of both substrates and zDHHC enzymes. Importantly, variation in acyl chain length and saturation can have profound consequences for protein localisation, membrane microdomain association, and downstream signalling. This underscores the need to consider acyl chain diversity when investigating the regulation and functional outcomes of protein S-acylation.

1.8 Features of S-acylated proteins and zDHHC enzyme substrate specificity

Despite extensive study, a universal consensus sequence for S-acylation has not been identified, and prediction algorithms still have only modest success in identifying likely S-acylated cysteines. However, the membrane proximity of cysteines, and therefore their proximity to zDHHC enzyme active sites, is undoubtedly a key factor determining whether they undergo S-acylation. For example, S-acylation of Src family kinases occurs adjacent to N-terminal myristoylation sites (Koegl et al., 1994), while S-acylation of Ras proteins is associated with C-terminal prenylation (Hancock et al., 1989, Chen et al., 2021). These dual lipidation events cooperate to stabilise membrane association, and in each case, the N-myristoylation or prenylation event is essential for subsequent S-acylation. A similar situation also exists for transmembrane proteins. For example, juxta-membrane cysteine positioning in claudins appears sufficient for modification, suggesting

accessibility-driven acylation rather than motif-based recognition (Rodenburg et al., 2017).

Although the zDHHC family comprises 23 closely related S-acyltransferases, how individual enzymes discriminate between substrates within complex cellular environments remains incompletely understood (Lemonidis et al., 2017b). Individual zDHHC enzymes are typically capable of modifying multiple substrates, and conversely, many substrates can be S-acylated by more than one zDHHC enzyme (Malgapo and Linder, 2021). Furthermore, numerous family members are co-expressed within the same cells and tissues, creating substantial potential for overlap and redundancy in substrate targeting (Wild et al., 2022, Wild et al., 2023). Defining the principles that organise these networks is therefore central to understanding the mechanistic basis of enzyme-substrate selectivity.

While spatial restriction provides one layer of selectivity, intrinsic molecular recognition mechanisms also contribute to substrate specificity. Some zDHHC enzymes, such as zDHHC3, are highly promiscuous and capable of modifying a broad range of substrates (Malgapo and Linder, 2021, Mesquita et al., 2024). One proposed explanation for the promiscuity of zDHHC3 and zDHHC7 is that these enzymes exist in a constitutively high-activity state and do not rely on clearly defined substrate interaction motifs. In this model, modification depends primarily on the accessibility and positioning of substrate cysteine residues within membranes rather than on specific protein-protein interactions (Lemonidis et al., 2014). However, it is important to note that the broad substrate selectivity of zDHHC3 and zDHHC7 has generally been reported through enzyme over-expression studies, and it is therefore possible that these enzymes have more restricted patterns of S-acylation at endogenous expression levels.

Some zDHHC family members do, however, display marked substrate selectivity. zDHHC17, for example, achieves specificity through its ankyrin repeat (ANK17) domain, which recognises substrates containing a defined zDHHC ankyrin-binding motif (zDABM), thereby coupling catalytic activity to direct protein-protein interaction (Lemonidis et al., 2015, Lemonidis et al., 2017a). zDHHC17's ANK domain was shown to mediate selective interactions with peripheral membrane proteins such as HTT, SNAP25 and CSP, indicating that this domain functions as an effective recruitment module for specific substrates. Deletion of this ANK region abolishes these interactions and its ability to S-acylate these substrates (Lemonidis et al., 2014). Conversely, appending the ANK domain onto zDHHC3 allowed this enzyme to S-acylate HTT, whereas wild-type zDHHC3 had no activity towards this protein (Huang et al., 2009). zDHHC13 similarly uses its ANK domain to engage SNAP25 and CSP, although in this case, interaction does not result in detectable S-acylation of these substrates (Lemonidis et al., 2014). zDHHC13 has a non-canonical DQHC motif, and it may be that this limits the set of binders that are able to be S-acylated by this enzyme. Indeed, adding a DQHC motif to zDHHC17 prevents the S-acylation of SNAP25 and CSP by this enzyme. This suggests a model in which motif-dependent substrate recruitment by ANK-containing zDHHC

enzymes represents a distinct and selective mechanism of enzyme-substrate interaction.

Additional interaction domains have been identified that contribute to protein interactions in other enzymes. For instance, zDHHC6 contains a Src homology 3 (SH3) domain that facilitates protein-protein interactions with regulatory partners (Fredericks et al., 2014). Furthermore, a PSD-95/Discs-large/ZO-1 homology (PDZ)-binding motif mediates substrate recruitment to zDHHC5, zDHHC8, and zDHHC14 and is predicted to function similarly in other family members (Thomas and Hayashi, 2013, Malgapo and Linder, 2021). In the case of zDHHC5, the C-terminal PDZ-binding domain of the enzyme specifically engages the third PDZ domain (PDZ3) of PSD-95. While PSD-95 itself was not found to be a substrate of zDHHC5, this interaction demonstrates how proteins can be recruited to zDHHC enzymes via PDZ-mediated binding (Li et al., 2010).

While zDHHC5 has been shown to interact with substrates through its PDZ-binding motif, a distinct substrate-interaction site has also been identified that regulates its association with specific targets (Howie et al., 2014, Plain et al., 2020). This region, located within the cytosolic C-terminal tail of the enzyme, interacts with the α -subunit of the Na^+/K^+ -ATPase, binding to its third intracellular loop (ICL) and helping position the regulatory protein phospholemman for S-acylation (Plain et al., 2020). The strength of this interaction is influenced by nearby post-translational modifications, including zDHHC20-mediated S-acylation of cysteine residues within the interacting region and O-GlcNAcylation of a serine residue within the surrounding region. Disruption of the zDHHC5- Na^+/K^+ -ATPase interaction reduces S-acylation of phospholemman, highlighting the functional importance of this recruitment mechanism. Because this binding site mediates interaction with a specific substrate complex, it represents a potentially more selective mechanism of zDHHC5-substrate recognition and may provide a strategy for selectively modulating the S-acylation of individual substrates.

Collectively, current evidence indicates that zDHHC enzyme-substrate networks are organised through a combination of spatial compartmentalisation, dynamic trafficking, targeting signals, catalytic activity states and defined interaction motifs. However, the relative contribution of each of these mechanisms, and how they integrate within specific cellular contexts, remains unresolved (Lemonidis et al., 2017b, Malgapo and Linder, 2021). Elucidating how these layers of regulation combine to generate selective yet flexible enzyme-substrate networks is therefore central to understanding the mechanistic basis of zDHHC-mediated substrate discrimination.

1.9 Transmembrane proteins are major targets of S-acylation

Transmembrane proteins are key to a vast number of cellular processes and are implicated in numerous diseases (Zauchar et al., 2021). They are a subgroup of integral membrane proteins, embedded within the lipid bilayer, and perform diverse functions,

including receptor activity, ion transport, and enzymatic catalysis. Integral membrane proteins comprise approximately 25 % of protein-coding genes and 20-30 % of the total mammalian proteome (Hegde and Keenan, 2022, Zaucha et al., 2021, Levental and Lyman, 2023). Some integral membrane proteins reside entirely within the membrane, others partially span it, and transmembrane proteins fully traverse the lipid bilayer. Cellular membranes are essential for compartmentalisation, yet selective transport and signalling across these barriers are essential for cellular function. Membrane proteins facilitate this by either directly transporting ions and molecules through channels or transporters, or by mediating ligand-activated signalling via receptors (Zaucha et al., 2021). As a result, transmembrane proteins are involved in many cellular processes such as metabolism, transport, immune responses, and signalling (Hegde and Keenan, 2022). However, their importance within cells also makes them vulnerable to pathological consequences arising from mutation, misfolding, or dysregulation (Sweeney et al., 2017).

Despite their importance, structural data for integral membrane proteins remain limited, with only around 24 % of human membrane proteins having full or partial Protein Data Bank structural data (Burley et al., 2019, Zaucha et al., 2021). Challenges in structural determination arise from hydrophobic membrane-spanning regions and the instability of these proteins outside their native lipid environment (Carpenter et al., 2008). X-ray crystallography requires solubilization in detergents, which can alter structure and protein-protein interactions (Birch et al., 2018). Most transmembrane regions adopt α -helical conformations, sequestering the hydrophilic protein backbone from the hydrophobic membrane, though some prokaryotic or organelle proteins form β -barrel structures (Noinaj et al., 2013).

Transmembrane proteins can contain multiple membrane-spanning domains, and the spatial arrangement of these domains is referred to as protein topology (Zaucha et al., 2021). They are often classified by the number and orientation of their transmembrane domains. Type I transmembrane proteins have a single domain with the N-terminus in the extracellular or luminal space and the C-terminus in the cytosol (Chataigner et al., 2020). Type II proteins also contain a single domain, but with the N-terminus in the cytosol and the C-terminus extracellular or luminal. Multi-pass transmembrane proteins span the membrane multiple times, creating complex topologies (Chou and Elrod, 1999).

The biomedical relevance of integral membrane proteins, particularly transmembrane proteins, is underscored by their involvement in disease and as drug targets. Approximately 60 % of therapeutic drugs act on membrane proteins (Overington et al., 2006). The cystic fibrosis transmembrane conductance regulator (CFTR) is an ion channel critical for water transport. Mutations in CFTR lead to mucus accumulation, inflammation, and impaired lung function. CFTR modulators, including potentiators and correctors, help restore protein function and trafficking, improving clinical outcomes, though they are not curative (Villamizar et al., 2019, Lopes-Pacheco, 2019). Receptor tyrosine kinases (RTKs) are single-pass transmembrane proteins with enzymatic activity, involved in processes such as proliferation, survival, and migration. Ligand binding activates downstream signalling cascades, such as MAPK, which can drive uncontrolled

proliferation if dysregulated, contributing to cancer. RTK inhibitors like bosutinib, gefitinib, and vemurafenib can slow tumour progression but are not curative due to resistance development (Saraon et al., 2021, Cohen et al., 2022). GPCRs, a multi-pass transmembrane receptor family, are targeted by roughly 34 % of FDA-approved drugs (Zhang et al., 2024). For example, muscarinic acetylcholine receptors (mAChRs), a type of GPCR, mediate bronchoconstriction in chronic obstructive pulmonary disease (COPD) and antagonists such as revefenacin improve airflow and reduce symptoms (Maqsood et al., 2019).

Many transmembrane proteins, including GPCRs, ion channels and plasma membrane receptors, undergo S-acylation. The structural complexity, functional diversity, and disease relevance of transmembrane proteins make them a critical class of S-acylation substrates and attractive targets for therapeutic intervention. Understanding their topology, trafficking, and post-translational regulation provides insight into the mechanisms governing selective modification and its broader cellular impact.

1.9.1 S-Acylation of single transmembrane proteins

Many Type I and Type II membrane proteins have been reported to undergo S-acylation, often in proximity to their transmembrane domain. CD4 is a Type I glycoprotein expressed on T cells and macrophages, and its S-acylation has been extensively characterised. Early work by Rose *et al.* using [³H] palmitate labelling in HeLa cells identified two cysteine residues near the C-terminal end of the transmembrane domain as sites of S-acylation (Crise and Rose, 1992). These cysteines were originally numbered C394 and C397, but in updated UniProt and SwissPalm databases, they correspond to C419 and C422. While C419 was initially described as part of the transmembrane domain, it is now known to be positioned immediately following it. Substitution of either C419 or C422 substantially reduced, and a double mutant completely abolished S-acylation, confirming the importance of both residues (Crise and Rose, 1992). Further studies in Jurkat T cells demonstrated that substitution of these cysteines with alanine completely abolished palmitate incorporation, whereas wild-type CD4 incorporated substantial amounts of palmitate (Fragoso et al., 2003). While the S-acylation sites of CD4 have been established, the functional consequences of this modification remain incompletely understood. CD4 acts as a co-receptor within the T cell receptor complex and plays a central role in immune responses. Conflicting effects of S-acylation on CD4 localisation have been reported (West et al., 2022). Truncation or substitution mutants lacking the C-terminal cysteine residues failed to localise to lipid rafts (Fragoso et al., 2003, Balamuth et al., 2004), and Lck binding was shown to further enhance CD4 partitioning into these domains, suggesting a cooperative effect (Fragoso et al., 2003). In contrast, other studies reported that S-acylation-deficient CD4 mutants exhibited little or no change in lipid raft association (Del Real et al., 2002, Popik and Alce, 2004). Together, these conflicting observations indicate that the effects of CD4 S-acylation on membrane localisation and function are still not fully understood, and the specific zDHHC enzymes responsible, as

well as the mechanisms by which S-acylation regulates CD4 activity, remain to be elucidated.

In contrast to the detailed information about S-acylation that is available for single transmembrane proteins such as CD4, the modification of many other single transmembrane proteins is poorly defined. One example is the single-pass transmembrane protein type III endosome membrane protein (TEMP), which is the focus of one chapter in this thesis. Although there is no published data reporting the S-acylation of TEMP, it was shown to interact with both zDHHC3 and zDHHC7 in the BioPlex Interactome (Huttlin et al., 2021). TEMP was identified as part of the FANTOM3 project, which aimed to perform functional analysis of cDNA data from the RIKEN Mouse Encyclopaedia Project (Maeda et al., 2006, Lizio et al., 2019, Hayashizaki, 2003). In humans, TEMP comprises 113 amino acids, forming a single transmembrane domain with a luminal N-terminus and cytosolic C-terminus (**Figure 1.6**). Conserved regions include the transmembrane domain (approximately 80 % conserved across species) and a C-terminal sequence, EDDDGFIEDNYIQPG, which is fully conserved (Aturaliya et al., 2012). This sequence is also reportedly found in the type I transmembrane protein Leucine-rich repeat containing Protein 19 (Lrrc19), although its specific function remains undefined (Chai et al., 2009). The protein also contains a predicted N-terminal glycosylation site and C-terminal [D/E]xxx[L/I] endosome sorting motif. TEMP expression is highest in the intestines, kidneys, and stomach (BioGPS/ SymAtlas) (Aturaliya et al., 2012).

TEMP was named for its localisation to endosomes and the plasma membrane. Using tagged expression constructs and fluorescence confocal microscopy, TEMP was shown to localise to both internal compartments and the plasma membrane (Aturaliya et al., 2012). Co-localisation studies with the early endosome proteins SNX1 and YFP-Rabankyrin-5 confirmed its presence in endosomes, and detailed analysis using Rab5 (early endosome), Rab7 (late endosome), and Rab4/Rab11 (recycling endosomes) indicated strong association with early and recycling endosomes but little with late endosomes. Live-cell microscopy in HeLa cells also revealed TEMP in tubular endosomes. Truncation of the [D/E]xxx[L/I] motif did not alter TEMP distribution, suggesting that other regions may contribute to its localisation. Overexpression of TEMP caused peripheral clustering of endosomes, potentially indicating a role in trafficking regulation, although experimental factors such as nocodazole treatment may have influenced these observations.

Although there are no reports of TEMP S-acylation, the reported interaction with two highly active zDHHC enzymes (zDHHC3 and zDHHC7) is of particular interest, as the mechanisms of substrate interaction by these enzymes are not well defined.

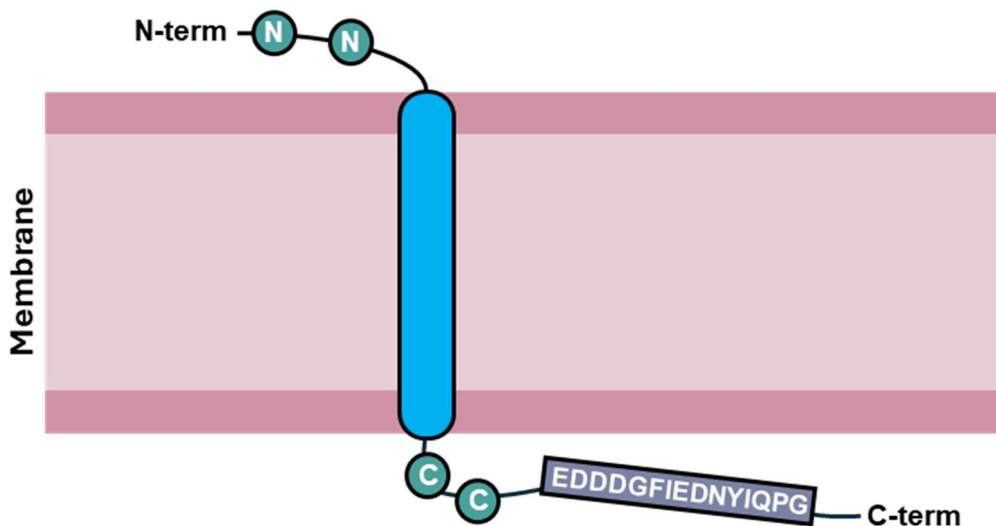


Figure 1.6. Diagram representing the structure of Type III Endosome Membrane Protein (TEMP). TEMP is shown embedded in the membrane with a single transmembrane domain (blue), C-terminal conserved domain (purple), and key amino acids (green).

1.9.2 S-Acylation of multi-transmembrane proteins

S-Acylation also plays an important role in regulating multi-pass transmembrane proteins. These proteins can be modified at multiple sites, including N- and C-terminal regions and on ICLs. Proteins with multiple membrane-spanning domains, such as the two-pass K5 ubiquitin ligase and tetraspanins with four transmembrane domains, highlight how S-acylation can influence protein stability, localisation, and interaction networks across diverse membrane environments.

K5, also known as modulator of immune response 2 (MIR2), is an E3 ubiquitin ligase encoded by Kaposi's sarcoma-associated herpesvirus (KSHV). It has transmembrane domains and cytosolic N- and C-termini, and plays a key role in ubiquitination and downregulation of cell surface proteins, including major histocompatibility complex I (MHC-I) and platelet endothelial cell adhesion molecule 1 (PECAM-1). Early studies in HeLa cells demonstrated that treatment with 2-BP reduced K5-mediated substrate downregulation, as measured by flow cytometry, suggesting a role for S-acylation. However, because 2-BP is not a reliable inhibitor of S-acylation, a biotin-switch assay was employed to confirm that K5 expressed in HEK293T cells is indeed S-acylated (Anania and Coscoy, 2011).

Functional analysis of cysteine residues revealed that C146, immediately following the second transmembrane domain, is critical for S-acylation. Substituting C146 with phenylalanine (C146F), a residue present at the corresponding position in the related

protein MIR1, reduced K5-mediated downregulation of MHC-I and PECAM-1. Biotin-switch assays confirmed that C146 is the S-acylation site, with no modification detected in the C146F mutant (Anania and Coscoy, 2011). Immunoprecipitation further showed that the C146F mutation disrupted the interaction between K5 and MHC-I, indicating that S-acylation at this residue is essential for substrate recognition. Finally, biotin-switch assays in TREx-BCBL1-Rta cells, human B-cell lymphoma-derived BCBL-1 cells latently infected with KSHV and engineered for inducible expression of Rta (Replication and Transcription Activator) a viral protein that triggers reactivation, confirmed K5 S-acylation in intact cells upon viral reactivation.

Tetraspanins are a family of multi-pass transmembrane proteins characterised by four transmembrane domains, which create two ECL, with the second loop typically larger. These proteins are widely distributed across cell types, often localising to the plasma membrane, and play diverse roles in processes such as cell adhesion, migration, and signal transduction (Reyes et al., 2018).

The tetraspanin CD9 has been implicated in cell proliferation, adhesion, and sperm-egg fusion (Reyes et al., 2018). [³H] palmitate labelling in the erythroleukemia cell line HEL demonstrated that CD9 incorporates palmitate, indicating S-acylation of the protein (Charrin et al., 2002). CD9 contains six cysteine residues located in cytosolic regions or near the cytosolic ends of transmembrane domains, making them potential sites for S-acylation. Site-directed mutagenesis and expression in CHO cells showed that mutation of all six cysteines (C9 in the cytosolic N-terminus; C78, C79, and C87 in the ICL; and C218 and C219 in the cytosolic juxta-membrane region adjacent to the final transmembrane domain) abolishes detectable palmitate incorporation, indicating that multiple cysteine residues collectively contribute to CD9 S-acylation (Charrin et al., 2002). However, this study did not resolve site-specific modification at the individual cysteine level. High-resolution native mass spectrometry later confirmed that CD9 exists as a heterogeneous population of multiply S-acylated species, directly demonstrating that all six cysteine residues can be modified in the mature protein (Neviani et al., 2020). CD9 typically carries at least three acyl groups, with the most abundant species containing up to five; while this reflects multiple S-acylation events, palmitoylation is predominant across the population, with strong contribution from C9, C87, and C219 (Neviani et al., 2020).

Co-expression experiments in HEK293T cells revealed that zDHHC2 most effectively enhanced CD9 S-acylation, while other zDHHC enzymes (zDHHC5, 7, 9, 11, and 15) had little effect (Sharma et al., 2008). Catalytically inactive zDHHC2 mutants did not enhance S-acylation, and similar results were obtained in the epidermoid carcinoma cell line A431, confirming the specificity of zDHHC2 in modifying CD9. The localisation of zDHHC2 was verified by staining with GM130, a marker of the Golgi matrix, suggesting that zDHHC2 is localised to the Golgi in this cell type. S-acylation by zDHHC2 is important for both the localisation and expression of CD9. Specifically, siRNA-mediated knockdown of zDHHC2 in HEK293T and breast cancer MDA-231 cells resulted in decreased CD9 expression, and flow cytometry analysis in HEK293T cells showed a

reduction in CD9 surface expression. In contrast, knockdown of other Golgi-localised zDHHC enzymes in HEK293T cells did not affect CD9 expression or S-acylation, suggesting that zDHHC2 is specifically responsible for the modification of this protein (Sharma et al., 2008).

In addition to CD9, several other tetraspanins are regulated by S-acylation, which influences their localisation, interactions, and function. CD151 is S-acylated on membrane-proximal cysteines, and loss of this modification reduces its association with other tetraspanins such as CD9 and CD63, alters its subcellular distribution, decreases stability during biosynthesis, and changes integrin-dependent cell morphology (Yang et al., 2002). CD81 is S-acylated on multiple juxta-membrane cysteines, and mutation of these residues reduces its interactions with partner proteins, including other tetraspanins and EWI-2, and can modulate susceptibility to hepatitis C virus entry (Delandre et al., 2009, Zhu et al., 2012). CD63 undergoes S-acylation that supports its association with CD9 and the integrin $\alpha\text{IIb}\beta\text{3}$ complex in activated platelets, facilitating its incorporation into tetraspanin-enriched microdomains (Israels and McMillan-Ward, 2010). These examples collectively highlight that S-acylation is a critical post-translational modification controlling tetraspanin membrane organisation, protein-protein interactions, and functional roles, and suggest that this modification may be a widespread regulatory mechanism across the tetraspanin family.

1.9.3 S-Acylation of GPCRs

GPCRs represent an important, large, and diverse group of multi-transmembrane receptors that are involved in a vast number of physiological functions, pathways, and diseases and are the target of approximately 30 % of FDA-approved drugs (Hauser et al., 2017). Structurally, GPCRs consist of seven transmembrane domains, an extracellular N-terminus and an intracellular C-terminus, producing three loops on both the intracellular and extracellular sides of the membrane, as well as a cytosolic C-terminal tail. These GPCRs share common structural features, including an extracellular ligand binding region, and an intracellular G protein binding region (Zhou and Wild, 2019). GPCRs are broadly divided into several classes based on sequence and structural homology, with class A (rhodopsin-like receptors) being the largest and most pharmacologically well-characterised group (Zhang et al., 2024). Other major classes include class B (secretin-like receptors) and class C (metabotropic glutamate-like receptors), which differ in their extracellular domain architecture and modes of ligand recognition and activation.

A common feature of GPCRs is the presence of one or more cysteine residues located in the C-terminal tail, typically positioned about 10-14 residues from the final transmembrane domain. Indeed, approximately 80 % of GPCRs have cysteine(s) located in this position (Escriba et al., 2007). The S-acylation of a cysteine in this location allows association of the C-terminus with the plasma membrane, essentially forming an additional ICL, which then has consequences for receptor conformation and protein interaction (Moench et al., 1994, Patwardhan et al., 2021). Notably, the first GPCR shown

to be S-acylated, bovine rhodopsin, was modified at two adjacent cysteine residues in this same region, highlighting the evolutionary conservation of this modification (Ovchinnikov Yu et al., 1988).

The β -adrenergic receptors are a family of GPCRs that have been extensively studied and represent an important class of drug targets. They are a subgroup of adrenoceptors that play a central role in regulating the sympathetic nervous system and are activated by catecholamines such as adrenaline and noradrenaline (Velmurugan et al., 2019). The β 2-adrenergic receptor (β 2AR) is expressed on the plasma membrane of a wide variety of cell types, including airway smooth muscle cells and skeletal muscle cells. Activation of β 2AR by adrenaline leads to physiological responses such as smooth muscle relaxation and bronchodilation. In contrast, the β 1-adrenergic receptor (β 1AR) is predominantly associated with cardiac tissue, where activation by noradrenaline and adrenaline regulates cardiac function.

The S-acylation of β 2AR has been well characterised. An initial site of S-acylation was identified at cysteine 341 within the cytoplasmic C-terminal region using [3 H] palmitate labelling (O'Dowd et al., 1989). This cysteine is located at a canonical GPCR S-acylation site, approximately 10 -14 amino acids downstream of the final transmembrane domain. Mutation of Cys341 reduced, but did not abolish, β 2AR S-acylation, indicating the presence of at least one additional modification site. Subsequently, an additional site of β 2AR S-acylation was identified at cysteine 265 within the ICL3 using acyl-RAC assays in HEK293T cells combined with cysteine-to-alanine mutagenesis (Adachi et al., 2016). Importantly, agonist stimulation with isoproterenol alters β 2AR S-acylation in a site-specific manner, reflecting differential regulation of distinct cysteine pools. C341 is believed to contribute to basal C-terminal membrane anchoring and shows relatively modest changes in turnover upon stimulation, whereas C265 within the ICL3 exhibits pronounced agonist-dependent dynamics, consistent with increased cycling of S-acylation and deacylation in the activated receptor state. This behaviour is thought to arise from agonist-induced conformational changes that differentially regulate accessibility of acyltransferases and thioesterases at each site (Loisel et al., 1996, Adachi et al., 2016).

The zDHHC enzymes responsible for S-acylating the C265 site of β 2AR were identified as the Golgi-localised enzymes zDHHC9, -14, and -18 through siRNA-mediated knockdown of the twenty human zDHHC enzymes detected in HEK293T cells by quantitative reverse-transcription PCR (Adachi et al., 2016). Knockdowns were performed in subfamilies based on zDHHC phylogenetic relationships. Combined knockdown of zDHHC9, -14, and -18 resulted in a marked reduction in C265 S-acylation, whereas individual knockdowns produced more modest effects. Conversely, overexpression of zDHHC9 or zDHHC14 enhanced isoproterenol-induced S-acylation at this site. Importantly, combined knockdown of these enzymes did not affect S-acylation at the C-terminal C341 site, indicating that distinct zDHHC enzymes mediate modification at different cysteine residues within the same receptor (Adachi et al., 2016).

The identification of agonist-induced S-acylation at C265 by Golgi-localised zDHHC enzymes supports a model in which a subset of activated β 2ARs traffic to the Golgi apparatus in addition to following the canonical post-activation pathway of internalisation, endosome sorting, and recycling or degradation. Consistent with this model, confocal immunofluorescence analysis revealed co-localisation of β 2AR with zDHHC9, -14, and -18 in the Golgi following isoproterenol stimulation (Adachi et al., 2016). Furthermore, plasma membrane-localised β 2AR was shown to undergo intracellular trafficking to the Golgi in response to agonist treatment. Disruption of Golgi integrity, which caused mis-localisation of zDHHC9, -14, and -18, led to a dose-dependent reduction in agonist-induced S-acylation at C265, demonstrating that Golgi localisation of these enzymes is critical for modification at this site.

In contrast to β 2AR, the S-acylation of β 1AR has been less extensively characterised. β 1AR exists in both immature and mature forms due to N-terminal O-glycosylation at serine residues S37 and S41 (Park et al., 2017). Using [3 H] palmitate labelling in HEK293T cells combined with cysteine-to-serine mutagenesis, S-acylation sites were identified at C-terminal residues C392, C393, and C414 (Zuckerman et al., 2011). The adjacent cysteines at positions C392 and C393 are highly conserved and are analogous to the primary C-terminal S-acylation site in β 2AR. Notably, an immature form of β 1AR was only detected when C392 and C393 were intact, suggesting that modification at these sites occurs early in the secretory pathway, prior to O-glycosylation, and contributes to the accumulation or detectability of a precursor form rather than affecting overall receptor expression or localisation. Mutation of the β 1AR S-acylation sites, either individually or in combination, did not significantly affect receptor stability or subcellular localisation (Zuckerman et al., 2011). However, as observed for β 2AR, there were clear differences in the dynamics of S-acylation at distinct cysteine residues. Pulse-chase experiments revealed that S-acylation at the C392/C393 site exhibited very slow turnover, whereas the modification at C414 was rapidly turned over (Zuckerman et al., 2011). Despite these insights, no zDHHC enzyme has yet been definitively identified as responsible for S-acylating β 1AR.

The vasopressin receptor family comprises three GPCRs that respond to the peptide hormone vasopressin, including vasopressin receptor 1A (V1aR) and vasopressin receptor 1B (V1bR). V1aR is expressed across a wide range of tissues, including the heart, kidney, and central nervous system, and is therefore involved in diverse physiological processes, including vascular contraction and glucose metabolism. In contrast, V1bR expression is more restricted, being predominantly localised to the pituitary gland and pancreas, where it is involved in the release of adrenocorticotrophic hormone and insulin (Wasilewski et al., 2016).

S-Acylation of V1aR has been characterised for the rat receptor but not the human protein. Rat V1aR contains three cysteine residues within the C-terminal region, two located 14 and 15 residues downstream of the final transmembrane domain, and a third located eight residues from the end of this domain. These cysteines are positioned at equivalent distances from the transmembrane domain in human V1aR, and the two

proteins are highly conserved in this region. Notably, all three residues fall within the canonical GPCR region commonly associated with S-acylation. Cysteine-to-glycine substitutions of the adjacent cysteine residues C371 and C372, followed by [³H] palmitate labelling in CHO cells, resulted in a significant but incomplete reduction in palmitate incorporation compared with the wild-type receptor (Hawtin et al., 2001). This finding indicates that C371 and C372 are sites of S-acylation, while also suggesting the presence of at least one additional modification site, potentially the third C-terminal cysteine at C365 or a cysteine located within an ICL. Consistent with dynamic regulation of this modification, stimulation of V1aR with arginine vasopressin increased S-acylation of both the wild-type and cysteine-to-glycine constructs, although the response was reduced for the mutant (Hawtin et al., 2001). Functional analyses revealed that substitution of C371 and C372 had only modest effects on ligand binding, as assessed by radioligand binding assays in HEK293T cells. Likewise, substitution of these residues did not markedly impair receptor signalling, as measured by inositol phosphate accumulation downstream of phosphoinositidase C activation (Hawtin et al., 2001). To date, the zDHHC enzymes responsible for S-acylating V1aR have not been identified.

In contrast to V1aR, S-acylation of V1bR has not yet been investigated. Similar to V1aR, V1bR contains a pair of adjacent cysteine residues within the C-terminal region located 14 and 15 residues downstream of the final transmembrane domain, as well as additional cysteines within ICLs. However, unlike V1aR, V1bR lacks a cysteine located eight residues from the transmembrane domain and instead contains a cysteine positioned further into the C-terminal tail. These structural differences may underlie distinct patterns of S-acylation and regulation between the two receptors.

1.10 C-C chemokine receptor type 5

1.10.1 CCR5 activation and signalling

While S-acylation has been identified as a key post-translational modification regulating the function, localisation, and signalling of various GPCRs, the effects of this modification can be highly receptor-specific. Among the many GPCRs expressed in immune cells, the C-C chemokine receptor type 5 (CCR5) is of interest due to its central role in chemokine recognition, immune cell migration, and signalling pathway activation. As this GPCR is a central focus of this thesis, CCR5 will be discussed in detail here, highlighting its structure, function, and regulatory mechanisms, including the emerging role of S-acylation in receptor regulation.

CCR5 is located on the surface of immune cells such as macrophages, T lymphocytes, and dendritic cells. Its ability to recognise chemokines allows migration of immune cells to the site of an infection along a chemokine gradient. The binding of ligands, including chemokine ligand 3 (CCL3), CCL4, CCL5 (also called RANTES), and CCL8, activates

CCR5 (Jasinska et al., 2022). As several other receptors can recognise the same chemokines as CCR5, it is generally thought of as a non-essential protein (Jansen and Beaumelle, 2022).

Activation of CCR5 allows interaction with heterotrimeric G proteins, which regulate cellular signalling pathways and can lead to transcription of genes involved in the immune response (Jasinska et al., 2022). CCR5 predominantly couples to G_{ai}, although signalling via additional G proteins such as G_{aq} has been reported in certain cellular contexts. Upon ligand binding, CCR5 undergoes conformational changes that promote GTP binding onto the G_α subunit and dissociation of G_α and G_{βγ}, initiating downstream signalling cascades involved in chemotaxis and immune cell activation (**Figure 1.7**) (Hamid et al., 2023).

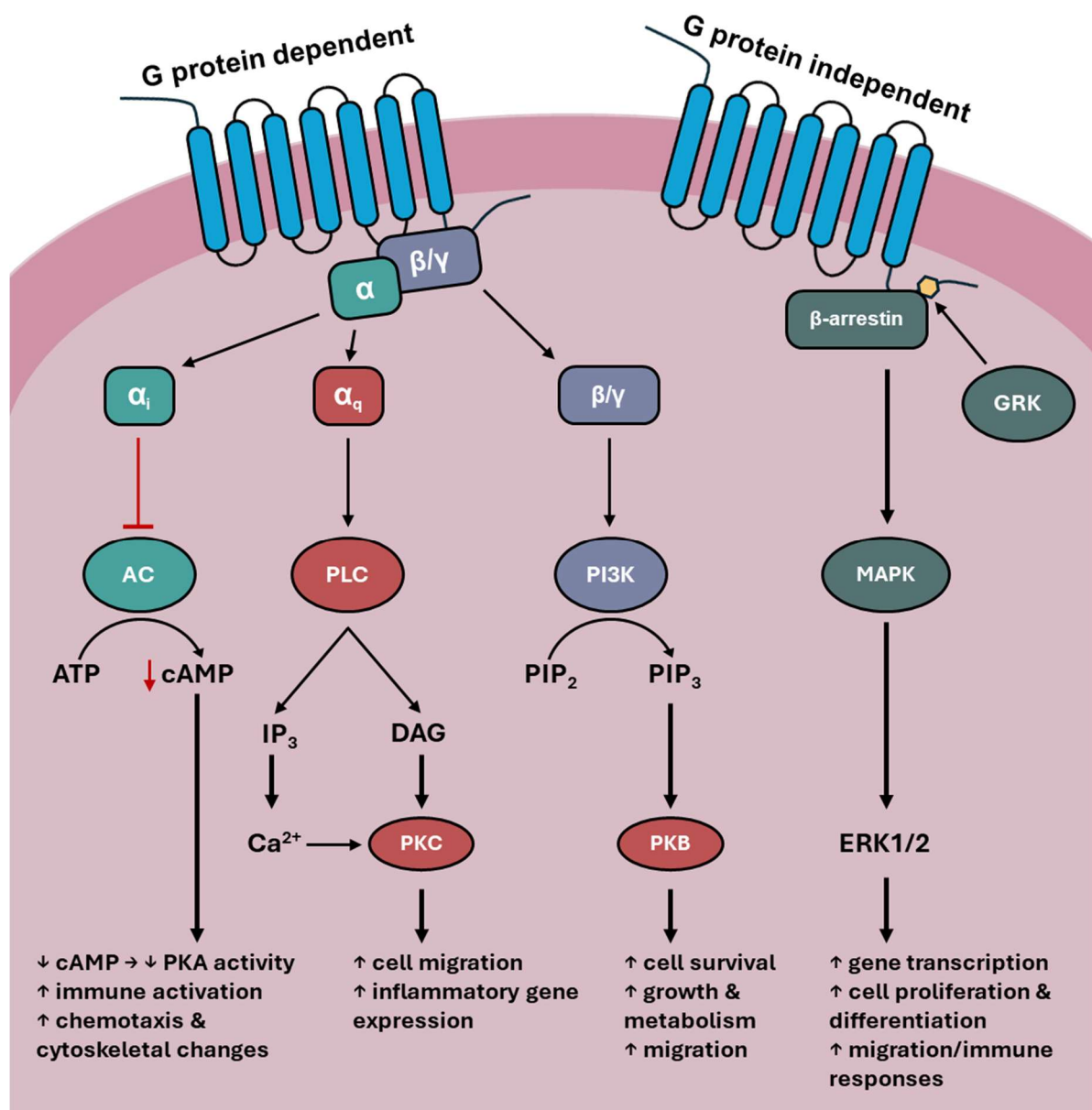


Figure 1.7. Molecular Mechanisms of CCR5 Signal Transduction. Schematic representation of CCR5 signalling via G protein-dependent and G protein-independent mechanisms. Upon ligand binding, CCR5 activates heterotrimeric G proteins, leading to dissociation into $G\alpha$ and $G\beta\gamma$ subunits. The $G\alpha_i$ subunit inhibits adenylyl cyclase (AC), reducing cyclic AMP (cAMP) levels, while $G\alpha_q$ activates phospholipase C (PLC) to generate inositol triphosphate (IP_3) and diacylglycerol (DAG), promoting Ca^{2+} release and protein kinase C (PKC) activation. The $G\beta\gamma$ complex activates phosphoinositide 3-kinase (PI3K), leading to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) production and downstream PKB signalling. In parallel, receptor phosphorylation by G protein-coupled receptor kinases (GRKs) promotes β -arrestin recruitment, resulting in receptor desensitisation and internalisation, as well as activation of G protein-independent pathways such as mitogen-activated protein kinase (MAPK)/ERK signalling.

Activation of G α i inhibits adenylyl cyclase (AC), reducing cyclic adenosine monophosphate (cAMP) levels and downstream PKA activity, thereby modulating transcriptional responses and cellular signalling outputs (Jasinska et al., 2022, Hamid et al., 2023). Concurrently, dissociated G $\beta\gamma$ subunits activate phosphoinositide 3-kinase (PI3K), leading to phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) production and activation of PKB, which drives downstream transcriptional regulators, including nuclear factor kappa B, and promotes cell survival and chemotactic responses. Activation of G α q stimulates phospholipase C (PLC), generating inositol trisphosphate (IP₃) and diacylglycerol (DAG), which mediate Ca²⁺ release from intracellular stores and activation of protein kinase C (PKC); PKC signalling subsequently activates mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathways, contributing to transcriptional responses associated with cell survival, proliferation, cytokine production, and chemotaxis. CCR5 signalling additionally regulates cytoskeletal dynamics through Rho family GTPases, promoting actin remodelling and cell migration.

Beyond G protein-dependent pathways, CCR5 signals through G protein-independent mechanisms, including β -arrestin-mediated receptor desensitisation, internalisation, and scaffolding of MAPK/ERK signalling complexes, contributing to sustained signalling and transcriptional regulation (Faivre et al., 2024, Hamid et al., 2023).

1.10.2 CCR5 structure

Building on the broader context of GPCR structure and regulation, CCR5 provides a compelling example of how structural features and post-translational modifications fine-tune receptor function. This protein is an example of a rhodopsin-like class A GPCR, the largest group within the family (Jasinska et al., 2022). Ligand recognition involves conserved N-terminal tyrosine residues and amino acids in ECLs 1 and 2. Ligand binding has been proposed, in 3D models with CCL5, to first require interaction between these residues and the core of the ligand. This is followed by the interaction of the CCL5 N-terminus with the region of CCR5 where the transmembrane domains have formed into a helical bundle (Arumugam et al., 2014).

The CCR5 receptor becomes desensitised following ligand binding due to the phosphorylation of the C-terminal domain on ICL3. Ligand dissociation then triggers receptor internalisation, followed by recycling to the cell surface (Mohamed et al., 2021). The presence of CCR5 as homo- and heterodimers has been observed through immunoprecipitation assays. However, there have been contrasting theories proposed regarding the formation of these dimers, with one theory suggesting that the dimers are formed due to ligand binding (Vila-Coro et al., 2000), and a second theory that these dimers are present constitutively (Benkirane et al., 1997). Issafras et al. utilised bioluminescence resonance energy transfer (BRET) assays to demonstrate constitutive oligomerisation of CCR5 in HEK293 cells (Issafras et al., 2002). Their findings showed that CCR5 exists as pre-formed oligomers in the absence of ligand stimulation, with no significant change observed following the addition of chemokines such as CCL5 or CCL4.

The authors proposed that discrepancies with earlier studies reporting ligand-induced oligomerisation may be due to methodological differences, particularly the use of denaturing conditions or chemical cross-linking, which can stabilise receptor interactions and influence their detection. These findings support the conclusion that CCR5 oligomerisation is an intrinsic and ligand-independent property of the receptor (Issafras et al., 2002). More recent structural and computational studies have further defined the molecular basis of CCR5 dimerization, identifying preferred transmembrane interfaces, particularly involving TM4-TM5 and TM6-TM7 helices (Di Marino et al., 2023). CCR5 dimers can adopt symmetric or asymmetric configurations, and these distinct assemblies may differentially regulate access to ligand and G protein binding sites. Together, these findings suggest that CCR5 oligomerisation is a constitutive property of the receptor that may function as an allosteric mechanism to modulate signalling output.

CCR5 undergoes multiple post-translational modifications (**Figure 1.8**) that regulate its structure and function. Disulfide bonds link cysteine 20 in the N-terminus with cysteine 269 in ECL3, and cysteine 101 in ECL1 with cysteine 178 in ECL2, with the latter disulphide bond necessary for maintaining proper receptor conformation as evidenced by loss of recognition by conformation-sensitive antibodies when mutated (Oppermann, 2004, Blanpain et al., 1999). CCR5 can also undergo heterogeneous tyrosine sulphation on tyrosine residues 3, 10, 14, and 15 located in the N-terminus. This plays a role in the binding of ligands to the receptor (Scurci et al., 2021). O-glycosylation occurs at serine residues 6 and 7, with at least one site glycosylated in the wild-type receptor, supporting β -chemokine binding (Bannert et al., 2001). Ligand binding also induces phosphorylation of serine residues 336, 337, 342, and 349 in the C-terminus via GPCR kinase and PKC (Venuti et al., 2017, Pollok-Kopp et al., 2003). This phosphorylation allows docking of β -arrestins, promoting their recruitment and activation (Pollok-Kopp et al., 2003, Oppermann, 2004). Once recruited, β -arrestins sterically block further G protein coupling, resulting in receptor desensitisation, while also initiating G protein-independent signalling pathways (Oppermann, 2004, Venuti et al., 2017). Phosphorylation and β -arrestin recruitment also directs CCR5 to the clathrin-mediated endocytic machinery, promoting receptor clustering and internalisation, with the receptor either recycled to the plasma membrane or targeted for degradation (Pollok-Kopp et al., 2003, Oppermann, 2004, Faivre et al., 2024). Through this coordinated series of events, CCR5 phosphorylation finely regulates both receptor availability at the cell surface and the balance between G protein-dependent and β -arrestin-dependent signalling pathways. Finally, CCR5 contains three S-acylated cysteine residues at positions 321, 323, and 324. These modifications are important for proper trafficking to the plasma membrane and for anchoring the C-terminus near the membrane, thereby influencing signalling pathways (Jansen and Beaumelle, 2022, Barmania and Pepper, 2013).

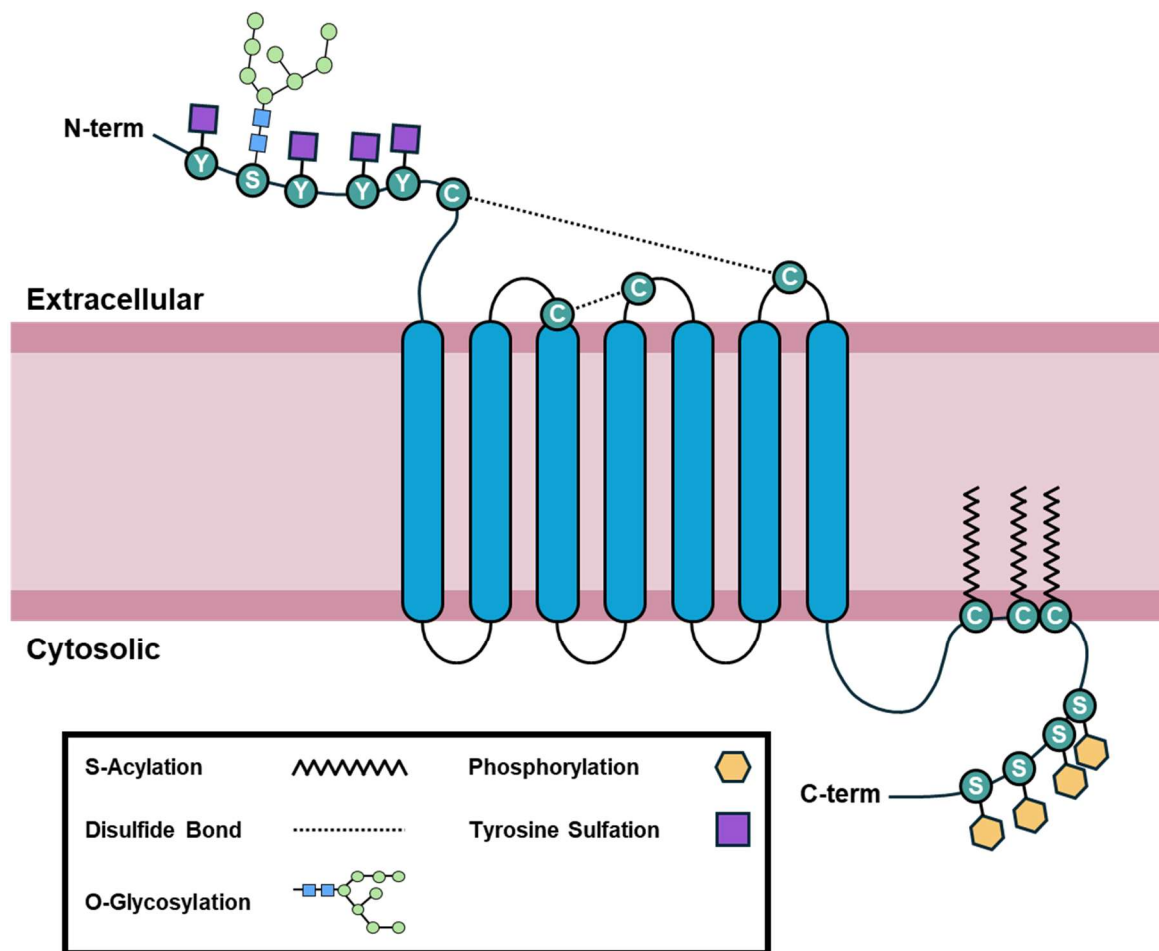


Figure 1.8. Structure and post-translational modifications of CCR5. CCR5 is depicted embedded within the plasma membrane, with its seven transmembrane domains shown in blue. Post-translationally modified residues are highlighted with green circles. A key indicates the type of modification for each residue, including disulfide bonds, tyrosine sulfation, O-glycosylation, phosphorylation, and S-acylation.

1.10.3 CCR5 O-glycosylation

CCR5 N-terminal O-glycosylation represents a key mechanism influencing receptor stability, ligand recognition, and protein-protein interactions, which are critical for immune responses and cell trafficking (Thompson and Wakarchuk, 2022). O-Glycosylation involves the attachment of oligosaccharides to the hydroxyl groups of serine or threonine residues. This distinguishes it from N-glycosylation, the most common form in eukaryotes, which attaches oligosaccharides to the nitrogen of asparagine residues (Apweiler et al., 1999). In mucin-type O-glycosylation, an initial N-acetylgalactosamine (GalNAc) residue is added to serine or threonine residues, after which the glycan is elongated through the stepwise addition of monosaccharides catalysed by glycosyltransferases in the Golgi apparatus (Thompson and Wakarchuk,

2022, Reily et al., 2019). These GalNAc-linked glycans are typically found on secreted and extracellular proteins, including membrane receptors such as CCR5, and biosynthesis is initiated after protein folding, in contrast to N-glycosylation which begins in the ER. In contrast, O-GlcNAcylation is a distinct form of O-linked glycosylation that involves the addition of a single N-acetylglucosamine (GlcNAc) residue to intracellular proteins and occurs at various locations throughout the cell including the nucleus and cytoplasm, catalysed by O-GlcNAc transferases (Torres and Hart, 1984, Holt and Hart, 1986, Cheng et al., 2024).

CCR5 glycosylation was initially investigated as potential N-glycosylation; however, treatment with either tunicamycin, which inhibits the first step of N-linked glycan precursor synthesis, or endoglycosidase F, which removes N-glycosylation through cleavage of glycoside bonds, did not alter the SDS-PAGE mobility of CCR5, suggesting N-glycosylation was not present (Farzan et al., 1999). The possibility of O-glycosylation was then investigated due to the presence of serine/threonine abundant regions on the protein, particularly on the N-terminus. Treatment with neuraminidase, which removes sialic acid, or O-glycosidases, which remove saccharides, resulted in increased mobility of CCR5 in SDS-PAGE demonstrating the presence of O-linked glycosylation containing sialic acid (Farzan et al., 1999).

The specific N-terminal O-glycosylation sites were identified using a truncation mutant removing residues 2-17, which include serine 6, serine 7, threonine 16, and serine 17. Expression of this mutant in Cf2Th cells showed a reduced molecular weight (~4-5 kDa) and abolished incorporation of galactose, a sugar commonly present in eukaryotic O-glycosylation (Bannert et al., 2001). Importantly, the truncation did not affect CCR5 trafficking to the cell surface. Other clusters of serine/threonine residues in the ECL were tested via alanine substitution, but these did not alter mobility, confirming that O-glycosylation is restricted to the N-terminus.

Further mutagenesis of serine/threonine residues revealed that mutation of serine 6 and 7 to alanine decreased molecular weight by ~2 kDa, consistent with glycosidase treatment, while mutation of threonine 16 and serine 17 had no effect. Individual residue mutations showed that either serine 6 or 7 could be O-glycosylated, but in wild-type CCR5, only one site is occupied at a time. Mutation of all four residues abolished galactose incorporation, confirming these as the primary O-glycosylation sites. Interestingly, minor galactose incorporation was still observed in the S6A/S7A double mutant, suggesting threonine 16 and serine 17 may be weakly glycosylated but at a lower efficiency or with smaller glycans. These patterns were consistent across multiple cell types, including HeLa, rhesus macrophages, and HEK293T cells, confirming conserved N-terminal O-glycosylation (Bannert et al., 2001).

To determine whether CCR5 O-glycosylation was GalNAc-linked, COSMC knockout (KO) cells, which impair elongation of mucin-type O-glycans beyond the initial GalNAc residue, were used. CCR5 expressed in COSMC KO HEK293T cells exhibited reduced molecular weight, supporting the presence of GalNAc-initiated O-glycosylation. Knockout of N-acetylgalactosaminyl-transferase (GALNT)-1, -2, and -3 had no effect in

HEK293T cells, suggesting functional redundancy among GalNAc-transferases. In contrast, knockout of GALNT1, 2, 7, 11, and 19 in CHO cells decreased CCR5 molecular weight, indicating that one or more of these enzymes contribute to CCR5 O-glycosylation in a cell-type-dependent manner (Verhallen et al., 2023).

Functionally, O-glycosylation of CCR5 has a pronounced effect on chemokine binding. Homologous competitive binding assays in Cf2Th cells demonstrated that the S6A/S7A double mutant exhibited markedly reduced binding of the chemokines CCL3 and CCL4, with CCL3 binding almost completely abolished. Analysis of single-residue mutants revealed that the substitution of serine 6 alone substantially decreased CCL4 binding, whereas the substitution of serine 7 had little to no effect, indicating that glycosylation at serine 6 is critical for efficient ligand recognition, while glycosylation of serine 7 may serve a minor or redundant role (Bannert et al., 2001). To further confirm that the effects on chemokine binding were specifically due to O-glycosylation, experiments were performed in CHO-derived Id1D cells, which lack sufficient UDP-galactose and UDP-GalNAc synthesis due to loss of epimerase activity. CCR5 expressed in these glycosylation-deficient conditions exhibited a reduced molecular weight of approximately 2 kDa, consistent with loss of O-glycan addition, and showed significantly decreased binding of both CCL3 and CCL4. Restoration of Gal and GalNAc in the media rescued both CCR5 molecular weight and ligand-binding capacity, directly linking O-glycosylation to chemokine affinity. Additionally, sialic acid, which is often incorporated at the termini of O-glycans, was shown to contribute to chemokine binding: treatment of CCR5-expressing Cf2Th cells with sialidase reduced binding of CCL3 and CCL4, suggesting that the negative charge introduced by sialylation may enhance interactions with positively charged residues on chemokines (Bannert et al., 2001). In contrast, O-glycosylation appeared to have minimal impact on HIV-1 entry. Although substitution of all four N-terminal serine/threonine residues slightly decreased viral entry, similar effects were observed when only threonine 16 and serine 17 were changed, indicating that this reduction is likely due to amino acid changes rather than the absence of O-glycans (Bannert et al., 2001).

Consistent with the functional importance of CCR5 glycosylation in chemokine binding, pulse-chase analysis in BaF3 cells stably expressing CCR5 demonstrated rapid receptor maturation. After 30 minutes of labelling with [³⁵S]methionine/cysteine, the immature, lower molecular weight form was no longer detectable, while the mature, higher molecular weight form persisted for several hours (~6 h), indicating efficient post-translational processing, including glycosylation (Petti et al., 2018). The prolonged stability of the mature form further supports the idea that glycosylation contributes to the formation of a stable, ligand-competent CCR5 population.

1.10.4.3 S-Acylation of CCR5

S-acylation of CCR5 represents a critical post-translational modification with major implications for receptor function, including trafficking, surface expression, and

signalling. This modification, primarily targeting a cluster of C-terminal cysteine residues, has attracted significant interest due to its potential to regulate CCR5 activity and stability.

Initial evidence for CCR5 S-acylation was provided by Blanpain *et al.*, who used [³H] palmitate metabolic labelling in CHO-K1 cells. Mutation of the three C-terminal cysteine residues (C321, C323, and C324) to alanine showed a visible reduction in palmitate incorporation after 4 hours of labelling, suggesting S-acylation was occurring on this cysteine cluster (Blanpain *et al.*, 2001). While double and triple cysteine mutants displayed a similar decrease compared to wild-type, the decrease in palmitate incorporation for the single cysteine mutants was predictably less extreme. When the cysteine residues were mutated individually, there was still a clear incorporation of [³H] palmitate with no visual difference between the individual cysteine residues, suggesting they were equally capable of being S-acylated (Blanpain *et al.*, 2001). However, these experiments assessed only the mature form of CCR5 and did not quantify S-acylation levels, making accurate comparison difficult.

In the same year, Percherancier *et al.* performed [¹⁴C] palmitate labelling in HEK293T cells lacking endogenous CCR5, reporting a 95 % reduction in palmitate incorporation in the triple cysteine-to-alanine mutant after 3 hours of labelling (Percherancier *et al.*, 2001). Consistent with the findings of Blanpain *et al.*, they observed no strong preference for any single cysteine, with individual substitutions of C321, C323, and C324 resulting in a 50 %, 35 %, and 30 % reduction, respectively (Blanpain *et al.*, 2001, Percherancier *et al.*, 2001). This data suggested that the other intracellular cysteine residues, C58 and C224, likely contribute minimally, if at all, to CCR5 S-acylation. Additional experiments showed that treatment of wild-type CCR5 with 100 µM 2-BP reduced the incorporation of palmitate to the same level as the triple cysteine mutant (Percherancier *et al.*, 2001). Contrasting findings were reported by Boncompain *et al.*, who observed only a ~50 % decrease in S-acylation of the triple cysteine mutant following 4 hours of [³H] palmitate labelling in HEK293T cells (Boncompain *et al.*, 2019). This data suggested that there may be additional cysteines outside the C-terminal cluster that contribute more significantly to CCR5 S-acylation than previously thought.

Functional consequences of these cysteine substitutions were assessed using fluorescence-activated cell sorting (FACS). In CHO-K1 cells, Blanpain *et al.* observed a ~50 % reduction in CCR5 surface expression of single cysteine mutants compared to wild-type (Blanpain *et al.*, 2001). This expression was further decreased in double and triple mutants, with the latter showing the greatest decrease. Similar patterns were observed in HEK293T cells by Percherancier *et al.* (Percherancier *et al.*, 2001). Boncompain *et al.* reported a more modest decrease in surface expression of CCR5 in HeLa cells, with ~20 % reduction for single mutants and for the C323/C324 double mutant, and ~50 % reduction for other double cysteine mutants (Boncompain *et al.*, 2019).

Subcellular localisation of CCR5 and its cysteine mutants was further investigated using confocal microscopy. Wild-type CCR5 exhibited strong plasma membrane expression in

CHO-K1 cells when detected using an antibody recognising an extracellular epitope, whereas the triple cysteine mutant was only detectable following cell permeabilisation, with signal predominantly retained in a perinuclear region, suggesting impaired trafficking (Blanpain et al., 2001). Constructs of these proteins, which had been fused to green fluorescent protein (GFP), confirmed increased membrane localisation for wild-type CCR5 compared to the mutant. In HeLa cells, wild-type CCR5 localised predominantly to the plasma membrane, displaying partial co-localisation with the raft microdomain marker ganglioside GM1 (Percherancier et al., 2001). In contrast, the triple cysteine-to-alanine mutant displayed a vastly different localisation with only punctate cytoplasmic staining and no visible expression on the plasma membrane.

FRAP experiments performed at the plasma membrane showed that wild-type CCR5 exhibited a greater degree of fluorescence recovery compared to the S-acylation-deficient mutant, consistent with increased membrane mobility and/or exchange with intracellular pools (Blanpain et al., 2001). S-Acylation of CCR5 shows some effects on receptor signalling. Ligand binding and Gi-mediated pathways, such as inhibition of cAMP accumulation, were generally preserved in cysteine mutants, indicating that receptor activation and basic G protein coupling can still occur (Blanpain et al., 2001). However, calcium mobilisation examined using an aequorin-based luminescence assay, following stimulation with CCL4, showed single cysteine mutants exhibited a moderate reduction in maximal calcium release (~50 % of wild-type), whereas double and triple cysteine mutants were severely impaired, with no detectable calcium signal even at the highest ligand concentration tested. In addition, [³⁵S] GTPγS binding assays, stimulated by CCL5, revealed somewhat reduced G protein coupling efficiency in membranes expressing non-acylated receptor (Blanpain et al., 2001). Overall, this indicates that S-acylation is not essential for receptor activation or Gi-mediated signalling but can influence the efficiency of downstream pathways. S-Acylation of CCR5 has also previously been implicated in the regulation of receptor stability. Pulse-chase experiments using [³⁵S]Met in HeLa cells showed a reduced half-life of ~1.5 hours for the triple cysteine mutant, compared to ~4.5 hours for wild-type CCR5, indicating that loss of S-acylation decreases receptor stability, likely in part through impaired trafficking and increased turnover (Percherancier et al., 2001).

More recently, zDHHC enzymes capable of S-acylating CCR5 were identified, providing a mechanistic link between receptor trafficking and its lipid modification. Using [³H] palmitate labelling in HEK293T cells, Boncompain *et al.*, demonstrated that zDHHC3, zDHHC7, and zDHHC15 increased CCR5 S-acylation (Boncompain et al., 2019). These enzymes are predominantly localised to the Golgi apparatus in HEK293T cells, supporting a model in which CCR5 undergoes S-acylation within intracellular secretory compartments during or after trafficking to the plasma membrane (Greaves et al., 2008, Greaves et al., 2010).

1.10.5 CCR5 S-acylation in disease

CCR5 is involved in the development of several cancers, including breast cancer and glioblastoma; however, human immunodeficiency virus 1 (HIV-1) is the disease most commonly associated with the receptor (Jiao et al., 2019, Oppermann, 2004). S-Acylation of CCR5 may influence its function in these disease contexts, affecting processes, particularly by influencing receptor trafficking.

HIV-1 strains can be defined according to the co-receptor utilised for entry into the cell. Virus strains which use C-X-C chemokine receptor type 4 (CXCR4) are defined as X4 strains, whereas those using CCR5 are called R5 HIV-1 strains (Mohamed et al., 2021). R5 strains are associated with early infection as they have a greater expression on CD4+ memory T cells compared to CXCR4 (Lin et al., 2002). Studies of long-term non-progressor (LTNP) HIV patients indicate that CCR5 is a critical factor in disease progression. In these individuals, CD4+ T cells typically display reduced CCR5 expression compared with HIV-positive patients who experience standard disease progression, suggesting that lower receptor levels may contribute to slower viral replication and delayed disease progression (Meijerink et al., 2014, Ostrowski et al., 1998). Some CCR5 ligands have been shown to provide a degree of immunity against R5 HIV-1 infections. CCL3 and CCL4 are upregulated in CD4+ T cells of LTNPs compared to patients with HIV-1, which has progressed to acquired immunodeficiency syndrome (Saha et al., 1998). This is believed to decrease infection through desensitisation by receptor blocking and inhibition of CCR5 recycling (Santa-Marta et al., 2013).

