

Novel chemical genetic tools for the proteomic profiling of S-acylation by ZDHHC enzymes.

A thesis presented for the degree of
Doctor of Philosophy of Imperial College London

By

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Statement of originality

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A mis padres

Novel chemical genetic tools for the study of ZDHHC dependent S-acylation.

Abstract

Post-translational modifications (PTM) represent the largest source of proteome diversity, regulating the function and fate of almost every eukaryotic protein. One of these is S-acylation, which refers to the post-translational covalent attachment of long-chain fatty acids onto thiol groups in surface-exposed cysteine residues. The combination of two enzyme classes, S-acyltransferases and thioesterases dynamically control the levels of this PTM in cellular proteins. The former, includes a family of 23 isoenzymes commonly known as ZDHHCs due to their conserved catalytic motif, which together S-acylate every eukaryotic protein. S-acylation has been found to participate in numerous cellular functions including protein localisation, trafficking, signalling or gene expression and consequently, misregulation of S-acylation has been associated to neurodegenerative diseases and cancer. To date, the tool set available to study the S-acylated proteome remains scarce and without small molecule modulators of individual ZDHHCs, their biological significance cannot be selectively detangled from the rest of the family. Here, I present the design and development of a novel chemical genetic approach to unravel substrate scope of ZDHHC20 via chemical proteomics. For this, ZDHHC20 was engineered to contain a mutation in the lipid binding domain, which allowed both the selective binding of a lipid probe and its transfer to substrates. This allowed the curation of the first substrate list for ZDHHC20 in HEK293T cells, several of which were validated in targeted assays. Their analysis depicted a strong involvement of ZDHHC20 in vesicular trafficking from the endoplasmic reticulum and Golgi apparatus. This relationship was further explored using ER-localised TurboID to compare ER-resident and secreted proteins in ZDHHC20 KO and wildtype backgrounds. These tools represent a novel and versatile perspective from which to explore the substrate scope of individual ZDHHC enzymes and their biological relevance in health and diseased cellular states.

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Statement of Collaboration

The progress presented in this thesis has been supported by collaborations with a group of researchers, most of whom are past or current members of the Tate group. Their contributions are noted throughout this document, and the key contributions are further summarized here:

- Dr. Tony Ocasio contributed to the initial set up of the chemical genetic system, generation of ZDHHC20 knockout lines, YnPalm proteomic analysis and enzyme kinetics experiments.
- Dr. James Siphthorp contributed proteomics experiments using the on-bead digestion protocol.
- Dr. Marc Baggelaar contributed the proteomics experiment with the on-bead hydrolysis protocol and synthesis of lipid probes.
- Christelle Soudy contributed design and synthesis of the lipid probes.
- Dr. Elisabeth Stork Saha contributed lipidomics data acquisition and manual curation of these results.
- Dr. Susana Palma (Metabolomics STP, The Francis Crick Institute) contributed the detection of acyl-CoA species in cells.
- Dr. Jack Houghton contributed quality control analyses of mass spectrometry samples.
- Zuzanna Jablonska contributed the microscopy experiments to localize TurboID constructs in cells.

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Abbreviations

2-BP	2-bromopalmitate
Å	Armstrong
Aa	Amino acid
ABE	Acyl-biotin exchange
Ac	Acetyl
ACN	Acetonitrile
ACSL	Long-chain fatty acid CoA ligases
APT	Acyl protein thioesterases
AS-ES	Analogue and electrophile-sensitive
Asp	Aspartic acid
BSA	Bovine serum albumin
Bz	Benzoyl
CAA	Chloroacetamide
CMA	Cyano-methacrylamide
CoA	Coenzyme A
cPr	Cyclopropanecarbonyl
CuAAC	Copper-catalysed alkyne-azide cycloaddition
Cys	Cysteine
DG	Diacylglycerols
DIPEA	N, n-diisopropylethylamine
DMSO	Dimethyl sulfoxide
ER	Endoplasmic reticulum
ERGIC	ER to Golgi intermediate compartment
ES	Electrophile sensitive
FA	Fatty acid
FLAG	DYKDDDDK peptide sequence
FTase	Farnesyltransferase
GGTase	Geranylgeranyl-transferase
GO	Gene ontology
HDFP	Hexadecyl-fluorophosphonate
His	Histidine
IFITM3	Interferon-induced transmembrane protein 3

Ile	Isoleucine
IP3	Inositol triphosphate
KD	Knock down
KDa	Kilodalton
KDEL	K-D-E-L amino acid tag
KDH	α -ketoglutarate dehydrogenase
KO	Knock out
LC	Liquid chromatography
LFQ	Label free quantification
MeOH	Methanol
MetAPs	Methionine aminopeptidases
MS	Mass spectrometry
MTBE	Methyl tert-butyl ether).
NADH	Nicotinamide adenine dinucleotide
NEM	N-ethylmaleimide
NES	Nuclear export signal
NH ₂ OH	Hydroxylamine
NK	Natural Killer
NLS	Nuclear localisation signal
NMT	<i>N</i> -myristoyl transferase
OBD	On-bead digestion
OBH	On-bead hydrolysis
PAT	Protein acyl transferase
PBS	Phosphate buffer saline
PC	Phosphatidylcholines
PDAC	Pancreatic ductal adenocarcinoma
Pdgfrb	Platelet-derived growth factor receptor beta
PE	Phosphatidyl ethanolamine
PG	Phosphatidylglycerols
Phe	Phenyl alanine
PTM	Post-translational modification
PTXQC	Proteomics quality control
RAC	Resin assisted capture
SDS	Sodium dodecyl sulphate
Ser	Serine

STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TM	Transmembrane helix
TMT	Tandem-mass-tag
TPP	Triphenyl phosphonium
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
WT	Wildtype
YnMyr	Alkyne myristate
YnPal	Alkyne palmitate
ZDHHC	Zinc-finger Asp-His-His-Cys motif

Chapter 1: Introduction

1.1. The importance of post translational modifications.

It is widely known that for any cell in an organism, the proteome is under constant dynamic regulation in response to internal and external stimuli and contrasts with the genome which is much more static and identical across all somatic cells ¹. It is also known that the size of an organism's proteome is a more direct measure of its complexity ^{2,3}. Cellular complexity begins at the genome level, where diversity is achieved through genetic mutations and variability starts to increase when the genome is transcribed. A single gene can give rise to several transcripts through alternative splicing, different transcription start sites, or differences in the processing of mRNA ^{4,5} (Fig. 1.1). During the translation process, different start sites can also give rise to several gene products, yet the proteome is greatly extended by co- and post-translational modifications (PTMs) ⁶ (Fig 1.1.). Modifications can occur on multiple amino acids and their combinatorial nature contrasts with the limited possibilities for genomic mutations, which are sometimes silent due to codon redundancies. Consequently, PTMs remain the greatest source of cellular variance, amplifying the chemical and functional diversity of a protein's primary sequence and consequently that of an organism's proteome by orders of magnitude (Fig 1.1) ^{7,8}. Notably, PTMs also have an impact on the diversity of the transcriptome, for example by mediating gene expression through histone modifications (i.e. methylation, acetylation or phosphorylation) which directly impact the degree of chromatin condensation ⁹.

There are approximately over 400 post-translational modifications reported to date which tightly regulate the function and fate of almost every eukaryotic protein, contributing to a whole range of cellular processes ¹⁰. A single modification to a protein's structure leads to a new protein state or proteoform ⁶ and by summing every protein PTM combination, the human proteome has been estimated to surpass 6 million proteoforms ¹¹. It is only when this multitude of protein states are considered that fundamental cellular processes in healthy and diseased states can really begin to be understood ⁷. Amongst these processes, those more dynamic in nature are often governed by reversible PTMs (i.e. phosphorylation and ubiquitination), which can

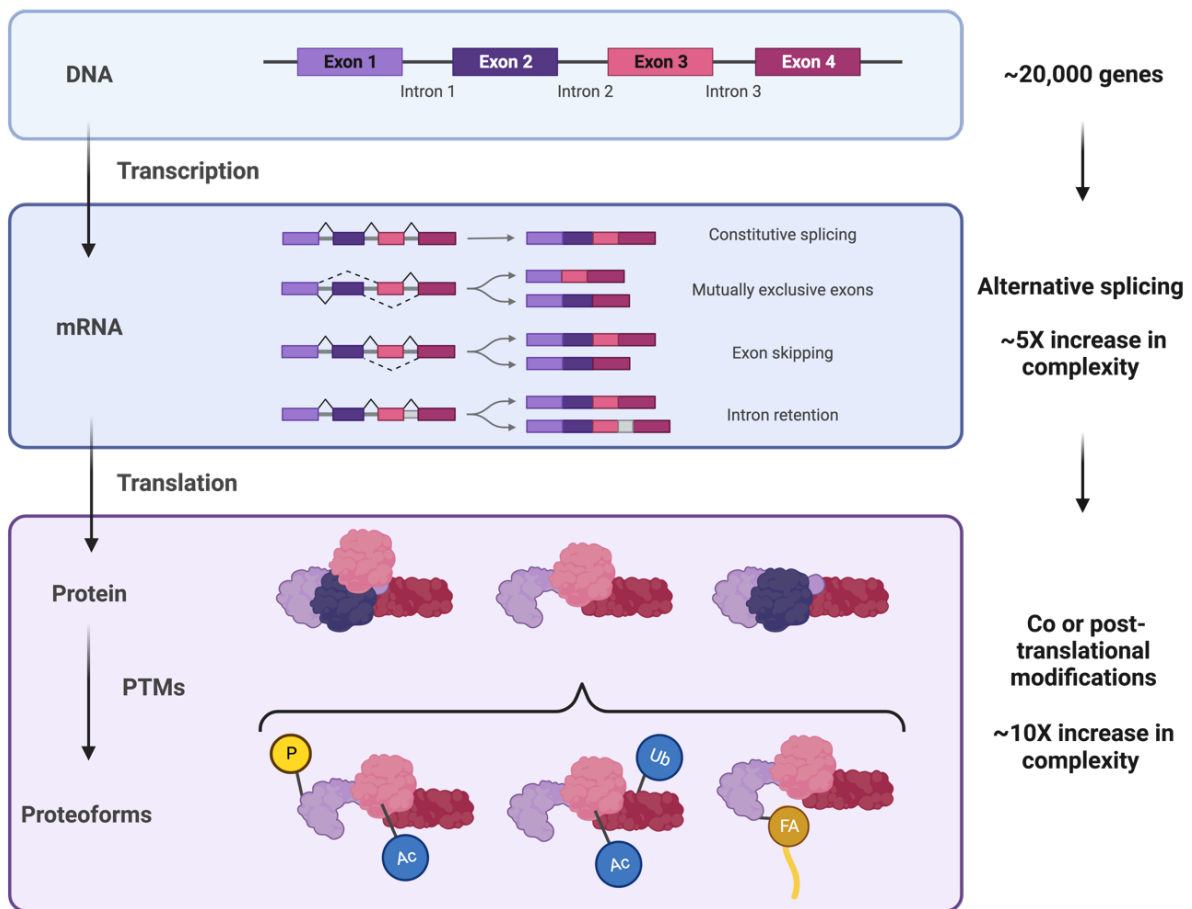


Figure 1.1. Post translational modifications as the biggest source of proteome complexity. Following the central dogma, DNA is transcribed to pre-mRNA, which is subject to alternative splicing giving rise to different mRNA isoforms and increasing approximately ~5X the complexity of the genome. In turn, these are translated to give rise to different protein isoforms, which can have divergent structures and functions. Finally, each of these protein isoforms could be differentially post-translationally modified increasing a further ~20 times the complexity of the proteome. Phosphorylation (P), acetylation (Ac), ubiquitination (Ub) and fatty acylation (FA). Figure created with BioRender.

participate in signalling cascades by acting as on/off switches for downstream signal relay ^{12–14}. On the contrary, irreversible PTMs are often found to dictate more static processes such as a protein's native localisation, yet they are equally decisive in dictating correct protein function (i.e. farnesylation, myristoylation)¹⁵.

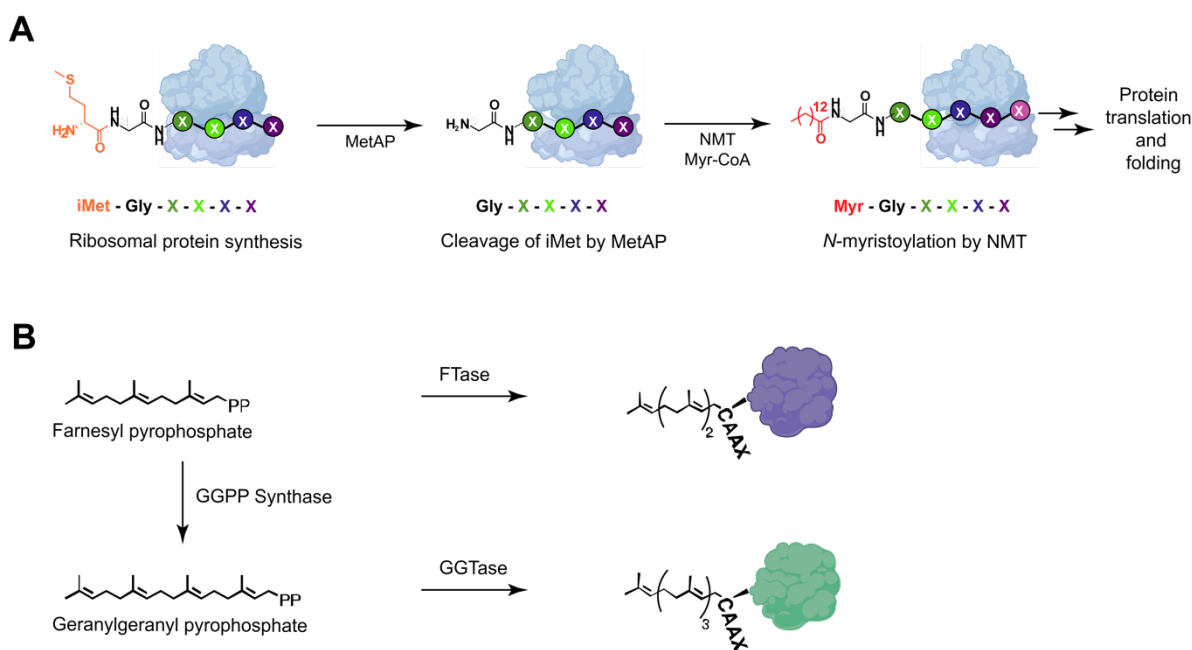
The importance of PTMs in cellular function is such, that significant efforts have been placed towards their understanding in normal and pathological conditions. This includes modern proteomics approaches for the high-throughput identification of PTMs and the amino acid modification sites ¹⁶ and in certain cases, in combination with chemical biology probes for endogenous substrates ^{17,18}. Furthermore, since

proteins remain the target of most drugs ¹⁹, chemical probes remain useful for target validation studies which are fundamental in the initial phases of drug discovery programs ²⁰.

1.2. Post translational protein lipidation.

Lipids are indispensable cellular building blocks. They constitute essential cellular membranes that separate the cellular content from the extracellular space and delimit cellular processes through organelle compartmentalisation ²¹. When post-translationally added to proteins, they are known for promote substrate association to cellular membranes. However, the addition of such hydrophobic groups has also been linked to the regulation of a wide range of cellular processes including of protein-protein interactions, protein localisation, stability, trafficking and also gene expression ^{7,15,22–26}. The development of chemical proteomic methods for the global profiling of lipidated substrates via mass spectrometry, has enabled the deconvolution the full scope of these PTMs in various settings including cells, pathogens and multicellular organisms ^{15,18,27}. Nevertheless, work still remains to understand the substrate scope and dynamic interplay between some of these lipidation events and the cross-talk with other non-lipid PTMs. From a pathological point of view, lipidation has been associated to viral infectivity, cancer, inflammation, developmental diseases and neurological disorders ^{28–32}. As such, lipid-modifying enzymes have been incorporated into drug-discovery programs with the aim of finding selective small molecule modulators of their function and gain a comprehensive understanding of their roles in more diverse contexts and potentially exploit them towards a therapeutic benefit ⁷.

Amongst all lipid PTMs, *N*-myristoylation, *S*-prenylation and *S*-acylation are the most well-studied PTMs due to their extensive contribution to cellular processes ^{7,15}. *N*-myristoylation, refers to the condensation of the fatty acyl group myristate (C14) from myristoyl-coenzyme A to the N-terminal glycine of substrate protein by two *N*-myristoyl transferase enzymes NMT1 and NMT2 which together modify approximately 400 substrates (Fig. 1.2. A) ³³. This occurs co-translationally at the ribosome after initiator methionine cleavage by methionine aminopeptidases (MetAPs), but can also occur post-translationally after protease digestion. *S*-prenylation occurs at C-terminal cysteines, this is the covalent attachment of



farnesyl (C15) or geranylgeranyl (C20) isoprenoids via a thioether linkage (Fig. 1.2 B)³⁴. Farnesyltransferase (FTase) and two geranylgeranyl-transferases (GGTase-1, GGTase-3), are responsible for the addition of isoprenoids and do so at a canonical C-terminal motif known as the 'CaaX' box (a: aliphatic amino acid)^{35,36}. Dysregulation of *S*-prenylation has been associated to several human pathologies including viral infections and cancer³⁷. Despite the fact that prenyltransferase inhibitors have been extensively investigated, they have all failed in clinical trials due to lack of efficacy and cytotoxicity³⁸. NMTs however, have been found a tractable drug target in pathogenic fungi, parasites and viral infections where NMT inhibitors could be novel potential therapeutics^{39–41}.

The two lipid PTMs discussed above are examples of irreversible lipidation at the *N*- and *C*-terminus respectively. In contrast, *S*-acylation, which refers to the covalent attachment of long chain fatty acids onto cysteine residues, is reversible. This PTM is discussed in more detail in the next sections of this introductory chapter as it is the main focus of this PhD thesis.

1.3. Protein S-acylation.

Protein S-acylation refers to the post-translational covalent attachment of a long chain fatty acid (FA) (C14-20), onto a free thiol residue on a surface-exposed cysteine. It is catalysed by a family of multiple-pass transmembrane enzymes: protein acyl-transferases (PATs), also known as ZDHHCs after their conserved zinc-finger Asp-His-His-Cys catalytic motif (Fig. 1.4) ^{7,15}. They are localised at lipid bilayers including the plasma membrane, ER and Golgi ⁴². This modification is often imprecisely referred to as S-palmitoylation or palmitoylation due to the prevalence of C16 fatty acids (palmitate) in cellular lipidic pools, even though incorporation of fatty acids with chain lengths from 14 to 20 can also occur ⁴³. Interestingly, direct autoacylation has been reported for TEADs, a family of transcription factors that modulate gene expression in response to the hippo signalling pathway ⁴⁴.

It has been commonly accepted that ZDHHCs S-acylate in a two-step or ping-pong mechanism, ever since kinetic studies identified an autoacylated form of these PATs both *in vitro* ⁴⁵ and *in cellulo* ⁴³. The current model for the catalytic mechanism of ZDHHCs is depicted in Figure 1.3. It begins with the binding of activated fatty acid CoAs to ZDHHCs, placing the thioester in proximity to the catalytic cysteine residue for a consequent thioesterification step that covalently loads the fatty acid onto the enzyme in an autoacylation reaction. A recent crystal structure shows human ZDHHC20 in complex with the activated CoA fatty acid supporting this model ⁴⁶. Next, when substrates come into close proximity, the fatty acid can be transferred to a surface-exposed cysteine in a trans-thioesterification step ⁴⁷. For substrates, no consensus sequence has been identified for S-acylation, contrary to other lipid PTMs such as S-prenylation. Moreover, to date, the overall mechanisms that govern substrate binding and recognition are largely unknown, with some ZDHHCs containing recognition domains for direct substrate interaction, which are discussed in the next section.

The consequences of S-acylation vary between substrates, for cytosolic proteins it increases their membrane affinity possibly changing their localisation and surrounding interactors ⁴⁸. For those already embedded in the membrane and/or with a prior lipid PTM, addition of a palmitoyl group can change their membrane localisation, to

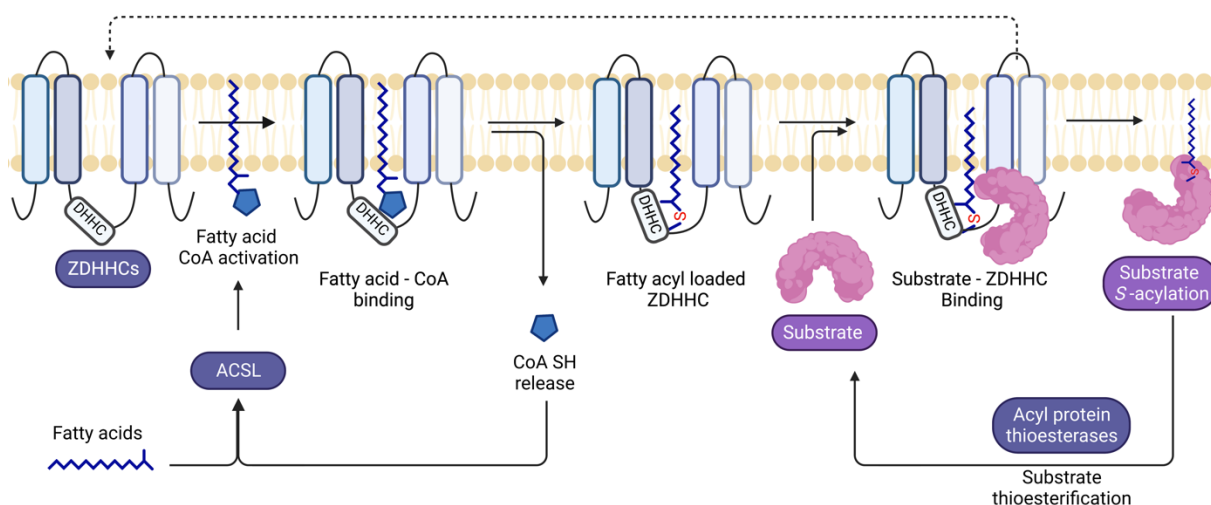


Figure 1.3. Biochemical pathway for substrate S-acylation. After binding of fatty acid CoAs, ZDHHC enzymes load (thioesterify) the fatty acid onto their catalytic Cys. Upon substrate binding a transfer (transthioesterification) step leads the enzyme back to its inactive state. Substrate thioester hydrolysis is performed by thioesterases, which participate in this dynamic cycle. Figure created with BioRender.

cholesterol rich areas (i.e. lipid rafts), induce a structural changes which can alter protein-protein interactions or preventing the addition of other PTMs ⁴⁹.

The uniqueness of S-acylation relies on its reversibility via the action of thioesterases which remove the fatty acid and return the protein to its non-S-acylated state (Fig. 1.4). The best characterized thioesterases are in the serine hydrolase superfamily: lysosomal PTT1 and PTT2 and cytosolic APT1 and APT2, and more recently the α/β hydrolase fold domain (ABHD) proteins (ABHD10 and ABHD17A-C) ^{50–55}. The combination of these with PATs creates a highly dynamic and tightly regulated system that is not present in the rest of the lipidation reactions, which are considered static. This reversibility can act as on-off switch comparable to other PTMs such as phosphorylation or ubiquitination, making it possible for S-acylation to participate in dynamic cellular processes like signalling and trafficking ^{14,56}. In other words, as a key response element to cellular stimuli.

1.3.1. Prevalence and significance of S-acylation

Estimations predict that 10% of genes in the human proteome (SwissPalm database) are S-acylated, a very high number compared to the estimated values of 1-4% for N-myristoylation ⁵⁷, which is indicative of the vast implication of S-acylation in essential

cellular processes in health and disease ^{58–60}. In fact, many oncogenic drivers have been found to rely on S-acylation for the regulation of their activity ^{24,61–63}. A study conducted by Bailey et al. identified 299 cancer-related genes, 78 of which had been found to be S-acylated ⁶⁴. Famous examples include the RAS family of GTPases (HRAS, NRAS and KRAS4a) which are also S-prenylated ^{65,66}, tumour suppressor SCRIB ⁶⁷ or EGFR whose localisation and dimerization has been found linked to S-acylation ^{68,69}. Another disease area vastly where this PTM has been demonstrated to play a key role are neuropathies, for example, Huntington's disease, Alzheimer's disease, mental disabilities or schizophrenia ^{28,70,71}. In neurons, ZDHHC enzymes and thioesterases dynamically regulate protein trafficking between the plasma membrane and intracellular compartments necessary for synaptic plasticity and correct function of the vesicle fusion machinery ^{23,72–74}. Neurotransmitters, post-synaptic receptors and multitude of vesicle-associated proteins are some of the many substrates of ZDHHCs in neuronal tissues ^{29,75–78}.

Finally, it is also worth highlighting that S-acylation also plays a role in the cellular immune response ⁷⁹, for example mediating lysosomal targeting of virus after infection ^{26,80}. In turn, several pathogens also rely on this PTM for essential survival mechanisms. For example, bacterial S-acylation has been found implicated in membrane maintenance, transport mechanisms, and signal transduction ^{81,82}. Furthermore, viruses also rely on their host lipidation machinery. Examples of viruses include Herpes Simplex virus whose structural integrity extensively depends on its host S-acylation machinery ⁸³, or SARS-CoV2 where evidence suggests that the S-acylation of the spike protein is responsible for virion membrane composition ⁸⁴. Overall, drugs designed to target protein S-acylation, could be promising anti-viral agents in the fight to tackle infectious diseases.

1.3.2. 3D structure of ZDHHCs

Proteins capable of S-acylation were firstly discovered around 20 years ago, when two yeast proteins with a common ZDHHC cysteine-rich domain were found to be able to transfer palmitoyl groups ^{85,86}. Later on, GODZ – a Golgi-specific PAT later renamed as ZDHHC3 – was found responsible for S-acylating the GABA-A receptor $\gamma 2$ subunit in cells ⁸⁷. The ZDHHC cysteine-rich domain of ZDHHC3 was used to search for homologous regions in the human genome, identifying 23 enzymes which, together

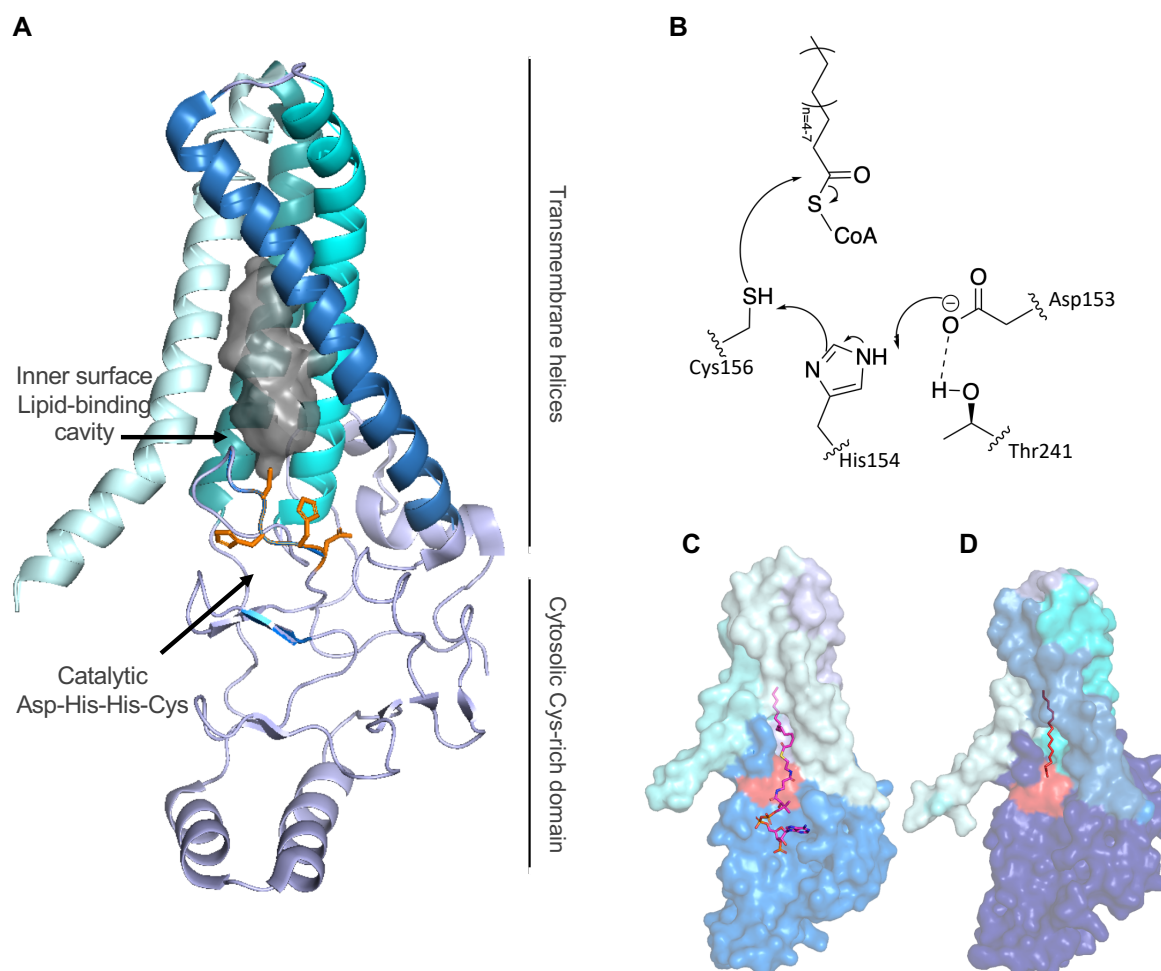


Figure 1.4 Structural features of human ZDHHC20. Based on the published crystal structures (pdb: 6BML; 7KHM) **A)** Crystal structure of human ZDHHC20 (pdb 6BML) with its four transmembrane helices forming a lipid binding cavity – its inner surface highlighted in grey. In orange the catalytic Asp-His-His-Cys motif located at the interface between the cytosol and the lipid bilayer. **B)** Proposed mechanism for nucleophilic attack by the thiolate anion onto the carboxylic acid of a fatty acid CoA. **C)** and **D)** Structures of lipid CoA and 2-BP respectively (7KHM; 6BML).

are responsible for the S-acylation of all substrates in the human proteome^{88,89}. These isoenzymes have a common topology comprised of at least four transmembrane helices; and a maximum of six in the case of ZDHHC13, 17 and 23. Their cysteine rich domain resides in between these transmembrane helices and is structured by the chelation of two zinc ions⁴⁷.

In 2018, Rana *et al.* in a key paper for the field, presented the first PAT crystal structures, those of human ZDHHC20 and zebrafish ZDHHC15, which revealed that these polytopic enzymes sit within the plasma membrane with their four transmembrane helices arranged in a conical fashion; creating a lipid cavity in the

middle that can harbour a long chain-fatty acid ⁴⁷ (Fig 1.4 A). The active site resides at the aqueous-membrane interface, with the catalytic cysteine residue pointing towards said lipid cavity and levelled to the hydrophobic part of the lipid bilayer (Fig 1.4 A). A close analysis of the crystal structures led Rana *et al.* to hypothesise that the amino acids in the catalytic triad participate both in the positioning of this cysteine residue and on its nucleophilic activation. In the case of ZDHHC20, Asp-153 and His-154 were found to be at a distance where hydrogen bonding can occur, with the latter putatively being capable of deprotonating the catalytic cysteine enhancing its nucleophilic attack on the fatty acid CoA (Fig. 1.4 B). During the trans-thioesterification step, proton transfer would be reversed from Asp-153, to stabilise the thiolate anion. With regards to His155, this was shown to be involved in the chelation of one of the two zinc atoms, and by doing so contributing to the arrangement of the catalytic Cys156. More recently, a publication revealed the crystal structure of the precatalytic complex of human ZDHHC20 bound to palmitoyl CoA, where the catalytic cysteine was mutated to serine (DHHS) to prevent fatty acid loading ⁴⁶. This structure, showed that Co-enzyme A – a charged headgroup based on a sugar phosphate – binds at the exposed aqueous surface of the enzyme and the lipid tail inserts in the pocket, shielding itself from the aqueous interface of the cytosol (Fig 1.4 C), yet not fully extended into the lipid cavity. The initial structure had 2-bromopalmitate (2-BP) – a highly reactive and unselective fatty acid-like small molecule ⁹⁰ – covalently bound. By comparison of the two, the fatty acid would only be fully inserted into the pocket only upon thioesterification onto the catalytic cysteine (Fig 1.4 D). Furthermore, it is yet unknown how the fatty acid inserts itself into the cavity, with a possibility being that the helices open up, letting the fatty acid CoA slide into the pocket whilst maintaining the CoA at the aqueous interface as per the crystal structure.

1.3.3. Substrate and fatty acid selectivity of ZDHHCs

Early structural studies based on single point amino acid mutations had shown that the residues that shape the cavity are responsible for the fatty acid selectivity of ZDHHCs. For example, Greaves *et al.*, using alkyne and azide tagged fatty acids, showed that Ile182 in ZDHHC3 dictates a fatty acid chain-length preference of C14 and C16, and an Ile182 to Ser mutation – present in ZDHHC7 – shifts the preferences towards length of C18 ⁴³. With this first study two things were highlighted: firstly, that

a bulkier isoleucine residue disfavours the loading of longer fatty acid chains; and secondly, that even PATs highly related in sequence such as ZDHHC3 and ZDHHC7 present different fatty acid selectivity ⁷. More recently, for ZDHHC20, the residue Tyr181 has been demonstrated exert fatty acid preferences of C14-C16. In this case, it has been proposed that Tyr181 is involved in a hydrogen bond with Ser29, closing the lipid cavity and disfavours the loading of longer fatty acids ⁹¹. Despite the fact that fatty acid preferences have been reported ^{43,45}, there is not much information on the extent by which saturation levels affect fatty acid preferences and no correlation has been found between them and PAT subcellular localization.

With regards to substrate selectivity, the main source of specificity is thought to be at the N- and C-terminal ends, where structural diversity within the family is mostly found ⁹². There are three main structural motifs found across acyltransferases: ankyrin motifs, PDZ (PSD-95, Dlg and ZO-1) motifs and SH3 domains. With a common evolutionary path, ZDHHC13 and ZDHHC17 contain ankyrin motifs at their cytosolic N-terminal tails (Fig. 1.5). These are 33-amino acid peptide sequences which are well known protein-protein interactor modules. In the case of ZDHHC17, they have shown to mediate the S-acylation of specific substrates such as DLK ⁹³ and SNAP25 ⁹⁴. Sequence analysis of ZDHHC17 substrates led to the identification of the following conserved amino acid motif: [AIPV][ITV]XX[CMQ][IP]X[KR], which mediates substrate binding and enables S-acylation at nearby cysteine residues ⁹⁵. This indicates that 'exosite' enzyme-protein interactions as the sole director of ZDHHC17 S-acylation. Bizarrely, ZDHHC13 – with a DQHC catalytic motif – is also capable of binding these substrates but it does not seem to increase their S-acylation levels of some of these substrates ⁹⁶, in spite of the co-localisation of both PATs at the Golgi. This implies that it might engage other functions apart from lipidation and that additional, yet unknown, structural motifs might participate its substrate recognition. Another widespread structural domain are PDZ sequences, found at the C-terminus of up to 9 PATs without a clear path for their evolutionary conservation, yet found to mediate the interaction and acylation of proteins such as postsynaptic density protein 95 (PSD-95) ⁹⁷ and GRIP1 ⁹⁸ by ZDHHC5, or SCRIB by ZDHHC7 ⁶⁷. Lastly, found at the C-terminus is the SH3 domain of the ER-localised ZDHHC6. Through this domain ZDHHC6 has been found to interact and acylate the inositol triphosphate (IP3) receptor, which also harbours a SH3 binding domain ⁹⁹. On Cys residues within this

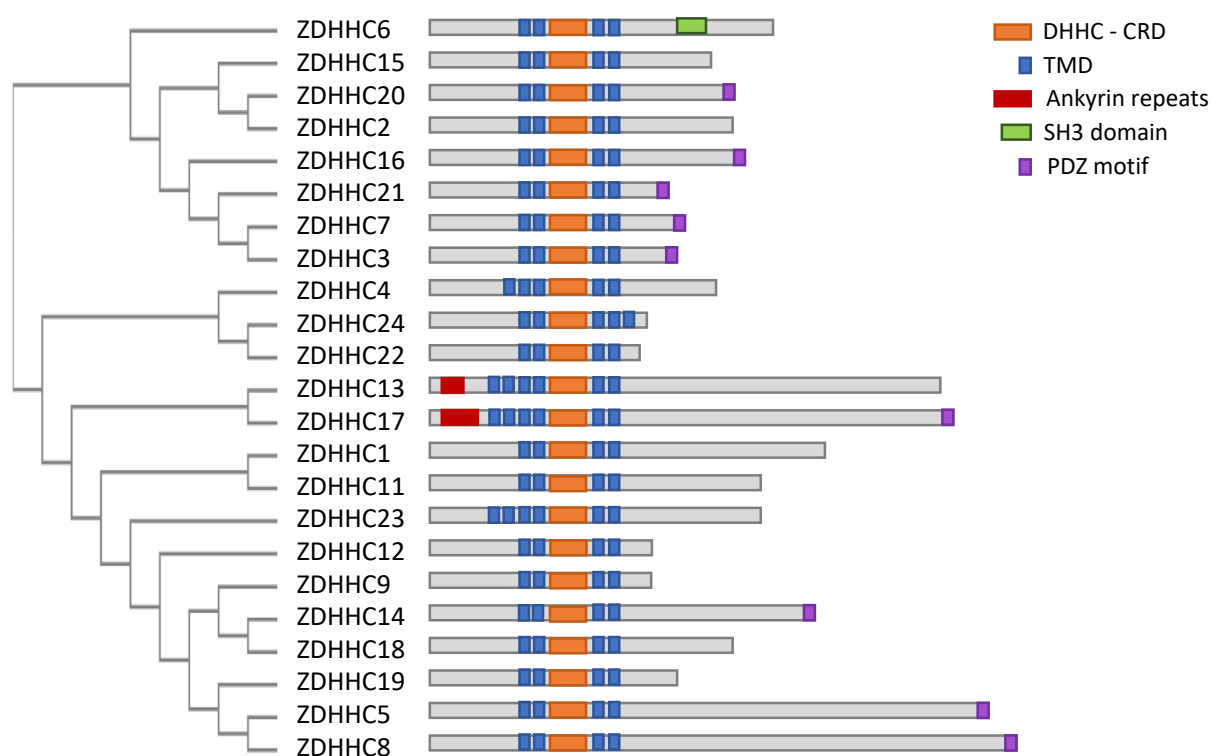


Figure 1.5. The ZDHHC acyltransferase family. Evolution of ZDHHC acyltransferases and their structural motifs

domain, ZDHHC6 has also been found to undergo S-acylation by ZDHHC16¹⁰⁰. This represents an example of how substrate binding can itself be regulated by modifications on the acyltransferase. Consequently, if PTMs on ZDHHCs itself are considered, the complexity of the question of how ZDHHCs exert their substrate selectivity increases. In fact, modifications found on ZDHHCs include phosphorylation, ubiquitination, S-acylation and there are examples in the literature that link them to substrate recognition, binding, and localisation¹⁰¹. For example, ZDHHC17 presents four tyrosine residues on its ankyrin repeat, which have been reported to be phosphorylated; of these the alanine mutant of Tyr67 presented reduced affinity for its substrate SNAP25⁹⁴. Phosphorylation also acts as an on/off switch mediating ZDHHC5 endocytosis from the plasma membrane¹⁰². These studies are not yet widespread and it might well be that different permutations of these PTMs are found for a single isoenzyme, or across tissues and disease states, which remains an open question in the field. Furthermore, the crosstalk between S-acylation and other lipid PTMs also remains understudied, yet recent evidence suggest a dependence of S-

acylation on the prior addition of a myristate group ¹⁰³. Future work on understanding the dependence mechanisms between lipid PTMs may shine light into the substrate scope and selectivity of sites of modification.