Resistance to HIV-1 can occur due to a lack of CCR5 expression through homologous mutations in CCR5 Δ 32 (Samson et al., 1996, Liu et al., 1996, Mirzabekov et al., 1999). The truncated nature of this CCR5 protein, due to the frameshift mutation, means that it lacks the S-acylated cysteine residues which are present on the wild-type CCR5. Therefore, the protein is unable to traffic beyond the ER and Golgi and is not expressed on the cell surface (Issafras et al., 2002, Venkatesan et al., 2001). There is also some benefit conferred to those heterozygous for this mutation with decreased plasma membrane CCR5 expression and subsequent decreased disease progression (Benkirane et al., 1997, Boncompain et al., 2019). Individuals with these mutations, and hence loss of CCR5 cell surface expression, do not appear to suffer any severe deleterious effects, possibly due to the alleged redundancy of CCR5. However, increased risk has been described in these individuals in regard to West Nile virus (Lim et al., 2008) and tick-borne encephalitis (Kindberg et al., 2008, Boncompain et al., 2019).

Due to the importance of CCR5 in the entry of HIV-1 into cells, the receptor has been targeted for the development of HIV therapies (Mohamed et al., 2021). Therapeutic strategies to target CCR5 during HIV-1 infection generally involve gene editing or blocking of CCR5 through the use of antagonists or antibodies. Maraviroc is an example of an approved drug targeting CCR5 in HIV-1 infection. It functions as an antagonist of the receptor, maintaining it in a conformation which does not allow binding of HIV-1 virus proteins. While therapies such as Maraviroc have shown potential, they are unable to effectively prevent all viral replication, and resistance can develop. Therefore, they

should be considered supplementary to pre-existing anti-retroviral therapies (Mohamed et al., 2021). The S-acylation of CCR5 cysteines is important in the trafficking of the protein to the plasma membrane. Understanding this post-translational modification in relation to CCR5 and its function could provide a potential target against which to develop therapies for HIV-1 infection

1.11 zDHHC enzyme specificity towards transmembrane proteins

For the vast majority of transmembrane proteins, the zDHHC S-acyltransferases that mediate their S-acylation are not well defined. As discussed above, for GPCRs such as CCR5 and β 2AR, the Golgi-localised enzymes zDHHC-3, -7, -9, and -15 have been implicated, however it is not clear if these enzymes play a wider role in the S-acylation of GPCRs.

More recent work identified the ER enzyme zDHHC6 as an enzyme with broad activity against single transmembrane proteins. zDHHC6 is an ER-localised enzyme with the canonical four-transmembrane DHHC architecture, modifying a range of proteins including calnexin, transferrin receptor, and the IP₃ receptor (**Figure 1.9**) (Lakkaraju et al., 2012, Senyilmaz et al., 2015, Fredericks et al., 2014). Interestingly, the stability and activity of zDHHC6 are regulated by the S-acylation of three cysteine residues in the C-terminal tails of the enzyme, C328, C329, C343, with cysteine-to-alanine substitution of these residues abolishing zDHHC6 S-acylation, suggesting these are the primary sites of S-acylation (Abrami et al., 2017). In cells where endogenous zDHHC6 was removed by shRNA, exogenous catalytically-inactive zDHHC6 was still S-acylated, suggesting that another zDHHC is responsible for this modification. Silencing of zDHHCs through siRNA revealed a 60 % decrease in zDHHC6 S-acylation when zDHHC16 was removed, and a similar loss was observed in CRISPR-cas9 zDHHC16 knockout studies, suggesting that zDHHC16 plays an important role in regulating the S-acylation and activity of zDHHC6 (Abrami et al., 2017).

As discussed previously, different zDHHC enzymes have specific structural features and domains linked to their interaction with substrates and other proteins. zDHHC6 contains a C-terminal SH3 domain, a non-catalytic motif typically involved in protein-protein interactions through recognition of proline-rich sequences. The SH3 domain of zDHHC6 contains the three conserved S-acylated cysteine residues, C328, C329, and C343. The SH3 domain facilitates interactions with accessory proteins such as selenoprotein K (SELENOK), which acts as a cofactor for zDHHC6 (Fredericks et al., 2014). In particular, the selenocysteine residue, an amino acid containing selenium in place of sulphur, at position 92 of SELENOK stabilises the acyl-zDHHC6 intermediate, preventing premature hydrolysis and enhancing the efficiency of acyl transfer to target proteins, including the IP₃ receptor (Fredericks et al., 2014, Fredericks et al., 2017). Microsomes lacking SELENOK exhibit markedly reduced S-acylation activity, underscoring its role in regulating catalytic efficiency *in vivo* (Fredericks et al., 2017). While the SH3 domain is

not strictly required for S-acylation *in vitro* as short transmembrane constructs lacking proline-rich motifs can still be modified by this enzyme, the domain may provide an interface for substrate recruitment and catalytic efficiency, expanding the range of substrates that can be efficiently recognized (Salaun et al., 2023, Fredericks et al., 2014, Fredericks et al., 2017, Meiler et al., 2013). This illustrates a broader principle in the zDHHC family where non-catalytic motifs, such as SH3 in zDHHC6 or ankyrin repeats in other members, can significantly influence substrate selectivity beyond the catalytic DHHC domain.

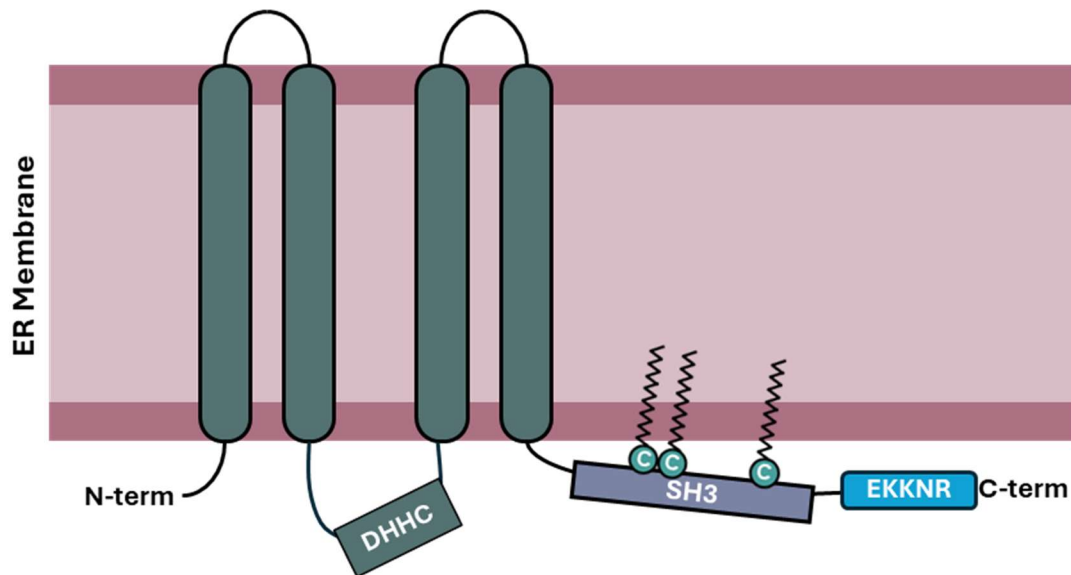


Figure 1.9. Schematic representation of zDHHC6. The enzyme zDHHC6 is depicted with four transmembrane domains (green) embedded in the ER membrane, with both N- and C-termini located in the cytosol. Key functional regions highlighted are the DHHC catalytic domain (green), the Src homology 3 (SH3) motif (purple), and the EKKNR motif (blue). Three S-acylated cysteine residues located within the SH3 domain are indicated.

Notably, zDHHC6 displays selectivity towards type I and type II single-pass transmembrane proteins when the target cysteine residue is positioned in close proximity to the membrane (Salaun et al., 2023). Using palmitic acid azide labelling combined with click chemistry and 5 kDa alkyne mPEG, zDHHC6 was shown to enhance S-acylation of transmembrane serine protease 2 (TMPRSS2) and bone marrow stromal antigen 2 (BST2), both of which contain a single transmembrane domain (Salaun et al., 2023). Although TMPRSS2 and BST2 could also be modified by zDHHC3 and zDHHC7, the greatest increase in S-acylation was observed with zDHHC6, consistent with preferential substrate engagement. In both proteins, S-acylation occurred at a single cysteine residue located at a juxta-membrane position. For TMPRSS2, the modified cysteine lies two amino acids within the transmembrane domain, whereas in BST2 it is positioned immediately adjacent to the membrane-spanning region. Repositioning the TMPRSS2

cysteine further into the transmembrane domain significantly reduced S-acylation, demonstrating that precise cysteine positioning relative to the lipid bilayer is critical for efficient modification.

These findings support a model in which zDHHC6 recognises substrates primarily through topological features rather than extended sequence motifs. Truncation analyses further reinforced this minimal recognition model. For both TMPRSS2 and BST2, the elements required for S-acylation were restricted to the transmembrane domain and adjacent basic residues (Salaun et al., 2023). A similar requirement was observed for transferrin receptor (TfR), a type II single-pass transmembrane protein that is S-acylated on two cytosolic cysteine residues located close to the membrane. Likewise, truncated constructs of several type I transmembrane proteins including calnexin, CD4, Ebola glycoprotein, VSV glycoprotein, and influenza A H7N1 hemagglutinin containing only the transmembrane domain and a short juxta-membrane region were sufficient for S-acylation by zDHHC6.

Across these examples, the unifying feature of zDHHC6 substrates is the presence of a single transmembrane domain and one or more cytosolic cysteine residues positioned immediately adjacent to the membrane. This recurring architectural theme suggests that zDHHC6 substrate selectivity is driven by membrane topology and cysteine accessibility within a defined spatial window at the cytosolic face of the bilayer. Additional reported substrates of zDHHC6 including interferon-induced transmembrane protein 3 (IFITM3), angulin-1, astrocyte elevated gene-1 protein (AEG1), and cytoskeleton-associated protein 4 (CKAP4) conform to this same structural pattern (McMichael et al., 2017) (Oda et al., 2020, Komaniecki et al., 2023, Sandoz et al., 2023). Only a limited number of reported zDHHC6 substrates deviate from this model. These include cytosolic proteins such as N-Ras and Myd88, as well as proteins in which the modified cysteine is positioned further from the transmembrane domain, such as IP3 receptor and gp78 (Qiu et al., 2022, Kim et al., 2019, Fredericks et al., 2014, Fairbank et al., 2012). Such cases appear to represent exceptions rather than the dominant mode of substrate recognition.

Together, these findings indicate that zDHHC6 achieves substrate selectivity through a combination of mechanisms: SH3-mediated recruitment that enhances catalytic efficiency for certain substrates, and intrinsic topological recognition of single-pass transmembrane proteins with juxta-membrane cysteines. This dual mode of substrate engagement provides a clear mechanistic example of how structural features and spatial constraints can encode selective enzyme-substrate recognition within broader S-acylation networks.

1.12 Project aims

This project aims to develop an improved understanding of the S-acylation of transmembrane proteins and to delineate the factors governing the recognition of these proteins by zDHHC enzymes, especially zDHHC6. Specifically, the project will explore the questions below.

- Chapter 3 will focus on CCR5 and examine the specific cysteines that are S-acylated in this protein, the enzymes involved in this modification and where in the cell S-acylation occurs, interplay between S-acylation and O-glycosylation, and effects of these post-translational modifications on CCR5 stability.
- Chapter 4 will examine if the broad activity of zDHHC6 towards single transmembrane proteins extends to multi-transmembrane proteins, including GPCRs, how cysteine position in these proteins affects recognition by zDHHC6, and the attachment of different chain length fatty acids by zDHHC enzymes and how this is affected by cysteine proximity to transmembrane domains.
- Chapter 5 will examine a novel single transmembrane protein interactor of zDHHC enzymes, TEMP, exploring zDHHC interaction specificity and the modification of this protein by S-acylation and glycosylation.

Overall, these areas of investigation seek to provide a new understanding about the S-acylation of transmembrane proteins and their recognition by the zDHHC enzyme family.

CHAPTER 2

Methods and Materials

Chapter 2 – Methods and Materials

2.1 Culture of HEK293T cells

2.1.1 HEK293T cell culture and passaging

Human Embryonic Kidney 293T (HEK293T) cells (CRL-3216, American Type Culture Collection, Virginia, USA) were split and plated for experiments when approximately 90 % confluent. Cells in T75 cm² flasks (15350591, Thermo Fisher Scientific UK Ltd., Loughborough, UK) were washed with 10 ml of warm sterile phosphate-buffered saline (PBS) (**Table 2.7. Buffers**). Cells were detached by incubating with 2.5 ml of warm TrypLE™ Express (12604-013, Gibco™, Paisley, UK) for 3 minutes at 37 °C, 5 % CO₂ in a Thermo BB15 incubator (12846716, Thermo Fisher Scientific UK Ltd., Loughborough, UK), followed by mechanical detachment. TrypLE™ Express was neutralised by the addition of 7.5 ml warm Dulbecco's Modified Eagle Medium (DMEM) + GlutaMAX™ (31966-021, Gibco™, Paisley, UK) supplemented with 10 % foetal bovine serum (FBS) (17479633, Gibco™, Paisley, UK). Cells were pelleted by centrifugation in a Heraeus Multifuge 3 S-R (75004371, Thermo Fisher Scientific UK Ltd., Loughborough, UK) for 3 minutes at 150 × g in a 15 ml centrifuge tube (05-408-129, Thermo Fisher Scientific UK Ltd., Loughborough, UK). The supernatant was discarded, and the cell pellet was resuspended in 10 ml DMEM + GlutaMAX™ with 10 % FBS, resulting in a 1 in 10 dilution.

To prepare new cultures, 0.5 ml of the resulting cell suspension was seeded into 10 ml of fresh DMEM + GlutaMAX™ with 10 % FBS in a T75 cm² flask, yielding a final 1:20 cell dilution. The flask was returned to the incubator for one week at 37 °C, 5 % CO₂.

2.1.2 Plating of HEK293T cells for experimental analysis

HEK293T cells were seeded onto either poly-D-lysine-coated 24-well plates (356414, Thermo Fisher Scientific UK Ltd., Loughborough, UK) for click chemistry and cycloheximide chase experiments, or uncoated 6-well culture plates (130184, Thermo Fisher Scientific UK Ltd., Loughborough, UK) for immunoprecipitation experiments. A 1:10 cell suspension was prepared as described in **Section 2.1.1** and further diluted to 1:50 by combining 3 ml of the 1:10 suspension with 12 ml of DMEM + GlutaMAX™ supplemented with 10 % FBS in a 30 ml polypropylene universal container (128CP, Sterilin Ltd., Newport, UK). For 24-well plates, 0.5 ml of the diluted suspension was added per well; for 6-well plates, 2 ml was added per well. Cells were incubated at 37 °C, 5 % CO₂ for 24 hours prior to transfection.

2.2 Plasmid constructs and mutagenesis

2.2.1 zDHHC plasmids

Plasmids encoding the zDHHC enzymes (mouse) were generated in a pEF-BOS-HA vector by Fukata *et al.* (Fukata *et al.*, 2004).

Catalytically inactive constructs of zDHHC3 (zDHHC3 CS) and zDHHC7 (zDHHC7 CS), in which the codon of the active site cysteine (DHHC) was substituted to a codon for serine (DHHS), were previously generated *via* site-directed mutagenesis in the Chamberlain lab (Greaves *et al.*, 2017). An endoplasmic reticulum-localised zDHHC3 construct (zDHHC3 EKKNR) with the ER retention sequence EKKNR from zDHHC6 fused to the C-terminus was also previously generated in the Chamberlain Lab (Gorleku *et al.*, 2011). All mutations were introduced into the pEF-BOS-HA-zDHHC plasmids (Fukata *et al.*, 2004).

2.2.2 Transmembrane protein plasmids

The sources of plasmids encoding transmembrane proteins investigated as potential substrates/interactors of zDHHC enzymes are described in the following tables (**Table 2.1, 2.2 & 2.3**). All plasmids were obtained from Addgene (<https://www.addgene.org/>), except type III endosome membrane protein (TEMP) and transmembrane serine protease 2 (TMPRSS2), which were synthesised by GenScript Gene Synthesis services (GenScript Biotech, Oxford, UK).

Table 2.1. G protein-coupled receptor (GPCR) plasmids					
Protein	Abbr.	Plasmid	Tag	Species	Source
C-C chemokine receptor type 5	CCR5	pCEP4-FLAG-CCR5	N-terminal FLAG	H. sapiens	A gift from Erik Procko (Addgene plasmid # 98949)
C-X-C chemokine receptor type 4	CXCR4	pCEP4-FLAG-CXCR4	N-terminal FLAG	H. sapiens	A gift from Erik Procko (Addgene plasmid # 98947)
G protein-coupled receptor 37	GPR37	GPR37-DuET	N-terminal FLAG C-terminal 1D4	H. sapiens	A gift from Thomas Sakmar (Addgene plasmid # 213282)
5-hydroxytryptamine receptor 2A	HTR2A	c-Myc-5-HT2A	N-terminal c-Myc	H. sapiens	A gift from Javier Gonzalez-Maeso (Addgene plasmid # 67944)
Oxytocin receptor	OXTR	hOT-GFP	C-terminal EGFP	H. sapiens	A gift from Christian Gruber (Addgene plasmid # 67848)
Purinergic receptor P2Y11	P2RY11	P2RY11-DuET	N-terminal FLAG C-terminal 1D4	H. sapiens	A gift from Thomas Sakmar (Addgene plasmid # 213364)
Vasopressin receptor 1A	V1aR	hV1aR-GFP	C-terminal EGFP	H. sapiens	A gift from Christian Gruber (Addgene plasmid # 67846)
Vasopressin receptor 1B	V1bR	hV1b-GFP-1	C-terminal EGFP	H. sapiens	A gift from Christian Gruber (Addgene plasmid # 67847)
Beta-1 adrenergic receptor	β 1AR	pcDNA3 Flag beta-1-adrenergic-receptor	N-terminal FLAG	H. sapiens	A gift from Robert Lefkowitz (Addgene plasmid # 14698)
Beta-2 adrenergic receptor	β 2AR	pcDNA3 Flag beta-2-adrenergic-receptor	N-terminal FLAG	H. sapiens	A gift from Robert Lefkowitz (Addgene plasmid # 14697)

Table 2.1. G protein-coupled receptor (GPCR) plasmids. Summary of GPCR plasmids used in this study, including the full protein name, abbreviation, plasmid name, tags, species and source. All plasmids were sourced from Addgene, and the original depositing laboratories and corresponding Addgene accession numbers are indicated.

Table 2.2. Transmembrane protein plasmids (1-4 transmembrane domains)					
Protein	Abbr.	Plasmid	Tag	Species	Source
Cluster of differentiation 4	CD4	pCMV-CD4-FLAG	C-terminal FLAG	H. sapiens	A gift from Suzanne Paradis (Addgene plasmid # 51604)
Cluster of differentiation 9	CD9	CD9-mEGFP	C-terminal EGFP	H. sapiens	A gift from Salvatore Chiantia (Addgene plasmid # 182864)
Kaposi's Sarcoma Associated Herpesvirus (KSHV) K5	K5	pCDNA4.TO-K5-2xCSTRE P	C-terminal Strep	Viral	A gift from Britt Glaunsinger (Addgene plasmid # 136154)
Type III Endosome Membrane Protein	TEMP	pcDNA3.1 TEMP-GFP	C-terminal EGFP	H. sapiens	Synthesised by GenScript Biotech Ltd, Oxford, UK
Transmembrane serine protease 2	TMPRSS2	pCS2-TMPRSS2-FLAG	C-terminal FLAG	H. sapiens	Previously generated (Salaun et al., 2023) Synthesised by GenScript Biotech Ltd, Oxford, UK
Tetraspanin-4	TSPAN4	pEGFP-N1-Tspan4	N-terminal EGFP	M. musculus	A gift from Li Yu (Addgene plasmid # 210323)

Table 2.2. Transmembrane protein plasmids (1-4 transmembrane domains). Summary of transmembrane protein (containing 1-4 transmembrane domain) plasmids used in this study, including the full protein name, abbreviation, plasmid name, tags, species and source. All plasmids were sourced from Addgene, and the original depositing laboratories and corresponding Addgene accession numbers are indicated.

All transmembrane mutant plasmid constructs were generated by GenScript Biotech (Oxford, UK) using the plasmids described above (**Table 2.1 & Table 2.2**). All transmembrane mutant plasmid constructs are detailed in the following table (**Table 2.3. Transmembrane plasmid constructs**).

Table 2.3. Transmembrane plasmid constructs	
Name	Modification
GPR37 C551/553A	Cystine to alanine substitution of C551 and C553 in the plasmid GPR37-DuET
TMPRSS2 A121C C123A	Alanine to cysteine substitution of A121 and cystine to alanine substitution of C123 in the plasmid pCS2-TMPRSS2-FLAG
TMPRSS2 C123T T125C	Cysteine to threonine substitution of C123 and threonine to cysteine substitution of T125 in the plasmid pCS2-TMPRSS2-FLAG
β2AR C329ins	Insertion of a cysteine residue immediately following the final transmembrane domain at position 329 in the plasmid pcDNA3 Flag beta-2-adrenergic-receptor
β2AR C329ins EKKNR	Insertion of a cysteine residue immediately following the final transmembrane domain at position 329 and insertion of the sequence EKKNR at the C-terminus of β2AR in the plasmid pcDNA3 Flag beta-2-adrenergic-receptor
β2AR EKKNR	Insertion of the sequence EKKNR at the C-terminus of β2AR in the plasmid pcDNA3 Flag beta-2-adrenergic-receptor

Table 2.3. Transmembrane plasmid constructs. Summary of transmembrane mutant constructs used in this study, including the name and description of the amino acid changes. All plasmids were generated by GenScript Biotech (Oxford, UK) of the plasmids GPR37-DuET (Addgene #213282), pCS2-TMPRSS2-FLAG (Salaun et al., 2023), pcDNA3 Flag beta-2-adrenergic-receptor (Addgene #14697)

2.2.3 CCR5 Plasmid Constructs

The CCR5 RUSH plasmid Str-KDEL_SBP-EGFP-CCR5 was kindly provided by Dr Gaëlle Boncompain (Boncompain et al., 2019).

All CCR5 mutant plasmid constructs were generated by GenScript Biotech (Oxford, UK) using the plasmid pCEP4-FLAG-CCR5 (**Table 2.1**). All CCR5 mutant plasmid constructs are detailed in the following table (**Table 2.4. CCR5 plasmid constructs**).

Table 2.4. CCR5 plasmid constructs	
Name	Modification
O-Gly 2	Serine to alanine substitution of potential glycosylation sites - S6, S7
O-Gly 4	Serine/threonine to alanine substitution of potential glycosylation sites - S6, S7, T16, S17
3CA	Cysteine to alanine substitution of known S-acylation sites - C321, C323, C324
6CA	Cysteine to alanine substitution of all identified potential S-acylation sites - C58, C213, C224, C321, C323, C324
58C	Backbone of 6CA with reintroduction of 58C by alanine to cysteine substitution – A58
213C	Backbone of 6CA with reintroduction of 213C by alanine to cysteine mutation – A213
224C	Backbone of 6CA with reintroduction of 224C by alanine to cysteine substitution – A224
58C 213C	Backbone of 6CA with reintroduction of 58C and 213C by alanine to cysteine substitution – A58, A213
58C 224C	Backbone of 6CA with reintroduction of 58C and 224C by alanine to cysteine substitution – A58, A224
213C 224C	Backbone of 6CA with reintroduction of 213C and 224C by alanine to cysteine substitution – A213, A224
C58/213/224A	Cysteine to alanine substitution of potential new S-acylation sites leaving only the previously known cysteines (on the C-terminus) - C58, C213, C224
C-15	Deletion of the sequence NYLLVFFQKHI AKRF from the C-terminus
C-18	Deletion of the sequence KFRNYLLVFFQKHI AKRF from the C-terminus
C-19	Deletion of the sequence EKFRNYLLVFFQKHI AKRF from the C-terminus
E302C	Glutamate to cysteine substitution of the amino acid immediately following the final transmembrane domain – E302
C302ins	Insertion of a cysteine immediately after the final transmembrane domain at position 302 – C302ins

Table 2.4. CCR5 plasmid constructs. Summary of CCR5 mutant constructs used in this study, including the name and description of the amino acid changes. All plasmids were generated by GenScript Biotech (Oxford, UK) of the plasmid pCEP4-FLAG-CCR5 (Addgene #98949).

2.2.4 TEMP plasmid constructs

All TEMP plasmid constructs were generated by GenScript Biotech (Oxford, UK) using the plasmid pcDNA3.1 TEMP-GFP (**Table 2.2**). All TEMP constructs are described in the table below (**Table 2.5. TEMP Plasmid Constructs**).

Table 2.5. TEMP plasmid constructs	
Name	Modification
C54A	Cysteine to alanine substitution of potential S-acylation site – C54
C57A	Cysteine to alanine substitution of potential S-acylation site – C57
C54A C57A	Cysteine to alanine substitution of both potential S-acylation sites – C54, C57
N2A	Asparagine to alanine substitution of potential glycosylation site – N2
N5A	Asparagine to alanine substitution of potential glycosylation site – N5
N2A N5A	Asparagine to alanine substitution of both potential glycosylation sites – N2, N5
1-83	Truncation of TEMP at E84 before C-terminal conserved domain
1-98	Truncation of TEMP at T99 after C-terminal conserved domain

Table 2.5. TEMP plasmid constructs. Summary of TEMP constructs used in this study, including the name and description of the amino acid changes. All plasmids were generated by GenScript Biotech (Oxford, UK) using the plasmid pcDNA3.1 TEMP-GFP.

2.3 Amplification of plasmid DNA

Competent *Escherichia coli* TOP10 cells (C404003, Invitrogen Ltd., Paisley, UK), stored at -80 °C, were thawed on ice. To 35 µl of the competent cells, 0.2 µg of plasmid DNA was added. The mixture was incubated on ice for 20 minutes, followed by heat shock in a 42 °C water bath for 1 minute. After heat shock, 200 µl sterile lysogeny broth (LB) (**Table 2.7. Buffers**) was added to the cells. The suspension was incubated at 37 °C for 1 hour with shaking at 250 rpm in a Classic C24 Incubated shaker (M1247-0005, New Brunswick Scientific, Hertfordshire, UK).

Cells were then plated on agar plates (11309283, Sterillin Ltd., Newport, UK) containing 100 µg/mL of either Ampicillin (A9518, Sigma-Aldrich Company Ltd., Poole, UK) or Kanamycin (J60668.09, Thermo Fisher Scientific UK Ltd., Loughborough, UK) depending on plasmid antibiotic resistance. Each plate consisted of 10 ml LB supplemented with 1.5 % agar powder (443570010, Thermo Fisher Scientific UK Ltd., Loughborough, UK). Plates were incubated overnight at 37 °C.

Individual colonies were picked using sterile pipette tips and cultured overnight at 37 °C in flasks containing 150 ml LB supplemented with 100 µg/mL Ampicillin or Kanamycin,

with shaking at 250 rpm. Plasmids were purified using the NucleoBond® Xtra Midi Prep Kit (740410.100, Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Plasmid concentrations were determined using a NanoDrop 2000. Plasmid DNA sequences were confirmed by University of Dundee DNA Sequencing & Services (Medical Sciences Institute, School of Life Sciences, University of Dundee, Dundee, UK).

2.4. Transfection of HEK293T cells

2.4.1 Transfection of HEK293T cells with PEI

Twenty-four hours after seeding HEK293T onto plates (**Section 2.1.2**), transient transfection was performed. For experiments investigating potential single-transmembrane zDHHC substrate proteins, polyethylenimine (PEI) (43896, Alfa Aesar Ltd., Heysham, UK) was used as the transfection reagent at a ratio of 2 µl PEI to 1 µg plasmid DNA.

Plasmid mixes were prepared in serum-free DMEM + GlutaMAX™. A total of 1 µg plasmid DNA per well was used for 24-well plates and 4 µg per well for 6-well plates, in 50 µl or 200 µl of media per well, respectively. PEI was added directly to the plasmid mixes, which were then vortexed and incubated at room temperature for 20 minutes. Following incubation, the mixes were vortexed again and added dropwise to the appropriate wells. Cells were incubated at 37 °C, 5 % CO₂ before experimental analysis was undertaken 24-hours post-transfection.

2.4.2 Transfection of HEK293T cells with Lipofectamine 2000

For experiments investigating potential multi-transmembrane zDHHC substrate proteins, Lipofectamine™ 2000 Transfection Reagent (LF) (11668019, Invitrogen Ltd., Paisley, UK) was used at a ratio of 2 µl LF to 1 µg plasmid DNA.

Plasmid mixes were prepared using the same protocol as for PEI (**Section 2.4.1**), with a modification to allow separate pre-incubation of LF. For each well of a 24-well plate, the plasmid DNA was diluted in 25 µL of serum-free DMEM + GlutaMAX™, while LF was diluted separately in 25 µL of the same medium. The LF solution was incubated at room temperature for 5 minutes, then mixed by gentle inversion and combined with the plasmid DNA solution. Volumes were scaled proportionally according to the number of wells. The resulting mixture was gently inverted again and incubated for 20 minutes at room temperature. Following incubation, the transfection mix was added to the cells which were then incubated for 24 hours at 37 °C, 5 % CO₂, prior to use in downstream experiments.

2.5. Fatty acid-azide labelling and click chemistry to study protein S-acylation

Fatty acid-azide labelling with click chemistry is a method used to visualise the S-acylation of proteins (**Figure 2.1**). In this method, cells expressing protein(s) of interest are incubated with azide-modified fatty acids (or unmodified fatty acids as a control). If a protein undergoes S-acylation, these fatty acid-azides are incorporated onto the cysteine residues. The fatty acid azide groups can then be reacted with an alkyne-tagged detection reagent. In our experiments, incubation with a 5 kDa alkyne-conjugated polyethylene (PEG) molecule enables a copper-catalysed binding “click” reaction between the azide and alkyne groups. This reaction results in a detectable band shift of S-acylated proteins when analysed by SDS-PAGE and immunoblotting.

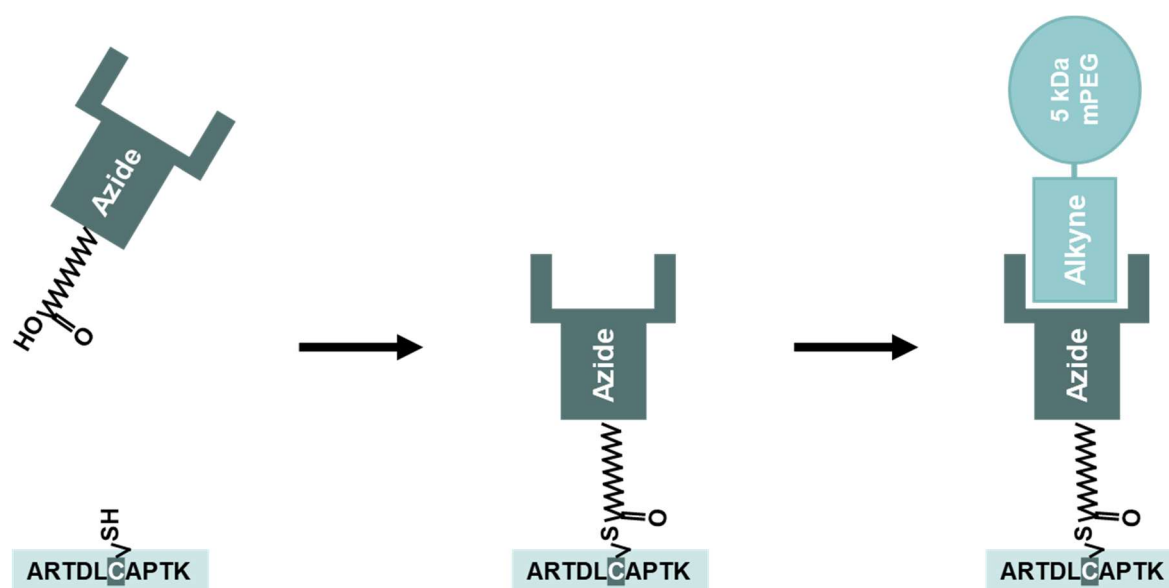


Figure 2.1. Click chemistry detection of S-acylation using fatty acid-azides and alkyne-tagged 5 kDa mPEG. Diagram representing the incorporation of an azide-modified fatty acid onto a protein cysteine and subsequent reaction of the azide with an alkyne fused to a 5 kDa PEG molecule.

Fatty acid or fatty acid-azide was added to an aliquot of warm serum-free DMEM + GlutaMAXTM media supplemented with 1 mg/ml fatty acid-free bovine serum albumin (BSA) (A7030, Sigma-Aldrich Company Ltd., Poole, UK) at a final concentration of 100 μ M. The fatty acids used were myristic acid (C14) (M3128, Sigma-Aldrich Company Ltd., Poole, UK), palmitic acid (C16) (P0500, Sigma-Aldrich Company Ltd., Poole, UK), and stearic acid (C18) (S4751, Sigma-Aldrich Company Ltd., Poole, UK), along with their corresponding fatty acid-azides: myristic acid-azide (C14-AZ), palmitic acid-azide (C16-AZ), and stearic acid-azide (C18-AZ) (C14/16/18-azide_DMSO Stock 50 mM, 500X; synthesised by Professor Nicholas Tomkinson, University of Strathclyde)(Greaves et al., 2017)

HEK293T cells were seeded in a 24-well plate (**Section 2.1.2**) and transfected with the relevant plasmids (**Section 2.4**). At 24 hours post-transfection, cells were washed with 0.5 ml warm PBS after which 0.5 ml of fatty acid or fatty acid-azide mix was added per well. Cells were incubated at 37 °C, 5 % CO₂ for 4 hours.

Following incubation, cells were washed with warm PBS and lysed with 100 µl of click lysis buffer (**Table 2.7. Buffers**). To each lysate, 80 µl of click chemistry reagent mix was added (**Table 2.7. Buffers**). Additionally, 20 µl 40 mM ascorbic acid (A15613, Alfa Aesar Ltd., Heysham, UK) was added to each lysate, and samples were rotated end-over-end for 1 hour at room temperature.

For SDS-PAGE, 67 µl of 4 x SDS sample buffer with 100 mM dithiothreitol (DTT) (R0862, Thermo Fisher Scientific UK Ltd., Loughborough, UK) was added to each sample.

2.6 Immunoprecipitation

ChromoTek GFP-Trap® Agarose immunoprecipitation (IP) beads (AB_2631357, Proteintech®, Manchester, UK) were used at 12 µl per sample. Beads were washed in 1 mL ice-cold PBS and pelleted by centrifugation at 3,000 × g for 3 minutes at 4 °C. The beads were then resuspended in 200 µl PBS per sample. For each sample, 200 µl of the resuspended beads were transferred into separate Eppendorf tubes (05-408-129, Thermo Fisher Scientific UK Ltd., Loughborough, UK) using 200 µl wide-bore graduated tips (154839, Starlab (UK) Ltd., Milton Keynes, UK). Beads were pelleted again by centrifugation (3,000 × g, 4 °C, 3 minutes), the PBS was removed, and the beads were kept on ice until use.

HEK293T cells were seeded in 6-well plates (**Section 2.1.2**) and transfected as described in **Section 2.4**. Cells were lysed in 280 µl PBS containing 1 % Triton X-100 (T8787, Sigma-Aldrich Company Ltd., Poole, UK) and protease inhibitor cocktail diluted 1:100. Lysis was carried out on ice for 30 minutes. Subsequently, 300 µl of cold PBS was added to dilute the Triton X-100 concentration. Lysates were centrifuged at 14,000 × g for 7 minutes at 4 °C to remove insoluble material. From each sample, 45 µl of clarified lysate was reserved as the “input” fraction. To this, 15 µl of 4× SDS sample buffer containing 100 mM DTT was added. The remaining 455 µl of lysate was transferred to the washed GFP-Trap beads, and incubated for 1 hour at 4 °C on a rotating wheel.

After incubation, the supernatant was removed, and beads were washed three times in cold PBS containing 0.2 % Triton X-100. Elution was performed by adding 50 µl of 0.1 M glycine (pH 2.0) (101196X, VWR International Ltd., Lutterworth, UK) to each tube, followed by end-over-end rotation at 4 °C for 30 minutes. Samples were centrifuged (3,000 × g, 4 °C, 3 minutes), and the supernatant was transferred to fresh tubes and labelled as “IP” samples.

To each IP sample, 17 μ l of 4x SDS sample buffer containing 100 mM DTT and 1 μ l saturated Tris (2 M) was added. Both “IP” and “Input” samples were heated on a heat block at 95 °C for 10 minutes. The “IP” samples were then centrifuged, and the supernatant transferred into new Eppendorf tubes for SDS-PAGE.

2.7. Fatty acid-azide labelling with retention using selective hooks (RUSH) and immunoprecipitation

The retention using selective hooks (RUSH) system allows trapping of proteins at specific organelles *via* a retention sequence, followed by their controlled release upon the addition of biotin (**Figure 2.2**). This enables the analysis of proteins at various stages of the trafficking pathway. The plasmid used in this experiment was Str-KDEL_SBP-EGFP-CCR5, which encodes an ER retention sequence (KDEL) fused to streptavidin, as well as the protein of interest (CCR5) fused to a streptavidin binding protein (SBP). Upon expression in cells, CCR5 is retained at the ER through the binding between SBP and streptavidin. The addition of biotin, which outcompetes SBP for binding to streptavidin, releases CCR5, allowing it to continue through its trafficking pathway.

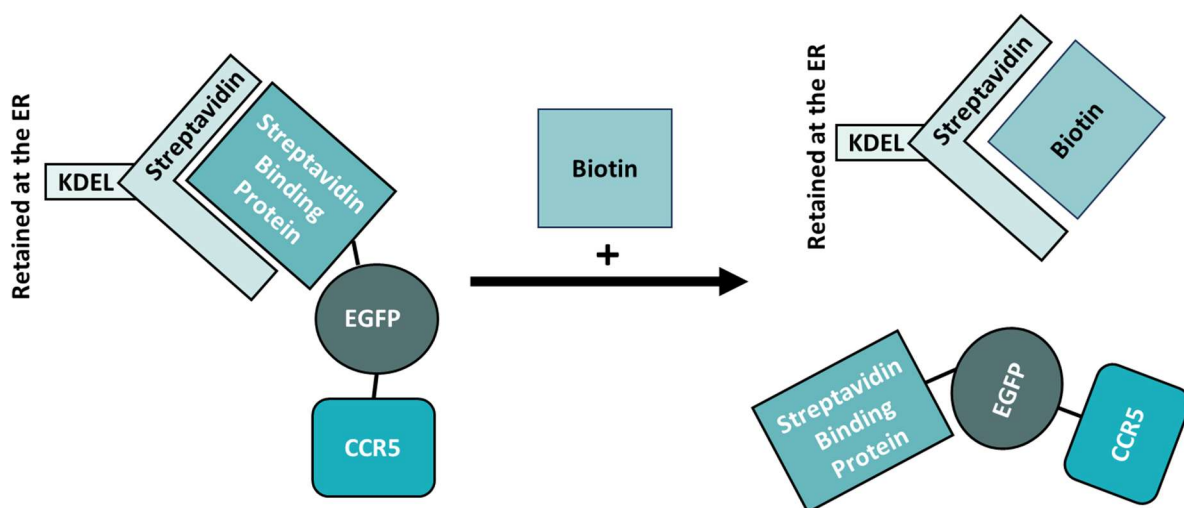


Figure 2.2. Retention Using Selective Hooks System. Diagram representing the mechanism of the RUSH system for CCR5 with the ER retention sequence KDEL. CCR5 is trapped at the ER until the addition of biotin allows its release.

When using the RUSH system, the transfection protocol for HEK293T cells seeded in a 24-well plate (**Section 2.1.2**) was modified to account for the lower expression levels of the Str-KDEL_SBP-EGFP-CCR5 plasmid. Instead of the standard 1 μ g of total DNA, 1.5 μ g

of the CCR5 RUSH plasmid was used. At 24-hours post-transfection, cells were labelled with PA or PA-AZ (**Section 2.5**). For the relevant wells, 100 μ M biotin (B4639-1G, Sigma-Aldrich Company Ltd., Poole, UK) was included in the fatty acid labelling mix to facilitate the release of CCR5 from the ER. Cells were then incubated at 37 °C, with 5 % CO₂ for 8 hours.

Following incubation, standard click chemistry protocols were carried out through the 1-hour incubation with the click mix. Thereafter, the IP protocol was followed (**Section 2.6**), with the exception that glycine elution was omitted. Instead, samples were eluted directly in sample buffer using a heat block at 95 °C. Samples were then centrifuged at 3,000 $\times g$ for 3 minutes at 4 °C, and the supernatant was transferred to fresh Eppendorf tubes for subsequent SDS-PAGE analysis. Using the IP protocol allowed for enrichment of CCR5 in immunoblotting analysis.

2.8 Cycloheximide-chase assays to study protein stability

Cycloheximide (CHX) (C-7698, Sigma-Aldrich Company Ltd., Poole, UK) was prepared at a final concentration of 50 μ g/ml in warm DMEM + GlutaMAX™ media supplemented with 10 % FBS. At 24 hours post-transfection, HEK293T cells seeded in a 24-well plate were washed with PBS and then incubated with 0.5 ml of the CHX mix per well. At the same time, a set of cells were lysed directly in 100 μ l of SDS sample buffer containing 25 mM DTT, representing the 0-hour time point.

After incubation for 8 hours at 37 °C and 5 % CO₂, CHX-treated cells were washed with warm PBS and lysed directly using 100 μ l of SDS sample buffer containing 25 mM DTT. Cells were scraped and transferred into 1.5 ml Eppendorf tubes using a wide-bore 200 μ l pipette tip. All samples (0-hour and 8-hour) were then heated at 95 °C on a heat block for 10 minutes before SDS-PAGE analysis.

2.9. SDS-PAGE

Acrylamide gels for SDS-PAGE analysis were prepared to contain a resolving gel and a stacking gel. For two 10 % (v/v) resolving gels, 5 ml of the 2 x resolving buffer (pH 8.9) (**Table 2.7. Buffers**) was mixed with 2.5 ml dH₂O, 2.5 ml 40 % acrylamide solution (J60868, Thermo Fisher Scientific UK Ltd., Loughborough, UK), 100 μ l 10 % (w/v) ammonium persulfate (APS) (A3678, Sigma-Aldrich Company Ltd., Poole, UK), and 10 μ l tetramethylethylenediamine (TEMED) (J63734.AE, Thermo Fisher Scientific UK Ltd., Loughborough, UK). The prepared resolving gel solution was poured immediately into gel cassettes, and 400 μ l isopropanol (34863, Sigma-Aldrich Company Ltd., Poole, UK) was gently layered on top to prevent bubble formation and facilitate gel polymerisation. Gels

were left to set for at least 30 minutes, after which the isopropanol was removed and the top of the gels washed with dH₂O.

The resolving gel was overlaid with a stacking gel. To prepare stacking gel solution for 4 gels, 4 ml of the 2 x stacking buffer (pH 6.8) (**Table 2.7. Buffers**) was mixed with 3.1 ml dH₂O, 0.9 ml 40 % acrylamide solution, 100 µl 10 % APS, and 10 µl TEMED. This stacking gel was poured immediately into the gel cassettes, and 15-well 1 mm combs were inserted.

The gels were placed in electrophoresis tanks that were filled with running buffer (**Table 2.7. Buffers**), and 15 µl of each sample was loaded per well, alongside 0.5 µl of a protein molecular weight marker (PL00002, Proteintech®, Manchester, UK). Gels were run at 80 V for 20 minutes, followed by 150 V until the dye had run off the gel.

2.10. Protein transfer

Following SDS-PAGE, gels were removed and briefly rinsed with transfer buffer (**Table 2.7. Buffers**). Nitrocellulose membranes (10600003, Cytivia Bioscience Ltd., Buckinghamshire, UK) measuring 6 cm x 9 cm were pre-soaked in transfer buffer for at least 15 minutes before use. A transfer cassette was assembled using sponges and chromatography paper (3030-681, Cytivia Bioscience Ltd., Buckinghamshire, UK) and soaked in transfer buffer.

Gels were placed onto the chromatography paper on the anode side of the cassette, then overlaid with the pre-soaked nitrocellulose membranes. Another sheet of chromatography paper and a sponge were placed on top, and the cassette was closed. The cassette was inserted into a transfer tank filled with transfer buffer, connected to a power supply, and the transfer was carried out at approximately 120 mA overnight.

2.11. Total protein stain

Following protein transfer (**Section 2.9**), nitrocellulose membranes were briefly rinsed in dH₂O and stained with LI-COR REVERT™ Total Protein Stain (TPS) (926-11010, LI-COR Biosciences UK Ltd., Cambridge, UK) for 5 minutes on a gentle shaker to visualise total protein content in each sample. Membranes were washed twice for 30 seconds each using a wash buffer (**Table 2.7. Buffers**). Stained membranes were imaged using a LI-COR Odyssey 9120 IR Imager (LI-COR Biosciences UK Ltd., Cambridge, UK) in the 700 nm channel.

To remove the TPS, membranes were incubated for 9 minutes in REVERT™ reversal solution (**Table 2.7. Buffers**). Membranes were then rinsed in dH₂O in preparation for immunoblotting.

2.12. Immunoblotting

Following TPS (**Section 2.11**), nitrocellulose membranes were incubated on a shaker in blocking solution containing 5 % semi-skimmed milk powder (Tesco PLC, Hertfordshire, UK) dissolved in PBS supplemented with 0.1 % Tween®20 (P1379, Sigma-Aldrich Company Ltd., Poole, UK) (PBS-T), at a volume of 20 ml per two membranes. Membranes were blocked for 45 minutes to prevent non-specific antibody binding.

After blocking, membranes were washed three times for 5 minutes each with PBS-T on a shaker. Primary antibody solution (15 ml per membrane; **Table 2.6 Antibodies for immunoblotting**) was then added and incubated as required. Following incubation, the primary antibody solution was removed, and membranes were washed three times with PBS-T. Secondary antibody (**Table 2.6. Antibodies for immunoblotting**) was then added and incubated for 45 minutes. Membranes were subsequently washed three times with PBS-T, scanned using the LI-COR Odyssey imaging system, and the results were recorded. Membranes were probed with additional primary antibodies as required.

Table 2.6. Antibodies for immunoblotting			
Antibody	Species	Dilution	Source
<i>Primary Antibodies</i>			
FLAG (THE™ DYKDDDDK Tag)	Mouse	1:2000	A00187, GenScript Biotech Ltd., Oxford, UK
GFP (JL8)	Mouse	1:5000	632381, Clonetechn, Takara Bio, London, UK
α-HA (3F10)	Rat	1:1000	11867423001, Roche, Hertfordshire, UK
Myc (9E10)	Mouse (also recognised by rat)	1:2000	Anti-c-Myc [9E10] - from Melina Fan (Addgene antibody #188875)
Strep (THE™ NWSHPQFEK Tag Antibody II)	Mouse	1:2000	A01732, GenScript Biotech Ltd., Oxford, UK
<i>Secondary Antibodies</i>			
IR DYE 680RD Anti-Rat	Goat	1:20,000	926-68076, LI-COR Biosciences UK Ltd., Cambridge, UK
IR DYE 800CW Anti Mouse	Donkey	1:20,000	926-32212, LI-COR Biosciences UK Ltd., Cambridge, UK

Table 2.6. Antibodies for immunoblotting. Table detailing all primary and secondary antibodies used in immunoblotting experiments, including species, working dilution, and supplier information. All antibodies were diluted in 15 ml PBS-T for use.

2.13 Buffers

Buffers used in this study, along with their composition, are detailed in the following table (**Table 2.7. Buffers**).

Table 2.7. Buffers	
Name	Components
Phosphate-buffered saline	1.3 M NaCl (10316943, Thermo Fisher Scientific UK Ltd., Loughborough, UK), 0.03 M KCl (P3911, Sigma-Aldrich Company Ltd., Poole, UK) 1 M NaH ₂ PO ₄ ·2H ₂ O (04269, Sigma-Aldrich Company Ltd., Poole, UK), 0.2 M KH ₂ PO ₄ (P0662, Sigma-Aldrich Company Ltd., Poole, UK).
Lysogeny broth	1 % tryptone (LPOO43B, Oxoid Ltd., Basingstoke, UK), 1 % NaCl, and 0.5 % yeast extract (LP0021B, Oxoid Ltd., Basingstoke, UK)
Click lysis buffer	50 mM Tris-HCl (T-3253, Sigma-Aldrich Company Ltd., Poole, UK) (pH 8.0), 0.5 % sodium dodecyl sulphate (SDS) (L6026, Sigma-Aldrich Company Ltd., Poole, UK) and protease inhibitor cocktail (P8340, Sigma-Aldrich Company Ltd., Poole, UK) diluted 1:100
Click chemistry reagent mix	200 µM 5 kDa alkyne-mPEG (methoxypoly(ethylene glycol)) (JKA3177, Sigma-Aldrich Company Ltd., Poole, UK) dissolved in dimethyl sulfoxide (DMSO) (D5879, Sigma-Aldrich Company Ltd., Poole, UK), 2 mM CuSO ₄ (451657, Sigma-Aldrich Company Ltd., Poole, UK), and 0.2 mM Tris((1-benzyl-4 triazolyl)methyl)amine (TBTA) (H66485, Alfa Aesar Ltd., Heysham, UK).
SDS sample buffer	200 mM Tris (BP152-1, Thermo Fisher Scientific UK Ltd., Loughborough, UK) (pH 6.8), 0.4 % bromophenol blue (A16899, Alfa Aesar Ltd., Heysham, UK), 40 % glycerol (A16205, Alfa Aesar Ltd., Heysham, UK), 8 % SDS
Resolving buffer	30 mM SDS, 4 mM ethylenediaminetetraacetic acid (EDTA) (17892, Thermo Fisher Scientific UK Ltd., Loughborough, UK), and 750 mM Tris base.
Stacking buffer	30 mM SDS, 4 mM EDTA, 250 mM Tris Base
Running buffer	25 mM Tris, 250 mM glycine, 0.1 % SDS
Transfer buffer	48 mM Tris base, 39 mM glycine, 0.9 mM SDS, and 20 % (v/v) methanol (MeOH) (32213, Sigma-Aldrich Company Ltd., Poole, UK)
Wash buffer	6.6 % acetic acid (27225, Sigma-Aldrich Company Ltd., Poole, UK) and 30 % MeOH in dH ₂ O.
Reverse solution	0.1 % NaOH (S/4800/60, Thermo Fisher Scientific UK Ltd., Loughborough, UK), and 30 % MeOH in dH ₂ O

Table 2.7. Buffers. Composition and application of buffers used in this study. The table summarises each buffer, including its components and concentrations

2.14 Data Analysis

Analysis and quantification of immunoblotting experiments were performed using LI-COR Image Studio™ Lite Version 5.2 software (LI-COR Biosciences UK Ltd, Cambridge, UK). Figures were prepared and statistical analyses were carried out using Microsoft Excel (Microsoft Ltd., WA, USA), Microsoft PowerPoint (Microsoft Ltd., WA, USA), and GraphPad Prism 8 (GraphPad Software Inc., CA, USA).

For click chemistry experiments, band intensities corresponding to acylated and non-acylated protein species were quantified from immunoblotting images. A lane-specific background signal was measured from a region lacking immunoreactive bands and subtracted from all corresponding intensity values. S-Acylation was calculated as the proportion of background-corrected acylated signal relative to total protein signal (acylated plus non-acylated). Each biological replicate comprised two technical replicates, from which mean % S-acylation values were calculated.

For immunoprecipitation assays, band intensities corresponding to GFP (protein of interest or GFP control) and HA-tagged zDHHC enzymes were quantified. Background correction was applied as described above. Interaction strength was calculated as the ratio of HA signal to GFP signal (HA/GFP), and values were normalised to the within-repeat mean across all conditions to enable comparison across independent experiments. Each condition was analysed in a single technical replicate, with mean normalised binding values derived from independent biological repeats.

For cycloheximide pulse-chase experiments, protein expression was assessed by quantifying total signal intensity at 0 hour and 8 hour timepoints. Background correction was applied to each lane, and values were normalised to total protein loading using the corresponding total protein stain. Protein stability was expressed as the percentage of signal remaining at 8 hours relative to 0 hours. Each biological replicate included two technical replicates, from which mean % protein remaining was calculated.

For proteins displaying multiple bands (e.g. TEMP), quantification was performed by defining a single region of interest encompassing the full observable banding pattern for the protein. Unless investigating this specifically, individual bands were not assigned to discrete molecular species in order to avoid assumptions regarding their identity, as these may represent different post-translationally modified forms, processing intermediates, or conformational variants. Total signal within the defined region was quantified and used consistently across conditions to enable comparative analysis.

For statistical analysis, ordinary one-way ANOVA with multiple comparisons was performed using GraphPad Prism 8. Statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Error bars represent the mean $\pm$ standard error of the mean (SEM), with the number of replicates stated in the relevant figure legends.

CHAPTER 3

Mechanisms of C-C Chemokine Receptor Type 5 (CCR5) S-Acylation

Chapter 3 - Mechanisms of C-C Chemokine Receptor Type 5 (CCR5) S-Acylation

3.1 Introduction

S-Acylation of the GPCR CCR5 has been previously studied due to its critical role in regulating receptor trafficking to the plasma membrane. As CCR5 functions both as a chemokine receptor and as a co-receptor for HIV-1 entry into cells, efficient delivery of CCR5 to the cell surface is essential for its biological activity. Post-translational modifications, particularly S-acylation of cysteine residues in the cytoplasmic C-terminal domain, have therefore emerged as key determinants of CCR5 maturation, stability, and function.

Early studies using metabolic [³H] palmitate labelling in CHO-K1 and HEK293T cells identified three C-terminal cysteine residues (C321, C323, and C324) as sites of CCR5 S-acylation. Cysteine to alanine substitutions at these positions demonstrated that all three residues could be S-acylated, with no strong preference for any single site (Blanpain et al., 2001, Percherancier et al., 2001). Combined substitution of all three cysteines was reported to nearly abolish S-acylation in these early studies; however, more recent work by Boncompain *et al.* (2019) reported that substitution of C321, C323 and C324 only decreased S-acylation by ~ 50 % in HEK293T cells, suggesting the presence of additional low-occupancy or context-dependent S-acylation sites (Boncompain et al., 2019). The enzymes that were able to catalyse CCR5 S-acylation were subsequently identified as the Golgi-localised zDHHC3, zDHHC7, and zDHHC15, highlighting the potential importance of the Golgi apparatus in the S-acylation of CCR5 (Boncompain et al., 2019).

S-Acylation has pronounced consequences for CCR5 trafficking and function. Flow cytometry analyses in CHO-K1, HEK293T, and HeLa cells consistently show reduced cell-surface expression across proteins with single, double, or triple cysteine to alanine substitutions (Blanpain et al., 2001, Percherancier et al., 2001, Boncompain et al., 2019). Confocal microscopy in both CHO-K1 and HeLa cells further revealed redistribution of these mutants from the plasma membrane to intracellular compartments, supporting the hypothesis that S-acylation promotes efficient delivery and/or retention of CCR5 at the cell surface (Blanpain et al., 2001, Boncompain et al., 2019). Additionally, CCR5 with triple cysteine substitutions exhibited impaired mobility when measured using fluorescence recovery FRAP analysis, which could underlie the observed defects in receptor trafficking (Blanpain et al., 2001).

S-Acylation also appears to play a role in regulating the stability of CCR5. S-Acylation has a well-established role in regulating protein stability. For example, S-acylation of the single-transmembrane protein Fas death receptor (FasR) stabilises the receptor at the plasma membrane, thereby allowing it to function effectively in cell death signalling.

When S-acylation is prevented, FasR is internalised and targeted for lysosomal degradation, resulting in a reduced receptor half-life (Rossin et al., 2015). A similar role for S-acylation in controlling protein stability has been identified for CCR5. Loss of S-acylation through the substitution of the three C-terminal cysteine residues led to a marked decrease in the receptor half-life from 4.5 hours to 1.5 hours and reduced steady-state surface expression. This suggests that S-acylation protects CCR5 from premature intracellular degradation, likely by promoting efficient trafficking to and retention at the plasma membrane and by limiting targeting to lysosomal degradation pathways (Percherancier et al., 2001).

Given that S-acylation affects CCR5 maturation and trafficking, it is important to consider how other post-translational modifications might influence CCR5 regulation or interact with S-acylation. CCR5 is known to undergo O-glycosylation at serine 6 and 7 within its N-terminal region (Bannert et al., 2001). This modification involves the attachment of GalNAc by specific GalNAc-transferases, and knockout of a combination of these enzymes abolished glycan addition, confirming that CCR5 carries a GalNAc-linked O-glycosylation (Verhallen et al., 2023). Unlike S-acylation, O-glycosylation does not appear to be essential for CCR5 trafficking, as deletion of the glycosylated region of the N-terminus did not affect plasma membrane expression (Bannert et al., 2001). However, O-glycosylation is functionally important as substitution of the O-glycan sites reduces ligand binding affinity, suggesting that the glycans contribute to proper receptor-chemokine interactions (Bannert et al., 2001). More broadly, O-glycosylation is known to exhibit crosstalk with phosphorylation, influencing receptor regulation and signalling outcomes (Wang et al., 2008). Such interactions underscore the importance of understanding how multiple post-translational modifications jointly shape CCR5 maturation, trafficking, and function within the secretory pathway.

These findings highlight that CCR5 is regulated by a complex network of post-translational events that occur during its biosynthetic processing. While the S-acylation of CCR5 has been investigated, there remain questions about the contribution of individual cysteine residues and the location of CCR5 S-acylation within the trafficking pathway. Furthermore, the extent to which the post-translational modifications of CCR5 influence one another, and how their interplay contributes to CCR5 stability and trafficking, remains incompletely understood.

3.2 Aims

- To identify and validate all the cysteine residues on CCR5 that undergo S-acylation and to confirm the specific zDHHC enzyme(s) responsible for catalysing CCR5 S-acylation.
- To determine the subcellular compartment(s) at which CCR5 S-acylation occurs, providing insight into how this modification fits into the receptor trafficking pathway.
- To investigate the relationship between CCR5 S-acylation and glycosylation, and to determine any interplay that could alter receptor maturation or stability.

Hypothesis

CCR5 S-acylation may be regulated by specific zDHHC enzyme(s) within distinct intracellular compartments, with this modification potentially influencing or being influenced by glycosylation during receptor maturation.

3.3 Results

3.3.1 Click-chemistry detection of S-acylation demonstrates increased CCR5 modification in the presence zDHHC3, zDHHC7, and zDHHC15

To independently validate previous studies that used [³H] palmitate labelling to identify the zDHHC enzymes capable of S-acylating CCR5, I first sought to confirm those findings using an alternative methodology. Establishing which zDHHC enzymes can modify CCR5 was important both for guiding subsequent experiments and for providing insight into where CCR5 S-acylation occurs within the secretory pathway. To achieve this, palmitic acid-azide labelling coupled with click chemistry was employed in transiently transfected HEK293T cells to screen a panel of zDHHC enzymes for potential activity towards CCR5. The panel included enzymes with diverse subcellular localisations (ER, Golgi apparatus, and plasma membrane), including zDHHC-2, -3, -6, -7, -15, and -17. Included in this panel were enzymes with broad substrate specificity, such as zDHHC3, as well as high specificity enzymes such as zDHHC17.

CCR5 was co-transfected with these zDHHC enzymes into HEK293T cells. The cells were then incubated with palmitic acid-azide, and cell lysates were reacted with an alkyne-tagged 5kDa mPEG (polyethylene glycol). The reaction of the azide and alkyne groups leads to attachment of mPEG to S-acylated proteins and a corresponding 5 kDa band-shift on SDS gels for every S-acylated cysteine (Salaun et al., 2020). Immunoblot analysis (**Figure 3.1A**) revealed several immunoreactive CCR5 species. A band at approximately the expected molecular weight for monomeric FLAG-CCR5 (~ 40 kDa) was observed just above the 35 kDa marker (Band 2), accompanied by a slightly lower molecular weight band at the 35 kDa marker (Band 1), which was hypothesised to represent an immature/less glycosylated form of the receptor (**Figure 3.1B**). A higher molecular weight band was detected above the 60 kDa marker (Band 4), consistent with a potential dimeric species of CCR5. In samples incubated with palmitic acid-azide (C16-AZ) (+), an additional double band pattern, similar to the monomer, was present around the 45 kDa marker (Band 3). As these bands were absent or markedly reduced in the corresponding palmitic acid-only controls (-), they are believed to represent the S-acylated forms of FLAG-CCR5 with the expected change in migration due to the attachment of the 5 kDa mPEG molecule following the click chemistry reaction.

To assess how co-expression of different zDHHC enzymes influenced CCR5 S-acylation, the proportion of S-acylated CCR5 (Band 3) relative to total CCR5 expression (sum of Band 1, Band 2, and Band 3) was quantified as a percentage (**Figure 3.1C**). In the absence of co-expressed zDHHC enzymes (Control), CCR5 displayed ~ 45 % S-acylation, indicating a relatively high level of modification by endogenous zDHHC enzymes in HEK293T cells. Co-expression with zDHHC3, zDHHC7, and zDHHC15 significantly increased CCR5 S-acylation. The largest increases were observed for zDHHC3 and zDHHC7, each producing an approximate 30 % rise above control levels, while zDHHC15 produced a more modest increase of ~ 20 %. Co-expression of CCR5 with zDHHC3 and

zDHHC7 also seemed to impact the appearance of the hypothesised CCR5 dimer (Band 4), which appeared less intense compared to the control, and samples with co-expressed zDHHC2, zDHHC6, zDHHC15, and zDHHC17. The presence of an additional faint band above this dimer, which is not in the control samples, suggests S-acylation of the proposed dimer (**Figure 3.1A**). The immunoblots in Figure 1A also confirmed that the co-expressed zDHHC enzymes themselves were S-acylated, consistent with the well-established auto-acylation characteristic of active zDHHC family members.