Despite the above-mentioned elements that dictate substrate specificity, current knowledge remains scarce and the means to enzyme-substrate recognition remain mostly elusive and consequently so does substrate selectivity across the family. Furthermore, it is currently believed that these enzymes present overlapping substrate scopes which makes it difficult to identify enzyme-substrate pairs, as the knockdown of a single ZDHHC enzyme often fails to show a reduction in substrate S-acylation levels ^{80,104}. Nevertheless, efforts have been placed to understand the common characteristics amongst sites of S-acylation in proteins. Salaun *et al.* ¹⁰⁵ classified them into three different cases:

- 1) Cysteine is adjacent to other sites of lipidation such as *N*-myristoylation ¹⁰³ and S-prenylation, this is the case for the RAS protein family ^{30,65}.
- 2) They are surrounded by other basic or hydrophobic residues with affinity for bilipid membranes ¹⁰⁵.
- 3) For integral membrane proteins, the location of Cys is proximal to transmembrane domains and approximately 8Å deep embedded in the membranes ¹⁰⁶.

In summary, it may well be that 'exosite' enzyme-protein interactions, in certain cases mediated by PTMs, but outside the catalytic site together with enzyme-substrate organelle distribution and tissue expression patterns are the only important drivers of substrate selectivity ^{94,95}. However, the identification and analysis of individual ZDHHC substrates at a whole proteome scale might unravel isoenzyme-specific consensus sequences, which may shine light on the mediators of S-acylation

1.4. Tools to study S-acylation

Historically, the first tools to study S-acylation were tritiated lipids, which were used in lengthy experiments with low sensitivity of detection, also with an associated health hazard due to exposure to radioactivity. In certain cases, tritiated lipids are still used as they are the best mimics of endogenous lipids in structure and physicochemical properties ¹⁰⁰. Nevertheless, with the development of bioorthogonal reactions such as, the copper-catalyzed alkyne-azide cycloaddition (CuAAC) and its application to study biological systems, soon the lipidation field shifted to the use of unobtrusive alkyne and azide tags on fatty acids together with biotin pulldown reagents, such as biotin azide or a protease-cleavable biotin analogue ¹⁰⁷. Alkynyl or azide fatty acids were soon demonstrated to be loaded to zDHHCs and metabolised as endogenous lipids and with similar selectivity profiles as endogenous fatty acids ⁴³. Consequently, since their development, these probes have been widely used in metabolic labelling experiments for visualization and identification of S-acylated proteins via in-gel fluorescence and mass spectrometry ¹⁰⁸.

To date, progress on the S-acylation field relies on a combination of biochemical and mass spectrometry assays in combination with small molecule modulators. Together, these have allowed to study this PTM in various cellular states, including *in vivo* tissue samples. These methods and their limitations are discussed in detail in the following sections as they constitute the foundations of our current knowledge.

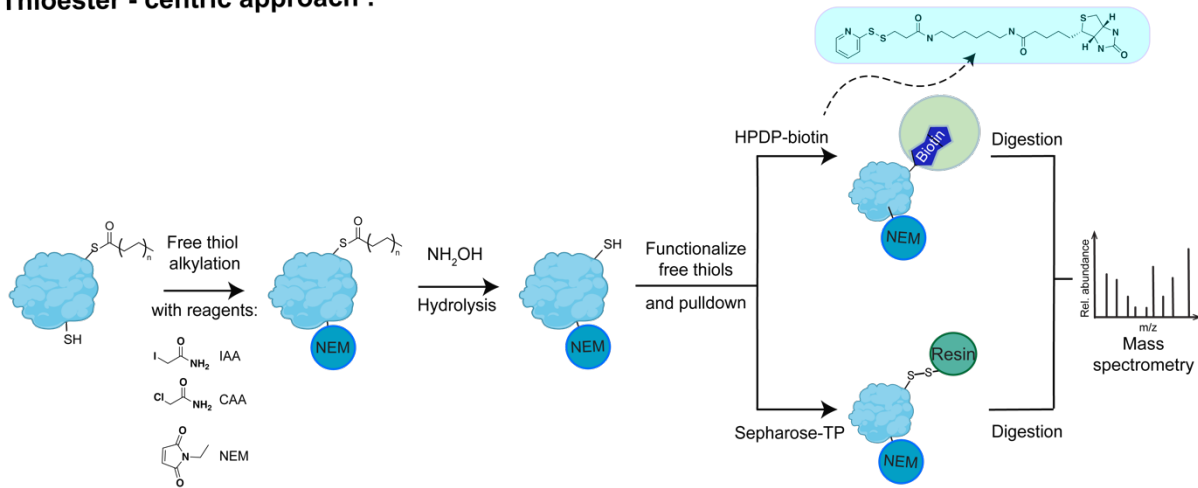
1.4.1. Mass spectrometry methods for S-acylation.

Studying protein lipidation via mass spectrometry presents one main challenge: lipid hydrophobicity, which prevents the facile isolation and characterization of lipidated peptides. Furthermore, S-acylation presents a further two analytical hurdles. Firstly, the extremely complex nature of the S-acylated proteome; containing substrates harboring multiple S-acylation sites, with different turnover times and sometimes in combination with other PTMs ¹⁰⁹. Secondly, the lability of the thioester bond, subject to hydrolysis in acidic or basic pH. This makes spontaneous thioester hydrolysis or transfer to other nucleophilic residues possible (i.e. O-acyl, N-acyl); potentially resulting in PTM loss during sample preparation protocols and a potential source of error in the identification of S-acylated proteins via mass spectrometry protocols ²⁷.

Nevertheless, two main mass spectrometry protocols have been developed to date to study S-acylation: 'lipid-centric' or 'thioester-centric' ^{7,22}. The latter is based on the hydrolysis of the S-acyl bond and the reaction of the free thiol with either resins, known as Acyl-RAC (resin assisted capture), or biotin reagents through the formation of a more stable thioether bond, named acyl-biotin exchange (ABE) (Fig.1.6) ^{110,111}. Thioester-centric methods are applicable for the study of biological samples where metabolic labelling is not possible. For example it has been used to profile S-acylated proteins in cardiac, brain or kidney tissues ^{50,112,113}, and in combination with gene-editing tools such as in PPT1 KO mouse brains, where approximately 10% of the S-acylated synaptic proteome presented increased acylation levels ⁵⁰. Furthermore, this method was soon modified to profile S-acylation sites, through the exchange of labile S-acyl groups with alkylating reagents (i.e. iodoacetamide or chloroacetamide) resulting in a carbamidomethyl bond, stable under standard mass spectrometry conditions ¹¹⁴. More recently, its combination with stable cysteine isotopic labelling has allowed both the large-scale identification of palmitoylation sites and the quantitative profiling of palmitoylation site changes ¹¹⁵. Nevertheless, cysteine-centric methods suffer from high background noise which hinders the identification of lowly abundant proteins. Reproducibility is impacted by off-target binding from unprotected thiol residues and cysteine oxidation during processing. Ethylenediaminetetraacetic acid (EDTA) has proven crucial to prevent cysteine oxidation by chelating the metals involved ¹¹⁶. The major drawback of this approach is the identification of S-acylated proteins irrespective of the size of the acyl chain (from C1) and false positives which may also originate from incomplete alkylation of free cysteines ^{117,118}.

The 'lipid-centric' approach relies on the metabolic incorporation of ω -alkynyl fatty acids and their use as handles to enrich for S-acylated proteins and their detection via mass spectrometry (Fig. 1.6) ^{7,15,22}. The basis of this method is the CuAAC reaction, used to link the alkyne group to biotin pulldown reagents ¹⁰⁷ (i.e. biotin-azide, biotin-TAMRA-azide) and isolate S-acylated proteins on streptavidin resin. It has been applied for the study of S-acylated proteome in mammalian cells ¹¹⁹, viruses ^{26,83} and parasites ¹²⁰. For example, in human dendritic cells, S-acylation has been linked to the cellular immune response, identifying the S-acylation of interferon-induced transmembrane protein 3 (IFITM3) ²⁶ and Toll-like receptors, involved in the response to viral and microbial pathogens, respectively ¹²¹. S-acylation in mammalian cells was

Thioester - centric approach :



Lipid-centric approach:

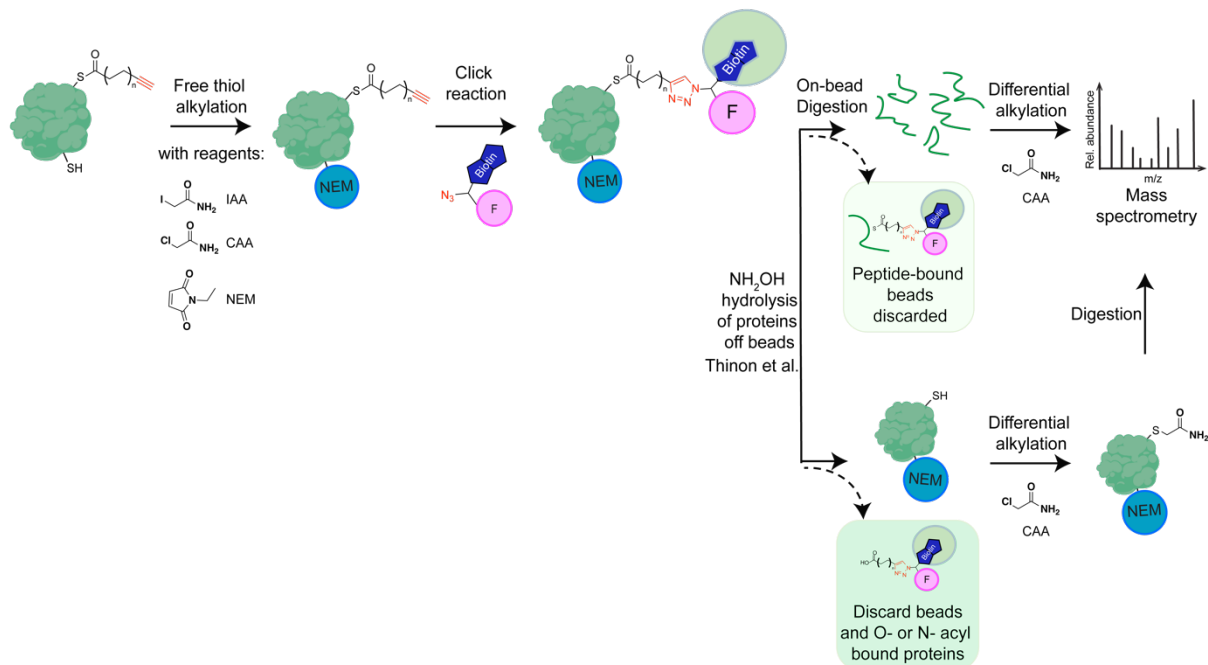


Figure 1.6. Proteomic methods to study S-acylation. Thioester-centric approach. This method relies on chemical modifications of the cysteine residue, both the reversibility of the thioester bond and the reactivity of the deprotected thiol. This reactivity is harnessed to enrich and isolate S-acylated proteins for their consequent mass spectrometry identification. IAA: iodoacetamide, CAA: chloroacetamide, NEM: N-ethylmaleimide. **Lipid-centric approach.** The basis of this approach is the combination of ω -alkynyl fatty acids and CuAAC chemistry (click reaction) to attach pulldown reagents onto S-acylated proteins to allow for their enrichment on the appropriate resin. More recently, with a thioester hydrolysis step to release proteins from beads, this protocol can allow for the selective mapping of sites of S-acylation¹²². NH_2OH : hydroxylamine.

also affected upon herpes simplex viral infection were alkyne-tagged fatty acids showed that 10-15% of the viral proteome relied on the host S-acylation machinery⁸³. More recently, proteomic analysis of HEK293T cells putatively showed S-acylation of

transcription factor STAT3, which putatively relies on this PTM to modulate its transcriptional activity; however, these findings have come into question in light of its subsequent retraction related to data implicating ZDHHC19 as its specific modifier ¹²³.

The main caveat of lipid centric datasets, is the metabolic incorporation of the fatty acids at other nucleophilic residues in proteins (i.e. lysine, serine) forming *N*- or *O*-acyl bonds, leading to false positives. To circumvent this caveat, the reversibility of the thioester bond has been harnessed and used to release only thioester-bound beads from streptavidin resin; thus, combining the strength of both methods ¹²². Furthermore, this also allows the direct tagging of modified Cys residues to selectively map lipid modification sites, increasing confidence levels of identified *S*-acylated substrates ¹²².

With either method, the reversibility of *S*-acylation makes it difficult to identify of the whole breadth of substrates with static experiments. Metabolic labelling only captures a fraction of the proteome that undergoes *S*-acylation within the time frame of the experiment. It does not capture the signal of proteins with turnover cycles that slower than the timeframe of the experiment and also that of those with fast turnover rates as their signal is diluted over time . Therefore, to capture the full scope of this PTM temporal profiling experiments are required ^{109,124}.

All in all, the wide-spread application of the above-mentioned methods for the high throughput identification of *S*-acylated substrates, has allowed the creation of a database, SwissPalm ¹²⁵, which centralizes *S*-acylated datasets of 81 different species and over 250 000 substrates, including approximately 5500 human proteins. This tool permits the curation and cross-referencing of palmitoyl-proteome datasets from the literature and contains annotated datasets to assess likelihood of a protein of interest to be a *bona fide* target of *S*-acylation; from proteins predicted to be *S*-acylated ^{126,127}, to those validated or identified multiple times in proteomic studies, with increasing substrate fidelity ¹²⁵.

1.4.2. Small molecule tools to study protein *S*-acylation.

S-acylation has been widely studied from the perspective of PTM removal, with the help of various inhibitors of fatty acyl thioesterases (Fig 1.7 A). The first compound developed was the beta-lactone Palmostatin B, initially designed as an inhibitor of the RAS thioesterase APT1 ¹²⁸, but later shown to react with a wide range of lipid

thioesterases in the serine hydrolase superfamily including FASN, PNPLA6, ABHD6, ABHD16A, and ABHD17A/B/C ⁵². Another promiscuous pan-inhibitor is the serine-reactive electrophilic hexadecyl-fluorophosphonate HDPP, which, via mass spectrometry, was found to react with 50 serine hydrolases in mouse T-cells ¹²⁴. HDPP also, increased alkynyl fatty acid incorporation into proteins by decreasing the rates of de-acylation ¹⁰⁹. Furthermore, a variant of the later was designed to selective inhibit mitochondrial thioesterases named MitoFP with a triphenyl phosphonium group for mitochondrial targeting ¹²⁹. MitoFP was shown to target ABHD10, increasing the S-acylation levels of its substrate PRDX5 stopping its antioxidant activity (Fig 1.7 A) ¹²⁹. Two isoform selective inhibitors ML348 and ML349, published in 2010, inhibit thioesterases APT1 and APT2 respectively (Fig 1.7 A) ^{130,131}. Despite sharing a piperazine scaffold, their structural make them adopt different binding modes to distort the catalytic sites and is responsible for their selectivity affecting the individual substrate scopes¹³². For example, inhibition of APT2 with ML439 showed restoration of Scribble localisation, which altered S-palmitoylation promotes pathways that drive malignancy; disrupting cell polarity, inhibiting apoptosis and mediating cancer progression. APT1 inhibition with ML348 in mice models of Huntington's disease, restores brain palmitoylation levels and reverses the neuropathology, locomotor deficits, and anxio-depressive behaviours of mice ¹³³.

Progress in understanding the roles and druggability of S-acylation in cellular pathways has been hampered by the lack of selective chemical inhibitors of ZDHHCs. To the detriment of the field, 2- bromopalmitate (2-BP) (Fig. 1.7 B) – first published as a β -oxidation inhibitor ^{134,135} and later shown to inhibit the S-acylation of kinases Lck and Fyn ¹³⁶ – continues to be incorrectly used as a ZDHHC inhibitor. Although, there is evidence clearly demonstrating its reaction with lipid-metabolizing enzymes but also with dozens of membrane-associated proteins at a higher rate than any ZDHHC ⁹⁰. Therefore, any result stemming from 2-BP could be a result of widespread interference with lipid metabolism due to its covalent promiscuity. More recently, another attempt was made to design and market cyano-myacrylamide (CMA) (Fig. 1.7 B) as an inhibitor of ZDHHCs, despite demonstrating via mass spectrometry that this compound reacts with 271 proteins, amongst which only one ZDHHC was found. The common strategy behind both of these cases is to target the catalytic cysteine of ZDHHCs by harnessing the hydrophobic nature of endogenous fatty acids as the

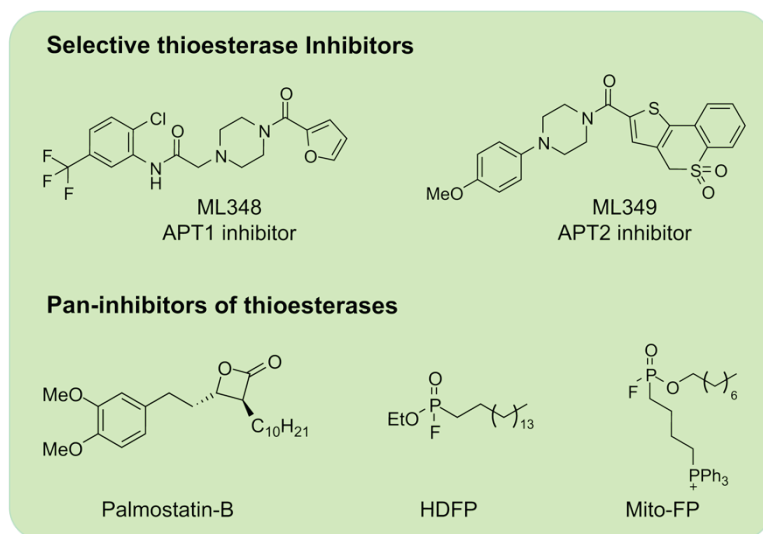
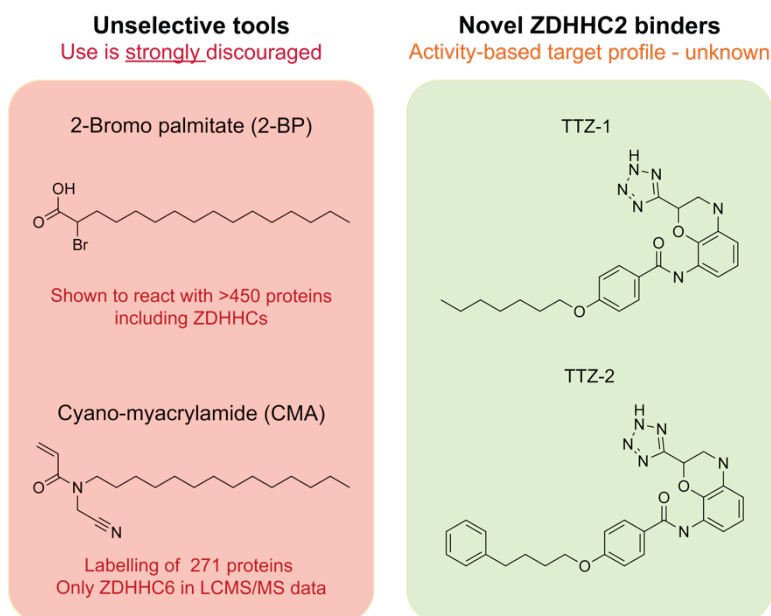
A**B**

Figure 1.7. Small molecule tools to study S-acylation. A) Structures of selective and pan-inhibitors of thioesterases. **B)** Tools used in the field to study ZDHHC enzymes. Right: unselective small molecules with established target profiles. Left: Structures of novel binders of ZDHHC2, without a known activity-based profile ¹³⁷.

driver behind selectivity and binding. The fact that this has proven unsuccessful even for designing pan-ZDHHC inhibitors, tells us that further structural complexity is needed putatively through the design of small-molecules with binding surfaces that include the hydrophobic Coenzyme A surface area.

Over the past year, initial efforts have been placed towards the identification of selective isoenzyme small molecule modulators. Salaun et al. presented a time-

resolved FRET based assay to monitor enzyme autoacylation using NBD-palmitoyl-CoA. This was applied for the screening of a library of approximately 350,000 compounds against ZDHHC2, identifying two hit compounds, TTZ-1 and TTZ-2, with a polar tetrazole core linked to an aliphatic chain (Fig 1.7 B) ¹³⁷. These compounds, showed inhibition of ZDHHC2 in vitro and of neuronal proteins PSD95 and SNAP25 in cells. An autoacylation assay was also used by, Qiu. *et al.* to screen around 1700 acrylamide-containing small molecules against ZDHHC20, with novel palmitoyl transferase probes (Fig. 1.7B) ¹³⁸. Despite the fact that no drug-like hits were detected, it shows the development of high throughput screening methods for ZDHHCs. Both of these reports focus on inhibiting the autoacylation reaction as a mean to stopping substrate acylation, yet assays that monitor substrate transfer could unravel a different class of small-molecule modulators screened at preventing protein binding to the isoenzymes. More structural diversity ought to be found governing substrate selectivity as opposed to autoacylation, which has a common mechanism and structural similarity. Consequently, high throughput assays aimed at inhibiting substrate transfer or binding could be more effective in finding isoenzyme selective inhibitors. In turn, these would aid the deciphering of the substrate scopes of individual PATs in a high-throughput manner which remains a challenge in the field.

Chapter 2:

Design, development and optimisation of a ZDHHC20 chemical genetic ‘bump and hole’ approach.

2.1. Introduction

With the rise of gene editing technologies, it has become easier to modulate protein function through genetic manipulations (i.e. knockouts or mutations). From a pharmacological point of view, finding small molecule modulators with high affinity and specificity towards a target protein remains costly and laborious. High-throughput phenotypic screens including thousands of compounds, often focus on addressing a specific disease-associated phenotype, and extensive characterisation is needed to comprehensively understand the cellular targets of a small molecule before the effects on a biological system can be interpreted. Multiple studies and techniques have been developed to address this limitation; that is, to profile the off-target effects of a drug and provide information on the origin of a pharmacological effect.

Another limitation of drug-developing programs is that it is estimated that only 10 to 20% of the proteome is considered to be druggable ¹³⁹. In the undruggable group are those proteins for which there are either no small molecule modulators or only unselective chemical tools ¹³⁹. This has prompted researchers to find alternative methods to study those proteins for which there are no selective small molecule modulators of their function. An example of this is chemical genetics, a technology that combines synthetic chemistry with the specificity of genetic tools to understand a section of the undruggable proteome ¹⁴⁰. This technique, also termed 'bump and hole' approach, is based on the engineering of a small molecule binding site – on a given protein – to selectively bind an unnatural synthetic ligand designed by steric complementarity (Figure 2.1. A). This results in a small molecule with selectivity that allows for the spatiotemporal modulation of a newly druggable target, which harbours a mutation that makes it analogue sensitive (AS) ¹⁴¹.

This approach has allowed the study of individual isoenzymes within a family of high structural similarity. For example, in the protein kinase superfamily composed of 535 members (2% of human genes) the introduction of analogue-sensitive mutations in the ATP binding pocket, in combination with synthetic ATP analogues, has allowed for the selective control and inhibition of a single kinase ^{140,142–144}. Furthermore, chemical genetic electrophile sensitive (ES) approaches have also been developed. These are

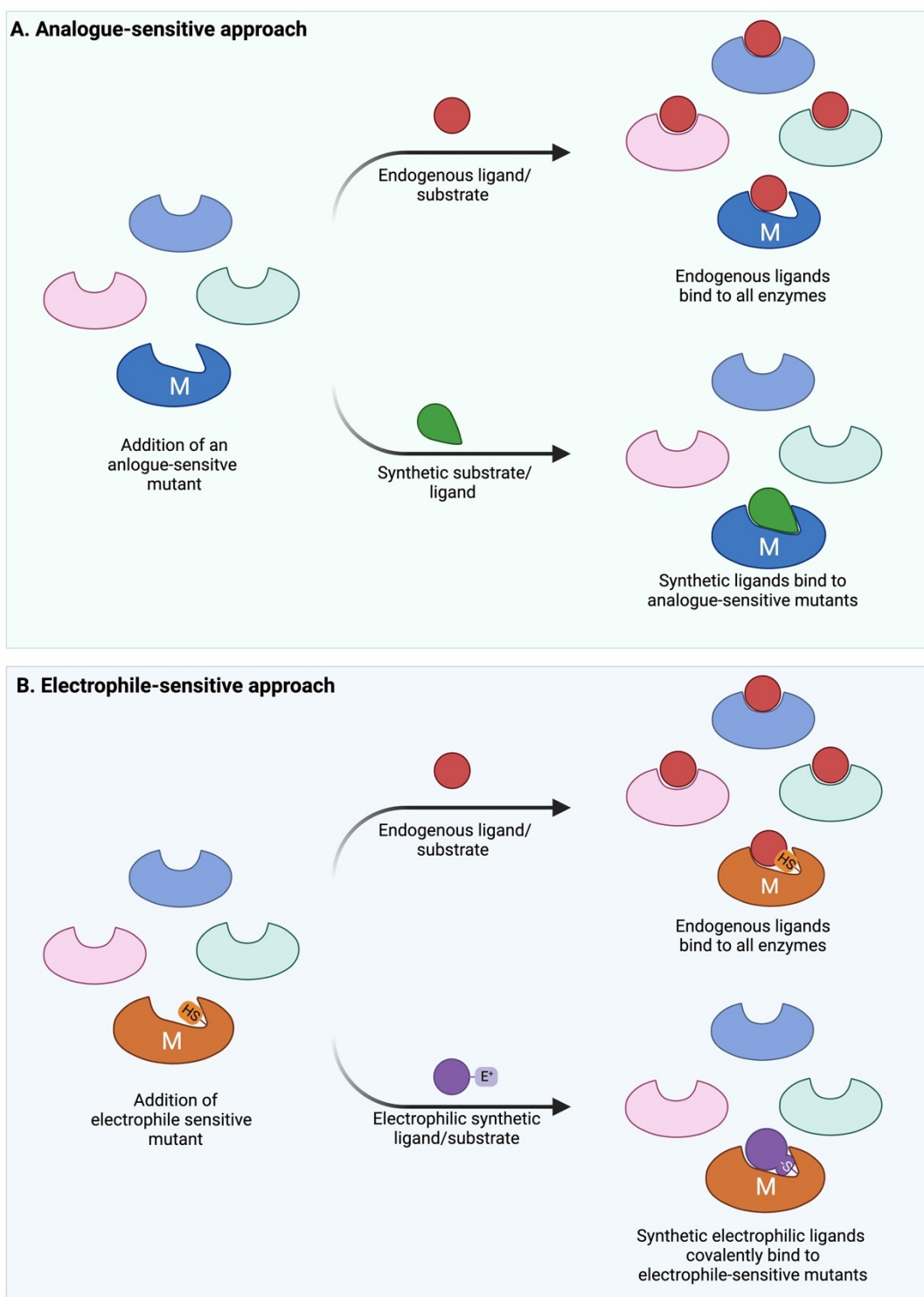


Figure 2.1. Chemical genetic ‘bump and hole’ approaches. **A)** In an analogue-sensitive (AS) approach, mutants bind to the endogenous ligands and those designed by synthetic complementarity. The synthetic ligands are not capable of binding to the wildtype enzymes due to steric clash. **B)** An electrophile sensitive (ES) approach is based on the introduction of a nucleophilic cysteine residue which can only form a covalent bond to a synthetic ligand bearing an electrophile (E^+). As the wildtype enzymes cannot form this covalent bond, selective inhibition of the mutant enzyme is achieved. Figure created with BioRender.

based on the introduction of nucleophilic cysteine residues and the complementary design of electrophilic ligands to achieve isoenzyme specific covalent inhibition ^{145–147}.

Chemical genetic systems have also proven useful for the characterisation of PTMs, one of the major challenges in postgenomic biology ¹⁴³. In this area, synthetic analogues have been modified to contain functional groups (i.e. alkyne handles) that allow for the isolation of PTM-modified proteins and their consequent analysis by mass spectrometry ^{148,149}. Such approaches have allowed for identification of kinase substrates and phosphorylation sites of a single isoenzyme ¹⁴² or the mapping of glycosylation sites of a glycosyl transferase isoenzyme in living cells ^{148,150}.

To date the bump and hole strategy has also been applied to other enzyme families such as methyltransferases and ADP-ribosyltransferases ^{151–154}. In all cases, the application has harnessed the common ability of these enzymes to bind to their endogenous co-factors or ligands. With these studies in mind, a similar approach was envisioned for the S-acyl transferase family of enzymes, with an analogue-sensitive mutations in their lipid-binding cavity and complementary lipid-binding probes (Figure 2.2.2). This strategy will contribute to the field in the following ways:

- 1) Helping to deconvolute isoenzyme-substrate pairs thus aiding to understand the implication of S-acylation in essential cell biology pathways. With this, contributing to the understanding of ZDHHC-related signalling networks where the downstream consequences of isoenzyme expression are not clear.
- 2) Identifying novel substrates by mass spectrometry, possibly buried as background signals in global S-acylation studies. By focusing on a subset of S-acylated proteins – those substrates of a single isoenzyme –, some peptides could become main signals instead of background.
- 3) Cell-type specific analysis of ZDHHC activity in co-culture models.¹⁵⁵
- 4) Providing tools to enable the field to modulate and study ZDHHC enzyme activity in disease relevant models.

In 2018, the publishing of the crystal structure of human ZDHHC20⁴⁷ greatly impacted the decision as to which isoenzyme to select for a proof of concept study, as decisions for analogue-sensitive mutations could be decided with the structure in mind.

Consequently, ZDHHC20 became the focus of this study. Over the course of this chapter the approach to apply the ‘bump and hole’ technology to ZDHHC20 will be explained. The progress will be broken down into five sections, starting on lipid probe design and synthesis and followed by mutant design, probe screening and optimisation, the validation of the probe’s mode of action and the design of an analogue and electrophile sensitive chemical genetic approach.

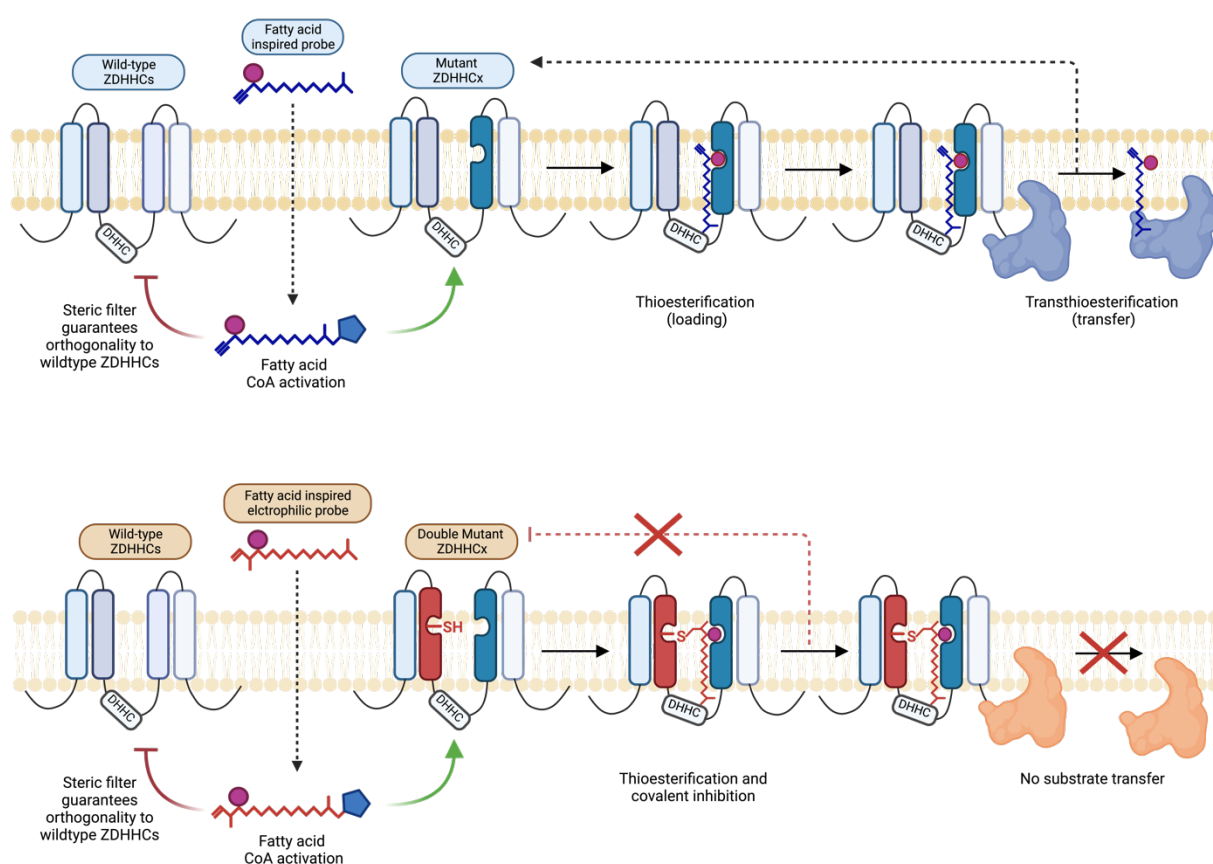


Figure 2.2. Application of the chemical genetic ‘bump and hole’ approach for the ZDHHC enzyme family. A) Chemical genetic ‘bump and hole’ strategy for the ZDHHC family. Fatty acid inspired probes, activated into their CoA forms are loaded (thioesterified) onto mutant ZDHHCs. Upon substrate binding probe S-acylation occurs and the mutant ZDHHC is regenerated. Substrate identification is allowed by cell lysis, click reaction, pulldown and substrate identification by mass spectrometry. **B)** Electrophile-sensitive (ES) chemical genetic approach for ZDHHCs. The complementary design of ES mutants – by mutation of an amino acid to cysteine – and electrophilic probes (i.e. acrylamide containing probes) would allow for the selective inhibition of ZDHHC mutants. Selective inhibition impedes substrate S-acylation. Figure created with BioRender.

2.2. Results and discussion.

2.2.1. Complementary design of mutants and probes.

2.2.1.1. The crystal structure of ZDHHC20: the basis for mutant design.

The generation of analogue-sensitive mutants of ZDHHC20 imposed serious requirements for mutant design: preservation of structural integrity to maintain both catalytic activity and substrate specificity. To prevent the loss of enzymatic activity and avoid any interference with the thioesterification (loading) step onto the enzyme or prior processing of the probe by other lipid metabolising enzymes (i.e. fatty acid CoA activation), mutations in the lipid binding cavity were envisioned distal to the catalytic site. This would help to minimise interference with enzymatic activity and substrate binding, a mechanism which remains poorly understood. However, S-acylation requires the interaction of incoming substrates with the catalytic cysteine and mutation of shallower residues could disrupt substrate binding or recognition to a greater extent.

With approximately eighteen residues shaping the inner cavity of ZDHHC20, there were numerous options for 'hole' generating mutants. The viability of several residues had already been profiled by Rana *et al.*, by comparison of enzyme activities on *in vitro* enzyme-coupled assays⁴⁷. Alanine mutants of residues near the active site, Phe171 and Trp158, had resulted in a drastic reduction or complete loss of catalytic activity, whereas residues further away from the catalytic site, Phe65 and Tyr181, resulted in a milder reduction of catalytic activity⁴⁷. In addition, mutation of the latter to Ala had been shown to shift the chain length preferences of ZDHHC20 from C16 to C18 and C20 fatty acids. Together with previous work on ZDHHCs¹⁵⁶, this provided a proof of principle for creating mutants with a shifted preferences towards lipid probes. Additionally, the selection of mutants was guided by the crystal structure of 2-BP bound covalently to ZDHHC20 (Figure 2.3. A). Also, 2-BP provided a lipid scaffold to overlay the design of our probes and assess both the suitability of each residue to generate a 'hole' and their possible steric complementarity with a synthetic probe⁴⁷. It should be noted, that an endogenous palmitic acid chain, thioesterified onto ZDHHC20, would be slightly shifted deeper into the enzyme pocket. With 2-BP the thioether bond forms to the alpha-carbon of the carboxylic acid.

Nine residues had been reported as closer interactors to the alkyl chain of 2-BP (Figure 2.3. A) ⁴⁷. Five of the bulkiest residues in the deep section of the pocket, were selected as candidates for analogue-sensitive mutants: Ile22, Phe174, Phe65, Tyr181 and Phe220 (Figure 2.3. B). Alanine mutants of the latter four residues (Phe174, Phe65, Tyr181 and Phe220) had presented a mild reduction of catalytic activity compared to the WT enzyme on *in vitro* enzymatic assays, with the mutant Tyr181Ala mutant presenting the highest reduction in activity ⁴⁷. In the case of Ile22, it had been reported not to present such tight contact with the fatty acid as the other four residues. Substitution of this residue for a bulky Trp resulted in a drastic reduction in catalytic activity ⁴⁷. Nevertheless, it was thought that an alanine mutant could create enough space to achieve steric complementarity with a ‘bump’ containing fatty acid-inspired probe. The remaining four residues, also reported to establish contact with the chain of 2-BP, were excluded in the study as they were considered not bulky enough (Val216, Ser29) and either a clear contact with the lipid chain was not observed, (Cys182) or they were too proximal to the catalytic cysteine (Trp158).