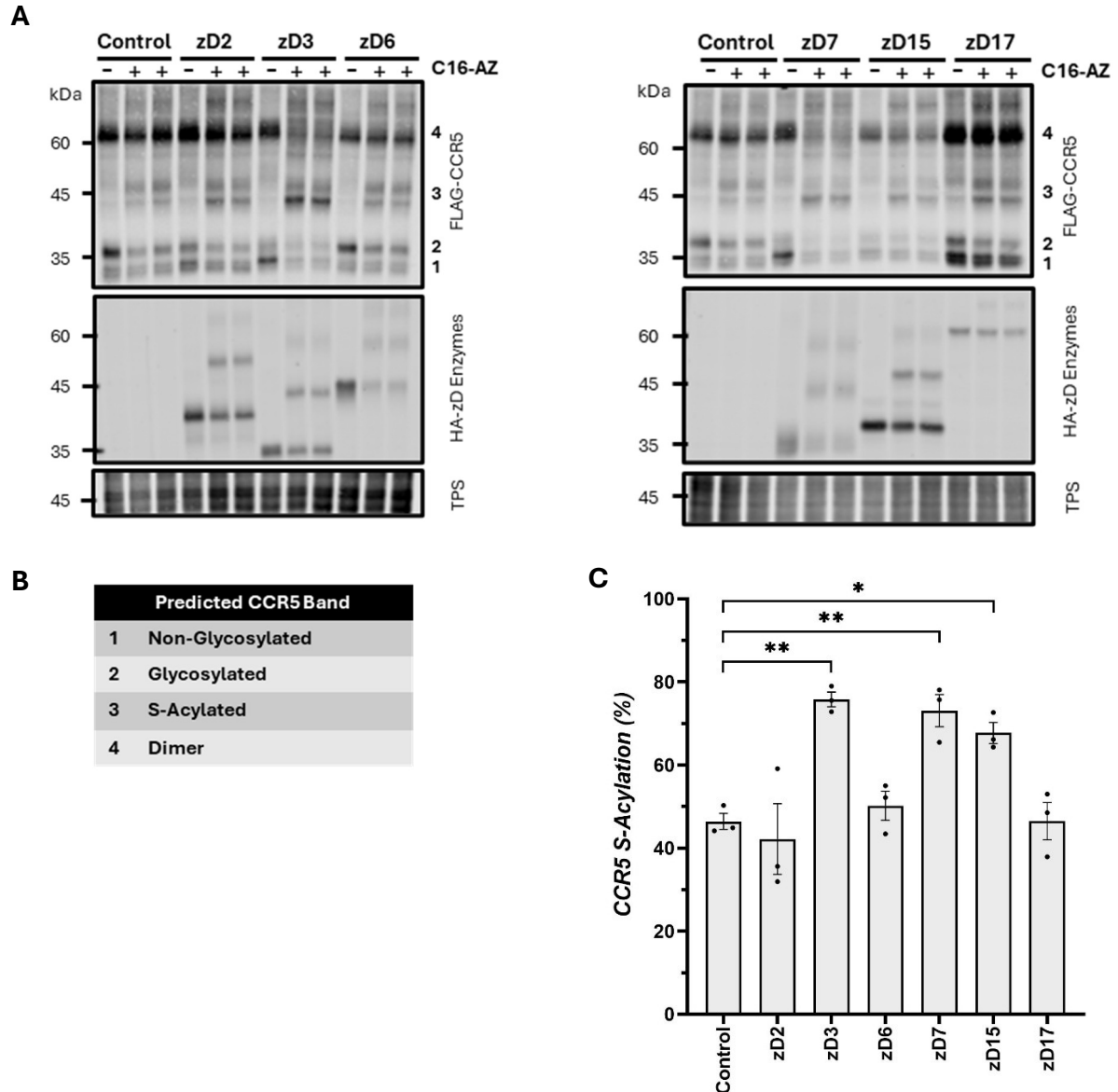


Figure 3.1. CCR5 S-acylation is enhanced by co-expression with zDHHC3, zDHHC7, and zDHHC15. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged CCR5 together with HA-tagged zDHHC enzymes (zDHHC-2, -3, -6, -7, -15, or -17) or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing CCR5 S-acylation (top; FLAG-CCR5), zDHHC enzyme expression (middle; HA-zD Enzymes), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Table of predicted CCR5 band identities corresponding to the numbered bands in the immunoblots. **(C)** Quantification of CCR5 S-acylation expressed as the mean percentage of the S-acylated band relative to the total CCR5 signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (* $p<0.05$, ** $p<0.01$).

3.3.2 O-Glycosylation-deficient CCR5 mutants shift CCR5 towards a lower molecular weight species while preserving S-acylation by endogenous zDHHC enzymes and co-expressed zDHHC3

As several distinct CCR5 bands were detected in the immunoblots of the previous experiment, it was important to determine what these species represented. In addition to S-acylation, CCR5 undergoes several other post-translational modifications, including N-terminal O-glycosylation (**Figure 3.2**). Based on findings from other groups, it was hypothesised that the bands at ~ 35 kDa (Band 1) and ~ 39 kDa (Band 2) (**Figure 3.1A**) reflected differences in O-glycosylation at serine residues 6 and 7 (Bannert et al., 2001). To test this, CCR5 N-terminal mutants were generated in which either two predicted O-glycosylation sites (S6 and S7; O-Gly2) or four sites (S6, S7, T16, and S17; O-Gly4) were replaced with alanine. In addition, it was of interest to investigate any possible interplay between S-acylation and O-glycosylation, as the influence of glycan modification on CCR5 S-acylation has not been fully examined. HEK293T cells were, therefore, transfected with these glycosylation-deficient mutants and compared to WT (WT) CCR5 following palmitic acid-azide labelling and click chemistry in the presence or absence of co-expressed zDHHC3.

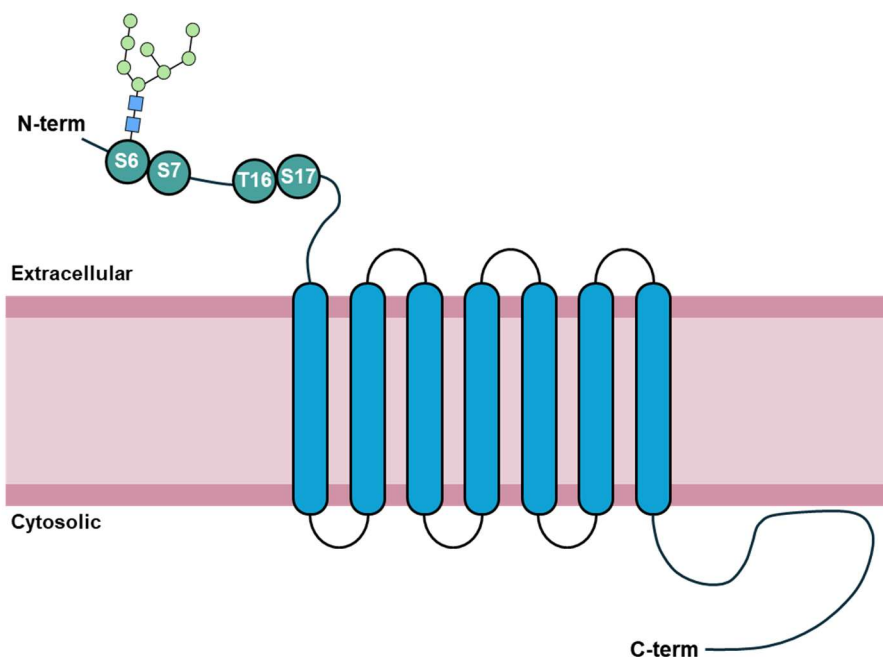


Figure 3.2. Schematic representation of CCR5 highlighting potential N-terminal O-glycosylation sites. CCR5 is shown embedded in the plasma membrane, with the seven transmembrane domains depicted in blue. The potential sites of N-terminal O-glycosylation (serine 6, 7, 17, and threonine 16) are indicated by green circles, with a representative O-glycan illustrated at serine 6.

When expressed without a co-transfected zDHHC enzyme (Control), both the O-Gly2 and O-Gly4 CCR5 mutants displayed a clear change in the immunoblot CCR5 band pattern relative to wild-type (WT) CCR5 (**Figure 3.3A**). The higher molecular weight band at ~ 39

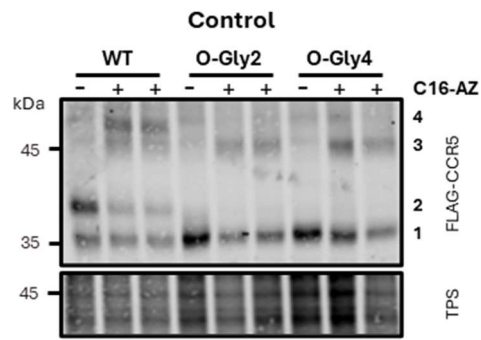
kDa (Band 2) observed for WT CCR5 was absent for both mutants in both control (-) and palmitic acid azide (+) samples, while the lower band at ~ 35 kDa (Band 1) remained. This indicates that the 35 kDa species likely represents a non-glycosylated form of CCR5. Quantification of the proportion of CCR5 expressed as the mature (glycosylated) non-acylated band (Band 2) relative to total non-acylated CCR5 expression (Band 1 and Band 2) in control samples showed a significant reduction from ~ 42 % in WT CCR5 to ~ 10 % in both O-Gly2 and O-Gly4 (**Figure 3.3C**). The absence of a significant difference between the two glycosylation mutants suggests that S6 and S7 constitute the principal O-glycosylation sites on CCR5.

The S-acylated CCR5 bands at ~ 45 kDa (Band 3) and ~ 50 kDa (Band 4) in the palmitic acid azide samples (+) when expressed without zDHHC enzyme (Control) revealed a similar change in band pattern to that observed for the non-acylated protein (**Figure 3.3A**). Specifically, the higher molecular weight band at ~ 50 kDa (Band 4) was reduced or absent in both O-Gly2 and O-Gly4 relative to WT. This suggests that glycosylation also contributes to the band pattern of the S-acylated CCR5 species revealed through click-PEG analysis. Consistent with this, quantification of the higher molecular weight S-acylated band (Band 4) relative to total expression of S-acylated CCR5 (Band 3 and Band 4) showed a significant decrease from ~ 61 % in WT CCR5 to ~ 41 % in both glycosylated mutants, again with no difference observed between O-Gly2 and O-Gly4 (**Figure 3.3D**).

When CCR5 was co-expressed with zDHHC3, a larger portion of the receptor was present in its S-acylated form at ~ 45 kDa (**Figure 3.3E**). Examination of the S-acylated banding pattern revealed only a single prominent band at ~ 45 kDa (Band 3) for WT, O-Gly2, and O-Gly4, in contrast to the two distinct bands observed for WT CCR5 under control conditions. This suggests that the lower molecular weight S-acylated band corresponds to a non-glycosylated form of CCR5, and that co-expression with zDHHC3 shifts the receptor predominantly into this species.

Substitution of the O-glycosylation sites did not appear to influence CCR5 S-acylation. There was no significant difference in S-acylation between WT, O-Gly2, or O-Gly4 when expressed under control conditions or when co-expressed with zDHHC3 (**Figure 3.3F**). As expected, co-expression with zDHHC3 significantly increased CCR5 S-acylation compared to control for all three constructs, indicating that loss of O-glycosylation does not impair the ability of CCR5 to undergo S-acylation.

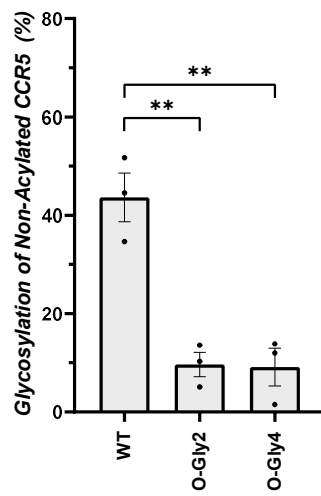
A



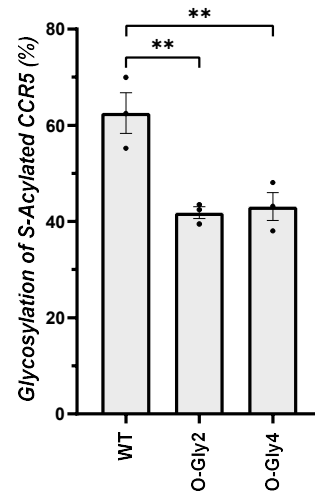
B

Predicted CCR5 Band	
1	Non-Glycosylated
2	Glycosylated
3	S-Acylated Non-Glycosylated
4	S-Acylated Glycosylated

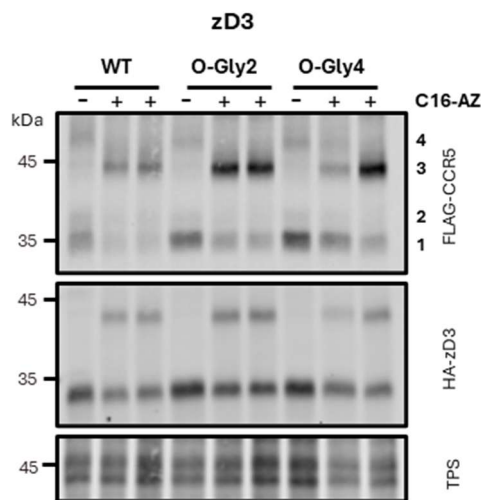
C



D



E



F

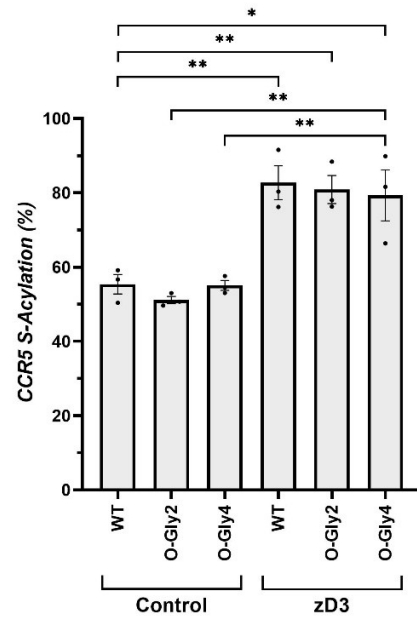


Figure 3.3. N-Terminal O-Glycosylation of CCR5 occurs at Serine 6 and Serine 7 and substitution of these residues does not impair CCR5 S-acylation. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged CCR5, either wild-type CCR5 (WT), a protein with serine to alanine substitutions at S6 and S7 (O-Gly2), or a protein with alanine substitutions at S6, S7, T16, and S17 (O-Gly4), together with HA-tagged zDHHC3 (zD3) or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblot showing CCR5 S-acylation (top; FLAG-CCR5) in the absence of zDHHC3 and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Table of predicted CCR5 band identities corresponding to the numbered bands in the immunoblots. **(C)** Quantification of CCR5 glycosylation in the absence of zDHHC3 co-expression, calculated from the non-acylated bands and expressed as the mean percentage of the glycosylated band relative to total CCR5 signal (Band 2/(Band 1 + Band 2)). **(D)** Quantification of CCR5 glycosylation in the absence of zDHHC3 co-expression, calculated from the S-acylated bands and expressed as the mean percentage of the glycosylated band relative to the total CCR5 signal (Band 4/(Band 3 + Band 4)). **(E)** Representative immunoblot showing CCR5 S-acylation (top; FLAG-CCR5), zDHHC3 co-expression (middle; HA-zD3), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(F)** Quantification of CCR5 S-acylation with or without zDHHC3 co-expression, expressed as the mean percentage of the S-acylated band relative to the total CCR5 signal (non-acylated plus acylated bands). For all quantified data, error bars represent $\pm$ SEM; n=3 independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (*p<0.05, **p<0.01).

3.3.3 Preventing O-glycosylation reduces CCR5 protein stability in an 8-hour cycloheximide pulse-chase assay

While it has been shown that CCR5 O-glycosylation does not impact receptor trafficking to or expression on the cell surface, the effect of O-glycosylation on CCR5 protein stability has not been clearly determined. Previous pulse-chase experiments by Petti *et al.* reported a rapid decrease in expression of an immature, non-glycosylated form of WT CCR5 within 30 minutes, suggesting that this form was rapidly converted into the mature glycosylated form (Petti *et al.*, 2018). However, to our knowledge, no study has directly examined the effect of preventing O-glycosylation on CCR5 stability over an extended period.

To address this, an 8-hour cycloheximide pulse-chase assay was conducted in HEK293T cells expressing either WT CCR5 or the O-glycosylation mutants O-Gly2 or O-Gly4. Cycloheximide treatment inhibits new protein synthesis, allowing the stability of the existing CCR5 protein to be monitored over time (Schneider-Poetsch *et al.*, 2010). Total CCR5 expression at 8 hours was quantified as a percentage of the expression at 0 hours. For consistency of quantification, the same total area was analysed for all samples, incorporating both immature and mature receptor bands.

WT CCR5 exhibited only a modest decrease in expression over the 8 hours, retaining ~ 80 % of the initial expression (**Figure 3.4B**). At 8 hours, the receptor predominantly appeared in the glycosylated form, consistent with the maturation of the immature protein into the mature receptor (**Figure 3.4A**). In contrast, the O-Gly2 mutant displayed a significant reduction in expression relative to WT, retaining only ~ 52 % of the protein after 8 hours of cycloheximide treatment (**Figure 3.4B**). A similar decrease was observed for the O-Gly4 mutant, with ~ 42 % expression remaining after cycloheximide treatment. No statistically significant difference in stability was detected between O-Gly2 and O-Gly4. These findings indicate that preventing O-glycosylation reduces the stability of CCR5, suggesting this modification contributes to maintaining receptor expression levels.

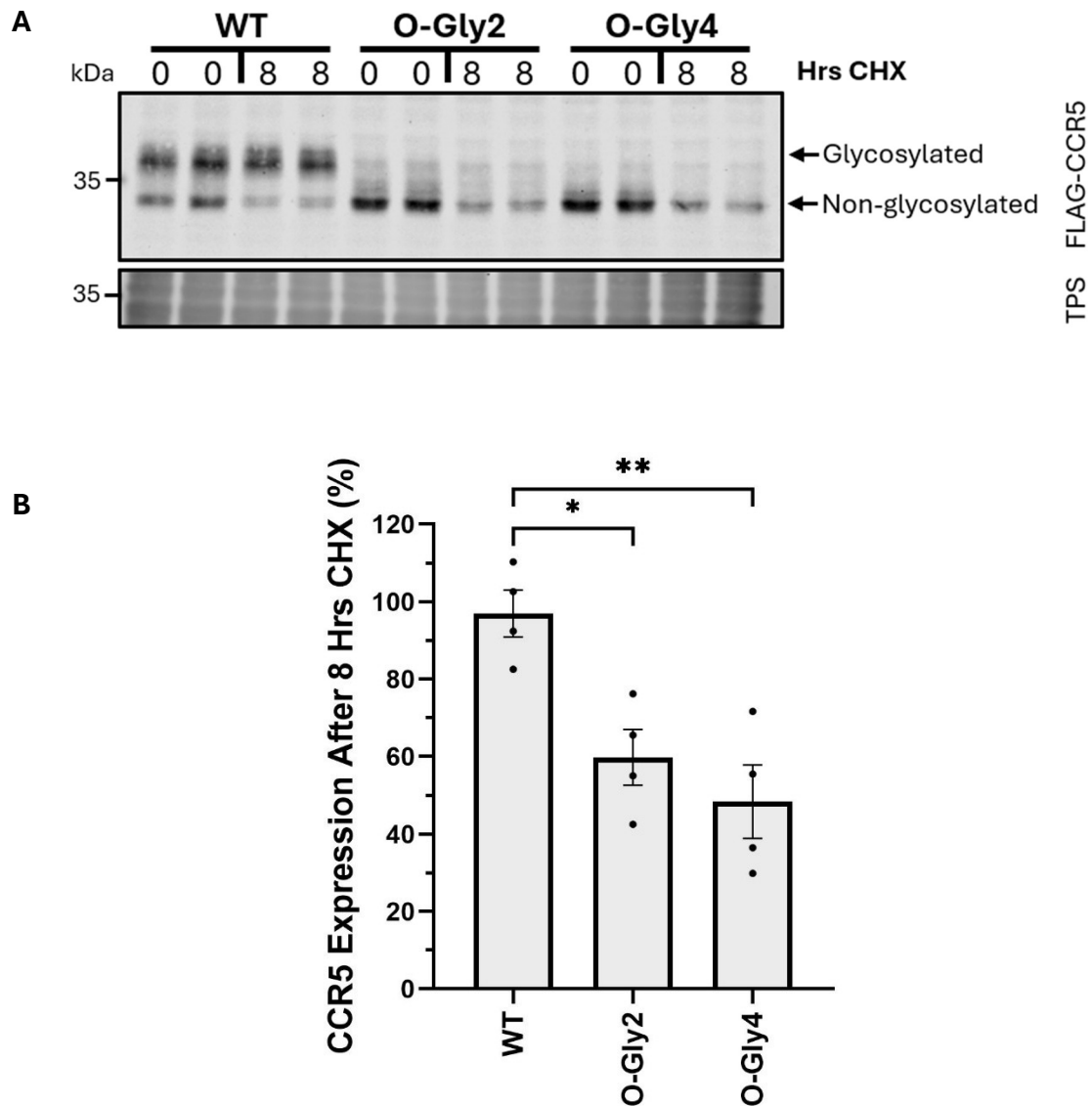


Figure 3.4. Substitution of N-terminal O-glycosylation sites at Serine 6 and Serine 7 reduces CCR5 stability. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged CCR5, either wild-type CCR5 (WT), a protein with serine to alanine substitutions at S6 and S7 (O-Gly2), or a protein with alanine replacements at S6, S7, T16, and S17 (O-Gly4). At 24 hours post-transfection, cells were treated with 50 μ g/ml cycloheximide (CHX). Samples were collected at 0 and 8 hours and analysed by SDS-PAGE and immunoblotting. **(A)** Representative immunoblot showing CCR5 expression (top; FLAG-CCR5) and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of CCR5 expression following 8 hours CHX treatment, expressed as the mean percentage of CCR5 remaining at 8 hours relative to expression at 0 hours. Error bars represent $\pm$ SEM; $n=4$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (* $p<0.05$, ** $p<0.01$).

3.3.4 Substitutions of the DHHC catalytic cysteine eliminates the zDHHC3/zDHHC7 dependent increase of CCR5 S-acylation

To strengthen the evidence that the observed increase in CCR5 S-acylation following co-expression with zDHHC3, zDHHC7 and zDHHC15 (**Figure 3.1**) resulted specifically from the catalytic activity of these enzymes, catalytically inactive versions of zDHHC3 and zDHHC7 were generated. In these proteins, the conserved cysteine residue within the DHHC motif, which is required for the auto-acylation step that initiates substrate S-acylation, was substituted with a serine, producing catalytically inactive forms (zD3CS and zD7CS). Comparing the activity of WT and catalytically inactive enzymes provides more evidence to determine whether the enhancement of CCR5 S-acylation is dependent on zDHHC catalytic function rather than other potential effects, such as protein overexpression or altered trafficking.

To assess this, HEK293T cells were co-transfected with WT CCR5 and either an empty vector control, the WT zDHHC3 or zDHHC7 enzymes, or their respective catalytically inactive versions. Palmitic acid-azide labelling followed by click chemistry was then used to quantify CCR5 S-acylation.

As expected from previous results, co-expression of CCR5 with either zDHHC3 or zDHHC7 resulted in a significant increase in CCR5 S-acylation compared to control (**Figure 3.5C**). In contrast, co-expression with zDHHC3CS or zDHHC7CS produced a marked reduction in CCR5 S-acylation relative to their WT counterparts. Importantly, the substitutions in the DHHC active site completely abolished the enhancing effect of zDHHC3 and zDHHC7, with CCR5 S-acylation levels indistinguishable from control.

When examining the immunoblots of CCR5, the loss of the hypothesised dimer (Band 4) can be observed when co-expressed with zDHHC3 or zDHHC7, but this effect is lost when co-expressed with the inactive zDHHC enzymes, suggesting this effect is indeed related to CCR5 S-acylation (**Figure 3.5A**). Interestingly, co-expression with the inactive zDHHC enzymes appeared to result in non-acylated CCR5 favouring expression as a non-glycosylated species (Band 1). Loss of catalytic activity in the mutant enzymes was also evident in immunoblots for the zDHHC enzymes, as they showed reduced S-acylation relative to their respective WT counterparts (**Figure 3.5A**).

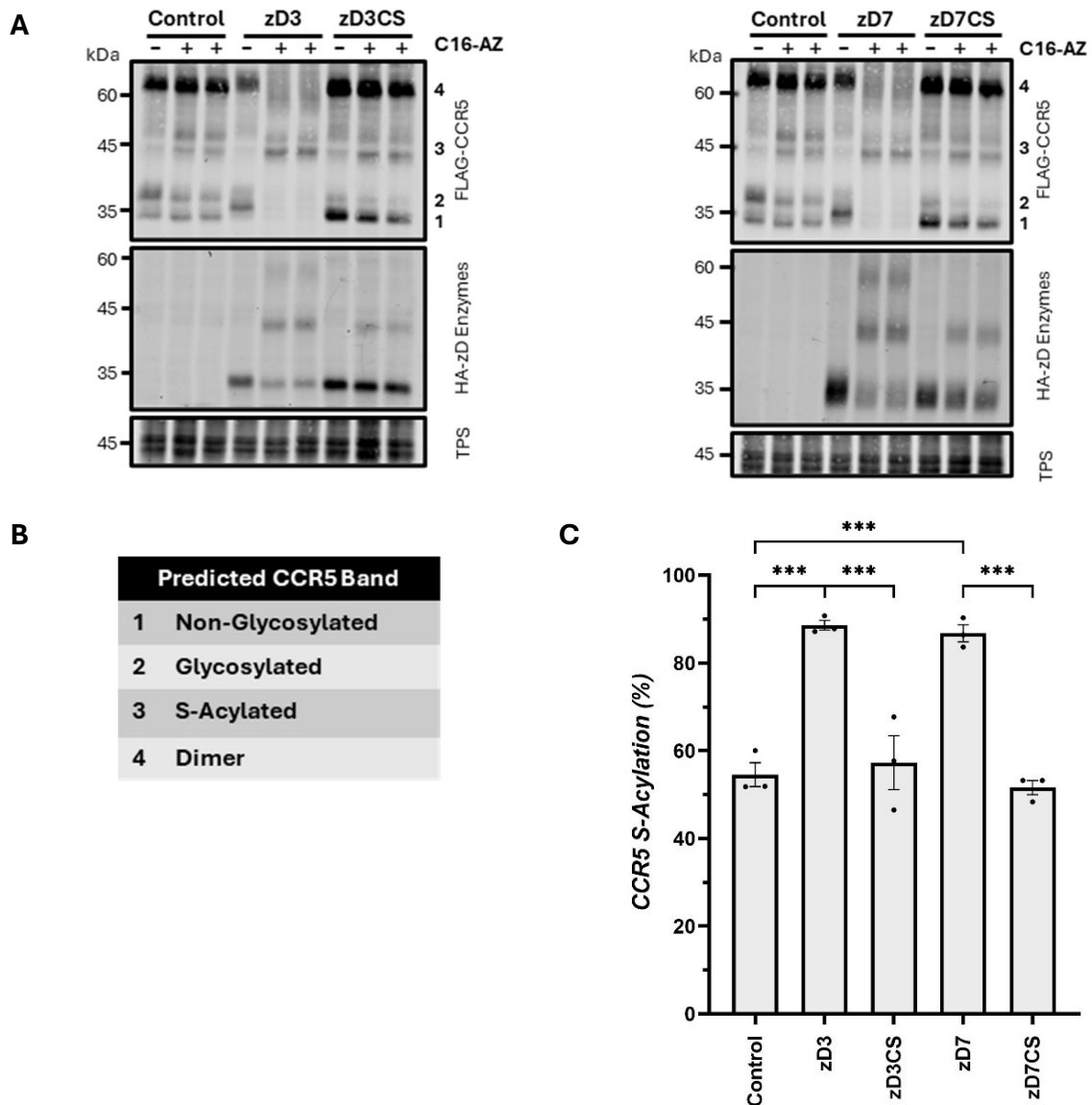


Figure 3.5. Substitution of the DHHC catalytic cysteine abolishes zDHHC3 and zDHHC7-mediated S-acylation of CCR5. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged CCR5 together with HA-tagged wild-type zDHHC3 or zDHHC7 (zD3 or zD7), enzymatically inactive cysteine to serine versions (zD3CS or zD7CS) or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE and immunoblotting. **(A)** Representative immunoblot showing CCR5 S-acylation (top; FLAG-CCR5), expression of wild-type or mutant zDHHC enzymes (middle; HA-zDHC Enzymes), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Table of predicted CCR5 band identities corresponding to the numbered bands in the immunoblots. **(C)** Quantification of CCR5 S-acylation expressed as the mean percentage of the S-acylated band relative to the total CCR5 signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (** $p < 0.001$).

3.3.5 S-Acylation of CCR5 by zDHHC3 is inhibited by the broad-spectrum zDHHC inhibitor 2-bromopalmitate

To further validate that the observed increase in CCR5 S-acylation upon co-expression with zDHHC3 was due to enzymatic S-acylation, palmitic acid-azide labelling was performed in the presence of the broad-spectrum S-acylation inhibitor 2-bromopalmitate (2-BP). 2-BP is a palmitate analogue which irreversibly blocks zDHHC auto-acylation and subsequent substrate S-acylation by covalently modifying the catalytic cysteine of the DHHC motif (Lan et al., 2021). Although widely used to inhibit protein S-acylation, 2-BP is known to be relatively non-specific, particularly at higher concentrations. Therefore, this experiment was intended as supporting evidence rather than a definitive measure of zDHHC3-mediated CCR5 S-acylation, due to the lack of a more specific inhibitor.

HEK293T cells transfected with WT CCR5, with or without co-expression of zDHHC3, were incubated with 0 μ M, 50 μ M, or 100 μ M 2-BP during the 4-hour palmitic acid-azide labelling period. CCR5 S-acylation was quantified as a percentage of S-acylated protein relative to total CCR5 expression. Incubation with 2-BP in cells expressing CCR5 and no zDHHC enzyme (Control) did not significantly alter basal S-acylation levels compared to non-treated cells, indicating the endogenous zDHHC activity was largely unaffected at these concentrations in this context.

As expected, co-expression of CCR5 with zDHHC3 in the absence of 2-BP resulted in a significant increase in CCR5 S-acylation relative to control (**Figure 3.6B**). Importantly, this increase in S-acylation was significantly reduced with 50 μ M 2-BP and further reduced with 100 μ M 2-BP, which effectively returned S-acylation levels to those observed in control cells. This provides further evidence that the increase of CCR5 S-acylation when co-expressed with zDHHC3 is dependent on enzymatic activity and that it can be inhibited by incubation with a broad-spectrum S-acylation inhibitor.

The effects of 2-BP were also evident in the immunoblots of zDHHC3 (**Figure 3.6A**). Auto-acylation of zDHHC3 was partially reduced after incubation with 50 μ M and completely abolished at 100 μ M 2-BP, consistent with inhibition of the enzyme's catalytic activity. However, high concentrations of 2-BP also appeared to produce off-target effects. Specifically, CCR5 expression levels were visibly reduced at 100 μ M 2-BP, suggesting that at this concentration the inhibitor may compromise protein stability or cell viability, reinforcing the need for caution when interpreting high-concentration 2-BP experiments.

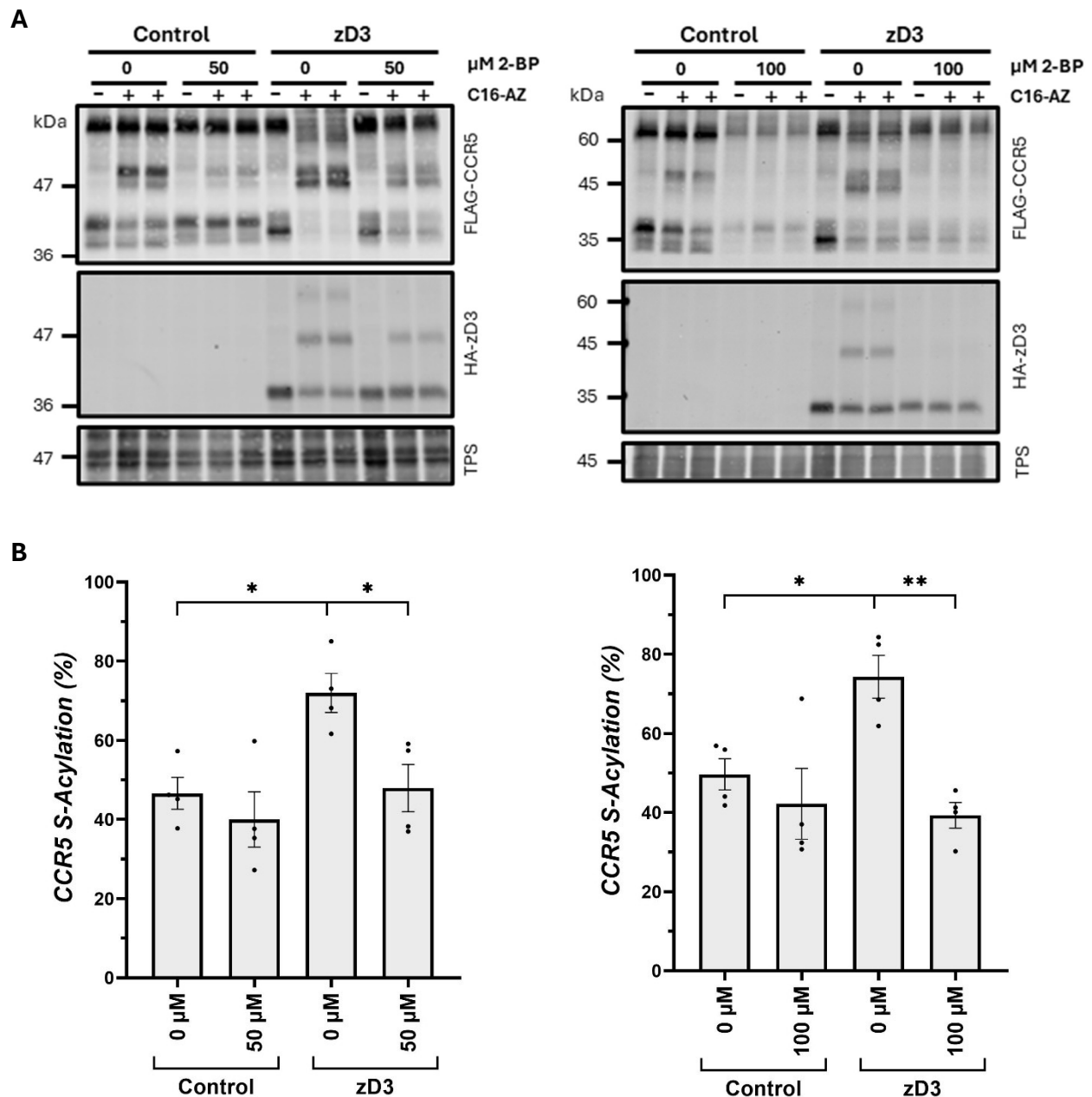


Figure 3.6. 2-Bromopalmitate inhibits zDHC3-mediated S-acylation of CCR5. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged CCR5 together with HA-tagged wild-type zDHC3 or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid (-) or palmitic acid azide (C16-AZ; +), in the presence or absence of 50 μ M or 100 μ M 2-bromopalmitate (2-BP), for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing CCR5 S-acylation (top; FLAG-CCR5), expression of zDHC3 (middle; HA-zD3), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of CCR5 S-acylation expressed as the mean percentage of the S-acylated band relative to the total CCR5 signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=4$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (* $p<0.05$, ** $p<0.01$).

3.3.6 CCR5 S-acylation increases following release from ER-trap in the RUSH trafficking system

The enzymes identified as capable of increasing CCR5 S-acylation (zDHHC3, zDHHC7, and zDHHC15) are all predominantly localised to the Golgi apparatus in HEK293T cells (Greaves et al., 2008, Greaves et al., 2010). This shared localisation suggests that the Golgi may represent a key site of CCR5 S-acylation during its trafficking pathway. However, both our own enzyme-identification experiment and those performed by others (Boncompain et al., 2019) relied on zDHHC overexpression, raising the possibility that the observed S-acylation patterns might not fully reflect the behaviour of CCR5 under endogenous conditions. To more directly determine when and where CCR5 becomes S-acylated during its normal trafficking route, CCR5 S-acylation should therefore be investigated without zDHHC co-expression.

To achieve this, the Retention Using Selective Hooks (RUSH) system was used. In this system, CCR5 fused to a streptavidin binding protein is retained in the ER through binding to streptavidin fused to an ER-retention hook (KDEL). Addition of biotin disrupts this interaction, releasing CCR5 from the ER and allowing it to resume trafficking through the cell (**Figure 3.7**). By combining the RUSH system with palmitic acid-azide labelling and click chemistry, and including biotin in a subset of samples during the initial 8-hour incubation, CCR5 S-acylation could be directly compared while trapped in the ER versus after its release and subsequent trafficking.

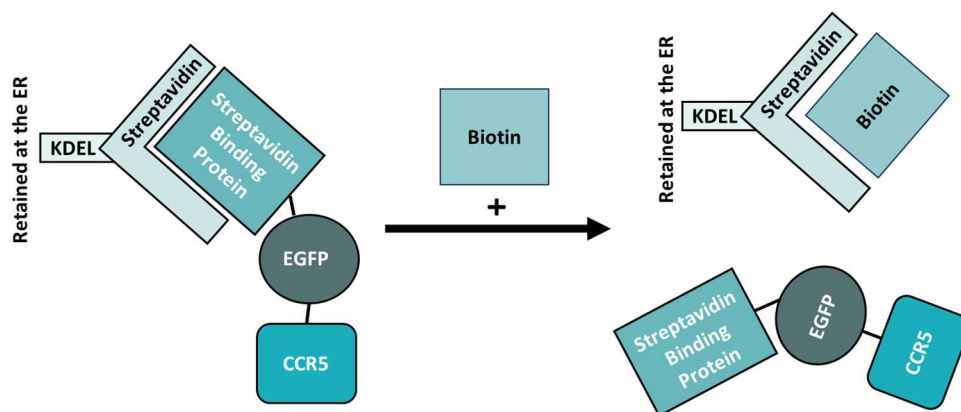


Figure 3.7. Schematic of the Retention Using Selective Hooks (RUSH) system. The diagram depicts the expression of Str-KDEL_SBP-EGFP CCR5, in which CCR5 fused to streptavidin binding protein (SBP) is retained in the ER through interaction with streptavidin fused to the ER retention sequence KDEL. Addition of biotin disrupts the streptavidin-SBP interaction, releasing CCR5 and allowing it to proceed through the secretory pathway.

As the expression of the CCR5 RUSH construct (Str-KDEL_SBP-EGFP-CCR5) was relatively low, the plasmid amount used for HEK293T cell transfection was increased to

1.5 μ g. Additionally, GFP immunoprecipitation was performed before SDS-PAGE to obtain a sufficiently strong CCR5 signal on immunoblots. S-Acylation of the immunoprecipitated CCR5 samples was quantified as a percentage of total CCR5 expression.

There was a significant increase, of approximately 3-fold, in the S-acylation of biotin-treated samples compared to CCR5 incubated without biotin (**Figure 3.8**). As this increase in CCR5 S-acylation was observed in the absence of zDHHC overexpression, it is reflective of the S-acylation driven solely by endogenous zDHHC enzymes. The increase observed with the addition of biotin suggests that there is increased CCR5 S-acylation at post-ER compartments.

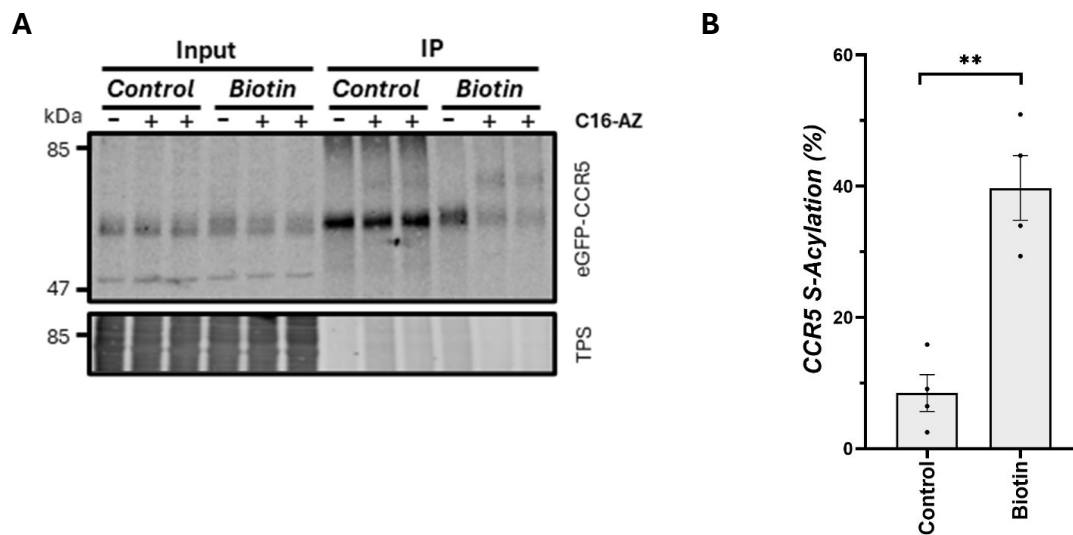


Figure 3.8. Release of CCR5 from ER retention using the Retention Using Selective Hooks (RUSH) system leads to increased S-acylation. HEK293T cells were co-transfected with the RUSH plasmid construct Str-KDEL_SPB-EGFP-CCR5. At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid (-) or palmitic acid azide (C16-AZ; +), in the presence or absence of 100 μ M biotin, for 8 hours. Samples were incubated with 5 kDa alkyne mPEG prior to immunoprecipitation (IP) using GFP-trap agarose beads and subsequent analysis by SDS-PAGE and immunoblotting. **(A)** Representative immunoblots showing CCR5 S-acylation (top; eGFP-CCR5) and total protein stain (bottom; TPS). Input samples (prior to IP) and IP samples are shown. Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of CCR5 S-acylation in IP samples expressed as the mean percentage of the S-acylated band relative to the total CCR5 signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=4$ independent experiments, shown as filled circles. Statistical significance was determined by unpaired t test (** $p<0.01$).

3.3.7 Substitution of cysteine residues C321, C323, and C324 with alanine did not decrease CCR5 S-acylation by zDHHC3 or zDHHC15

CCR5 contains three C-terminal cysteine residues (C321, C323, and C324), which have previously been identified as sites of S-acylation (**Figure 3.9**) (Blanpain et al., 2001, Percherancier et al., 2001, Boncompain et al., 2019). However, the precise contribution of these cysteines to the overall S-acylation of CCR5 remains unclear. Different studies report varying degrees to which replacement of these residues disrupts S-acylation, with some reporting complete loss of S-acylation while other groups report a decrease of ~ 50 %. Importantly, all previous analyses relied on radioactive palmitate labelling, a technique that, while effective, is less sensitive than other more recent approaches used to interrogate S-acylation.

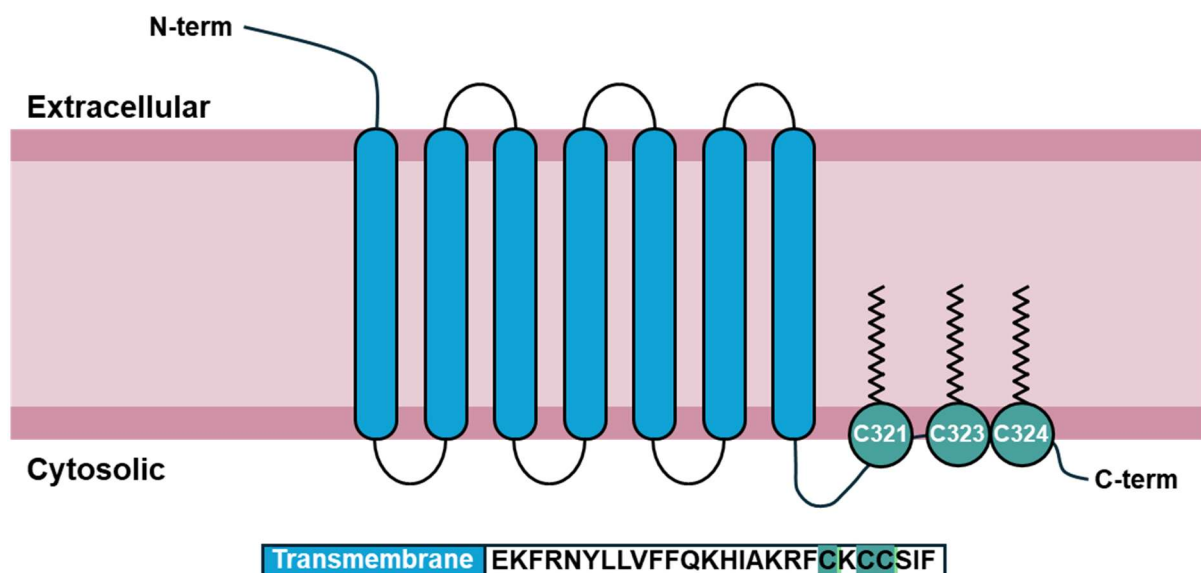


Figure 3.9. Schematic representation of CCR5 highlighting C-terminal S-acylation sites. CCR5 is shown embedded in the plasma membrane, with the seven transmembrane domains depicted in blue. The sites of C-terminal S-acylation (cysteine 321, 323, and 324) are indicated by green circles, with representative fatty acyl chains attached (zig-zag lines).

In an effort to resolve these discrepancies and more sensitively quantify the contribution of the three CCR5 C-terminal cysteines, the S-acylation of these residues was investigated using palmitic acid-azide labelling coupled with click chemistry, which provides improved sensitivity when compared to radiolabelling. To determine the contribution of these residues, a triple cysteine-to-alanine protein (C321A, C323A, C324A) was generated, which will be referred to as CCR5 3CA. The S-acylation of CCR5 3CA was then compared to WT CCR5 in the presence or absence of co-expressed

zDHHC3 or zDHHC15 in HEK293T cells. Alanine was chosen as the replacement residue because it is structurally small and non-reactive, allowing removal of the cysteine residue without introducing large side chains or other chemical functionalities that might disrupt the folding or trafficking of the receptor. Additionally, alanine was the residue substituted in previous studies, and thus allowed for better comparison of the results generated by the different methodologies.

There was no significant difference in S-acylation between WT CCR5 and the CCR5 3CA mutant when co-expressed with either zDHHC3 or zDHHC15 (**Figure 3.10**). Likewise, no significant difference was observed between WT and CCR5 3CA under conditions without zDHHC enzyme overexpression (Control). For both WT and CCR5 3CA, S-acylation increased significantly when co-expressed with zDHHC3 compared to the control and when co-expressed with zDHHC15 compared to the control. Therefore, in our hands, mutation of the three C-terminal cysteine residues did not decrease CCR5 S-acylation.

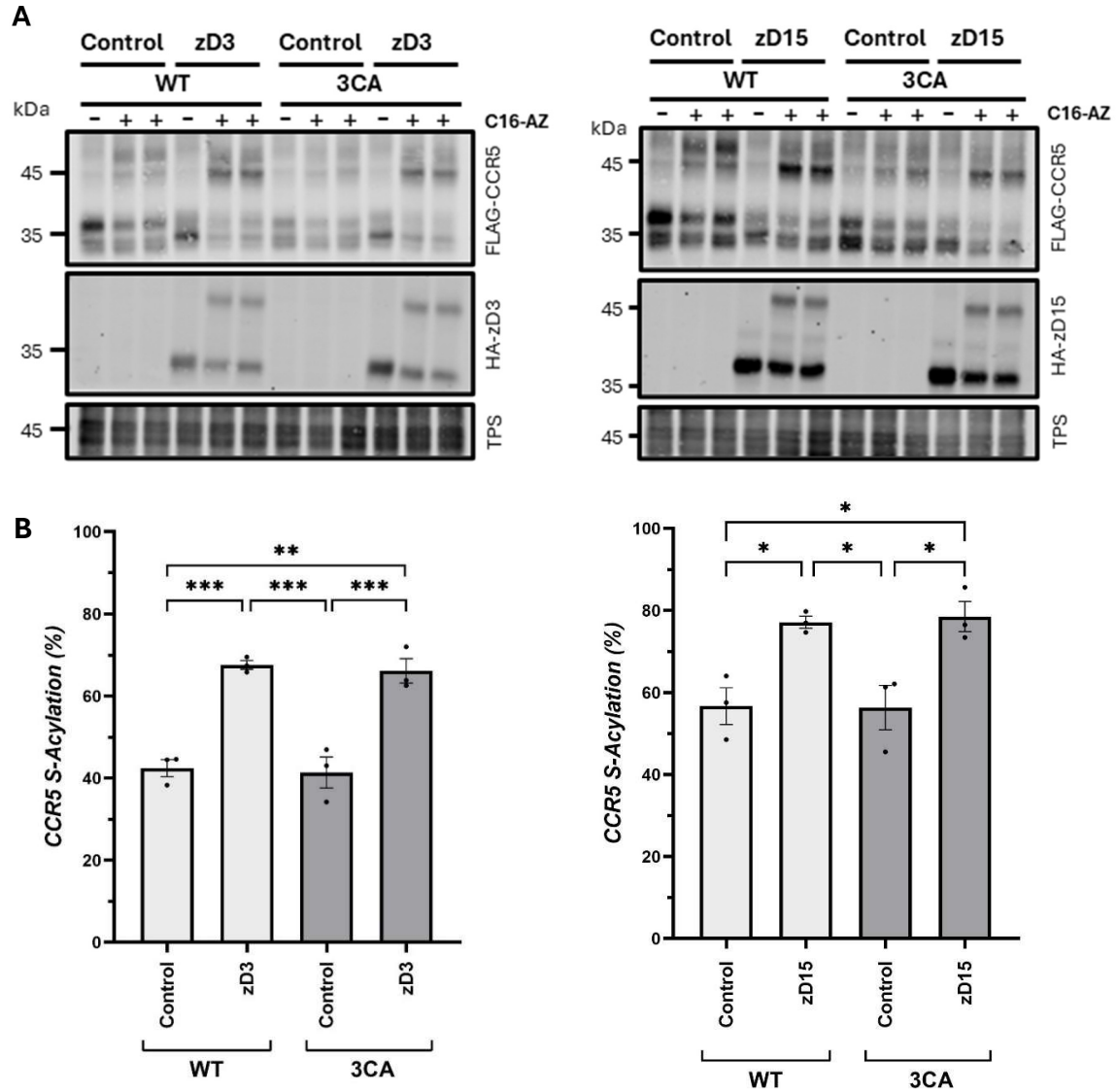


Figure 3.10. Substitution of C-terminal cysteines C321, C323, and C324 does not abolish zDHC3- and zDHC15-mediated S-acylation of CCR5. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged CCR5, either wild-type (WT) or a protein with alanine substitution of the C-terminal residues C321, C323, C324 (3CA), together with HA-tagged zDHC3, zDHC15 or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE and immunoblotting. **(A)** Representative immunoblots showing CCR5 S-acylation (top; FLAG-CCR5), expression of zDHC3/zDHC15 (middle; HA-zD3/HA-zD15), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of CCR5 S-acylation expressed as the mean percentage of the S-acylated band relative to the total CCR5 signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

3.3.8 Combined substitution of six candidate cysteine residues decreases zDHHC-mediated CCR5 S-acylation

As substitution of the three previously identified CCR5 S-acylation sites (C321, C323, C324) did not affect CCR5 S-acylation, it was hypothesised that additional cysteine residues in this protein may also undergo S-acylation. To explore this possibility, we first identified three further cysteine residues that are structurally positioned to be accessible to zDHHC enzymes. These were C58, located at the end of the first transmembrane domain; C213, positioned six residues inside the fifth transmembrane domain from the cytosolic interface; and C224, situated within the third intracellular loop (Figure 3.11). The proximity of these residues to cytosol-facing regions made them plausible candidates for zDHHC-mediated modification.

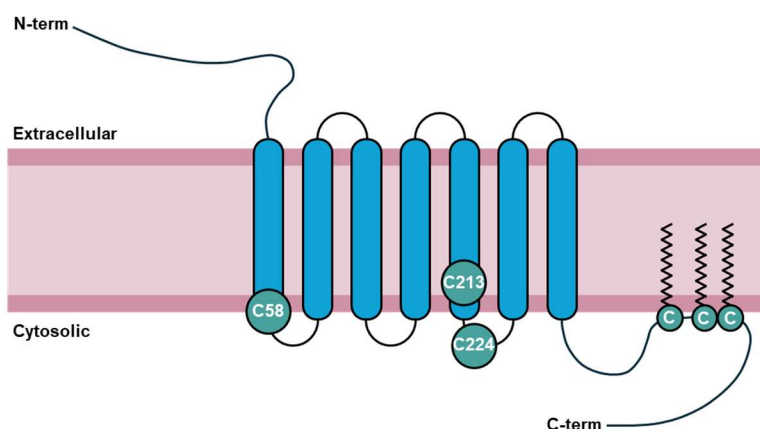


Figure 3.11. Schematic representation of CCR5 highlighting candidate novel S-acylation cysteine residues. CCR5 is shown embedded in the membrane, with the seven transmembrane domains depicted in blue. The sites of C-terminal S-acylation (cysteines 321, 323, and 324) and potential novel non-C-terminal cysteine residues (C58, C213, C224) are indicated by green circles

To test whether any of these additional cysteines contributed to overall CCR5 S-acylation, a protein with cysteine-to-alanine replacements was generated in which the three previously described C-terminal cysteines (C321, C323, C324) and these newly identified candidate sites (C58, C213, C224) were simultaneously replaced. This construct will be referred to as CCR5 6CA. The S-acylation of CCR5 6CA was assessed using palmitic acid-azide labelling with click chemistry in HEK293T cells co-expressing either zDHHC3 or zDHHC15. Levels of S-acylation were compared to those of WT CCR5 and the previously generated CCR5 3CA mutant.

CCR5 6CA exhibited a significant reduction in S-acylation compared to both WT CCR5 and the CCR5 3CA mutant when co-expressed with zDHHC3 (Figure 3.12). Whereas WT and CCR5 3CA displayed ~ 70 % S-acylation, the CCR5 6CA mutant showed only ~ 30 % S-acylation. A comparable decrease in S-acylation was observed when CCR5 6CA was co-expressed with zDHHC15, further supporting the conclusion that these additional cysteine residues contribute to CCR5 S-acylation.

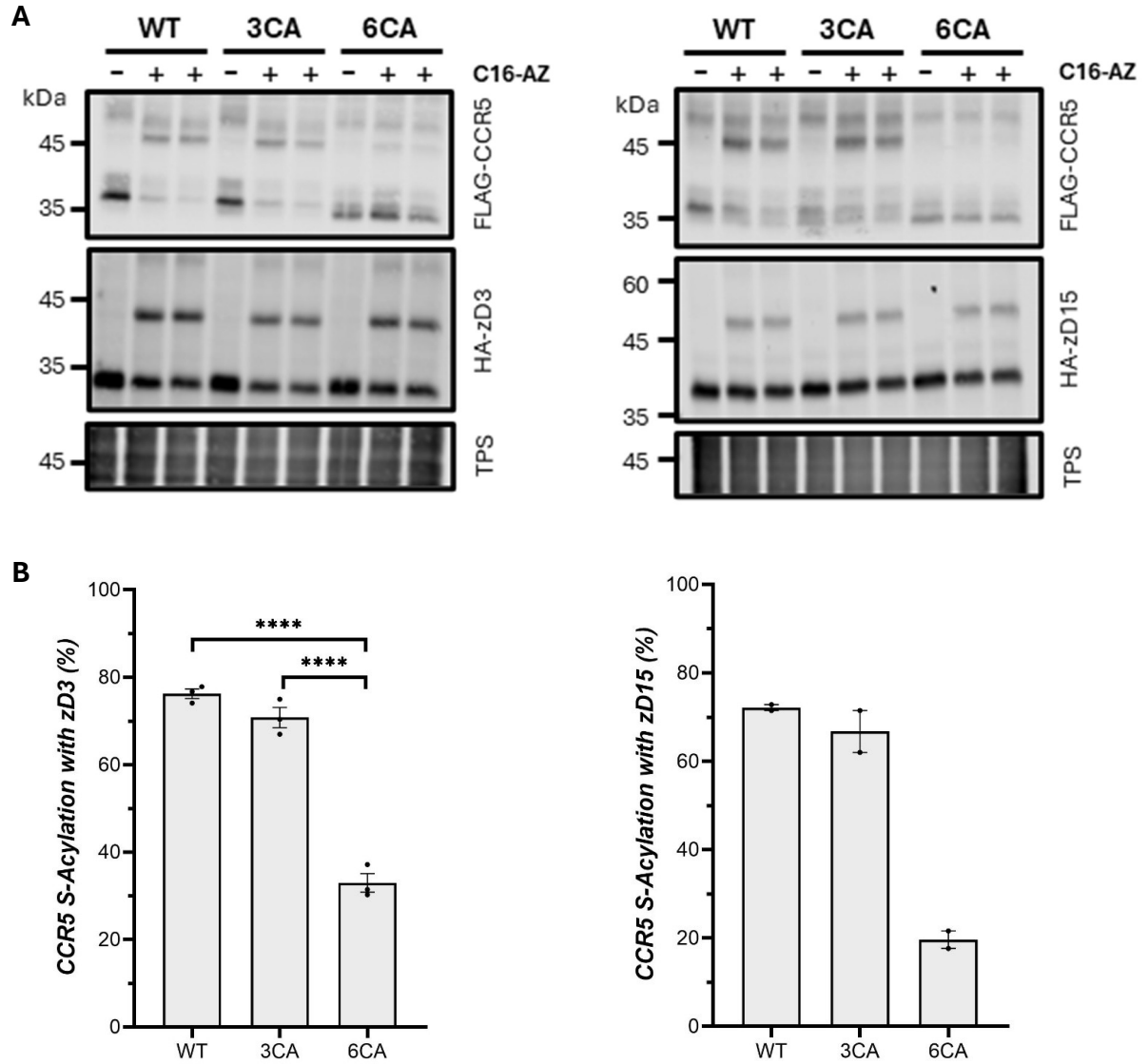


Figure 3.12. Combined replacement of six cysteine residues abolishes zDHC3- and zDHC15-mediated S-acylation of CCR5. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged CCR5, either wild-type (WT), or a protein with alanine substitution of three C-terminal residues C321, C323, C324 (3CA), or a protein with substitution of six residues 58C, 213C, 224C, 321, C323, and C324, together with HA-tagged zDHC3, zDHC15 or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE and immunoblotting. **(A)** Representative immunoblots showing CCR5 S-acylation (top; FLAG-CCR5), expression of zDHC3/zDHC15 (middle; HA-zD3/HA-zD15), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of CCR5 S-acylation expressed as the mean percentage of the S-acylated band relative to the total CCR5 signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=3$ (zD3) and $n=2$ (zD15) independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (**** $p<0.0001$).

3.3.9 S-Acylation of the CCR5 C-terminal cysteines (C321, C323, C324) is enhanced by zDHHC3 when other candidate cysteine residues are removed

As no difference was observed in the S-acylation of CCR5 between the WT and 3CA mutant, but a clear decrease was detected in the 6CA mutant, it became necessary to determine the specific contribution of the previously identified C-terminal cysteine residues (C321, C323, C324) to CCR5 S-acylation. In the context of the 6CA results, the data suggested that the newly identified cysteine residues contributed substantially to overall S-acylation, raising the question of whether the modification of C321, C323 and C324 had been overestimated in earlier studies.

To address this, a CCR5 protein was designed in which C58, C213, and C224 were each substituted to alanine, thereby eliminating these potential S-acylation sites while leaving the three C-terminal cysteine residues intact. This construct allowed the evaluation of the capacity of the C-terminal region to be S-acylated, in the absence of other modifiable cysteines. The S-acylation of this CCR5 C58/213/224A protein was assessed using palmitic acid-azide labelling combined with click chemistry in HEK293T cells co-expressing no zDHHC enzyme (Control), zDHHC3, zDHHC6, or zDHHC15.

The S-acylation of the CCR5 C58/213/224A protein was significantly increased when co-expressed with zDHHC3 compared to control or zDHHC6, rising from ~ 30 % to ~ 50 % (**Figure 3.13B**). Although zDHHC15 did not significantly increase S-acylation, there was also no statistically significant difference between zDHHC3 and zDHHC15, likely due to the variation observed across the replicates. When analysing the immunoblot, a clear S-acylated band at ~ 42 kDa was visible when the CCR5 C58/213/224A protein was expressed with zDHHC3, whereas this band was faint or absent in the control, zDHHC6, and zDHHC15 conditions (**Figure 3.13A**), supporting the quantitative analysis.