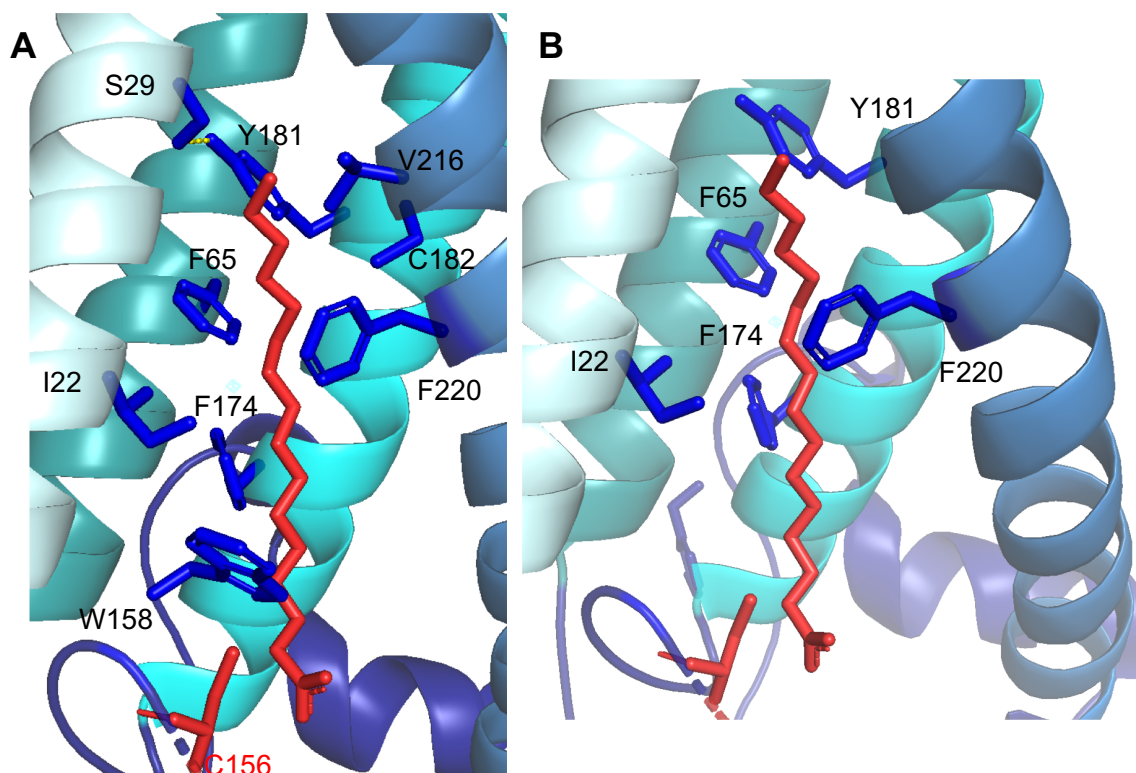


Figure 2.3. Analysis of the lipid pocket of ZDHHC20 from its crystal structure to assess residues for mutation. (pdb:6BML). A) Residues (dark blue) inside the lipid binding cavity that interact with the alkyl chain of 2-BP (in red) as calculated by Rana *et al.* Also, in red, catalytic cysteine-156. Yellow dotted lines represent the polar contacts calculated in Pymol, the hydrogen bond between Ser29 and Tyr181. **B)** The five candidate mutants (dark blue) selected to generate analogue-sensitive constructs for the generation of a chemical genetic system for ZDHHC20. 2-BP and catalytic cysteine in red.

2.2.1.2. Design and synthesis of a library of lipid probes

The design of a chemical genetic system for ZDHHC20 also imposes serious requirements for probe design. From the perspective of chemical functionality, the lipid probes had two indispensable requirements: a 'bump' – to achieve selectivity against the wild-type (WT) enzyme and the rest of the enzyme family –, and an alkyne handle, which allows the ligation of fluorescent reporters and/or affinity handles (e.g. TAMRA, biotin) through CuAAC. The latter was a key functionality for isoenzyme-dependent PTM studies and the downstream sample analysis of S-acylated proteins via mass spectrometry.

In order to mimic the physicochemical properties of fatty acids (i.e. lipophilicity and cell permeability), these two functional elements were envisioned to be incorporated onto a long alkyl chain which, in turn, would harbour a terminal carboxylic acid for thioesterification onto the catalytic cysteine (Fig. 2.4) To link the two chemical functionalities (alkyne handle and bump) to an alkyl chain, a tertiary amine was firstly designed to avoid the introduction of any chiral centres in the probes. However, the basicity of tertiary amines would result in probes with a terminal positive charge and impede their insertion into lipid bilayers. Therefore, it was decided to attach the 'bump' in the structure through the formation of an amide (Figure 2.4).

To create synthetic diversity, a variation in chain length was included in the probe library with 16, 18 and 20 atom long probes, the most common lipid chain lengths

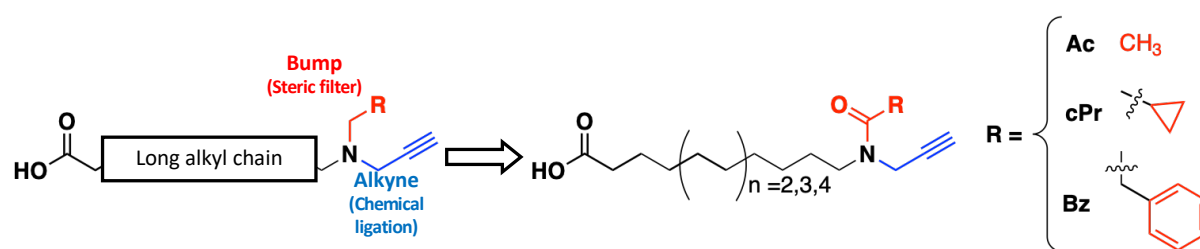
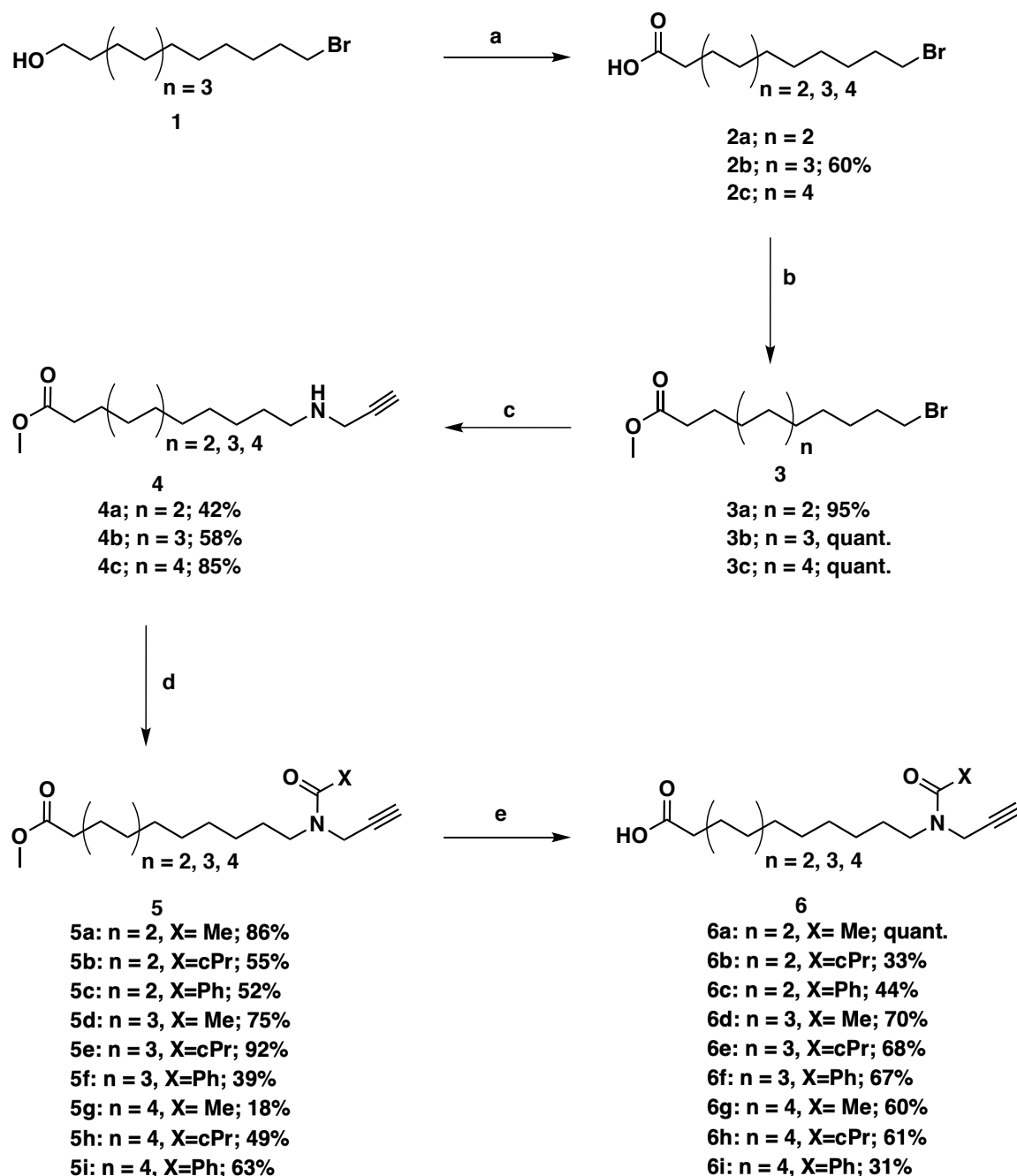


Figure 2.4. Structure of lipid probes and build-up of structural diversity for probe library. Left, probe structure containing all the necessary chemical functionalities (steric filter and chemical ligation handle) for the chemical genetic system to be applied. Right, in order to avoid the introduction of charges and chirality, the probe was optimised to link all elements via an amide group. The library was optimised to contain alkyl chains of 16, 18 or 20 atoms in length and three steric filters were included as bumps: acetyl (Ac) cyclopropanecarbonyl (cPr) and benzoyl (Bz).



Scheme 1. Synthetic route for the generation of all lipid probes. **a)** Jones oxidation – only 18-atom long probes had this step. **b)** MeOH, cat. H₂SO₄, rt. **c)** Propargyl amine, triethylamine, acetonitrile, 80°C, 4h. **d)** DIPEA, DCM, acyl chloride (acetyl chloride (X=Me), cyclopropylacetyl chloride (X=cPr), benzoyl chloride (X=Bz)), room temperature. **e)** MeOH, LiOH, room temperature, 4h. Percentage yields per compound included within the figure.

found in endogenous S-acylation⁴³. This was thought to allow for variation of the height at which the ‘bump’ was positioned, at different distances with respect to the catalytic cysteine. Furthermore, to increase the degree of orthogonality to the wild-type enzyme, probes with bumps of increasing bulkiness were included for each chain-length with small (acetyl, Ac), medium (cyclopropanecarbonyl, cPr) and large

(benzoyl, Bz) groups. This resulted in a library of nine lipid probes, from the combination of 3 chain lengths and 3 bumps. Each probe was named after its length and its bump i.e. 18-Bz: 18 atom long with a benzoyl bump.

Synthesis of these novel probes was carried out by Principal Laboratory Research Scientist Christelle Soudy (Medicinal Chemistry STP, The Francis Crick Institute). For the synthesis of these novel probes a synthetic route was designed (**Scheme 1**) to allow for the parallel synthesis of all probe chain lengths, with 'bump' functionalisation at the latest step to reduce the complexity of library synthesis. The synthesis of all probes (16,18,20) began with the protection of the carboxylic acids as methyl esters in quantitative yields to give compounds **3a-c**. The 18-atom long probes required an additional step as carboxylic acid **2b** was not commercially available, instead it was obtained via a Jones oxidation of alcohol **1**, also in quantitative yield. Propargyl amines were used to incorporate the alkyne handle and generate secondary amines **4a-c** in a nucleophilic S_N2 reaction with the alkyl halide. This conversion was performed routinely with compounds **4a-c** being the main product of the reaction – dual addition was not detected and yields of the reaction ranged from 42-85% conversion. 'Bump' functionalisation would take place at the late stages via reaction of a secondary amine with an acyl chloride – a building block of which there was a lot of diversity commercially available but most importantly all of our desired 'bumps' – in the presence of N, N-Diisopropylethylamine (DIPEA) to give compounds **5a-i**.

All synthetic routes concluded with the deprotection of the methyl ester under basic conditions, to give our final probes with quantitative yields. The route was not further optimised, as it was deemed sufficiently robust to produce all analogues with the desired quantities.

2.2.2. In-cell screening of mutants and probes.

2.2.2.1. ZDHHC20 [Y181G] as candidate for an analogue-sensitive chemical genetics approach.

In preparation for in cell assays, human ZDHHC20 was FLAG-tagged at its C-terminal tail, allowing for immunoprecipitation and immunoblotting of the construct after transient cell expression. From this construct single point mutagenesis was performed to create alanine mutants of the five chosen residues. As mentioned earlier, out of all alanine mutants Tyr181Ala had been reported to be the least active ⁴⁷. Therefore, a glycine mutant was included in case increasing flexibility would be preferable to restore enzymatic activity. To these, a DHHS construct was added as a negative control for enzyme loading, as the substitution of the catalytic Cys for Ser had been reported to result in a catalytically dead enzymes, incapable of loading palmitoyl-CoA ^{47,156}. In total, with the wild-type enzyme, eight constructs were made to screen for an optimal analogue sensitive mutant.

Firstly, the autoacylation activity for WT ZDHHC20 and its catalytic dead mutant was tested. Constructs ZDHHC20[C156S] and WT ZDHHC20 were transiently expressed in HEK293Ts cells and subsequently metabolically labelled with YnPal, an alkyne tagged analogue of palmitate (C16:0), which had been shown to have comparable uptake to that of the endogenous fatty acid by ZDHHCs ^{43,122}. Constructs were FLAG immunoprecipitated, CuAAC to TAMRA-azide and analysed by in-gel fluorescence analysis (Figure 2.5 A). The autoacylation activity for the WT enzyme was determined in a concentration dependent manner (Figure 2.5 B and C) with a half maximal loading concentration of 12.6 μ M. Residual signal observed for ZDHHC20[C156S] (Figure 2.5 B), was attributed to previously reported S-acylation at non-catalytic cysteine residues mediated by endogenous ZDHHCs including the WT enzyme ¹⁵⁷. Time-dependent S-acylation of WT ZDHHC20 with YnPal was explored. It was found that after a sharp increase in loading, the signal reached its maximum at 2 h and fluctuated, only slightly, up to the end of the assay (Figure 2.5 D and E). In this instance, the signal of peripheral labelling more stable in nature, might be superseding that of the catalytic Cys causing signal saturation. To confirm that lipids were S-acylating onto the proteins and not

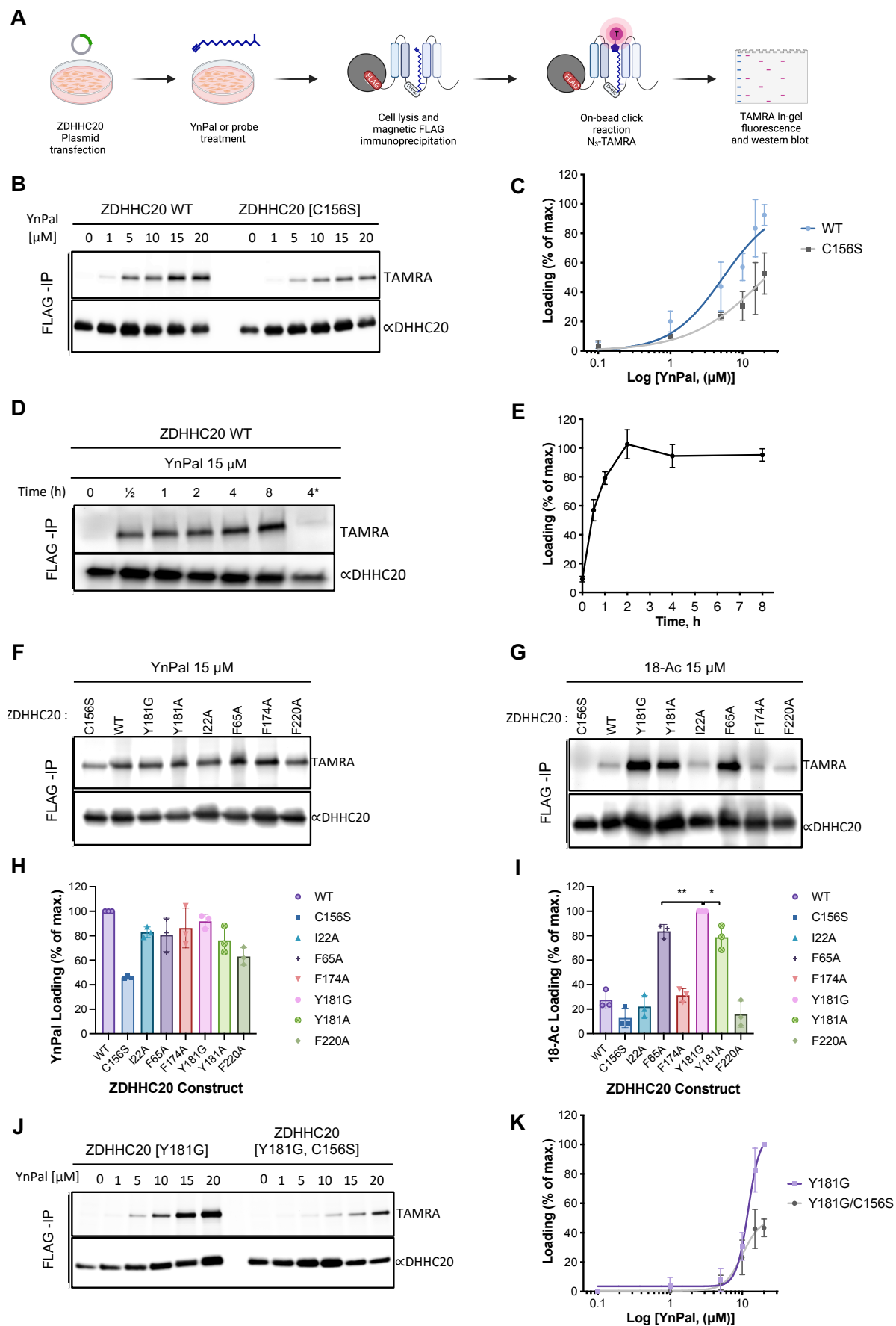


Figure 2.5. Screening assays, looking for candidates for ZDHHC20 analogue-sensitive mutants.
A) Scheme for metabolic labelling experiments with overexpressing ZDHHC20. Figure created with

BioRender. **B)** In cell-based loading assays in HEK293T cells, over expressing ZDHHC20-WT and ZDHHC20[C156S] and treated with various concentrations of alkyne palmitate, immunoprecipitated (IP:FLAG) and CuAAC to TAMRA-azide. Labelling of the catalytic mutant ZDHHC20[C156S], is thought to correspond to peripheral S-acylation by endogenous ZDHHC's. **C)** Quantification of n of 3 replicates of A. Signal fitted to plots using Prism 9.0. **D)** Time-dependent S-acylation signal of ZDHHC20 WT. (*) corresponds to a hydroxylamine (HA) treated sample, indicating a thioester dependence of the signal. **E)** Quantification of n of 3 replicates of C. **F-I)** Analysis of analogue-sensitive mutants of ZDHHC20 (C-terminally FLAG-tagged) overexpressed in HEK293Ts treated with 15 μ M YnPal (**F-H**) or 15 μ M 18-Ac (**G-I**). Samples were once again immunoprecipitated (IP: FLAG), clicked with TAMRA-azide and analysed by in-gel fluorescence and western blot. **J)** Dose-response curve of ZDHHC20[Y181G] and ZDHHC20[Y181G; C156S] with YnPal, samples were immunoprecipitated and subject to CuAAC with TAMRA-azide. **K)** Quantification of J. All images were quantified by pixel densitometry analysis using ImageJ, plots were made using Prism 9.0 and correspond to n=3 biological replicates. P-values were determined using Prism 9.0 unpaired t-test statistics module (ns (not statistically significant), $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ and ****, $P \leq 0.0001$). Panels B, C, F, G, J and K were kindly provided by Dr. Tony Ocasio.

covalently binding through N-acyl or O-acyl bonds, we looked at the stability of this signal after treatment with hydroxylamine (HA), which reacts with thioesters cleaving off the fatty acids (Figure 2.5 and Appendix Fig. 8.1 B). As expected HA treatment led to signal loss, corresponding to the cleavage (hydrolysis) of the fatty acid.

After understanding signal changes for the WT enzyme, each of the six FLAG-tagged ZDHHC20 mutants were transiently expressed in HEK293Ts. Similar sample processing and in-gel fluorescence analysis showed that all mutants were capable of autoacylating YnPal (Figure 2.5 F and H). Of all six mutants, ZDHHC20[Y181G] showed the highest auto-acylation signal – on average, only 8% lower than the wild-type signal. This result contrasted with the Ala mutant, ZDHHC20[Y181A], with a 25% reduction in activity compared to the WT enzyme and in accordance with *in vitro* enzymatic assays which had shown a 30-40% reduction in catalytic activity for this mutant ⁴⁷.

The conditions of this screen were repeated with the lipid bumped probe 18-Ac, 18-atoms in chain length with the smallest bump in the library. Pleasingly, the results presented the first evidence that WT ZDHHC20 could load the designed probes onto its structure (Figure 2.5 G and I). Interestingly, the reduction in signal of ZDHHC20 [C156S] and the wildtype enzyme points towards the inability of endogenous enzymes to use this probe to S-acylate at peripheral residues. Overall, two residues were found to be amenable for analogue-sensitive mutations Phe65 and Tyr181. When looking at the crystal structure, both residues sit on transmembrane helix (TM) 2 and TM3

respectively, two of the more ordered or less flexible helices – with lower B-factors (Appendix Fig 8.2); indicating that mutations in areas of increased rigidity could be preferred to achieve steric complementarity with the ‘bumped’ probes. Residue Tyr181, could only be complemented with a glycine but not an alanine residue, following the preference observed in the YnPal screen. With 18-Ac, the ZDHHC20[Y181G] mutant presented on average, a selectivity window of 70% with respect to the WT enzyme; making it the best mutant to load both the lipid probe and YnPal. The auto-acylation of this mutant was compared to that of the WT enzyme with YnPal in a concentration dependent manner (Figure 2.5 J and K) which resulted in a half-maximal loading value of 12.7 μ M, comparable to the WT enzyme (Figure 2.5 C and K). The mutant ZDHHC20 [Y181G] was taken forward for optimisation experiments against the whole probe library.

Collectively, this data served as the first evidence that mutations on the lipid binding cavity preserved sufficient structural integrity to maintain autoacylation activity. Thus far, the probes were uptaken by cells, permeating the lipid bilayer, and being used by both point mutants and wild type enzymes in autoacylation reactions.

2.2.2.2. Modifying assay conditions for the lipid probe screen.

A considerable difference in labelling signal had been observed with the 18-Ac probe between WT and mutant ZDHHC20[Y181G] – from here onwards referred to simply as mutant or mutant enzyme. Nevertheless, the rest of the probe library was screened against this mutant to determine whether steric complementarity could be achieved. A two-step screening strategy was adopted, depicted in Figure 2.6 A. Firstly, mutant- to-wildtype orthogonality was assessed by varying the chain length of the probe featuring the bump with the least steric bulk (Ac). Secondly, with the optimal chain length, those probes with bumps of increasing bulkiness were screened to both achieve the maximal steric complementarity to the mutant and orthogonality to the wildtype enzyme.

After metabolic labelling (4h) and sample processing as described before, fluorescence analysis of probes with the smallest bump and increasing chain lengths (16-Ac, 18-Ac and 20-Ac) revealed that a Y181G mutation shifts the chain length preference of the ZDHHC20 to 18 and 20 atoms. This corroborated the function of the Tyr residue to delimitate the inner edge of the lipid pocket – as mentioned before, an

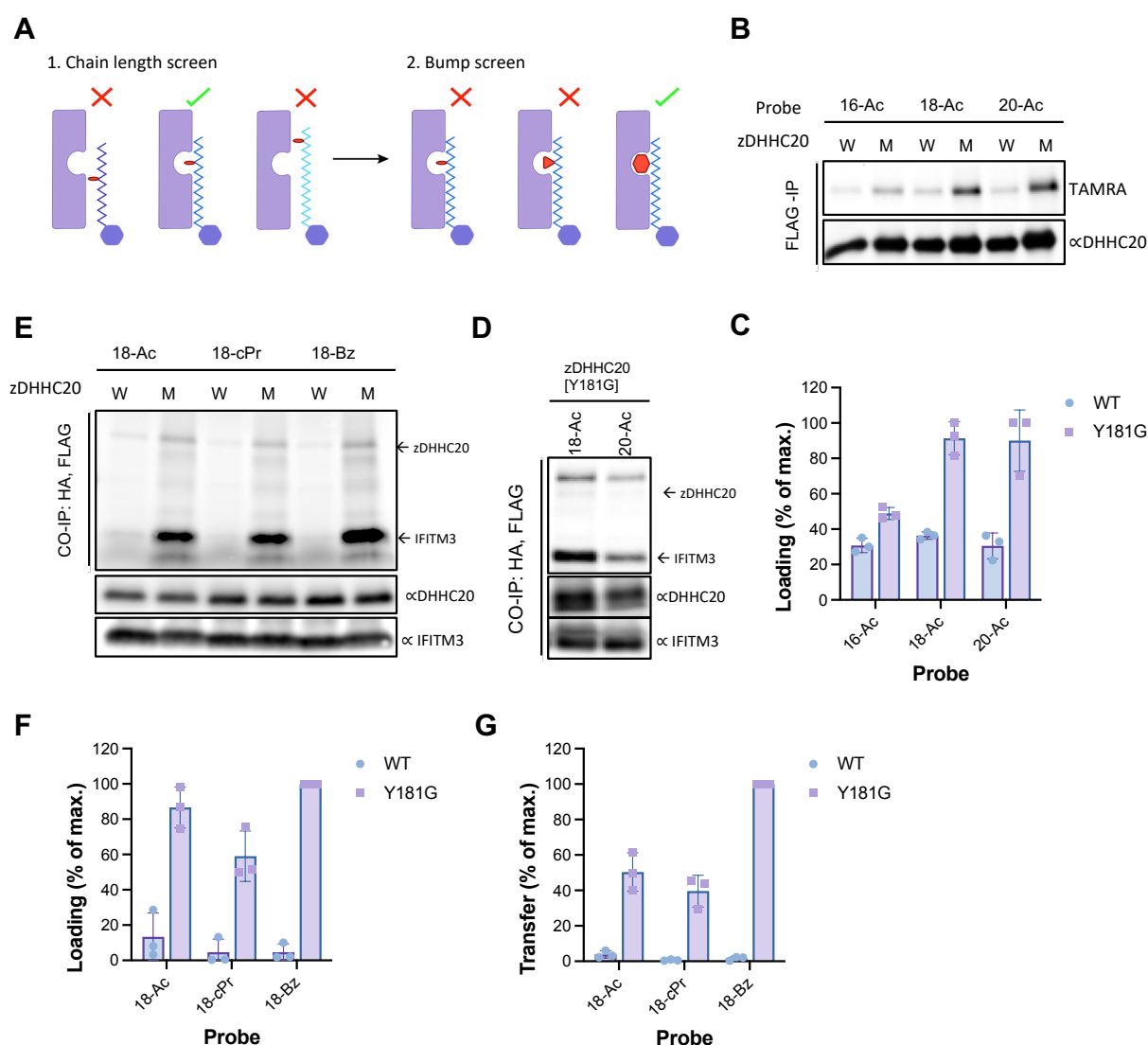


Figure 2.6. Probe library screen against mutant ZDHHC20 [Y181G] **A**) Diagram depicting the step-wise screening of the probes to achieve maximal steric complementarity against the mutant ZDHHC20[Y181G]. **B**) Optimisation of chain length with smallest bump (Ac). Loading assays in HEK293T cells, over expressing ZDHHC20-WT (W) or mutant ZDHHC20[Y181G] (M) and treated with 15 μ M of probes. In-gel and western data of samples after an immunoprecipitation (IP:FLAG) and CuAAC to TAMRA-azide. **C**) Quantitation of 3 independent biological replicates of B. **D**) Loading and transfer assays of ZDHHC20 [Y181G] with 18-Ac and 20-Ac. HEK 293T cells overexpressed with substrate IFITM3. In-gel fluorescence images correspond to a co-immunoprecipitation (FLAG and HA) after a click (CuAAC) reaction to TAMRA-azide. **E**) Bump screen of 18-atom long probes. In-gel fluorescence images of HEK293T cells overexpressing ZDHHC20-WT (W) or ZDHHC20[Y181G] (M) and treated with 15 μ M of probes. **F-G**) Quantification of 3 biological replicates of loading (**F**) or transfer (**G**) signal. All images were quantified by pixel densitometry analysis using ImageJ, plots were made using Prism 9.0 and correspond to n=3 independent biological replicates. Panels B, E and D were kindly provided by Dr. Tony Ocasio.

Ala mutant had been reported to shift in fatty acid preferences to stearic (C18:0) and arachidic (C20:0) ⁴⁷. Both the 18-atom and the 20-atom long probes presented similar orthogonality windows to the wildtype enzyme (Figure 2.5 B and C). At this stage, it remained unknown whether any of these probes could be transferred on to ZDHHC20 substrates – a key requisite for S-acylation studies – or whether they stayed bound to the enzyme and not transferred. To differentiate between the two probes, a substrate was to be co-overexpressed with the two ZDHHC20 constructs (WT and mutant) and similar in-gel fluorescence analysis would allow to assess any differences in the efficiency of S-acylation. Three proteins were initially considered for transfer assays: IFITM3, GobX and VAMP3. The latter, had been found to be S-acylated by mass spectrometry in HeLa cells metabolically labelled with YnPal ¹²² and GobX, a bacterial E3 ubiquitin ligase, had been reported to require zDHHC20 S-acylation and localization at the Golgi membrane ¹⁵⁸. As for IFITM3 its activity increased only by overexpression of zDHHC20 due to lysosomal colocalization of the two proteins ⁸⁰.

Co-expression of only VAMP3 or IFITM3 with ZDHHC20 WT led to a signal increase of these proteins compared to the empty vector control (Appendix Fig 8.1. C and D). Of these, IFITM3 was chosen as the model substrate to observe transfer of the fatty acid probes as it was the most validated ZDHHC20 substrate ^{26,80,159}. When co-expressing the enzymes ZDHHC20 WT and ZDHHC20 [Y181G] with IFITM3 and metabolic labelling with both 18-Ac and 20-Ac, it was found that 18-Ac was more readily transferred onto IFITM3 compared to 20-Ac (Figure 2.5 D) thus pointing towards the 18-atom length as more suitable for S-acylation PTM studies.

With IFITM3 overexpression incorporated into the assays, and a chain length preference of 18-atoms, the bump screen was performed with probes: 18-Ac, 18-cPr and 18-Bz. After a 4-hour probe incubation in HEK293T cells co-overexpressing ZDHHC20 (WT or [Y181G]) and IFITM3, co-immunoprecipitation, CuAAC ligation and in-gel fluorescence analysis the C18-Bz as it presented the maximal loading and transfer signal compared to the less bulky 18-Ac and 18-cPr (Figure 2.5 E-G). The combination of 18-Bz probe and ZDHHC20[Y181G] point mutant was taken forward for further optimisation of the labelling window through loading and transfer assays with respect to the wildtype enzyme and future mass spectrometry based PTM studies.

2.2.2.3. Optimisation of the selectivity window in favour of ZDHHC20[Y181G].

In preparation for mass spectrometry based PTM studies, the selectivity window of the mutant, with respect to the wildtype enzyme with 18-Bz, was studied in similar metabolic labelling experiments as those described previously. Initially, the mutant enzyme was thought to retain the ability to load and transfer endogenous lipids and essential lipids present in cell culture media were suspected to compete for enzyme loading with the lipid probe. Indeed, although mutant-selective 18-Bz labeling was clearly observed in media supplemented with 10% fetal bovine serum (FBS), optimal labeling intensity was achieved at lower serum levels 0.5% (Fig. 2.7 A); implying that the mutant enzyme retains the structural capabilities to load endogenous lipids. The loading and transfer signals of the wildtype enzyme with 18-Bz did not increase significantly at 0.5% FBS, yet those for the mutant presented increased signals and the selectivity window became more pronounced. This effect was probe-dependent and not seen with YnPal treatment, putatively because 18-Bz is not as easily incorporated into peripheral Cys by endogenous ZDHHC's. Overall, the reduction of essential lipids present in FBS, is thought to help increase the influx of the probe to the lipid pool, helping their uptake and transfer by the mutant enzyme.

Next, the mutant-to-wildtype enzyme selectivity window was assessed with increasing probe concentration and time of labelling. At the highest 18-Bz probe concentration (50 μ M), it was found that the WT enzyme could only reach a maximal loading signal of 20%. In other words, an 80% increase of loading was seen for the mutant enzyme with respect to the WT enzyme signal (Figure 2.7 C and D). The maximal fold increase (mutant/wildtype signal) between ZDHHC20[Y181G] and WT ZDHHC20 was found at 10-15 μ M (Appendix Fig. 8.4A and B); and as a result, was considered the best concentration at which to dose cells for labelling experiments. The steric complementarity of the selected bump-mutant pair was further validated by measuring the loading signal of the medium bump probe 18-cPr, which showed increased loading of the WT enzyme, up to 40% at 50 μ M (Appendix Fig. 8.4C and D).

The time dependence of labelling was profiled at 15 μ M 18-Bz. A sharp increase in labelling was found only 30 minutes after dosing, reaching a maximal signal at 4 hours which remained constant until the end of the assay (8 hours) (Figure 2.7 C and D).

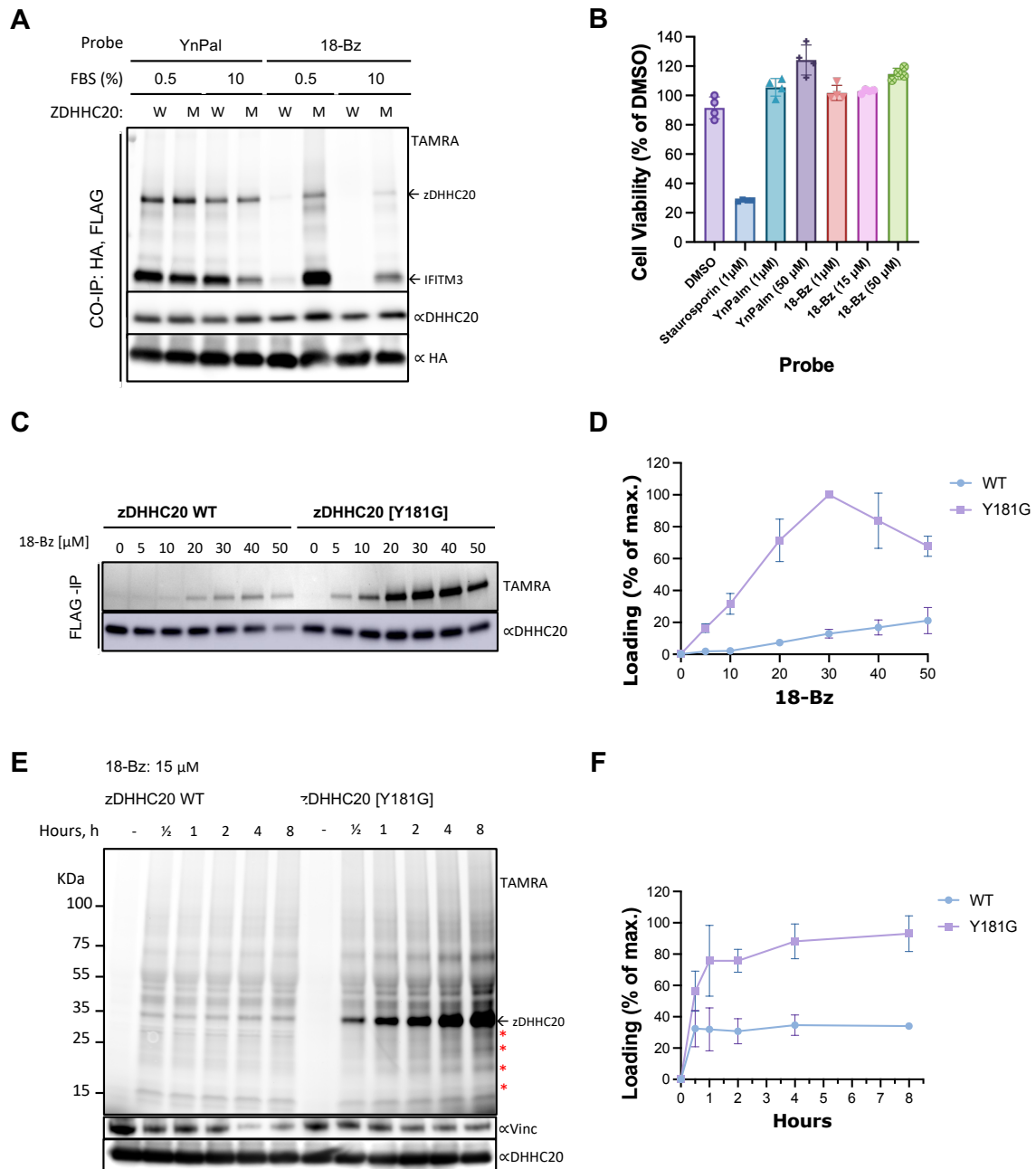


Figure 2.7. Optimisation of selectivity window between ZDHHC20[Y181G] and 18-Bz with respect to wildtype enzyme labelling. A) Impact of FBS on 18-Bz probe uptake compared to YnPal. HEK293T cells overexpressing ZDHHC20-WT (W) or ZDHHC20[Y181G] (M) and treated with 15 μ M of YnPal or 18-Bz and samples co-immunoprecipitated (FLAG and HA) and analysed via in-gel fluorescence after a CuAAC reaction to TAMRA-azide. **B)** Cell viability (CellTiter-Blue[®] assay, Promega) after 18-Bz or YnPal treatment. Probes showed no toxicity compared to DMSO control and staurosporin (50nM) as positive control. Plots represent n=4 independent experiments. **C)** HEK293T cells overexpressed with ZDHHC20 WT or ZDHHC20[Y181G] and incubated with varying concentrations of 18-Bz. Samples were subject to immunoprecipitation prior to in-gel fluorescence and western blot analysis. **D)** Quantification of n=3 biological replicates of C. **E)** Time-dependent S-acylation signal of HEK293T cells treated with 18-Bz. Novel bands (red stars) depict selectivity of probe transfer to endogenous substrates. **F)** Quantitation of ZDHHC20 constructs signal of n= 3 biological replicates of E. Selectivity window stabilises at. 4h. Images were quantified by pixel densitometry analysis using ImageJ, plots

were made using Prism 9.0 and unless otherwise stated, correspond to n=3 biological replicates. Panel A was kindly provided by Dr. Tony Ocasio.