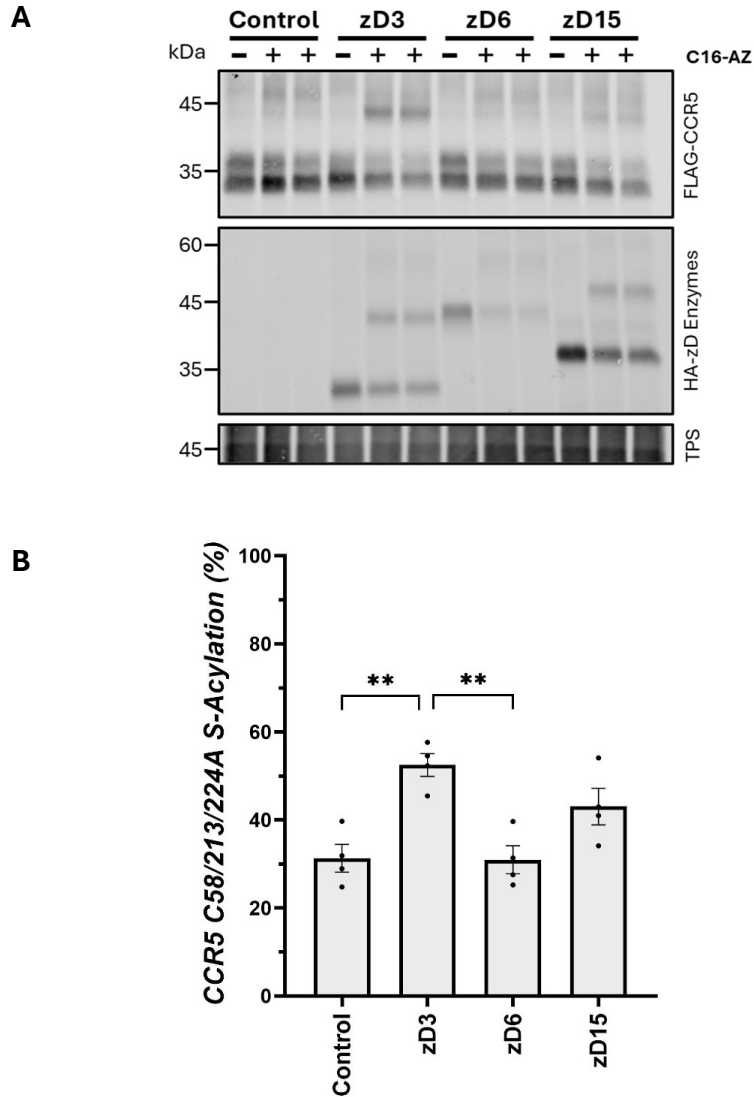


Figure 3.13. zDHHC3 mediates S-acylation of CCR5 at the C-terminal cysteine residues C321, C323, and C324. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged CCR5 with cysteine to alanine substitutions at the potential novel S-acylation sites 58C, 213C, and 224C together with HA-tagged zDHHC3, zDHHC6, zDHHC15 or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing CCR5 S-acylation (top; FLAG-CCR5), expression of zDHHC enzymes (middle; HA-zD Enzymes), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of CCR5 S-acylation expressed as the mean percentage of the S-acylated band relative to the total CCR5 signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (** $p<0.01$).

3.3.10 Pairwise reintroduction of C58 with either C213 or C224 restores CCR5 S-acylation by zDHHC3 in the CCR5 6CA backbone

Building upon the previous findings, which demonstrated that C-terminal cysteine residues contribute only partially to total CCR5 S-acylation, it became important to determine the specific contribution of the three newly proposed S-acylation sites (C58, C213, and C224). The substantial loss of S-acylation in the CCR5 6CA mutant suggested that these other sites play a major role, but the individual contribution of each site, or combinations of sites, remained unclear.

To investigate this, a set of CCR5 proteins was generated using the CCR5 6CA backbone, to remove the influence of the C-terminal cysteines, in which the cysteine residues were selectively reintroduced in pairs. This approach allowed evaluation of whether particular combinations of the novel cysteines were sufficient to restore S-acylation and to identify which residues are most efficiently targeted by zDHHC enzymes. Three proteins were created, each restoring a different pair of the novel cysteine residues, 58C/213C, 58C/224C, and 213C/224C. The S-acylation of these constructs was determined using palmitic acid-azide labelling with click chemistry when co-expressed with zDHHC3 in HEK293T cells.

When compared with the CCR5 6CA mutant, both the 58C/213C and 58C/224C proteins exhibited a significant increase in S-acylation in the presence of zDHHC3 (**Figure 3.14**). Notably, the level of S-acylation in these two proteins was not significantly different from that observed in WT CCR5 or in the CCR5 3CA mutant, indicating that reintroduction of cysteine 58 paired with either 213 or 224 was sufficient to restore substantial S-acylation. In contrast, there was no significant increase in S-acylation of the 213C/224C mutant relative to the CCR5 6CA mutant, and both remained significantly decreased compared with WT CCR5 and the CCR5 3CA mutant.

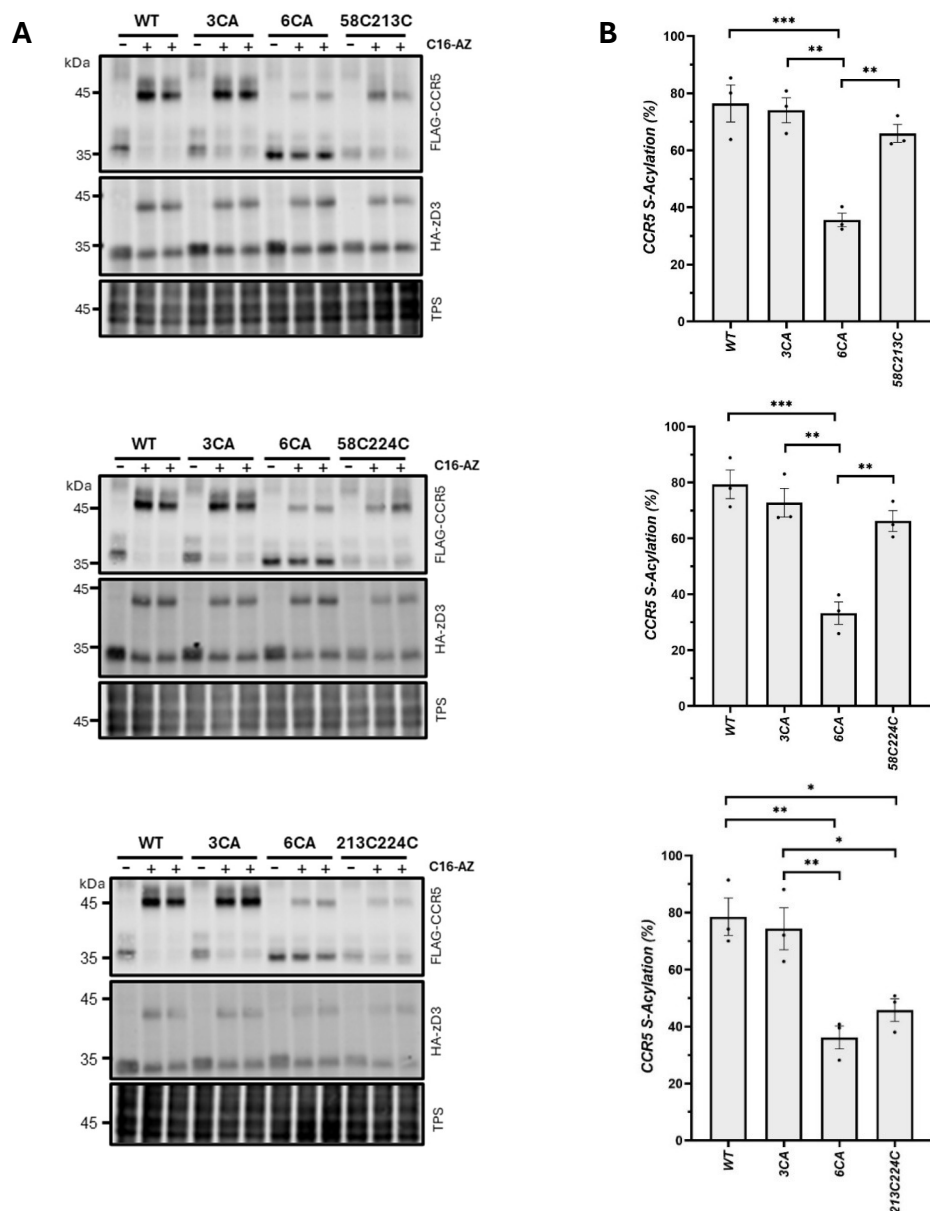


Figure 3.14. CCR5 S-acylation mediated by zDHH3 is restored by pairwise reintroduction of non-C-terminal cysteine residues, dependent on the presence of C58. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged CCR5 based on the 6CA mutant (C58/213/224/321/323/324A) with pairwise reintroduction of cysteine residues by alanine to cysteine replacements at the potential novel S-acylation sites 58C, 213C, and 224C, together with HA-tagged zDHH3. At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing CCR5 S-acylation (top; FLAG-CCR5), expression of zDHH3 enzyme (middle; HA-zD3), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of CCR5 S-acylation expressed as the mean percentage of the S-acylated band relative to the total CCR5 signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

3.3.11 Reintroduction of C58, but not C213 or C224, partially restores zDHHC3-mediated CCR5 S-acylation in the CCR5 6CA protein

The pairwise reintroduction experiment indicated that the proteins containing C58 paired with either C213 or C224 restored substantial S-acylation, whereas the 213C/224C protein did not. This pattern strongly suggested that C58 may be the primary S-acylation site among the newly identified cysteine residues. To directly test this hypothesis and to determine whether any of the three novel sites could independently support S-acylation, proteins were generated using the CCR5 6CA backbone. In these constructs, individual cysteine residues were reintroduced at positions 58, 213, or 224, thereby allowing assessment of the ability of each residue alone to serve as a site of CCR5 S-acylation.

The S-acylation of the three single-cysteine mutants (58C, 213C, 224C) was evaluated by palmitic acid-azide labelling combined with click chemistry in HEK293T cells co-expressing zDHHC3. The S-acylation of these mutants was compared to cells expressing WT CCR5, CCR5 3CA, or CCR5 6CA.

When compared to the CCR5 6CA mutant, there was no significant increase in S-acylation of the 213C or 224C proteins (**Figure 3.15**), indicating that neither of these residues alone is sufficient to support detectable S-acylation under these conditions. In contrast, the 58C protein displayed a significant increase in S-acylation relative to the 6CA construct, rising from ~ 30 % to ~ 60 %. Despite this increase, the S-acylation of the 58C protein remained significantly lower than that of the WT CCR5, with an approximate 20 % reduction, suggesting that although C58 is a major contributor, full S-acylation requires additional cysteine residues. While there appeared to be a similar decrease in CCR5 58C S-acylation when compared to the CCR5 3CA mutant, this decrease was not significant.

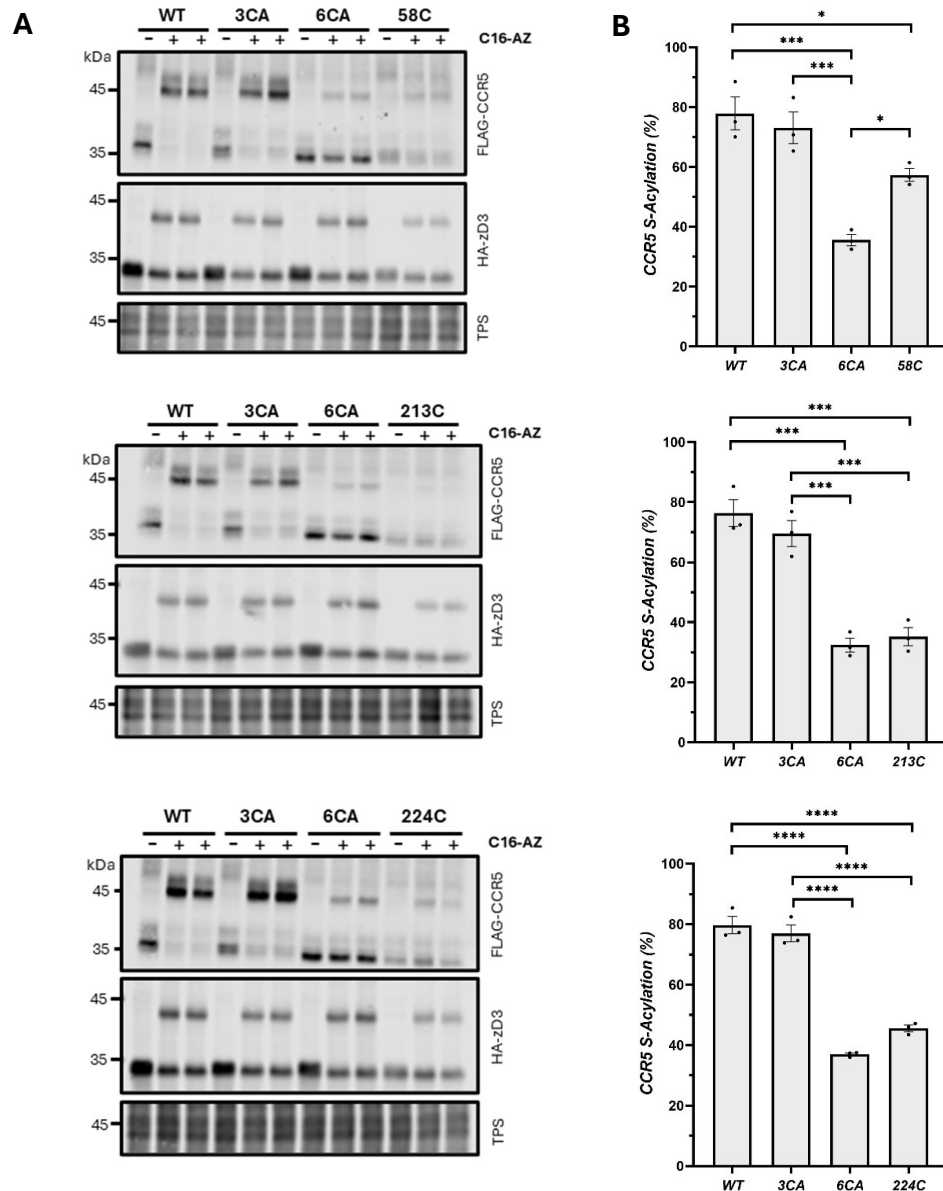


Figure 3.15. CCR5 S-acylation mediated by zDHH3 is partially restored by reintroduction of the non-C-terminal cysteine residue C58 but not C213 or C224. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged CCR5 based on the 6CA mutant (C58/213/224/321/323/324A) with individual reintroduction of cysteine residues by alanine to cysteine replacement at the potential novel S-acylation sites 58C, 213C, and 224C, together with HA-tagged zDHH3. At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing CCR5 S-acylation (top; FLAG-CCR5), expression of zDHH3 enzyme (middle; HA-zD3), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of CCR5 S-acylation expressed as the mean percentage of the S-acylated band relative to the total CCR5 signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (* $p<0.05$, *** $p<0.001$, **** $p<0.0001$).

3.3.12 Substitution of CCR5 S-acylation sites does not significantly alter receptor stability or glycosylation

After identifying cysteine residues that impact the S-acylation of CCR5, it was of interest to determine whether these residues also have a functional role in CCR5 stability or if they influence CCR5 glycosylation, which could indicate effects on receptor trafficking or maturation. As it was previously observed that CCR5 glycosylation impacts receptor stability (**Figure 3.4**), it was therefore of interest to investigate whether the presence or absence of S-acylation sites also influences CCR5 stability. S-Acylation is known to affect the stability of many proteins, including CCR5, and substitution of the C-terminal cysteine residues of CCR5 has been shown to decrease receptor half-life (Percherancier et al., 2001). As additional candidate cysteine residues for S-acylation were identified in this study, it was of interest to determine whether these residues similarly impact CCR5 stability.

To address this, the stability of CCR5 3CA, CCR5 C58/213/224A, and CCR5 6CA was compared to WT CCR5 using an 8-hour cycloheximide chase assay in HEK293T cells. No zDHHC enzymes were co-expressed, allowing analysis of receptor stability in the presence of only endogenous S-acylation machinery. CCR5 expression at 8 hours was then quantified as a percentage of expression at 0 hours to assess receptor stability. In addition, the proportion of mature glycosylated CCR5 relative to total CCR5 expression was determined at both 0 and 8 hours post-cycloheximide treatment to assess potential effects of these cysteine substitutions on CCR5 maturation.

No significant differences in stability were observed between the CCR5 proteins with cysteine substitutions and WT CCR5 following 8 hours of cycloheximide treatment (**Figure 3.16B**). Although there was a relatively high variability in expression levels between experiments, even after normalising to TPS, a consistent trend was observed across replicates. While both the immunoblots (**Figure 3.16A**) and quantified data (**Figure 3.16B**) suggested a decrease in expression of the CCR5 3CA mutant at 8 hours, this reduction was not statistically significant and likely appeared more pronounced due to greater variability in expression levels of the other mutants.

Similarly, no clear differences in glycosylation were observed between WT CCR5 and any of the cysteine mutants at either 0 or 8 hours post-cycloheximide treatment (**Figure 3.16C**). Both WT CCR5 and the cysteine mutants exhibited the expected increase in glycosylation at 8 hours compared to 0 hours, indicating that a greater proportion of the expressed CCR5 had undergone maturation during the incubation period. These results suggest that disruption of S-acylation at these cysteine residues does not impair CCR5 glycosylation or maturation over the time course examined.

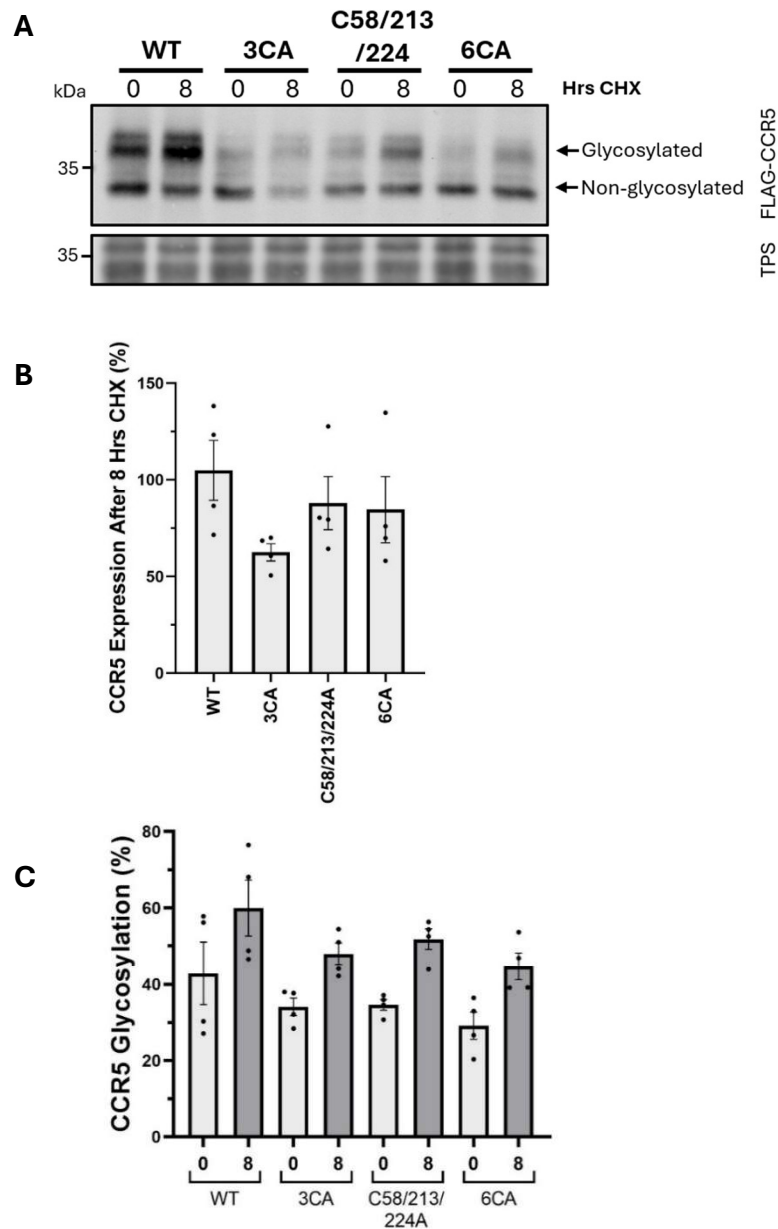


Figure 3.16. Disruption of CCR5 S-acylation sites does not alter receptor stability or glycosylation. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged CCR5, including wild-type CCR5 (WT), or proteins with cysteine to alanine substitutions, C321/323/324A (3CA), C58/213/224A, or C58/213/224/321/323/324A (6CA). At 24 hours post-transfection, cells were treated with 50 μ g/ml cycloheximide (CHX). Samples were collected at 0 and 8 hours and analysed by SDS-PAGE and immunoblotting. **(A)** Representative immunoblot showing CCR5 expression (top; FLAG-CCR5) and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of CCR5 expression following 8 hours CHX treatment, expressed as the mean percentage of CCR5 remaining at 8 hours relative to expression at 0 hours. **(C)** Quantification of CCR5 glycosylation at 0 and 8 hours post-CHX treatment. Error bars represent $\pm$ SEM; $n=4$ independent experiments, shown as filled circles. No statistically significant differences were detected by one-way ANOVA.

3.3.13 Co-Expression with zDHHC3 does not alter the stability of CCR5 with cysteine substitutions but affects receptor glycosylation

As the previous experiment examined the stability of CCR5 proteins with cysteine substitutions in the presence of only endogenous S-acylation enzymes and identified no significant differences, it was of interest to determine whether increasing CCR5 S-acylation through co-expression of zDHHC3 would influence these outcomes. In addition, potential effects on CCR5 glycosylation were assessed, as in the previous experiment.

The stability of CCR5 3CA and CCR5 6CA was compared with WT CCR5 when co-expressed with zDHHC3 using an 8-hour cycloheximide chase assay in HEK293T cells. CCR5 expression at 8 hours relative to the initial 0-hour time point was quantified to assess receptor stability. In parallel, the proportion of CCR5 present as the mature glycosylated receptor after 8 hours of incubation was determined to evaluate potential effects on receptor maturation.

No significant difference in stability was observed for any of the proteins with cysteine substitutions compared to WT CCR5 following 8 hours of cycloheximide treatment. This is consistent with the results obtained in the absence of zDHHC co-expression (**Figure 3.17B**), suggesting that, under these experimental conditions, replacement of the candidate S-acylation cysteine residues does not measurably affect CCR5 stability.

In contrast, a significant difference in CCR5 glycosylation was observed following 8 hours of cycloheximide treatment (**Figure 3.17C**). Glycosylation of both the CCR5 3CA and CCR5 6CA proteins was significantly reduced compared with WT CCR5. While the decrease observed for the 3CA protein was modest (approximately 5 - 10 %), a more pronounced and significant reduction of ~ 20 % was observed for the 6CA protein, with the difference between 3CA and 6CA also reaching significance. These results indicate that, under conditions of enhanced S-acylation through zDHHC3 co-expression, maturation of CCR5 proteins with cysteine substitutions is delayed or reduced relative to WT CCR5.

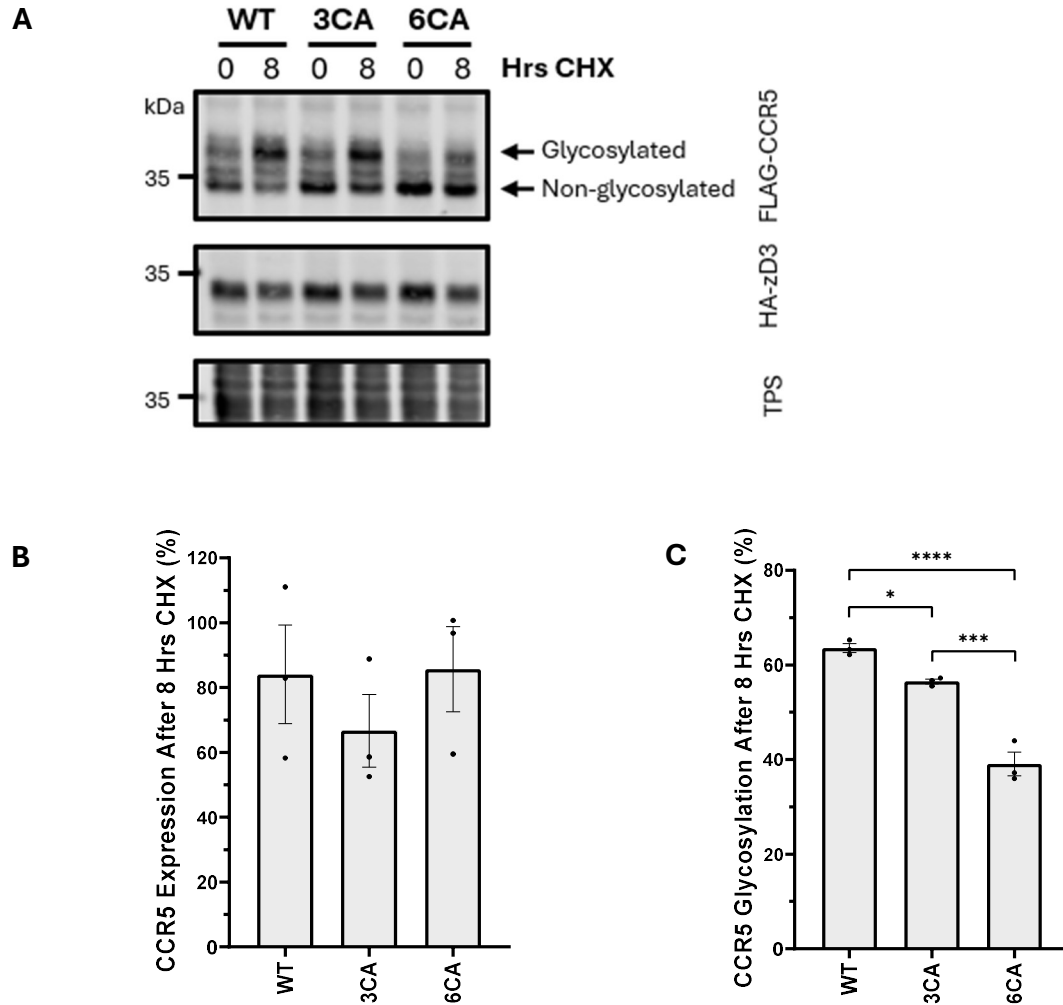


Figure 3.17. Disruption of CCR5 S-acylation sites does not affect receptor stability but alters glycosylation upon co-expression with zDHHC3. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged CCR5, including wild-type CCR5 (WT), and proteins with cysteine to alanine substitutions C321/323/324A (3CA), or C58/213/224/321/323/324A (6CA). zDHHC3 was co-transfected. At 24 hours post-transfection, cells were treated with 50 μ g/ml cycloheximide (CHX). Samples were collected at 0 and 8 hours and analysed by SDS-PAGE and immunoblotting. **(A)** Representative immunoblot showing CCR5 expression (top; FLAG-CCR5), expression of zDHHC3 enzyme (middle; HA-zD3), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of CCR5 expression following 8 hours of CHX treatment, expressed as the mean percentage of CCR5 remaining at 8 hours relative to expression at 0 hours. **(C)** Quantification of CCR5 glycosylation at 8 hours post-CHX treatment. Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (* $p<0.05$, *** $p<0.001$, **** $p<0.0001$).

3.4 Discussion

3.4.1 Summary

Collectively, the findings presented in this chapter are broadly consistent with the central hypothesis that specific zDHHC enzymes and intracellular localisation contribute to CCR5 S-acylation, while extending this hypothesis by suggesting that multiple cysteine residues and crosstalk with glycosylation also shape receptor maturation. CCR5 maturation, trafficking, and stability appear to be shaped by the coordinated and context-dependent interplay between S-acylation and O-glycosylation, and likely other modifications not investigated in this study. S-Acylation appears to be initiated during post-ER trafficking, potentially at the Golgi, and is mediated predominantly by Golgi-associated zDHHC enzymes. However, CCR5 S-acylation is not restricted to the canonical C-terminal cysteine residues and instead involves additional sites that may be differentially targeted by zDHHC enzymes. In contrast, N-terminal O-glycosylation appears to be a more robust determinant of receptor stability. The observation that alterations in S-acylation can influence glycosylation patterns under conditions of elevated zDHHC enzymatic activity highlights an unanticipated level of crosstalk between these modifications. Together, this data supports a model in which CCR5 regulation is distributed across multiple modification sites and pathways, providing adaptability to receptor trafficking and function, while also highlighting the importance of cellular environment and experimental approach in interpreting post-translational regulation.

3.4.2 Insights into the potential subcellular site of CCR5 S-acylation

Post-translational S-acylation is a critical regulatory mechanism for many GPCRs, influencing their trafficking, stability, and signalling capabilities. For CCR5, S-acylation has been implicated in efficient plasma membrane localisation and function. Identification of the enzymes responsible for CCR5 S-acylation and the intracellular site at which this modification occurs is therefore essential for understanding how CCR5 maturation and trafficking is regulated. The results of the zDHHC enzyme screen conducted in this study (**Figure 3.1**) are consistent with those reported by Boncompain *et al.* (2019), supporting the conclusion that zDHHC3, zDHHC7, and zDHHC15 are the primary enzymes responsible for CCR5 S-acylation (Boncompain *et al.*, 2019). Although our experimental screen was limited to a subset of zDHHC enzymes (zDHHC-2, -3, -6, -7, -15, and -17), Boncompain *et al.* performed a more comprehensive analysis of the zDHHC family. The convergence of findings from these independent studies strengthens confidence in the selectivity of these enzymes for CCR5.

Importantly, zDHHC3, zDHHC7, and zDHHC15 are all predominantly localised to the Golgi apparatus in HEK293T cells, supporting a model in which CCR5 undergoes S-acylation during transit through the Golgi as part of its maturation (Ohno *et al.*, 2006,

Greaves et al., 2008, Greaves et al., 2010). The Golgi localisation of CCR5 S-acylation is particularly significant given that CCR5 must efficiently traverse the secretory pathway to reach the plasma membrane, where it performs its physiological functions. S-Acylation at this stage may contribute to proper folding, membrane partitioning, or protection from premature degradation, therefore influencing both receptor stability and downstream signalling competence. The agreement between our data and previous studies, obtained using distinct experimental approaches, provides strong evidence that Golgi resident zDHHC enzymes play a central role in regulating CCR5 S-acylation and potentially its subsequent trafficking and surface expression.

Further support for the direct catalytic involvement of zDHHC3 and zDHHC7 in the S-acylation of CCR5 was provided by mutational analysis of the DHHC domain. Mutation of the catalytic cysteine residue to serine (zDHHC3CS and zDHHC7CS) abolished the enhancement of CCR5 S-acylation and resulted in loss of the characteristic S-acylation band (**Figure 3.5**). This strongly suggests that CCR5 modification occurs via the canonical two-step “ping-pong” mechanism, in which auto-acylation of the DHHC cysteine is a prerequisite for substrate S-acylation (Jennings and Linder, 2012). These findings reduce the likelihood that the observed effects arise from indirect mechanisms such as altered CCR5 folding, trafficking, or stability caused by zDHHC overexpression. They also oppose a role for the non-catalytic functions of zDHHC enzymes, such as scaffolding effects, and minimise the possibility that other endogenous enzymes are responsible for the increased S-acylation observed.

While earlier experiments relied primarily on overexpression of zDHHC enzymes, the use of the RUSH system enabled control over CCR5 trafficking and provided additional insight into the localisation of CCR5 S-acylation without the need for zDHHC overexpression. Using this system, increased CCR5 S-acylation was observed following release from ER retention, consistent with the modification occurring post-ER (**Figure 3.8**). When considered alongside the identification of CCR5 S-acylation by Golgi zDHHC enzymes, this provides support for the hypothesis that CCR5 S-acylation occurs at the Golgi. This approach is particularly valuable because overexpression of zDHHC enzymes, including zDHHC3, has been reported to cause enzyme mis-localisation, potentially confounding interpretation (Malgapo and Linder, 2021). By avoiding zDHHC overexpression, the RUSH experiments more closely reflect endogenous cellular conditions and therefore strengthen the physiological relevance of the findings.

A limitation of the RUSH system as applied here is that it does not allow precise identification of the specific organelle in which CCR5 S-acylation occurs. Future studies could address this by using alternative retention signals that selectively trap CCR5 in defined compartments, allowing direct comparison of S-acylation levels across distinct stages of the secretory pathway. Another potential issue with the use of RUSH is that the interaction of the streptavidin-binding protein with streptavidin could inhibit S-acylation by altering the conformation of CCR5. Therefore, it would also be useful to repeat the RUSH experiments with CCR5 containing the tags at the opposite end, and also using different hook proteins. Additionally, the use of knockdown or knockout models targeting

zDHHC3, zDHHC7, and zDHHC15, either individually or in combination, would provide stronger evidence that these enzymes are required for CCR5 S-acylation under endogenous conditions. Such approaches would also help to address potential functional redundancy within the zDHHC family.

Beyond enzymatic identification, future work should explore the functional consequences of Golgi-mediated CCR5 S-acylation, including its impact on plasma membrane localisation, ligand-induced signalling, and degradation. Given the clinical importance of CCR5, particularly in HIV-1 infection, a more detailed understanding of the zDHHC enzymes involved and how they regulate receptor S-acylation may reveal new opportunities for therapeutic intervention targeting receptor maturation or trafficking.

3.4.3 Exploring the interplay between CCR5 O-glycosylation and S-acylation

Analysis of CCR5 migration patterns revealed N-terminal O-glycosylation at S6 and S7, consistent with previously reported modification sites (Bannert et al., 2001). The observed shifts in molecular weight, together with the absence of additional changes between O-Gly2 and O-Gly4 mutants, support the conclusion that S6 and S7 represent the predominant O-glycosylation sites of CCR5 in HEK293T cells (**Figure 3.3**).

Beyond confirming known glycosylation sites, this study examined a previously unexplored aspect of CCR5 regulation in the potential interplay between O-glycosylation and S-acylation. While both post-translational modifications have been individually characterised in the context of CCR5 maturation and function, their combined influence has not been directly investigated. The data presented here indicate that N-terminal O-glycosylation is not required for efficient CCR5 S-acylation, as substitution of S6 and S7 did not result in a reduction in overall S-acylation levels (**Figure 3.3**). This suggests that these two modifications occur largely independently within the secretory pathway, rather than one being strictly dependent on the other.

Current evidence suggests that CCR5 S-acylation occurs post-ER, most likely in the Golgi. Similarly, CCR5 O-glycosylation has been identified as a GalNAc-type O-glycosylation, which is initiated in the Golgi after the majority of protein folding has taken place (Bannert et al., 2001). This differentiates GalNAc O-glycosylation, as the majority of other forms of glycosylation are predominantly initiated in the ER (Moremen et al., 2012). Previous knockout studies have implicated GalNAc transferase 1 as the primary enzyme responsible for CCR5 O-glycosylation, with possible limited involvement of GalNAc transferase 11, at least in CHO cells (Verhallen et al., 2023). However, the diversity of GalNAc transferase isoforms and the reliance on recombinant expression systems limit the extent to which these findings can be generalised across cell types and tissues, making it difficult to determine the exact location in the Golgi where this modification is initiated. This variability highlights the importance of considering cell-specific glycosylation landscapes when interpreting CCR5 post-translational modification patterns.

Although O-glycosylation did not alter total CCR5 S-acylation, a change in the distribution of S-acylated CCR5 species was observed. In control conditions, S-acylated CCR5 was detected in both glycosylated and non-glycosylated forms, indicating that S-acylation can occur independently of glycosylation. This suggests that O-glycosylation contributes to the distribution of S-acylated CCR5 species and raises the possibility of context-dependent crosstalk during receptor maturation. One explanation is that S-acylation occurs at different stages of trafficking, with only a subset of modified receptors progressing to full glycan maturation, or that glycosylation state influences subsequent modification and processing. S-acylation may therefore stabilise distinct CCR5 conformations with different accessibility to glycosylation machinery. Overall, while O-glycosylation and S-acylation are not strictly interdependent, they may indirectly influence one another by shaping CCR5 conformation, trafficking kinetics, and time spent within specific Golgi compartments.

The observed reduction in steady-state stability of the glycosylation mutants compared with WT CCR5 further supports a functional role for O-glycosylation in receptor maintenance (**Figure 3.4**). Although CCR5 glycosylation has been shown to influence receptor maturation and functional outcomes such as ligand binding (Bannert et al., 2001), to our knowledge direct quantification of glycosylation-dependent changes on receptor stability, cell surface expression, or localisation has not previously been demonstrated. O-Glycosylation may therefore contribute to CCR5 stability indirectly by promoting efficient maturation through the secretory pathway and reducing the likelihood of quality-control-mediated degradation of incompletely processed receptor populations. Notably, clear examples of O-glycosylation directly regulating GPCR stability remain limited; however, analogous roles for glycosylation have been demonstrated for N-linked modifications. For example, in the μ -opioid receptor, alterations in N-glycosylation, such as those arising from the A118G polymorphism, lead to reduced receptor protein stability and expression levels, consistent with increased degradation (Huang et al., 2012). These findings have potential implications for CCR5 function, as altered stability or glycan composition may influence the balance of receptor populations available for signalling, including effects on ligand binding capacity and susceptibility to internalisation.

Several limitations should be acknowledged about the analysis of the glycosylation mutants of CCR5. The cycloheximide pulse-chase assay used here does not distinguish between proteasomal and lysosomal degradation pathways, nor does it capture dynamic trafficking events such as recycling. Future work could employ pulse-chase analysis in combination with specific inhibitors of proteasomal or lysosomal degradation to help clarify the mechanisms underlying the reduced stability of O-glycosylation mutants. Additionally, direct measurement of CCR5 trafficking kinetics, for example, using live cell imaging, would provide further insight into how O-glycosylation influences receptor maturation and surface expression. Here, the use of the RUSH system could allow the trafficking of glycosylation-deficient CCR5 to be compared with WT CCR5 following their release from ER trapping. Finally, extending these analyses to physiologically relevant cell types, such as primary T cells, would be important to determine whether the observed

interplay between O-glycosylation, S-acylation, and stability is conserved under endogenous expression conditions.

3.4.4 Evidence supporting additional sites of CCR5 S-acylation

For CCR5, three C-terminal cysteine residues have previously been identified as the sites of S-acylation (C321, C323, C324) with functional consequences for receptor trafficking and stability (Blanpain et al., 2001, Percherancier et al., 2001). Unexpectedly, substitution of all three known C-terminal S-acylation sites to alanine did not result in a detectable reduction in overall CCR5 S-acylation in this study (**Figure 3.10**). This observation contrasts with previous studies and suggests that CCR5 may undergo S-acylation at additional cysteine residues beyond those classically described.

One possible explanation for this discrepancy is the limited sensitivity of the previously used radiolabel-based S-acylation assays, which may not detect partial changes in modification as reliably as click-chemistry based assays (Yap et al., 2010). Although it might be tempting to attribute the remaining S-acylation of the CCR5 protein with alanine substitution of the C-terminal cysteines to overexpression of zDHHC enzymes, this protein was still S-acylated in the absence of zDHHC enzyme co-expression. This reveals that endogenous enzymes are sufficient to maintain baseline CCR5 S-acylation when the C-terminal cysteines are replaced.

These observations raise the possibility that S-acylation at previously unidentified additional cysteine residues could represent a compensatory response when the primary C-terminal cysteine residues are unavailable. However, it is more likely that these residues are bona fide additional S-acylation sites within CCR5 that have not previously been identified, and that they are modified under normal conditions alongside the three well-characterised C-terminal cysteines. This would imply that CCR5 S-acylation is more expansive than previously appreciated and that zDHHC3 and zDHHC15 are not strictly limited to modifying the C-terminal tail. An alternative, though not mutually exclusive, explanation is that mutation of the C-terminal cysteines alters CCR5 conformation in a way that exposes additional cysteine residues to zDHHC enzymes, thereby facilitating S-acylation at non-canonical sites. Such structural rearrangements could complicate the interpretation of mutagenesis studies, particularly under conditions of overexpression.

The near-complete loss of S-acylation observed in the 6CA mutant provides strong evidence that additional cysteine residues beyond the C-terminus contribute to CCR5 S-acylation (**Figure 3.12**). These additional cysteines (C58, C213, C224) are located in regions of the receptor that are positioned near membrane interfaces or within intracellular loops, making them accessible to zDHHC enzymes. The residual signal detected in the 6CA mutant is likely attributable to background labelling, as a comparable signal was observed in control samples labelled with palmitic acid alone. Structural analysis of CCR5 further supports this interpretation, as no other cysteine residues are positioned in a manner compatible with S-acylation, being either

extracellular or located within transmembrane domains. This strengthens the case that the identified sites account for the majority of CCR5 S-acylation.

Consistent with previous studies, targeted experiments confirmed that the CCR5 C-terminal cysteines are indeed substrates of zDHHC3, reinforcing their role as S-acylation sites (**Figure 3.13**) (Boncompain et al., 2019). However, when only the C-terminal cysteines were present, overall S-acylation appeared lower than WT CCR5. This suggests that cysteine residues outside of the C-terminal tail could make a substantial contribution to total CCR5 S-acylation. Interestingly, zDHHC15 did not enhance S-acylation of the CCR5 protein with cysteines only present in the C-terminus, despite having activity towards WT CCR5 and the 3CA mutant in earlier experiments. This finding might indicate site-specific preferences for different zDHHC enzymes or structural contexts within CCR5. Alternatively, zDHHC15 activity may depend on a CCR5 conformation that is disrupted when only the C-terminal cysteines are present.

It should also be noted that zDHHC15 activity showed considerable variation between experiments and appeared sensitive to factors such as cell passage number. This inconsistency may have contributed to the observed lack of S-acylation in certain mutant contexts and represents an important technical limitation of the study. Future work using more controlled expression systems or endogenous tagging approaches may help clarify zDHHC15-specific effects.

Reintroduction of cysteine residues into the 6CA backbone provided further insight into the contribution of CCR5 S-acylation sites. Pairwise rescue experiments revealed that mutants containing C58 restored S-acylation (**Figure 3.14/Figure 3.15**). However, full restoration to WT levels required the presence of additional cysteines, suggesting that C58 functions as the primary non-C-terminal S-acylation site, with C213 and C224 potentially playing supportive or cooperative roles. These residues may enhance S-acylation efficiency through additive effects or by stabilising a receptor conformation that favours modification of C58. In GPCRs such as the β 2AR conformational effects of S-acylation which alter other post-translational modifications have been proposed. In this receptor, S-acylation of the conserved C-terminal cysteine (C341) has been shown to constrain the conformation and accessibility of the cytoplasmic tail, thereby regulating downstream phosphorylation and effector interactions (Moffett et al., 1993, Moffett et al., 1996).

The identification of multiple S-acylation sites raises important questions regarding their functional significance. Distinct cysteine residues may mediate different aspects of CCR5 biology such as trafficking, membrane microdomain partitioning, signalling, or receptor turnover. Previous studies have shown that substitution of the C-terminal cysteines affects CCR5 trafficking, but it is conceivable that S-acylation at intracellular loop cysteines serves separate or complementary roles, potentially in fine-tuning receptor behaviour in response to the cellular environment (Blanpain et al., 2001, Percherancier et al., 2001, Boncompain et al., 2019). Notably, one of the identified sites, C224, resides within intracellular loop 3 (ICL3), a region that is involved in the G_i binding interface in cryo-EM structures of ligand-bound CCR5 (Isaikina et al., 2021). In other receptor

systems, ICL3 has been implicated in regulating G protein coupling efficiency and subtype specificity (Sadler et al., 2023).

Several limitations in this part of the study should be acknowledged. The reliance on overexpression systems may influence receptor conformation and enzyme accessibility, underscoring the need for validation in endogenous models. Further studies employing mass-spectrometry-based proteomics would be invaluable for directly confirming modification of individual cysteine residues and assessing how S-acylation patterns change across mutant combinations or cellular conditions. Previous work has employed native mass spectrometry to provide site-specific information on S-acylation stoichiometry of membrane proteins, and this approach could also be employed for CCR5 (Rodenburg et al., 2017). Finally, functional assays examining trafficking, signalling, and ligand responsiveness of site-specific mutants would be critical to determine how individual S-acylation events contribute to CCR5 function and regulation.

3.4.5 CCR5 stability was not impacted by S-acylation but there was interplay with glycosylation

Although S-acylation has previously been implicated in regulating CCR5 stability, replacement of all identified candidate S-acylation sites did not result in a measurable change in overall receptor stability over an 8-hour cycloheximide treatment period (**Figure 3.16**). This was unexpected, particularly given prior reports demonstrating reduced CCR5 half-life following mutation of the C-terminal cysteine residues (Percherancier et al., 2001). In the present study, even the complete replacement of six cysteine residues with alanine failed to produce a clear destabilising effect, suggesting that S-acylation may not be a dominant determinant of CCR5 stability under the experimental conditions used here.

One possible explanation for this discrepancy lies in differences in experimental sensitivity and design. Previous studies reporting reduced CCR5 half-life employed metabolic pulse-chase labelling with [³⁵S] methionine in HeLa cells, a method that allows direct tracking of newly synthesised protein pools. In contrast, the cycloheximide based assay used here inhibits global protein synthesis and measures bulk degradation of the existing receptor population. Notably, WT CCR5 in this study remained highly stable over the eight-hour period, in contrast to earlier reports showing a half-life of approximately 4.5 hours. These differences may suggest that S-acylation is particularly important for the stability of CCR5 in the early stages of its life cycle (e.g. during trafficking through the secretory pathway) but may become less important when the protein is localised at the plasma membrane. Again, the RUSH system could allow compartment-specific stability of CCR5 to be explored by trapping of the proteins at different cell organelles.

It is also possible that CCR5 stability is governed by overlapping or compensatory mechanisms that obscure the contribution of individual post-translational modifications. In earlier experiments, O-glycosylation was shown to play a more prominent role in maintaining CCR5 stability. In the present experiments, no substantial

differences in glycosylation were observed between the cysteine mutants under basal conditions, suggesting that receptor maturation through the secretory pathway remained largely intact. This may explain why stability was preserved despite disruption of S-acylation, and supports a model in which glycosylation exerts a stronger influence on CCR5 stability than S-acylation under steady state conditions.

Several methodological limitations further complicate interpretation. Cycloheximide treatment induces cellular stress and broadly alters protein homeostasis, potentially affecting degradation pathways in a non-physiological manner (Dai et al., 2013). In addition, this assay reports total cellular protein levels and cannot distinguish between receptors at the plasma membrane, within intracellular compartments, or trafficking through the secretory pathway. If S-acylation primarily regulates CCR5 surface retention, recycling, or localised degradation at the plasma membrane these effects would not be readily detected by this approach.

The absence of a stability defect was also observed when cysteine mutants were co-expressed with zDHHC3, indicating that enhanced S-acylation does not substantially alter CCR5 turnover in the timescale examined (**Figure 3.17**). However, in contrast to basal conditions, a clear reduction in receptor glycosylation was observed under zDHHC3 co-expression. One explanation for this difference is that endogenous S-acylation activity may be insufficient to reveal functional consequences of cysteine substitution, whereas overexpression of zDHHC3 increases the extent or rate of S-acylation, thereby amplifying the dependence of CCR5 maturation on these sites. Under these conditions, loss of S-acylation sites may become rate-limiting for efficient trafficking through the secretory pathway, resulting in reduced acquisition of mature glycosylation. This effect appeared to scale with the degree of S-acylation disruption, as the reduction in glycosylation was more pronounced in the 6CA mutant compared to the 3CA mutant. This suggests that multiple cysteine residues contribute in an additive or cooperative manner, potentially by stabilising CCR5 conformation or promoting efficient progression through intracellular compartments. Increased demand on proper folding or trafficking under conditions of elevated S-acylation may therefore expose defects that are not apparent at basal enzyme levels. This appears consistent with previous experiments in which enhanced S-acylation appeared to preferentially shift CCR5 expression towards a non-glycosylated form.

Interestingly, despite the reduction in glycosylation observed under conditions of elevated S-acylation, this did not translate into a detectable decrease in receptor stability. This appears contradictory to earlier observations showing that glycosylation deficient CCR5 mutants are less stable. However, even in the most affected S-acylation mutant, glycosylation levels remained much higher than the near complete loss of glycosylation that occurred with the glycosylation mutants. This remaining glycosylation may be sufficient to preserve receptor stability over the assay window. This suggests the existence of a threshold effect, whereby partial glycosylation is adequate to maintain stability, while more severe disruption is required to trigger accelerated degradation.

Together these findings suggest a model in which S-acylation does not act as a primary regulator of CCR5 bulk stability, but instead modulates receptor maturation and processing in a context dependent manner. The functional consequences of S-acylation may therefore be more apparent at the level of trafficking kinetics, membrane compartmentalisation, or signalling efficiency rather than overall protein abundance.

Future studies should aim to address these limitations by employing more sensitive and spatially resolved approaches. Pulse-chase analysis combined with inhibitors of lysosomal and proteasomal degradation could help distinguish between degradation pathways. Live cell imaging approaches would allow direct assessment of plasma membrane stability and recycling. Finally examining these effects in endogenous expression systems would be necessary to determine if the effects are reflective of native CCR5 regulation.

3.4.6 Key Takeaways

- CCR5 S-Acylation is mediated by Golgi-associated zDHHC enzymes (zDHHC3, zDHHC7, and zDHHC15), with evidence for modification during post-ER trafficking
- S-Acylation of CCR5 is not limited to the three previously characterised C-terminal cysteine residues (C321, C323, C324), with C58 identified as a key additional S-acylation site.
- N-Terminal O-glycosylation at Ser6 and Ser7 contributes to CCR5 stability but does not appear to influence S-acylation.
- Enhanced S-acylation can reveal disruptions in CCR5 glycosylation, suggesting potential context-dependent crosstalk between these modifications.
- CCR5 exhibited sustained stability over 8 hours, with receptor stability appearing largely resistant to perturbations in S-acylation.

CHAPTER 4

Mechanisms of zDHHC Enzyme-Substrate Specificity in Transmembrane Proteins

Chapter 4 - Mechanism of zDHHC Enzyme-Substrate Specificity in Transmembrane Proteins

4.1 Introduction

Although S-acylation is a widespread modification affecting a diverse range of proteins, the principles governing zDHHC substrate specificity and the organisation of broader substrate networks remain incompletely defined (Salaun et al., 2023). Individual zDHHC enzymes are typically capable of S-acylating multiple substrates, and conversely, many substrates can be modified by more than one zDHHC enzyme (Malgapo and Linder, 2021). A deeper understanding of how these complex enzyme-substrate networks are regulated, particularly within protein families such as GPCRs, which are frequently implicated in disease and heavily targeted pharmacologically, could enable the development of novel therapeutic strategies or intervention points.

One important determinant of substrate specificity is the subcellular localisation of zDHHC enzymes. Due to cellular compartmentalisation, the membrane in which a particular zDHHC enzyme resides restricts the pool of accessible substrates. However, zDHHC localisation is not static and can vary between cell types or in response to cellular activity. Certain zDHHC enzymes, such as zDHHC3, are highly promiscuous and are capable of S-acylating a broad range of substrates (Malgapo and Linder, 2021, Mesquita et al., 2024). In the absence of clear conserved interaction motifs, a proposed explanation for the promiscuity of zDHHC3 and zDHHC7 is that these enzymes exist in a constitutively high-activity state. In this model, S-acylation is driven primarily by the accessibility and positioning of substrate cysteine residues, rather than by specific protein-protein interactions (Salaun et al., 2023). In contrast, other family members, including zDHHC17, display a much higher degree of substrate selectivity (Lemonidis et al., 2017b). In this case, specificity is mediated by the interaction of substrates containing a zDHHC ankyrin-binding motif (zDABM) with the ankyrin-repeat domain of zDHHC17.

Interestingly, zDHHC6 has been shown to exhibit selectivity towards type I and II single-pass transmembrane proteins (Salaun et al., 2023). The common feature shared by these zDHHC6 substrates, regardless of topology, is the presence of a single transmembrane domain and one or more cytosolic cysteine residues located in close proximity to the membrane. This observation suggests a potential model for zDHHC6 substrate selectivity. Only a small number of reported zDHHC6 substrates deviate from this model, including cytosolic proteins such as NRas (Qiu et al., 2022).

Given these findings, it is of particular interest to determine whether the apparent rules governing zDHHC6 substrate selectivity are limited to single-pass transmembrane proteins or whether they extend to more complex multi-pass transmembrane proteins. Large and functionally critical protein families such as GPCRs represent an especially compelling class of potential substrates. GPCRs comprise a large and diverse group of

multi-pass transmembrane receptors involved in a wide array of physiological processes and disease states, and are the target of ~ 30 % of FDA approved drugs (Hauser et al., 2017). A common feature of GPCRs is the presence of one or more cysteine residues in the C-terminal tail, typically located 10 to 14 residues downstream of the seventh transmembrane domain. Indeed, ~ 80 % of GPCRs possess a cysteine in this position (Escriba et al., 2007). S-Acylation of these cysteine residues promotes association of the C-terminus with the plasma membrane, effectively forming an additional intracellular loop that influences receptor conformation and protein-protein interaction (Moench et al., 1994).

If zDHHC6 requires cysteine residues to be positioned in close proximity to the final transmembrane domain, this may preclude the C-terminal cysteine residues found in many GPCRs from serving as zDHHC6 substrates. Moreover, as zDHHC6 is predominantly localised to the ER, this localisation may play a key role in shaping its substrate repertoire during early stages of protein trafficking. In contrast, many well-characterised S-acylated GPCRs are modified by zDHHC enzymes localised to the Golgi (Adachi et al., 2016, Boncompain et al., 2019). Investigating the potential S-acylation of GPCRs and other multi-pass transmembrane proteins therefore provides an opportunity to test and refine current models of zDHHC6 substrate selectivity and to expand understanding of how S-acylation regulates major transmembrane signalling proteins.

Furthermore, evidence suggests that transmembrane architecture, specifically the positioning of cysteine residues relative to transmembrane domains, may also influence the lipid selectivity of S-acylation. Although current data is limited, for some single-pass viral transmembrane proteins, it has been shown that cysteines located within or immediately adjacent to the transmembrane domain often preferentially incorporate longer, saturated lipids such as stearate, whereas cysteines positioned further into cytoplasmic regions are more commonly modified by palmitate (Veit et al., 1991, Kordyukova et al., 2008, Kordyukova et al., 2010, Brett et al., 2014). This spatial dependence raises the possibility that multi-pass transmembrane proteins, such as GPCRs, may exhibit distinct patterns of acyl chain selection at different cysteine sites, shaped by local membrane environment and topology. Exploring whether similar positional rules govern lipid selectivity in multi-pass proteins would not only extend current models of zDHHC substrate recognition, but also provide deeper insight into how S-acylation contributes to the regulation of complex transmembrane signalling proteins.

4.2 Aims

- To investigate whether zDHHC6 is capable of S-acylating transmembrane proteins containing multiple transmembrane domains, or whether its apparent substrate preference is restricted to single-pass transmembrane proteins.
- To assess the importance of cysteine residue positioning relative to the transmembrane domain in determining substrate S-acylation by zDHHC6.
- To evaluate whether the ER localisation of zDHHC6 contributes to its substrate selectivity and S-acylation activity.
- To examine whether transmembrane topology and cysteine positioning relative to transmembrane domains influence lipid selectivity during S-acylation, including in multi-pass transmembrane proteins.

Hypothesis

zDHHC6 may preferentially S-acylate substrates according to their transmembrane topology, cysteine positioning, and ER localisation, with these factors also potentially influencing lipid selectivity.

4.3 Results

4.3.1 S-Acylation of the single-transmembrane protein CD4 is increased by zDHHC3 and zDHHC6

The S-acylation of cluster of differentiation 4 (CD4), a single-transmembrane glycoprotein expressed on T cells and macrophages, has been well characterised in previous studies. The S-acylation occurs at two cysteine residues, C419 and C422, located in the C-terminus of CD4, immediately following the transmembrane domain (Figure 4.1) (Crise and Rose, 1992, Fragoso et al., 2003). Additionally, a short construct of CD4, including only the transmembrane domain and a short juxta-membrane section, has been shown to be S-acylated by the enzyme zDHHC6 (Salaun et al., 2023). However, this has not yet been confirmed for the full-length CD4 protein.

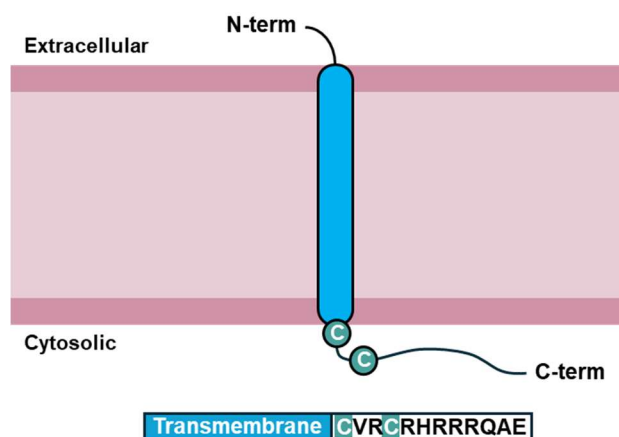


Figure 4.1. Schematic representation of CD4 highlighting C-terminal S-acylation sites. CD4 is shown embedded in the plasma membrane, with the transmembrane domain depicted in blue. The sites of C-terminal S-acylation (cysteine 419 and 422) are indicated in green. A schematic is included displaying a short sequence of the C-terminus indicating the positioning of the S-acylated cysteines.

Given the established role of zDHHC6 in S-acylating single-transmembrane proteins, I sought to confirm this finding with full-length CD4. In particular, I aimed to verify whether CD4, when expressed in HEK293T cells, could be S-acylated by zDHHC6, as a truncated version of the protein has previously been shown to be a substrate of the enzyme (Salaun et al., 2023). To explore this, FLAG-tagged full-length CD4 was co-expressed with HA-tagged zDHHC6. In addition, zDHHC3 and zDHHC15 were also included in the analysis based on their activity against other transmembrane proteins, including CCR5 (Chapter 3. Figure 3.1). To investigate CD4 S-acylation, palmitic acid azide labelling was used with detection of S-acylation by click chemistry.

S-Acylation of CD4 was clearly detected under all experimental conditions tested, including the control condition, without any co-expressed zDHHC enzymes (**Figure 4.2A**). This suggests that endogenous zDHHC enzymes were capable of S-acylating this protein. Two distinct bands corresponding to S-acylation were observed, which is consistent with the presence of two C-terminal cysteine residues, C419 and C422, which have previously been identified as S-acylation sites (Crise and Rose, 1992, Fragoso et al., 2003). The greatest increase in S-acylation was observed when CD4 was co-expressed with zDHHC6, with a much more prominent second band of S-acylation under these conditions.

When quantifying S-acylation as a percentage of total CD4 expression, a clear increase in S-acylation was noted in the presence of both zDHHC3 and zDHHC6 compared to control (**Figure 4.2B**). S-Acylation of CD4 increased significantly from ~ 55 % with control to around 70 % with zDHHC3 and ~ 85 % with zDHHC6. In contrast, no significant increase in CD4 S-acylation was observed when CD4 was co-expressed with zDHHC15.

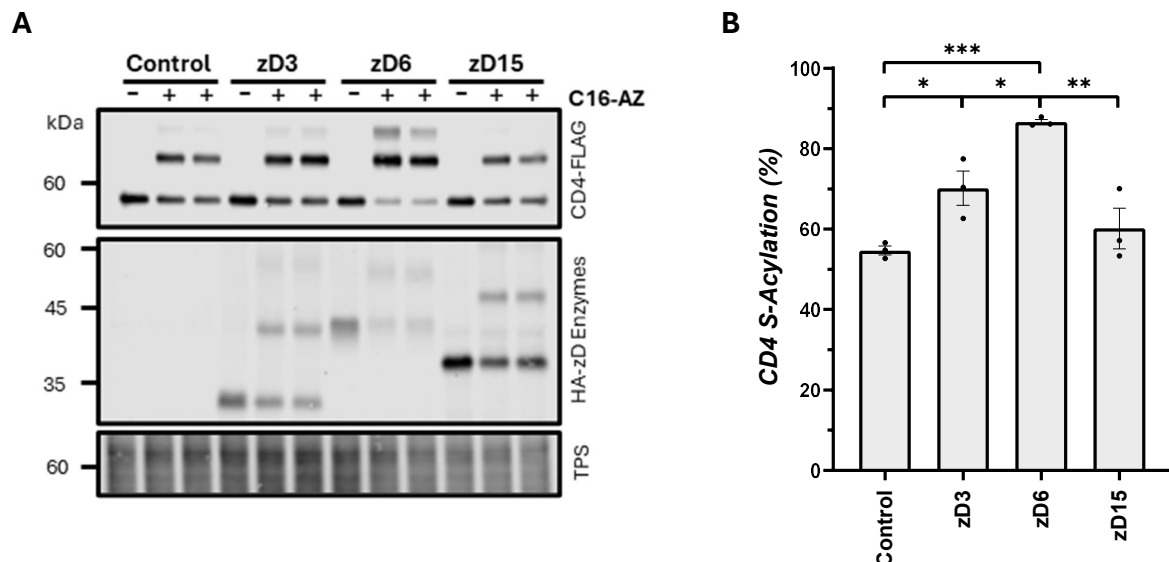


Figure 4.2. CD4 S-acylation is enhanced by co-expression with zDHHC3 and zDHHC6. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged CD4 together with HA-tagged zDHHC3, zDHHC6, zDHHC15 or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing CD4 S-acylation (top; CD4-FLAG), zDHHC enzyme expression (middle; HA-zD Enzymes), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of CD4 S-acylation expressed as the mean percentage of the S-acylated band relative to the total CD4 signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; n=3 independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (*p<0.05, **p<0.01, ***p<0.001).