The background signal for the WT enzyme reached an average maximal signal of approximately 30% with respect to the mutant enzyme. It is believed that, as seen before for 18-Ac (Fig 2.5. G, I), this orthogonality is also extended to endogenous enzymes minimizing peripheral loading of 18-Bz. Furthermore, time-dependent labelling and in-gel fluorescence analysis of the whole proteome showed novel bands appearing over time pointing towards endogenous selective transfer of the probe by the mutant enzyme (Appendix Fig. 8.3). Finally, cell viability was maintained for 48h after a dosing with 18-Bz at 50 μ M, ruling out any toxicity within the shorter times of our assays (Fig. 2.7.B). Overall, it was then established that the conditions to achieve the greatest selectivity window between mutant and WT enzymes were 15 μ M 18-Bz, 8h and 0.5% FBS. These represented the foundation conditions for proteomics experiments discussed in Chapter 3.

2.2.3. Validating the mode of action of the lipid probes: enzyme kinetics, metabolomics and lipidomics.

2.2.3.1. ZDHHC20 [Y181G] and 18-Bz enzyme kinetics.

Despite having observed orthogonality to the wildtype enzyme with the novel mutant-probe pair, it remained unknown how the kinetic parameters of loading and transfer compared to those of the wildtype-endogenous lipid pair. To measure the kinetics of fatty acid loading onto acyl transferases, one assay had been previously described and used in the literature to measure the rate of autopalmitoylation of acyltransferases, an α -ketoglutarate dehydrogenase (KDH) enzyme coupled *in vitro* assay^{47,160}. This assay, measures the release of CoA-SH after thioesterification of the fatty acid CoA (loading) onto the acyltransferase. The resulting CoA-SH is then used to produce an equimolar amount of reduced nicotinamide adenine dinucleotide (NADH) a fluorescent product of the oxidation of α -ketoglutarate to succinyl-CoA by α -ketoglutarate hydrogenase in the presence of NAD⁺ (Figure 2.8 A). For this assay, 18-Bz-CoA was chemically synthesised by activation of the carboxylic acid with 1,1'-carbonyldiimidazole and reacted with CoA-SH, synthesis was carried out by Christelle

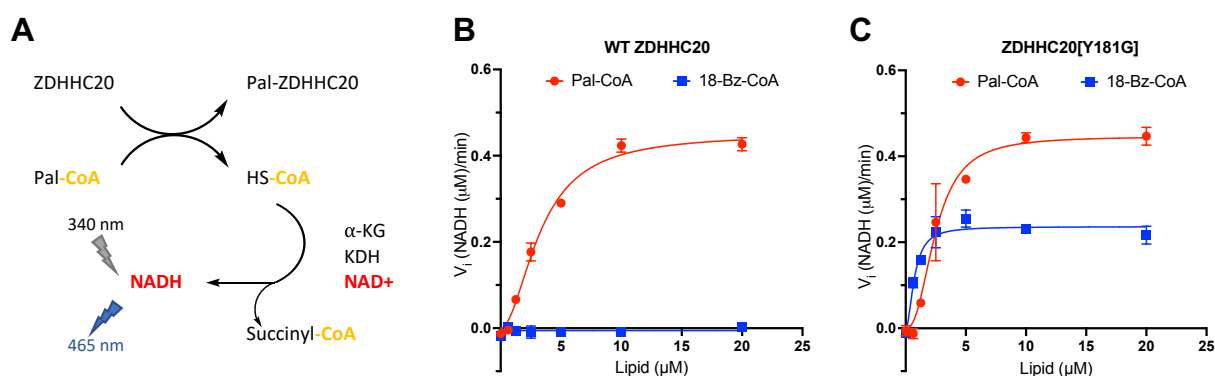


Figure 2.8. Establishing the enzyme kinetics for ZDHHC20 [Y181G] and 18-Bz **A)** KDH-enzyme coupled assay. ZDHHC20 autoacylation was measured by Coenzyme A (CoA-SH) release, which is oxidized to Succinyl-CoA and used to produce equimolar amounts of reduced NADH (fluorescent absorption: 340nm; emission 465nm), by α -ketoglutarate dehydrogenase enzyme (KDH) along with its substrates α -ketoglutarate (α -KG), thiamine pyrophosphate (TPP) and NAD⁺. **B-C)** NADH-enzyme coupled assay of DHHHC20 WT or ZDHHC20[Y181G] treated with Pal-CoA (red) or 18-Bz-CoA (blue). **B-C)** Michaelis-Menten plots generated from average reaction rate ($n = 3$) (NADH generated (μ M)/min) $\pm$ S.D. vs. lipid concentration (μ M) using Prism 9.0; basal rate in absence of enzyme was subtracted from each corresponding reaction rate. Panels B and C were kindly provided by Dr. Tony Ocasio.

Soudy, senior research associate in the Tate Group. The following steps were performed by Dr. Tony Ocasio. FLAG-tagged ZDHHC20 WT and ZDHHC20[Y181G] were expressed in mammalian cells and purified by FLAG agarose affinity chromatography (Chapter 6: Materials and Methods). Initially, the kinetic parameters of YnPal-CoA were tested and found to be similar for both WT and ZDHHC[Y181G], with a catalytic efficiency (K_{cat}/K_m) of 6.8 ± 0.3 and $7.6 \pm 0.3 \mu\text{M} \cdot \text{min}^{-1}$, respectively (Figure 2.8 B,C). Assays with 18-Bz-CoA showed a catalytic efficiency for ZDHHC20[Y181G] of $16.0 \pm 1.0 \mu\text{M} \cdot \text{min}^{-1}$ better than that found for YnPal-CoA (Figure 2.8 B, C and Appendix Table 8.5). No measurable activity was observed for WT ZDHHC20 with 18-Bz-CoA, reinforcing probe orthogonality to the endogenous enzyme *in vitro*. As previously reported and expected⁴⁷, both catalytically dead mutants DHHS had no measurable activity, due to their inability to thioesterify the fatty acids onto their structure.

2.2.3.2. Metabolic fate of lipid probes: metabolomics and lipidomics.

From the beginning of the project, it was considered necessary to investigate the metabolism of our probes in cells. Initially, the focus was to understand which probe

species was the lipid source for the mutant enzyme. As mentioned previously, fatty acids are activated into their Coenzyme A adducts prior to thioesterification onto ZDHHC enzymes ⁴⁶. From a more recent crystal structure of the pre-catalytic complex of ZDHHC20 bound to palmitoyl CoA, it can be inferred that the bivalent recognition of both the lipid and the polar headgroup is essential for catalysing fatty acid thioesterification onto acyltransferases ⁴⁶. Therefore, to determine whether the lipid probes were metabolised into their CoA adducts following the endogenous metabolic pathway, the focus turned to mass spectrometry.

The main challenge behind the detection of lipid CoAs is the instability and reactivity of the thioether bond which is highly electrophilic and subject to hydrolysis at high temperature or basic pH ¹⁶¹. Several publications had reported methods for fatty-acid CoA extraction and analysis by UPLC-MS/MS with a sensitivity to detect up to picomolar amounts from cell extracts ^{162,163}. Amongst these methods, a methanol-chloroform-water extraction was chosen as a starting point as it prevented the use of buffers, which could cause the hydrolysis of lipid-CoAs. This method was tested to extract metabolites from untreated HEK293T lysates, spiked with 1 nM of either chemically synthesized 18-cPr-CoA or commercially available palmitate-CoA (C18:0-CoA). Comparison of the chromatograms of DMSO versus spiked extracts showed additional peaks corresponding to the mass of 18-cPr CoA and C18:0-CoA; confirming, in our hands, the suitability of the method for extraction and detection of lipid-CoAs (data not shown). Next, HEK293T cells were treated for 2 hours with 18-Bz (20 μ M) and after metabolite extraction, extracts were analyzed by LC-MS/MS. 18-Bz-CoA was detected by mass spectrometry with equal retention time and fragmentation pattern as its chemically synthesized standard demonstrating probe activation inside cells (Figure 2.9 A, B). This result suggests that long-chain fatty acid CoA ligases (ACSL), responsible of activating endogenous fatty acids, are also capable of activating these fatty acid-like probes.

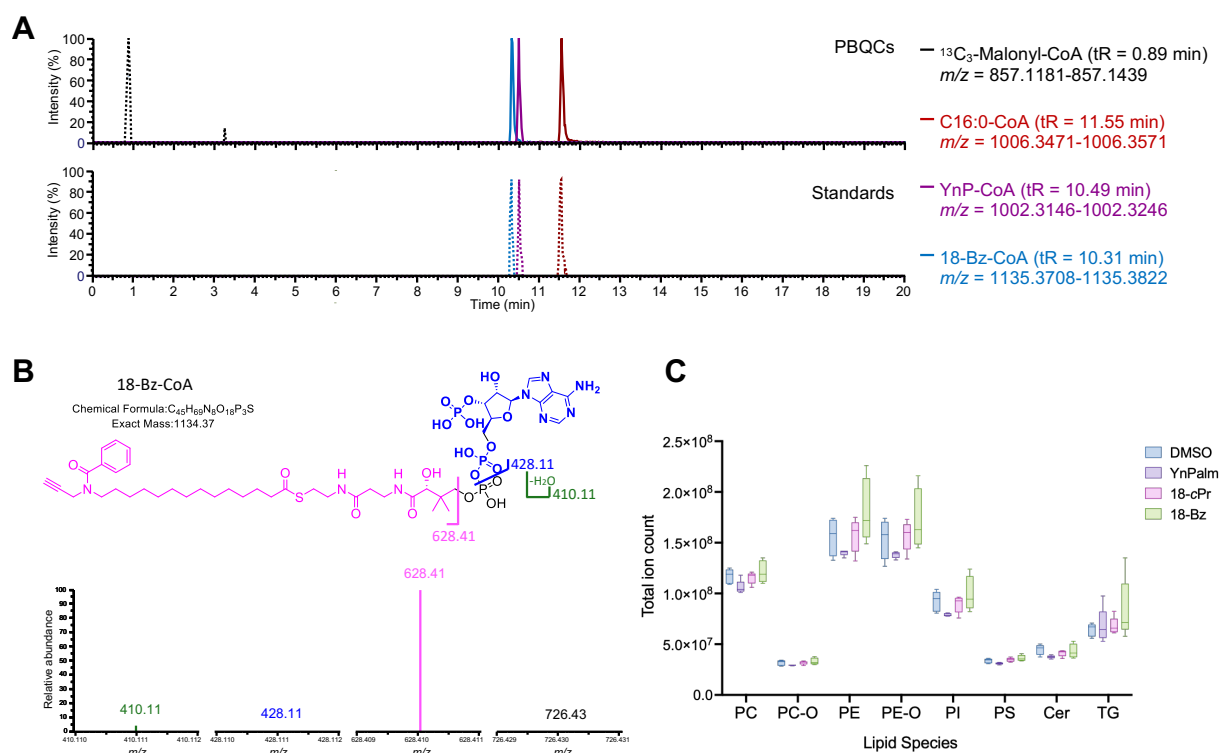


Figure 2.9. Lipidomic and metabolomic analysis of cellular probe metabolism in HEK293 cells.

A) Probes and probe-CoA molecules were detected by LC-HRMS in positive and negative modes. Like features from sample pooled biological controls (PBQCs, solid lines, upper panel) and authentic standards (dotted lines, lower panel) had identical retention times (shown) and fragmentation (not shown). $^{13}\text{C}_3$ -Malonyl-CoA (black dotted line) was used as an internal standard. Retention times (tR) and mass ranges (m/z) used for each molecule are shown. **B)** Upper panel show chemical structures, formulae, and exact masses. Lower panels show the spectrum of the major LC-MS/MS product ion of 18-Bz-CoA, with colours equivalent to fragmentations depicted in the upper panels. Abundance is shown relative to the most abundant product ion m/z 628.41. **C)** The average total ion count $\pm$ S.D. of a selection of the most abundant cellular lipid species, plotted using Prism 9.0. Panels A and B were kindly provided by Dr. Susana Palma (Metabolomics STP, Francis Crick Institute).

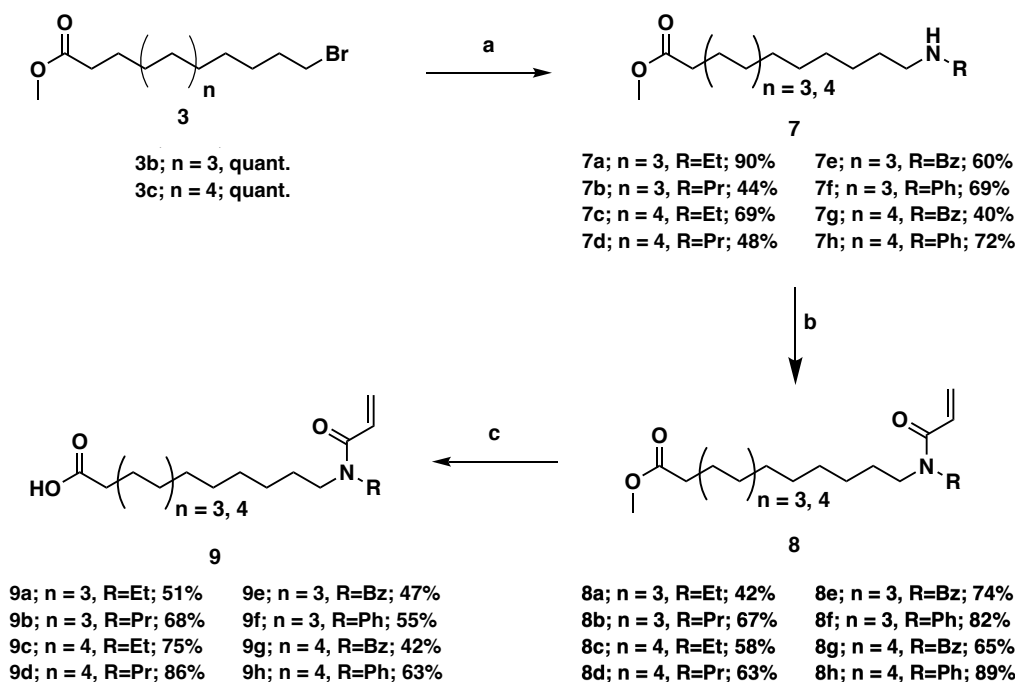
Beyond fatty acid CoA activation, the bigger picture of the metabolic fate of our probes in cells soon came into question. The *in-situ* activation of fatty acids to CoA esters is a step that also occurs for the metabolic incorporation of fatty acids into several, more complex, lipid structures for example, diacylglycerols (DG), phosphatidylcholines (PC) and phosphatidylglycerols (PG). Moreover, it is known that lipid biosynthesis has an impact on cellular physiology and it has been shown that cells adapt their lipidome, cellular lipid composition, in response to different stimuli^{164–166}. Therefore, to mass spectrometry was used to assess whether the lipid probes could be incorporated into other lipid species and cause changes in the lipidome. Metabolites of HEK293 cells treated with 18-cPr and 18-Bz (20 μM), were extracted using H_2O : MeOH: methyl tert-butyl ether (MTBE). This is the preferred method of extraction for lipidomics due to the

faster and cleaner lipid recovery – the lower density of MTBE makes the lipid phase form as the upper layer easing the collection and minimizing contamination ¹⁶⁷. In collaboration with Dr. Elisabeth Stork Saha (Kings College London), UPLC-MS/MS analysis and manual data curation revealed the incorporation of both 18-cPr and 18-Bz into several lipid species including phosphatidyl ethanolamine (PE), PCs and PGs (Appendix Fig 8.5). Analysis of the major classes of endogenous lipids, revealed no significant changes compared to the DMSO control samples. Similarly, when looking within individual lipid families, the 20 most abundant lipids did not suffer any changes in detection (Figure 2.9 C). Collectively, this data implies that treatment with the designed probes remained orthogonal to the main pathways of lipid metabolism and that incorporation into the metabolite pool did not significantly perturb lipid equilibrium.

2.2.4. An electrophile-sensitive approach for selective ZDHHC20 inhibition.

2.2.4.1. Design and synthesis of electrophilic probes for covalent targeting

An unmet need in the field is to selectively inhibit the activity of a single ZDHHC isoenzyme with small molecules. Therefore, given the degree of orthogonality achieved with the analogue sensitive approach, an analogue and electrophile-sensitive (AS-ES) method was envisioned (Fig. 2.1 B). In order to implement this technique, the main requirements for probe design were firstly, to maintain the steric filter ('bump') for orthogonality to the wildtype enzyme and secondly to contain an electrophilic warhead, to which a nucleophilic cysteine residue could covalently bind. Previous ES approaches had made use of electrophilic groups such as, vinyl sulphones, acrylamides or fluoromethyl ketones ¹⁴⁶. Yet, for the first generation of inhibitors, non-substituted acrylamides were chosen as warheads – addition of a Michael acceptor to a cysteine thiol group is a common modality of targeted covalent inhibitor. Furthermore, their easy incorporation into the synthetic pathway made them optimal options for a proof of concept study. In first instance, alkyne handles were excluded from the structure as experiments with this inhibitory probes would be designed to measure the reduction in labelling with YnPal or 18-Bz.



Scheme 2. Synthesis of electrophilic-fatty acid like probes for an analogue and electrophile sensitive approach. a) KI, DMF, H₂N-R (Et: ethanolamine, Pr: propylamine, Ph: aniline, Bz: benzylamine), 4h, rt; b) acryloyl chloride DIPEA, DCM; c) LiOH, THF, 3h. Percentage yields per compound included within the figure.

As for structural diversity, only two chain lengths were selected for the inhibitory probes, 18 and 20 atoms, as those represented the shift in preferences seen for ZDHHC20 [Y181G]. The lessons learned for this mutant were also applied to the choice of bumps focusing on aromatic (Ph: phenyl, and Bz: benzoyl) groups and aliphatic (Et: ethyl, Pr: propyl) for comparison purposes. The probes were named to reflect the chain length, electrophilic warhead and bump. For example, 18-Acr-Bz would be 18 atoms long, have an acrylamide warhead and a benzoyl bump.

The pathway for the inhibitory probes was derivatised from compound 2 (Scheme 2), common intermediate to the synthesis of probes (Scheme 1, Section 2.2.1), to which aliphatic or aromatic bumps were incorporated as primary amines in a similar fashion as the propargyl amine reacted with the lipid probes. Secondary amines **7a-7h** were then reacted with acryloyl chloride, which in turn was subject to ester hydrolysis, under basic conditions, to give the final compounds. In this final step, a side reaction was observed: the 1,4 addition of a hydroxyl group to the Michael acceptor for compounds **9e-9h** (data not shown) with aromatic 'bumps' possibly exerting an electron-withdrawing effect on the acrylamide increasing its electrophilic character and the

nucleophilic attack by hydroxyl groups. This side reaction remained at minor approximately under 10% and was removed via mass-directed reverse-phase purification.

2.2.4.2. Mutant screen and inhibition with bump probes

An analogue-sensitive and electrophile sensitive approach was envisioned, with the introduction of a 'hole' and a Cys residue by mutation. This would ensure a high degree of orthogonality towards the wildtype enzyme but also reduce off-target covalent binding to other lipid processing enzymes as seen with unselective compounds 2-BP and CMA (see section 1.4.2). Designing double mutants of ZDHHC20 imposes similar requirements as those established previously for the single point mutants: the preservation of overall structural integrity to ensure the enzyme is catalytically active and capable of substrate S-acylation. Out of all residues in the lipid binding cavity, only those far away from the catalytic cysteine but also in the proximity of the mutation [Y181G] were considered, to accommodate the 'bump' and to covalently react with the acrylamide warhead.

Four residues fulfilled in first instance the requirements (Figure 2.9 A), however, the focus turned to Ser29 since this residue sits on the opposite helix of that of Tyr181, mutated in ZDHHC20[Y181G] mutant. Sterically, this facilitates the accommodation of both the bump and the warhead given the putative bond angles between them of approximately 109.5 °C (sp^3 hybridisation), the bond angles of a tertiary amine. In wild-type ZDHHC20, Ser29 is thought to form a hydrogen bond to Tyr181, dictating fatty acid chain-length selectivity ⁴⁷. Elimination of this bond in the analogue-sensitive ZDHHC20 [Y181G] mutant had shown to preserve catalytic activity (Sections 2.2.2 to 2.2.5). As Ser29 does not interact with any other residues, a dual mutant ZDHHC20 [Y181G,S29C] was considered a good starting point for proof of principle inhibition studies with our designed inhibitory probes. Furthermore, an analogous residue in ZDHHC15 had been shown amenable to mutation to cysteine. Previous work in our group had shown that in the case of human ZDHHC15, the expression of an analogue-sensitive mutant (zDHHC15 [Y184G]), was improved by mutation of Ser35 to a cysteine residue (manuscript in preparation, data not shown). In the crystal structure

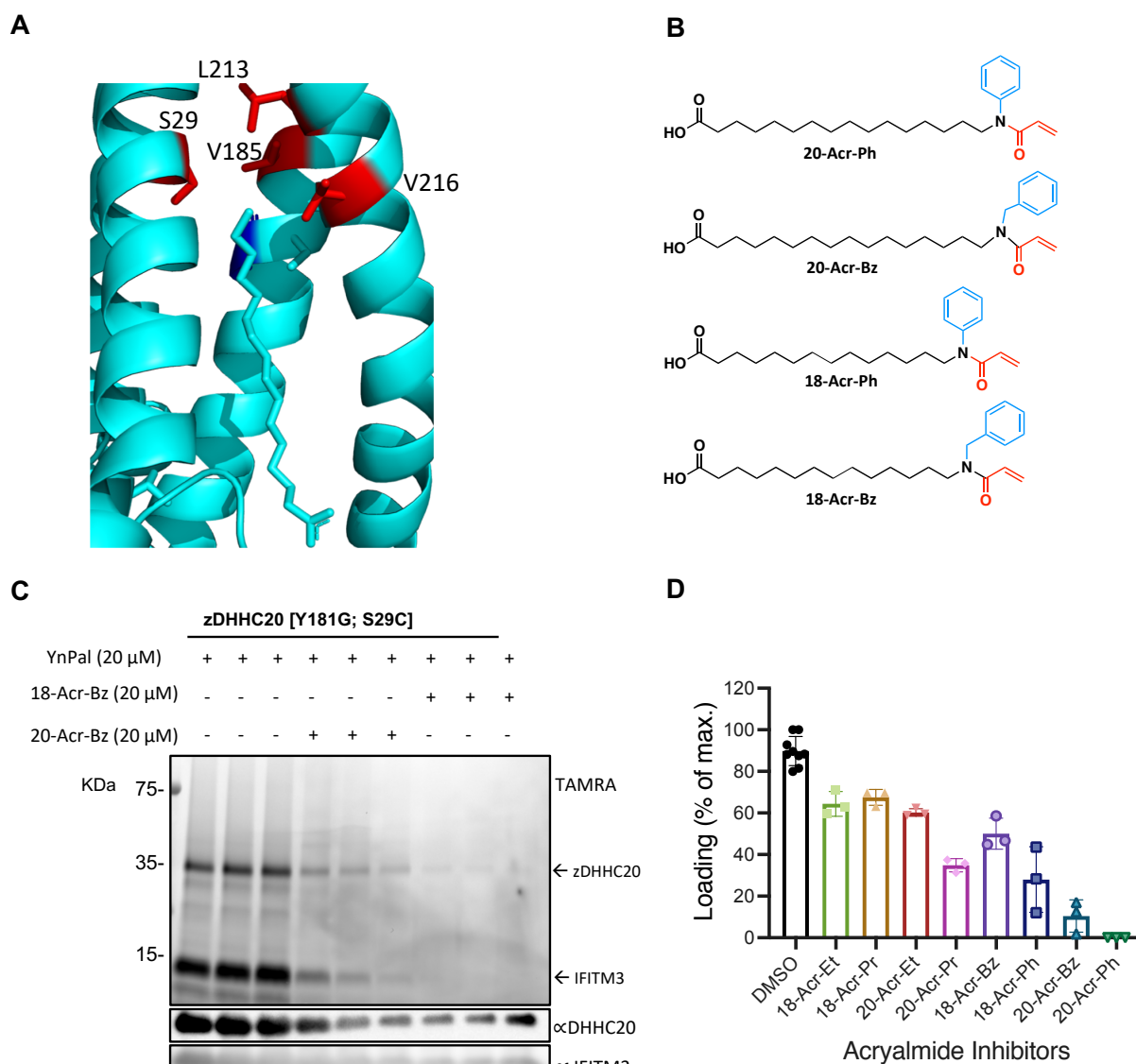


Figure 2.10. Analogue and electrophile sensitive mutants and their inhibition with acrylamide probes. **A)** Structure of ZDHHC20 [Y181G] (pdb:6BML). Highlighted in red, the residues suitable to mutation for implementation of an analogue and electrophile-sensitive chemical genetic system. **B)** Chemical structures of the aromatic acrylamide inhibitory probes. **C)** HEK293T cells labelled with alkyne palmitate (20 μ M) for 4h and inhibited with compounds (20 μ M) for 3h. In-gel fluorescence analysis after FLAG immunoprecipitation and click reaction with TAMRA-azide. Figure contains three technical replicates of the same biological replicate. Images of the other probes are included in Appendix Fig 8.6. **D)** Quantification of technical replicates of C and Appendix. Fig. 8.6. All images were quantified by pixel densitometry analysis using ImageJ, plots were made using Prism 9.0.

of zebrafish ZDHHC15, this serine residue also forms a hydrogen bond to Tyr184, analogous to that of ZDHHC20⁴⁷.

Having rationally decided on a mutation, it was introduced into the ZDHHC20[Y181G] plasmid for transient expression. Firstly, ZDHHC20 [Y181G,S29C] overexpressed HEK293T cells were metabolically labelled (4 h) over a concentration range (0-50 μ M) with 18-cPr and 18-Bz. FLAG-immunoprecipitation, click reaction and in-gel fluorescence analysis revealed that both [Y181G] and [Y181G, S29C] present similar lability to load the probes with the double mutant, preserving orthogonality to the wild-type enzyme (data not shown). After establishing that the selectivity window of the double mutant was similar to that of ZDHHC20[Y181G], the focus turned to the inhibitory probes. Initial experiments were set up to measure loss of YnPal labelling. In this case, cells overexpressing ZDHHC20[Y181G, S29C] were treated with YnPal for 1h, followed by inhibitory probe dosing at equal concentration for another 3 h (4 h from YnPal dosing). After a FLAG-immunoprecipitation and standard in-gel fluorescence analysis, a reduction YnPal-autoacylation signal for all probes was observed from a single biological replicate. The highest reduction in YnPal signal was observed for bulkier aromatic inhibitory probes and of these, the longer chain lengths caused the greatest reduction in labelling.

This line of research was interrupted in 2020 due to the coronavirus pandemic. Once research activity was resumed, the focus of the project changed and this investigation was not continued in favour of other work. The data presented in this section remains preliminary and further validation is needed for both, compounds and mutants to establish an analogue and electrophile sensitive system for ZDHHC20.

2.3. Chapter summary and future work

The work presented in this chapter, shows the step-by-step development of the first chemical genetic system for ZDHHC20. In line with the project's aims, this study shows a great effort towards expanding the set of tools available to study S-acylation, and will be of great benefit to expand the chemical tool set available in the field. The data presented provided the sufficient evidence to support the hypothesis that this novel chemical genetic system works orthogonally to wildtype ZDHHC metabolism and with comparable catalytic activity to the wildtype enzyme.

The point mutants of ZDHHC20 harbouring a 'hole' or enlarged lipid cavity were tested for activity towards endogenous lipid analogues and chemically synthesized bumped probes. Out of five residues tested, mutation of only two showed orthogonal labelling to the wildtype enzyme with our set of probes and comparable labelling to the wildtype enzyme with YnPal. These locations, namely Phe65 and Tyr181, harboured their mutations at more static helices, as dictated by the crystal structure, indicating that overall helix rigidity aids to withstand structural integrity and the loading of lipid probes. Interestingly, the residue most amenable for mutation Tyr181, showed better loading with YnPal when mutated to a more flexible glycine versus a more rigid alanine. Once removing the Tyr181-Ser29 hydrogen bond, the increase in flexibility of a glycine residue compared to alanine might allow Ser29 to find additional polar contacts and a higher degree of stability. Selectivity with 18-Ac was also improved with a glycine mutation, yet this might be due to steric clash of the bump with the alanine side chain. Furthermore, the alanine mutant had been shown to have reduced catalytic activity compared to the wildtype⁴⁷, yet in our hands the tyrosine to glycine mutant ZDHHC20[Y181G] had comparable activity to the wildtype enzyme in the same assay.

The systematic screening of probes, one variable at a time (length and bump), allowed for a rapid in-cell screening of the probe library against mutant ZDHHC20[Y181G], establishing 18-Bz as the best probe. Residual labelling of the wildtype enzyme (~20%) was not considered problematic, as ensuring some residual labelling of the WT enzyme was necessary to calculate fold changes of labelling, and especially important for proteomic analysis. After the optimisation of the assay conditions (FBS,

concentration and time), no further significant information could be gathered due to the limitations of in-gel fluorescence analysis.

The kinetic parameters of the wildtype and mutant enzyme with endogenous lipids indicate that the wildtype enzyme retains almost equal activity with YnPal. Nevertheless, it is worth pointing out that the enzymatic assay used does not provide a rate of substrate transfer. For this, other assays could be used such as a modification of the acyl-clip assay, previously developed in our group to assess the by the hedgehog O-acyltransferase (HHAT) ¹⁶⁸. A crystal structure of the mutant enzyme with the probe 18-Bz would be of added value to understand any structural changes caused by that the mutation or structural adaptation to fit the bump probe and the basis for the orthogonality observed.

The robustness of this chemical genetic system also lies on the fact that it only requires a point mutation on a single enzyme. The development of other chemical genetic systems, sometimes requires the mutation of a second enzyme, to convert the precursor analogue into a metabolite that can be used as substrate. Thorough analysis of metabolites from cell extracts confirmed the activation of probes into CoA adducts (18cPr-CoA and 18-Bz-CoA). This reaction could be performed by the ACSL enzyme family, which would not be susceptible to the increased bulkiness and complexity of the probes. Overall, the probes would follow the endogenous metabolic pathway, and would bind to ZDHHC20 as CoA adducts. The publishing of structure of the pre-catalytic complex of human ZDHHC20 with palmitoyl CoA, would indicate that lipid probe CoAs with increased binding affinity to the acyltransferase are the source of lipid probe for ZDHHC20[Y181G]. These activated CoA species can be also used to build up more complex lipid species as shown by the of lipidomic analysis. This opens the door to the use of similar probes as starting points to study other fatty acid metabolizing enzyme families, such as ACSLs. The lack of major perturbations in the abundant lipids classes remains a surprising result since the lipidome have been reported for subtle changes in lipid composition.

With regards to the electrophile sensitive approach, further work is needed to establish the system. It is believed, that double mutant ZDHHC20[Y181G,S29C] is a good candidate given the preliminary data presented yet a comparison with the wildtype enzyme is needed to assess any changes in catalytic activity.

Overall, the present work includes the development of a chemical genetic system for ZDHHC20, with a thorough and extensive validation of both mutants and probes. Furthermore, orthogonality of the system has been established through various methods. The following chapter will show that orthogonality is preserved in substrate transfer, through the development and optimisation of proteomic protocols.

Chapter 3:

Mass Spectrometry based identification of ZDHHC20 substrates with chemical genetics.

3.1. Introduction.

The involvement of post-translational lipidation in essential cellular processes pushed in the 2010s for the overall development of methods for the global profiling of lipidated proteins. A key breakthrough in this field was the prior development of bioorthogonal chemistry (click chemistry) and its widespread application for the study of biological systems^{169–171}. This paved the way for the development of tagged lipids (i.e. ω -alkynyl fatty acids)¹⁰⁸, which can be metabolically incorporated as fatty acids onto cellular proteins, and used as tool reagents for the large-scale global profiling of lipidated substrates via mass spectrometry¹⁷².

Amongst all lipid PTMs, S-acylation stands out due to the reversible nature of the thioester bond, subject to hydrolysis by thioesterases (i.e. ATPs), making it participate in dynamic cellular functions such as, trafficking, localisation and signalling^{37,52,156–158}. This reversibility is also a major challenge for mass spectrometry analysis. PTM loss can occur during sample preparation due to changes in temperature or pH, via the transfer of fatty acids to other nucleophilic residues forming new N-acyl or O-acyl bonds or due to the activity of thioesterases in lysates. Moreover, mass spectrometry profiling of S-acylated proteins suffers from two other analytical hurdles: peptide hydrophobicity and the complex nature of the S-acylated proteome^{7,22}. The latter corresponds to 10% of the total proteome⁶⁰, with protein substrates varying in turnover rates and stoichiometric levels¹⁰⁹, usually harbouring multiple S-acylation sites and sometimes in combination with other lipid PTMs^{86,103}. Peptide hydrophobicity, a common challenge for lipid PTMs, prevents the facile isolation and characterization of lipidated peptides, with reduced low water solubility and ionization²⁷. To counter these challenges, two proteomic approaches have been developed referred to as either ‘lipid centric’ or ‘thioester centric’^{22,114} (discussed in Chapter 1). Despite each method having its own limitations, their extensive use spurred the creation of a database for S-acylated proteins, called SwissPalm¹²⁵. This tool permits the curation and cross-referencing of palmitoyl-proteome datasets from the literature and contains annotated datasets to assess likelihood of a protein of interest to be a *bona fide* target of S-acylation. Nowadays, this centralised database contains information on the S-acylated proteome of 81 different species and a sum of approximately 260,000 substrates. In the human proteome, of all proteins predicted to be S-acylated only ~27% have been

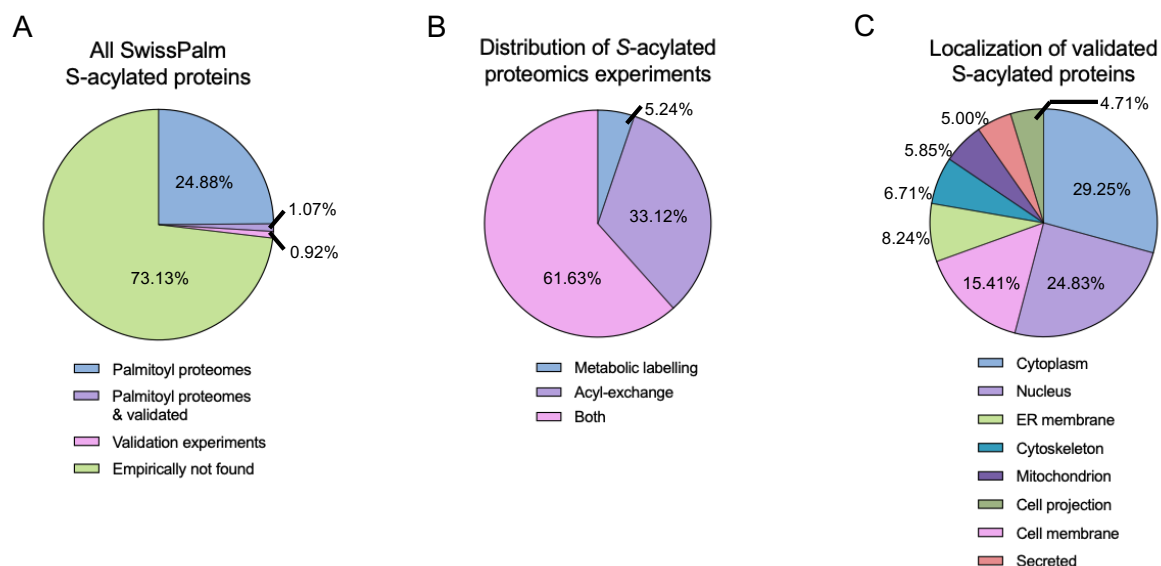


Figure 3.1. Analysis of human S-acylated proteins annotated in SwissPalm. A) Distribution of proteins predicted to be S-acylated. B) Break-down of methods used to identify S-acylated proteins. More data has been gathered using acyl-exchange metabolic labelling. C) Cellular localisation of proteins validated to be S-acylated spans a wide distribution of organelles. All data source from the SwissPalm data base ¹²⁵.

validated, (Fig. 3.1 A) with ~25% identified via mass spectrometry and with preference for acyl-change methods (Fig. 3.1 B). The functional diversity of these substrates can be inferred from their localisation which spans all cellular organelles (Fig. 3.1 C), and can also be translated into a high degree of functional complexity.

In total, only 89 human proteins have a known ZDHHC, representing only 1.63% of the reported S-acylated proteome (high confidence hits). The aim of this project was to address this challenge by harnessing the power of chemical genetics, creating a high-throughput method to identify the substrates of individual ZDHHCs, starting with ZDHHC20. Previously, other PTMs have been studied by using mass spectrometry to identify either substrates enriched by analogue-sensitive (AS) enzymes over wildtype ones ^{154,176}, or using AS inhibitors for a selective inhibition AS enzymes ^{144,177}. Using the former approach, this chapter describes the steps that were taken to optimise and combine the chemical genetic system, explained in Chapter 2, with mass spectrometry for the identification of ZDHHC20 substrates. Some of these are biochemically validated and all are used to understand the biological functions that ZDHHC20 S-acylation is implicated.

3.2. Results and discussion

3.2.1. Development of a proteomic protocol to profile ZDHHC20 substrates using chemical genetics.

3.2.1.1. An on-bead hydrolysis protocol for substrate identification.

The optimisation of parameters to maximize the selectivity window for mutant ZDHHC20[Y181G] versus wildtype enzyme, presented in Chapter 2, laid out an initial set of conditions for the discovery of novel ZDHHC20 substrates through chemical proteomics. Initially, a 'lipid-centric' ⁷ sample processing approach was taken, making use of the installed alkyne handles on the lipid probes for substrate enrichment and consequent mass spectrometric analysis. Initially, steps for the direct identification of sites of fatty S-acylation were included, following a study published by Thinon *et al* ¹²². In summary, following CuAAC ligation to biotin-azide, free (non-acylated) cysteines are capped with *N*-ethylmaleimide (NEM) and enriched on a streptavidin resin. NH₂OH hydrolysis, releases thioester-bound proteins from the resin, allowing the specific capping of previously S-acylated Cys with chloroacetamide (CAA) and the identification of these sites during mass spectrometry analysis (Fig. 3.2). The protocol, which was termed 'on-bead hydrolysis' (OBH), allowed to circumvent the following caveats or analytical hurdles present when using metabolic labelling to study S-acylation:

- 1) Thioester hydrolysis during sample preparation is addressed by the early capping of free Cys with *N*-ethylmaleimide. This de-risks any later PTM loss, which in any case, is chemically hydrolysed and differentially tagged from initially free Cys, also allowing site identification via mass spectrometry.
- 2) Identification of sites of S-acylation, which is not possible from S-acylated peptides as they are too hydrophobic and have low ionization. The sites of modification are valuable information for validation of novel substrates.
- 3) A considerable reduction in background from non-specifically bound proteins or those modified via probe incorporation via *N*- or *O*-acylation – which remain bound to the resin. This is one of the main caveats of a 'lipid centric' approach.