4.3.2 S-Acylation of the two-transmembrane protein K5 is increased by both zDHHC3 and zDHHC6

As zDHHC6 has been shown to S-acylate single-transmembrane proteins, where a transmembrane domain and a juxta-membrane region appear to be sufficient for modification, it was of interest to explore whether zDHHC6 could also modify multi-transmembrane proteins. This raised the question of whether the increased size and complexity of multi-transmembrane proteins might affect the apparent substrate selectivity of zDHHC6. To address this, a protein with two transmembrane domains and cysteine residues positioned in a way that might be favourable for zDHHC6-mediated S-acylation was identified.

K5, an E3 ubiquitin ligase encoded by Kaposi's sarcoma-associated herpesvirus (KSHV), is a multi-pass transmembrane protein with two transmembrane domains and cytosolic N- and C-termini. Notably, K5 is S-acylated at cysteine 146, which is located immediately after the second transmembrane domain (**Figure 4.3**). This modification is involved in the interaction of K5 with certain substrates, such as major histocompatibility complex I, suggesting that C146 plays an important functional role (Anania and Coscoy, 2011). The location of C146 makes K5 a particularly attractive candidate for investigating whether zDHHC6 could modify multi-transmembrane proteins, as it possesses a cysteine residue in a position that might be accessible for S-acylation by zDHHC6.

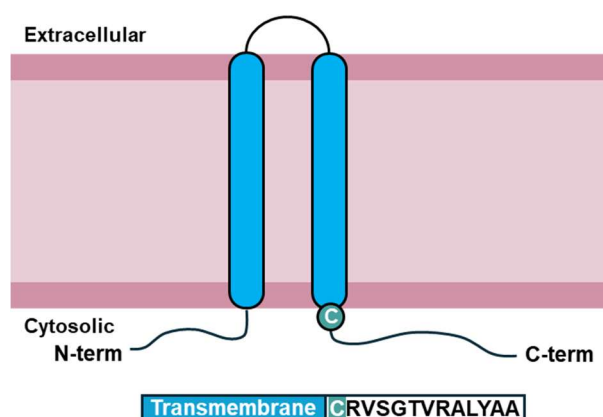


Figure 4.3. Schematic representation of K5 highlighting the C-terminal S-acylation site. K5 is shown embedded in the plasma membrane, with the transmembrane domains depicted in blue. The site of C-terminal S-acylation (cysteine 146) is indicated in green. A schematic is included displaying a short sequence of the C-terminus indicating the positioning of the S-acylated cysteine.

Although K5 contains additional cysteine residues in its N-terminus and another near its C-terminus, previous biotin-switch assays showed that substitution of the canonical cysteine at position 146 resulted in a loss of S-acylation, suggesting it is indeed the primary site of modification (Anania and Coscoy, 2011). However, the specific zDHHC enzymes responsible for S-acylating K5 remain unknown. To address this, Strep-tagged K5 was co-expressed in HEK293T cells with zDHHC3, zDHHC6, and zDHHC15. The S-

acylation of K5 was then assessed using palmitic acid azide labelling followed by click chemistry to determine whether any of these zDHHC enzymes could mediate the modification of K5.

When K5 was co-expressed with zDHHC3, zDHHC6, or zDHHC15, an increase in S-acylation was observed compared to the control condition, where K5 was expressed without any zDHHC enzyme. A single S-acylated band was detected in the zDHHC co-expression samples, which was absent in the control, indicating that the zDHHC enzymes were indeed modifying K5 (**Figure 4.4A**). Quantification of the S-acylation levels revealed a significant increase in K5 modification when expressed with both zDHHC3 and zDHHC6 relative to the control (**Figure 4.4B**). The largest increase in S-acylation was observed with zDHHC3, where the percentage of modified K5 increased from ~ 10 % in the control to ~ 35 %. Co-expression with zDHHC6 also led to an increase in S-acylation, reaching ~ 30 %. Although a slight increase in S-acylation was seen with zDHHC15, this change was not statistically significant, suggesting that zDHHC15 may have minimal effect on K5 modification.

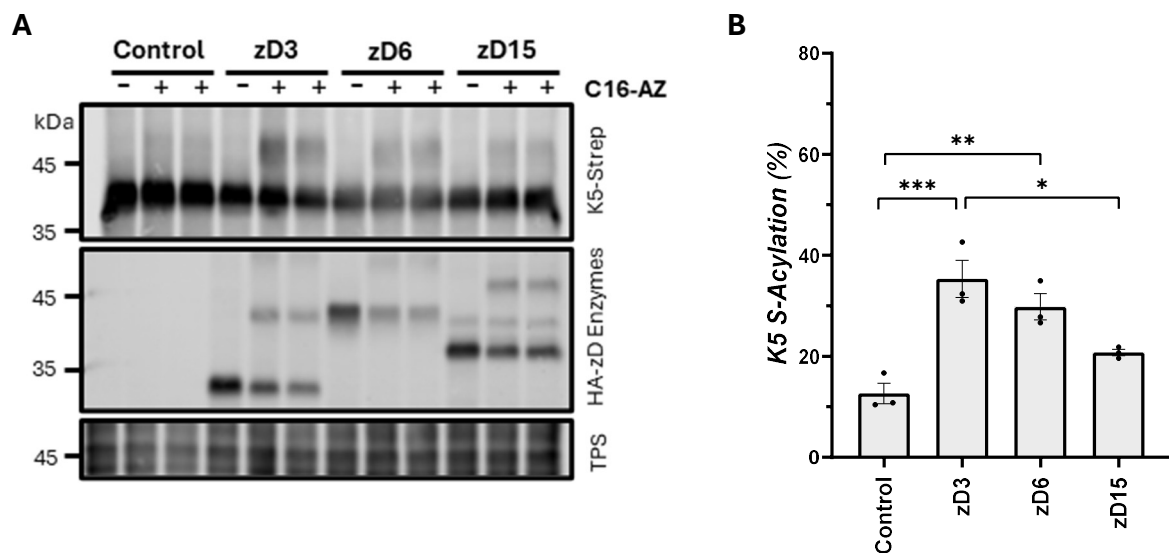


Figure 4.4. K5 S-acylation is enhanced by co-expression with zDHHC3 and zDHHC6. HEK293T cells were co-transfected with plasmids encoding Strep-tagged K5 together with HA-tagged zDHHC3, zDHHC6, zDHHC15 or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing K5 S-acylation (top; K5-Strep), zDHHC enzyme expression (middle; HA-zD Enzymes), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of K5 S-acylation expressed as the mean percentage of the S-acylated band relative to the total K5 signal (non-acylated plus acylated band). Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

4.3.3 Differential S-acylation of tetraspanins by zDHHC6

To further assess whether zDHHC6 is capable of modifying larger and more structurally complex multi-pass transmembrane proteins, the S-acylation of two tetraspanins, CD9 and tetraspanin 4 (TSPAN4), was examined. Tetraspanins are characterised by the presence of four transmembrane domains and represent a potentially more complex class of substrates than the two-transmembrane protein K5. This prompted an investigation into whether zDHHC6 could similarly modify proteins with more transmembrane domains and multiple potential S-acylation sites.

CD9 is a tetraspanin containing six S-acylated cysteine residues. These are located at positions C9 in the cytosolic N-terminus, C78, C79, and C87 in the intracellular loop, and C218 and C219 in the final transmembrane domain proximal to the C-terminus (**Figure 4.5**) (Charrin et al., 2002, Neviani et al., 2020). These cysteine residues are positioned 3, 1, 2, 0, 3, and 2 residues respectively from transmembrane-cytosol boundaries. Previous studies have demonstrated that CD9 can be S-acylated by zDHHC2, a Golgi-localised zDHHC enzyme, whereas no clear change in S-acylation was observed following co-expression with zDHHC5, -7, -9, or -11, or -15 (Sharma et al., 2008).

TSPAN4 is also a tetraspanin, however its S-acylation has been less extensively characterised. Like CD9, TSPAN4 contains multiple cysteine residues located in the N-terminus, intracellular loop, and C-terminus, which are potentially accessible to zDHHC enzymes (**Figure 4.5**). Although palmitoyl proteomics studies have suggested possible S-acylation of TSPAN4, this modification has not been experimentally validated, and no specific zDHHC enzymes have previously been identified as capable of S-acylating TSPAN4 (Thinon et al., 2018).

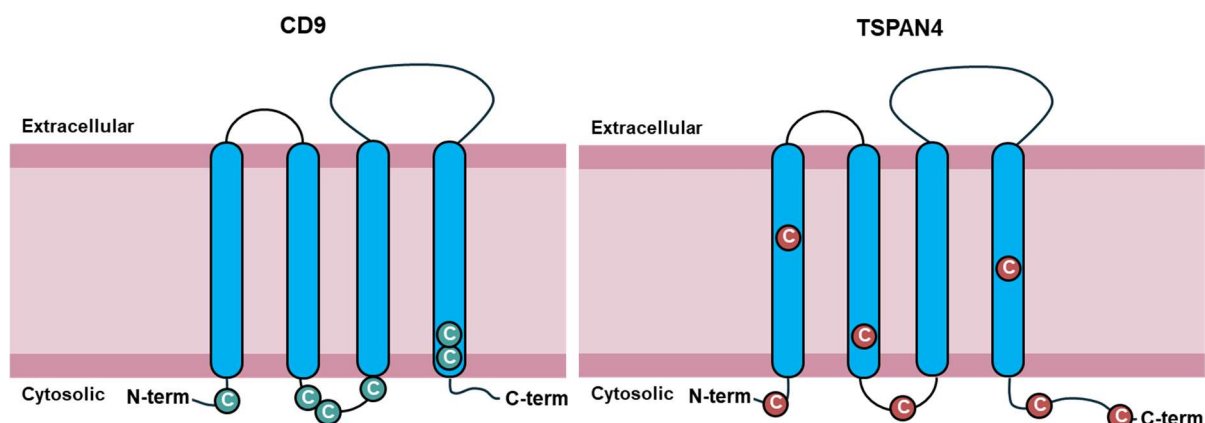


Figure 4.5. Schematic representation of CD9 and TSPAN4 highlighting cysteine residues. CD9 and TSPAN4 are shown embedded in the plasma membrane, with the transmembrane domains depicted in blue. The six known S-acylated cysteine residues are depicted in green for CD9 (C9, C78, C79, C87, C218, and C219). As sites of TSPAN4 S-acylation have not been established the cysteines located in the cytosolic side of transmembrane domains or cytosolic regions have been depicted in red (C5, C25, C72, C81, C211, C228, and C237).

To determine whether zDHHC6 can S-acylate tetraspanin proteins, GFP-tagged CD9 or TSPAN4 were co-expressed with HA-tagged zDHHC3, zDHHC6, or zDHHC15 in HEK293T cells. Protein S-acylation was assessed using palmitic acid azide labelling followed by click chemistry-based detection.

CD9 exhibited clear S-acylation under control conditions in the absence of exogenous zDHHC expression, with two distinct S-acylated bands observed, consistent with modification by endogenous enzymes (**Figure 4.6A**). Co-expression with zDHHC3 resulted in a statistically significant increase in CD9 S-acylation, rising from ~60 % under control conditions to ~80 % (**Figure 4.6B**). In contrast, no increase in CD9 S-acylation was detected following co-expression with either zDHHC6 or zDHHC15.

Similarly, TSPAN4 displayed detectable S-acylation under control conditions, with multiple S-acylated species observed (**Figure 4.6A**). Co-expression with zDHHC3 significantly increased TSPAN4 S-acylation from ~55 % to around 75 % (**Figure 4.6B**). In contrast to CD9, co-expression with zDHHC6 also resulted in a comparable and statistically significant increase in TSPAN4 S-acylation. No increase in TSPAN4 S-acylation was observed upon co-expression with zDHHC15.

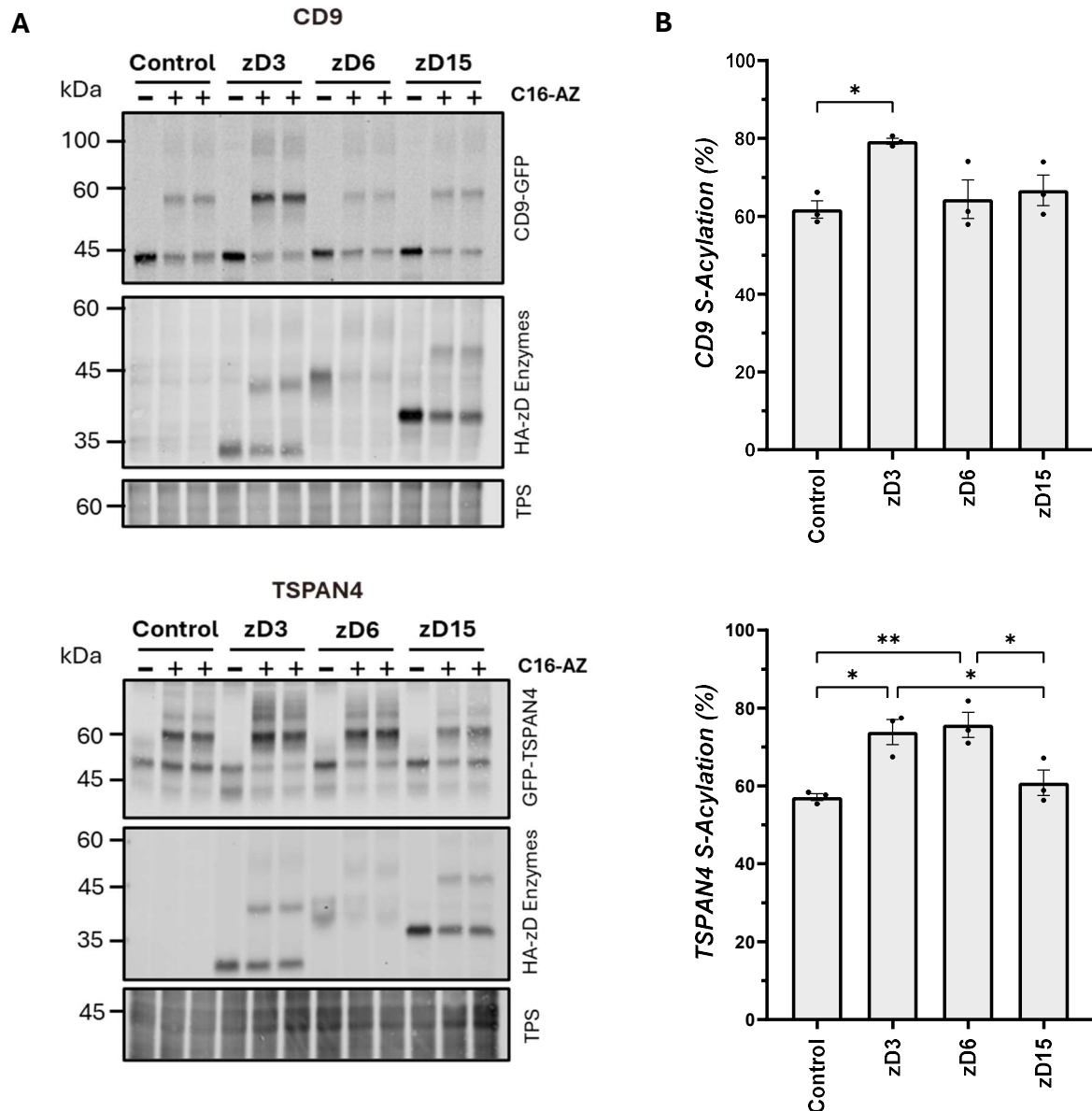


Figure 4.6. TSPAN4 S-acylation is enhanced by co-expression with zDHHC3 and zDHHC6, while CD9 S-acylation is only enhanced by zDHHC3. HEK293T cells were co-transfected with plasmids encoding GFP-tagged CD9 or TSPAN4 together with HA-tagged zDHHC3, zDHHC6, zDHHC15 or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing CD9 or TSPAN4 S-acylation (top; CD9-GFP/GFP-TSPAN4), zDHHC enzyme expression (middle; HA-zD Enzymes), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of CD9 or TSPAN4 S-acylation expressed as the mean percentage of the S-acylated band relative to the total signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (* $p<0.05$, ** $p<0.01$).

4.3.4 S-Acylation of β 2AR, but not β 1AR, was increased by zDHHC3 and zDHHC15

GPCRs represent one of the most important classes of multi-pass transmembrane proteins due to their large and complex structures and their central roles in physiology. Given their structural complexity, it was of particular interest to investigate the S-acylation of GPCRs and to determine whether specific zDHHC enzymes display activity towards this class of proteins. In GPCRs, cysteine residues within the C-terminal tail are typically positioned at a greater distance from the final transmembrane domain, allowing the C-terminus to associate with the membrane. If proximity of cysteine residues to the transmembrane domain is a key determinant of zDHHC6-mediated S-acylation, it would therefore be hypothesised that zDHHC6 would be unlikely to efficiently S-acylate these more distal C-terminal cysteines.

β -Adrenergic receptors are a well-characterised subfamily of GPCRs that play essential roles in sympathetic nervous system signalling and are major therapeutic targets. Despite their close relationship, β 1AR and β 2AR exhibit distinct regulatory features including differences in their patterns of S-acylation.

S-Acylation of β 2AR has been extensively characterised and occurs at multiple sites, including a conserved C-terminal cysteine and an additional cysteine within an intracellular loop (**Figure 4.7**) (O'Dowd et al., 1989, Loisel et al., 1996, Adachi et al., 2016). Modification at the intracellular loop site is mediated by a subset of Golgi-localised zDHHC enzymes, zDHHC9, zDHHC14, and zDHHC18 (Adachi et al., 2016). In contrast, S-acylation of β 1AR is less well understood, while several C-terminal cysteines have been identified as modification sites with distinct turnover kinetics, the enzymes responsible remain unknown (**Figure 4.7**) (Zuckerman et al., 2011).

As both β 1AR and β 2AR are closely related GPCRs which are both known to be S-acylated, they were selected as an initial model for investigating GPCR S-acylation. Although the zDHHC enzymes responsible for S-acylation of the intracellular loop cysteine of β 2AR have been identified, these enzymes do not modify the C-terminal cysteine, suggesting that additional zDHHC enzymes may be responsible for C-terminal S-acylation. It should be noted that β 1AR also contains a cysteine residue located immediately proximal to the penultimate transmembrane domain, however this cysteine has not been identified as a site of S-acylation.

The S-acylation of FLAG tagged β 1AR and β 2AR was therefore examined by co-expressing these receptors in HEK293T cells with HA tagged zDHHC3, zDHHC6, or zDHHC15 followed by palmitic acid azide labelling and detection using click chemistry.

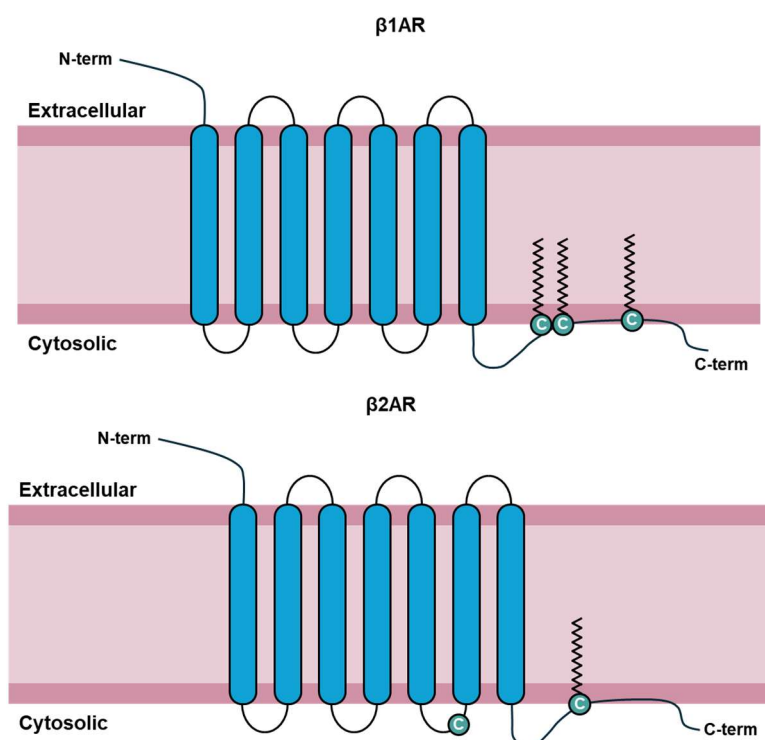


Figure 4.7. Schematic representation of β 1AR and β 2AR highlighting S-acylation sites. β 1AR and β 2AR are shown embedded in the plasma membrane, with the transmembrane domains depicted in blue. Cysteine residues known to be S-acylated are indicated in green: C392, C393, and C414 for β 1AR, and C265 and C341 for β 2AR.

β 1AR was detected as two distinct bands by immunoblotting and displayed a clear S-acylation signal under all experimental conditions (**Figure 4.8A**). To quantify S-acylation, both bands around the 60 kDa marker, which were also present in (-) samples, were included as non-acylated bands. Under control conditions, S-acylation of β 1AR was ~ 40 %, suggesting relatively high baseline S-acylation. Co-expression with zDHHC3, zDHHC6, or zDHHC15 did not result in a significant change in β 1AR S-acylation (**Figure 4.8B**). Although substantial variability in β 1AR was observed between independent experiments, no consistent trends were evident that would suggest enzyme-dependent changes in β 1AR S-acylation.

In contrast to β 1AR, β 2AR S-acylation was clearly influenced by co-expression with specific zDHHC enzymes. Baseline S-acylation of β 2AR under control conditions was comparatively low, at ~ 15 %, and the S-acylation signal was less pronounced, suggesting limited modification by endogenous zDHHC enzymes in this experimental system. Co-expression with zDHHC3 resulted in a significant increase in β 2AR S-acylation to ~ 60 % (**Figure 4.8B**). A significant increase was also observed following co-expression with zDHHC15, reaching around 45 % S-acylation; however, this condition exhibited greater variability between independent experiments. In contrast, co-expression with zDHHC6 did not produce a significant change in β 2AR S-acylation compared with control conditions.

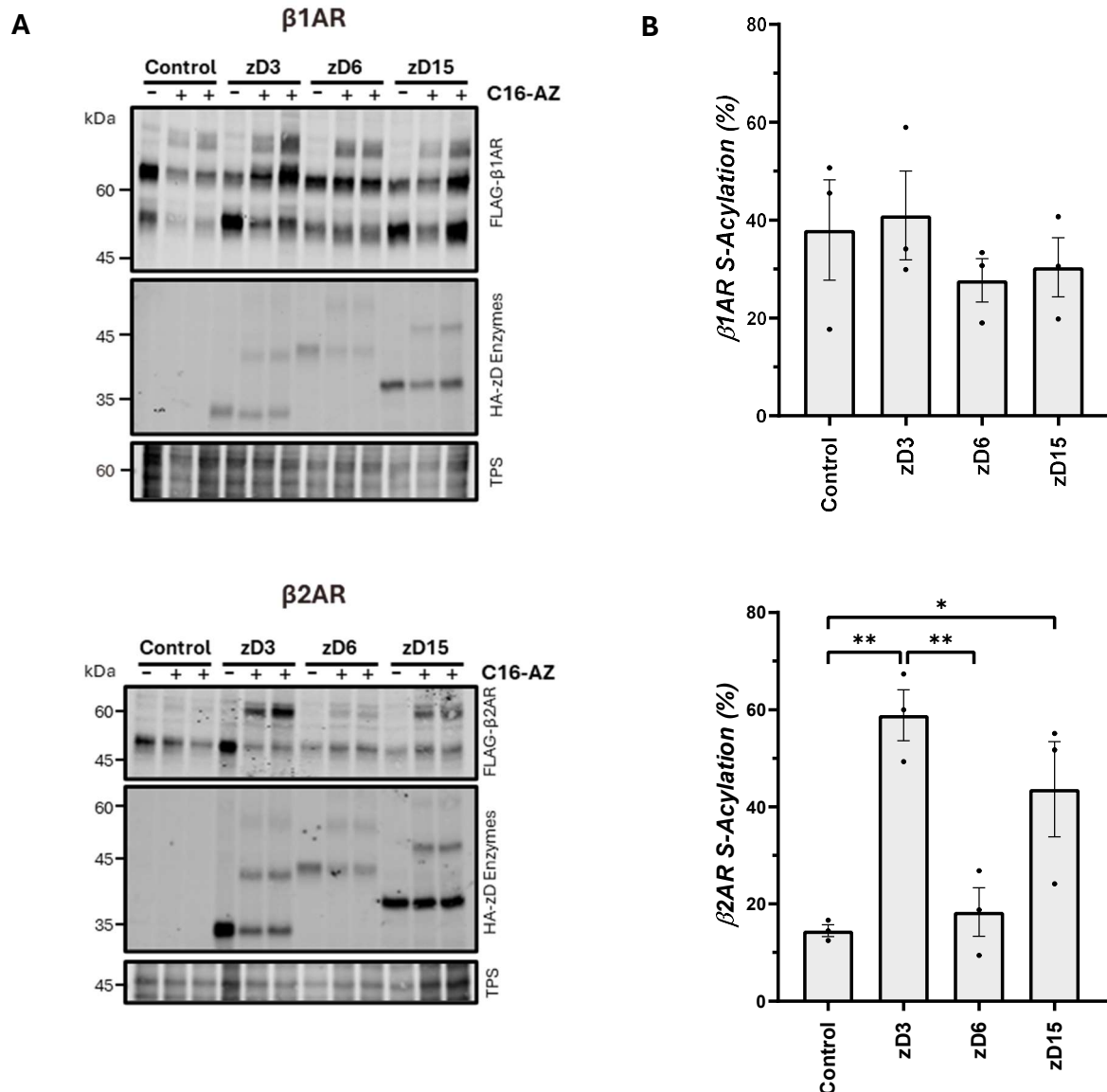


Figure 4.8. β2AR S-acylation is enhanced by co-expression with zDHHC3 and zDHHC15, while no effect was identified for β1AR. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged β1AR or β2AR together with HA-tagged zDHHC3, zDHHC6, zDHHC15 or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μM palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing β1AR or β2AR S-acylation (top; FLAG-β1AR/FLAG-β2AR), zDHHC enzyme expression (middle; HA-zD Enzymes), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of β1AR or β2AR S-acylation expressed as the mean percentage of the S-acylated band relative to the total signal (non-acylated plus acylated bands). Error bars represent ± SEM; n=3 independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (*p<0.05, **p<0.01).

4.3.5 S-Acylation of V1aR is increased by co-expression with zDHHC3 and zDHHC15, but V1bR S-acylation was not detected

Given the differential S-acylation of β 1AR and β 2AR by specific zDHHC enzymes, and the potential importance of cysteine positioning and accessibility in determining substrate selectivity, it was of interest to extend this analysis to additional, closely related GPCRs. The vasopressin receptor family provides a useful model in this context, as its members share high structural similarity yet differ subtly in the number and positioning of cytosolic cysteine residues. The family comprises three GPCRs, including vasopressin receptor 1A (V1aR) and vasopressin receptor 1B (V1bR) (Koshimizu et al., 2012).

S-Acylation of V1aR has been characterised in the rat receptor, although modification of the human protein has not been directly examined. Rat V1aR contains three cysteine residues within the C-terminal region, one present eight residues from the end of the transmembrane domain (C365) and two located 14 and 15 residues downstream of the final transmembrane domain (C371 and C372). Previous studies have identified that C371 and C372 are S-acylated, and suggested the presence of at least one additional modification site, potentially involving the third C-terminal cysteine at C365 or a cysteine located within an intracellular loop (Hawtin et al., 2001). Importantly, all three cysteine residues fall within the canonical GPCR C-terminal region commonly associated with S-acylation. These cysteines are conserved in human V1aR and are positioned at equivalent distances from the transmembrane domain, reflecting a high degree of sequence conservation within this region (**Figure 4.9**). However, the zDHHC enzymes responsible for V1aR S-acylation have not been identified.

S-acylation of V1bR has not been investigated to date. Like V1aR, V1bR contains a pair of adjacent cysteine residues within the C-terminal region located 14 and 15 residues downstream of the final transmembrane domain, as well as additional cysteines within intracellular loops (**Figure 4.9**). However, unlike V1aR, V1bR lacks a cysteine located closer to the transmembrane domain and instead contains a cysteine positioned further into the C-terminal tail. These differences in cysteine positioning may give rise to distinct patterns of S-acylation and differential sensitivity to specific zDHHC enzymes.

The S-acylation of GFP-tagged V1aR and V1bR was examined by co-expressing each receptor in HEK293T cells with HA-tagged zDHHC3, zDHHC6, or zDHHC15. S-Acylation was assessed using palmitic acid azide labelling followed by click chemistry-based detection.

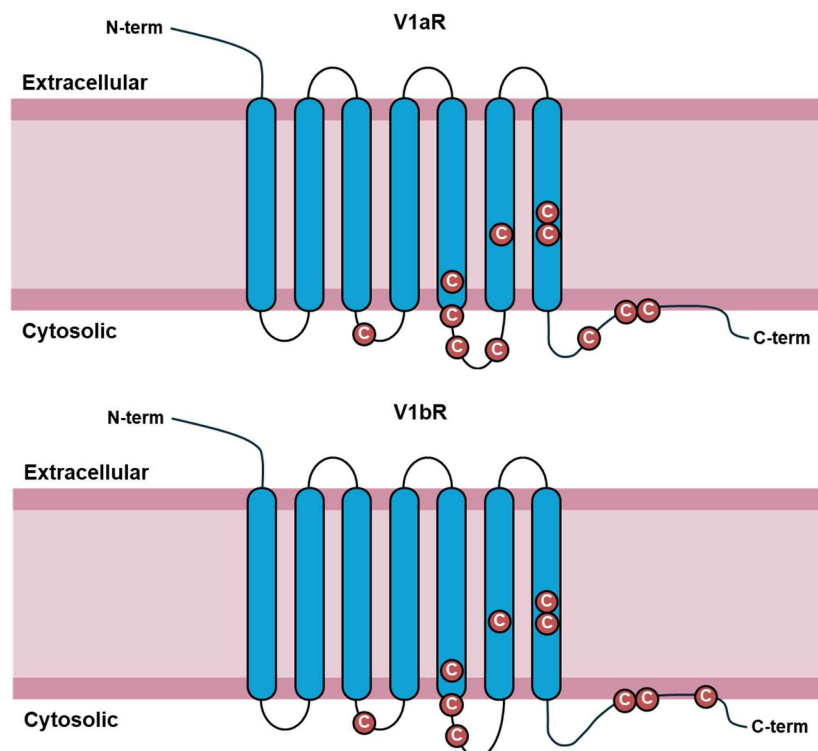


Figure 4.9. Schematic representation of V1aR and V1bR highlighting cysteine residues. V1aR and V1bR are shown embedded in the plasma membrane, with the transmembrane domains depicted in blue. As sites of S-acylation have not been established, the cysteines located in the cytosolic side of transmembrane domains and cytosolic regions have been highlighted in red. The cysteine residues highlighted are: C154, C235, C240, C245, C275, C303, C342, C343, C359, C365, and C366 for V1aR, and C137, C218, C223, C227, C293, C332, C333, C355, C356, and C385 for V1bR.

When analysed by immunoblotting, V1aR was detected as a single band and exhibited minimal S-acylation under control conditions (**Figure 4.10A**). In contrast, a clear increase in V1aR S-acylation was observed following co-expression with zDHHC3 and zDHHC15, as evidenced by the appearance of a distinct S-acylated band. Quantitative analysis revealed a significant increase in V1aR S-acylation from ~ 15 % under control conditions to around 30 % and 35 % when co-expressed with zDHHC3 and zDHHC15, respectively (**Figure 4.10B**). No significant difference was observed between the effects of zDHHC3 and zDHHC15. In contrast, co-expression with zDHHC6 did not result in a significant change in V1aR S-acylation compared with control conditions.

In immunoblots, V1bR migrated as two distinct bands, consistent with differential processing or additional post-translational modifications (**Figure 4.10A**). However, no clear or quantifiable S-acylation signal was detected for V1bR under any of the conditions tested. Although a very faint signal was occasionally observed, this did not exceed background levels upon quantitative analysis, and no consistent differences in S-acylation were detected between control and zDHHC co-expression conditions.

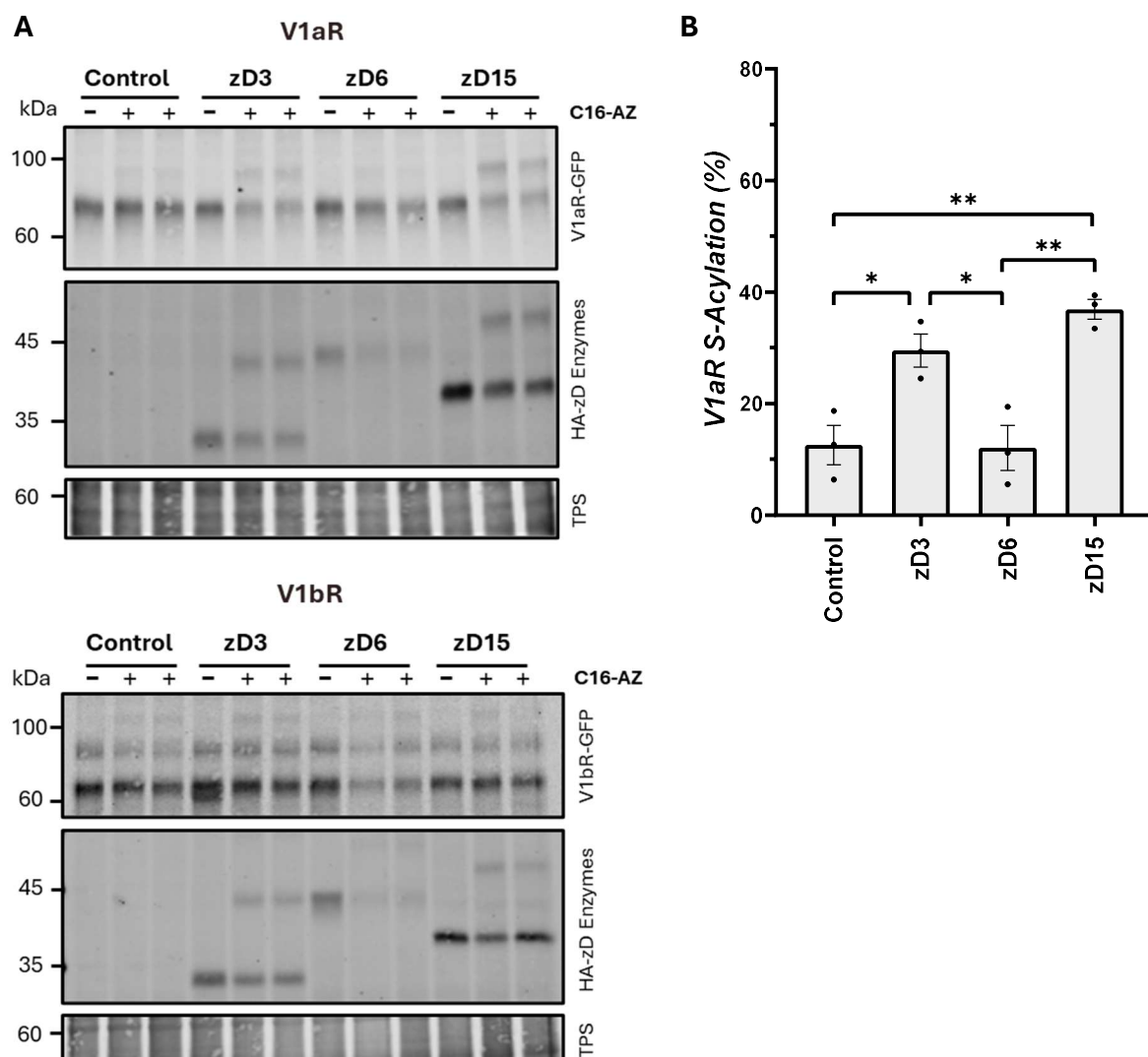


Figure 4.10. V1aR S-acylation is enhanced by co-expression with zDHC3 and zDHC15 while no S-acylation was identified for V1bR. HEK293T cells were co-transfected with plasmids encoding GFP-tagged V1aR or V1bR together with HA-tagged zDHC3, zDHC6, zDHC15 or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing V1aR or V1bR S-acylation (top; V1aR-GFP/V1bR-GFP), zDHC enzyme expression (middle; HA-zD Enzymes), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of V1aR S-acylation expressed as the mean percentage of the S-acylated band relative to the total signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (* $p<0.05$, ** $p<0.01$).

4.3.6 S-Acylation of the GPCRs OXTR, 5HTR2A, and CXCR4 was increased by zDHHC3 and zDHHC15 but not zDHHC6

To extend the analysis of GPCR S-acylation and to determine whether the Golgi-localised zDHHC enzymes zDHHC3 and zDHHC15 display activity across a broader range of GPCRs, additional receptors were selected for investigation. Candidate GPCRs were chosen based on availability of constructs, successful expression in preliminary experiments, and the presence of cytosolic cysteine residues positioned in regions compatible with S-acylation. This approach aimed to generate a more diverse dataset encompassing GPCRs with varying cysteine distributions, cellular functions, and regulatory features, therefore enabling a broader assessment of zDHHC enzyme substrate selectivity.

The oxytocin receptor (OXTR) is a GPCR expressed in both the central nervous system and peripheral tissues, where it binds the neuropeptide oxytocin and regulates a range of physiological processes including uterine contraction and lactation (Jurek and Neumann, 2018). Although S-acylation of OXTR has not been previously reported, the receptor possesses several structural features predictive of this modification. Specifically, OXTR contains a pair of cysteine residues located 14 and 15 amino acids downstream of the final transmembrane domain within the C-terminal tail, corresponding to the canonical GPCR S-acylation region. In addition, OXTR contains a distal C-terminal cysteine and multiple cysteine residues located within or adjacent to intracellular loops, all of which are potentially accessible to zDHHC enzymes (**Figure 4.11**). These features make OXTR a strong candidate for S-acylation and a useful model for assessing zDHHC activity against GPCRs lacking prior experimental characterisation.

The 5-hydroxytryptamine receptor 2A (5HTR2A) is a serotonin receptor widely expressed throughout the central nervous system which has key functions in cognition and behaviour (Fryc et al., 2025). Evidence for S-acylation of 5HTR2A has been reported through palmitoyl-proteomics of mouse forebrain proteins, although this modification has not been independently validated at the individual protein level (Collins et al., 2017). The identified cysteine residue is positioned 13 amino acids downstream of the final transmembrane domain, consistent with the canonical GPCR S-acylation sites. The corresponding cysteine is conserved in the human 5HTR2A protein, suggesting potential functional relevance. In addition to this site, 5HTR2A contains several other cytosolic cysteine residues distributed throughout intracellular loops that could potentially serve as substrates for zDHHC enzymes (**Figure 4.11**). To date, no zDHHC enzymes have been identified as mediators of 5HTR2A S-acylation, making this receptor a valuable candidate for further investigation.

C-X-C chemokine receptor type 4 (CXCR4) is a chemokine receptor expressed in a wide range of tissues and cell types, particularly within the immune system, where it plays a key role in cell migration and survival (Rueda et al., 2025). S-Acylation of CXCR4 has not previously been reported. In contrast to the other GPCRs examined in this study, CXCR4 lacks cysteine residues within its C-terminal tail. Instead, it contains two cysteine residues located within the third intracellular loop, which may be accessible to zDHHC

enzymes and serve as alternative sites of S-acylation (**Figure 4.11**). CXCR4 is also known to undergo additional post-translational modifications, including N-terminal N-glycosylation at asparagine 11 (Chabot et al., 2000). Furthermore, potential O-glycosylation sites have been identified at serine residues 5 and/or 9, and substitution of these sites reduces ligand binding to CXCL12 as well as downstream signalling (Pan et al., 2025). The presence of multiple regulatory post-translational modifications suggests that CXCR4 may be subject to complex post-translational regulation, making it an informative candidate for examining whether S-acylation contributes to this regulatory landscape.

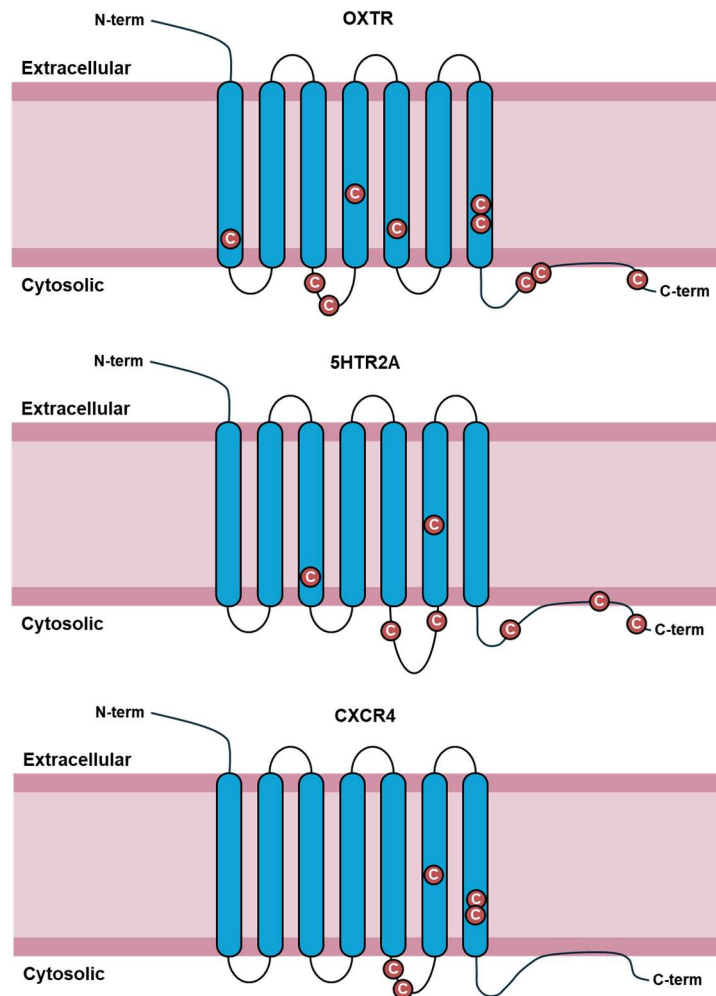


Figure 4.11. Schematic representation of OXTR, 5HTR2A, and CXCR4 highlighting cysteine residues. OXTR, 5HTR2A, and CXCR4 are shown embedded in the plasma membrane, with the transmembrane domains depicted in blue. As sites of S-acylation have not been established, the cysteines located in the cytosolic side of transmembrane domains and cytosolic regions have been highlighted in red. The cysteine residues highlighted are: C59, C138, C142, C164, C219, C323, C324, C346, C347, and C383 for OXTR; C167, C268, C322, C337, C397, C443, and C470 for 5HTR2A; C222, C224, C255, C299, and C300 for CXCR5.

Together, the inclusion of OXTR, 5HTR2A, and CXCR4 allowed for the evaluation of zDHHC3- and zDHHC15-mediated S-acylation across GPCRs with diverse cysteine positioning and regulatory contexts. This expanded dataset provided an opportunity to determine whether the activity of Golgi-localised zDHHC enzymes observed for β 2AR and V1aR extends more broadly across the GPCR superfamily. To achieve this, these GPCRs were co-expressed in HEK293T cells with zDHHC3, zDHHC6, or zDHHC15 and S-acylation was detected by palmitic acid azide labelling and click chemistry methods.

OXTR was detected by immunoblotting as two distinct bands under control conditions, with the lower molecular weight species being more prominent (**Figure 4.12A**). Co-expression with zDHHC3 or zDHHC15 resulted in the appearance of multiple S-acylated bands that were not observed in control samples, suggesting the presence of more than one site of S-acylation. Due to the relatively high molecular weight of OXTR, band separation was limited and the exact number of S-acylated species could not be clearly resolved. Quantitative analysis revealed a significant increase in OXTR S-acylation following co-expression with both zDHHC3 and zDHHC15, increasing from ~ 10 % under control conditions to around 25 % and 30 %, respectively (**Figure 4.12B**). No significant difference was observed between zDHHC3 and zDHHC15-mediated S-acylation, and co-expression with zDHHC6 did not result in a significant change compared to control.

For 5HTR2A, a detectable S-acylation signal was present under control conditions, indicating modification by endogenous zDHHC enzymes. Co-expression with zDHHC3 or zDHHC15 led to an intensification of this S-acylation signal and the appearance of an additional S-acylated band, consistent with modification at more than one cysteine residue (**Figure 4.12A**). Quantification showed a significant increase in 5HTR2A S-acylation from ~ 30 % under control conditions to around 55 % and 50 % following co-expression with zDHHC3 and zDHHC15, respectively (**Figure 4.12B**). As observed for OXTR, no significant change in S-acylation was detected upon co-expression with zDHHC6.

CXCR4 exhibited two clearly resolved bands on immunoblots, with the higher molecular weight species predominating under control conditions and no detectable S-acylation signal observed (**Figure 4.12A**). Upon co-expression with zDHHC3, and to a lesser extent zDHHC15, a clear S-acylation band became detectable. This was accompanied by an apparent shift in the relative abundance of the receptor towards the lower molecular weight species. Quantitative analysis revealed a significant increase in CXCR4 S-acylation from ~ 5 % under control conditions to around 20 % following co-expression with either zDHHC3 or zDHHC15 (**Figure 4.12B**). As with the other GPCRs examined, co-expression with zDHHC6 did not result in a significant change in CXCR4 S-acylation.

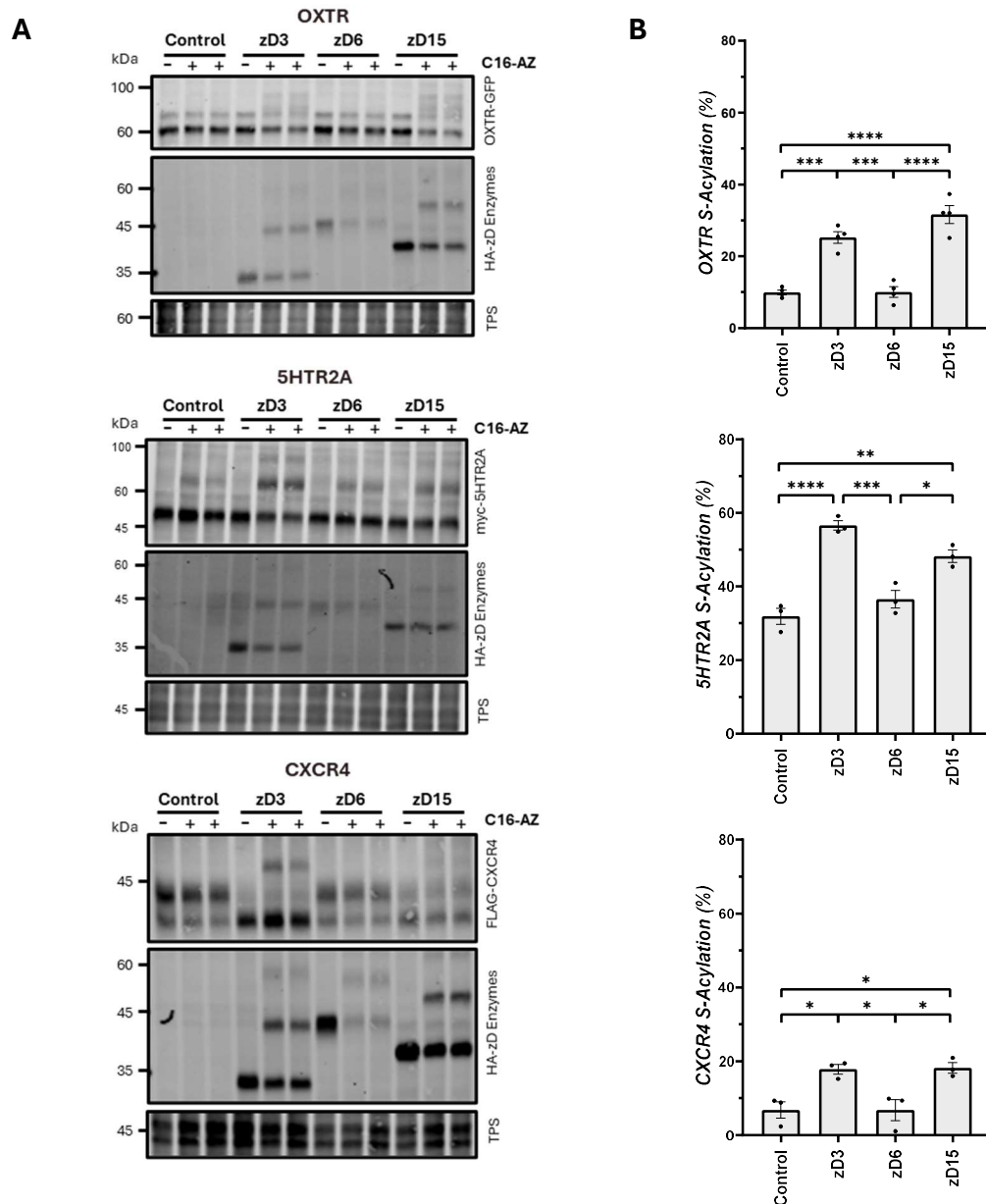


Figure 4.12. S-acylation of OXTR, 5HTR2A, and CXCR4 is enhanced by co-expression with zDHHC3 and zDHHC15. HEK293T cells were co-transfected with plasmids encoding GFP-tagged OXTR, myc-tagged 5HTR2A, or FLAG-tagged CXCR4 together with HA-tagged zDHHC3, zDHHC6, zDHHC15 or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing OXTR, 5HTR2A, or CXCR4 S-acylation (top; OXTR-GFP/myc-5HTR2A/FLAG-CXCR4), zDHHC enzyme expression (middle; HA-zD Enzymes), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of OXTR, 5HTR2A, or CXCR4 S-acylation expressed as the mean percentage of the S-acylated bands relative to the total signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

4.3.7 GPR37 S-acylation is increased by zDHHC3, zDHHC6, and zDHHC15 but no S-acylation was identified for P2RY11

The previous experiments demonstrated that zDHHC6 did not enhance S-acylation of several GPCRs, with S-acylation instead being mediated predominantly by the Golgi-localised enzymes zDHHC3 and zDHHC15. Notably, all GPCRs examined in these experiments lacked cysteine residues positioned immediately adjacent to the final transmembrane domain, a feature that was present in the single-pass and two-transmembrane proteins identified as zDHHC6 substrates. The only exception was β 1AR, which contains a cysteine near the final transmembrane domain that is not known to be S-acylated.

To directly test whether cysteine proximity to the final transmembrane domain influences zDHHC6-mediated S-acylation of GPCRs, two receptors were identified that contain cysteine residues positioned immediately adjacent to the final transmembrane domain. Such cysteine positioning appears uncommon among GPCRs, substantially limiting the number of suitable candidate proteins. Nonetheless, examining GPCRs with this architecture provided an opportunity to determine whether the presence of juxta-membrane cysteines is sufficient to permit zDHHC6-mediated S-acylation in GPCRs.

GPR37 is predominantly expressed in the central nervous system and has been implicated in maintaining neuronal homeostasis, although its precise physiological roles and endogenous ligands remain incompletely characterised (Liu et al., 2025). To date, S-acylation of GPR37 has not been investigated. Notably, GPR37 contains a high density of cytosolic cysteine residues distributed across intracellular loops and the C-terminal tail, many of which are positioned in locations predicted to be accessible to zDHHC enzymes. In particular, GPR37 possesses cysteine residues at juxta-membrane positions within intracellular loops, as well as two cysteines located at the immediate end of the final transmembrane domain, one positioned two residues from the end of the transmembrane segment and another located directly adjacent to it in the C-terminus. Additionally, a cluster of six cysteine residues is present ~ 11 residues downstream of the final transmembrane domain, falling within the canonical GPCR C-terminal S-acylation region, followed by an additional cysteine three residues later and a terminal cysteine at the extreme C-terminus (**Figure 4.13**). This unique cysteine architecture makes GPR37 a strong candidate for assessing whether juxta-membrane cysteines enable zDHHC6-mediated S-acylation of GPCRs.

The P2Y purinoceptor P2RY11 is an ATP-activated GPCR expressed on immune cells and has been implicated in immune modulation (Andersen et al., 2023). In contrast to GPR37, P2RY11 lacks cysteine residues within intracellular loops but contains a distinct cluster of cysteine residues immediately following the final transmembrane domain. Specifically, P2RY11 possesses three consecutive cysteine residues directly adjacent to the transmembrane domain, followed by an additional cysteine three residues downstream. Unlike most GPCRs examined in this study, P2RY11 does not contain cysteine residues within the typical distal C-terminal S-acylation region (**Figure 4.13**). S-acylation of P2RY11 has not previously been reported, however, the exclusive localisation

of cysteine residues to the juxta-membrane region provides a simplified system in which to directly assess the importance of cysteine positioning for zDHHC6 activity, independent of intracellular loop or distal C-terminal cysteines.

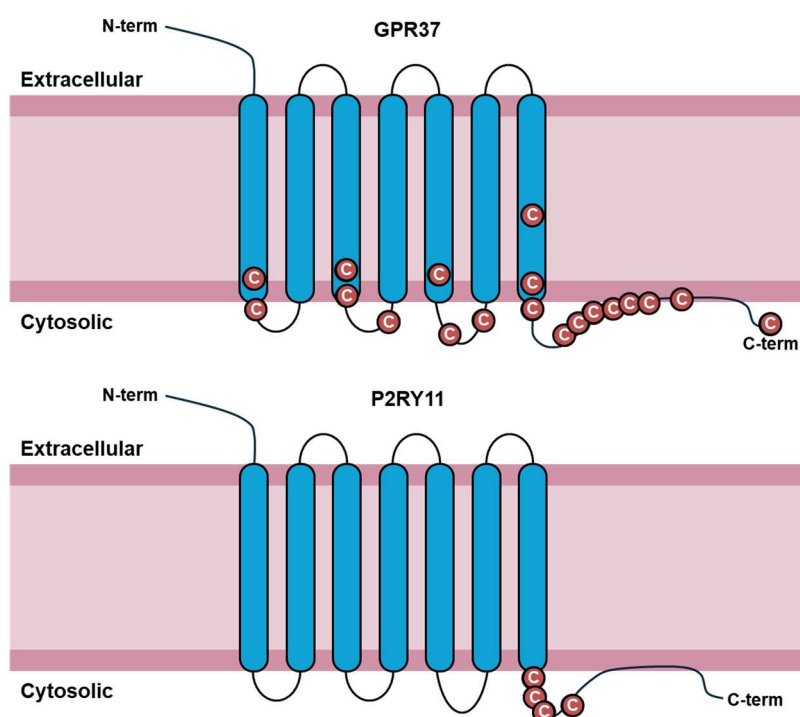


Figure 4.13. Schematic representation of GPR37 and P2RY11 highlighting cysteine residues. GPR37 and P2RY11 are shown embedded in the plasma membrane, with the transmembrane domains depicted in blue. As sites of S-acylation have not been established, the cysteines located in the cytosolic side of transmembrane domains and cytosolic regions have been highlighted in red. The cysteine residues highlighted are: C284, C287, C353, C356, C376, C461, C476, C492, C543, C551, C553, C563, C564, C565, C566, C567, C568, C571, and C613 for GPR37; and C330, C331, C332, and C335 for P2RY11.

Together, GPR37 and P2RY11 provide complementary models to test the hypothesis that zDHHC6-mediated S-acylation requires cysteine residues positioned immediately adjacent to the final transmembrane domain. These receptors allow direct evaluation of whether juxta-membrane cysteine placement is sufficient to overcome the apparent inability of zDHHC6 to modify conventional GPCR substrates. To test this, FLAG-tagged GPR37 or P2RY11 were co-expressed in HEK293T cells with HA-tagged zDHHC3, zDHHC6, or zDHHC15. S-Acylation of the receptors was assessed using palmitic acid azide labelling followed by click chemistry for detection of S-acylation.

When analysed by immunoblotting, GPR37 showed no detectable S-acylation under control conditions (**Figure 4.14A**). In contrast, co-expression with each of the zDHHC

enzymes tested resulted in the appearance of S-acylated species. Co-expression with zDHHC3 or zDHHC15 produced three distinct S-acylated bands, with the uppermost band being most clearly resolved following zDHHC3 expression. In comparison, co-expression with zDHHC6 resulted in the appearance of a single, comparatively faint S-acylated band. These differences suggest that GPR37 undergoes S-acylation at multiple cysteine residues and that the ability of individual zDHHC enzymes to modify these sites differs.

Quantitative analysis revealed a significant increase in GPR37 S-acylation from ~ 35 % under control conditions to around 50 % following co-expression with zDHHC6, 70 % with zDHHC15, and 80 % with zDHHC3 (**Figure 4.14B**). The magnitude of S-acylation differed significantly between enzymes, with zDHHC3 and zDHHC15-mediated S-acylation both significantly greater than that observed with zDHHC6, and zDHHC3 producing a significantly higher level of S-acylation than zDHHC15.

In contrast to GPR37, no detectable S-acylation of P2RY11 was observed under any of the conditions tested (**Figure 4.14A**). Nevertheless, P2RY11 was consistently detected as three distinct bands by immunoblotting, suggesting the presence of post-translational modification or differential processing independent of S-acylation.

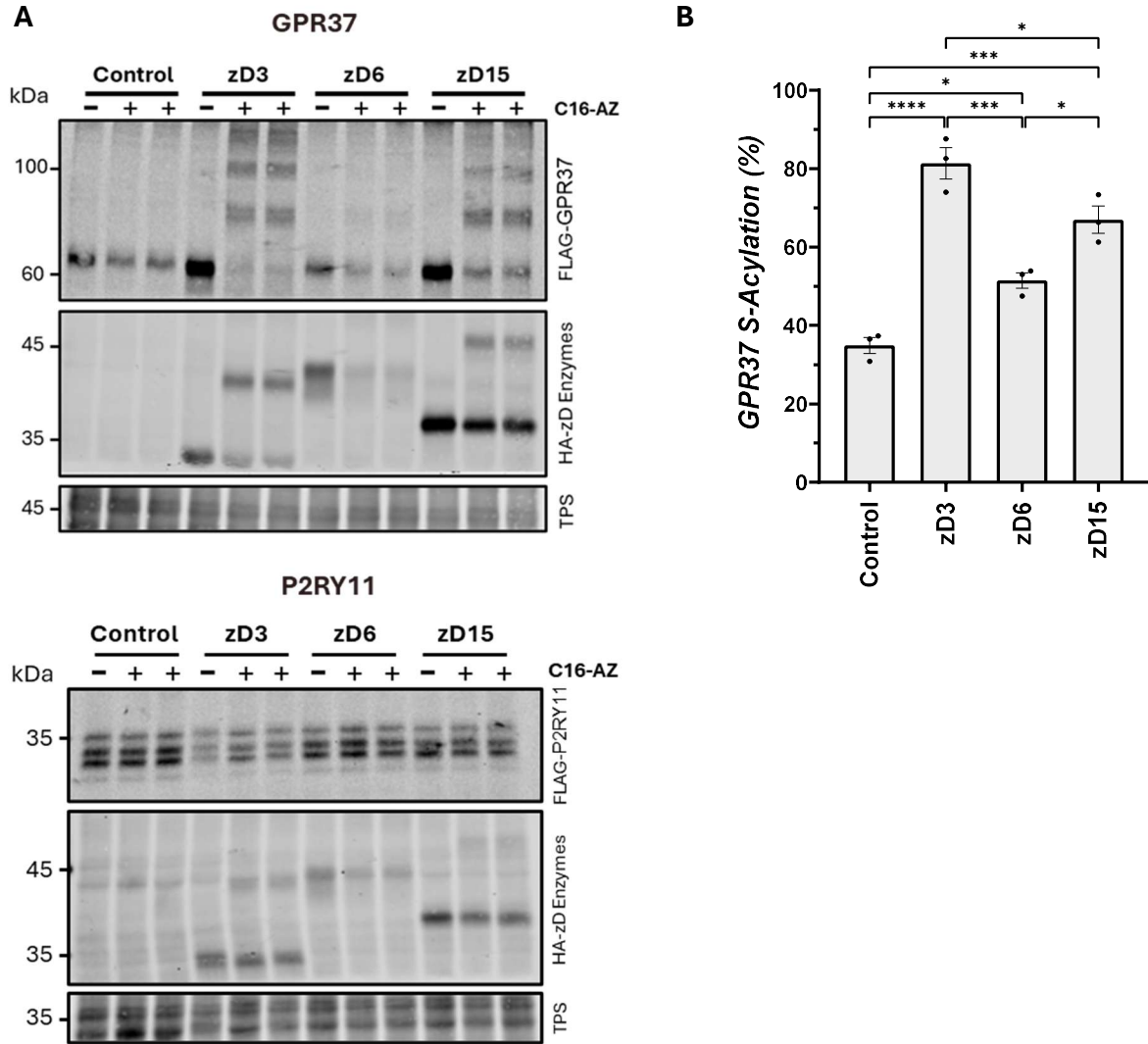


Figure 4.14. S-Acylation of GPR37 is enhanced by co-expression with zDHHC3, zDHHC6, and zDHHC15, but S-acylation of P2RY11 was not detected. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged GPR37 or P2RY11 together with HA-tagged zDHHC3, zDHHC6, zDHHC15 or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing GPR37 or P2RY11 S-acylation (top; FLAG-GPR37/FLAG-P2RY11), zDHHC enzyme expression (middle; HA-zD Enzymes), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of GPR37 S-acylation expressed as the mean percentage of the S-acylated bands relative to the total signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (* $p<0.05$, *** $p<0.001$, **** $p<0.0001$).

4.3.8 Determining the contribution of juxta-membrane cysteine residues to GPR37 S-acylation

Following the observation that co-expression of GPR37 with zDHHC enzymes revealed distinct S-acylation patterns, most notably a simplified single-band profile with zDHHC6 compared to multiple bands with zDHHC3 and zDHHC15, it was important to investigate whether specific cysteine residues underlie this apparent site selectivity. The more restricted acylation pattern observed with zDHHC6 suggested that this enzyme may preferentially target a limited subset of cysteine residues within GPR37.

GPR37 contains multiple cysteine residues within its intracellular regions that could potentially serve as substrates for S-acylation. However, systematic interrogation of all possible sites would require extensive mutagenesis. To refine this analysis, attention was focused on cysteine residues positioned in close proximity to the final transmembrane domain, as juxta-membrane cysteines have been implicated in selective recognition by zDHHC6. In particular, two C-terminal cysteine residues, C551, located two residues from the C-terminal end of the final transmembrane domain, and C553, positioned immediately following this transmembrane domain within the C-terminal tail, were identified as strong candidates. This positioning is notable because it mirrors that of established zDHHC6 substrates such as CD4 and K5, where S-acylated cysteines are located at or immediately adjacent to the membrane interface. In contrast, GPCRs not identified to be substrates of zDHHC6 typically had C-terminal cysteines at more distal positions further into the cytoplasmic tail. This spatial distinction provided a rationale to test whether proximity to the final transmembrane domain is a determinant of zDHHC6-mediated S-acylation of GPR37.

To assess the contribution of these residues, a double cysteine-to-alanine substitution construct of GPR37, C551/553A, was generated. FLAG-tagged wild-type or alanine-substituted GPR37 constructs were co-expressed in HEK293T cells with HA-tagged zDHHC3, zDHHC6, or zDHHC15 to evaluate enzyme-specific effects on receptor S-acylation. In parallel, wild-type and substitution construct receptors were co-expressed with either an empty HA vector control or HA-zDHHC3, which had previously been shown to robustly enhance GPR37 S-acylation, to determine the impact of these mutations on overall acylation levels. S-Acylation of GPR37 was assessed using metabolic labelling with palmitic acid azide followed by click chemistry-based detection.

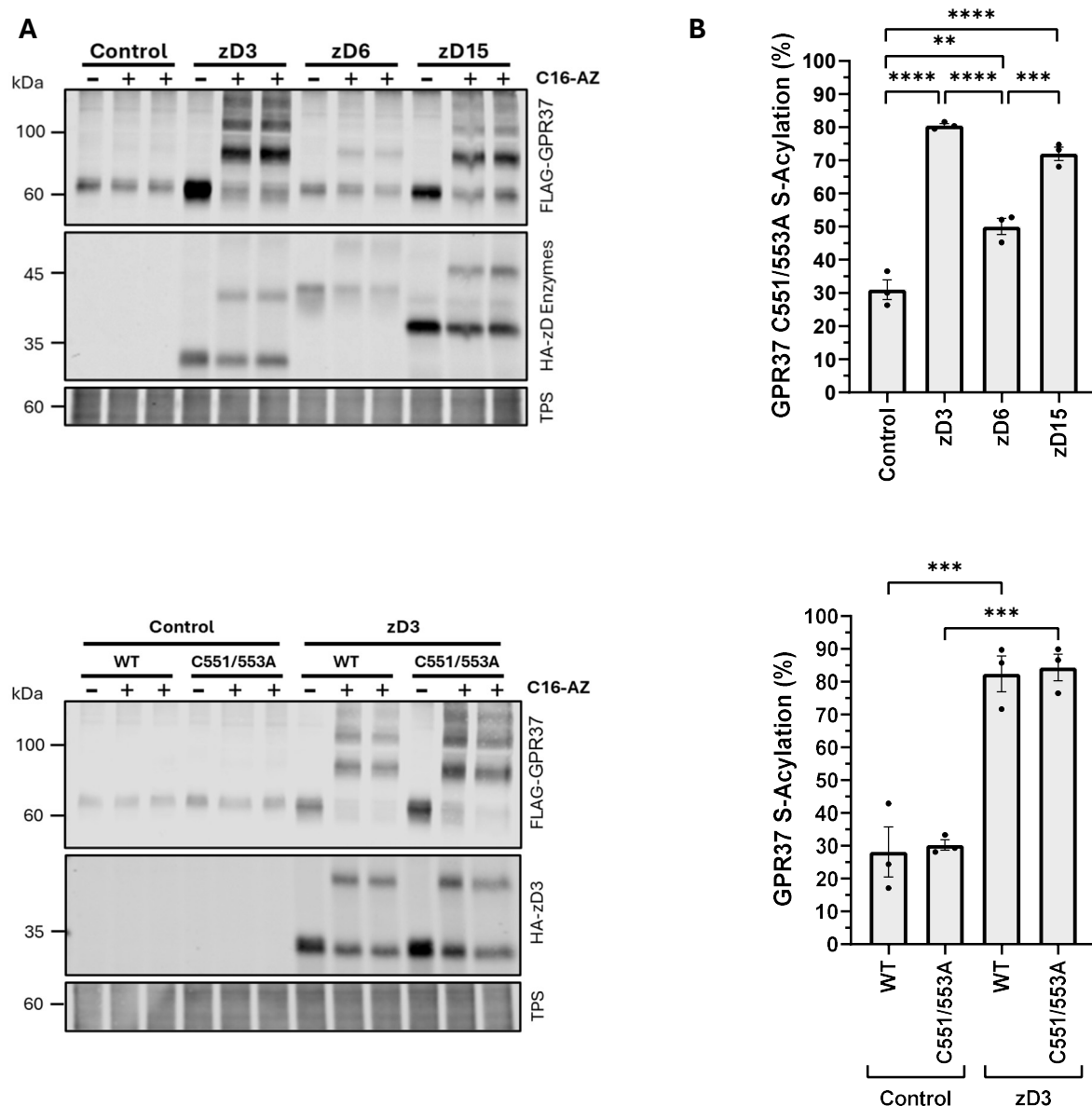


Figure 4.15. Contribution of juxta-membrane cysteines to GPR37 S-acylation by zDHHC enzymes. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged wild-type GPR37 or the cysteine-to-alanine mutant (C551/553A) together with HA-tagged zDHHC3, zDHHC6, zDHHC15, or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing S-acylation of wild-type and C551/553A GPR37 (top; FLAG-GPR37), expression of zDHHC enzymes (middle; HA-zDHHC enzymes/HA-zD3), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of GPR37 S-acylation expressed as the mean percentage of S-acylated signal relative to total protein (non-acylated plus acylated bands) for wild-type and C551/553A constructs under each condition. Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

To determine whether the juxta-membrane cysteine residues C551 and C553 contribute to GPR37 S-acylation, the C551/553A mutant was assessed following co-expression with zDHHC enzymes previously shown to enhance acylation of the wild-type protein. Consistent with observations for wild-type GPR37, co-expression of the C551/553A construct with zDHHC3, zDHHC6, and zDHHC15 resulted in a significant increase in S-acylation relative to control conditions (**Figure 4.15A**). The pattern of acylation also remained unchanged, with zDHHC3 and zDHHC15 producing multiple S-acylated species, while zDHHC6 generated a single predominant S-acylated band.

Quantification confirmed that S-acylation of the C551/553A mutant increased from approximately 30% under control conditions to ~50% with zDHHC6, ~70% with zDHHC15, and ~80% with zDHHC3 (**Figure 4.15B**). While all three enzymes significantly enhanced S-acylation, the increases observed with zDHHC3 and zDHHC15 were significantly greater than zDHHC6, with no significant difference detected between zDHHC3 and zDHHC15. The persistence of a clear zDHHC6-mediated S-acylation signal following mutation of C551 and C553 indicates that these residues are not essential sites for zDHHC6-dependent modification and suggests that additional cysteine residues within GPR37 can serve as substrates for this enzyme.

Comparison of the C551/553A mutant with wild-type GPR37 revealed no detectable differences in S-acylation patterns at the level of immunoblotting (**Figure 4.15A**). Quantitative analysis supported this observation, showing a significant increase in S-acylation for both wild-type and mutant GPR37 upon co-expression with zDHHC3 relative to control, but no significant difference between the two constructs under either condition (**Figure 4.15B**). Together, these data indicate that substitution of C551 and C553 is not sufficient to reduce overall S-acylation of GPR37, suggesting that these juxta-membrane cysteines are not major determinants of total receptor acylation and that other cysteine residues likely compensate for their loss.

4.3.9 Analysis of the S-acylation of CCR5 with cysteine residues moved closer to the transmembrane domain

Although mutation of the juxta-membrane cysteine residues C551 and C553 did not reduce overall GPR37 S-acylation or alter the enzyme-specific pattern observed with zDHHC6, these findings do not exclude the possibility that cysteine positioning relative to the membrane plays a broader role in determining zDHHC6 substrate selectivity. In particular, GPR37 contains multiple additional cysteine residues in its proximal C-terminal region, and redundancy among these sites may mask the contribution of any individual residue. This raises the possibility that zDHHC6 recognition may depend less on specific amino acids and more on the spatial relationship between the final transmembrane domain and the downstream cysteine-containing region. In wild-type CCR5, the C-terminal cysteines targeted for S-acylation are located ~ 20 residues downstream of the transmembrane domain, a distance typical of many GPCRs and thought to promote membrane anchoring of the C-terminus. It was therefore hypothesised that this spacing may prevent access of zDHHC6 to these cysteines.

To directly test whether reducing the distance between the final transmembrane domain and the C-terminal cysteine residues could enable zDHHC6-mediated S-acylation, a series of CCR5 truncation constructs was generated. These constructs were based on the CCR5 C58/213/224A backbone, in which intracellular loop cysteine residues had been substituted with alanine to eliminate potential confounding S-acylation sites. Three variants were produced in which 15 (C-15), 18 (C-18), or 19 (C-19) amino acids were removed from the region between the end of the final transmembrane domain and the C-terminal cysteine residues (**Figure 4.16**).

The C-15 construct retained the short sequence EKFR immediately following the transmembrane domain in an attempt to preserve aspects of the native membrane environment and minimise disruption to protein structure. In contrast, the C-18 and C-19 constructs were designed to position the cysteine residues as close as possible to the transmembrane domain, testing whether more extreme repositioning was required to overcome the apparent barrier to zDHHC6 activity. FLAG-tagged constructs were co-expressed in HEK293T cells with HA-tagged zDHHC3, zDHHC6, or zDHHC15, and S-acylation was assessed using palmitic acid azide labelling followed by click chemistry.



Figure 4.16. Schematic representation of CCR5 deletion constructs designed to reposition cysteine residues C321, C323, and C324 closer to the final transmembrane domain. Constructs were generated using the CCR5 C58/C213/C224A backbone. Deletions of 15 (C-15), 18 (C-18), or 19 (C-19) residues were introduced into the CCR5 C-terminus between the end of transmembrane domain 7 (TMD7) and the S-acylated cysteine residues C321, C323, and C324, thereby moving these cysteine residues closer to the end of TMD7.

On immunoblots, all three CCR5 truncation constructs migrated at the expected molecular weight just below the 35 kDa marker (**Figure 4.17A**). Compared with wild-type CCR5, there was a noticeable reduction in the proportion of higher molecular weight species, potentially consistent with altered glycosylation. S-acylated species were detected at ~ 45 kDa. For all three constructs, no clear S-acylation was observed under control conditions or upon co-expression with zDHHC6. In contrast, S-acylation by zDHHC3 was readily detected for all constructs. Notably, zDHHC15-mediated S-acylation was clearly visible for the C-18 and C-19 constructs but not for C-15.

Quantitative analysis supported these observations. For the C-15 construct, S-acylation increased significantly from ~ 20 % under control conditions to around 45 % upon co-expression with zDHHC3, with no significant change observed with either zDHHC6 or zDHHC15 (**Figure 4.17B**). For the C-18 construct, S-acylation increased significantly from ~ 30 % to around 40 % with both zDHHC3 and zDHHC15, but remained unchanged with zDHHC6. Similarly, S-acylation of the C-19 construct increased significantly from ~ 20 % under control conditions to ~ 45 % with zDHHC3 and to around 35 % with zDHHC15, with no significant effect observed following co-expression with zDHHC6.

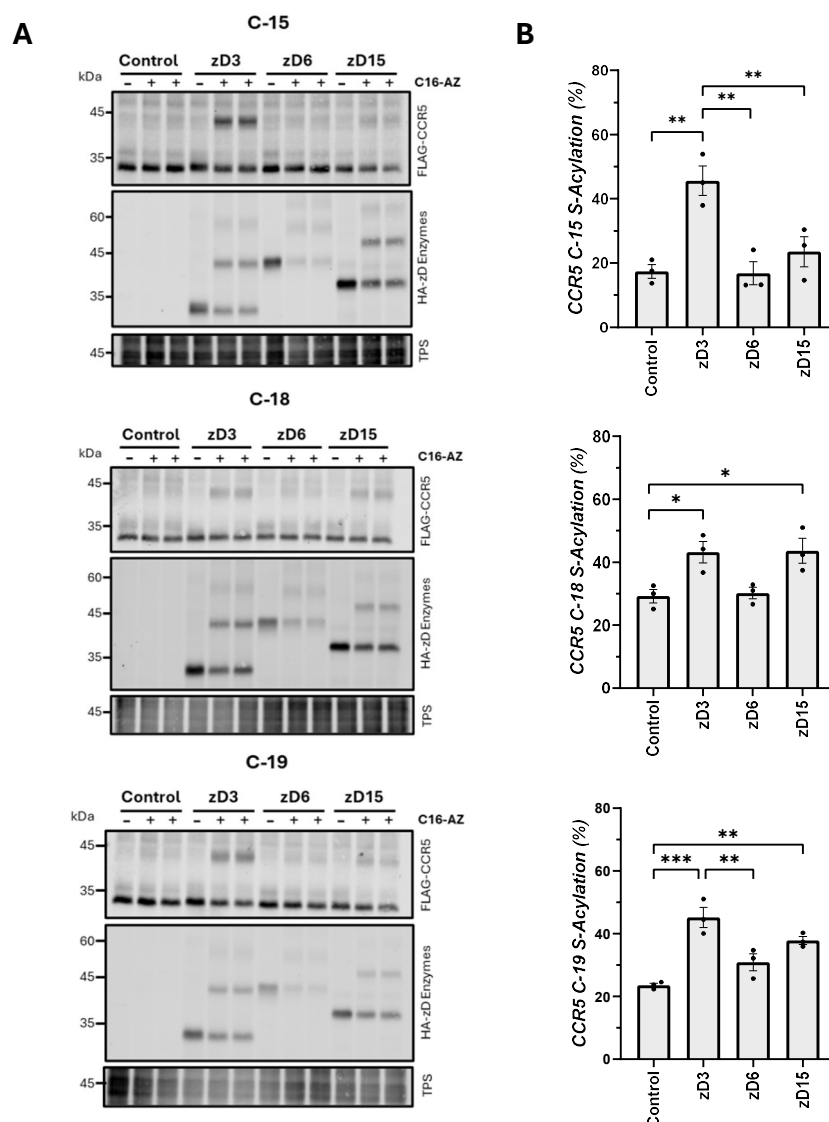


Figure 4.17. S-Acylation of CCR5 C-15, C-18, and C-19 was enhanced by co-expression with zDHHC3 but only C-18 and C-19 S-acylation was increased by zDHHC15. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged constructs of CCR5 with either 15, 18, or 19 amino acids removed between the final transmembrane domain and the C-terminal cysteine residues, C-15, C-18, or C-19, together with HA-tagged zDHHC3, zDHHC6, zDHHC15, or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing C-15, C-18, or C-19 S-acylation (top; FLAG-C-15/C-18/C-19), zDHHC enzyme expression (middle; HA-zD Enzymes), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of C-15, C-18, or C-19 S-acylation expressed as the mean percentage of the S-acylated bands relative to the total signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

4.3.10 Effect of introducing a juxta-membrane cysteine on CCR5 S-acylation by zDHHC6

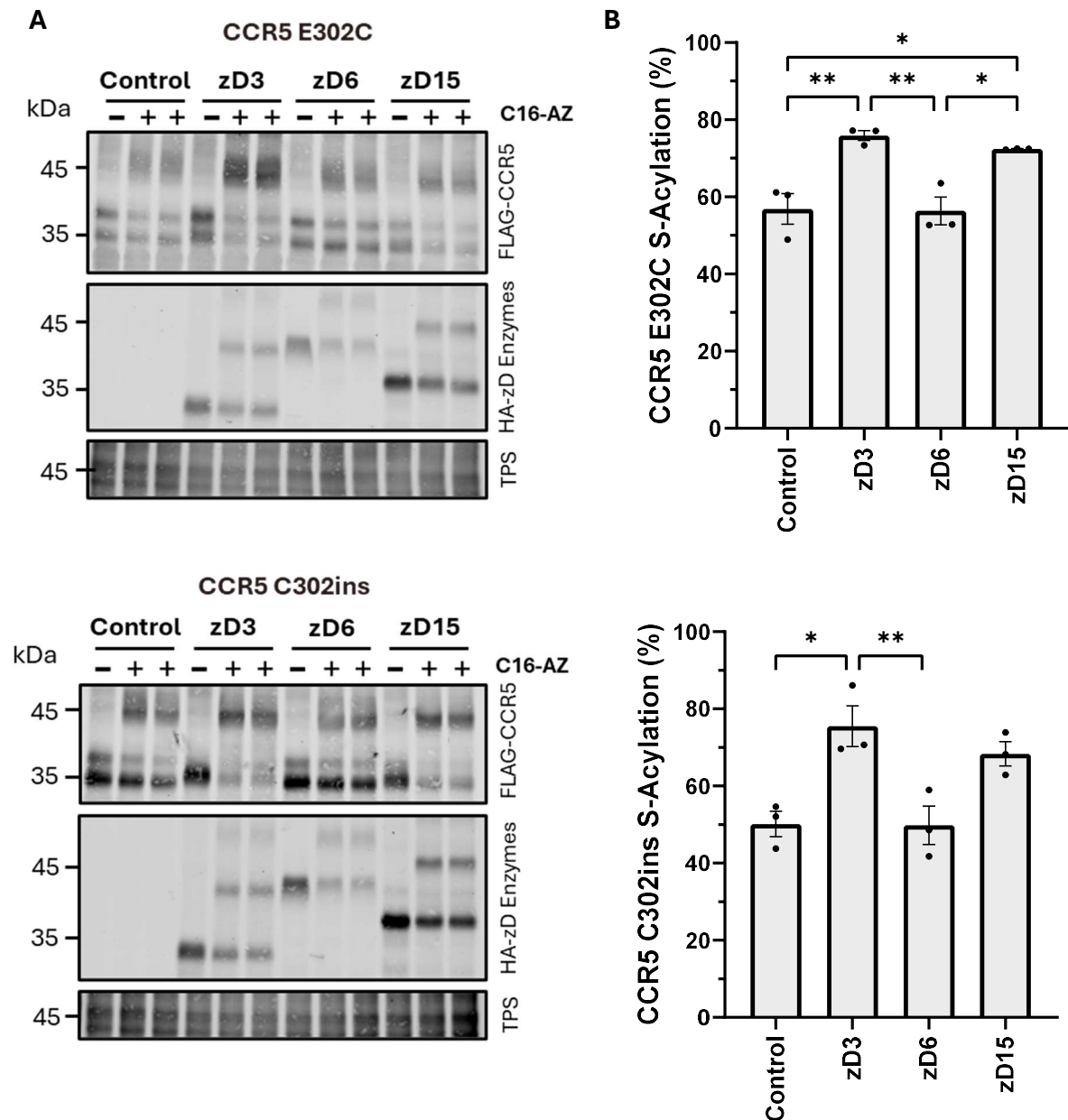
Although removal of the amino acids between the final transmembrane domain and the C-terminal S-acylated cysteine residues did not alter CCR5 S-acylation by zDHHC6, this approach may have inadvertently disrupted local structural features at the membrane interface. In particular, truncation of this region could alter transmembrane boundary definition, protein folding, or accessibility of downstream cysteine residues, potentially masking any effects of altered cysteine positioning.

To address this limitation and directly test whether the presence of a juxta-membrane cysteine is sufficient to influence zDHHC6-mediated S-acylation, a complementary approach was taken. CCR5 constructs were engineered to introduce a cysteine residue immediately adjacent to the end of the final transmembrane domain. Two variants were produced: one in which the glutamic acid at position 302 was substituted with cysteine (E302C), and a second construct in which a cysteine was inserted directly after the final transmembrane domain (C302ins). The C302ins construct was specifically designed as a control to retain the native negatively charged glutamic acid at position 302, thereby preserving the local electrostatic environment at the membrane boundary while introducing a juxta-membrane cysteine for comparison.

FLAG-tagged CCR5 wild-type, E302C, and C302ins constructs were co-expressed in HEK293T cells with HA-tagged zDHHC3, zDHHC6, zDHHC15, or an empty HA vector control. S-acylation was then assessed using metabolic labelling with palmitic acid azide followed by click chemistry-based detection, enabling comparison of enzyme-dependent S-acylation across constructs with altered juxta-membrane cysteine positioning.

For the CCR5 E302C construct, S-acylation was significantly increased relative to control conditions, rising from approximately 55 % under baseline conditions to ~75 % following co-expression with zDHHC3 and ~70 % with zDHHC15 (**Figure 4.18B**). In contrast, co-expression with zDHHC6 did not produce a significant change compared to control. Accordingly, S-acylation levels observed with both zDHHC3 and zDHHC15 were significantly higher than those observed with zDHHC6.

For the CCR5 C302ins construct, S-acylation increased significantly from approximately 50 % under control conditions to ~75 % upon co-expression with zDHHC3 (**Figure 4.18B**). Co-expression with zDHHC15 also increased S-acylation to approximately 70 %, although this did not reach statistical significance compared to control. No significant change was observed with zDHHC6. Consistent with this, S-acylation with zDHHC3 was significantly higher than that observed with zDHHC6.



4.18 Juxta-membrane cysteine introduction maintains CCR5 S-acylation by zDHHC3 but fails to confer zDHHC6 activity. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged CCR5 wild-type, E302C, or C302ins constructs together with HA-tagged zDHHC3, zDHHC6, zDHHC15, or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing CCR5 S-acylation (top; FLAG-CCR5), zDHHC enzyme expression (middle; HA-zD Enzymes), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of CCR5 S-acylation expressed as the mean percentage of the S-acylated bands relative to the total signal (non-acylated plus acylated bands) for wild-type, E302C, and C302ins constructs. Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (* $p<0.05$, ** $p<0.01$).

4.3.11 Testing the effect of juxta-membrane cysteine positioning on β 2AR S-acylation by zDHHC enzymes

While introduction of a juxta-membrane cysteine in CCR5 did not confer responsiveness to zDHHC6, it also did not have any significant impact on S-acylation mediated by zDHHC3 or zDHHC15. This was not unexpected, as wild-type CCR5 is already robustly S-acylated by both enzymes under the experimental conditions used, suggesting that the system is likely near saturation with respect to detectable S-acylation. Consequently, the addition of a single juxta-membrane cysteine does not appear sufficient to measurably alter the overall capacity of zDHHC3 or zDHHC15 to modify CCR5.

Importantly, these findings indicate that the introduction of a membrane-proximal cysteine does not intrinsically enhance or disrupt zDHHC3- or zDHHC15-mediated S-acylation in this context, but they do not exclude the possibility that such effects could be observed in receptors with different baseline acylation characteristics or cysteine architectures.

To test this, a second GPCR was selected in order to determine whether the same manipulation produces comparable outcomes in a receptor with a more defined and lower basal S-acylation profile. β 2AR provides a well-characterised model in which S-acylation occurs at defined sites, including a conserved C-terminal cysteine located approximately 10-14 amino acids from the final transmembrane domain and an additional intracellular loop cysteine. While the intracellular loop site is known to be modified by a subset of Golgi-localised zDHHC enzymes, including zDHHC9, zDHHC14, and zDHHC18, the enzymes responsible for C-terminal S-acylation remain less clearly defined.

Given this spatially and mechanistically distinct S-acylation pattern, β 2AR was used to assess whether introducing a juxta-membrane cysteine can influence S-acylation in a receptor with more defined modification sites and lower basal signal. A β 2AR C329ins construct was therefore generated, in which a cysteine was inserted immediately adjacent to the final transmembrane domain. This construct was co-expressed with zDHHC3, zDHHC6, zDHHC15, or an empty vector control in HEK293T cells, and S-acylation was assessed using metabolic labelling with palmitic acid azide followed by click chemistry-based detection.

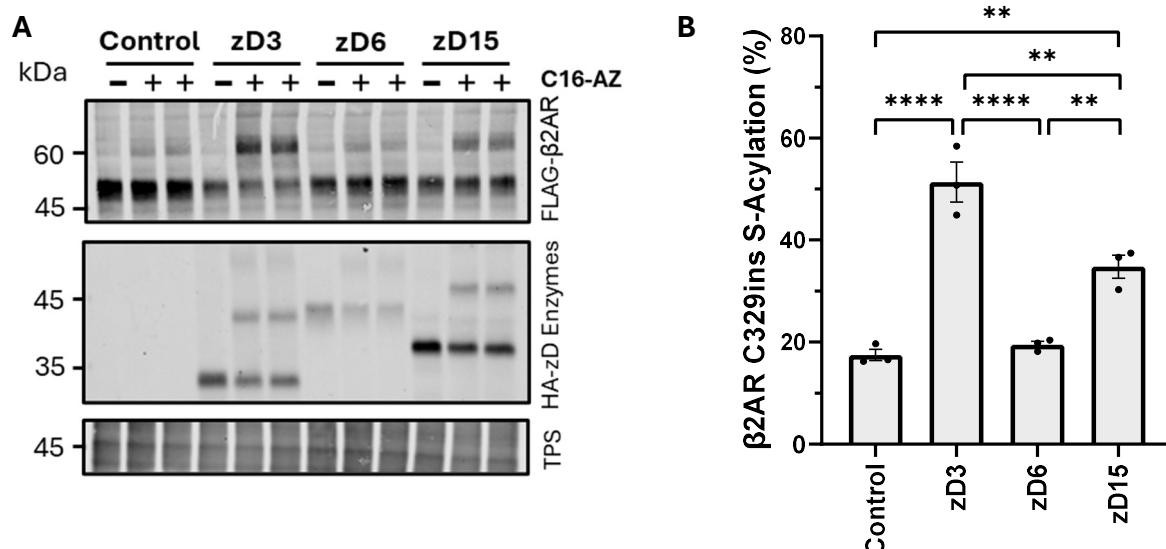


Figure 4.19. Introduction of a juxta-membrane cysteine does not enable zDHHC6-mediated S-acylation of β2AR and does not alter zDHHC3- or zDHHC15-dependent S-acylation. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged β2AR wild-type or the C329ins construct together with HA-tagged zDHHC3, zDHHC6, zDHHC15, or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μM palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing β2AR S-acylation (top; FLAG-β2AR), zDHHC enzyme expression (middle; HA-zD Enzymes), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of β2AR S-acylation expressed as the mean percentage of S-acylated bands relative to total signal (non-acylated plus acylated bands) for wild-type and C329ins constructs. Error bars represent ± SEM; n=3 independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (**p<0.01, ****p<0.0001).

Consistent with observations for wild-type β2AR, co-expression with zDHHC3 and zDHHC15 resulted in significant increases in S-acylation compared to control conditions (**Figure 4.19**). S-Acylation increased from approximately 15 % under control conditions to ~30 % with zDHHC15 and ~50 % with zDHHC3, with the increase observed for zDHHC3 being significantly greater than that for zDHHC15. In contrast, co-expression with zDHHC6 did not result in any detectable increase in S-acylation relative to control.

These findings mirror those observed for CCR5, further supporting the conclusion that zDHHC6 does not mediate S-acylation of these GPCRs under the conditions tested, even when a juxta-membrane cysteine is introduced.

4.3.12 The effect of zDHHC3 re-location to the ER on the S-acylation of CCR5 and β 2AR

Previous experiments indicated that the majority of GPCRs examined, including CCR5 and β 2AR, were S-acylated by the Golgi-localised zDHHC enzymes zDHHC3 and zDHHC15, whereas the ER-localised enzyme zDHHC6 showed little or no activity toward these receptors. This suggests that zDHHC enzymes with activity against GPCRs are enriched at the Golgi, and that zDHHC localisation thereby determines the timing of GPCR S-acylation (at the Golgi). However, an alternative explanation is that GPCRs are not able to undergo S-acylation at the ER, even if this compartment contained active enzymes. To directly test whether GPCRs can be S-acylated at the ER, a zDHHC3 construct containing a C-terminal ER retention signal (EKKNR) was employed to redirect the enzyme from the Golgi to the ER (Gorleku et al., 2011). FLAG-tagged CCR5 or β 2AR was co-expressed in HEK293T cells with HA-tagged wild-type zDHHC3, or zDHHC3 EKKNR and receptor S-acylation was assessed using palmitic acid azide labelling followed by click chemistry detection.

For CCR5, immunoblot analysis revealed comparable levels of S-acylated receptor when co-expressed with either wild-type zDHHC3 or zDHHC3 EKKNR (**Figure 4.20A**). Quantification confirmed a significant increase in CCR5 S-acylation relative to control conditions, rising from ~40 % with empty vector to ~60 % with either zDHHC3 or zDHHC3 EKKNR (**Figure 4.20B**). No significant difference was observed between the wild-type and ER-retained zDHHC3 constructs.

A similar pattern was observed for β 2AR. Although overall receptor expression appeared reduced in samples co-expressed with zDHHC3 EKKNR, as indicated by fainter immunoblot bands, the proportion of S-acylated β 2AR was not detectably altered by enzyme re-localisation (**Figure 4.20A**). Quantitative analysis showed a significant increase in S-acylation from ~30 % under control conditions to ~70 % following co-expression with either wild-type zDHHC3 or zDHHC3 EKKNR, with no significant difference between the two enzyme variants (**Figure 4.20B**).

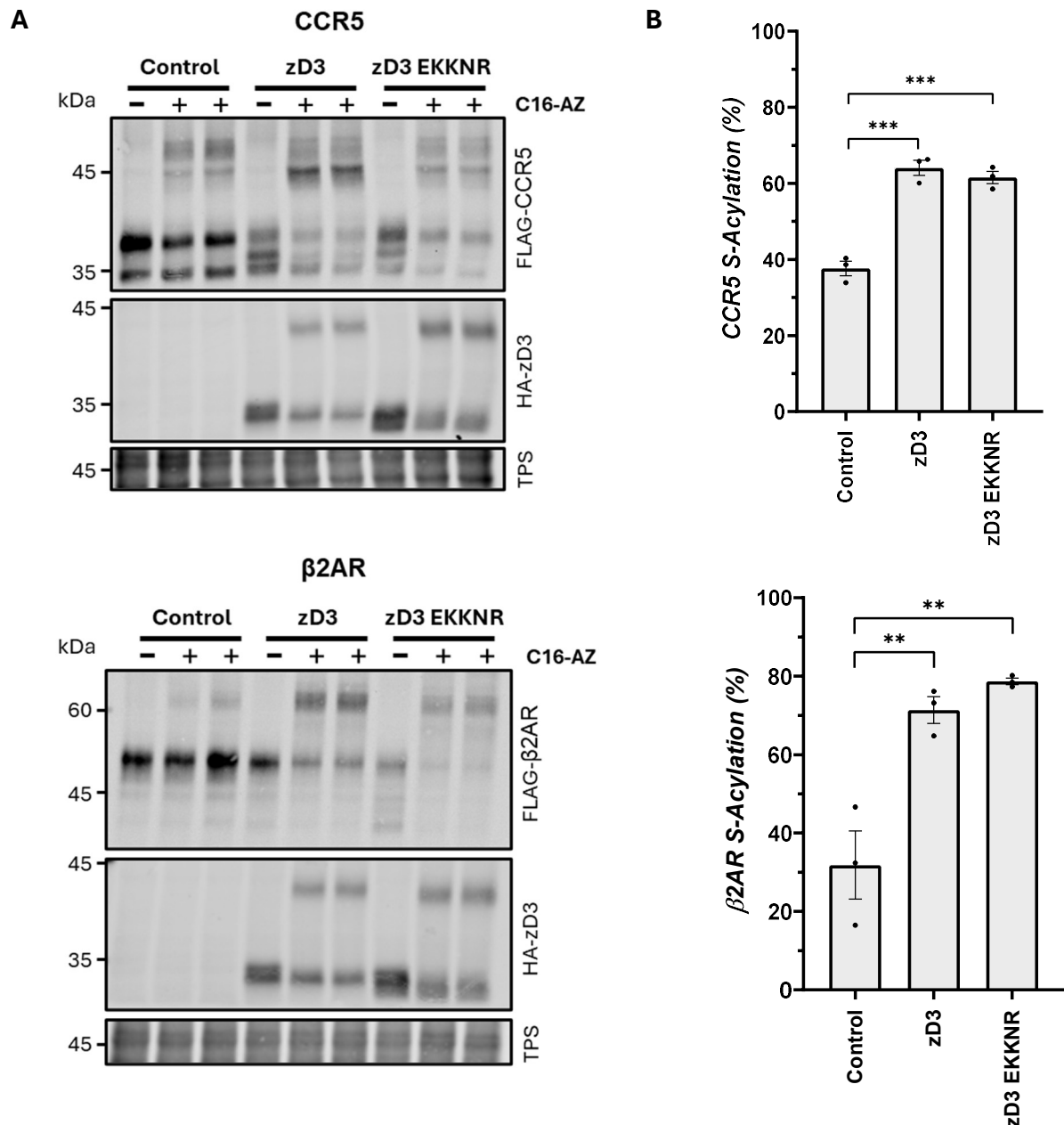


Figure 4.20. CCR5 and β2AR S-acylation by zDHHC3 is not altered by relocation of the enzyme to the ER. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged constructs of CCR5 or β2AR together with HA-tagged zDHHC3, ER-localised zDHHC3 EKKNR, or an empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 μM palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing CCR5 or β2AR S-acylation (top; FLAG-CCR5/FLAG-β2AR), zDHHC enzyme expression (middle; HA-zD Enzymes), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of CCR5 or β2AR S-acylation expressed as the mean percentage of the S-acylated bands relative to the total signal (non-acylated plus acylated bands). Error bars represent ± SEM; n=3 independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (*p<0.01, ***p<0.001).

4.3.13 Expression of β 2AR with zDHHC3 (EKKNR) does not alter stability of the GPCR

In the preceding experiment, co-expression of β 2AR with ER-retained zDHHC3 (zDHHC3 EKKNR) appeared to reduce overall receptor expression despite producing levels of S-acylation comparable to wild-type zDHHC3. This raised the possibility that re-localisation of zDHHC3 to the ER might alter the stability or turnover of β 2AR, potentially through changes in the timing or compartment of S-acylation. To investigate whether zDHHC3 localisation influences β 2AR stability, a cycloheximide chase assay was performed.

FLAG-tagged β 2AR was co-expressed in HEK293T cells with HA-tagged wild-type zDHHC3 or zDHHC3-EKKNR. Protein synthesis was inhibited by cycloheximide, and β 2AR expression levels were assessed by immunoblotting at 0 hours and after 8 hours. Receptor abundance at 8 hours was quantified relative to the corresponding 0-hour time point for each condition.

Immunoblot analysis revealed a clear reduction in β 2AR expression following 8 hours of cycloheximide treatment in all conditions tested (**Figure 4.21A**). Quantification showed that ~ 40 % of β 2AR remained after 8 hours, with no significant differences observed between cells expressing empty vector, wild-type zDHHC3, or zDHHC3-EKKNR (**Figure 4.21B**). This data indicates that neither overexpression of zDHHC3 nor its re-localisation to the ER measurably alters β 2AR stability over the time course examined.

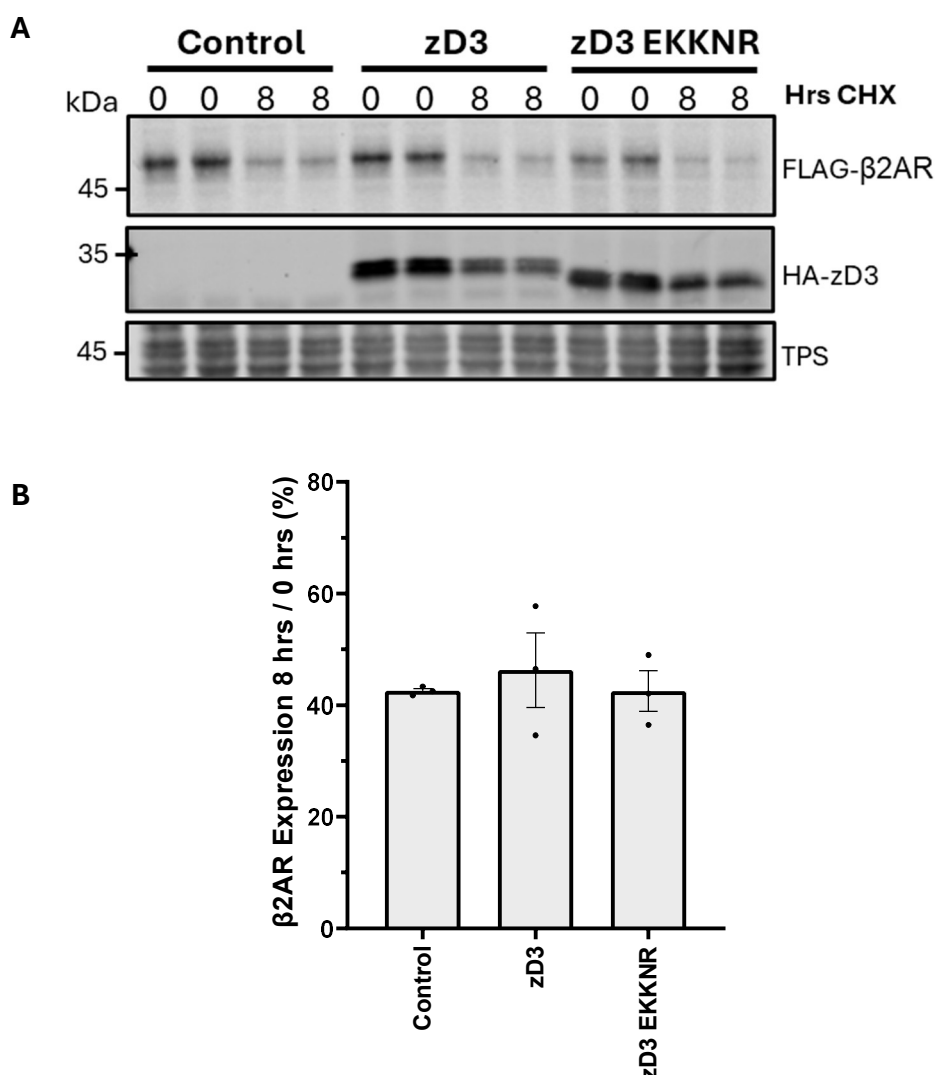


Figure 4.21. β 2AR stability is not altered by expression with zDHHC3 or ER-localised zDHHC3 EKKNR. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged β 2AR together with HA-tagged zDHHC3, ER-localised zDHHC3 EKKNR, or an empty HA vector (control). At 24 hours post-transfection, cells were treated with 50 μ g/ml cycloheximide (CHX). Samples were collected at 0 and 8 hours and analysed by SDS-PAGE and immunoblotting. **(A)** Representative immunoblot showing β 2AR expression (top; FLAG- β 2AR), zDHHC3 enzyme expression (middle; HA-zDHHC3) and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of β 2AR expression following 8 hours CHX treatment, expressed as the mean percentage of β 2AR remaining at 8 hours relative to expression at 0 hours. Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA.

4.3.14 Analysis of whether trapping β 2AR at the ER limits its S-acylation by endogenous zDHHC enzymes

As the GPCRs examined in this study exhibited a preference for S-acylation by Golgi-localised zDHHC enzymes, it was of interest to determine whether subcellular localisation of the substrate itself influences S-acylation efficiency. Previous experiments demonstrated that redirecting zDHHC3 to the ER using the C-terminal ER retention sequence EKKNR did not impair its ability to S-acylate either CCR5 or β 2AR. This indicates that zDHHC3 retains catalytic activity outside of its typical Golgi localisation and suggests that enzyme localisation alone is not the primary determinant of substrate modification.

Given this, the localisation of the GPCR substrate was investigated as a potential limiting factor. If S-acylation predominantly occurs in the Golgi, restricting a GPCR to the ER would be expected to reduce or prevent its modification. Supporting this, previous experiments using the RUSH system with CCR5 (**Chapter 3, Figure 3.8**) demonstrated that S-acylation increased upon release of the receptor from the ER, suggesting that trafficking beyond the ER enhances access to the relevant S-acylation machinery.

To directly test whether Golgi localisation is required for efficient GPCR S-acylation, a β 2AR construct containing a C-terminal EKKNR ER retention sequence was generated. In addition, a β 2AR C329ins construct and a combined C329ins EKKNR construct were included. Although previous experiments showed that introduction of a juxta-membrane cysteine at this position did not enhance S-acylation or confer responsiveness to zDHHC6, these experiments were conducted under conditions where the receptor was free to traffic through the secretory pathway. It therefore remained possible that the lack of effect reflected limited access of ER-localised enzymes, such as zDHHC6, to the introduced cysteine.

By retaining the receptor within the ER, the C329ins EKKNR construct provides a means to test whether spatial proximity to ER-resident zDHHC enzymes enables S-acylation of a cysteine that is otherwise not utilised. Inclusion of the C329ins construct alongside the wild-type and EKKNR-tagged receptors also allows direct comparison to determine whether any effects observed are due to altered localisation, cysteine positioning, or a combination of both. Experiments were performed in the absence of co-expressed zDHHC enzymes to assess the contribution of endogenous acyltransferases under these conditions. Together, this approach allows evaluation of whether the inability of zDHHC6 to S-acylate β 2AR is due to intrinsic substrate features or reflects spatial segregation between enzyme and substrate within the cell.

FLAG-tagged β 2AR wild-type, C329ins, EKKNR, and C329ins EKKNR constructs were expressed in HEK293T cells in the absence of co-expressed zDHHC enzymes to assess the contribution of endogenous acyltransferases. At 24 hours post-transfection, cells were incubated with palmitic acid azide, followed by click chemistry-based labelling with alkyne mPEG. Samples were then analysed by SDS-PAGE to assess S-acylation levels and compare the effects of altered receptor localisation and cysteine positioning.

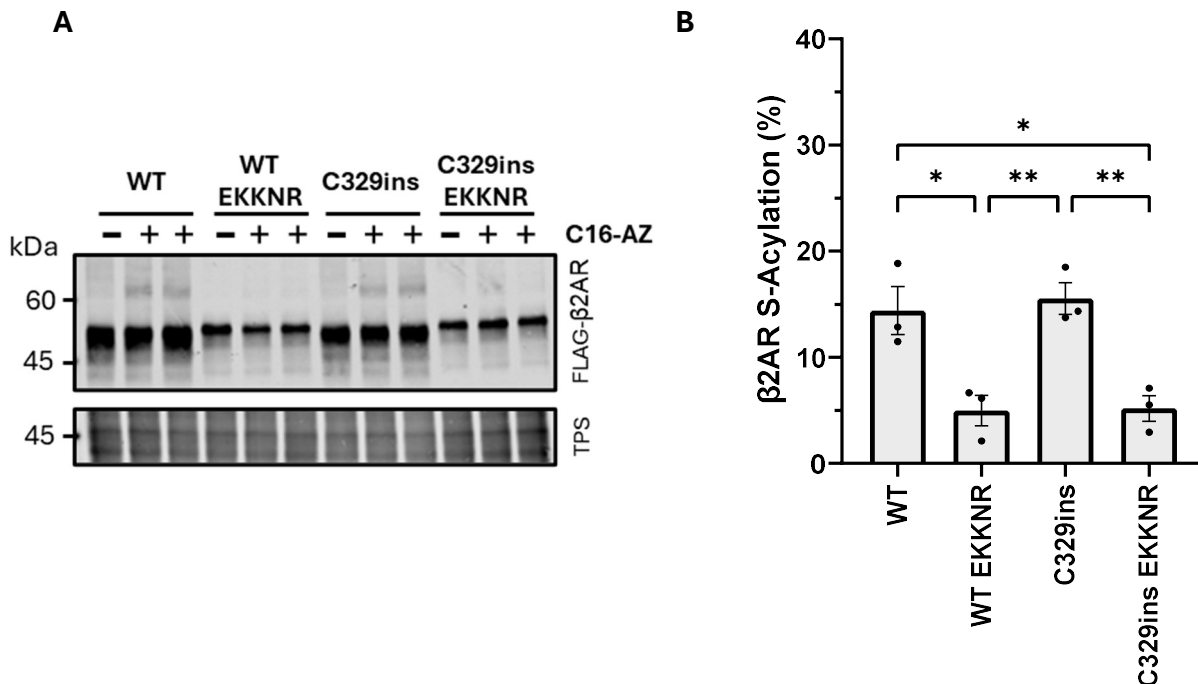


Figure 4.22. Effect of ER retention and juxta-membrane cysteine introduction on β2AR S-acylation. HEK293T cells were transfected with plasmids encoding FLAG-tagged β2AR wild-type (WT), C329ins, WT EKKNR, or C329ins-EKKNR constructs. At 24 hours post-transfection, cells were incubated with 100 μM palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblot showing β2AR S-acylation (top; FLAG-β2AR) and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of β2AR S-acylation expressed as the mean percentage of S-acylated bands relative to the total signal (non-acylated plus acylated bands) for each construct. Error bars represent ± SEM; n=3 independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (*p<0.05, **p<0.01).

A clear effect of the EKKNR ER retention sequence on β2AR S-acylation was evident from the immunoblot analysis (**Figure 4.22A**). Both wild-type and C329ins β2AR displayed a distinct S-acylated band under control conditions, whereas no detectable S-acylation signal was observed for either of the EKKNR-tagged constructs. Quantification confirmed this observation, with S-acylation levels of approximately 15% for both wild-type and C329ins β2AR. In contrast, S-acylation of the EKKNR-tagged constructs was significantly reduced to approximately 5%, representing a substantial decrease compared to their respective non-ER-retained counterparts (**Figure 4.22B**). These results indicate that retention of β2AR within the endoplasmic reticulum markedly reduces its S-acylation, supporting the conclusion that efficient modification of this receptor is dependent on trafficking beyond the ER.

4.3.15 Analysis of fatty acid selectivity of zDHHC3, zDHHC6, and zDHHC15

The results presented so far show that different transmembrane proteins are modified by distinct zDHHC enzymes in co-expression experiments. I next set out to determine if the fatty acids added to these transmembrane proteins by endogenous enzymes match the fatty acid selectivity profile of the enzymes that modify them in co-expression assays.

S-Acylation is known to exhibit fatty acid selectivity, with previous studies demonstrating that individual zDHHC enzymes display distinct preferences for fatty acyl-CoA chain length. In particular, zDHHC3 and zDHHC15 have been reported to preferentially utilise C16:0 substrates over C18:0, whereas the fatty acid selectivity profile of zDHHC6 has not been comprehensively characterised (Greaves et al., 2017).

The fatty acid selectivity of zDHHC3, zDHHC6, and zDHHC15 was compared by expressing each enzyme individually in HEK293T cells, followed by metabolic labelling with fatty acid azide probes of defined chain length (C14:0, C16:0, or C18:0) and detection using click chemistry-based analysis. This approach enables the intrinsic lipid preferences of each zDHHC enzyme to be assessed in isolation from substrate-specific effects, providing a direct comparison of enzyme-dependent fatty acid selectivity.

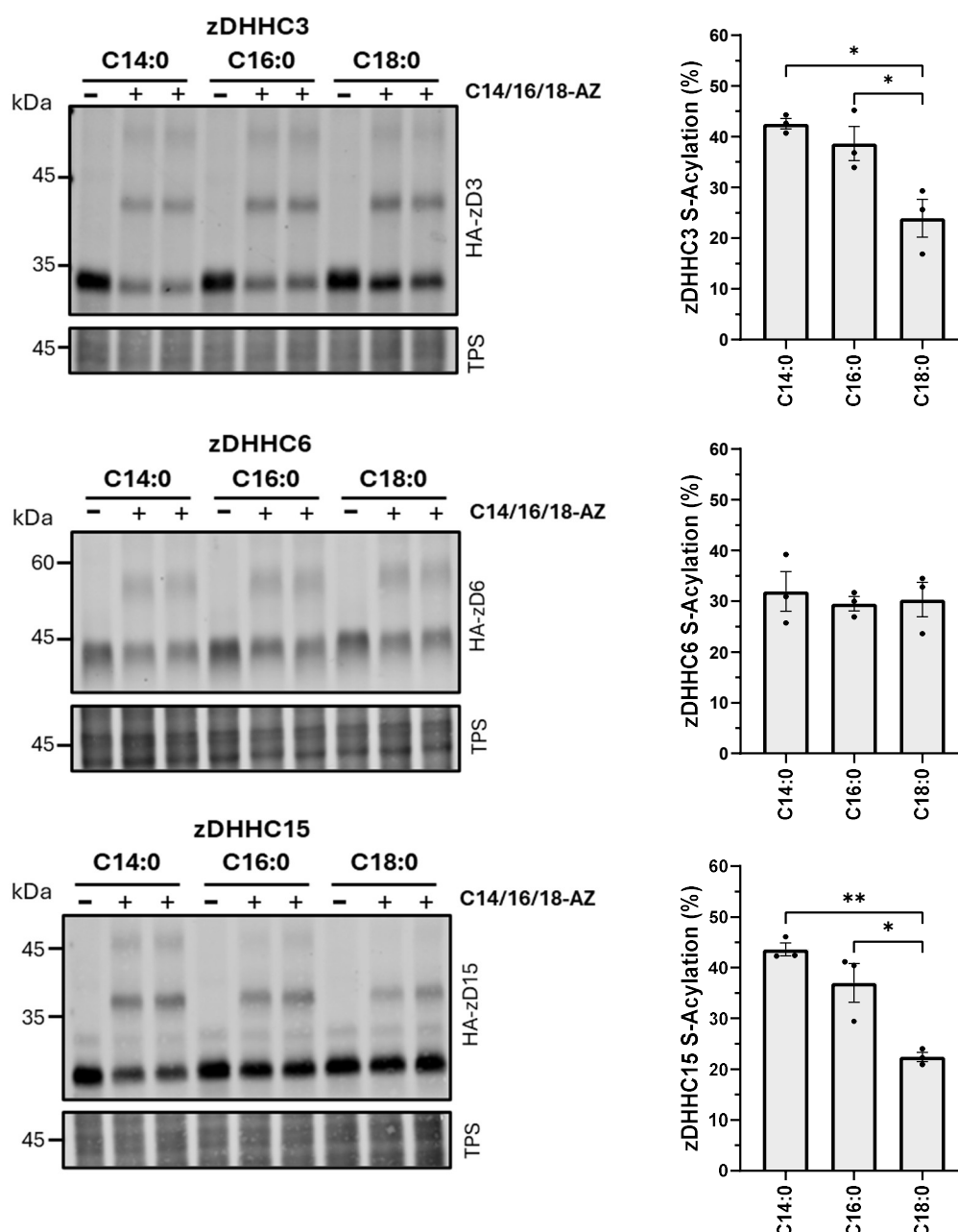


Figure 4.23. Fatty acid selectivity of zDHHC3, zDHHC6, and zDHHC15 assessed by metabolic labelling with fatty acid azide probes. HEK293T cells were transfected with plasmids encoding HA-tagged zDHHC3, zDHHC6, or zDHHC15. At 24 hours post-transfection, cells were incubated with 100 μ M fatty acid control (-) or fatty acid alkyne probes of defined chain length (C14:0-AZ, C16:0-AZ, or C18:0-AZ) for 4 hours. Labelled samples were subjected to click chemistry-based conjugation with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing zDHHC enzyme S-acylation patterns (top; HA-zDHHC3/zDHHC6/zDHHC15) and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of zDHHC S-acylation expressed as the mean percentage of S-acylated species relative to total signal (non-acylated plus acylated bands) for each fatty acid condition. Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (* $p<0.05$, ** $p<0.01$).

For zDHHC3, immunoblot analysis revealed a consistent S-acylation pattern when cells were labelled with C14:0 and C16:0, with a single non-acylated band and two distinct higher molecular weight bands corresponding to S-acylated species (**Figure 4.23A**). In contrast, C18:0 labelling resulted in a visibly stronger non-acylated band relative to the total signal. Quantification confirmed this trend, with S-acylation levels of approximately 40 % for both C14:0 and C16:0, compared to a significant reduction to ~25 % with C18:0 (**Figure 4.23B**), indicating reduced utilisation of the longer-chain fatty acid by zDHHC3.

For zDHHC6, no obvious differences were observed across the three fatty acid conditions at the level of the immunoblots, with a single dominant S-acylated band present in all cases (**Figure 4.23A**). Quantification supported this observation, with S-acylation remaining relatively constant at approximately 30 % for C14:0, C16:0, and C18:0, and no statistically significant differences detected between conditions (**Figure 4.23B**). This suggests that, under these experimental conditions, zDHHC6 does not display strong selectivity for fatty acid chain length.

In contrast, zDHHC15 displayed a more pronounced dependence on fatty acid chain length. C14:0 and C16:0 labelling both produced two distinct S-acylated bands on immunoblots, consistent with efficient incorporation of these fatty acids (**Figure 4.23A**). However, C18:0 resulted in a clear reduction in S-acylated species. Quantification showed S-acylation levels of approximately 45 % with C14:0 and ~35 % with C16:0, with no significant difference between these two conditions. In comparison, C18:0 resulted in a significant reduction in S-acylation to approximately 20 % (**Figure 4.23B**), indicating reduced utilisation of the longer-chain fatty acid by zDHHC15.

These findings are consistent with previous reports that zDHHC3 and zDHHC15 preferentially utilise C16:0 fatty acyl-CoA over C18:0 substrates, while extending this observation by demonstrating a lack of strong chain-length selectivity for zDHHC6 under the conditions tested, therefore supporting the idea that individual zDHHC enzymes possess distinct and intrinsic fatty acid preference profiles.

4.3.16 Analysis of fatty acid selectivity patterns of CD4, CD9, and CCR5

The observed enzyme-specific differences in fatty acid utilisation by zDHHC3, zDHHC6, and zDHHC15 suggest that zDHHC enzymes possess intrinsic preferences for fatty acyl-CoA chain length, with zDHHC3 and zDHHC15 displaying reduced utilisation of C18:0 compared with C14:0 and C16:0, while zDHHC6 showed no clear chain-length dependence under the conditions tested. Given that zDHHC enzymes exhibit both distinct subcellular localisation and substrate selectivity, these properties may together influence not only whether a protein is S-acylated, but also the nature of the lipid moiety incorporated onto distinct substrates.

To determine whether enzyme-dependent fatty acid selectivity is reflected at the level of physiological substrates, the incorporation of fatty acids of differing chain length was next examined for CD4, CD9, and CCR5. These proteins were selected based on their defined zDHHC enzyme dependencies established in earlier experiments. CD4 is predominantly modified by zDHHC6, which is primarily associated with ER localisation, whereas CD9 and CCR5 are not substrates for zDHHC6 and instead undergo S-acylation mediated by Golgi-localised enzymes, including zDHHC3 and zDHHC15.

All three proteins exhibit detectable basal S-acylation under endogenous conditions without co-expression of exogenous zDHHC enzymes, allowing fatty acid incorporation to be assessed in a system that reflects native enzyme availability and compartmentalisation. This approach therefore enables direct comparison of fatty acid profile across substrates that are preferentially modified by ER-associated (zDHHC6) versus Golgi-localised (zDHHC3 and zDHHC15) enzymes, and provides a framework to assess whether enzyme-specific fatty acid preferences are translated into distinct substrate lipid signatures in cells.

FLAG-tagged CCR5 or CD4, or GFP-tagged CD9, were expressed in HEK293T cells and metabolically labelled with azide-linked myristic, palmitic, or stearic acid analogues. Following labelling, incorporated lipids were detected using click chemistry, allowing direct comparison of lipid incorporation across proteins with distinct transmembrane architectures.

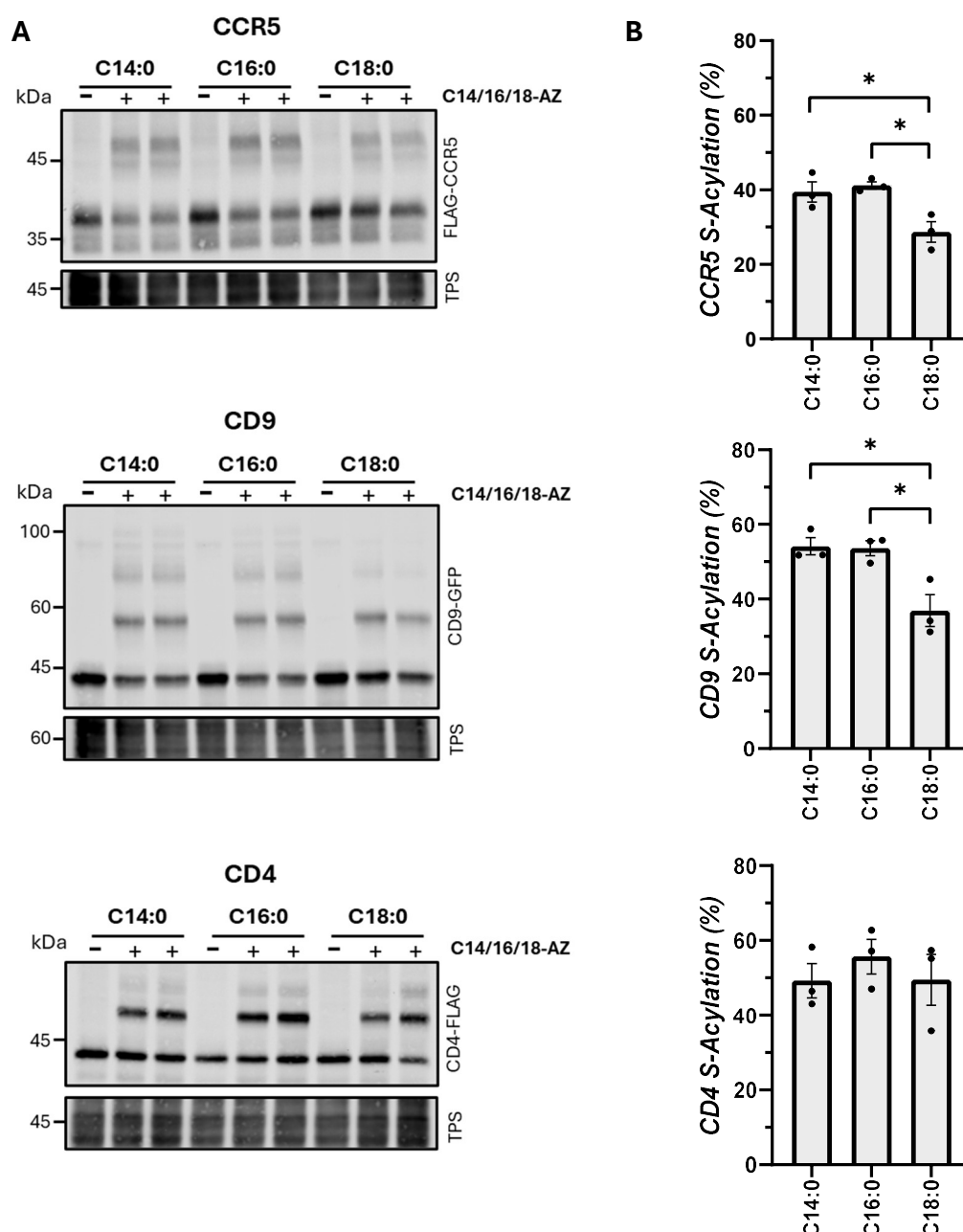


Figure 4.24. CCR5 and CD9 preferentially incorporate C14:0 and C16:0 over C18:0, while CD4 shows no lipid preference. HEK293T cells were transfected with plasmids encoding FLAG-tagged constructs of CCR5 or CD4 or GFP-tagged CD9. At 24 hours post-transfection, cells were incubated with 100 mM myristic acid (C14:0), palmitic acid (C16:0), or stearic acid (C18:0) control (-) or myristic acid azide, palmitic acid azide, or stearic acid azide (C14/16/18-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing CCR5, CD9 or CD4 S-acylation (top; FLAG-CCR5/CD9-GFP/CD4-FLAG) and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of CCR5, CD9 or CD4 S-acylation expressed as the mean percentage of the S-acylated bands relative to the total signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA ($*p<0.05$).

Immunoblot analysis revealed a clear reduction in S-acylation of both CCR5 and CD9 when cells were labelled with stearic acid (C18:0) compared with myristic (C14:0) or palmitic acid (C16:0) (**Figure 4.24A**). For CD9, this decrease was most pronounced in the upper S-acylated band, whereas the lower band showed comparatively little change, suggesting that only a subset of modification sites or acylated species is sensitive to fatty acid chain length.