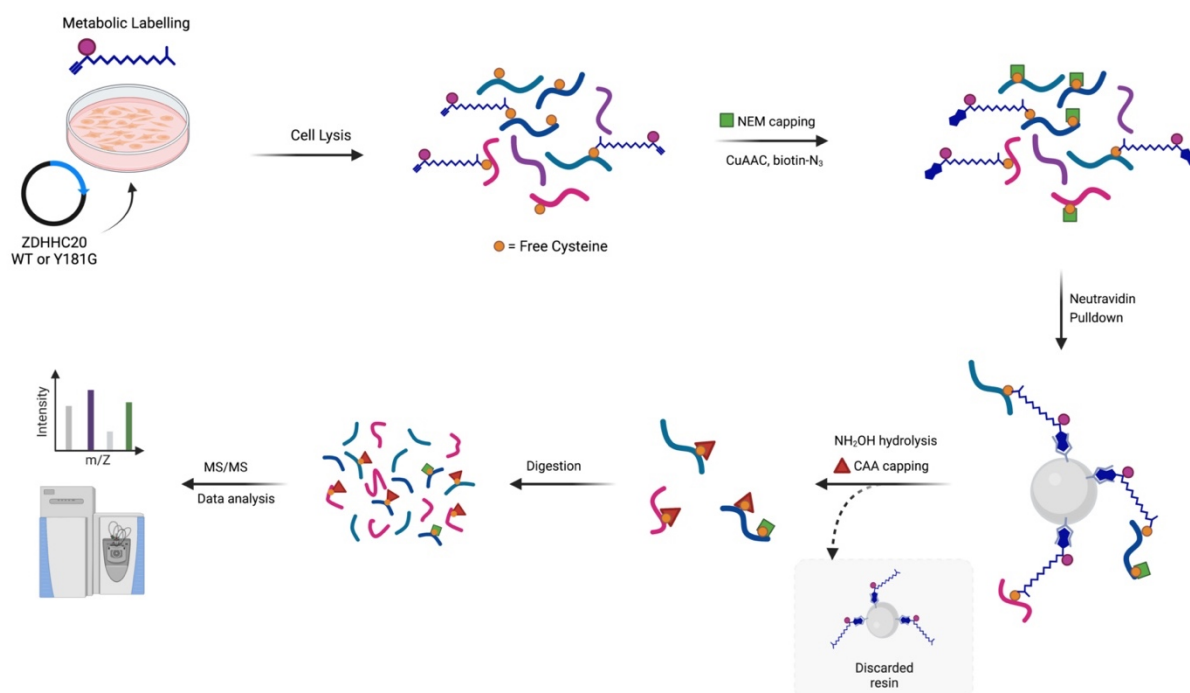


Figure 3.2. Proteomics on-bead hydrolysis protocol for the analysis of S-acylated proteins. Initial protocol for the identification of substrates using the chemical genetics system developed for ZDHHC20. After transient enzyme overexpression (ZDHHC20 WT or [Y181G]), proteins are metabolically labelled with lipid probes, which are enzymatically added to Cys residues through S-acyl bonds. After cell lysis, free Cys are capped with *N*-ethylmaleimide (NEM) and S-acylated proteins are clicked via the lipid probe to biotin-azide. This allows the isolation of S-acylated proteins on neutravidin agarose beads. Thioester bonds are then hydrolyzed off the beads and free thiols are capped with chloroacetamide (CAA). Finally, after trypsin digestion, peptide mixtures are analyzed via mass spectrometry for protein identification. Figure created with BioRender.

In a first experiment, conditions were set up to harness the orthogonality of the system. It was deemed sufficient to compare the wildtype enriched substrates to those enriched by the mutant ZDHHC20[Y181G]. The wildtype samples should only contain background labelling by the wildtype enzyme and other acyltransferases in the family. During data analysis, this would constitute the baseline signal from which to calculate differences and significances thereof necessary to identify mutant-enriched substrates. With regards to the type of sample quantification, label free quantification (LFQ) was the method chosen to simplify sample processing steps and minimize costs. With this in mind, in collaboration with Dr. Marc Baggelaar, HEK293 cells transiently expressing ZDHHC20[Y181G] or ZDHHC20 WT, were labelled with 15 μ M 18-Bz, for 8h and at 0.5% FBS. After sample processing, following the OBH protocol and nanoLC-MS/MS, the data was searched by LFQ with MaxQuant and analyzed

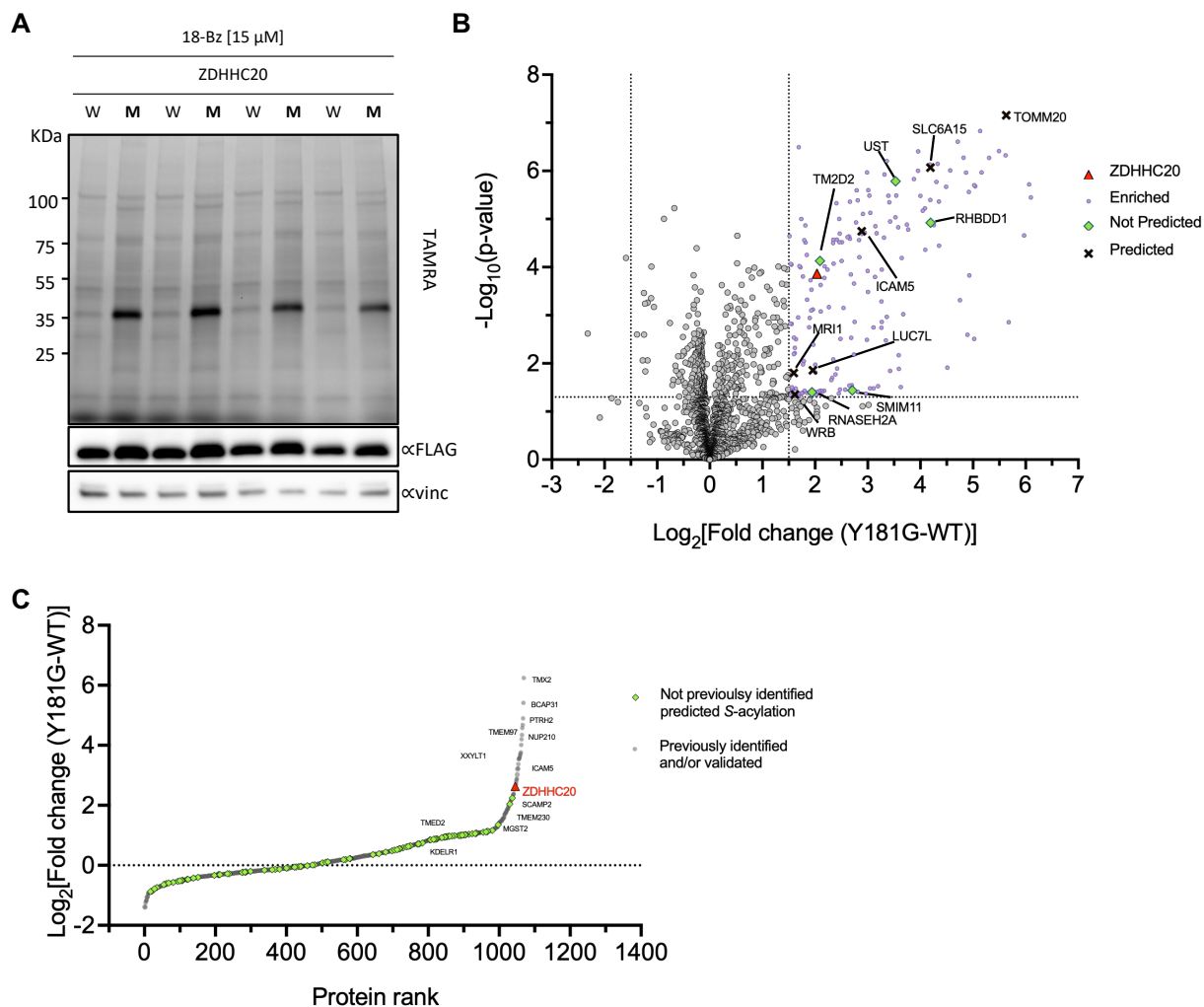


Figure 3.3. Identification of ZDHHC20[Y181G] substrates using an on-bead hydrolysis sample preparation protocol. A) In gel fluorescence analysis of lysates of HEK293 cells overexpressing mutant ZDHHC20[Y181G] (M) or wildtype (W) constructs and metabolically labelled with 18-Bz for 8h. Bands correspond to 18-Bz modified substrates clicked to TAMRA-azide. Imaging performed by Dr. Marc Baggelaar. B) Volcano plot depicting ZDHHC20[Y181G] enriched substrates from A. Most enriched substrates had been previously identified in proteomics experiments. C) S-plot of proteins ranked by Log₂ (fold change) depicting proteins those not previously identified as being S-acylated and of these, whether their S-acylation is either *in-silico* predicted or not. Panel A was kindly provided by Dr. Marc Baggelaar.

with the Perseus software (method detailed in Chapter 6). In brief, LFQ intensities were Log₂ transformed, contaminants were removed, and proteins groups were filtered to as to contain 3/4 valid values in the ZDHHC20[Y181G] condition. Missing values, not detected mostly in the wildtype technical replicates, were imputed following a normal distribution to mimic values at the lower limit of detection. To those protein groups remaining in the dataset, a two-sample student t-test ($S_0 = 0.1$, FDR = 0.05) was performed, comparing the two sets of conditions (ZDHHC20 WT and ZDHHC20

[Y181G]), to identify significantly enriched proteins for the mutant acyltransferase over the wildtype. Moreover, to identify statistically significant proteins, a p-value of 0.05 was set as cut-off and with regards to biological significance, a $\log_2$ (fold change) of 1.5 was used as it allowed to shortlist only proteins enriched for ZDHHC20[Y181G] by almost three-fold change, for further analysis and potential validation (Fig. 3.3.A). With these parameters, a total of 191 proteins were found to be significantly enriched, amongst which, ZDHHC20 was also found (Fig. 3.3.A). In this enriched group, 180 proteins had been previously reported to be modified with only 11 proteins newly identified as S-acylated. Of the later, a subset of 6 annotated as predicted (SLC6A15, TM2D2, TOMM20, LUC7L, MRI1, WRB) and five as not predicted (ICAM5, RHBDD1, UST, SMIM11, RNASEH2A) in SwissPalm ¹²⁵ (Fig. 3.3.A). One false positive was identified (JAGN1) with no Cys residues, and on first instance detected due to its co-precipitation with another substrate, yet no known interactor was found amongst its substrate scope. Furthermore, two proteins were identified as enriched in the wildtype conditions: acyl-coenzyme A thioesterase 8 (ACOT8) and peroxisomal carnitine O-octanoyltransferase (CROT), involved in the hydrolysis of acyl-CoAs and the beta-oxidation of fatty acids respectively. It is possible that their enrichment is the product of probe metabolism and not directly related to their S-acylation by ZDHHC20, yet both are reported to be S-acylated and have been found to be S-acylated in previous proteome profiling experiments. Finally, analysis conducted by Dr. Marc Baggelaar showed this protocol to be useful for the identification of sites of S-acylation with 99 novel sites detected and 28 previously reported (data not shown).

Overall, this first experiment validated the preservation of acyltransferase activity for the analogue-sensitive point mutant and the orthogonality of the approach all the way up to substrate transfer; beyond the optimization experiments presented in Chapter 2. Furthermore, with 94% of enriched substrates annotated as S-acylated previously reported, the ability of 18-Bz to be covalently incorporated into Cys residues is confirmed. Finally, those substrates not identified previously, speak towards the putative ability of this novel system to focus into a subsection of the S-acylated proteome and detect substrates that could otherwise be masked in global S-acylation studies.

3.2.1.2. Contaminants: a recurring problem and inflexion point for sample processing.

Unfortunately, the experiment presented in the previous section could not be replicated due to the sudden detection of contaminants in consequent biological replicates. Despite not making previous data invalid, this did raise questions on the robustness of the OBH protocol. Mass spectrometry contaminants are a common feature underlying experiment failure and as such, they have been extensively profiled ¹⁷⁸. In small quantities, those of protein origin – peptide fragments – do not represent a major concern. However, small molecule contaminants, such as detergents or plasticizers can have a major effect on chromatography and peptide identification. These types of contaminants, deposit on the LC column – sometimes rendering it unusable – and coat the inner components of the mass spectrometer, diminishing sensitivity and suppressing the ionization of peptide fragments ¹⁷⁹. The contaminants also compete for MS/MS scans with peptides lowering the peptide signals and also affecting the chromatographic behavior of peptides during liquid chromatography separation, causing peak broadening and fluctuations in retention times.

The analysis of contaminants arising from OBH protocols was performed by searching within the MS1 signal, mass-to-charge ratios of common contaminants using a published list ¹⁸⁰. Two major problems were identified in different OBH sample sets. Firstly, high quantities small molecule contaminants, specifically detergents (tween 60, tween 80, triton X-100, triton 101) and polyethylene glycol (PEG), common in small quantities as it leaks from any plastic containers (Fig. 3.5 A). It is difficult to pinpoint the origin as there their presence was variable across samples and could have been introduced due to the contamination of communal laboratory reagents. Secondly, a retention time shift and peak broadening was observed for peptide fragments of trypsin and avidin, characteristic of mobile phase changes and possibly due to the presence of negatively charged hydrophobic detergents, such as SDS – not identified on positive m/z scans ¹⁸¹. A possible origin for traces of the later was thought to be the use of the surfactant ProteaseMax® (Promega) to aid protein hydrolysis off the resin and which had been marketed as compatible with most mass spectrometry protocols. Its use had been justified, due to reports stating that the use of detergents aids the solubilization of proteins on beads needed to make thioester bonds accessible for their cleavage

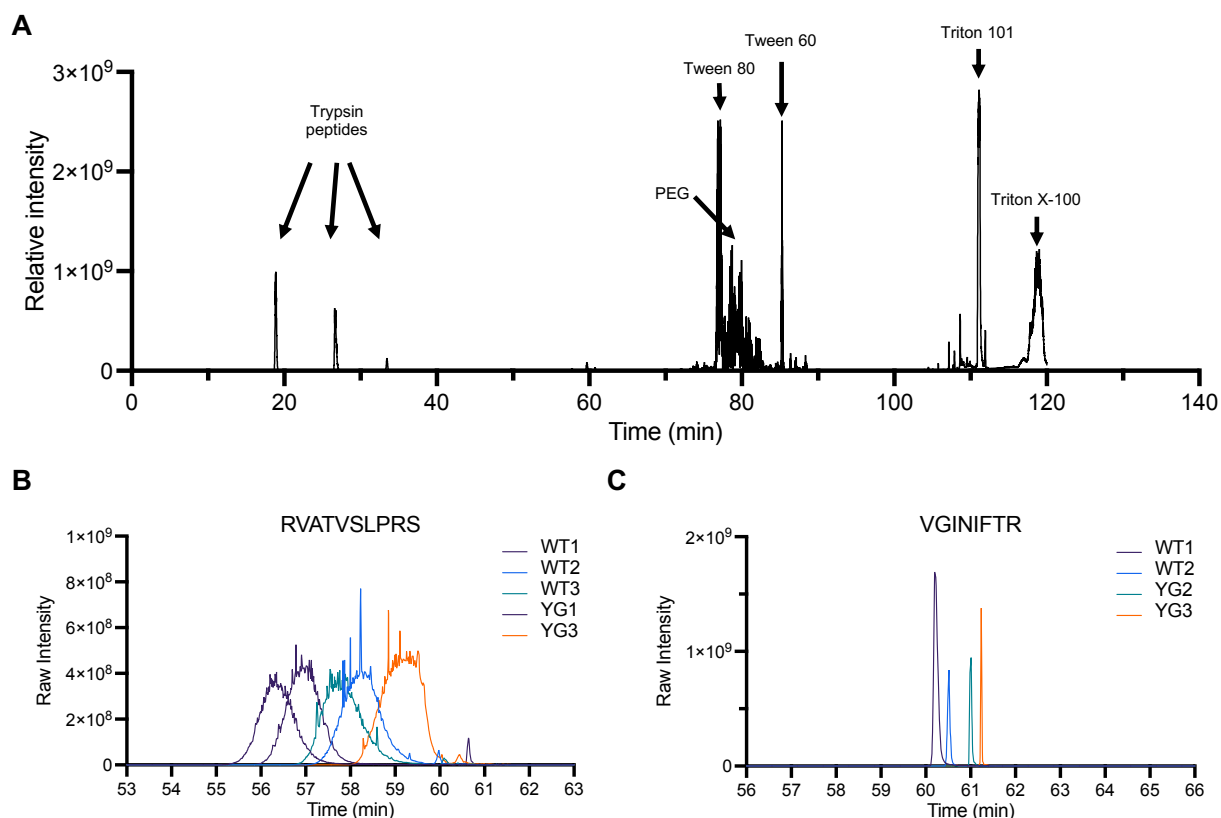


Figure 3.4. MS1 contaminant analysis of samples processed with the OBH protocol. A) MS1 m/z search of common mass spectrometry contaminants. Sample corresponds to a technical replicate of a sample overexpressing ZDHHC20 wildtype enzyme, treated with 18-Bz for 8h and processed with the OBH protocol as described in Chapter 6. B) and C) Trypsin fragments experience retention time shifts and peak broadening due to the putative presence of contaminants.

with NH_2OH ¹²². Other detergents such as Rapigest, utilized in the literature, were tested in the Tate group, yet similar contaminations were detected in several test samples which led to experiments being discarded as a result (data not shown).

Continuous identification of detergents prevented full experiments to be analyzed and put into question the robustness of the protocol and led to its modification to avoid the presence of detergents or surfactants in the final steps of the protocol. An on-bead digestion protocol was proposed, where the release of enriched proteins from beads would be accomplished through an enzymatic digestion step using LysC, a protease that cleaves proteins on the C-terminal side of lysine residues. To minimize avidin peptide contamination from beads and achieve optimal signal-over-background noise, a method had been previously developed in the group to chemically dimethylate

commercial Neutravidin resin. This decreases the affinity of LysC for those residues, lowering the amount of avidin-derived tryptic peptides and improving enriched peptide identification ³³. Furthermore, the robustness of this protocol had been extensively tested and successfully used in our group for the study of irreversible lipid PTMs mainly *N*-myristoylation and *S*-prenylation ^{27,34,41}. Although justified, this change of protocol did not permit the gathering of information for site identification. A peptide would remain covalently bound to the resin through its lipid-modified cysteine group. Thioester hydrolysis off the beads allowed the hypothetical removal of off-target incorporation of the lipid-probe into other nucleophilic residues (*N*-acyl or *O*-acyl bonds). Nevertheless, for the OBD protocol, this off-target should be accounted for by the wildtype sample group as it ought to be present in those samples as well.

An initial test of the OBD protocol revealed no traces of detergents or plasticizers on the MS1 signal (data not shown). Next, with the help of Dr. Jack Houghton, after raw data search with the Perseus software output tables were subject, to a more extensive quality control analysis using an R-based QC pipeline called Proteomics Quality Control (PTXQC) ¹⁸². This more extensive MS2 analysis showed on average, low relative intensities for tryptic and avidin peptide. More importantly, peptide identification, peak width and retention time were found stable (Appendix Fig. 8.7) across samples. Furthermore, peptide protein group and LFQ intensity distribution was on average higher for samples expressing ZDHHC20[Y181G]. This indicates that probe selectivity can be seen not only at the pulldown level, but also on the amount of information collected at the mass spectrometry level (Appendix Fig. 8.7). In comparison, analysis of two previous experiments with the OBH protocol revealed a significant drop in peptide identification and lower MS/MS scans half way through the sample runs, at a similar time point where the tween signal was detected in the MS1 signal (Fig. 3.5 A, Appendix Fig. 8.7). This is indicative that the presence of detergents is hampering the mass spectrometer detection of peptides across these samples. Another worrying feature, which confirmed the importance of the protocol change, was a peak width fluctuation across samples and over time, which is indicative of suboptimal chromatography. Moreover, the OBH method was found to contain higher peptide protease missed cleavage sites, all samples presented approximately over 35% of peptides with miss-cleavage sites compared to less than 25% in the OBD sample set (Appendix Fig. 8.7). This showed digestion conditions were suboptimal,

possibly due to the combination of residual ProteaseMax[®] and NH₂OH from thioester hydrolysis. Furthermore, high miss-cleavages are associated with poorer identification rates, as some spectra cannot be matched to *in-silico* database. Overall, the OBD protocol was found to give significantly more robust data without the any contaminants and no chromatographic issues.

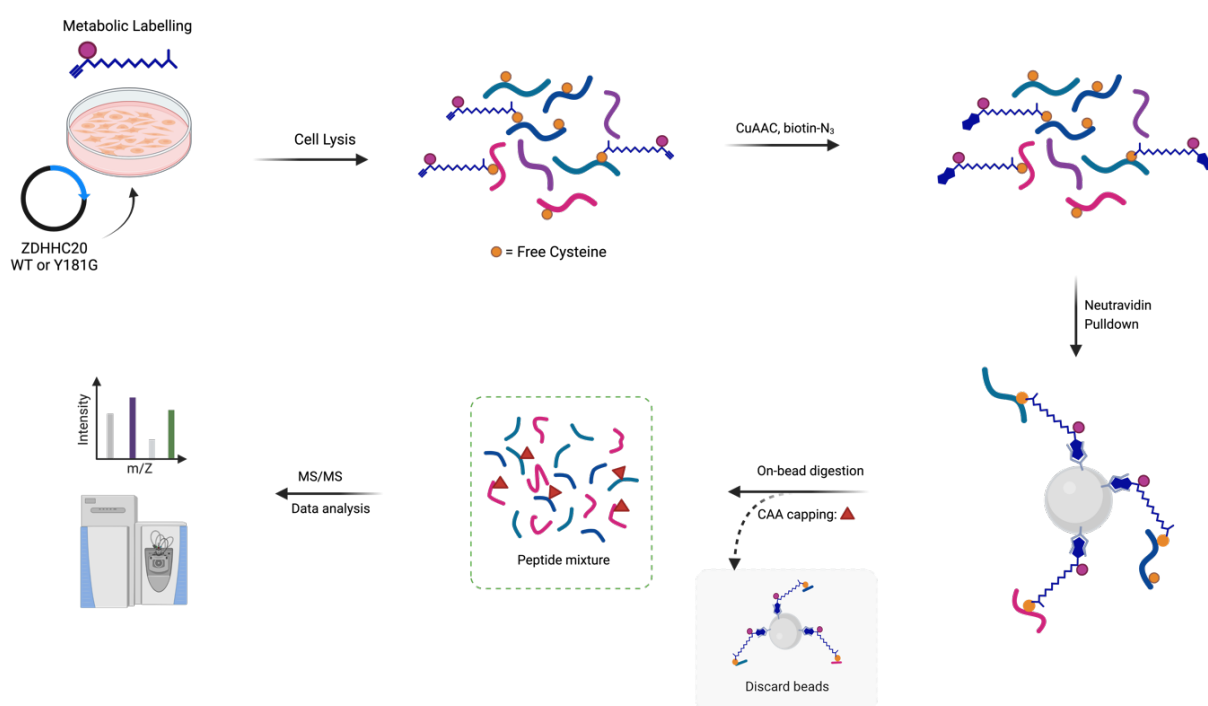


Figure 3.5. Simplification of mass spectrometry sample preparation protocol to avoid the introduction of contaminants in samples. A) Modification of the on-bead hydrolysis protocol for a robust identification of substrates using chemical genetics. S-acylated proteins are isolated after a CuAAC reaction to biotin on neutravidin agarose beads. An on-bead digestion, allows for the identification of enriched substrates via mass spectrometric analysis. Figure created with BioRender.

3.2.2. Proteomic identification and analysis of ZDHHC20 substrates in HEK293 cells

3.2.2.1. Identification and analysis of ZDHHC20[Y181G] substrates with an on-bead digestion protocol.

The chemical genetics OBD protocol was employed for the processing of metabolically labelled samples with the probe 18-Bz. Overall probe incorporation was detected across all samples (Fig.3.6 A), with selectivity of 18-Bz for mutant ZDHHC20[Y181G] confirmed by selective pulldown of the mutant enzyme and no enrichment for the wildtype one (Fig. 3.6. A). Analysis of MS1 *m/z* ratios revealed no traces of detergents or apparent chromatographic issues, such as those detected with OBH protocol. Avidin peptides were detected as contaminants but in low quantities. Furthermore, as explained above, proteomics quality control analysis showed all technical replicates to be adequate for data processing. Raw data was searched with the MaxQuant software, and analyzed with Perseus both as previously described (Chapter 6. Methods). Statistical analysis of the data, with a two-sample student T-test ($S_0 = 0.1$ FDR =0.05) revealed a clear enrichment of proteins with zDHHC20[Y181G] (Fig. 3.6 B). Over half of the protein groups detected were found to be positively enriched for ZDHHC20 [Y181G] towards mutant showing a clear selectivity for post-translational addition of the probe by the mutant enzyme.

To shortlist putative substrates, the significance cut-offs previously established were maintained. A list of 164 proteins were found to be significantly enriched for the mutant ZDHH20 [Y181G]; of these, only 35 (21%) were identified by the OBH method, which indicates a low overlap in substrate identification between both methods. Comparison of fold changes of hits in common between both methods revealed a very low correlation between methods with a R^2 of 0.1331 (Fig. 3.6 C). Nevertheless, the dynamicity of S-acylation and possible variability of cell states due to transient transfection could well be responsible for such difference. Next, to determine the ability of the probe and protocol to enrich for S-acylated proteins, this proteomics OBD dataset was cross-referenced to a SwissPalm list, containing all human proteins identified by proteomics experiments and their prediction annotations for S-acylation. Of all proteins identified by mass spectrometry, approximately 76% had already

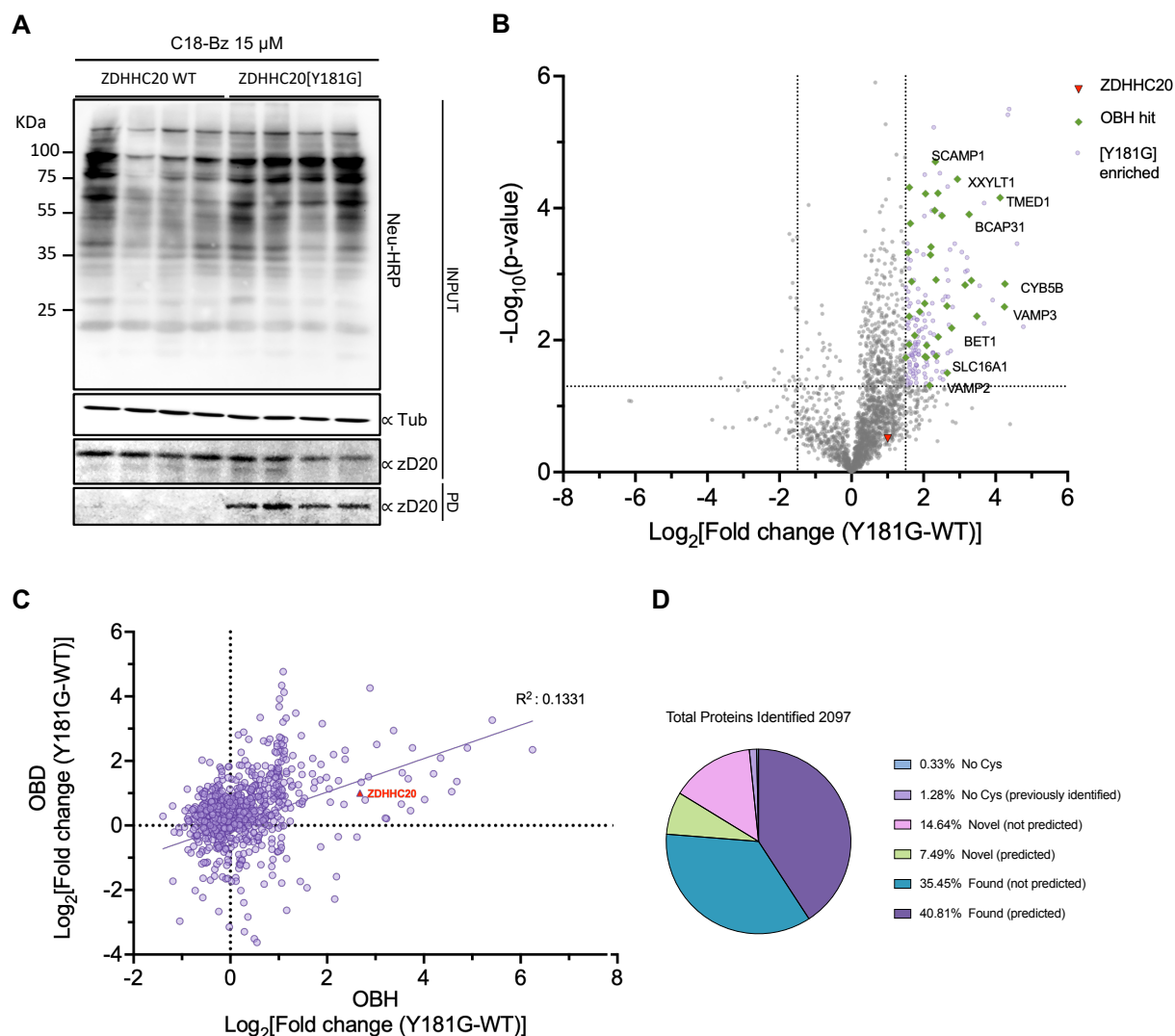


Figure 3.6. Substrate identification and analysis of ZDHHC20[Y181G] with an on-bead digestion protocol. **A)** Western blot analysis of HEK293T cells transiently transfected with WT ZDHHC20 or ZDHHC20[Y181G] and metabolically labelled with 18-Bz probe (15 μ M) for 8h. After a CuAAC reaction to biotin-azide, input lysates were analyzed via Western blot. After pulldown with Neutravidin agarose beads, gel-loading buffer was added to a fraction of beads for pulldown (PD) analysis. Neutravidin-HRP (Neu-HRP); ZDHHC20 (zD20); Tubulin (tub). **B)** Chemical proteomic analysis of ZDHHC20 substrates of A, processed using the OBH workflow. Enrichment of ZDHHC20 indicates selective loading for ZDHHC20[Y181G] over WT ZDHHC20. Significantly enriched ZDHHC20 [Y181G] substrates are highlighted in purple; substrates in common with OBH workflow are highlighted in green; ZDHHC20 highlighted as a red triangle. **C)** Comparison OBD and OBH protocols through the Log2(fold changes) of mutant ZDHHC20[Y181G] (Y181G) over wildtype ZDHHC20 (WT). Linear regression and Pearson R-squared values calculated using Prism9. **D)** Percentage of all proteins identified in B based on their SwissPalm annotation.

been reported by others to be S-acylated at least in one palmitoyl proteomics experiment and of those not priorly reported, 7.5% were predicted to be modified but not previously found (Figure 3.6 D). As stated before, this lack of previous detection,

speaks towards the ability of the developed chemical genetic system to detect novel substrates, putatively by fractionating the S-acylated proteome and focusing into the substrates of ZDHHC20. More interestingly approximately 15% of proteins not predicted to be S-acylated were identified, giving an indication that enzyme overexpression might be S-acylating proteins that are not endogenously modified. Contrary to the initial OBH experiment, ZDHHC20 was not found to be significantly enriched in this first experiment, possibly due to its lower expression (Fig. 3.6 A), a consequence of the variability present in transient transfections.

The experiment was repeated twice with the help of Dr. James Siphthorp and in-gel fluorescence and proteomic analysis of other two biological replicates showed higher overall ZDHHC20 expression (mutant and wildtype) via in-gel fluorescence and enrichment of ZDHHC20[Y181G] after proteomic analysis (Appendix Fig. 8.8). Analysis of substrates from all experiments revealed a higher overlap between every two replicates than with the OBH, and 21 common hits amongst the three replicates (Fig. 3.7 A and Appendix Table 8.7). Substrates found in 2 out of 3 replicates were considered high confidence hits and several were picked for technology validation. For this, post-doctoral researchers in the group Dr. James Siphthorp and Dr. Tony Ocasio, cloned cysteine to alanine mutants of several substrates and after their overexpression with either wildtype or ZDHHC20[Y181G] enzymes and metabolic labelling with 18-Bz their S-acylation pattern was analyzed (Fig. 3.7 B-E). From these experiments, the S-acylation pattern of PI4K2A, confirmed the reported sites of modification showing that the combination of analogue sensitive mutant and bumped probe preserve site-specificity (Fig. 3.7 D). For BCAP31 and VAMP3 sites Cys23 and Cys76 had been identified in the OBH experiment and alanine mutants of these showed almost no incorporation of 18-Bz by in-gel fluorescence. Additionally, metabolic labelling of XXYLT1 showed specificity for labelling with ZDHHC20[Y181G], thus validating it as a substrate for this mutant.

Our group later developed chemical genetic systems for isoenzymes ZDHHC7 and ZDHHC15 which underwent similar optimization steps as those presented in Chapter 2 for ZDHHC20. The substrates of these acyltransferases were also profiled by mass spectrometry by other group members (data not shown). Therefore, with this data in hand the specificity of the detected hits towards modification by ZDHHC20 was assessed. Cross-comparison of the high confidence substrates of ZDHHC7,

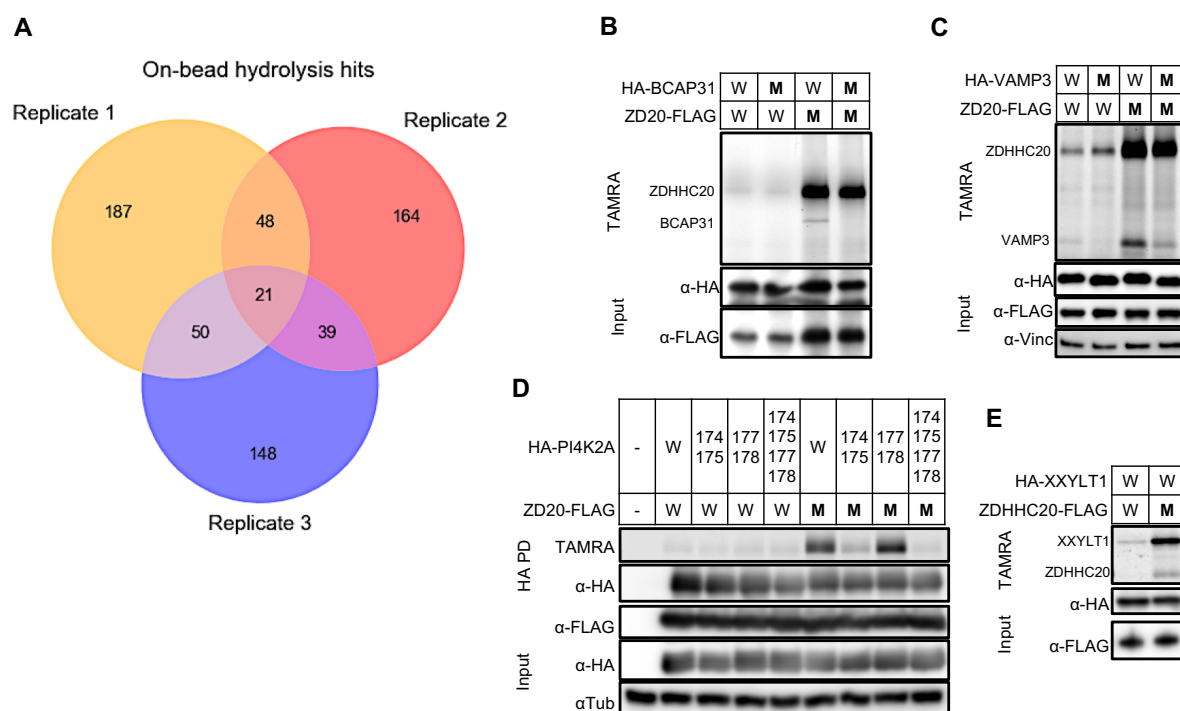


Figure 3.7. Cross comparison of ZDHHC20 putative substrates and the validation of high confidence hits. **A)** Venn diagram comparing the enriched substrates for ZDHHC20[Y181G] across three biological replicates using the on-bead digestion protocol. **B-E).** Overexpression of putative ZDHHC20 substrates or their cysteine mutants (**M**) with wildtype ZDHHC20 or ZDHHC20[Y181G] mutant (**M**); metabolically labelling with 18-Bz [15μM]. HA-PD refers to pulldown with HA beads. TAMRA refers to in-gel fluorescence signal and Input refers to western blots showing loading signal across. Experiments performed by postdoctoral researchers in the group Dr. James Sipthorp and Dr. Tony Ocasio. **B)** Metabolic labelling of HA-tagged BCAP31 and its Cys23 to Ala mutant **C)** Metabolic labelling of HA-VAMP3 and its Cys76 to Ala mutant **D)** Metabolic labelling of PI4K2A, two Cys to Ala double mutants and a no Cys mutant confirms the biological mimic of S-acylation patterns by ZDHHC20[Y181G] **E)** XXYLT1 metabolic labelling confirms it is a ZDHHC20 substrate. Panels B, C, D and E were kindly provided by Dr. Tony Ocasio and Dr. James Sipthorp.

ZDHHC15 and ZDHHC20, allowed to identify those that were found exclusively for each of these acyltransferases. For ZDHHC20, 33 substrates were exclusively found as high-confidence in its datasets for example, VAMP3, VAMP8 and PI4K2A (Appendix Table 8.8). The rest of the substrates of ZDHHC20 were found to be part of overlapping substrate scopes for example, BCAP31 found to be a ZDHHC15 substrate, or SEC22B common substrate with ZDHHC7 (Appendix Table 8.8). A group of seven substrates were found to be readily S-acylated by all isoenzymes (Appendix Table 8.8). Yet, it is worth pointing out that the overexpression conditions could have led to off-target S-acylation, and as a consequence the detected substrate scopes are distorted and do not fully capture the endogenous scope of each of the

acyltransferases. It is also possible, that enzyme mislocalization due to overexpression is enlarging the breadth of the substrate scopes, in some instances reported to be governed by substrate proximity and accessibility of Cys residues. Consequently, it might well be that under endogenous expression levels, the substrate scopes are more reduced, less overlapping and found to be more consistent using chemical genetics. Another possibility is that the overlap is biologically necessary upon variation of ZDHHC expression levels, for example, across cell types and tissues, or disease states. Following the same train of thought, those substrates that have selective acyltransferase under overexpression conditions could constitute exclusive substrates for ZDHHC20, yet for this to be fully confirmed the profile of all isoenzymes in the family would have to be discovered. Finally, there is a risk that the introduction of the mutation is affecting the substrates that each ZDHHC is targeting.

3.2.2.2. Analysis of ZDHHC20 substrates identified via chemical genetics.

From a general perspective, S-acylation and ZDHHCs have been linked to a wide range of biological functions such as, protein localization, transport, signaling or gene expression. These links have been established on an individual basis, linking a ZDHHC to a substrate. Nevertheless, the extent of the involvement of individual isoenzymes on the correct unfolding of these processes remains unknown. With studies establishing a relationship between the S-acylation of IFITM3 and EGFR by ZDHHC20, this enzyme has been linked to two biological functions: cellular viral immunity and cellular signaling.

With a list of putative ZDHHC20 substrates – those found in at least 2 out of 3 biological replicates – a unique opportunity was presented to assess any other biological functions that ZDHHC20 could take part in. For this, putative substrates were subject to functional enrichment analysis using the STRING database ('Search Tool for the Retrieval of Interacting Genes/Proteins')^{96,183}. This online tool, allows to relate any two proteins based on their association to a biological function or a cellular component, based on for example, experimental data (i.e. biochemical or genetic) or previously curated pathway or protein-complex information (i.e. KEGG annotation¹⁸⁴). This evidence is used to calculate interaction scores for associated proteins – a calculated probability of the association being true¹⁸³. For the substrate list in

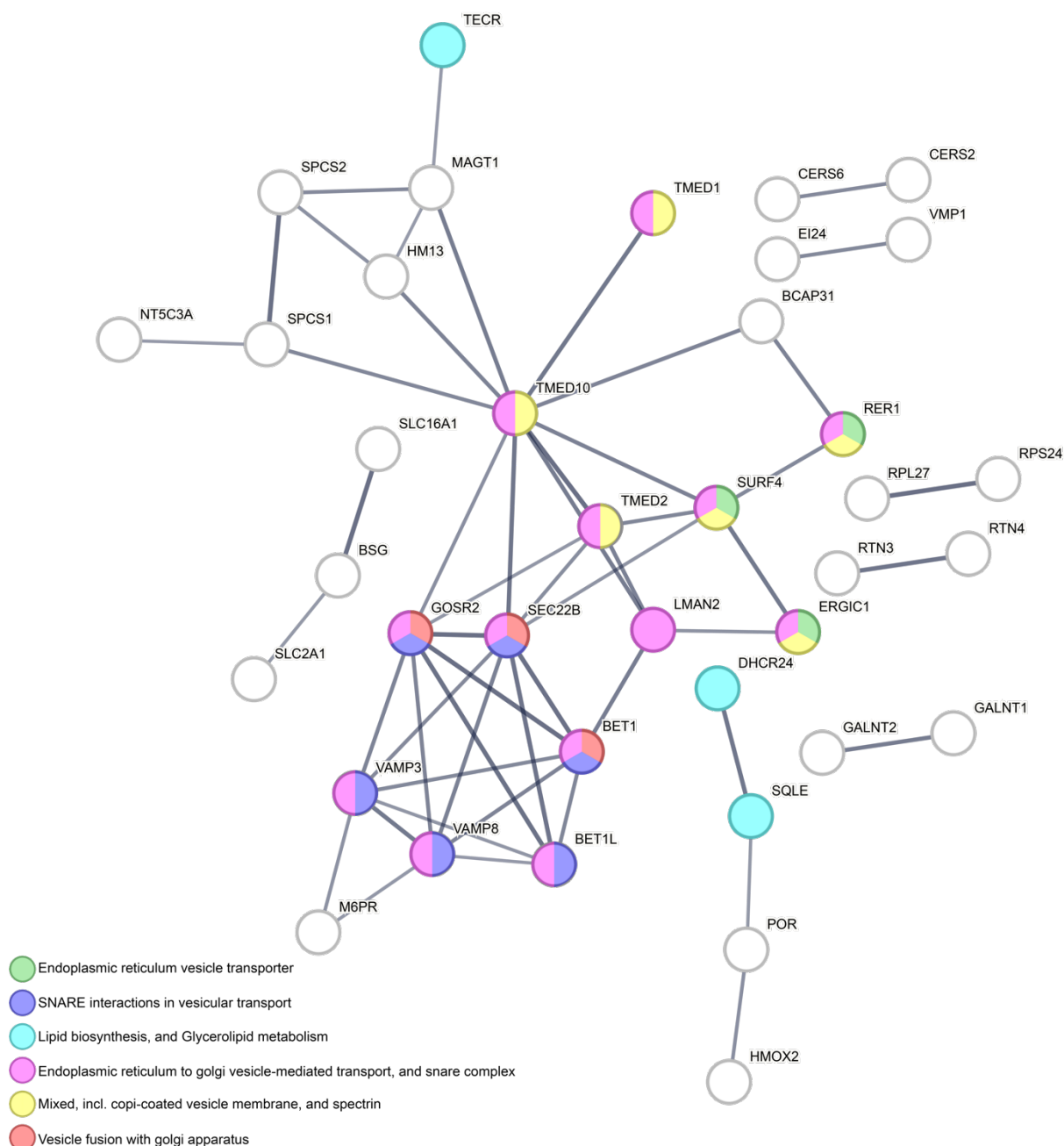


Figure 3.8. STRING analysis of ZDHHC20 high-confidence substrates identified with the chemical genetic system and the OBD protocol. Cluster map was calculated in the <https://string-db.org/> website, and searching for associations of high confidence ZDHHC20 substrates¹⁸³. Highlighted in colour are the STRING clusters with the highest number of associations and nodes (proteins). Associations included experimental data, gene association and previous pathway curations. Nodes without any associations were removed. Thick lines represent very high confidence association scores (90% confidence of association) and thin lines represent high confidence association scores (70% confidence of association).

question, a 70% confidence in association was set as cut off, which includes only high-confidence relationships. The resulting association network (Fig. 3.8), shows the most

redundant functional associations for ZDHHC20 substrates to be: i) endoplasmic reticulum vesicle transporter; ii) SNARE interactions in vesicular transport; iii) Lipid biosynthesis and glycerol lipid metabolism; iv) Endoplasmic reticulum to Golgi-vesicle mediated transport and snare complex; v) COPI-coated vesicle membrane; and vi) vesicle fusion (Fig. 3.8 A). The dominant cluster of proteins (Fig. 3.8, in pink) is related to the transport of proteins between the Endoplasmic Reticulum and the Golgi apparatus, and the SNARE complex – responsible for the fusion of vesicles at the plasma membrane. The role of S-acylation in the controlled fusion and excision of biological membranes has been well studied, and is of great importance in neuronal signaling and development where it has been extensively profiled ^{23,73,185,186}. Another large cluster was formed by COPI-coated vesicle membrane proteins (Fig 3.8 in yellow), where the TMED family of cargo receptors – important regulators of protein transport – was found overrepresented. Certain proteins were found to be strongly associated to this cluster, an example being the previously validated BCAP31 – reported to be one of the most abundant ER proteins acting as a chaperone for misfolded proteins and directing them to the ERAD pathway ¹⁸⁷.

To further explore the distribution of the putative ZDHHC20 substrates, statistical overrepresentation analyses of gene ontology (GO) terms, for cellular compartments and biological processes, were performed using the online tool PANTHER ¹⁸⁸. Using as reference all genes from the human genome, overrepresented cellular components were found to encompass the endomembrane system, which includes vesicles, the ER, the Golgi apparatus, the ER to Golgi intermediate compartment (ERGIC) and the plasma membrane (Fig. 3.9 A). This is in accordance with reports of the localization of ZDHHC20 at the ER Golgi and plasma membrane depicting enzyme-substrate co-localization⁴². The localisation of hits was found to further encompass GO terms for the SNARE complex, vacuoles and the cell periphery, which represents the plasma membrane but also secretory vesicles. With regards to overrepresented GO terms for biological processes, these were found to be in accordance with the former analysis, with substrates participating in the organization of the endomembrane system and the anterograde and retrograde protein transport through the ER – Golgi intermediate compartment (ERGIC) (Fig. 3.9 B).

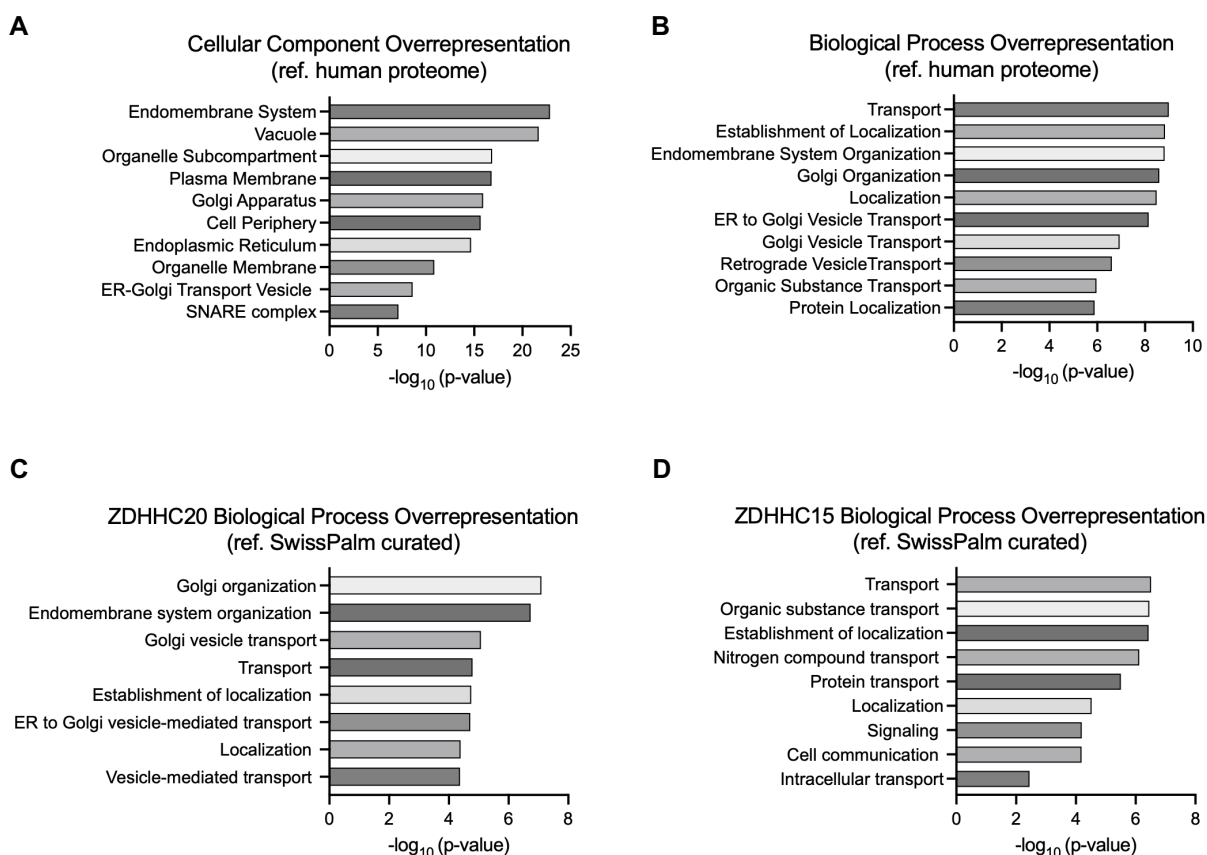


Figure 3.9. PANTHER overrepresentation analysis of putative ZDHHC20 substrates. A-B) Respectively: cellular component and Biological process overrepresentation compared to the human proteome, used as reference list. **C-D)** Biological process overrepresentation of ZDHHC20 and ZDHHC15 substrates respectively, using a SwissPalm list of validated hits as reference. Analysis was performed using the PANTHER¹⁸⁸ online tool with statistical analysis and p-values were determined using the Fisher's Exact test.

Next, to discard the possibility of such profiles being the specific to general S-acylation, the analysis was repeated using as reference a list of S-acylated proteins from SwissPalm; curated to contain those predicted and found in at least 2 palmitoyl proteomics experiments. This analysis revealed a similar profile of enriched biological functions for ZDHHC20 substrates within the S-acylated proteome (Fig.3.9.C), corroborating the possibility of those substrates being specific to this acyltransferase. Finally, similar analysis was performed for substrate lists obtained with the chemical genetic systems developed for ZDHHC15 and ZDHHC7 and curated by Dr. James Siphthorp. This analysis revealed gene ontology annotations and additional functions for ZDHHC15 such as signaling and cell junctions and despite the fact that ZDHHC15 substrates were also found to be involved in transport, GO terms were much more

general and no association to ER-Golgi classical secretion was found. Due to the smaller substrate list size for ZDHHC7, no statistically significant biological functions were found to be overrepresented.

In summary, a degree of specificity in the biological processes of putative ZDHHC20 substrates was detected, implying that this isoenzyme participates in specific cellular pathways putatively through the co-localisation with its substrates. However, it is worth pointing out that these results remain true within the conditions of the experiments. It is expected that the overexpression levels of this assay, may impact the distribution of pathways that ZDHHC20 is involved in. As previously explained, these conditions could result in the expansion of its substrate scope. Therefore, it would be extremely interesting to assess the ability of the chemical genetic system to detect substrates of ZDHHC20 under near-endogenous expression levels. Furthermore, substrates may change under the effect of external environmental cues, which may trigger specific signaling pathways and the S-acylation of a specific subset of substrates. Alternatively, different cellular models may have varying isoenzyme substrate scopes. All in all, chemical genetics is proving to be a technology that can help answer these questions.

3.2.2.3. Investigating possible PTM dependencies for ZDHHC20 substrates.

Lastly, this dataset was analyzed to understand a potential cross-talk between ZDHHC20 S-acylation and other PTMs. For this, the list of putative ZDHHC20 substrates was cross-referenced with firstly with datasets for *N*-myristoylation and S-prenylation, gathered from experimental evidence produced in the group and manually curated by Dr. Wouter Kallemeijn. A low overlap was found, not surpassing 10% of the hits (Fig.10 A and Appendix Table 8.6). Next, utilizing online databases for glycosylation (uniprot.org) and phosphorylation (PhosphoSitePlus®¹⁸⁹) the hits were further cross referenced showing a 30% and 85% overlap respectively.

The co-occurrence of S-acylation and phosphorylation in substrate has been reported for several proteins previously. A clear example is the case of EGFR, where S-acylation is thought to fold the C-terminal tail preventing receptor autophosphorylation⁶⁹. The reversibility of both PTMs, acting in certain instances as

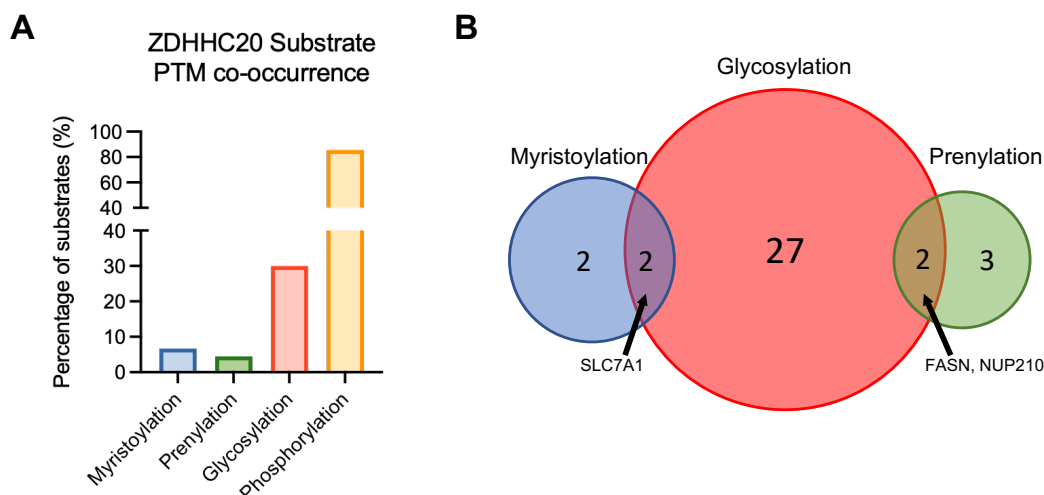


Figure 3.10. Analysis of ZDHHC20 chemical genetic hits with other PTMs. A) Percentage of substrates found to be post translationally modified by other enzymes, after cross referencing with internal *N*-myristoylation and *S*-prenylation lists (provided by Dr. Wouter Kallemeijn), or publicly available datasets for glycosylation (uniprot.org) or phosphorylation (PhosphoSitePlus®¹⁸⁹). **B)** Overlap in PTM annotations for *S*-acylated protein. This depicts a potential co-regulation of protein activity by multiple PTMs or differences in protein activity by distinct proteoforms.

on/off switches of signal relay in metabolic pathways, indicates that these might exist in distinct proteoforms each responsible of a distinct protein function not co-existing at the same time. With regards to glycosylation and to the best of our knowledge, there are no proteome-wide studies on its co-regulation with *S*-acylation. The fact that glycosylation occurs mostly in the endoplasmic reticulum (ER) and Golgi apparatus¹⁹⁰, might hypothetically mean a higher degree of co-occurrence with ZDHHC20 *S*-acylation. Example of co-occurrence of these PTMS is the SARS-CoV-2 spike protein (modified by ZDHHC20)⁸⁴, and other cellular surface receptors such as CD63 CD47, both found to be putative substrates of ZDHHC20 in the mass spectrometry data. After cross-referencing all the substrates with these PTMs, no overlap was seen between *N*-myristoylation and *S*-prenylation (Fig. 10 B), which is thought to be due to the inherent irreversibility of these modifications – reported to trigger the partitioning of their substrates into lipid bilayers – but also due to the selective localisation of these at the *N*-terminus (*N*-myristoylation) or *C*-terminus (*S*-prenylation). To these irreversible PTMs, the addition of reversible *S*-acylation, with its dynamic nature ought to impart additional regulatory functions.

To conclude, it is worth pointing out that with this analysis does not imply the co-occurrence of these PTMs in the same proteoforms, and it might well be that

different patterns of modification have varying functional implications. Further studies would be needed to investigate the repercussions of ZDHHC20 S-acylation, and the lack of it, on the regulation by other PTMs. This could be done achieved via the combination of the developed chemical genetic system with for example pan-kinase inhibitors, NMT inhibitors such as IMP1088 ⁴⁰ or inhibitors of prenyl-transferases. Alternatively, although more laborious, this could be studied in a case by case basis via mutation of sites of modification and a consequent functional analysis.

3.2.3. Comparison of the chemical genetics with biochemical approaches.

With regards to isoenzyme selective profiling of S-acylated proteomes, the only tools available are genetic knockout (KO) or knockdown (KD). These methods identify substrates only if their modification is lost under KO/KD conditions. As mentioned before, it has been reported that other S-acyltransferases can compensate for this loss resulting difficult to identify novel substrates. Moreover, to validate the utility of any newly developed technology it is good practice to compare it to the gold-standard in the field. Therefore, it was deemed necessary to compare gene-editing methods to the developed chemical genetic system to assess the robustness of the method. To perform this comparison, the proteome of ZDHHC20 KO HEK293 cells was profiled using YnPal. For this, Dr. Tony Ocasio generated ZDHHC20 KO HEK293 single cell cloned cell lines using CRISPR/Cas9 (for details see Chapter 6). Two of these, were confirmed via western blot (Fig. 11. A) and sequenced. The DNA around the target site was amplified by PCR showing a base insertion (KO1) and deletion (KO2) which led to a frameshift and consequent ZDHHC20 KO; validating each clonal cell line (Fig. 3.11 B).

After clonal cell line validation, these were used to assess differences in the S-acylated proteome between KO and wildtype cell lines. In order to do this, cells were metabolically labelled with YnPal (20 μ M), and lysates were processed with the OBD protocol. After sample quality testing and data analysis as described earlier (Sections 3.2.1 and 3.2.2), hardly any substrate enrichment was detected for either of the two KO samples tested when comparing them to the wildtype, including no sign of ZDHHC20 enrichment for the wildtype samples (Fig. 3.11 C). This lack of enrichment could indicate that either the rest of the isoenzymes in the background are able to overcompensate for the loss of ZDHHC20; or that highly modified proteins in the system are hampering the detection of any substrates putatively S-acylated to a lower extent, that may be differing in the background. Those hits identified with the chemical genetics method were found to hardly change between conditions and were part of the background signal. Alternatively, it might well be that the use of gene-editing methods are suitable more complex biological settings (i.e. *in vivo*) where acyltransferase substrate pairs have been detected. Moreover, less redundancy might

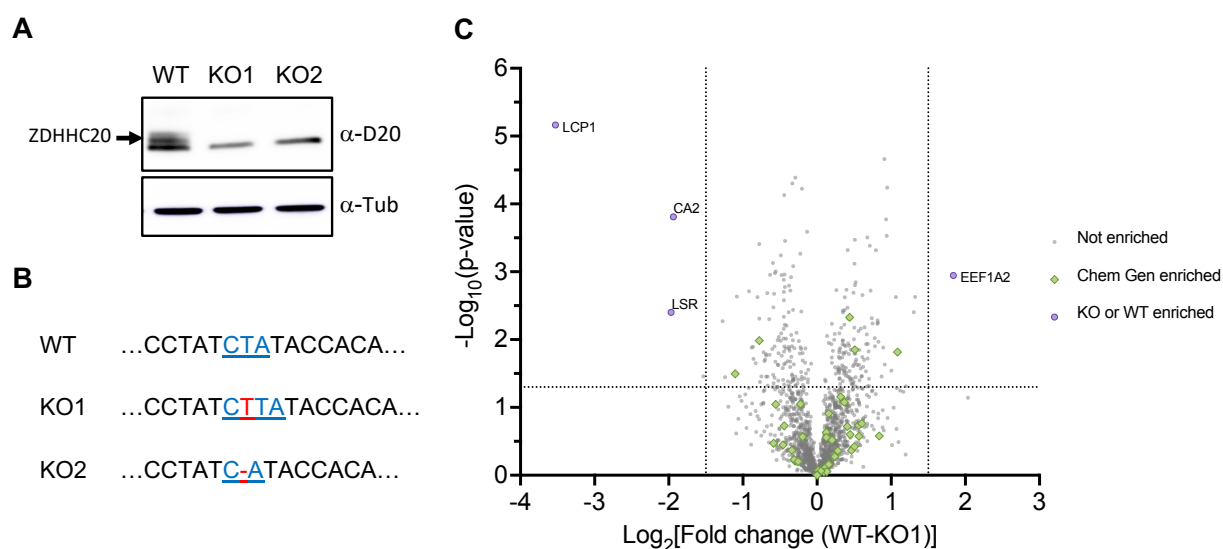


Figure 3.11. Profiling of ZDHHC20 KO HEK293 clones and their analysis via mass spectrometry. **A)** Western blot comparing wildtype to two KO clones showing disappearance of ZDHHC20 band. **B)** Type of frameshift present in the ZDHHC20 KO cell lines. Genetic knockout of KO1 is by base insertion and that of KO2 through base deletion. **C)** Volcano plot of HEK293T and ZDHHC20 KO1 cell lines metabolically labelled with 20 μM YnPal and processed using the on-bead digestion protocol. Cross-referencing of chemical genetic hits shows lack of downregulation of hits in ZDHHC20 KO1 cell line. Panel A was kindly provided by Dr. Tony Ocasio.

be observed in non-diseased tissues or different disease models as the level of plasticity of cancer cells might contribute towards a faster adaptation upon genetic editing. All in all, this suggests that the chemical genetic approach developed, which could be applied to all acyltransferases, can complement existing methods whilst having the power to bypass any redundancy and compensatory effects.

3.2.4. Generation of ZDHHC20 [Y181G] mutant stable cell lines.

Despite the initial success in applying the technology, it started to become apparent that a degree of doubt remained on substrate identification due to the enzymatic overexpression. To remedy some of the flaws of the overexpression system (i.e. fluctuation in enzyme expression, S-acylation of substrates not modified under endogenous levels, mislocalization), I moved towards the generation of cell lines stably expressing the different ZDHHC20 constructs. To achieve maximal control on enzyme expression, a doxycycline-inducible system was envisioned, which would

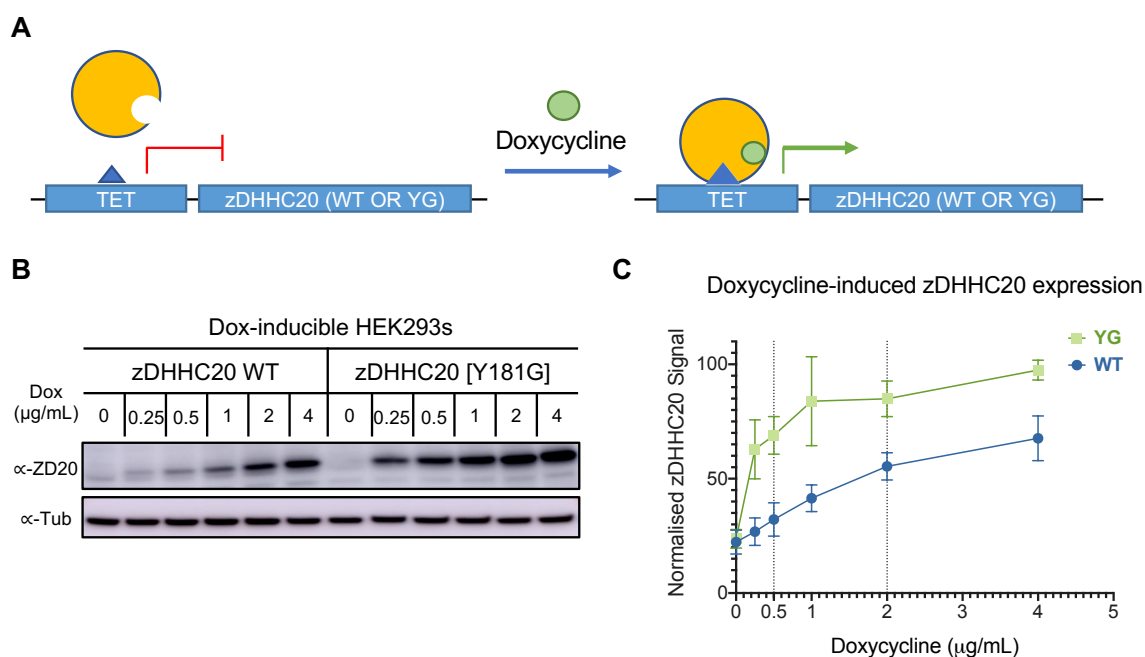


Figure 3.12. Stable doxycycline inducible cell lines. the stable expression of ZDHHC20 WT and [Y181G]. **A).** Scheme depicting doxycycline induced promoter activation. **B)** Western blot dose-response curves of ZDHHC20 WT and ZDHHC20[Y181G] doxycycline-inducible cell lines. **C)** Quantification of n of 3 replicates of B, normalized to tubulin loading controls. Dotted vertical lines indicate the approximate dosing times to achieve similar expression levels.

allow the titration of enzyme expression with doxycycline (Fig. 3.12 A). For this, ZDHH20[Y181G] and ZDHHC20 WT constructs were amplified by PCR and Gibson assembled into an empty pLVX tet-on vectors (second generation lentiviral delivery plasmids). After lentiviral production, HEK293 cells were infected with either ZDHHC20 WT or ZDHHC20 [Y181G] viral particles and the population was selected with antibiotic. A first attempt at single cell cloning was unsuccessful, and single cell clonal lines were found to have unstable expression over time. In some cases, expression was lost despite culturing under maintenance levels of antibiotic (data not shown). In a second attempt, cell lines were not single-cell cloned and after an initial round of antibiotic selection, construct expression was seen to increase with doxycycline concentration (Fig 3.12 B). This trend was replicated over 3 passages, and doxycycline concentrations were found to express of both constructs at similar levels (Fig. 3.12 C). Unfortunately, yet again fluctuations in expression were seen in later passages. Consequently, these efforts were paused in lieu of designing other strategies for the stable expression of the constructs including a CRISPR/Cas9 to knock in the point mutation Y181G.

3.3. Chapter summary and future work

This Chapter presents the application of the chemical genetic system developed in Chapter 2, in combination with proteomic protocols for the identification of ZDHHC20 substrates via mass spectrometry. Initial efforts, though successful in enriching ZDHHC20 [Y181G] modified proteins over wildtype, proved to be unreproducible due to recurrent problems with contaminants. After their exhaustive profiling, which pinpointed a plausible origin for said contaminants, the protocol is successfully changed and proteomic analysis of ZDHHC20 [Y181G] substrates is performed in triplicate. This allowed the generation of a list of 90 ZDHHC20 substrates, which were reproducibly enriched by the mutant enzyme at least 2 of 3 biological replicates. With a reversible PTM, those substrates found in a single experiment are thought to be a consequence of either fluctuations in construct expression due to overexpression, or due to subtle differences in cellular status, not visible or quantifiable by eyesight. Nevertheless, with a shortlist of 90 putative substrates of ZDHHC20, the functional association was studied to reveal a majority of them can be associated to vesicular protein transport from the ER to the Golgi. Moreover, functional enrichment analysis showed a degree of specificity of the functions to ZDHHC20 amongst the S-acylated proteome. In a final proteomics effort, the ability of the field's gold standard, that is gene-editing methods, was tested to identify loss in S-acylation of ZDHHC20 substrates in KO cell lines after YnPal treatment. This method proved unsuccessful, further supporting, at this stage the ability of the developed chemical genetic technology to identify acyltransferase-substrate pairs.

It is worth re-stating that these substrates are considered 'valid' or 'true' under the biological setting studied: overexpressed HEK293T cultured in monolayer with the increased abundance of ZDHHC20 in cells potentially leading to mislocalization. It is expected that any changes or environmental cues would lead to the discovery of novel substrate profiles even under the same experimental settings. Furthermore, one of the main limitations of using metabolic labelling to study any PTM is that it can only capture the fraction of proteins that are modified within the time-frame of the experiment. In the cases presented in this chapter, the system would only be capable of detecting a fraction of the ZDHHC20 substrate scope with a S-acylation cycle within the time frame of the experiments. Those substrates with faster cycles might experiment changes

that are lost within a steady state bulk analysis; and those with longer S-acylation cycles are excluded from the analysis. Previous studies have addressed this issue by profiling S-acylation in time, with the help of thioesterase inhibitors and isotopic labelling or tandem-mass-tag (TMT) ^{109,124}, resulting in the profiling of proteins with varying speeds of S-acylation cycles. Similar experiments would be proposed to assess the time-dependent ZDHHC20 S-acylation using chemical genetics, in combination with TMT to circumvent the issue arising with missing values. Furthermore, since 85% of the ZDHHC20 substrates identified are reported to be glycosylated, peptide identification might be improved by enzymatic removal of this PTM. This could facilitate protein pulldown and enzymatic digestion giving a higher protein coverage, increasing hit identification.

In the future, one remaining question that should be further explored: whether the substrate scope of mutant ZDHHC20 [Y181G] is identical to that of the wildtype enzyme or whether that point mutation has a structural impact on enzyme structure and consequently on substrate identification. That is, whether the similar substrates are enriched using YnPalm with ZDHHC20 WT or ZDHHC20 [Y181G]. To do this, one could compare the YnPalm labelling patterns for both enzymes by mass spectrometry, with similar enrichment profiles indicating comparable substrate interactions and minimal or negligible structural changes. Otherwise, yet much laborious would be obtaining the crystal structure of the point mutant to visualize any structural changes.

With regards to proteomic substrate analysis, current and future efforts lie on the generation of cell lines with lower expression levels. Recent work in the project has already been shifted towards the generation of stable cell lines using Flp-In™-293 cell lines, which allows a fast generation of isogenic stable cell lines; and future work has been envisioned to use CRISPR/Cas9 approaches to introduce a single point mutation in the gene locus of ZDHHC20. This will allow the profiling of ZDHHC20 at endogenous levels and, in the case of the later, under the endogenous promoter. Once these cell lines have been established, it will be interesting to study changes in substrate profiles under different exogenous cues. Furthermore, it would be intriguing to further understand the implications of PTM co-occurrence with other lipid and non-lipid modifications, especially glycosylation with 85% of the detected ZDHHC20 hits having been reported to be modified with sugars.

Overall, sufficient progress has been made towards the validation of the mutant ZDHHC20[Y181G] in combination with 18-Bz probe, as a powerful tool to deconvolute the substrate scope of the wildtype enzyme. This study marks the foundations upon which an even more robust chemical genetic approach can be built. Finally, it provides the field with a novel tool, pushing and encouraging the use for the deconvolution of S-acylation in different disease models.

Chapter 4:

Using proximity labelling to understand the role of ZDHHC20 in trafficking and secretion.

4.1. Introduction.

Analysis of the ZDHHC20 substrates detected via mass spectrometry with the chemical genetic technology (Chapters 2 and 3) had demonstrated an overrepresentation of proteins involved in protein trafficking with origin in the ER and Golgi. To date and to the best of our knowledge, there has been no global analysis on the effects of S-acylation, on cellular protein localisation, trafficking and secretion; and much less so for single ZDHHCs. In parallel, our collaborators in the Downward group had identified that ZDHHC20 could be a potential target for pancreatic ductal adenocarcinoma (PDAC), as it was found to exert a protective effect against tumour cell clearance by natural killer (NK) cells *in vivo*. In mice with an immune system containing NK cells, ZDHHC20 KO cell lines resulted in tumours of reduced size and number, and in mice lacking NK cells the opposite effect was observed (data not shown). Furthermore, high ZDHHC20 levels were found to be an unfavourable prognostic marker in patients with renal and pancreatic cancer (<https://www.proteinatlas.org>)^{191,192}, establishing a relationship to real patient data. In pancreatic cancer, patients with high ZDHHC20 expression presented an average reduction in survival of 1.5 years (Fig. 4.1). Taken together this results indicated a possible involvement of ZDHHC20 in cell-to-cell communication, putatively through the direct or indirect (i.e. through a signalling cascade) action of a ZDHHC20 substrate or collection of substrates. It is known that NK cell recognition of tumour cells occurs via the integration of multiple ligand-receptor interactions and cell-contact independent signals^{193,194}. Tumour cells secrete to the extracellular environment growth factors, cytokines and exosomes which impact NK cell response, whilst NK cell exosomes containing cytolytic molecules lead to tumour cell killing^{134,135}. Hypothetically, the lack of S-acylation could impact the correct trafficking of receptors to the plasma membrane or the secretion of factors to intracellular space, breaking down the defensive line for evading NK cell recognition.

Most relationships between S-acylation and protein trafficking and localisation have been established on an individual basis, by analysing the effects of mutating out single or multiple cysteine residues (i.e. STAT3⁵⁹, p53²⁴, NRAS¹⁹⁶)¹⁷³. Alternative methods are therefore required for a global understanding of the implications of ZDHHC20 in different trafficking pathways or substrate localisation. For this, a proximity-based

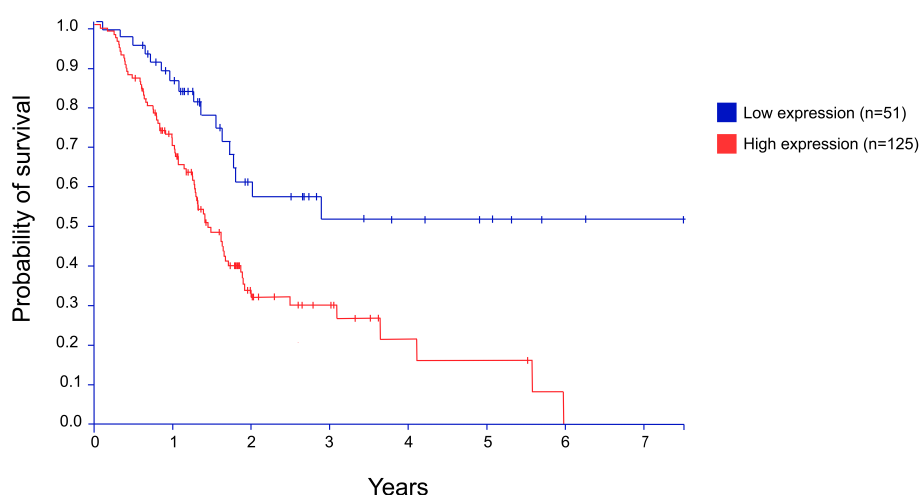


Figure 4.1. ZDHHC20 as an unfavourable prognostic marker for pancreatic cancer patients.

Kaplan-Meier plot, was obtained from The Human Protein Atlas ^{191,192}. It represents the correlation between ZDHHC20 mRNA expression levels and patient survival. Patient groups were divided based on the level of expression. The cut-off for high expressing patients was set at 13.78 FPKM which was the median expression of patients that had survived the disease. X-axis shows survival in years and y-axis shows the probability of survival with 1.0 corresponding to 100%.

labelling strategy was envisioned. Previous work using small peptide sequences as tags to direct biotin ligases to specific compartments had extensively demonstrated their ability to achieve spatial mapping of a protein to cellular organelles including the extracellular space ^{197–202}. Their precision is achieved due to the short spatial life time of the reactive biotin species, a radius of approximately 1-10 nm in living cells, and the ability to isolate modified proteins with enrichment methods for their analysis by mass spectrometry ²⁰³. Alternative biochemical methods based on density centrifugation for subcellular fractionation, suffer from organelle cross-contamination and loss of material, which limits the specificity and depth of the studies compared to proximity labelling ²⁰⁴.

Proximity labelling methods have proven useful in the profiling the proteome of membrane-less organelles, organellar contact spots or specific cellular locations such as the mitochondrial intermembrane space, synaptic clefts or the secreted proteome ^{200,205}. Profiling the secreted proteome has its own specific challenges due to the fact that the quantity of protein of interest is small compared to the amount of serum albumin proteins. Therefore, the direct tagging of secreted proteins for their later isolation on solid resins has already proven to be a successful method.

We proposed that, by mapping proteins to organelles, one could monitor the trafficking of proteins from the ER, through the secretory pathway and into the extracellular space. Moreover, in combination with genetic (KD, KO) methods, this method would allow to identify any faults in trafficking or secretory pathways that could be associated to the phenotype observed with ZDHHC20 KO tumours in mice models. This chapter presents the use of a proximity labelling strategy to identify proteins trafficked from the ER and Golgi to the plasma membrane and/or secreted to the extracellular space. The aim of this project was to apply this strategy to both wildtype and ZDHHC20 KO cells to establish whether this acyltransferase is implicated in protein trafficking and secretion.