Quantification confirmed a significant reduction in C18:0 incorporation for both proteins (**Figure 4.24B**). For CCR5, S-acylation decreased from approximately 40 % with C14:0 and C16:0 to ~30 % with C18:0. A more pronounced effect was observed for CD9, where S-acylation decreased from ~50 % with C14:0 and C16:0 to ~35 % with C18:0. No significant differences were detected between C14:0 and C16:0 for either protein.

In contrast, CD4 displayed no obvious differences in S-acylation across all three fatty acid conditions, with quantitative analysis confirming no statistically significant variation between C14:0, C16:0, or C18:0 labelling. This indicates that CD4 S-acylation is relatively insensitive to fatty acid chain length under these experimental conditions.

Together, this suggests that substrate S-acylation can exhibit distinct fatty acid selectivity profiles that are dependent on the protein context and likely reflect the underlying zDHHC enzyme responsible for modification.

4.3.17 Influence of cysteine position relative to the transmembrane domain on zDHHC6 fatty acid selectivity

The previous experiments showed that proteins modified by zDHHC3 and zDHHC15 preferentially incorporate C16:0 over C18:0 fatty acids, consistent with the reported fatty acid preferences of these enzymes (Greaves et al., 2017). In contrast, CD4, a substrate of zDHHC6, showed no preference between the two fatty acid chain lengths, suggesting that zDHHC6-mediated S-acylation is less sensitive to fatty acid identity under these conditions.

In addition, previous studies suggest that the spatial position of S-acylated cysteine residues relative to transmembrane domains can influence fatty acid selectivity. In single-pass transmembrane proteins, cysteines located within or immediately adjacent to the transmembrane region are more frequently associated with incorporation of longer, saturated fatty acids such as stearate, whereas cysteines positioned further into the cytoplasmic region tend to preferentially incorporate palmitate (Veit et al., 1991) (Kordyukova et al., 2008, Kordyukova et al., 2010, Brett et al., 2014). This raises the possibility that cysteine positioning may act in concert with enzyme identity to determine the final acyl chain composition of modified proteins.

To investigate this in a defined enzyme context, TMPRSS2 was selected as a model single-pass transmembrane substrate for zDHHC6 (**Figure 4.25**) (Salaun et al., 2023). The aim was to determine whether cysteine position relative to the transmembrane domain influences acyl chain specificity in a zDHHC6-dependent manner. TMPRSS2 was chosen due to its relatively simple S-acylation profile, containing a single well-defined S-acylation site, allowing direct interpretation of changes in fatty acid incorporation without confounding contributions from multiple modified residues.

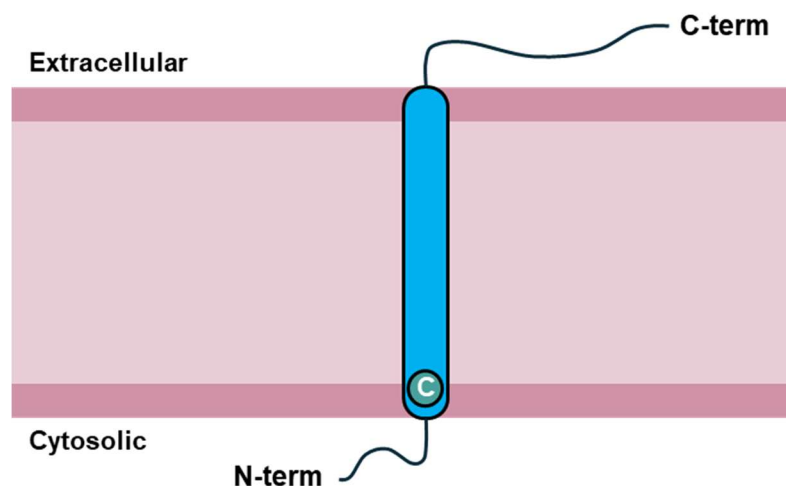


Figure 4.25. Schematic representation of TMPRSS2 highlighting the S-acylation site. TMPRSS2 is shown embedded in the plasma membrane, with the transmembrane domain depicted in blue. The site of S-acylation (cysteine 123) is indicated in green.

To assess this, the native S-acylated cysteine (C123), located two residues within the transmembrane domain, was repositioned relative to the membrane interface to evaluate the effect of spatial context on fatty acid selectivity (**Figure 4.26**). In one construct, the cysteine was shifted towards the cytoplasmic side, immediately preceding the transmembrane domain (A121C C123A), while in a second construct it was moved further into the transmembrane region (C123T T125C). As TMPRSS2 exhibits minimal basal S-acylation under these conditions, FLAG-tagged constructs were co-expressed with HA-tagged zDHHC6 to enable detectable modification. Cells were then metabolically labelled with either palmitic acid azide or stearic acid azide, followed by click chemistry-based detection, allowing direct assessment of how cysteine position relative to the transmembrane domain influences fatty acid selectivity mediated by zDHHC6.

TMPRSS2 WT

---PKSPSGTVCTSKTKKA **LC**ITLTTLGTFLVGAALAAGLL

TMPRSS2 A121C C123A

---PKSPSGTVCTSKTKK**C** LAITLTTLGTFLVGAALAAGLL

TMPRSS2 C123T T125C

---PKSPSGTVCTSKTKKA **LTIC**LTTLGTFLVGAALAAGLL

Figure 4.26. Schematic representation of TMPRSS2 constructs with the S-acylation site repositioned. A segment of the TMPRSS2 sequence is shown, including part of the N-terminal cytosolic region followed by the transmembrane domain (blue). The S-acylation site (cysteine 123) is highlighted in yellow. The position of this cysteine is indicated for TMPRSS2 WT, TMPRSS2 A123C C123A, and TMPRSS2 C123T T125C.

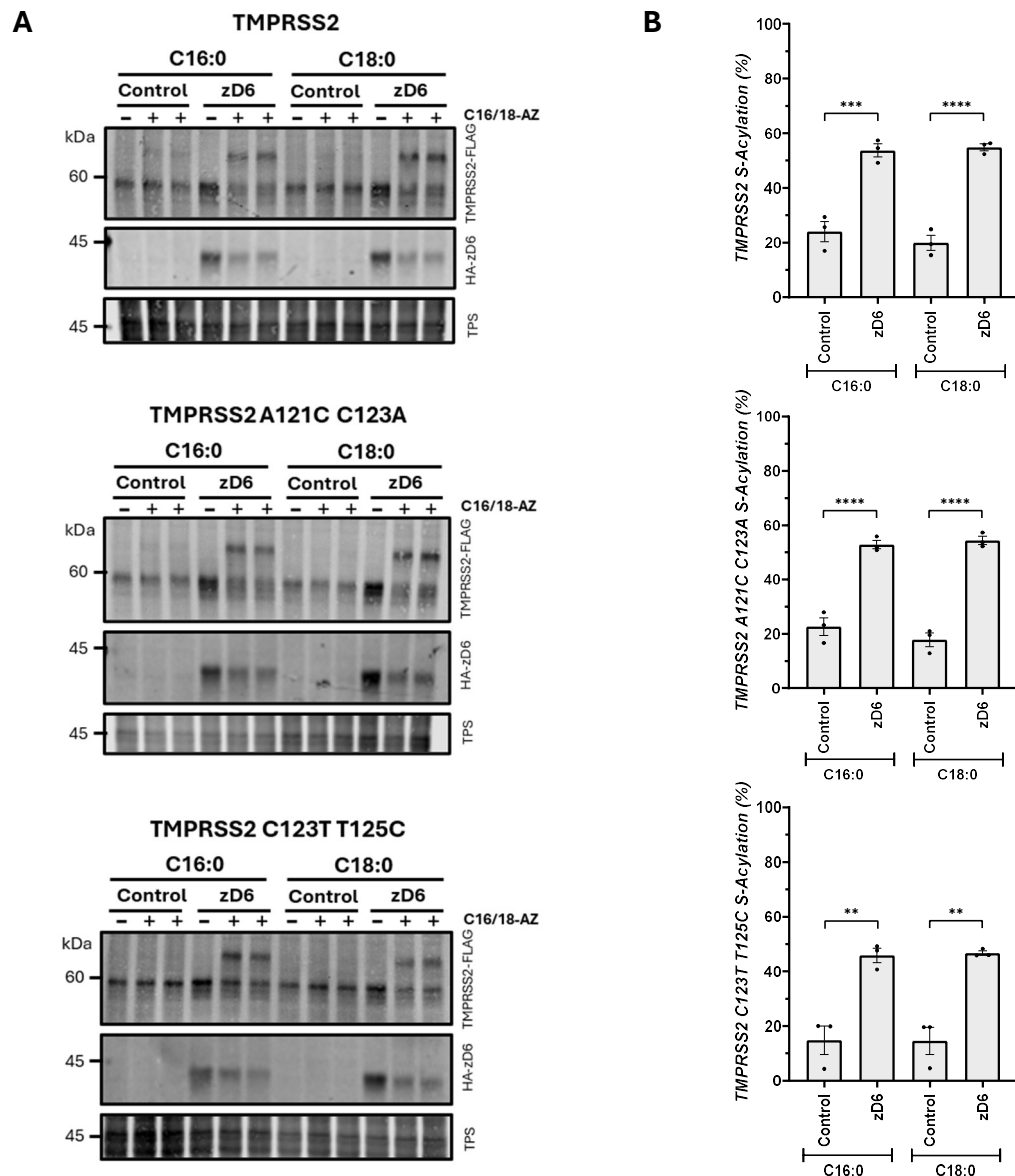


Figure 4.27. Moving the cysteine residue of TMPRSS2 relative to the transmembrane domain does not alter lipid selectivity. HEK293T cells were co-transfected with plasmids encoding FLAG-tagged constructs of TMPRSS2 either wild-type, or with the cysteine residue moved further into the cytoplasm (A121C C123A) or further into the transmembrane domain (C123T T125C) together with HA-tagged zDHHC6 or empty HA vector (control). At 24 hours post-transfection, cells were incubated with 100 Mm palmitic acid (C16:0) or stearic acid (C18:0) control (-) or palmitic acid azide or stearic acid azide (C16/18-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE. **(A)** Representative immunoblots showing TMPRSS2 S-acylation (top; TMPRSS2-FLAG), zDHHC6 enzyme expression (middle; HA-zDHHC6), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of TMPRSS2 S-acylation expressed as the mean percentage of the S-acylated bands relative to the total signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

For all TMPRSS2 constructs, co-expression with zDHHC6 resulted in a clear and significant increase in overall S-acylation. A single S-acylated band was detected for each construct in the presence of zDHHC6, whereas no detectable signal was observed under control conditions (**Figure 4.27A**). This pattern was consistent for both C16:0 and C18:0 labelling across all constructs.

Quantification confirmed that zDHHC6 increased TMPRSS2 S-acylation by approximately 25 % relative to empty vector controls, with no significant differences observed between C16:0 and C18:0 incorporation (**Figure 4.27B**). This indicates that zDHHC6 efficiently modifies TMPRSS2 regardless of fatty acid chain length under these conditions.

However, in contrast to expectations based on previous reports of cysteine-position-dependent lipid selectivity, no significant differences were observed between constructs in which the cysteine was repositioned relative to the transmembrane domain. This suggests that, in the context of TMPRSS2, altering cysteine position by two residues is not sufficient to influence fatty acid selectivity mediated by zDHHC6.

Together with the lack of fatty acid preference observed for CD4 and the absence of a C16:0 versus C18:0 bias in zDHHC6-mediated modification, these findings suggest that zDHHC6-driven S-acylation is relatively insensitive to both fatty acid chain length and modest changes in cysteine positioning relative to the membrane interface.

4.4 Discussion

4.4.1 Summary

This study began with the working hypothesis that juxta-membrane cysteine residues in transmembrane proteins might be a key determinant of zDHHC6 substrate selectivity, based on precedent from single-pass membrane proteins where proximity to the membrane was a feature of all modified proteins (Salaun et al., 2023). This provided a starting framework for examining how zDHHC enzymes recognise and modify diverse membrane proteins, particularly multi-pass receptors such as GPCRs.

Across the experimental dataset, this proximity-based model was not supported as a general determinant of zDHHC6 activity. In multiple GPCRs and other membrane proteins, cysteines positioned near transmembrane boundaries were neither sufficient nor predictive of zDHHC6-mediated S-acylation. Likewise, engineered repositioning of cysteines and manipulation of transmembrane context did not reliably confer zDHHC6 responsiveness, indicating that simple spatial optimisation is not enough to reprogram enzyme specificity.

Instead, a clearer pattern emerged in which enzyme identity was the dominant factor. zDHHC3 and zDHHC15 consistently acted as broadly permissive Golgi-associated enzymes, efficiently modifying a wide range of GPCRs and producing relatively consistent patterns of fatty acid incorporation. In contrast, zDHHC6 displayed a more restricted activity profile that could not be explained by cysteine proximity alone, and even substrates that were made structurally “favourable” under the original model did not become zDHHC6 targets.

This interpretation was reinforced by lipid selectivity analyses. Fatty acid usage was strongly enzyme-dependent, with zDHHC3 and zDHHC15 showing a consistent bias against longer acyl chains, whereas zDHHC6 showed comparatively little discrimination. This pattern was conserved across the small number of substrates that were examined, indicating that lipid selectivity is primarily an intrinsic property of the enzyme rather than being dictated by substrate topology. This was further reinforced by the finding that the acyl chains added to TMPRSS2 did not change as the modified cysteine was moved between cytosolic and membrane positions.

This supports a model in which zDHHC-mediated S-acylation is governed by a combination of enzyme identity, subcellular context, and higher-order substrate compatibility. Within this framework, zDHHC3 and zDHHC15 function as dominant, broadly acting modifiers within the secretory pathway, whereas zDHHC6 appears to act on a more restricted subset of substrates defined by factors beyond simple proximity rules.

4.4.2 Single-pass, multi-pass, and tetraspanin proteins collectively refine a juxta-membrane model for zDHHC6 selectivity

Previously, zDHHC6-mediated S-acylation of the single-pass transmembrane protein CD4 had only been demonstrated using a truncated construct (Salaun et al., 2023). In this study, full-length CD4 was efficiently S-acylated, with the strongest increase observed upon co-expression with zDHHC6, although zDHHC3 also promoted S-acylation (**Figure 4.2**). The presence of two distinct S-acylated species was consistent with previous reports identifying two C-terminal cysteine residues as modification sites (Crise and Rose, 1992).

At first glance, the strong S-acylation of CD4 by zDHHC6 appeared consistent with a juxta-membrane model in which zDHHC6 preferentially targets single-pass membrane proteins containing cysteine residues positioned close to the transmembrane domain. This model is supported by multiple established zDHHC6 substrates, including calnexin, transferrin receptor, and IFITM3, which are single-pass membrane proteins containing cysteine residues located in close proximity to their transmembrane domains (Lakkaraju et al., 2012, Senyilmaz et al., 2015, McMichael et al., 2017, Abrami et al., 2017, Salaun et al., 2023). Similar architecture is also observed in TMPRSS2, further reinforcing the idea that zDHHC6 preferentially targets membrane-proximal cysteines in relatively simple transmembrane contexts (Salaun et al., 2023). However, the comparable activity of zDHHC3 indicates that cysteine proximity alone is not sufficient to define enzyme specificity. Instead, CD4 appears to be accessible to multiple zDHHC enzymes, with relative contributions likely influenced by enzyme localisation and expression level.

Extension of these analyses to a two-pass transmembrane protein, K5, further demonstrated that zDHHC6 can act on more complex membrane architectures (**Figure 4.4**). S-Acylation of K5 has previously been reported at a single cysteine residue (C146) located immediately following the second transmembrane domain within the C-terminal region (Anania and Coscoy, 2011). The appearance of a single S-acylated band in the present study is consistent with modification at a single site, in agreement with these findings. Notably, while this study identified the site of S-acylation, the zDHHC enzymes responsible for this modification were not defined, and the current data therefore extend these observations by demonstrating that both zDHHC3 and zDHHC6 are capable of modifying K5 under these conditions. In contrast, zDHHC15 did not enhance K5 S-acylation. These findings indicate that zDHHC6 is not limited to single-pass substrates, but can also modify more complex membrane architectures, provided that suitably positioned cysteine residues are accessible within the membrane environment. Despite identifying K5 as a two-pass transmembrane substrate for zDHHC3 and zDHHC6, I was not able to identify additional well-characterised examples of two-pass transmembrane proteins with experimentally defined S-acylation sites and assigned zDHHC enzymes in the current literature. It is possible that such proteins exist but have not yet been investigated or clearly annotated in terms of both modification sites and responsible enzymes. As a result, K5 represents one of the few experimentally tractable examples of this topology within the present study, and broader conclusions regarding S-acylation of

two-pass membrane proteins remain limited by the availability of comparable characterised systems.

Following the observation that zDHHC6 can S-acylate a two-pass transmembrane protein, the analysis was extended to larger multi-pass membrane proteins such as tetraspanins to assess how increased structural complexity influences enzyme activity. CD9, a well-characterised tetraspanin extensively S-acylated by the Golgi-localised enzyme zDHHC2, did not show increased S-acylation upon zDHHC6 co-expression despite containing multiple juxta-membrane cysteine residues (**Figure 4.6**) (Sharma et al., 2008). In contrast, co-expression with zDHHC3 produced a clear increase in CD9 S-acylation, representing a finding not previously reported in overexpression studies. Sharma et al. observed no obvious change in CD9 S-acylation following zDHHC3 siRNA-mediated knockdown, although zDHHC3 had not been assessed in an overexpression context in that work. This discrepancy may reflect differences between overexpression and knockdown approaches, where overexpression could enhance non-physiological enzyme-substrate interactions, whereas knockdown effects may be masked by redundancy among Golgi-localised zDHH enzymes. In agreement with previous overexpression data, zDHHC15 did not increase CD9 S-acylation in the current study, despite both zDHHC3 and zDHHC15 being Golgi-localised enzymes (Sharma et al., 2008).

In contrast to CD9, TSPAN4, which had not previously been experimentally confirmed as S-acylated, was clearly modified by zDHHC6 (**Figure 4.6**). As with CD9, multiple S-acylated species were observed, suggesting the presence of more than one modification site; however, definitive assignment of the number and location of these sites would require site-directed mutagenesis. TSPAN4 was also S-acylated by zDHHC3 but not by zDHHC15, further supporting the idea that individual zDHH enzymes exhibit distinct substrate preferences that extend beyond shared subcellular localisation and likely reflect additional structural or contextual determinants.

Although both CD9 and TSPAN4 contain cytoplasmic cysteine residues near transmembrane domains, their spatial organisation differs markedly. CD9 contains multiple cysteines positioned extremely close to transmembrane boundaries, including within intracellular loops and at transmembrane interfaces, whereas the closest cysteine in TSPAN4 is positioned approximately five residues from a membrane boundary. Structural context may further contribute to these differences. CD9 is known to partition into tetraspanin-enriched microdomains, where it engages in extensive protein-protein and protein-lipid interactions characteristic of organised tetraspanin assemblies, which may restrict enzyme access (Reyes et al., 2018, Huang and Yu, 2022). In addition, its short C-terminal tail may limit conformational flexibility required for productive enzyme engagement. In contrast, TSPAN4 is also a member of the tetraspanin family and is therefore expected to localise to tetraspanin-enriched microdomains; however, its organisation within these structures has been less extensively characterised than canonical tetraspanins such as CD9. It may therefore present a comparatively more permissive environment for zDHHC6 activity, although this remains speculative in the

absence of direct evidence for differences in microdomain organisation or enzyme accessibility.

Across single-pass, multi-pass, and tetraspanin substrates, a consistent pattern is observed in which zDHHC6 can modify structurally diverse membrane proteins, although its activity is not determined solely by the presence of juxta-membrane cysteine residues. Instead, S-acylation likely depends on a combination of substrate and enzyme-specific factors, as well as features of the surrounding membrane environment, although the relative contribution of each of these factors remains speculative. These findings refine the original juxta-membrane model, suggesting that while cysteine proximity to transmembrane domains may contribute to substrate compatibility, it is not sufficient on its own to predict zDHHC6-mediated S-acylation.

4.4.3 GPCR S-acylation is predominantly mediated by Golgi-localised zDHHC3 and zDHHC15, whereas zDHHC6 exhibits restricted activity across GPCRs tested

Extending the analysis from tetraspanins to a broad panel of seven-transmembrane GPCRs enabled evaluation of zDHHC enzyme selectivity within a structurally conserved yet highly diverse protein family. Despite their shared topology, GPCRs vary considerably in sequence, trafficking routes, and post-translational modification patterns, providing a background to assess whether common structural principles govern zDHHC recognition or whether S-acylation is primarily driven by receptor-specific features and enzyme localisation, or more likely a combination of both.

A key outcome of this study is the identification of several GPCRs for which S-acylation could be directly demonstrated and for which the responsible zDHHC enzymes were previously unknown, thereby extending the GPCR lipidation network. Across the receptor panel, β 2AR, V1aR, OXTR, 5HTR2A, and CXCR4 all showed robust increases in S-acylation upon co-expression with the Golgi-localised enzymes zDHHC3 and zDHHC15 (**Figures 4.8, 4.10, 4.12**), supporting their role as broadly acting GPCR S-acyltransferases. Indeed, this profile was also seen with CCR5 in Chapter 3.

Within this dataset, OXTR is established here as a previously unreported S-acylated GPCR, while CXCR4 is similarly identified as a novel member of the chemokine receptor family undergoing S-acylation. For 5HTR2A, prior palmitoyl-proteomic analysis of mouse forebrain tissue had suggested S-acylation, although this had not been independently validated at the level of the individual protein (Collins et al., 2017). The modified cysteine reported in those datasets lies 13 amino acids downstream of the final transmembrane domain and is conserved in the human receptor; however, this site has not been experimentally confirmed, and other cysteine residues remain potential candidates in the absence of site-directed mutagenesis. For V1aR, S-acylation has been characterised in the rat receptor but had not been directly assessed in the human protein. Previous work identified C371 and C372 as modified residues and proposed additional S-acylation at either C365 or a cysteine within an intracellular loop (Hawtin et al., 2001). The present data extend these findings by demonstrating S-acylation of the human V1aR and

identifying zDHHC3 and zDHHC15 as enzymes capable of enhancing this modification. In contrast, S-acylation of β 2AR is well established and occurs at a conserved C-terminal cysteine together with an additional intracellular loop cysteine (O'Dowd et al., 1989, Loisel et al., 1996, Adachi et al., 2016). Notably, intracellular loop S-acylation of β 2AR has been shown to be mediated by Golgi-localised zDHHC enzymes, including zDHHC9, zDHHC14, and zDHHC18 (Adachi et al., 2016), consistent with the broader pattern observed here for zDHHC3 and zDHHC15 across multiple GPCRs.

β 1AR and V1bR showed no detectable changes in S-acylation under any of the conditions tested. More broadly, zDHHC6 did not enhance S-acylation for most GPCRs examined in this panel. A key feature of the receptors analysed is that they generally do not contain cysteine residues positioned immediately adjacent to the final transmembrane domain. Instead, cysteines are typically located within intracellular loops, at more distal positions in the C-terminal tail, or at the cytoplasmic ends of non-terminal transmembrane domains. Within this context, CXCR4, despite lacking a canonical juxta-membrane C-terminal cysteine, was still efficiently S-acylated by zDHHC3 and zDHHC15, demonstrating that intracellular loop cysteines can act as functional substrates and reinforcing that GPCR S-acylation is not restricted to C-terminal juxta-membrane motifs. Similarly, the presence of multiple S-acylated species in OXTR and 5HTR2A is consistent with multi-site modification within individual receptors.

This data indicates that the presence of juxta-membrane cysteines alone is not sufficient to confer zDHHC6-dependent S-acylation in GPCRs. However, because most GPCRs analysed here do not naturally contain cysteine residues positioned immediately adjacent to the final transmembrane domain, these experiments do not directly address whether such residues, when present, would support zDHHC6-mediated modification. In many GPCRs, S-acylation sites are located within the proximal C-terminal tail, typically several residues downstream of transmembrane domain seven, where they are thought to contribute to anchoring the C-terminus to the inner leaflet of the membrane and stabilising receptor conformation (Patwardhan et al., 2021). This anchoring role may help explain why cysteine residues are often not positioned directly at the end of the final transmembrane domain, as residues immediately adjacent to the membrane interface may be sterically constrained or partially embedded within the lipid bilayer, limiting their accessibility to zDHHC enzymes. Instead, cysteines located slightly further into the C-terminal tail are likely more exposed and therefore more accessible for modification. Although zDHHC6 did not enhance S-acylation for the majority of GPCRs tested, this should not be interpreted as evidence of a general inability of the enzyme to modify GPCRs, but rather reflects the limited set of receptors examined.

In contrast to this general pattern, GPR37 represents a particularly informative exception. Unlike most GPCRs examined, GPR37 contains cysteine residues positioned juxta-membrane to the final transmembrane domain alongside a broader intracellular cysteine network. GPR37 was robustly S-acylated by zDHHC3 and zDHHC15, and uniquely also displayed detectable zDHHC6-mediated S-acylation, albeit with a more restricted pattern of modification (**Figure 4.14**). This makes GPR37 the only GPCR in this study in

which zDHHC6-dependent S-acylation is observed, highlighting that zDHHC6 can act on GPCRs, but only in a limited subset of cases identified here.

These findings suggest that zDHHC3 and zDHHC15 function as broadly acting GPCR S-acyltransferases within the Golgi, while zDHHC6 exhibits a comparatively restricted activity profile across the GPCRs tested. Importantly, this work not only defines enzyme-substrate relationships across a diverse GPCR panel but also identifies several GPCRs for which the modifying enzymes were previously unknown, expanding the current map of GPCR S-acylation. Overall, the data support a model in which GPCR S-acylation is primarily governed by enzyme localisation and substrate accessibility within the secretory pathway, rather than predictable rules based on cysteine positioning alone.

4.4.4 Structural determinants of zDHHC6 S-acylation in GPCRs

Following the observation that most GPCRs tested were not efficiently modified by zDHHC6, despite being robust substrates for zDHHC3 and zDHHC15, targeted structural manipulations were used to test whether GPCR architecture could be made permissive to zDHHC6 activity. This was motivated by the working hypothesis that cysteine proximity to membrane interfaces contributes to zDHHC6-mediated S-acylation, and by the behaviour of GPR37, which uniquely showed zDHHC6-dependent modification.

To test this, CCR5 was used as a model to either reposition endogenous cysteines closer to the membrane via C-terminal truncation or introduce engineered juxta-membrane cysteines in CCR5 and β 2AR. In all cases, these changes failed to confer zDHHC6-dependent S-acylation (**Figure 4.17, 4.18, and 4.19**). In parallel, mutagenesis of GPR37 C551/C553 did not abolish the zDHHC6-dependent pattern, indicating that modification is not driven solely by these juxta-membrane residues and is likely distributed across other intracellular cysteines (**Figure 15**).

Together, these approaches demonstrate that neither repositioning existing cysteines nor introducing juxta-membrane cysteines is sufficient to reprogram GPCRs into zDHHC6 substrates. These findings refine the original hypothesis by showing that, although juxta-membrane positioning can contribute to S-acylation in some single-pass proteins, it does not translate directly to GPCRs. Instead, zDHHC6-dependent modification appears restricted to a limited subset of receptors, with GPR37 as the only clear example identified here. While the basis for this selectivity may involve additional structural or contextual factors, these were not directly resolved in this study. Overall, membrane proximity alone is insufficient to explain zDHHC6 activity across GPCRs, indicating that further determinants are required.

4.4.5 Influence of subcellular localisation on zDHHC enzyme selectivity

A central question arising from the preceding experiments was whether the subcellular localisation of zDHHC enzymes contributes to their apparent selectivity toward GPCR substrates. Given that zDHHC3 and zDHHC15 are primarily Golgi-localised, it was hypothesised that their activity toward GPCRs might depend on access to post-ER compartments during receptor trafficking (Greaves et al., 2010).

To test this directly, zDHHC3 was experimentally re-localised to the ER using a C-terminal EKKNR retention motif (Gorleku et al., 2011). This approach allowed assessment of whether restricting enzyme localisation to an earlier secretory compartment would impair its ability to modify GPCR substrates. ER-retained zDHHC3 remained fully competent to S-acylate both CCR5 and β 2AR (**Figure 4.20**). This indicates that GPCR S-acylation by zDHHC3 is not strictly dependent on Golgi localisation under these experimental conditions, and that catalytic activity can be maintained outside its predominant endogenous compartment. This is consistent with previous studies, which found zDHHC3 EKKNR was still able to efficiently S-acylate its substrates SNAP25 and CSP (Gorleku et al., 2011). This finding also suggests that the inability of zDHHC6 to S-acylate GPCRs does not arise due to an inability of these proteins to undergo modification at this compartment.

With this approach however, it should be noted that the added EKKNR motif may mediate incomplete retention and transient trafficking to the Golgi cannot be excluded. Consistent with this, the EKKNR motif may not fully prevent escape from the ER, and overexpression could further obscure localisation-dependent constraints. However, as there was no detectable difference in activity of WT and EKKNR mutant zDHHC3 against GPCRs, the result is consistent with there being no specific constraint that prevents GPCR S-acylation from occurring at the ER. A secondary observation was a reduction in steady-state β 2AR levels upon co-expression with ER-retained zDHHC3. However, cycloheximide chase experiments did not reveal a clear change in overall degradation kinetics (**Figure 4.21**). Because this assay measures total cellular protein, it cannot distinguish between intracellular and plasma membrane pools, and therefore may not capture compartment-specific effects or rapid early trafficking events occurring within the shorter time frame of the labelling experiments. As a result, subtle effects of altered enzyme localisation on receptor trafficking or stability cannot be excluded. Therefore, future work might explore this question more rigorously using metabolic labelling experiments with radiolabelled methionine.

After establishing that enzyme re-localisation does not abolish GPCR S-acylation, the next question was whether restricting substrate localisation within the secretory pathway influences the extent or site of S-acylation. This was tested using ER retention of β 2AR, with and without an engineered juxta-membrane cysteine (C329ins), allowing assessment of both (i) the contribution of receptor trafficking to S-acylation and (ii) whether increased cysteine proximity to the membrane could enhance modification at the ER. Under control conditions, both wild-type β 2AR and the C329ins variant exhibited comparable basal levels of S-acylation, consistent with earlier experiments showing that

introduction of a juxta-membrane cysteine alone does not substantially alter overall receptor modification (**Figure 4.22**). In contrast, the addition of the EKKNR ER retention sequence resulted in a marked reduction in S-acylation for both constructs. This demonstrates that retention of β 2AR at the ER significantly limits its S-acylation, indicating that efficient modification of this receptor normally occurs after exit from the ER. This finding is consistent with a model in which β 2AR S-acylation predominantly occurs at post-ER compartments, where zDHHC3 and zDHHC15 are enriched. It also aligns with previous RUSH-based experiments on CCR5 (**Chapter 3 Figure 3.8**), which showed increased S-acylation upon ER release, supporting the idea that trafficking into later secretory compartments enhances access to relevant zDHHC enzymes. It should be noted that the expression of the β 2AR EKKNR constructs was decreased compared to the wild-type. This reduction may reflect general effects associated with ER retention on receptor handling and steady-state levels, including altered distribution within cellular compartments and differences in overall protein processing. However, this does not change the finding that S-acylation was decreased, as S-acylation was determined relative to total expression. Importantly, the similarity between wild-type and C329ins β 2AR in both trafficking-competent and ER-retained conditions further indicates that introducing a juxta-membrane cysteine does not enhance S-acylation. This reinforces the conclusion that cysteine positioning alone is not a major limiting factor in determining GPCR S-acylation in this system.

Future work should aim to more precisely define the compartmental origin of β 2AR S-acylation, for example, through compartment-restricted enzyme targeting, site-specific mutagenesis, and mass spectrometry-based mapping. In addition, real-time trafficking approaches could help resolve whether S-acylation occurs predominantly during post-ER transit. Together, these approaches would provide a more complete understanding of how spatial organisation of both enzyme and substrate governs GPCR S-acylation.

4.4.6 Lipid selectivity in S-acylation of multi-transmembrane proteins

Following the observation that zDHHC6 activity toward GPCRs is restricted and not readily explained by juxta-membrane cysteine positioning alone, attention was next directed towards whether enzyme identity might also influence an additional layer of specificity, namely the fatty acid composition of S-acylated substrates. This represents a further extension of the broader question emerging from this chapter: if cysteine position and receptor architecture are insufficient to predict zDHHC6 activity, do they also influence the nature of the lipid modification itself, or is this instead primarily determined by the enzyme?

To address this, incorporation of myristic (C14:0), palmitic (C16:0), and stearic (C18:0) acids was compared across substrates with distinct zDHHC enzyme dependencies. These experiments were designed to determine whether fatty acid selectivity is an intrinsic property of individual zDHHC enzymes or whether it is modulated by substrate architecture and transmembrane organisation. To first establish baseline enzyme

behaviour, zDHHC3, zDHHC6, and zDHHC15 were analysed in isolation. zDHHC3 and zDHHC15 both showed reduced incorporation of C18:0 relative to C14:0 and C16:0, consistent with a preference for shorter acyl chains and in agreement with previous reports (**Figure 4.23**) (Greaves et al., 2017). In contrast, zDHHC6 showed no significant differences in incorporation between fatty acid chain lengths, indicating comparatively weak discrimination under these conditions. Together, these findings suggest that zDHHC3 and zDHHC15 exhibit more restricted fatty acid usage, whereas zDHHC6 is comparatively permissive.

This enzyme-specific behaviour was reflected at the substrate level. For multi-transmembrane proteins primarily modified by Golgi-localised enzymes, CCR5 and CD9, stearic acid incorporation was consistently reduced relative to myristic and palmitic acid (**Figure 4.24**). Notably for CD9, this effect appeared to be largely confined to one of the two S-acylated species, suggesting that individual modification sites or lipidated states may differ in their sensitivity to acyl chain length. These patterns are consistent with modification by zDHHC3 and zDHHC15 and reflect the enzyme-intrinsic preference for C16:0 over C18:0 observed in the enzyme-only assays. In contrast, CD4, which is primarily modified by zDHHC6, showed no detectable differences in incorporation between C14:0, C16:0, or C18:0. This is consistent with the absence of strong fatty acid discrimination by zDHHC6 in the enzyme-only experiments and suggests that CD4 S-acylation is relatively insensitive to acyl chain length under these conditions. Direct examples of substrate-intrinsic preference for different acyl chain lengths are limited in mammalian systems. However, viral glycoproteins such as influenza haemagglutinin exhibit site-dependent differences in acyl-chain incorporation that influence membrane partitioning (Kordyukova et al., 2008), while tetraspanins such as CD9 display multiple S-acylated states consistent with heterogeneous lipid modification across individual cysteine residues (Sharma et al., 2008). Together, these studies suggest that lipid composition can vary across modification sites, although the extent to which this reflects substrate-intrinsic preference versus enzyme- and membrane-dependent effects remains unclear.

To extend this analysis beyond substrate identity and directly test whether spatial features of the substrate influence lipid choice, cysteine positioning was next examined using TMPRSS2 relocation mutants in the context of zDHHC6-mediated S-acylation. Although zDHHC6 increased overall S-acylation of all constructs, no differences were observed between C16:0 and C18:0 incorporation regardless of whether the cysteine was positioned closer to or further within the transmembrane domain (**Figure 4.27**). This directly tests the positional hypothesis raised earlier in the chapter and indicates that, at least in this system, cysteine proximity to the membrane does not measurably influence fatty acid selectivity. This contrasts with previous work in simpler single-pass membrane proteins, particularly influenza haemagglutinin, where cysteine positioning relative to the membrane interface has been shown to determine acyl-chain identity. In HA, a transmembrane-border cysteine preferentially incorporates stearate (C18:0), whereas more cytoplasmic juxta-membrane cysteines are predominantly modified with palmitate (C16:0), indicating that spatial positioning can act as a key determinant of lipid selection

in simpler membrane architectures (Kordyukova et al., 2008, Brett et al., 2014). Taken together, these findings suggest that positional rules governing acyl-chain choice in single-pass systems may not be broadly applicable to more complex substrates or to zDHHC6-mediated reactions in the context examined here. A caveat here is that we do not have evidence that the TMPRSS2 amino acid changes result in a change in membrane-insertion depth of the target cysteine.

This suggests zDHHC3 and zDHHC15 consistently impose a preference against stearate that is reflected in their substrates, whereas zDHHC6 exhibits minimal chain-length discrimination, and this behaviour is maintained across both enzyme-only and substrate-based assays. However, these conclusions remain provisional given the limited number of substrates and enzymes analysed, and the possibility that additional protein classes or physiological contexts may reveal more nuanced contributions of substrate architecture to lipid selectivity.

4.4.7 Key Takeaways

- Juxta-membrane cysteine proximity alone does not appear to be a general determinant of zDHHC6 substrate selectivity.
- zDHHC3 and zDHHC15 exhibit relatively broad substrate permissivity across multiple GPCRs, efficiently modifying diverse targets, whereas zDHHC6 appears to act primarily on single-pass transmembrane proteins but also shows some selective activity against multi-transmembrane proteins.
- Differences in fatty acid incorporation suggest that lipid selectivity may be largely enzyme-intrinsic, rather than dictated by substrate topology.

CHAPTER 5

Type III Endosome Membrane Protein

Chapter 5 - Type III Endosome Membrane Protein

5.1 Introduction

An important area of research in the S-acylation field is to discover new substrates and regulators of zDHHC enzymes. The BioPlex database is a large-scale resource containing information from immunoprecipitation-mass spectrometry experiments used to profile cellular protein-protein interactions (Huttlin et al., 2021). In a bid to identify new protein interactions of zDHHC enzymes, BioPlex was explored. A notable interaction was identified between Type III endosome membrane protein (TEMP; C1orf210) and two highly-conserved zDHHC enzymes, zDHHC3 and zDHHC7 (**Figure 5.1**).

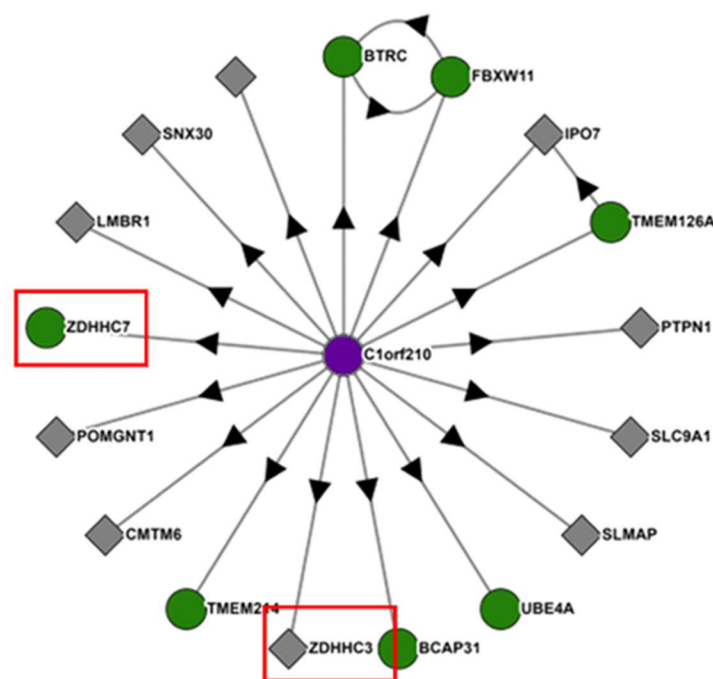


Figure 5.1. TEMP (C1orf210) interaction network derived from the BioPlex dataset. Grey diamonds represent prey proteins, while green circles represent bait proteins. zDHHC3 and zDHHC7 are highlighted by red boxes. Data accessed from the BioPlex resource: <https://bioplex.hms.harvard.edu/>

TEMP is a poorly characterised protein, with only a single publication to-date (Aturaliya et al., 2012). This study reported that TEMP localises to both the plasma membrane and endosomal compartments, indicating that it traffics through multiple membrane systems and may participate in processes linked to protein localisation or turnover. TEMP

was especially interesting in the context of this thesis, as it is a single-pass transmembrane protein.

TEMP contains several structural features which suggest it could be regulated by post-translational modifications. These include two cytosolic juxta-membrane cysteine residues, which may serve as sites of S-acylation, and a predicted N-terminal glycosylation site (**Figure 5.2**) (Aturaliya et al., 2012). Notably, the positioning of these cysteine residues immediately adjacent to the transmembrane domain is consistent with known features of zDHHC6 substrates, supporting the hypothesis that TEMP may be a zDHHC6 substrate. However, the protein was only present in the Bioplex networks of zDHHC3 and zDHHC7, not zDHHC6.

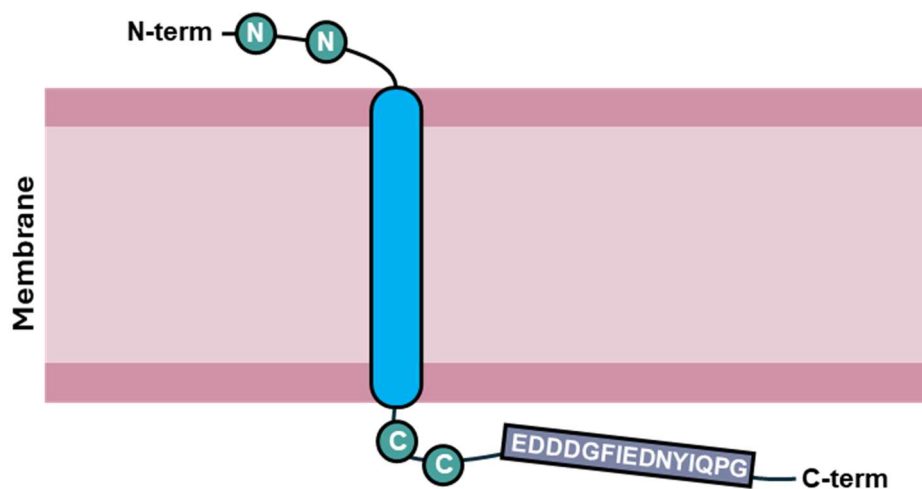


Figure 5.2. Diagram representing the structure of Type III Endosome Membrane Protein (TEMP). TEMP is shown embedded in the membrane with a single transmembrane domain (blue), C-terminal conserved domain (purple), and key amino acids (green).

The stable interaction of TEMP with zDHHC3 and zDHHC7, sufficient to withstand immunoprecipitation recovery, is unusual for these enzyme isoforms, which have been suggested to have only weak or transient interactions with their substrates (Lemonidis et al., 2014). Thus, it was unclear if TEMP was acting as a typical substrate of these enzymes. In addition, the fact that TEMP displayed characteristics that make it a likely substrate of zDHHC6 generated further interest in this protein. Therefore, this chapter set out to investigate the interactions and post-translational modifications of TEMP in more detail.

5.2 Aims

- Confirm that TEMP interacts with zDHHC3 and zDHHC7 and test whether this interaction is specific for these isoforms
- Establish whether TEMP is S-acylated by zDHHC3, zDHHC7 or zDHHC6
- Explore the interplay between S-acylation and glycosylation of TEMP
- Examine if glycosylation and S-acylation impact the stability of TEMP

Hypothesis

TEMP interacts with multiple zDHHC enzymes, is a novel S-acylated protein, and is a potential substrate of zDHHC6.

5.3 Results

5.3.1 *TEMP associates with multiple zDHHC enzymes*

In a search for novel proteins that interact with zDHHC enzymes, TEMP (also known as C1orf210) was identified as a protein interacting with both zDHHC3 and zDHHC7 using data from the BioPlex resource. The identification of TEMP association with zDHHC3 and zDHHC7 in the BioPlex dataset prompted several key questions. First, it was important to determine if the co-immunoprecipitation of TEMP with zDHHC3 and zDHHC7 could be confirmed, and also to test if TEMP interacts with other zDHHC enzymes.

For this, co-immunoprecipitation assays were performed in HEK293T cells. GFP-tagged TEMP or an empty GFP control construct were co-expressed with a panel of zDHHC enzymes, followed by immunoprecipitation using GFP-trap beads. The panel included zDHHC3 and zDHHC7, as well as additional enzymes selected to assess interaction specificity. zDHHC6 was included based on its reported preference for single-pass transmembrane proteins (Salaun et al., 2023), while zDHHC2 and zDHHC17 were chosen to represent zDHHC enzymes with differing subcellular localisations and substrate profiles.

Consistent with the BioPlex data, GFP-TEMP co-immunoprecipitated both zDHHC3 and zDHHC7 (**Figure 5.3**), supporting the validity of these previously reported associations. Notably, TEMP was also found to associate with zDHHC2 and zDHHC17, indicating that its interaction profile is not restricted to the enzymes identified in the interactome dataset. In contrast, no interaction was detected between TEMP and zDHHC6 under the conditions tested.

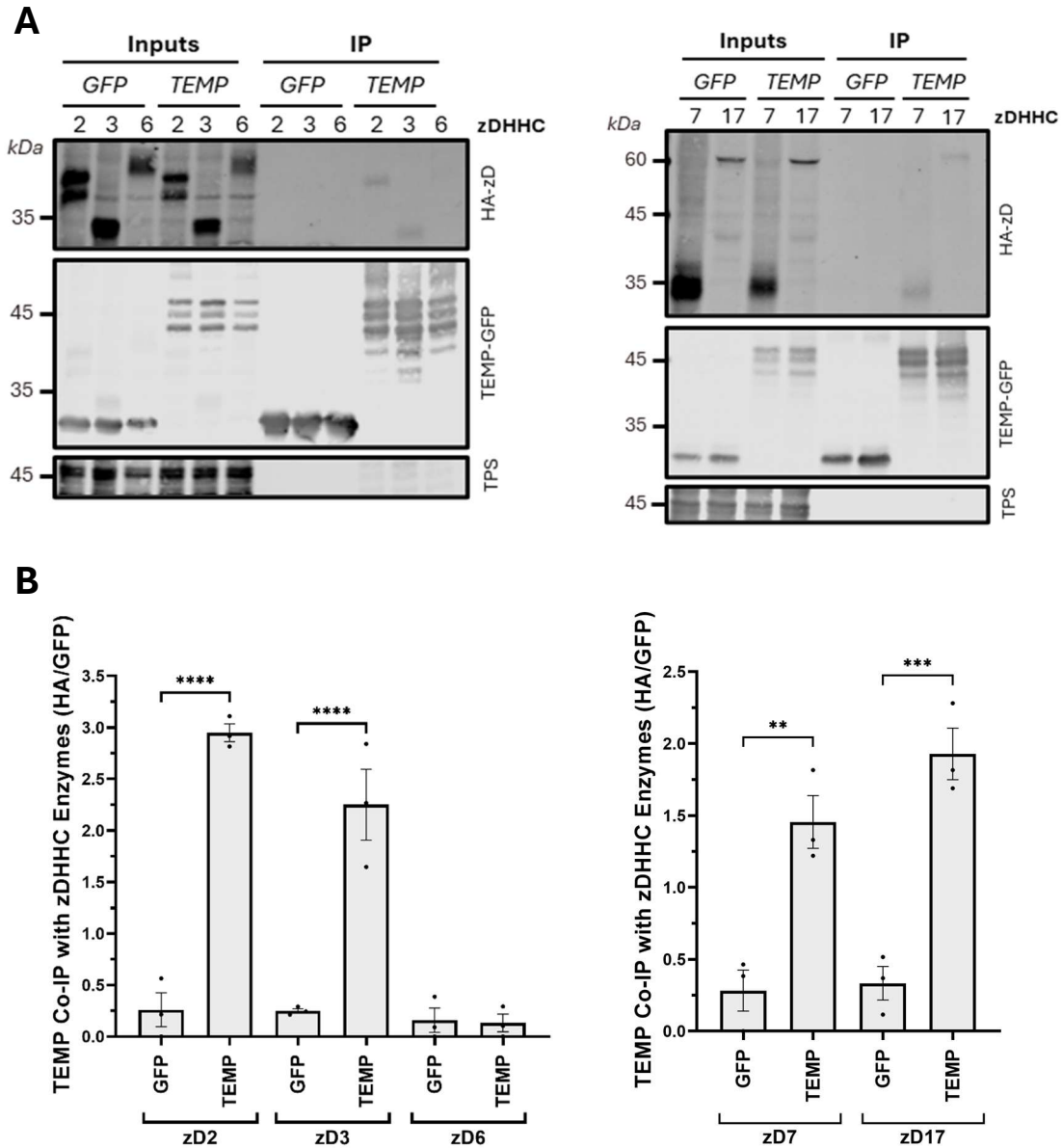


Figure 5.3. Co-immunoprecipitation analysis of TEMP interactions with zDHC enzymes. HEK293T cells were co-transfected with pcDNA3.1-TEMP-eGFP (TEMP) or empty control pcDNA3.1-eGFP (GFP), together with pEF-BOS-HA-zDHC2, -zDHC3, -zDHC6, -zDHC7, or -zDHC17 (zD2, zD3, zD6, zD7, zD17). Lysates were subjected to immunoprecipitation (IP) using GFP-Trap beads. Samples were collected before incubation with beads (“input”) and after immunoprecipitation (“IP”). **(A)** Representative immunoblot showing zDHC expression (top; HA-zD), GFP/TEMP expression (middle; TEMP-GFP), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of zDHC binding to TEMP in GFP-Trap immunoprecipitates, expressed as HA signal over GFP and normalised to within-repeat mean. Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

5.3.2 Interaction of TEMP with zDHHC3 does not require the DHHC catalytic cysteine

Building on the finding that TEMP associates with multiple zDHHC enzymes, the importance of the catalytic activity of zDHHC3 for TEMP interaction was assessed. The catalytic cysteine within the DHHC domain of zDHHC enzymes is essential for S-acylation of target substrates. If the interaction between TEMP and zDHHC3 depended on catalytic activity rather than non-catalytic binding interfaces, the catalytic cysteine might be necessary for association.

To test whether TEMP association depends on zDHHC3 catalytic activity, GFP-tagged TEMP or an empty GFP control was co-expressed with either wild-type zDHHC3 or a catalytically inactive construct in which the essential DHHC cysteine was replaced by serine (zDHHC3CS). Following expression, GFP-TEMP complexes were immunoprecipitated using GFP-trap beads, and the presence of co-precipitated zDHHC3 or zDHHC3CS was detected by HA immunoblotting (**Figure 5.4**).

TEMP efficiently pulled down both wild-type zDHHC3 and the zDHHC3CS mutant, indicating that the catalytic cysteine is not required for physical association with TEMP (**Figure 5.4**). This suggests that active site fatty acid loading on zDHHC3 is not required for the interaction of this enzyme with TEMP. It should be noted that the GFP signal from the empty control plasmid was relatively strong compared with the zDHHC enzymes, leading to some bleed-through into the HA detection channel. Additionally, the zDHHC3CS mutant appeared to be expressed at higher levels than wild-type zDHHC3 in the input samples, which may have contributed to higher levels (compared with wild-type zDHHC3) in the immunoprecipitated material.

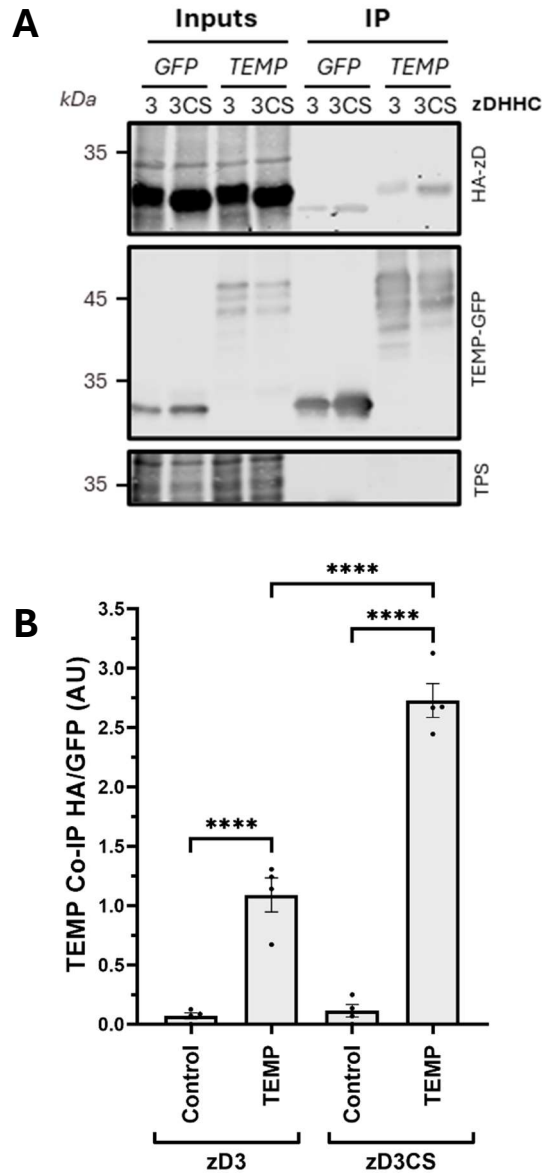


Figure 5.4. TEMP co-immunoprecipitates with both wild-type and catalytically inactive zDHHC3. HEK293T cells were co-transfected with pcDNA3.1-TEMP-eGFP (TEMP) or empty control pcDNA3.1-eGFP (GFP), together with pEF-BOS-HA-zDHHC3 (zD3) or a catalytically inactive cysteine-to-serine construct, zDHHC3CS (zD3CS). Lysates were subjected to immunoprecipitation (IP) using GFP-Trap beads. Samples were collected before incubation with beads (“input”) and after immunoprecipitation (“IP”). **(A)** Representative immunoblot showing zDHHC expression (top; HA-zD), GFP/TEMP expression (middle; TEMP-GFP), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of zDHHC binding to TEMP in GFP-Trap immunoprecipitates, expressed as HA signal over GFP and normalised to the within-repeat mean. Error bars represent $\pm$ SEM; $n=4$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (**** $p<0.0001$).

5.3.3 Mapping the C-terminal determinants of TEMP-zDHHC2 interaction

To understand the regions of TEMP that are involved in the interaction with zDHHC enzymes, a cytosolic sequence at its C-terminus found to be entirely conserved across all species (amino acids 84-EDDDGFIEDNYIQPG-98), the function of which is currently unknown, was examined. The C-terminus of TEMP includes amino acids 51-113. To investigate whether the conserved 84-98 region contributes to the association of TEMP with zDHHC enzymes, truncation constructs of TEMP were generated. One construct removed the conserved sequence entirely by truncating at amino acid 83 (TEMP 1-83), while a second construct retained the conserved region but removed the extreme C-terminal residues beyond it (TEMP 1-98) (**Figure 5.5**).

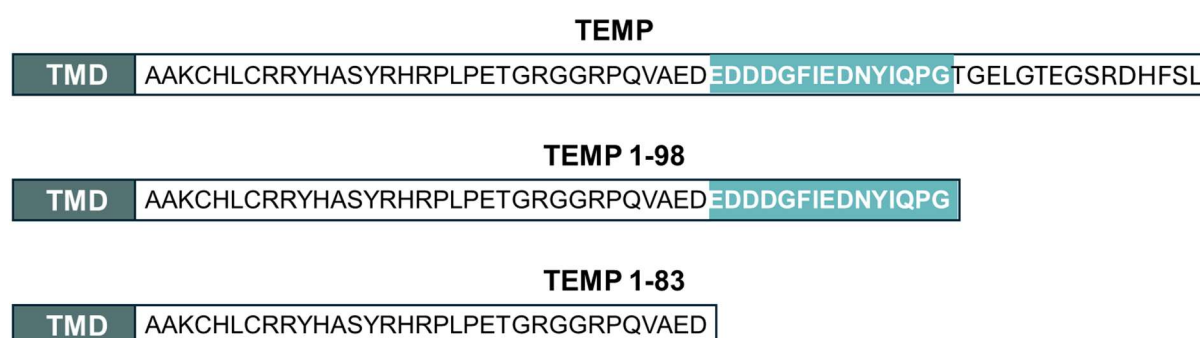


Figure 5.5. Schematic representation of TEMP C-terminal truncation constructs. TEMP is depicted with its transmembrane domain (TMD; green), followed by the C-terminal region (amino acids 51-113), including the conserved C-terminal domain (blue). Three constructs are shown: full-length wild-type TEMP, TEMP truncated after the conserved domain (TEMP 1-98), and TEMP truncated prior to the conserved domain (TEMP 1-83).

GFP-tagged wild-type TEMP and the truncation constructs were co-expressed with HA-zDHHC2 in HEK293T cells, as zDHHC2 had previously shown robust binding to the wild-type protein (**Figure 5.3**). GFP-trap immunoprecipitation was performed to compare the ability of each construct to pull down zDHHC2 (**Figure 5.6**).

On immunoblots, both truncation constructs migrated at lower apparent molecular weights than wild-type TEMP, consistent with the loss of C-terminal residues (**Figure 5.6A**). TEMP 1-83 appeared at approximately 38 kDa, compared to ~43 kDa for wild-type TEMP, while TEMP 1-98 migrated only slightly faster than wild-type. Analysis of the co-immunoprecipitation samples revealed a reduction in zDHHC2 pull-down for both truncation constructs compared to wild-type. Quantitative analysis indicated that the decrease was statistically significant for TEMP 1-98, whereas TEMP 1-83 did not reach significance (**Figure 5.6B**). This may be due to the lower expression of the 1-83 construct in the input samples, although this remains speculative.

Importantly, the disruption of binding in the construct lacking residues 99-113 (beyond the conserved domain) suggests that amino acids in this region are important for zDHHC interaction. Residues in the 84-97 region may also be important but this cannot be verified from the results of this experiment.

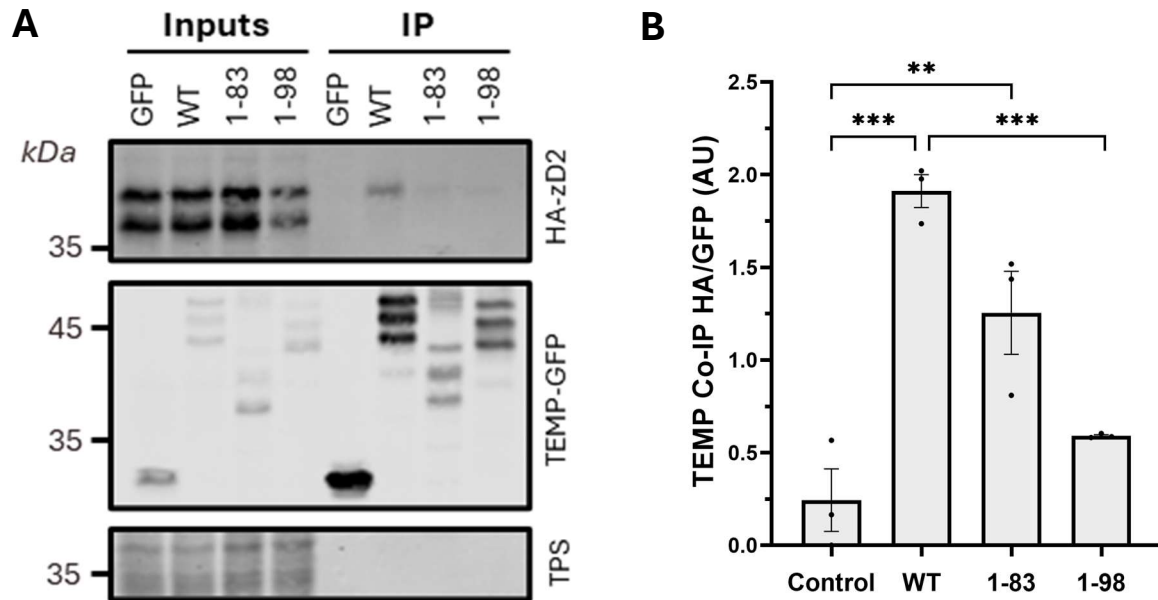


Figure 5.6. Co-immunoprecipitation of TEMP truncations demonstrates a role for the extreme C-terminal region in zDHHC2 interaction. HEK293T cells were co-transfected with pcDNA3.1-TEMP-eGFP wild-type (WT), TEMP truncation constructs (1-83 and 1-98), or empty control pcDNA3.1-eGFP (GFP), together with pEF-BOS-HA-zDHHC2 (zD2). Lysates were subjected to immunoprecipitation (IP) using GFP-Trap beads. Samples were collected before incubation with beads (“input”) and after immunoprecipitation (“IP”). **(A)** Representative immunoblot showing zDHHC2 expression (top; HA-zD2), GFP/TEMP expression (middle; TEMP-GFP), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of zDHHC2 binding to TEMP in GFP-Trap immunoprecipitates, expressed as HA signal over GFP and normalised to the within-repeat mean. Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (** $p<0.01$, *** $p<0.001$).

5.3.4 TEMP is a substrate of zDHHC6

Following the confirmation that TEMP associates with multiple zDHHC enzymes and that it contains two C-terminal cysteine residues that are potentially accessible to zDHHC-mediated modification, it was next examined if TEMP undergoes S-acylation. Based on the previous co-immunoprecipitation experiments, zDHHC2, zDHHC3, and zDHHC7 were selected for S-acylation analysis, while zDHHC6 was also included due to its reported preference for single-pass transmembrane proteins (Salaun et al., 2023).

To assess S-acylation, palmitic acid azide labelling followed by click chemistry was performed in HEK293T cells expressing GFP-tagged TEMP together with each HA-tagged zDHHC enzyme (**Figure 5.7**). Several features of TEMP expression on immunoblots complicated the interpretation of these experiments. Non-acylated TEMP consistently migrated as three distinct bands, suggesting that additional post-translational modifications may influence its molecular weight. S-acylated TEMP similarly appeared as multiple bands, with notable differences depending on the co-expressed zDHHC enzyme.