4.2. Results and discussion

4.2.1. A proximity labelling strategy to study the role of ZDHHC20 in trafficking and secretion.

Several proximity labelling approaches have been developed to date, the advantages and disadvantages of these were compared in order to determine which would be more suitable for our application (Table 3.1). Soon, the ascorbate peroxidase APEX was found to be unsuitable to monitor the secreted proteome (secretome) due to its short labelling time and the toxicity associated to both phenol and peroxide-containing reagents. Instead, *E. Coli* BirA-derived biotin ligases were found appropriate as they allow for more extensive labelling times without any toxicity complications, which has made them amenable to *in vivo* labelling (i.e. flies, parasites or mice) ^{206–208}. Long labelling times would be needed to accumulate proteins secreted to the extracellular space ^{199,202}. Amongst all, TurboID was chosen as it had been shown to be more active than other BirA-derived ligases over different cellular compartments with signal observed 10 minutes after biotin treatment ¹⁹⁸.

Several constructs had already been validated in the literature with tags (short peptide sequences) which, direct TurboID to compartments such as, the nucleus, the plasma membrane, the ER or the cytosol (Fig. 4.2 A and B) ^{198,209}. In fact, ER-localised TurboID has been used for the *in vivo* profiling of the secreted proteome of

Table 3.1. Advantages and disadvantages of the most common proximity labelling methods.

Ligase	Labelling time	Advantages	Disadvantages
APEX2	< 1 min	High temporal resolution and high cellular activity in most cellular compartments.	Toxicity of H ₂ O ₂ , low permeability of biotin-phenol. Not compatible with <i>in vivo</i> applications
BioID	18h	Only requires biotin treatment, compatible with <i>in vivo</i> applications.	Slow kinetics lead to poor temporal resolution
TurboID	<10 min		Highest activity promiscuous ligase.
miniTurboID	<10 min		Lower catalytic activity than TurboID.

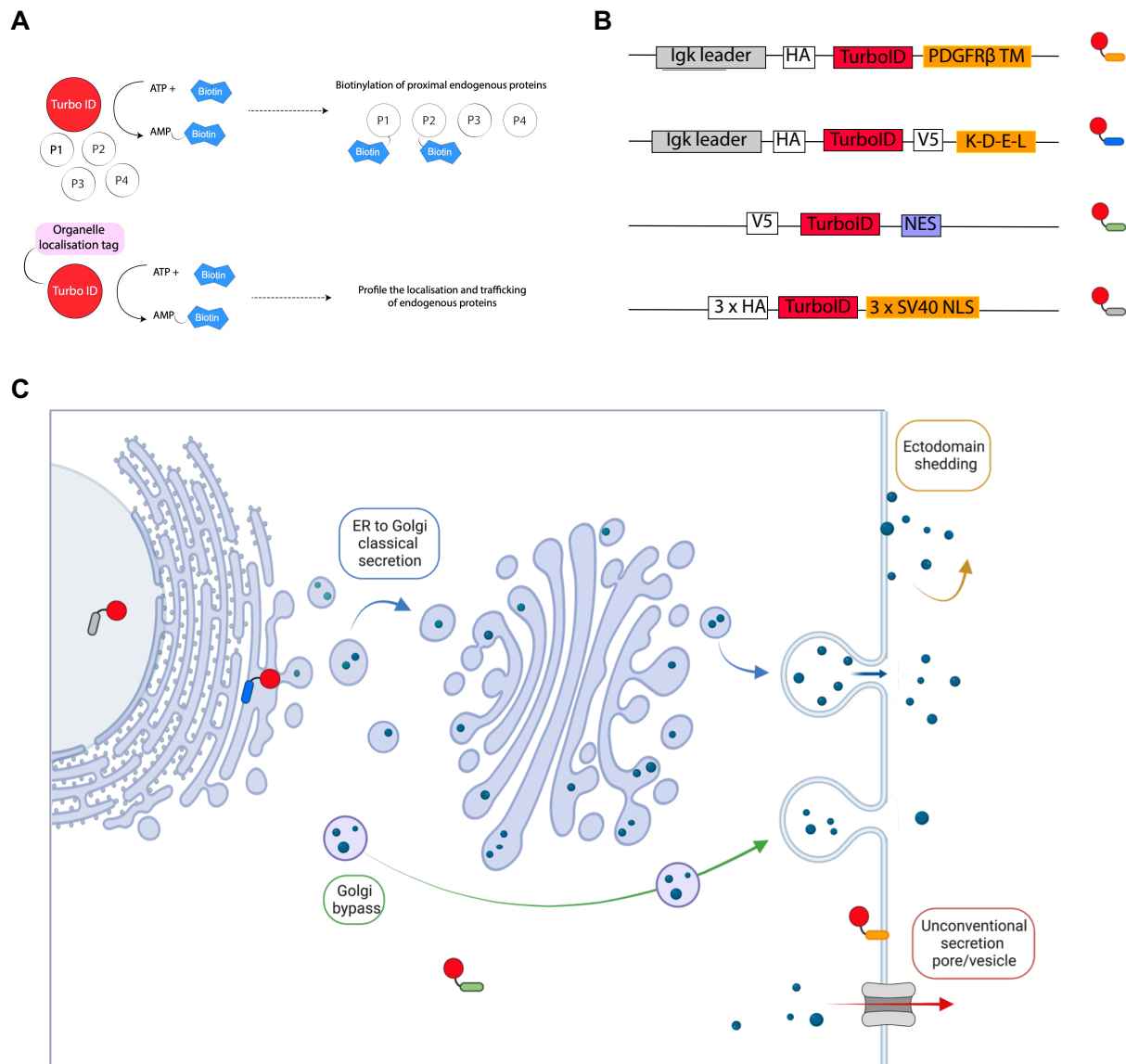


Figure 4.2. Proximity labelling constructs to monitor trafficking and secretion. **A)** Scheme for TurboID tagging to control ligase cellular localisation. **B)** components of constructs. In yellow localisation tags, in red TurboID, in white tags for western blot and immunofluorescence detection and in grey IgK leader sequences for ER internalisation. **C)** Classification of pathways of secretion with TurboID constructs localised to their organelles. Figure created with BioRender.

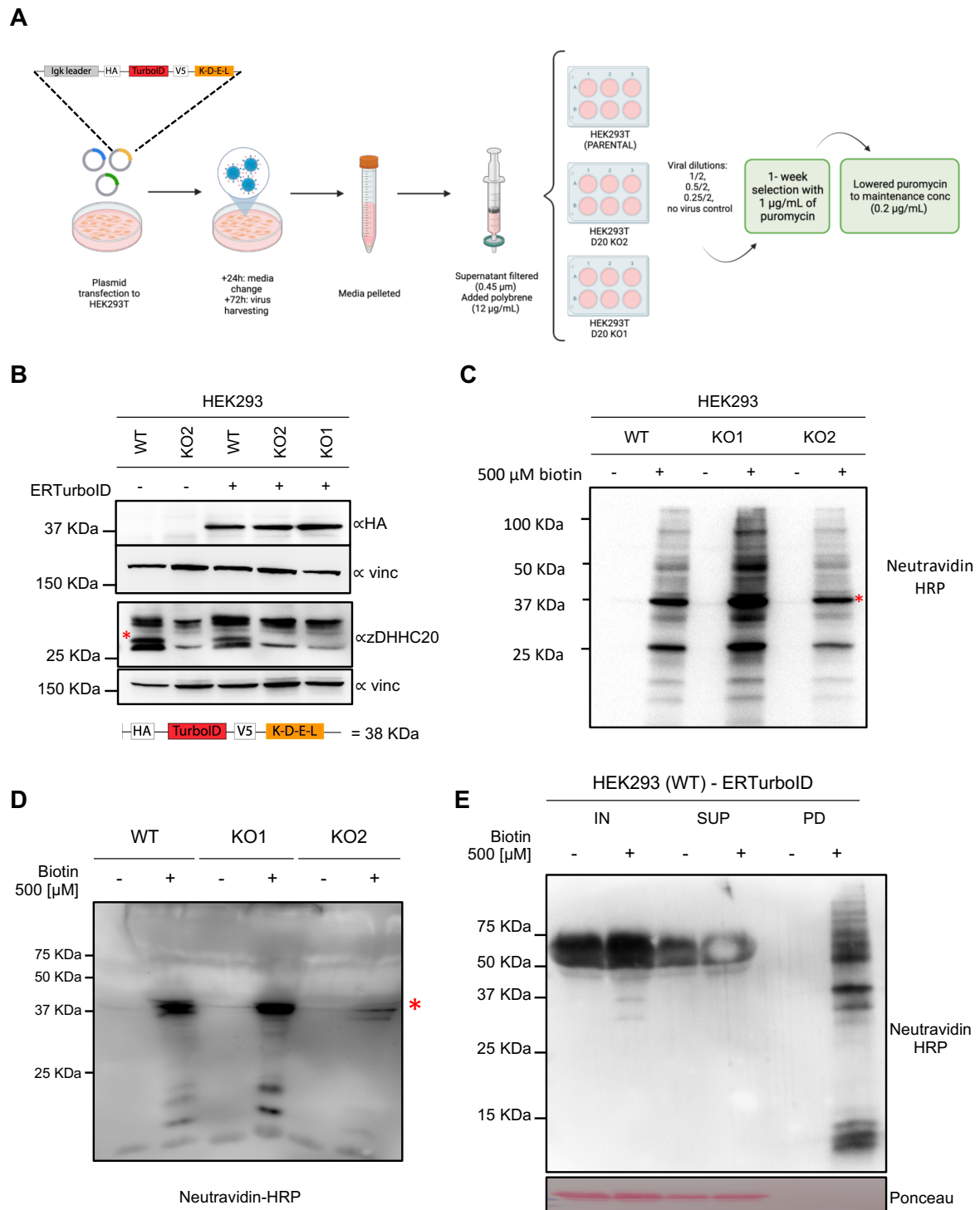
hepatocytes in mice; showing a high degree of sensitivity, capable of detecting lowly abundant proteins²⁰². Consequently, for this work, the initial focus was placed on already-validated TurboID constructs; including control constructs for nuclear localised proteins (nucTurboID), and three others: the cytoplasm (cytoTurboID), ER (ERTurboID) and plasma membrane (PmTurboID), to monitor biotinylation in the respective organelles. In brief, cytoTurboID and nucTurboID contain a C-terminal nuclear export signal (NES) and nuclear localisation signal (NLS) respectively (Fig 4.2 B). The ERTurboID and PmTurboID constructs, both contain an N-terminal IgK

(immunoglobulin kappa) leader sequence, which directs a nascent polypeptide chain from the ribosome to the sec61 translocon for ER internalisation and is cleaved by proteases in the ER lumen ²¹⁰. Once in the ER lumen the ERTurboID construct relies on the C-terminal amino acid sequence, K-D-E-L. This peptide sequence allows the construct to bind to the KDEL receptors which are in charge of retrograde transport from the Golgi apparatus back to the ER, thus ensuring correct localisation of the ligase ^{199,211}. The plasma membrane construct contains a C-terminal sequence corresponding to a transmembrane domain of platelet-derived growth factor receptor beta (*Pdgfrb*); leading to insertion in the lipid bilayer and localisation at the internal face of the plasma membrane ^{199,202,212}. All constructs contained additional peptide tags (V5 or FLAG) for western blot or immunofluorescence analysis. In combination, these constructs allow to monitor classical ER to Golgi secretion as well as unconventional secretory pathways like Golgi bypass or ectodomain shedding (Fig. 4.1 B) ²⁰².

Finally, with regards to the choice of cell line it was decided to continue working with HEK293 due to the ability of cross-referencing the ZDHHC20 chemical genetic hits in this cell line. Furthermore, due to the *in vivo* observations with ZDHHC20 KO we decided to include the two HEK293 ZDHHC20 KO cell lines presented in Chapter 3, Despite no major differences were observed in the S-acylated proteome between WT and KO cell lines, organelle-based fractionation could aid spectral deconvolution, revealing more subtle changes between cell lines.

4.2.2. Establishing ER-TurboID HEK293 cell lines.

The TurboID constructs were already extensively validated in previous publications, enabling us to proceed directly to utilising them. Therefore, all the TurboID constructs – a gift from Alice Ting’s laboratory – were amplified by PCR (see Appendix Table 8.2 for a full list of primers) and inserted by Gibson assembly into a third-generation lentiviral vector for constitutive stable expression. The plasmid sequences were confirmed by sanger sequencing and a diagnostic restriction digest. Cloning into a third generation lentiviral pBABE-puro expression vector (Appendix Fig. 8.9) was attempted but this resulted primarily in vector recombination. Furthermore, doxycycline inducible expression vectors were considered but such level of control



4.3. ERTurboID cell line generation and validation. A) Scheme for the production of lentiviral cell lines HEK293 cell lines. Figure created with BioRender. **B)** Western blot confirmation of integration and expression of ERTurboID in HEK293 wildtype and ZDHHC20 KO1 and KO2. **C)** Neutravidin-HRP western blot signal of ERTurboID cell lines treated with 500 µM biotin for 16h. **D)** ERTurboID media (10 µL) of samples in C. Red asterisk red star corresponds to ER-TurboID construct (~38KDa) which leaks into the media and is self-biotinylated. Red asterisk corresponds to signal at molecular weight of ERTurboID. **E)** Pulldown analysis of 1mL of media of ERTurboID wildtype HEK293 cells treated with 500 µM of biotin for 16 h.

over construct expression and the consequent fine-tuning of expression across cell lines, was thought unnecessary. This decision was supported by numerous reports stating the lack of toxicity of the ligase seen even in the presence of biotin and *in vivo*, which also supported a stable construct expression. A pJLM1 second generation lentiviral vector for constitutive expression (Appendix Fig. 8.9) was chosen and all TurboID constructs were constructed by Gibson assembly.

With the lentiviral vectors in hand, the initial focus was centred around the ER-TurboID construct which, had been reported to capture most trafficking and secretory pathways and to yield the broadest coverage of the secretome, *in vivo*^{199,202}. Therefore, HEK293FT cells were transfected with pLJM1-ERTurboID, envelope and packaging plasmids, producing lentivirus for the transduction of HEK293s and the two ZDHHC20 KO HEK293 cell lines (Fig. 4.3 A). As both KO cell lines had been originally single-cell cloned, after puromycin selection ERTurboID populations were maintained and not subject to a second round of selection. Consequently, avoiding a result bias due to measuring clonal-specific effects unrelated to the KO of ZDHHC20 but linked to a specific clone of a clone. After a selection period of one week, cell lines were maintained at a lower concentration of puromycin (0.2 µg/mL) to exert a selective pressure on the population and prevent fluctuations of construct expression (i.e. due to epigenetic regulation).

Once the cell lines had been established, ERTurboID expression and intracellular ligase activity were confirmed via western blot (Fig. 4.3 B and C). Intracellularly, a band corresponding to auto-biotinylation of ERTurboID was detected at approximately 38 KDa, along with multiple bands across the whole mass-range indicating biotin incorporation into a diverse range of proteins. After a 16h biotin treatment, the media was collected and after a 10-fold concentration it was analysed via western blot with bands confirming the presence of biotinylated proteins in the secretome (Fig. 4.3 D). One of the bands at 38 KDa is thought to correspond to ERTurboID secretion. A recent publication has demonstrated that the KDEL peptide signal is insufficient to retain the construct in the ER¹⁹⁹. In this publication the authors demonstrate that a transmembrane tag of the sec61b translocon retains TurboID intracellularly to a greater extent. However, secretion of TurboID could serve as both a positive control during proteomic analysis and aid data normalisation.

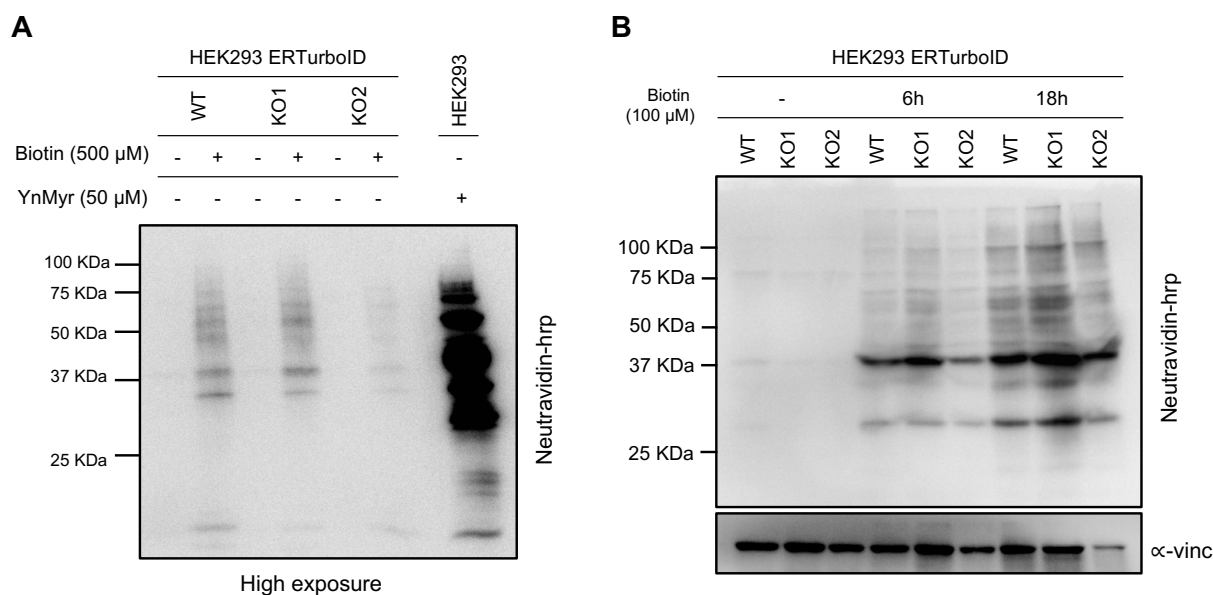


Figure 4.4. Assessment of the biotinylation signal from lysates and media. A) Comparison of the pulldown signal from 1 mL of media of ERTurboID cell lines (500 μ M biotin for 16h) with that of a HEK293 lysate (15 μ g at 1 mg/mL) of cells treated with 50 μ M YnMyr for 16h and clicked to biotin-azide. After pixel densitometry analysis with ImageJ, signal from WT and KO1 correspond to approximately 15% of YnMyr signal and KO2 to 8%. Image showed is a highly exposed image, as lower exposures failed to show lower signals. **B)** Biotinylation signal over time, HEK293 ERTurboID cell lines treated for 0, 6h and 16h with biotin (100 μ M) shows accumulation of signal over the maximum time point measured.

A foreseen challenge for proteomics sample processing was excess biotin in the media. Excess biotin would compete for binding to the neutravidin resin during enrichment of biotinylated proteins. To prevent this, the media was exchanged with PBS containing 0.2% (3 x media sample volume), using spin-columns with 5 KDa cut-off filters. This step aimed to simultaneously remove unreacted biotin, and concentrate the sample volume prior to isolation on neutravidin resins. A small-scale test using 3 mL of media confirmed immunoprecipitation of biotinylated proteins with neutravidin magnetic beads and revealed that biotinylation modified proteins over a wide mass range suggesting biotinylation and secretion of a wide scope of proteins (Fig. 4.3 E). The final challenge were serum albumin proteins, contained in high amounts in media, as they could stick to the beads and their peptides hinder the detection of lower expressed signals. Analysis of the PBS buffer (0.2% SDS) used for washing beads, showed minimal presence of BSA (Appendix Fig. 8.10).

Next, in order to quantify how much signal was obtained from 1 mL of media, pulldown signals were compared to that of a control YnMyr treated sample (15 μ g), clicked to biotin-azide. Analysis of pixel density of western blot images revealed that pulldown

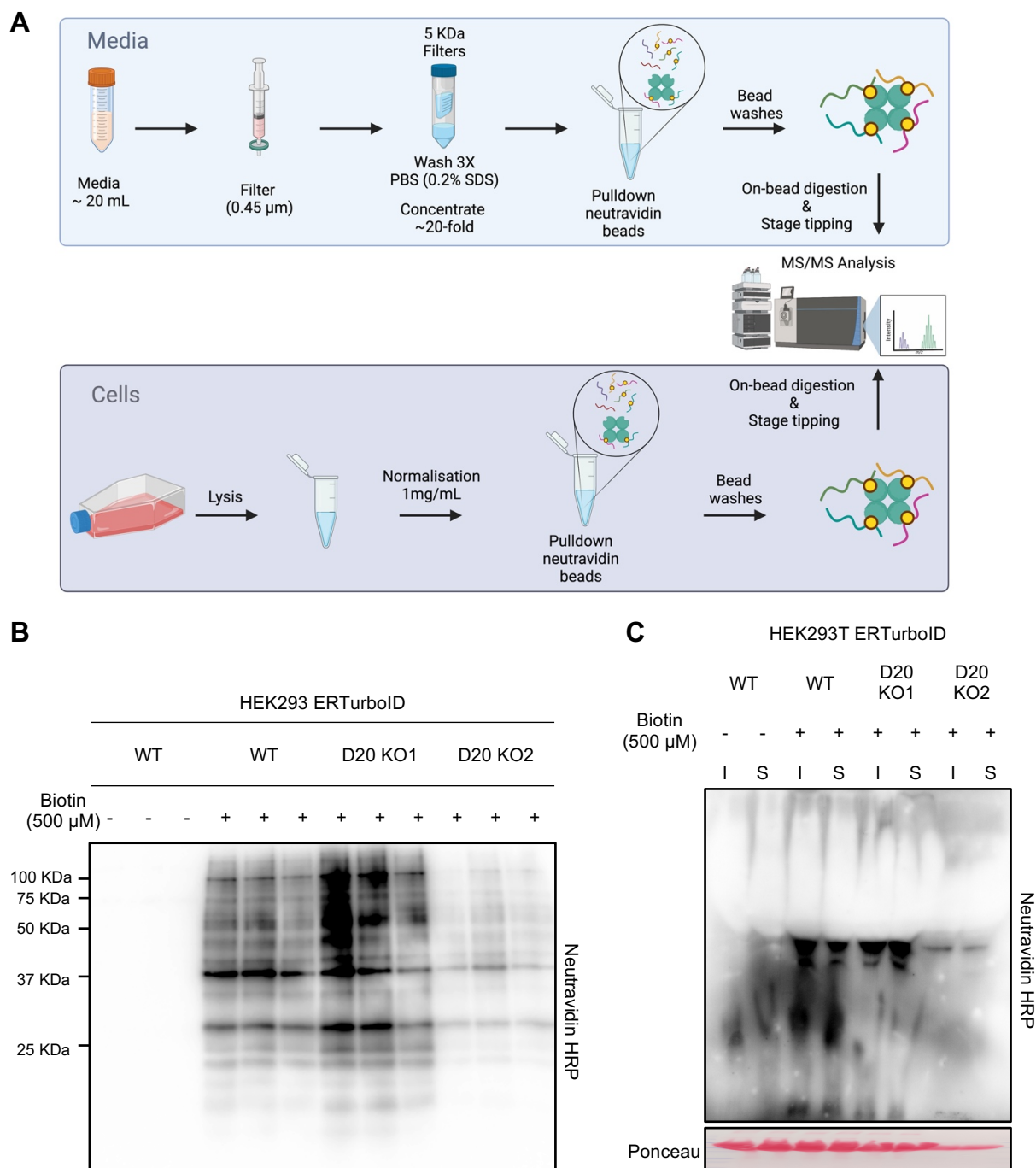
signals were 10-15 % that of the control YnMyr (Fig. 4.4 A). Therefore, it was approximated that 10-20 mL of media would be needed to obtain sufficient signal for mass spectrometry profiling of the secretome, with the additional challenge that further increasing the amount of media increases the total amount of BSA. Finally, in preparation for a proteomics experiment, the time-dependence of the signal was explored in both cells and media treated with 100 μ M biotin – to prevent signal saturation in earlier time points. Over a time-course of 16h the signal from lysates increased without apparent signal saturation (Fig. 4.4.B and Appendix Fig. 8.10). In the case of the secreted proteins, the biotinylation signal remained below the limit of detection in western blot chemiluminescence with our instrument, up to the 16 h time point (data not shown).

Construct localisation was checked by confocal microscopy in cardiac mesenchymal cells by Zuzanna Jablonska – a PhD student in the Tate group. Immunofluorescence analysis, showed localisation of the ER-Turbo construct in the perinuclear region, co-localising with calnexin – an integral ER protein used as marker for this organelle (Appendix Fig. 8.11). Overall, these results proved in our hands ER-TurboID activity the ability of this construct to biotinylate secreted proteins and allowed to proceed towards the proteomic analysis of the cells lines.

4.2.3. Proteomic profiling of ER-TurboID cell lines

Previous results were used to establish an initial set of conditions for proteomics cell line analysis, which would provide adequate signal of biotinylated proteins for both cells and the secretome (Fig 4.3 and Fig 4.4). Secreted proteins and intracellular biotinylation would be processed separately. Nevertheless, for both, pulldown would be performed with di-methylated neutravidin beads and after on-bead digestion, peptides would be de-salted and analysed by nanoLC-MS/MS (Fig.4.5. A), as described in section 3.2.1.

The ERTurboID HEK293 wildtype and the two ZDHHC20 KOs were seeded in triplicate. An additional set of control samples corresponding to ERTurboID HEK293, without biotin were included as controls. The latter would account for background labelling for all samples, with the assumption that this signal for the two KO cell lines would be comparable to that of the WT ones and independent from ZDHHC20



expression. Samples were cultured in T175 flasks with 25 mL of media and after treatment with biotin (500 µM, 16 h), media and lysates were frozen for later processing. Prior to mass spectrometry processing, the presence of biotinylated

Figure 4.5. Mass spectrometric processing of samples from ERTurboID cells. **A)** Sample-processing scheme for mass spectrometry analysis of secreted (top) and in-cell or lysates (bottom) of ERTurboID biotinylated proteins. Figure created with BioRender. **B)** HEK293 ERTurboID lysates of cells treated with 500 µM biotin for 16h and pulled-down with neutravidin dimethylated resin. Signal corresponds to approximate 5% of all beads, boiled with 2X Laemmli buffer, remaining beads were processed following scheme in A. **C)** Media signal of samples in B. Input (I) and supernatant (S) of

media pulldown with dimethylated neutravidin agarose beads. No difference in input and supernatant suggests conditions for pulldown are suboptimal.

proteins was confirmed before and after neutravidin pulldown via western blot (Fig. 4.5 A). In the case of ZDHHC20 KO2 a slight decrease in biotinylation signal was observed (Fig. 4.5.A), confirming loss of construct expression over passages in accordance with previous observations (Fig. 4.4 A). This indicated further deviation in construct expression within the population, despite selective pressure being exerted with a low concentration of puromycin. This clone was maintained for further analysis as normalisation strategies would help circumvent signal differences across replicates.

4.2.3.1. Analysis of intracellular biotinylation signal in ER-TurboID cell lines.

Samples from cell lysates were processed as described in the previous section and analysed by nano-LCMS/MS, and raw data was searched using the MaxQuant software. Final data analysis was done using the Perseus software in a similar way to that described in Chapter 3. Briefly, all contaminants were filtered, raw intensities were $\log_2$ transformed and normalised firstly, to the sample mean, and secondly against ERTurboID intensities. Technical replicates were grouped together and only protein groups with at least two valid values (2/3) were kept for further analysis, and missing values were then imputed following a normal distribution at the limit of detection. Those protein groups remaining in the dataset were subject to different two-sample student t-tests ($S_0=0.1$, FDR=0.05), to compare all sample groups.

Subsequent analysis was initially focused on the comparison of biotinylation activity between WT and DMSO treated ERTurboID lysates, where a total of 370 protein groups were identified. Firstly, a two-sample student t-test was performed on ERTurboID HEK293s with and without biotin treatment. Here, a clear enrichment was detected upon biotin treatment (Fig. 4.6 A), with ERTurboID (BirA) detected amongst the enriched proteins and acting as a positive control. More interestingly, a high number of those enriched hits, 42%, are predicted to be S-acylated in accordance with reports suggesting that this PTM is added to proteins in the ER and Golgi where most acyltransferases are found ⁴². Of these, it is worth highlighting three: BCAP31, IKBIP and CKAP4, which were found to be substrates of ZDHHC20 with the chemical genetic

system presented in Chapter 3. All three are transmembrane proteins which have been found to co-localize in the ER, which depicts construct localisation in the ER.

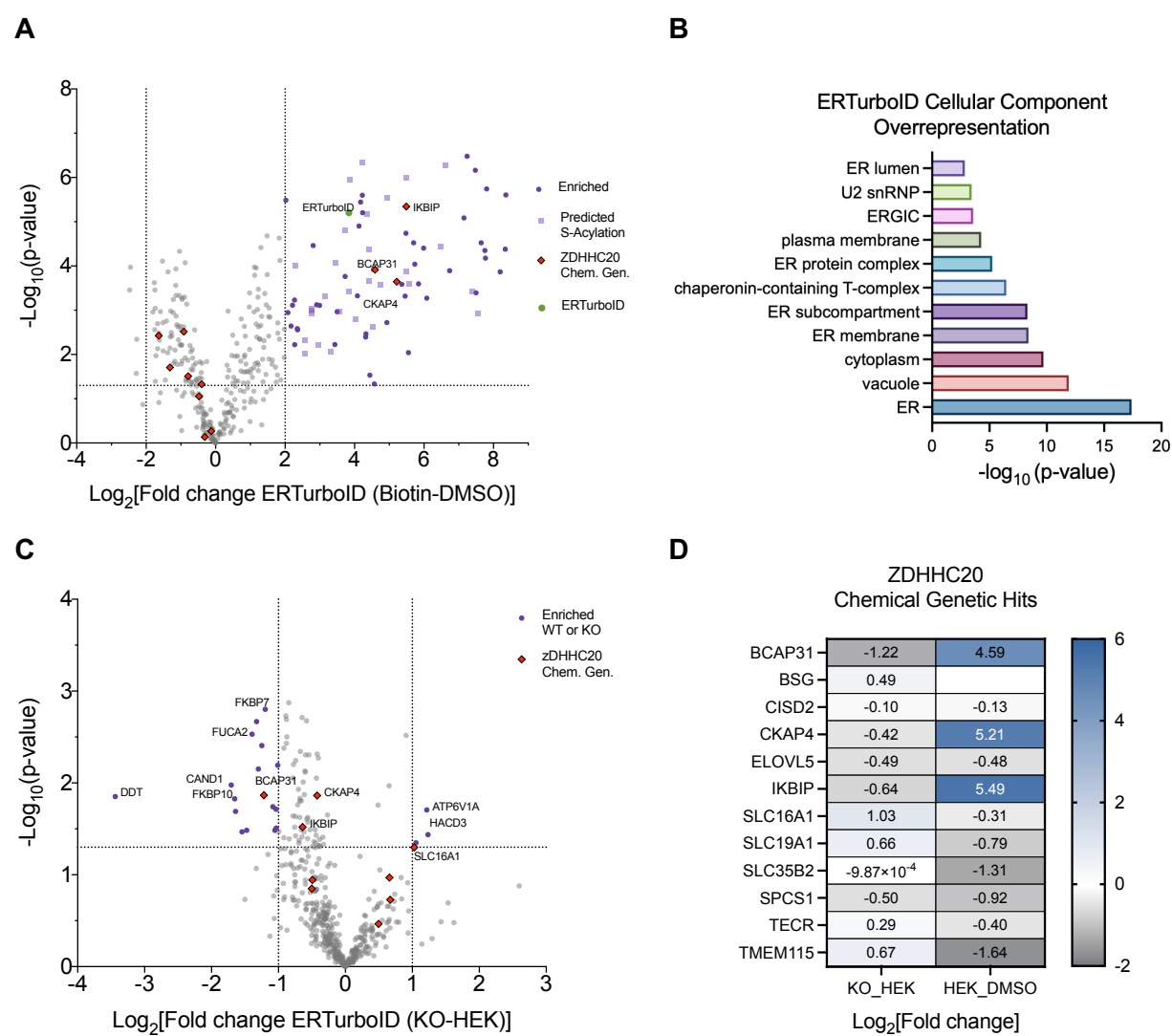


Figure 4.6. Proteomic analysis of cellular signal from ERTurboID cell lines. A) Volcano plot of HEK lysates of Figure 4.4.B. Comparison of DMSO and biotin treated samples of the cell line ZDH20 WT ERTurboID. In red ZDHHC20 proteomics chemical genetics hits, in light purple hits predicted to be S-acylated. **B)** Cellular component overrepresentation analysis of enriched proteins of A. Analysis was performed using the PANTHER online tool with statistical analysis and p-values were determined using the Fisher's Exact test. Only top eight enriched GO terms (p-values <0.05) are shown. **C)** Volcano plot comparing and against all KO samples, n of 3 of KO1 and KO2. In red ZDHHC20 proteomics chemical genetics hits of the chemical genetics and in purple those overall enriched or depleted in the KOs. **D)** Hit map comparing the log₂ (fold changes) for the chemical genetic hits depicted in figures A and Cm the colour scale was adjusted to the maximal and minimal values.

Next, all wildtype enriched substrates were subject to cellular component overrepresentation analysis (Fig. 4.6 B) against the whole human proteome, as previously described in section 3.2.2.2. This demonstrated the enrichment of ER-associated proteins (lumen, membrane, ERGIC or ER-protein complex) and was

found indicative of construct localisation in ER and preferred biotinylation of ER-associated proteins. Furthermore, the enrichment of proteins annotated as being localised at the plasma membrane and vacuoles, which are likely trafficked through the ER further validates the system. Interestingly, the chaperonin-containing T-complex and the U2 snRNP were also identified, which might still be the result of biotinylation in the ER, post-synthesis and folding, prior to their trafficking to the nucleus or diffusion into the cytosol. Overall, 51% of proteins detected were associated to ER structures and 34% to the plasma membrane.

The following analysis on this dataset was a comparison between biotin treated conditions. After a student t-test as previously described fold-changes and p-values were plotted to assess statistical and biological significance thresholds to determine enriched and downregulated substrates (Fig. 4.6 C). In this instance, the fold-changes were found to be much subtler – an indication of comparable ERTurboID biotinylation levels for both cell lines and overlap of biotinylation activity between WT and KO cells. Therefore, biological significance cut-offs were moved to a less stringent fold change of 2 ($\log_2 2=1$), with statistical significance kept at p-value <0.05. As a result, 17 proteins were found to be downregulated in the ZDHHC20 KO cell lines and four upregulated. More interestingly, those three proteins highlighted earlier, also detected via chemical genetics: BCAP31, IKBIP and CKAP4, were found to be downregulated in the KO samples (Fig.4.6.D). BCAP31 is an abundant ER protein which acts as a chaperone for misfolded proteins prior to their degradation¹⁸⁷; and CKAP4 has been found to participate in the anchoring of the endoplasmic reticulum to microtubules²¹³. Finally and more relevant to the ZDHHC20 KO phenotype *in vivo*, IKBIP a proapoptotic gene²¹⁴, was found to be amongst a list of genes upregulated in myelogenous leukaemia lymphoblasts upon treatment with human NK cell extracellular vesicles¹⁹⁵.

Assuming ERTurboID ligase activity is independent of ZDHHC20 expression, the downregulation of proteins is then dependent of protein concentration in the ER. If these results proven to be reproducible, it would indicate that loss of ZDHHC20 S-acylation leads to reduced levels of substrates in the ER either due to mislocalization or instability and a consequent degradation. As no differences were observed between WT and KO cell lines with YnPalm treatment (Fig. 3.11), it could well be that these subtle changes were buried amongst the signal from highly S-acylated proteins. Furthermore, enrichment and downregulation of ZDHHC20 substrates shows that

organelle-based fractionation using ER-localised TurboID could be complementary in detecting changes resulting from the KO of a ZDHHC compared to whole proteome S-acylation studies. Unfortunately, further replicates are needed to validate these results.

4.2.3.2. Analysis of secretome of ER-TurboID cell lines

To profile the secretome, the media was filtered, to remove cell debris, and on spin columns (cut-off 5 KDa) excess biotin was washed and samples were concentrated. Due to the high concentration of protein in the concentrated media, the beads were thoroughly washed four times with 0.2% SDS in PBS. and another four without detergent in preparation for on-bead digestion (OBD).

A flaw in the processing of the secreted proteome had already been identified on the western blot pulldown checks, which had shown most biotinylation signal had not been removed from the supernatant. Consequent raw data searches and data analysis revealed 60 proteins had been isolated (Appendix Table 8.9). Of the proteins identified, 75% had been annotated to be localised in exosomes or secreted to the extracellular matrix (Appendix Table 8.9), including ERTurboID. However, a very high number of missing values, with proteins often detected in only one replicate, and overall low intensity signals, made it impossible to perform any type of statistical data analysis. As a first attempt, the data and strategy was considered promising despite the protocol being sub-optimal and requiring further optimisation. At this stage, two reasons were thought to be behind experimental failure:

- 1) Residual biotin present in the media, which had not been efficiently removed with the 5 KDa cut-off filters, was hampering biotinylated protein isolation.
- 2) The sheer abundance of BSA was preventing the successful pull-down of proteins through off-target binding to the solid resin.

It is believed that after optimisation of the pulldown conditions, proximity labelling could well be a powerful method to isolate the secreted proteome and assess any potential differences that may result from the KO of ZDHHC20. Unfortunately, this project was paused due to the time constraints associated to the end of this PhD.

4.3. Summary and future work

This chapter presents a proximity-based labelling strategy which could help elucidate the cellular mechanisms that rely on ZDHHC20, in both healthy and diseased states. In HEK293 ZDHHC20 KO cells a decrease in biotinylation levels of three ER-localised proteins, which were also found to be ZDHHC20 substrates using chemical genetics was detected. Putatively, this indicates a relationship between this acyltransferase and protein localisation. However, the results are preliminary and further research is needed for their corroboration in the case of intracellular signal, and further optimization of the protocol with regards to the secretome isolation. At the sample processing level, current and future efforts lie in finding an optimal solvent-based precipitation method to remove excess biotin from the media, improving the pulldown and mass spectrometry identification of secreted proteins. Addition of a glycan removal step with a glycosylase, may also facilitate the later enrichment of biotinylated proteins both in the secretome and intracellularly. Furthermore, it will be interesting to profile other compartments, using the other TurboID tagged constructs, to assess whether ZDHHC20 KO leads to a mislocalization of substrates or proteins to another cellular organelle (i.e. from the ER to the plasma membrane).

The secretome is important not only for the basic understanding of human biology but also for the identification of potential targets for future diagnostics and therapies. To date, pancreatic cancer remains difficult to diagnose with an on average 5-year patient survival rate of 11% ²¹⁵. Therefore, understanding how ZDHHC20 protects tumour cells from NK cell killing, could lead to novel diagnostic tools or therapies, and overall improvement of patient survival.