With both the control and zDHHC2, two bands at approximately 60 kDa were present in control samples lacking palmitic acid azide (-), which were at the same position as the major S-acylated band that appeared in cells incubated with palmitic acid azide (+) (**Figure 5.7A**). These 60 kDa bands in the control samples were less evident when TEMP was co-expressed with zDHHC3, zDHHC6, or zDHHC7. Furthermore, there were three main S-acylated bands that were observed, with the middle band migrating at roughly 60 kDa. The intensity of these bands differed with the zDHHC enzyme that was expressed: all three bands had a similar intensity with zDHHC6, whereas the upper and lower bands were more intense for zDHHC3 and zDHHC7, and the lower and middle bands were more prominent with zDHHC2.

Quantitative analysis included the region containing all three of the S-acylated bands to capture all potential S-acylated species. Under these conditions, a statistically significant increase in S-acylation was observed only when TEMP was co-expressed with zDHHC6 compared to the control (**Figure 5.7B**). While the immunoblots suggest that TEMP may also undergo S-acylation in the presence of zDHHC3 and zDHHC7, the variability in banding patterns and overlapping signals in control samples prevents definitive conclusions.

Overall, these results indicate that TEMP can be S-acylated, with zDHHC6 demonstrating the most robust effect under the conditions tested. The observation of multiple S-acylated species and the complex banding patterns further suggests that TEMP may be subject to additional post-translational modifications that influence its apparent molecular weight.

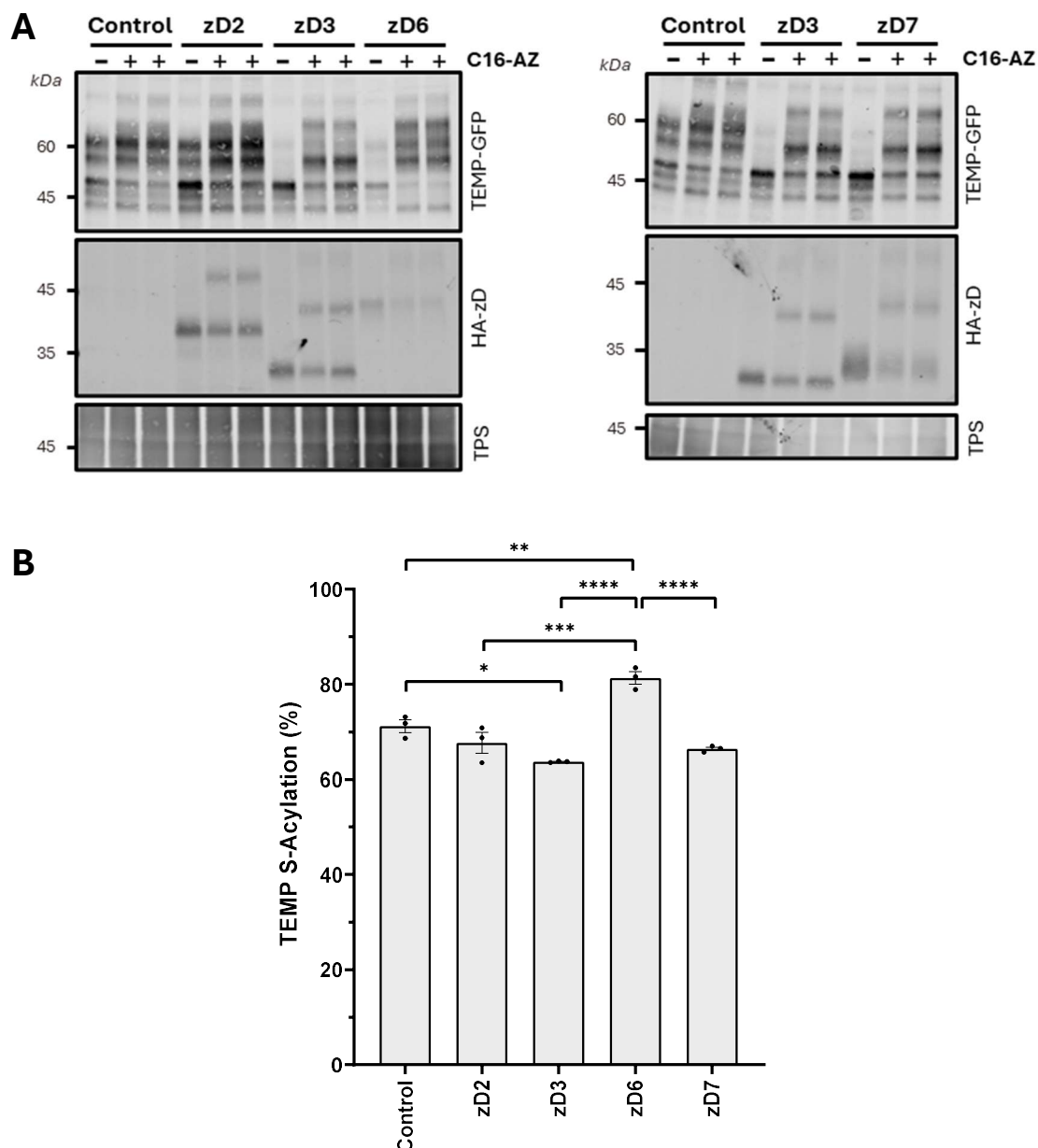


Figure 5.7. S-Acylation of TEMP is significantly increased in the presence of zDHHc6. HEK293T cells were co-transfected with pcDNA3.1-TEMP-eGFP (TEMP) together with pEF-BOS-HA-zDHHc2, -zDHHc3, -zDHHc6, -zDHHc7 (zD2, zD3, zD6, zD7) or empty vector (Control). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE and immunoblotting. (A) Representative immunoblot showing TEMP S-acylation (top; TEMP-GFP), zDHHc expression (middle; HA-zD), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. (B) Quantification of TEMP S-acylation expressed as the mean percentage of the S-acylated bands relative to the total TEMP signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (** $p<0.001$).

5.3.5 TEMP band heterogeneity is partially due to N-terminal modification

Following the identification of multiple TEMP band species and evidence of S-acylation, the possibility that some of these species represented glycosylated forms was investigated. Previous work has reported that TEMP contains an N-terminal region predicted to be glycosylated, with residues N2 and N5 identified as likely candidate sites based on annotations from the UCSC Genome Browser (Aturaliya et al., 2012, Kuhn et al., 2007). To test this, GFP-tagged TEMP constructs were generated in which these asparagine residues were substituted with alanine individually (N2A, N5A) or in combination (N2AN5A), and expression patterns in HEK293T cells were compared to wild-type TEMP.

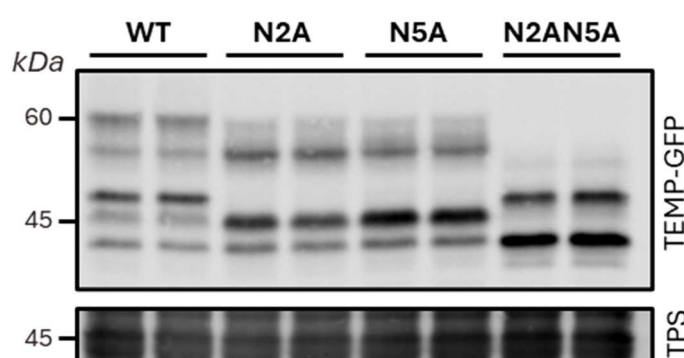


Figure 5.8. Substitution of predicted N-terminal glycosylation sites (N2 and N5) alters TEMP expression patterns. HEK293T cells were transfected with pcDNA3.1-TEMP-eGFP wild-type (WT) or asparagine-to-alanine substitution constructs of predicted N-terminal glycosylation sites (N2A, N5A, N2AN5A). Cells were lysed directly in SDS sample buffer with 25 mM DTT, resolved by SDS-PAGE and examined by immunoblotting. Representative immunoblot showing TEMP S-acylation (top; TEMP-GFP) and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left.

Wild-type TEMP consistently migrated as three bands around the 45 kDa marker, with additional bands observed at higher molecular weights (**Figure 5.8**). In the single substitution constructs (N2A or N5A), the banding pattern became less complex, and this was further enhanced for the double substitution construct (N2AN5A), with only two main bands present. This change in banding pattern for the different asparagine mutants strongly suggests that both N2 and N5 are glycosylated, with glycosylation at each site contributing to the distinct banding pattern observed for wild-type TEMP.

5.3.6 Impact of TEMP N-terminal modification on S-acylation

The potential for the N-terminal modification of TEMP to influence its S-acylation was investigated. Palmitic acid azide labelling with click chemistry was used to investigate the S-acylation of the N2A, N5A, and N2AN5A constructs compared to wild-type TEMP in HEK293T cells when co-expressed with empty vector control or HA-tagged zDHHC6 (Figure 5.9).

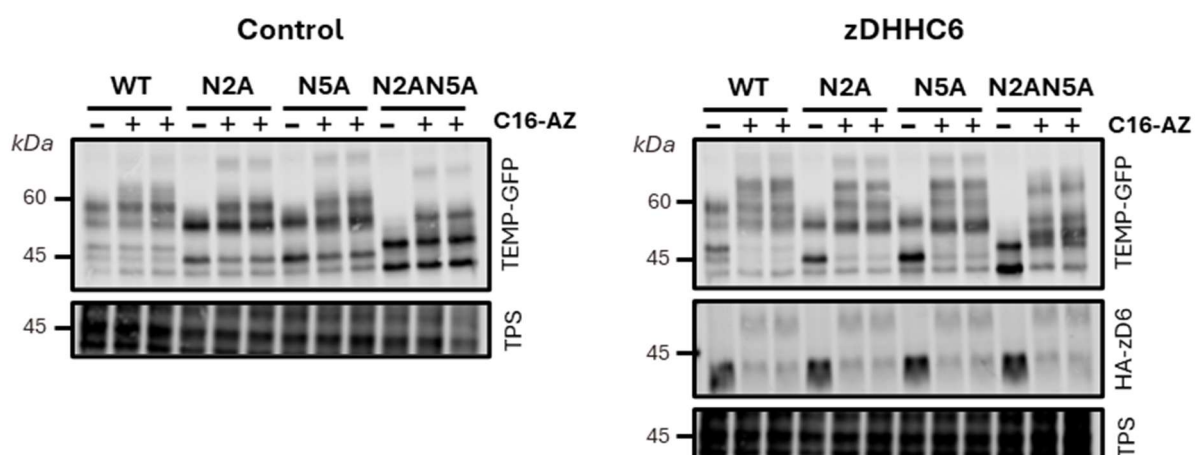


Figure 5.9. TEMP S-acylation does not appear altered by disruption to N-terminal modification. HEK293T cells were transfected with pcDNA3.1-TEMP-eGFP wild-type (WT) or asparagine-to-alanine substitution constructs at predicted N-terminal glycosylation sites (N2A, N5A, N2AN5A). At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE and immunoblotting. Representative immunoblot showing TEMP S-acylation (top; TEMP-GFP) and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left.

No obvious differences in S-acylation were observed when the N2A, N5A, or N2AN5A constructs were co-expressed with control or zDHHC6 compared to wild-type TEMP (Figure 5.9). However, the altered expression pattern of the N2AN5A construct, combined with the multiple overlapping bands, complicated quantitative interpretation. These results suggest that N-terminal glycosylation does not overtly interfere with TEMP S-acylation under the conditions tested, although subtle effects cannot be excluded.

These experiments identify N2 and N5 as putative glycosylation sites on TEMP and help explain some of the multiple bands observed in previous expression and S-acylation analyses. The presence of multiple post-translational modifications, including glycosylation and S-acylation, likely contributes to the complex banding pattern of TEMP and may influence its interactions with zDHHC enzymes or other cellular partners.

5.3.7 TEMP stability is unaffected by disruption of the N2/N5 modification sites

Post-translational modifications such as glycosylation can influence protein stability, folding, and trafficking, potentially affecting TEMP's cellular function and interactions with zDHHC enzymes. Given that it is proposed that the putative glycosylation sites N2 and N5 contributed to TEMP band heterogeneity, it was important to determine whether loss of this modification alters protein turnover. An 8-hour cycloheximide chase assay was therefore performed in HEK293T cells to compare the stability of proposed glycosylation-deficient TEMP constructs (N2A, N5A, N2AN5A) to wild-type TEMP.

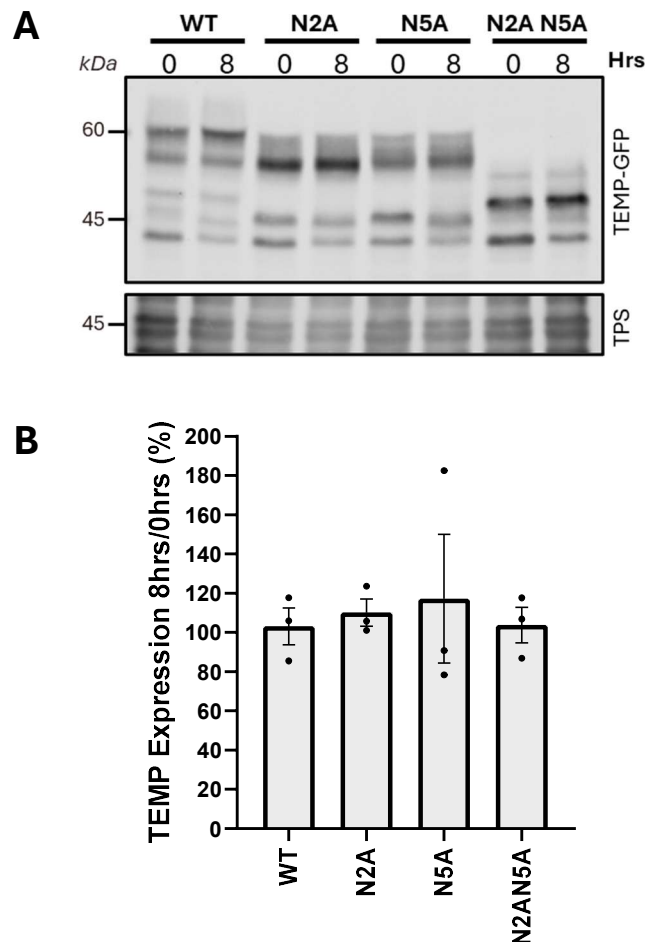


Figure 5.10. TEMP N-terminal substitution constructs (N2A, N5A, N2AN5A) display similar stability to wild-type in an 8-hour cycloheximide chase. HEK293T cells were transfected with pcDNA3.1-TEMP-eGFP wild-type (WT) or asparagine-to-alanine substitution constructs at predicted N-terminal glycosylation sites (N2A, N5A, N2AN5A). At 24 hours post-transfection, cells were treated with 50 μ g/ml cycloheximide (CHX). Samples were collected at 0 and 8 hours and analysed by SDS-PAGE and immunoblotting. **(A)** Representative immunoblot showing TEMP expression (top; TEMP-GFP) and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of TEMP expression following 8 hours CHX treatment, expressed as the mean percentage of TEMP remaining at 8 hours relative to expression at 0 hours. Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA.

Across all constructs, TEMP expression remained relatively constant, with 90-100 % of the initial protein level detected at the end of the assay (**Figure 5.10**). No clear or statistically significant differences in stability were observed between WT and any of the glycosylation mutants. These results suggest that proposed N-terminal glycosylation at residues N2 and N5, while contributing to TEMP band heterogeneity, does not substantially affect protein stability under these experimental conditions.

5.3.8 Mapping the S-acylation sites of TEMP

As TEMP was confirmed to be S-acylated by zDHHC6, it was of interest to determine which cysteine residue(s) serve as the sites of modification. TEMP contains only two cysteines, C54 and C57, positioned in the C-terminus near the transmembrane domain and thus likely accessible to zDHHC enzymes (**Figure 5.2**). To determine the contribution of each cysteine, GFP-tagged alanine substitution constructs were generated individually (C54A, C57A) and in combination (C54AC57A). S-acylation of these constructs was assessed using palmitic acid azide labelling followed by click chemistry, in HEK293T cells co-expressing either control or HA-tagged zDHHC6 (**Figure 5.11**).

Analysis revealed a modest but statistically significant decrease in S-acylation (~10 %) for the C54A construct compared to wild-type TEMP, whereas the C57A construct did not show a significant change (**Figure 5.11**). The combined C54AC57A construct exhibited the greatest reduction, with % S-acylation decreased by approximately 30 % relative to wild-type with control and around 40 % relative to wild-type when co-expressed with zDHHC6. This data suggests that C54 is the primary site of S-acylation, but C57 can act as a secondary or compensatory site if C54 is unavailable. Although the double cysteine mutant only showed a 30-40% decrease in S-acylation, the remaining S-acylation signal is likely attributed to non-acylated bands that migrate at the same size as the S-acylated bands (i.e. also visible in negative control samples).

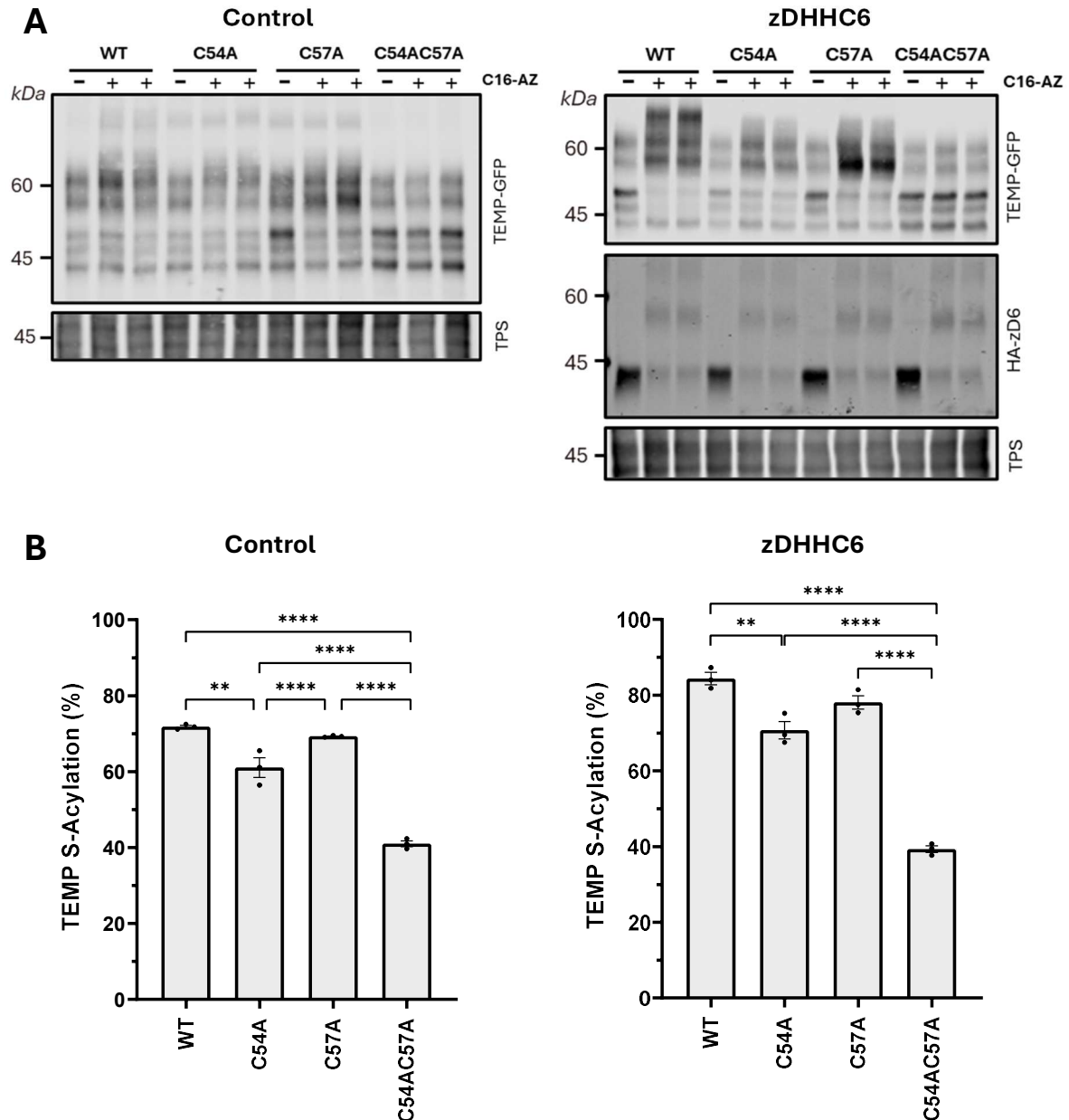


Figure 5.11. C54 is the primary site of S-acylation on TEMP, with C57 acting as a secondary site. HEK293T cells were co-transfected with pcDNA3.1-TEMP-eGFP wild-type (WT) or cysteine-to-alanine substitution constructs of predicted C-terminal S-acylation sites (C54A, C57A, or C54AC57A), together with empty vector (control) or HA-zDHHC6. At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE and immunoblotting. **(A)** Representative immunoblot showing TEMP S-acylation (top; TEMP-GFP), zDHHC6 expression (middle; HA-zD6), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of TEMP S-acylation expressed as the mean percentage of the S-acylated bands relative to the total TEMP signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (** $p<0.01$, **** $p<0.0001$).

5.3.9 C-terminal S-acylation does not significantly affect TEMP stability

Having identified S-acylation of TEMP on its C-terminal cysteine residues, it was of interest to investigate whether this modification affects protein stability. S-Acylation can influence folding, trafficking, and turnover of many transmembrane proteins, so assessing the stability of cysteine-substituted TEMP constructs provides insight into the functional consequences of S-acylation. To address this, an 8-hour cycloheximide chase assay was performed to compare the stability of wild-type TEMP with the C54A, C57A, and C54AC57A constructs in HEK293T cells, expressed in the absence of exogenous zDHHC enzymes (**Figure 5.12**).

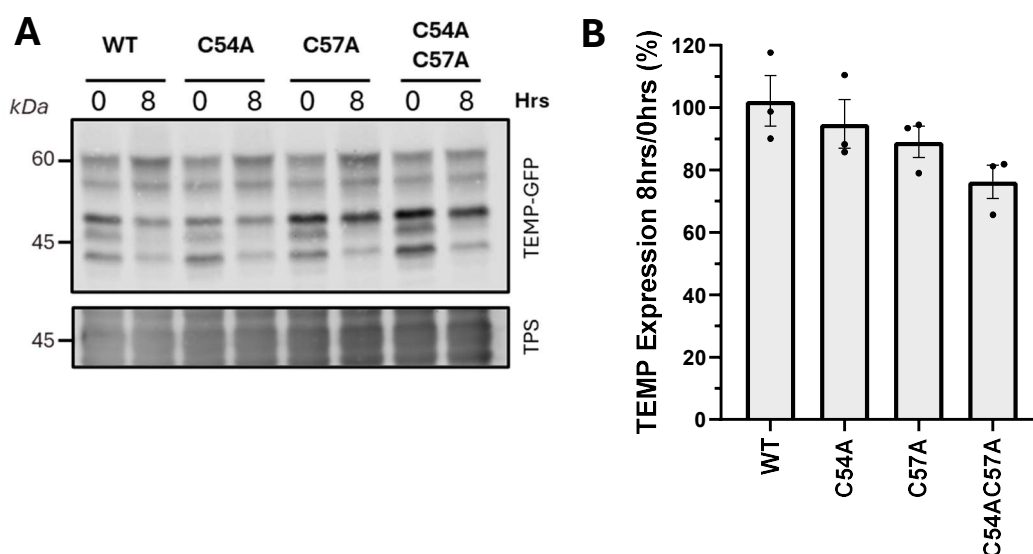


Figure 5.12. TEMP cysteine-substituted constructs show similar stability to wild-type in an 8-hour cycloheximide assay. HEK293T cells were transfected with pcDNA3.1-TEMP-eGFP wild-type (WT) or cysteine-to-alanine substitution constructs at predicted C-terminal S-acylation sites (C54A, C57A, or C54AC57A). At 24 hours post-transfection, cells were treated with 50 μ g/ml cycloheximide (CHX). Samples were collected at 0 and 8 hours and analysed by SDS-PAGE and immunoblotting. **(A)** Representative immunoblot showing TEMP expression (top; TEMP-GFP) and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of TEMP expression following 8 hours CHX treatment, expressed as the mean percentage of TEMP remaining at 8 hours relative to expression at 0 hours. Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA.

Analysis showed minimal loss of wild-type TEMP over 8 hours, with approximately 90 % of protein remaining (**Figure 5.12**). Although there was a trend towards reduced stability for the C54AC57A protein, no significant differences in stability were observed for any of the cysteine-substituted constructs relative to wild-type TEMP, indicating that loss of C-terminal S-acylation sites does not substantially affect protein turnover under these experimental conditions.

5.3.10 Role of the C-terminal domain in TEMP S-acylation

Previous immunoprecipitation experiments showed that the C-terminal region of TEMP may be involved in zDHHC2 interaction. To investigate if this region is also important for S-acylation by zDHHC6, palmitic acid azide labelling followed by click chemistry was used to compare S-acylation of wild-type TEMP, 1-83, and 1-98 truncation constructs when co-expressed in HEK293T cells with either control or HA-tagged zDHHC6 (**Figure 5.13**).

Analysis revealed that the 1-83 construct, which removes the conserved domain and additional C-terminal residues, exhibited a significant decrease in S-acylation compared to wild-type TEMP (**Figure 5.13**). In cells expressing only endogenous zDHHC enzymes (control), S-acylation decreased from approximately 80 % in wild-type to around 50 % in the 1-83 construct. Co-expression with zDHHC6 also resulted in reduced S-acylation for 1-83, although the decrease was more modest (~10 % compared to wild-type and ~5 % compared to 1-98). In contrast, the 1-98 construct, which retains the conserved domain but truncates residues downstream, showed no significant change in S-acylation relative to wild-type.

These results indicate that disruption of the C-terminus can impair the ability of zDHHC enzymes to S-acylate TEMP. The conserved domain may play a role in supporting efficient S-acylation, as the 1-98 construct, which preserves this region, does not exhibit the same decrease. However, the more substantial truncation in the 1-83 construct may contribute to the observed effect, so it remains unclear whether the conserved domain itself is directly involved in S-acylation.

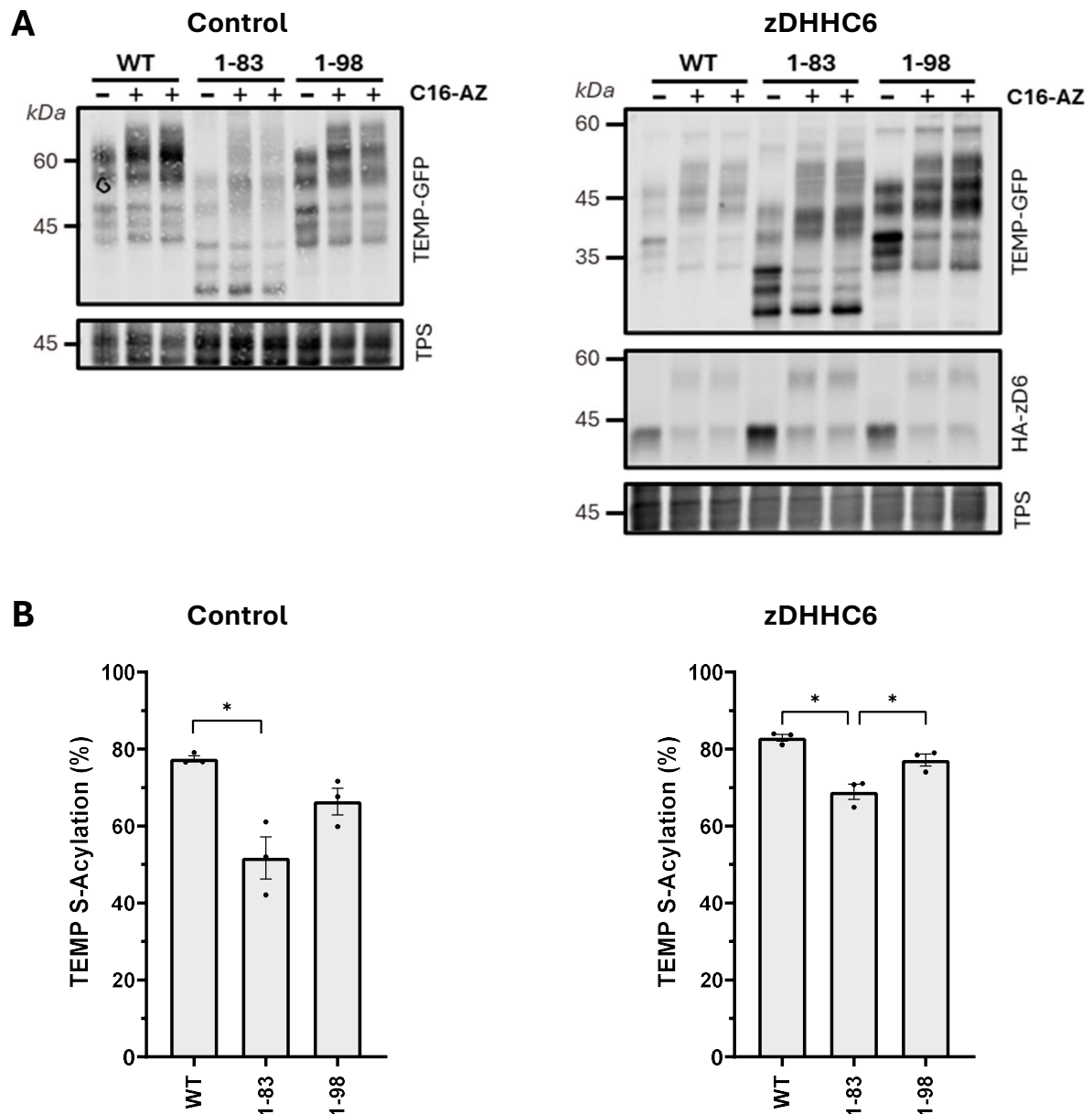


Figure 5.13. TEMP S-acylation efficiency is diminished by removal of the conserved C-terminal domain. HEK293T cells were co-transfected with pcDNA3.1-TEMP-eGFP wild-type (WT) or truncation constructs (1-83 or 1-98), together with empty vector (control) or HA-zDHHC6. At 24 hours post-transfection, cells were incubated with 100 μ M palmitic acid control (-) or palmitic acid azide (C16-AZ; +) for 4 hours. Labelled samples were incubated with 5 kDa alkyne mPEG and analysed by SDS-PAGE and immunoblotting. **(A)** Representative immunoblot showing TEMP S-acylation (top; TEMP-GFP), zDHHC6 expression (middle; HA-zD6), and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of TEMP S-acylation expressed as the mean percentage of the S-acylated bands relative to the total TEMP signal (non-acylated plus acylated bands). Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA (* $p<0.05$).

5.3.11 C-terminal truncation does not affect TEMP stability

Given that the C-terminal region of TEMP appears to influence binding with zDHHC enzymes and can impact S-acylation efficiency, it was important to determine whether this domain also affects protein stability. To test this, an 8-hour cycloheximide chase assay was performed in HEK293T cells expressing wild-type TEMP or the C-terminal truncation constructs 1-83 and 1-98 (**Figure 5.14**).

Analysis showed that although there was a trend towards reduced stability of the 1-83 and 1-93 mutants, there were no statistically significant differences observed compared to wild-type TEMP (**Figure 5.14**). These results suggest that, despite its potential importance for enzyme binding and S-acylation, the C-terminal region, including the conserved domain, does not significantly influence TEMP stability under these experimental conditions.

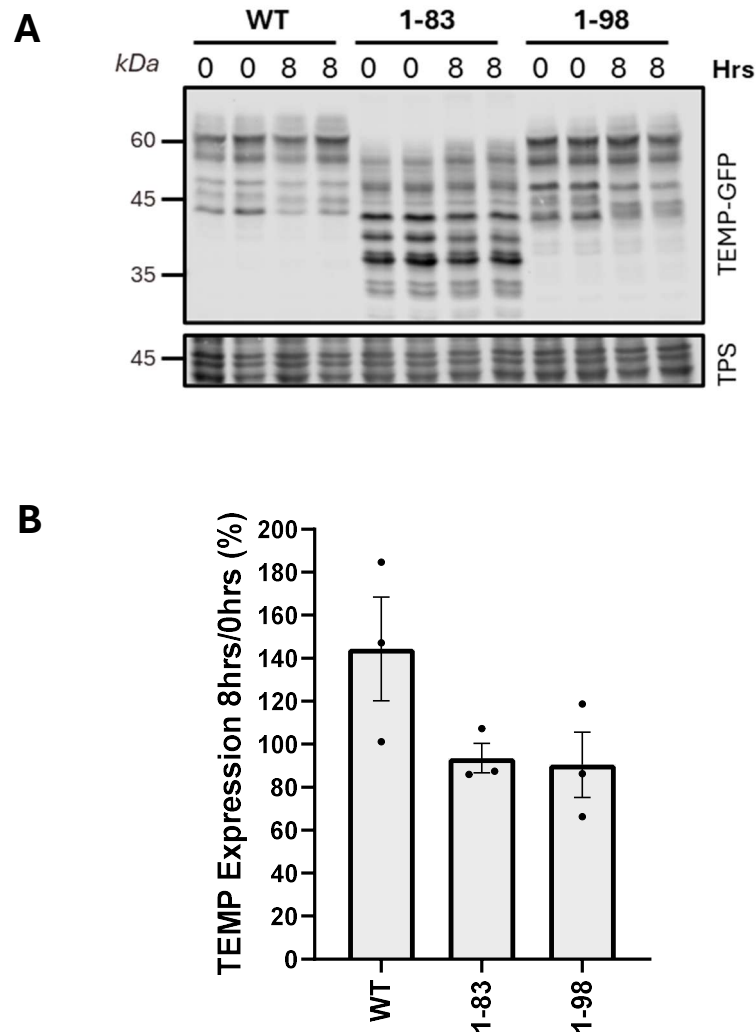


Figure 5.13. Cycloheximide chase analysis of C-terminal truncation mutants of TEMP. HEK293T cells were transfected with pcDNA3.1-TEMP-eGFP wild-type (WT) or truncation constructs (1-83 or 1-98). At 24 hours post-transfection, cells were treated with 50 μ g/ml cycloheximide (CHX). Samples were collected at 0 and 8 hours and analysed by SDS-PAGE and immunoblotting. **(A)** Representative immunoblot showing TEMP expression (top; TEMP-GFP) and total protein stain (bottom; TPS). Molecular weight markers (kDa) are indicated on the left. **(B)** Quantification of TEMP expression following 8 hours CHX treatment, expressed as the mean percentage of TEMP remaining at 8 hours relative to expression at 0 hours. Error bars represent $\pm$ SEM; $n=3$ independent experiments, shown as filled circles. Statistical significance was determined by one-way ANOVA.

5.4 Discussion

5.4.1 Summary

Overall, the results presented here support the central hypothesis that TEMP interacts with multiple zDHHC enzymes and is a novel S-acylated protein, while extending this hypothesis by identifying zDHHC6 as a TEMP S-acyltransferase and defining the primary sites of S-acylation. TEMP associates with multiple zDHHC enzymes, including zDHHC2, zDHHC3, zDHHC7, and zDHHC17, with binding potentially influenced by structural elements in the extreme C-terminal region rather than the conserved domain or the catalytic site of the enzymes. TEMP was also established as a substrate of zDHHC6, with C54 serving as the primary site of S-acylation and C57 acting as a secondary or compensatory site. Investigation of the predicted N-terminal glycosylation sites, N2 and N5, suggests that these residues contribute to TEMP's higher-molecular-weight species, although the modification remains unconfirmed and does not appear to substantially affect protein stability or S-acylation. Together, these findings clarify the interaction and modification landscape of TEMP and provide a foundation for future studies to explore how S-acylation and glycosylation influence its cellular roles.

5.4.2 TEMP binding to zDHHC enzymes

Co-immunoprecipitation experiments provide strong evidence that TEMP associates with multiple zDHHC enzymes, expanding its interaction profile beyond the initial observations in the BioPlex dataset (**Figure 5.3**). The confirmed interactions with zDHHC3 and zDHHC7 validate the high-throughput data, demonstrating that these associations can be reproduced under controlled experimental conditions. Notably, additional interactions with zDHHC2 and zDHHC17 indicate that TEMP may engage broadly with the zDHHC family, rather than being restricted to a narrow subset of enzymes. The observation that TEMP selectively co-immunoprecipitates with only the higher molecular weight species of zDHHC2 suggests that this interaction may depend on a specific state of zDHHC2. Although the identity of the two bands has not been determined, the slower-migrating species could reflect a post-translationally modified form of the protein. This species could represent the auto-acylated form of the enzyme, but it is unlikely that this is linked to binding, as mutation of the active site cysteine did not affect co-immunoprecipitation with TEMP. The preferential association of TEMP with this higher molecular weight band raises the possibility that TEMP recognises a distinct subpopulation of zDHHC2 rather than the total cellular pool.

This broad interaction profile suggests two possibilities. TEMP could act as a promiscuous substrate, capable of being recognised by multiple zDHHC enzymes. Alternatively, as several proteins have been identified as accessory/regulatory proteins for zDHHC enzymes, it is possible that TEMP also has a similar role. For example, GCP16

stabilises zDHHC9 and is important for its activity against substrates such as H-Ras (Nguyen et al., 2023, Swarthout et al., 2005) and selenoprotein K improves zDHHC6 catalytic efficiency by stabilising the acyl-enzyme intermediate (Fredericks et al., 2017). While GPC16 is a soluble protein, selenoprotein K is a single-transmembrane protein, like TEMP, demonstrating that such proteins can also have a regulatory function in zDHHC activity.

Given that zDHHC enzymes often display overlapping substrate specificity and partially intersecting membrane localisations, both biochemical compatibility and spatial proximity likely contribute to the observed associations. As TEMP is located on endosomes and the plasma membrane, it is possible that it interacts with zDHHC enzymes during its biosynthetic trafficking through the secretory pathway. It is notable that zDHHC2, which had the highest level of association with TEMP, is localised to similar membrane compartments (Greaves et al., 2011).

Interestingly, no interaction with zDHHC6 was detected, despite TEMP possessing juxta-membrane cysteine residues characteristic of known zDHHC6 substrates (Salaun et al., 2023). This may reflect a transient or low-affinity interaction, which is common for enzyme-substrate relationships and could evade detection by co-immunoprecipitation. This is the case for SNAP25, which is S-acylated by zDHHC3 and zDHHC7, but only very low interaction was detected by pull-down assays, suggesting this interaction is transient (Lemonidis et al., 2014). It is also important to recognize that co-immunoprecipitation does not distinguish between direct and indirect interactions; the observed associations may occur through multiprotein complexes, membrane microdomains, or scaffold proteins rather than direct binding. Furthermore, overexpression in HEK293T cells can enhance weak or non-physiological interactions, highlighting the need to interpret these findings in the context of cellular overexpression.

The binding of TEMP to a catalytically inactive zDHHC3 construct (zDHHC3CS) demonstrates that the DHHC catalytic cysteine is not required for stable association, suggesting that the activity state of zDHHC enzymes is likely not important for TEMP binding (**Figure 5.4**). However, given the interaction of TEMP with a broad range of enzymes, it is still possible that the interaction detected through co-immunoprecipitation experiments is with the DHHC-CRD. As only zDHHC6 showed activity against TEMP in quantified data from click chemistry experiments, it is possible that a higher level of binding to zDHHC enzymes actually has a negative effect on S-acylation. Indeed, this has previously been shown for the zDHHC17-SNAP25 interaction, where SNAP25 mutants displaying increased co-immunoprecipitation with zDHHC17 were less S-acylated by the enzyme (Salaun et al., 2020).

Analysis of C-terminal truncation constructs of TEMP further refines the understanding of structural contributions towards binding to zDHHC enzymes. The 1-83 construct, lacking the conserved C-terminal sequence, and the 1-98 construct, retaining the conserved domain but removing residues 99-113, both showed reduced pull-down of zDHHC2; however, only the 1-98 construct was significant (**Figure 5.6**). The reduced interaction with 1-98 indicates that residues 99-113, immediately downstream of the

conserved domain, contribute to binding, whereas the conserved sequence itself is not essential; however, these residues are lacking in the 1-83 construct, and this was not significantly reduced. The apparent lower expression of 1-83 relative to that of wild-type and 1-98 may however, distort the results for this construct. Nevertheless, we can conclude that part of the C-terminal region of TEMP is likely important for zDHHC interaction. More detailed analysis of the C-terminal region, likely involving more specific truncation/substitution constructs, would be required to accurately identify any impact of the C-terminus on zDHHC binding.

Together, these observations have important implications. First, TEMP can bind zDHHC enzymes independently of S-acylation, suggesting that it may act not only as a substrate but potentially as a regulator or scaffold. Second, the identification of specific C-terminal residues potentially important for binding provides a foundation for targeted mutational studies to define the minimal interface and to assess how these residues influence S-acylation efficiency or enzyme specificity.

To fully determine the nature and physiological relevance of TEMP-zDHHC interactions, several avenues for further investigation would be required. Direct binding assays using purified proteins could determine whether these associations are direct or mediated through other proteins or membrane microdomains. Assessing interactions at endogenous expression levels using specific antibodies would provide a more physiologically relevant perspective, mitigating potential artefacts of overexpression. Visualisation of potential co-localisation of TEMP with zDHHC enzymes could determine if they are ever present in the same subcellular compartments. Live-cell techniques, including FRET, could also provide insight into the spatial and temporal dynamics of TEMP-zDHHC interactions, including transient or compartment-specific associations that are difficult to capture by co-immunoprecipitation. Finally, functional studies are needed to explore the consequences of TEMP binding on zDHHC activity, localisation, or substrate competition, which would clarify whether TEMP functions primarily as a substrate, a scaffold, or a regulator within S-acylation pathways. TEMP knockdown experiments using siRNA could be used to investigate potential impacts on zDHHC localisation, substrate S-acylation or cell phenotypes to begin to determine potential functional consequences. Collectively, these approaches would provide a more comprehensive understanding of TEMP's role in modulating zDHHC enzyme function.

5.4.3 TEMP S-acylation

The S-acylation experiments demonstrate that TEMP can be modified by zDHHC enzymes, with zDHHC6 producing the most robust and statistically significant effect under the conditions tested (**Figure 5.7**). This observation is consistent with the known preference of zDHHC6 for single-pass transmembrane proteins and supports the idea that TEMP is a potential substrate for this enzyme (Salaun et al., 2023). Importantly, these results highlight that physical association with a zDHHC enzyme does not necessarily equate to efficient enzymatic modification, as TEMP associated with several enzymes in

co-immunoprecipitation experiments, but was only significantly S-acylated by zDHHC6, which was not pulled down. This distinction underscores the need to complement binding studies with functional assays to assess enzymatic activity. As zDHHC6 is located at the ER, this suggests that TEMP is S-acylated at this compartment before moving to the endosomes and plasma membrane (Aturaliya et al., 2012). Therefore, it is possible that S-acylation of newly-synthesised TEMP at the ER is important for trafficking to post-ER compartments. A role for S-acylation in regulating other single transmembrane proteins has been reported. For example, the single-transmembrane protein Lyn kinase requires S-acylation to reach the plasma membrane and disruption of this modification results in the protein being retained at the ER/Golgi (Kinoshita-Kikuta et al., 2020, Morinaga et al., 2017).

Several limitations of the current analysis should be noted. TEMP consistently expressed as multiple bands in both non-acylated and S-acylated forms, likely reflecting additional post-translational modifications, or partial degradation. These overlapping bands complicated the interpretation of S-acylation, particularly for zDHHC3, and zDHHC7, where potential modification was difficult to distinguish from background. In an attempt to mitigate the issues caused by these multiple bands, palmitic acid azide labelling and click chemistry with a fluorescent alkyne label was attempted to avoid the complication of additional band shifts, however the immunoblots resulted in large, unclear fluorescence signals, which were less distinct than the mPEG immunoblots (data not presented). Additional methods of detecting S-acylation, such as Acyl-RAC or ABE could be useful to clarify the S-acylation status of TEMP.

Despite these limitations, mapping of S-acylation sites identified C54 as the primary site of modification, with C57 able to act as a secondary or compensatory site when C54 is unavailable (**Figure 5.11**). The partial reduction in S-acylation in the C54A construct, and the near-complete loss in the double C54AC57A construct, support a model in which zDHHC6 preferentially targets C54 but retains flexibility to modify nearby cysteines. It is also possible that these cysteine residues perform different roles. For example, in H Ras, Cys181 is the primary S-acylation site required for Golgi-to-plasma membrane trafficking as visualised by fluorescent microscopy and supported by biochemical fractionation. Cys184 S-acylation can support plasma membrane delivery but results in inefficient trafficking, as shown by reduced and delayed plasma membrane accumulation and altered subcellular distribution, indicating less efficient Golgi-to-plasma membrane transport. However, once at the plasma membrane, S-acylation of Cys184 contributes to membrane microdomain organization, as shown by membrane domain partitioning analyses, illustrating that adjacent cysteines can play different roles in trafficking efficiency and membrane organisation (Roy et al., 2005). The location of TEMP cysteine residues is consistent with the known preference of zDHHC6 for juxta-membrane cysteines adjacent to the transmembrane domain (Salaun et al., 2023). The residual S-acylation observed in C54A suggests that zDHHC6 can accommodate multiple cysteine sites, potentially ensuring TEMP modification under conditions in which one site is inaccessible. Direct confirmation of modification at C54 and C57 using mass

spectrometry or alternative site-specific approaches would strengthen these conclusions.

Cycloheximide chase experiments indicate that S-acylation of TEMP at its C-terminal cysteines does not substantially affect protein stability over an 8-hour period. Constructs C54A, C57A, and C54AC57A all displayed stability comparable to wild-type TEMP, suggesting that under these experimental conditions, C-terminal S-acylation is not a major determinant of protein turnover (**Figure 5.12**). It remains possible that TEMP stability could be influenced under different cellular contexts or stress conditions, however, wild-type TEMP appeared inherently very stable over this time period, which could mask any subtle differences. Future studies could investigate the effect of S-acylation on TEMP localisation, interactions with zDHHC enzymes, or other functional properties beyond protein half-life.

Analysis of C-terminal truncation constructs further highlights regions of TEMP required for S-acylation (**Figure 5.13**). The 1-83 construct, which removes the conserved C-terminal domain and additional residues, exhibited a significant decrease in S-acylation compared to wild-type TEMP, whereas the 1-98 construct, which retains the conserved domain but removes only residues downstream, showed no significant change. This suggests that the conserved domain may support S-acylation indirectly, help maintain a C-terminal conformation favourable for modification, or that larger truncations disrupt S-acylation efficiency. These findings align with the earlier observation that the C-terminal region influenced binding to zDHHC2, indicating that both enzyme association and S-acylation are affected by the structural integrity of the extreme C-terminus. Further fine truncations or point mutations within the conserved domain could clarify its potential specific contribution to S-acylation. The C-terminal 15-amino-acid motif EDDDGFIEDNYIQPG in TEMP has also been reported to occur in the cytoplasmic region of LRRC19 orthologs, indicating a conserved sequence motif shared between these unrelated proteins (Aturaliya et al., 2012). This motif is not currently annotated as a domain in sequence databases, and the closest sequence identified in LRRC19 is DDDGFIEDKYI, which is shorter than the TEMP sequence and has a K in place of an N. Therefore, it is difficult to conclude that the sequence is present in both proteins. Nevertheless, the conserved nature of this motif in TEMP suggests functional importance.

This supports a model in which TEMP's C-terminal region, including residues immediately downstream of the conserved domain, plays a role in facilitating zDHHC enzyme binding and promoting efficient S-acylation. However, these modifications do not appear to strongly affect protein stability under the experimental conditions tested, suggesting that the primary functional consequences of S-acylation and C-terminal integrity may involve trafficking, subcellular localisation, or interaction networks rather than turnover (**Figure 5.14**). Future work using endogenous TEMP expression, stress conditions, or additional functional assays will be essential to fully define the biological relevance of these modifications.

5.4.4 TEMP glycosylation

Analysis of the TEMP immunoreactive banding patterns, combined with alanine substitution of the predicted N-terminal glycosylation sites (N2 and N5), indicates that these residues contribute to the higher-molecular-weight species observed in SDS-PAGE. The consensus sequence for N-linked glycosylation is N-X-S/T (where X is any amino acid except proline), and TEMP has this sequence in the N-terminus at N2 (NET) and N5 (NKT) (Zielinska et al., 2010, Taguchi et al., 2021). Substitution of individual sites partially reduced these bands, while substitution of both residues (N2AN5A) resulted in the loss of an intermediate band, consistent with the possibility that both N2 and N5 undergo modification (**Figure 5.8**). These observations suggest that the N-terminal region contributes to TEMP band heterogeneity, likely through a post-translational modification such as glycosylation.

However, it is important to emphasise that these experiments do not provide definitive evidence of glycosylation. Only the putative sites were mutated, and no direct assay for glycan addition, such as PNGase F treatment or mass spectrometry, was performed (Zielinska et al., 2010, Bi and Tian, 2024). Therefore, it remains possible that the observed changes in band patterns are due to indirect effects of the mutations on protein folding, stability, or modifications at nearby residues rather than glycan attachment.

Additionally, while disruption of these potential glycosylation sites removed many of the unidentified TEMP band species, at least one species remained undefined. This suggests the presence of an additional modification. TEMP contains a predicted phosphorylation site on tyrosine 94 in its C-terminus, which could account for this species (Ochoa et al., 2020). While individual tyrosine kinases often have preferences for certain sequence or structural contexts, these preferences differ widely between kinases, and there is no single consensus sequence that applies across the family (Yaron-Barir et al., 2024). As substrate specificity depends on both kinase-specific preferences and structural interactions, the potential phosphorylation of this C-terminal tyrosine in TEMP cannot be reliably predicted and would have to be confirmed experimentally.

Cycloheximide chase experiments indicate that substitution of N2, N5, or both residues does not substantially alter TEMP stability over the timescale tested (**Figure 5.10**). This suggests that any modification at these sites is unlikely to play a major role in regulating protein turnover under these experimental conditions. Similarly, analysis of S-acylation in the N-terminal mutants revealed no clear differences compared to wild-type TEMP, indicating that potential N-terminal modification does not overtly interfere with C-terminal S-acylation by zDHH6, however this could not be determined quantitatively due to the multiple, ill-defined band species present (**Figure 5.9**).

These findings suggest that the N-terminal region of TEMP may undergo modification that influences its mobility, but the exact nature of this modification remains unconfirmed. The data is consistent with a model in which N2 and N5 contribute to post-translational heterogeneity, but further experiments are needed to conclusively determine whether

these residues are glycosylated. Approaches such as enzymatic de-glycosylation or mass spectrometry would provide direct evidence of glycan addition, while functional studies could clarify whether N-terminal modification affects TEMP trafficking, interactions, or other cellular roles.

5.4.5 Key takeaways

- TEMP interacts with multiple zDHHC enzymes, but is only clearly and efficiently S-acylated by zDHHC6.
- C54 and C57 are the S-acylation sites of TEMP
- The C-terminus of TEMP may be important for zDHHC binding and S-acylation efficiency.
- Predicted N-terminal glycosylation sites (N2, N5) contribute to band heterogeneity
- S-acylation and N-terminal modifications do not significantly affect TEMP stability.

CHAPTER 6

Discussion

Chapter 6 - Discussion

6.1 Integrated summary of key findings

Across this thesis, S-acylation of several transmembrane proteins was characterised, alongside investigation of the substrate selectivity and functional roles of specific zDHHC enzymes, contributing to a broader understanding of how protein S-acylation is regulated within the secretory pathway.

Using CCR5 as a model GPCR, S-acylation by zDHHC3, zDHHC7 and zDHHC15 was confirmed in HEK293T cells, including modification of three C-terminal cysteine residues. Application of the RUSH system demonstrated increased S-acylation following ER exit, providing strong evidence that CCR5 is modified later in its trafficking pathway, consistent with the Golgi localisation of these enzymes. In addition to confirming known sites, a novel S-acylation site at C58 was identified, expanding the current understanding of CCR5 modification. Furthermore, mutational analysis of proposed O-glycosylation sites revealed reduced protein stability, indicating that these sites are functionally important. This extends previous knowledge, where glycosylation of CCR5 had primarily been linked to ligand binding, by suggesting an additional role in maintaining protein stability.

Building on this, substrate selectivity of zDHHC enzymes toward transmembrane proteins was investigated, and the known repertoire of S-acylated proteins and their modifying enzymes was expanded. Previously unreported S-acylation events and associated zDHHC enzymes were identified across a range of proteins, particularly within the GPCR family. zDHHC6 was shown to S-acylate not only single-pass transmembrane proteins but also multi-pass proteins such as K5, TSPAN4 and GPR37, albeit with reduced efficiency, indicating that while not strictly limited by membrane topology, its activity is influenced by substrate complexity.

Importantly, the positioning of juxta-membrane cysteine residues, previously proposed to predict zDHHC6 activity in single-pass proteins, did not reliably extend to multi-pass proteins. This suggests that such structural determinants are context-dependent and likely reflect the simpler architecture of single-transmembrane proteins rather than a universal mechanism of substrate recognition.

In contrast, GPCRs displayed a broader preference for Golgi-localised enzymes, particularly zDHHC3 and zDHHC15. This was supported by trafficking-based experiments, where both CCR5 and β 2AR showed reduced S-acylation upon ER retention in the absence of zDHHC overexpression, indicating that these observations reflect endogenous enzyme activity rather than experimental artefacts. Together, these findings emphasise the importance of subcellular localisation in regulating S-acylation, particularly for GPCRs.

Additionally, differences in lipid substrate preference between zDHHC enzymes were identified, with zDHHC3 and zDHHC15 favouring shorter acyl chains, while zDHHC6

showed no clear preference. This trend was also reflected in an initial analysis of known substrates, suggesting that enzyme identity may influence the lipid composition of modified proteins, although further investigation with larger datasets is required.

Finally, TEMP was characterised as a novel S-acylated protein and identified as a single-pass transmembrane substrate of zDHHC6. Despite challenges associated with multiple observed protein species, TEMP was shown to be likely S-acylated at two cysteine residues and to contain two probable N-glycosylation sites on its N-terminus, indicating that it undergoes multiple post-translational modifications. Interaction studies revealed binding to several zDHHC enzymes, including zDHHC3, zDHHC7, zDHHC2 and zDHHC17. Notably, no detectable binding to zDHHC6 was observed despite TEMP being a substrate, while binding to zDHHC2 occurred in the absence of detectable S-acylation. These findings suggest that TEMP may have additional roles in modulating zDHHC enzyme activity or organisation, which warrants further investigation.

Overall, this work expands the catalogue of S-acylated transmembrane proteins, identifies new enzyme-substrate relationships, particularly among GPCRs, and provides insight into how enzyme localisation, substrate structure, and lipid preference collectively influence S-acylation within the cell.

6.2 Experimental limitations and technical considerations

Several technical and experimental limitations should be considered when interpreting the findings of this study.

A major challenge throughout this work was the expression and detection of transmembrane proteins, many of which appeared as multiple bands on immunoblots. This was particularly evident for proteins such as TEMP, which consistently presented as multiple species, likely reflecting different post-translational modifications such as glycosylation. As a result, considerable time was required to determine which bands corresponded to relevant protein forms, and this complexity may have limited the sensitivity of detecting subtle changes in S-acylation. In some cases, modest effects may have gone undetected due to overlapping or poorly resolved bands. These challenges were compounded by variability in protein expression, particularly for larger constructs, which was influenced by factors such as the passage number of HEK293T cells. Reduced expression in higher passage cells affected reproducibility and was especially notable in experiments involving zDHHC15, where enzymatic activity appeared more variable under suboptimal conditions. This issue was further exacerbated in experiments using the RUSH system, as these constructs are relatively large and not optimised for immunoblot-based analysis, making consistent and robust expression more difficult to achieve.

More broadly, determining enzyme-substrate specificity across zDHHC enzymes proved challenging. Protein selection was constrained by the availability of plasmids (e.g. through Addgene) and reliable antibodies, and required cross-referencing with

databases such as SwissPalm to identify likely S-acylated candidates. Although a large number of proteins were screened, many were excluded due to poor expression, lack of detectable S-acylation, or no observable change upon co-expression with zDHHC enzymes. While this limited the overall dataset, it also reflects the inherent difficulty in defining substrate specificity, particularly as different protein classes showed distinct trends, for example, many GPCRs preferentially associated with zDHHC3 and zDHHC15 rather than zDHHC6. As such, the dataset, while informative, does not fully capture the breadth or complexity of zDHHC enzyme selectivity.

A further limitation lies in the reliance on overexpression systems. While useful for detecting S-acylation and enzyme-substrate relationships, overexpression may exaggerate modification levels or introduce non-physiological interactions. Similarly, the extensive use of mutagenesis to identify modification sites introduces the possibility of unintended effects on protein structure, stability, or trafficking, which could indirectly influence S-acylation.

Finally, this study primarily focused on biochemical characterisation and did not extensively investigate functional consequences or physiological relevance. While multiple S-acylation sites were identified on proteins such as CCR5, including both C-terminal and intracellular loop cysteines, their distinct functional roles remain unclear. Determining how these modifications influence receptor signalling, trafficking, or stability would be an important next step. Additionally, although this work provides foundational insight into S-acylation and zDHHC enzyme selectivity, it does not directly address how these processes contribute to disease states. Further studies in more physiologically relevant systems will be required to establish clearer biological and clinical relevance.

Overall, while these limitations do not undermine the core conclusions of the study, they highlight important areas for methodological improvement and future investigation.

6.3 Future directions and outstanding questions

This study provides a foundation for understanding S-acylation of transmembrane proteins and zDHHC enzyme selectivity, but it also raises several mechanistic and conceptual questions that warrant further investigation.

A key next step is to directly link S-acylation to protein trafficking using imaging-based approaches. While it is well established that S-acylation can influence protein trafficking and membrane organisation, the specific consequences for individual proteins identified in this study remain unclear. Confocal or live-cell imaging of fluorescently tagged wild-type and mutant constructs would allow the effects of S-acylation site mutations and glycosylation changes to be visualised in real time, providing clearer insight into how these modifications contribute to differences in localisation and protein stability.

For CCR5 in particular, a major unresolved question is the precise subcellular compartment in which S-acylation occurs. Although the data presented here strongly support a post-ER modification consistent with Golgi-localised zDHHC enzymes, this has not been directly demonstrated. Defining the exact site of modification would strengthen this model. In addition, CCR5 may exist as distinct molecular populations defined by their post-translational modification status, including potentially glycosylated-only, S-acylated-only, or dual-modified forms. Investigating whether these represent functionally distinct receptor pools could reveal a more complex regulation of receptor maturation and stability.

Extending these findings to more physiologically relevant systems is also important. This study relies heavily on HEK293T cells, yet both zDHHC expression patterns and GPCR trafficking behaviour can vary between cell types depending on their endogenous protein expression profiles. Examining S-acylation in more appropriate cellular models for specific receptors would therefore help determine whether the enzyme-substrate relationships observed here are broadly conserved or context-dependent.

A further priority is the systematic investigation of zDHHC enzyme selectivity across a broader range of substrates. While this study identified clear trends, particularly the preference of many GPCRs for zDHHC3 and zDHHC15 rather than zDHHC6, fully resolving enzyme specificity will require either a more targeted or a substantially expanded experimental approach. One option would be a focused strategy centred on well-defined protein families, such as GPCRs or single-pass transmembrane receptors, allowing controlled comparisons across related substrates. Alternatively, a larger-scale systematic screening approach could be undertaken, although this would require significant optimisation and resources. In either case, combining curated datasets such as SwissPalm with experimental validation provides a useful starting point for prioritising candidate substrates, but must be supported by direct biochemical confirmation, given the variability in predictive accuracy.

Finally, the role of proteins such as TEMP in S-acylation networks remains to be determined. Although identified here as a single-pass transmembrane substrate of zDHHC6, TEMP also interacts with multiple zDHHC enzymes, including those for which it is not a clear substrate. This raises the possibility that TEMP may not function solely as a substrate, but could also influence the organisation, accessibility, or activity of S-acylation machinery. Systematic co-expression experiments combining TEMP, specific zDHHC enzymes, and established substrates could help determine whether TEMP acts as a regulatory or scaffolding component within these pathways.

Overall, these future directions would move the field beyond static identification of S-acylated proteins toward a more integrated understanding of how S-acylation is dynamically regulated in space and time, and how it contributes to the functional diversity of membrane proteins.

6.4 Conclusions

This thesis has systematically characterised S-acylation across a range of transmembrane proteins and provided new insight into the determinants of zDHHC enzyme selectivity. It expands the catalogue of S-acylated substrates and identifies previously unreported enzyme-substrate relationships, particularly among GPCRs, which show a strong association with Golgi-localised zDHHC enzymes such as zDHHC3 and zDHHC15.

Using CCR5 as a model receptor, this work supports a model in which S-acylation occurs predominantly post-ER and acts in concert with other post-translational modifications to regulate receptor maturation and stability. In addition, TEMP was identified as a novel single-pass transmembrane S-acylated protein and shown to associate with multiple zDHHC enzymes, including zDHHC6. Its ability to bind several zDHHC family members suggests a potential role as an interactor within the S-acylation machinery rather than solely a conventional substrate, further broadening the scope of zDHHC interaction networks.

Overall, these findings demonstrate that zDHHC substrate selectivity is not governed by a single structural determinant, but instead emerges from an interplay between protein architecture, subcellular localisation, and cellular context. Together, this work provides a framework for understanding how S-acylation is organised across diverse membrane protein classes and establishes a foundation for future studies aimed at linking these biochemical mechanisms to functional and physiological outcomes.

CHAPTER 7

References

Chapter 7 – References

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