Chapter 5:

General Conclusions and Future Perspectives

5.1. Thesis conclusions.

S-acylation is a key PTM involved in a myriad of cellular processes (see section 1.3.1) Over the past decade, progress in the S-acylation field has been hampered by the lack of novel tools. This work, presents the development and application of novel tools for studying ZDHHC20 isoenzyme dependent biology. The findings and conclusions presented throughout this thesis are summarized hereunder.

Firstly, it can be concluded that ZDHHC20 and probably the rest of the ZDHHC enzymes in the family are amenable to analogue sensitive mutations – necessary to implement a chemical genetic strategy – within the lipid-binding cavity. For the best mutant found, ZDHHC20[Y181G], it is believed that the interference with substrate binding is minimal as Tyr181 resides in the deep end of the lipid binding cavity. Putatively far away from any binding domains, although further work is needed to fully validate the extent of the structural changes that the mutation causes. Furthermore, data presented in Chapter 2 demonstrates that this mutation allows the uptake of lipid probes, whilst preserving enzymatic activity in vitro and in metabolic labelling experiments. Secondly, a key milestone behind the success of this technology is the design of the lipid probes, which are metabolically added across the S-acylated proteome. Their orthogonality against wildtype ZDHHC20 is probably achieved by a combination the alkyne and the steric filter. Specifically, the probe 18-Bz was found to present the highest degree of orthogonality to wildtype ZDHHC20 in cells without any cellular toxicity. A major achievement for these probes is their metabolism into very few lipid classes, whilst still being converted to their CoA adducts which, is necessary to preserve their binding affinity to ZDHHCs. Thirdly, and very encouragingly, the orthogonality window obtained throughout the technology optimization phase, was propagated to substrate transfer in cells. This was one of the major achievements of the project, it demonstrated that substrate identification for ZDHHCs using chemical genetics is possible. In combination with mass spectrometry, chemical genetics allowed to shortlist 90 proteins as substrates of ZDHHC20 and depicted a strong involvement of this enzyme in protein trafficking, localisation and secretion.

Finally, preliminary results strongly suggest that organelle profiling using proximity labelling, can allow to detect changes in protein localisation caused by the KO of

ZDHHC20. The hypothesis is that fractionation reduces the complexity of mass spectrometry data, allowing to detect smaller changes. Despite further optimization is needed, this approach has been applied for secretome profiling and in combination with gene-editing methods (KO or KD) could permit to profile the relationship between ZDHHCs and secretion at a cellular level.

Overall, the work presented throughout this thesis demonstrates that novel and established approaches can be powerful tools to understand isoenzyme-specific ZDHHC biology. Prior to this research, chemical genetics had proven a powerful approach to deconvolute isoenzyme-substrate selectivity in protein families with high structural similarity and without selective small molecule modulators. From this work, it can be concluded that chemical genetic systems could help understand biological processes associated to individual ZDHHCs.

5.2. Future perspectives.

The application of chemical genetics to understand the substrate scope of ZDHHCs is still a novel technology in the field. Immediate efforts on this line of research should be initially focused on technology validation in lower enzyme-expressing systems. Also, the design of chemical genetic systems for the remaining members of the ZDHHC family – project which has already started in the Tate group – could further elucidate the factor which govern substrate selectivity in the family. Investigations in other cell lines should also bring valuable insights on the essentiality functions of these enzymes across different tissues. Furthermore, in the future, this approach has the power to enable cell-specific biorthogonal tagging, to study these enzymes in co-culture models and understand how S-acylation is impacted by changes in the microenvironment or exogenous factors. Another, question remains unanswered in the field is the interdependence of lipid and non-lipid PTMs. Novel tools, can help study the unexplored level of complexity behind PTM crosstalk and substrate profiles across the cell. Finally, after the publishing of the first high-throughput screens against ZDHHCs, more results derived from these efforts will likely be reported in the future. These will probably bring novel small molecule modulators of ZDHHCs

Overall, this progress will be fueled by the need to understand essential biological questions linked to S-acylation to contextualize the lipid modification in different

biological settings. In turn, this could unravel how atypical substrate S-acylation events occur in disease contexts, giving insights into druggable vulnerabilities in S-acylation, thus creating a therapeutic interest that could lead into novel drug-discovery programs.

Chapter 6:

Materials and Methods

6.1. General procedures

Cell culture and compound preparation. HEK293T, and HEK293FT cells were grown in Dulbecco's Modified Eagle Medium supplemented with GlutaMAX™ and 10% v/v FBS; at 37°C and 5% CO₂. All cell lines were provided by the Francis Crick Cell Service facility where their short-tandem repeat (STR) profile was validated. Cell stocks were thawed by incubation at 37°C, transferred to 15 mL falcon tubes containing 10 mL of fresh media, spun (5 min at 200 x g) to remove residual DMSO and pipetted back to an appropriate-sized flask. Cells were cultured for up to 15 passages, after which they were disposed. Trypsin-EDTA 0.25% (Gibco; 25200056) was used for splitting and passaging. Frozen cell stocks were prepared from low-passaged cells; after trypsinisation cells were dissolved in FBS (10% DMSO) and put in a Mr. Frosty™ overnight at -80°C and stocks were transferred to a -150°C freezer for long term storage.

Stock solutions of synthetic transfer (or inhibitory) probes and alkyne palmitic acid were aliquoted and dissolved in DMSO and stored at -20°C. For cell line generation a puromycin dihydrochloride (Life Technologies) stock solution (10 mg/mL) was prepared with sterile Mili-Q H₂O, aliquoted and stored at -20°C.

General transfection procedure for transient construct expression. All transfections were performed from the same trypsinised cell suspension and reagent mixture to ensure equal distribution of transfection materials across experimental replicates. Briefly, the transfection mixture was prepared by sequential addition of plasmid DNA (1 µg per 1x10⁶ cells), OPTI-MEM (100 µL per 1x10⁶ cells) and FuGENE® (PROMEGA) reagent (3 µl per 1 µg DNA), and incubated at room temperature for 15-20 min. Meanwhile, cells were trypsinised, counted and the total number of cells was added to the transfection mixture. Fresh media was added so that the concentration of cells was 1x10⁶ cells per mL.

Copper catalysed azide-alkyne cycloaddition (CuAAC) or click reaction. Protein concentration from metabolically labelled lysates was measured using a BCA protein assay (DC Reagent Assay; Bio-Rad). All samples were normalised to a concentration of 1-2 mg/mL. A click mix was prepared as described previously²¹⁶ by sequential mix of 10mM of capture reagent (AzT, AzB), 50mM of CuSO₄, 50 mM tris(2-carboxyethyl)phosphine (TCEP) and 10 mM tris(benzyltriazolylmethyl)amine (TBTA)

in a 1:2:2:1 ratio. Per 100 μ L of lysate, 6 μ L of click mix were added. The reaction was allowed to proceed at room temperature for 1 hour after which the reaction was quenched through the addition of 5 mM EDTA (from a 100x stock), and excess reagents were removed with a MeOH:CHCl₃ precipitation.

Methanol-Chloroform precipitation. To protein lysates, 1 sample volume of methanol and 0.25 volumes of chloroform were added and after mixing samples were centrifuged for 5 min 4000 x g. The solvent mixture was separated in two phases with the proteins pelleted in a disc at the biphasic interphase. The top aqueous-methanol layer was carefully discarded, one sample volume of methanol was added and the sample was sonicated and spun at 7500 x g for 5 mins at 4°C. After two additional methanol washing steps (500 μ L), the samples are centrifuged (7500 x g for 5 mins at 4°C) to re-forming the pellet. After the final removal of methanol, the pellet was allowed to air dry before resuspension in 100 μ L of 2% SDS in a buffer of choice, then diluted to 0.2% for subsequent processing.

SDS-PAGE and Western Blot.

Normalised lysates were diluted with 4X Laemmli loading buffer (Bio-Rad) containing β -mercaptoethanol (1/10 in 4X buffer) and boiled for 5 mins at 95°C. Affinity-resin eluates were boiled in 2X Laemmli loading buffer and loaded directly onto polyacrylamide gels.

Approximately 10 μ g per sample were loaded per well on polyacrylamide gels (10-12%) and proteins were separated by gel electrophoresis with a TRIS glycine SDS buffer (BIORAD). Gels were transferred onto PVDF or nitrocellulose membranes using the Trans-Blot Turbo System (Bio-Rad) with settings: 2.5A, 25V, 7 min. After transfer, membranes were stained with Ponceau S solution (Sigma Aldrich) – to check for general loading and transfer efficiency –, imaged and washed with TBST (2 x 10 min). Membrane blocking was performed for 1h in TBST containing 5% semi skimmed milk or 3% bovine serum albumin (BSA). Afterwards, membranes were incubated with the corresponding primary antibody (see Chapter 6 for antibody information) in blocking solution overnight at 4°C. Membranes were then washed with TBST (3 x 10 mins) before incubating with secondary HRP or fluorophore conjugated antibody for 1h at room temperature. Membranes were then washed again with TBST (3 x 10 mins) before developing using the Luminata Crescendo Western HRP substrate (Milipore) and

imaged using an Amersham Imager 680, ImageQuant LAS 4000 scanner or fluorescent detection using a LICOR Odyssey CLx. Protein band intensity was quantified by pixel densitometry analysis using ImageJ 1.50c and data was plotted using Prism 9.0.

Mouse-derived monoclonal antibodies for FLAG[®] M2 (F1804), HA-epitope (HA-7) and α -tubulin (T5168) and rabbit-derived polyclonal antibodies for ZDHHC20 (Atlas Antibodies, HPA014702), BCAP31 (Atlas Antibodies, HPA003906) and V5-epitope were purchased from Sigma-Aldrich. Mouse monoclonal anti-GFP (GF28R) antibody was purchased from Genexon LTD and rabbit-derived polyclonal antibodies against vinculin (42H89L44) and calnexin (ab22595) were purchased from Thermo Fisher Scientific and Abcam, respectively. Secondary antibodies were HRP-conjugated, polyclonal goat-derived antibodies against mouse and rabbit (Dako, Agilent Technologies) and IRDye 800CW goat anti-mouse (ab216772). Western blot analysis was accomplished through SDS-PAGE of cell lysates or affinity-resin eluates in 1X Laemmli loading-buffer (Bio-Rad) containing β -mercaptoethanol (1/20 in 4X buffer) and transfer of protein onto PVDF or nitrocellulose using the Trans-Blot Turbo System (Bio-Rad). Secondary HRP-conjugates were visualized after addition of ECL Prime Western Blotting Substrate and chemiluminescent detection in an Amersham Imager 680 or fluorescence detection using a LICOR Odyssey CLx. Quantitation of western blot protein intensity was performed by densitometry using ImageJ 1.50c or Image Studio Lite and data were plotted using Prism 9.0.

In-gel fluorescence. Lysates treated with lipid probes were normalised, clicked to AzT and loaded into an SDS-PAGE gel, run at 160V for 1h and imaged using a Typhoon FLA 9500 imager with excitation and emission wavelengths of 532/575 nm. Autoacylation signals were quantified by pixel densitometry analysis using ImageJ 1.50c and data was plotted using Prism 9.0.

6.2. Molecular Biology and Cloning.

Plasmids and subcloning

Human C-terminally Myc-FLAG-tagged ZDHHC20 (C-FLAG-D20) was purchased from Origene Technologies, catalogue number MR205665. Plasmids containing point mutants of ZDHHC20, including the catalytic dead C156S and the 'hole' mutants were

obtained by mutagenesis of C-FLAG-D20 described below. The C-FLAG-pcDNA3 'empty' expression vector (Addgene 20011) was used as a negative control for FLAG-tagged ZDHHC expression. pCMV-HA-hIFITM3 (Addgene 58397) was used for overexpression experiments. For the generation of stable cell lines, empty vectors pLVX-TetOne-Puro (Clontech), pLJM1 (Addgene 91980) and pBABE-puro (Addgene 1764) were used as starting points for the subcloning of delivery lentiviral vectors. Proximity labelling TurboID plasmids, 3xHA-TurboID-NLS_pCDNA3 (Addgene 107171), pAAV-TBG-ER-TurboID (Addgene 149415), pAAV-TBG-Cyto-TurboID (Addgene 149414), pAAV-TBG-mem-TurboID (Addgene 149413) were obtained from addgene and used for the subcloning of the protein constructs into lentiviral vectors.

All constructs were built using the NEBuilder HiFi Assembly Kit, with linearized vectors and PCR fragments produced with the Q5® Hot Start High-Fidelity 2X Master Mix and the appropriate primer sets in Appendix Table 8.2. pLVX-TetOne-Puro was linearized using restriction sites BamHI and EcoRI and pLJM1 vector (Addgene 91980) at sites BmtI and EcoRI. For a complete list of vectors produced throughout this work see Appendix Table 8.1.

Mutagenesis. All mutagenesis reactions were carried out using the QuikChange II Site-Directed or Lightning MultiSite-Directed Mutagenesis Kits (Agilent) according to the manufacturer's instructions. See Appendix Table 8.3 or a list of mutations and mutagenic primer sets. All plasmids were sent to GATC Biotech or Genomics Equipment Park (The Francis Crick Institute) for Sanger sequencing to confirm mutations. Mutagenic primers were purchased from Sigma-Aldrich.

Molecular cloning – lentiviral cell line generation. Lentiviral plasmids pLVX-C-FLAG-D20 or pLVX ERTurboID together with packaging and viral envelope plasmids pCMV-Delta-8.2 (Addgene 12263) and pCMV-VSV-G (Addgene 8454) were transfected into HEK293-FT cells using FuGENE® (PROMEGA) as transfection reagent, following the manufacturer's instructions. Media was changed after 24h and lentiviral particles were harvested 48h after the first media change. Media was then filtered (0.2 µm filter), polybrene was added to a final concentration of 12 µg/mL and solution was diluted to different concentrations with fresh media (1/2, 1/3, 1/4, 1/8). Doxycycline inducible HEK293 cells were prepared by transduction of 2.5×10^5 low-

passage cells with lentivirus, followed by puromycin selection at $1.0 \mu\text{g ml}^{-1}$ for 1 w. Next, cells were cultured with $0.25 \mu\text{g ml}^{-1}$ puromycin and, after 4 or more passages, and then screened by immunoblot with antibodies against FLAG or HA epitopes.

6.2. Cellular and biochemical assays.

6.2.1. zDHHC20 autoacylation and substrate S-acylation in cells

Immunoprecipitation. Cell lysates were incubated with magnetic FLAG and/or HA tagged beads ($3 \mu\text{L}$) overnight at 4°C . Using magnetic racks, the cell lysate was removed and the beads were washed with PBS (x2). Click reactions were performed on-bead using the standard click mix. Beads were boiled with Laemmli buffer and proteins analysed via SDS-PAGE.

Substrate Palmitoylation Assay. HEK293Ts were trypsinised and cell concentration was determined. Transfection mixtures were prepared as described above using zDHHC20 plasmids ($1 \mu\text{g}$ per transfection x number of transfections) with or without IFTM3 ($0.5 \mu\text{g}$ per transfection x number of transfections). 1×10^6 cells times the number of experiments were added to mixture and was adjusted as described above with DMEM media. Transfection and cell attachment were allowed to proceed overnight. Then the cell media was removed and replaced with probe containing DMEM media (0.5% FBS) for the desired time. Finally, cells were washed with PBS, lysed (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% glycerol, 2% DDM, 0.5 mM TCEP, $10 \mu\text{M}$ Palmostatin B, 0.05 U/ μL benzonase and 1X protease inhibitor tablet) for 1h at 4°C while rotating. Insoluble cell debris was removed by centrifugation (13000 rpm, 10 min). Cell lysate was transferred to fresh Eppendorf tubes for immunoprecipitation. Immunoprecipitation was performed as described above, followed by an on-bead click reaction, addition of Laemmli buffer and SDS-PAGE analysis of the samples.

Inhibition Assay in HEK293T Cells. HEK293T confluent cells were trypsinised and the number of cells per mL was determined. Transfection mixtures containing ZDHH20 enzyme plasmid ($1 \mu\text{g}$ per transfection), were prepared as per the general procedure. Then, 10^6 cells per transfection were added and additional media was added to make up 1mL of mixture per well of experiment. Cells were plated in 12-well plates and incubated at 37°C overnight. After then, the media was aspirated and replaced with

DMEM (0.1% FCS). Inhibitors were dosed at 25 μ M concentration for 4h. After the 1st hour the cyclopropyl probe was dosed at the same concentration. Finally, cells were detached by pipetting, and the media was removed after cell centrifugation (2000 g for 2 min). Cells were lysed (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% glycerol, 2% DDM, 0.5 mM TCEP, 10 μ M Palmostatin B, 0.05 U/ μ L benzonase and 1X protease inhibitor tablet) for 1h at 4 °C while rotating. Insoluble cell debris was removed by centrifugation (13000 rpm, 10 min). Cell lysate was transferred to fresh Eppendorf tubes for immunoprecipitation. Immunoprecipitation was performed as described above, followed by an on-bead click reaction, addition of Laemmli buffer and SDS-PAGE analysis of the samples.

Testing ERTurboID activity in cells and media.

All ERTurboID HEK293T cells were cultured with DMEM media in 6 well plates. When cells reached 50% density, their media was changed with DMEM supplemented with 500 μ M biotin to induce biotinylation (3 mL of media per 6-well). After 16 h, the media was collected and placed on ice. Media (3 mL) was placed on 5 KDa Protein concentrator filters (Pierce), and washed three times with 2 mL of PBS (spun at 4000g x 30min 4 °C). The resulting concentrate was diluted to 1 mL and transferred to newly labelled Eppendorf tubes and 30 μ L of magnetic neutravidin beads (Pierce) was added. Pulldown was allowed to proceed at room temperature for 1 hour. In parallel, to cells was with PBS, lysis buffer (50 mM HEPES pH 7.4, 0.5% NP-40, 0.25% SDS, 10 mM NaCl, 2 mM MgCl₂, cOmplete protease inhibitor (Roche) and 0.05 U/ μ L benzonase) was added. After a BCA normalization, 100-200 μ g were used for pulldown with neutravidin magnetic beads.

After immunoprecipitation, the beads were then washed firstly with PBS 0.2% SDS (3 x 1mL) and secondly with PBS (3 x 1mL). Finally, beads were spun 2000 rpm, all supernatant was removed and Laemmli buffer (30 μ L) was added. Beads were boiled at 95°C for 10 mins and samples loaded on an acrylamide gel for SDS-PAGE and western blotting.

6.2.2. DHHC20 Enzyme Kinetics.

Protein Purification. For each FLAG-ZDHHC20 construct, 2 dishes of HEK293T cells were prepared and transfected with calcium phosphate transfection mix. To a 10 cm tissue culture dish was added 6×10^6 HEK293T cells in 8 mL DMEM culture media. Cells were allowed to settle o/n in a 37 °C incubator. To prepare 1 calcium phosphate transfection mix, 10 µg of C-FLAG-D20 construct and water was added to a 1.5 ml Eppendorf tube to a volume of 436 µl. This solution was topped up with 64 µl of 2M CaCl_2 to give a final volume of 500 µl, which was slowly added dropwise and with continuous bubbling to 500 µl 2X HBSS (273.8 mM NaCl, 9.4 mM KCl, 1.5 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 15 mM glucose, 42 mM HEPES (free acid) pH 7.05 in MilliQ- H_2O , filter sterilized) in a sterile 30 ml polystyrene tube. This 1 ml solution was allowed to incubate at RT for 5 m before use. To a 10 cm plate of HEK293T cells was added 1 ml of the DNA mixture. The mix was added dropwise and evenly across the media surface to ensure maximal cell coverage with precipitated DNA. The tissue culture dish was returned to the 37 °C incubator and left to incubate for 72 h before harvesting cells for protein purification.

After 72 h, cells were dislodged manually using an automatic pipette, collected in a 50 ml falcon tube and pelleted at $200 \times g$ for 5 m. The media was decanted, and cells washed 3X with cold PBS. The cell pellet was dislodged and mixed with 5 ml 2% DDM buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% glycerol, 2% DDM, 0.5 mM TCEP, 10 µM Palmostatin B and 1X protease inhibitor tablet), vortexed thoroughly and allowed to incubate at 4 °C with constant rotation. After 4 h, lysate was centrifuged for 20 m at $20,000 \times g$ and 4 °C. 1 ml anti-FLAG M2 Affinity Gel suspension was equilibrated in 5 ml 1% DDM buffer before use. The cleared lysate was added to 1 volume (~ 5 ml) of 0% DDM buffer before being added to cold (chilled on ice), pre-equilibrated resin, after which the suspension was allowed to mix o/n at 4 °C. After incubation, the lysate-resin mix was added to an Econo-Pac gravity-flow chromatography column (Bio-Rad); the lysate container was rinsed thoroughly with 1% DDM buffer to transfer all residual resin to the column. In the cold room and on ice, the resin was washed 3X each with ice-cold buffers W1 (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% DDM, 2 mM TCEP and 1X protease inhibitors), W2 (25 mM HEPES pH 7.5, 500 mM NaCl, 0.2% DDM, 2 mM TCEP and 1X protease inhibitors)

and W3 (W2 with 25 mM NaCl). After washing, FLAG-ZDHHC20 was eluted with 5 X 0.5 ml portions of buffer W3 containing 0.25 mg ml⁻¹ 3X FLAG-peptide (MDYKDHDGDYKDHDIDYKDDDDK, Crick Peptide Chemistry STP stock peptide). FLAG-ZDHHC20 containing fractions were pooled and buffer exchanged with W3 simultaneously with concentration using an Amicon Ultra 15 ml Spin-Column with 50 KDa cut-off.

Enzyme-coupled ZDHHC20-autoacylation assay. Autoacylation reactions were carried out as previously described (Banerjee, Science, 2018) with a few exceptions. Reactions were prepared in a Corning 96-w black half-area plate. In one well was prepared a pre-start mix with the indicated concentration of fatty acid- or probe-CoA, 2 mM α -ketoglutaric acid, 0.25 mM NAD (β -nicotinamide adenine dinucleotide, oxidized) and 0.2 mM thiamine pyrophosphate in 25 μ l reaction buffer (25 mM MES, pH 6.8, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.2 mM DDM and 1X protease inhibitors). The reaction was started by addition of 25 μ l ZDHHC master mix containing 20 nM ZDHHC20 and 2 μ l of α -ketoglutarate dehydrogenase (KDH, Sigma-Aldrich, K1502). In the first reaction step, ZDHHC20 autoacylation leads to production of free CoA-SH, which is then converted to NADH in the next step by KDH. NADH production was monitored using the fluorescence module (Ex. 360/Em. 465) of the Tecan Spark Multimode Microplate Reader. Note that 18-Bz-CoA exhibited weak, but measurable, activity in KDH reactions without ZDHHC20 enzyme. Therefore, all rates from reactions with ZDHHC20 and 18-Bz-CoA were adjusted by subtraction of the ZDHHC20-independent rates from the corresponding total rates of reactions with ZDHHC20.

6.3. Quantitative Mass Spectrometry-Based Proteomics

6.3.1. Sample preparation and processing

On-bead hydrolysis proteomics Cells were concurrently plated and transfected with 1 μ g/mL of the appropriate ZDHHC construct using 3 μ L of FuGENE[®] per 1 μ g of DNA in a 10 cm dish. After 6 h the media was refreshed and cells were incubated o/n. Cells were treated with 15 μ M of the appropriate probe in 0.5% FBS media for 8 h. Cells were then washed 3X with PBS then lysed in SDS lysis buffer (50 mM HEPES pH 7.4,

0.5% NP-40, 0.25% SDS, 10 mM NaCl, 2 mM MgCl₂, cOmplete protease inhibitor (Roche) and 0.05 U/ μ L benzonase). Lysates were adjusted to 2 mg/mL, using 2 mg per condition, then subjected to a click reaction (see section 6.1) using biotin-PEG3-azide (Sigma) and small portion taken to be clicked with TAMRA azide, following the same protocol, for analysis by western blot and in-gel fluorescence. The click reaction was quenched with 5 mM EDTA followed by chloroform/methanol precipitation. For those samples where no site ID was performed protein samples were suspended in 50 mM HEPES pH 7.4 containing 1% SDS then precipitated with chloroform/methanol. For experiments including site identification of lipidation proteins were solubilized in 1 mL 50 mM triethanolamine pH 7.5, 4% SDS, 5 mM EDTA. TCEP (10 mM) was added to samples and incubated for 20 mins at RT with agitation. To these solutions 25 mM *N*-ethylmaleimide (NEM, from 1M stock in EtOH) was added and incubated for 2h at RT with agitation. Samples were then precipitated with chloroform/methanol. All protein samples were then suspended in 50 mM HEPES pH 7.4 containing 0.2% SDS. Labelled proteins were enriched using NeutrAvidin agarose beads (Thermo Fisher) using 40 μ L (50% slurry) per condition. The beads were equilibrated by washing in 0.2% SDS in 50 mM HEPES pH 7.4 prior to incubation with the samples for 3h at RT with agitation. Beads were then washed twice with 0.2% SDS in 50 mM HEPES pH 7.4 and four times with 50 mM HEPES pH 7.4. Beads were then suspended in 20 μ L of 50 mM triethanolamine pH 7.5, 4 mM EDTA and 0.5% ProteaseMax (Promega). To this was added 10 μ L of the NH₂OH cleavage solution (100 μ L 8.16 M NH₂OH in 50 mM TEA pH 8.0, 30 μ L 500 mM triethanolamine pH 7.5, 2.6 μ L 500 mM EDTA, 197 μ L water) giving a final NH₂OH concentration of 0.82 M, and the samples were incubated at RT for 2 h with agitation. Following this, 100 μ L of 50 mM HEPES pH 8.0 containing 5 mM TCEP was added and the beads pelleted and 120 μ L of supernatant taken. 20 mM chloroacetamide was added to the supernatant and they were incubated at RT for 15 mins. Samples were then diluted with 400 μ L 50 mM HEPES pH 8 and digested with 0.3 μ g of trypsin (Promega) o/n at 37 °C. Samples were acidified with 0.5% (v/v) trifluoroacetic acid (TFA), flash frozen and lyophilized. Samples were dissolved in water containing 0.5% TFA and loaded onto stage tips containing three SDB-XC poly(styrenedivinyl-benzene) copolymer discs (Merck). The stage tipping procedure was carried out as described by Rappsilber, et al. Peptide samples were eluted in 55% acetonitrile in water and the solvent removed by incubation in an Eppendorf Concentrator plus at 45 °C.

Samples in which NEM capping was not performed were dissolved in water containing 0.1% TFA, ready for LC-MS/MS analysis. For samples in which NEM capping for site identification was included, prior to LC-MS/MS analysis, 3 X fractionation using stage tips loaded with 3 layers SDB-RPS discs (3M™ Empore™) was performed. Peptides were loaded on the solid phase in 0.5% TFA, and subsequently washed with 60 µL 0.2% TFA (3x). Peptides were subsequently eluted using 3 elution buffers (60 µL); buffer 1 (100 mM ammonium formate, 40% (v/v) MeCN, 0.5% (v/v) formic acid), buffer 2 (150 mM ammonium formate, 60% (v/v) MeCN, 0.5% (v/v) formic acid), buffer 3 (5% (v/v) ammonium hydroxide, 80% (v/v) MeCN). Samples were dried (Eppendorf Concentrator plus at 45 °C) and then dissolved in water containing 0.1% TFA. Peptides were analyzed on a Q-Exactive mass spectrometer (Thermo Fisher) coupled to an Ultimate3000 LC (Thermo Fisher) using an Easy Spray Nano-source. The instrument was operated in data dependent acquisition mode selecting the 10 most intense precursor ions for fragmentation.

On-bead digestion proteomics. Cells were simultaneously plated and transfected with 1 µg/mL of the appropriate ZDHHC construct using 3 µL of FuGENE per 1 µg of DNA in a 10 cm dish. After 6 h the media was refreshed and cells were incubated o/n. Cells were treated with 15 µM of the appropriate probe in 0.5% FBS media for 8 h. Cells were then washed 3X with PBS then lysed in SDS lysis buffer (50 mM HEPES pH 7.4, 0.5% NP-40, 0.25% SDS, 10 mM NaCl, 2 mM MgCl₂, cOmplete protease inhibitor (Roche) and 0.05 U/µL benzonase). The concentration of lysates was adjusted to 2 mg/mL, using 1-2 mg per condition, then subjected to a click reaction using biotin-PEG3-azide (Sigma) and small portion taken for analysis by western blot using Neutravidin-HRP (ThermoFisher). The click reagent mixture was prepared as described in section 6.1 and 64 µL were added per 1 mL of lysate and the samples incubated for 1 h at RT (1200 rpm). The reaction was quenched with 5 mM EDTA followed by chloroform/methanol precipitation. Pellets were washed by sonication in 200 µL of MeOH, and re-pelleted by centrifugation (1200 rpm). Protein pellets were suspended in 50 mM HEPES pH 7.4 containing 1% SDS and shaken until fully dissolved. Percentage of SDS was lowered to 0.2% using 50 mM HEPES pH 7.4 and samples were enriched using NeutrAvidin agarose beads priorly dimethylated (Thermo Fisher) using 40 µL (50% slurry) per condition. The beads were equilibrated by washing in 0.2% SDS in 50 mM HEPES pH 7.4 prior to incubation with the samples

for 3 h at RT (1200 rpm). Beads were then washed three times with 0.2% SDS in 50 mM HEPES pH 7.4 and four times with 50 mM HEPES pH 8. Following this, beads were resuspended in 150 μ L of HEPES pH 8 and 0.4 μ g of LysC were added and digestion was allowed to proceed at 37°C for 2h with gentle shaking. Next, beads were pelleted (2000 x g, 3 min) and the supernatant (100 μ L) was transferred to newly labelled Eppendorf tubes. Beads were washed with 100 μ L of HEPES pH 8 and after centrifugation (2000 x g, 3 min), the supernatants were combined. Reduction and alkylation steps were performed by addition of 5 mM TCEP (from a fresh 10X stock) and 10 mM CAA (chloroacetamide, from a fresh 10X stock); samples were shaken at room temperature in the dark for 10 min. Following this, 0.2 μ g of trypsin was added and digestion was allowed to proceed O/N at 37°C with mild shaking. The next morning, the samples were acidified with TFA until the pH of solution was lower than 3. Samples were de-salted using Peptide Desalting Spin Columns (Pierce™) following the manufacturers protocol. Peptide samples were eluted in 55% acetonitrile in water and the solvent removed on a Savant SPD1010 SpeedVac Concentrator (Thermo Fisher) at 45 °C and stored at -80 °C until submission. Peptides were analyzed on a Q-Exactive mass spectrometer (Thermo Fisher) coupled to an Ultimate3000 LC (Thermo Fisher) using an Easy Spray Nano-source. The instrument was operated in data dependent acquisition mode selecting the 10 most intense precursor ions for fragmentation.

ERTurboID On-bead digestion proteomics. Cell lines, ERTurboID HEK293T (n=8) ERTurboID ZDHHC20 KO1 (n=4) HEK293T and ERTurboID ZDHHC20 KO2 HEK293T (n=4), were cultured in T175 flasks until ~50 % confluency with DMEM Glutamax supplemented with 10% FBS. Then, media was changed to 30 mL of DMEM media supplemented with 500 μ M biotin, with the exception of four ERTurboID HEK293T flasks (no biotin controls). After 24 h, the media was collected and placed on ice. Media (15 mL) was placed on 5 KDa Protein concentrator filters (Pierce) spin filters, which had been washed with 10 mL of PBS 0.2 % SDS (spun at 4000g x 40min 10-15 oC). Excess biotin in the media was removed with 3 washes with 20 mL PBS 0.2 % SDS and concentrated to a total of 1-2 mL. 108 μ L of bead mix (50:50 dimethylated agarose beads: plain agarose beads) was added pulldown was performed for 4h at room temperature with shaking (1200 rpm). Next, beads were spun at 2000 rpm for 5 mins the large centrifuge and the supernatant was carefully removed.

Beads were transferred to newly Labelled LowBind Eppendorf tubes and washed: 3 times with 1mL 1% SDS PBS pH 7.4, 3 times with 1 mL HEPES 50 mM pH 7.4, and twice with 1 mL HEPES 50 mM pH 8.1. The next steps were performed as described above on the on-bead digestion protocol.

Cells were washed with PBS (x2) and dislodged from the flasks with gentle pipetting. Cells were spun (0.2 x g 5 min), supernatant PBS removed and protein pellets were frozen and stored at -80°C. Pellets were lysed with SDS lysis buffer (50 mM HEPES pH 7.4, 0.5% NP-40, 0.25% SDS, 10 mM NaCl, 2 mM MgCl₂, cOmplete protease inhibitor (Roche) and 0.05 U/ μ L benzonase). Lysates were normalized to 1 mg of protein, samples were precipitated using the chloroform: methanol protocol and resuspended with 100 μ L of 50 mM HEPES pH 8 containing 1% SDS and shaken until fully dissolved. Percentage of SDS was lowered to 0.2% using 50 mM HEPES pH 7.4 and samples were enriched using NeutrAvidin agarose beads priorly dimethylated (Thermo Fisher) using 40 μ L (50% slurry) per condition. The following steps were performed as described above on the on-bead digestion protocol

6.3.2. Proteomic Data Analysis and Bioinformatic analysis

6.3.2.1. Proteomics Searches and Data Analysis

RAW files were uploaded into MaxQuant (version 1.6.7.0) and searched against human proteome (uniprot.org) using the built-in Andromeda search engine. Cysteine carbamidomethylation was selected as a fixed modification and methionine oxidation and acetylation of protein N terminus as variable modifications and where appropriate cysteine NEM modification was included as a variable modification. Trypsin/P was set as the digestion enzyme, up to two missed cleavages were allowed and a false discovery rate of 0.01 was set for peptides, proteins and sites with match between runs selected. Data was quantified using LFQ with a minimum ratio count = 2.

Data analysis was performed using Perseus (version 1.6.2.1). MaxQuant proteingroups.txt output files were uploaded and filtered against contaminants, reverse and proteins identified by site. A base 2 logarithm was applied to all LFQ intensities and data filtered to contain over 75% (chemical genetics) or 65% (YnPalm proteomics and ERTurboID proximity labelling) valid values in the positive condition.

Missing values were imputed from a normal distribution (width = 0.3, downshift = 1.8). Data was normalized by each condition by subtracting the median value from each column. A two-sample student T-test was performed comparing the various sets of condition groupings ($S_0 = 0.1$, FDR = 0.01) for all proteins remaining in the dataset and the results analyzed according to their statistical significance.

6.3.2.2. Bioinformatic – PANTHER overrepresentation analysis.

The online bioinformatic tool PANTHER ¹⁸⁸ was used to perform statistical overrepresentation analyses of a ZDHHC20 substrate lists obtained using the chemical genetic system, containing gene hits present in at least 2 of 3 HEK293T OBD analyses (MUT vs WT). Overrepresentation analyses were performed using gene ontology terms for cellular compartment or protein class. As reference two lists were used a default list of genes from the human genome, or a manually curated list representing the human S-acylated proteome. This list was obtained from SwissPalm dataset 5 (palmitoylation validated proteins or found in palmitoyl-proteomes using 2-independent methods) and curated to contained those proteins also predicted to be S-acylated to give a total of 2812 unique genes. Statistical analysis and p-values were determined using the Fisher's Exact test. The false discovery rate (FDR) was calculated by the Benjamin-Hochberg procedure. By default, a critical value of 0.05 is used to filter results.

6.4. Lipidomics

6.5.1. Lipid extraction

HEK293T cells were seeded in 6-well plates, grown to 70% confluency and treated with bumped fatty acid probes (15 μ M) for 3 hours. Cells were dislodged into their growth media by pipetting and pelleted by centrifugation (500 x *g*, 5 min). The cell pellet was washed twice by resuspending in ice-cold PBS and pelleting by centrifugation. Subsequently, the cells were resuspended in ice-cold 150 mM ammonium bicarbonate (500 μ L). An aliquot (10%) was kept aside for protein concentration determination and the remaining sample snap-frozen in liquid nitrogen and stored at -80°C until further processing. For protein concentration determination, cells were lysed in M-PER™ Mammalian Protein Extraction Reagent (Thermo

Scientific) and protein content determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific) as per the manufacturer's instructions. An aliquot equivalent to 100 µg protein per sample were used for lipid extraction. Lipid were extracted by the methyl-tert-butyl ether (MTBE) method with minor modifications¹⁶⁷. Extractions were performed in glass vials fitted with Teflon-lined caps using MS-grade solvents and water. Glass pipettes were used to handle any MTBE-containing solutions or lipid extracts. Methanol (1.5 mL) was added and the protein sample vortexed. MTBE (5 mL) was added and the mixture was incubated for 1h at room temperature on a shaker. Phase separation was induced by the addition of water (1.25 mL) followed by incubation for 10 min at room temperature. The sample was centrifuged (1,000 x g, 10 min) and the upper organic phase collected. The lower aqueous phase was re-extracted by addition of 1.67 mL of solvent mixture comprising MTBE/methanol (10:3, v/v) and 0.32 mL water. The samples were vortexed, incubated for 10 min and centrifuged (1000 x g, 10 min). The upper phase was recovered, and the combined organic phases were evaporated at 37°C under a stream of nitrogen and stored at -20°C. Before analysis, lipid extracts were reconstituted in 100 µL loading buffer (isopropanol/water/acetonitrile, 2:1:1, v/v/v). Blank control extraction was performed on a 200 µL aliquot of 150 mM ammonium bicarbonate solution. Quality control (QC) samples were prepared by pooling a small aliquot of all experimental samples after resuspension in loading buffer.

The following steps (sections 6.5.2 – 6.5.4) were performed by Dr. Elisabeth Stork Saha at Kings College University:

6.5.2. Ultra-high-performance liquid chromatography-mass spectrometry analysis of lipid extracts

Ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS) analysis was performed on a 1290 Infinity II UHPLC system coupled to a 6550 iFunnel quadrupole time-of-flight (QTOF) mass spectrometer (Agilent Technologies). The reversed-phase chromatography protocol was optimised with minor modifications from Cajka and Fiehn²¹⁷. Extracted lipids were separated on an Acquity UPLC CSH C18 column (130 Å, 1.7 µm, 2.1 x 100 mm) fitted with an Acquity UPLC CSH C18 VanGuard pre-column (130 Å, 1.7 µm, 2.1 mm x 5 mm) (both Waters). The column was maintained at 65°C at a flowrate of 0.6 mL/min. The mobile phases used were

60:40 (v:v) acetonitrile/H₂O (solvent A) and 10:90 (v:v) acetonitrile/isopropanol (solvent B). Solvent A and B were supplemented with 10 mM ammonium formate and 0.1% formic acid for ESI positive mode and with 10 mM ammonium acetate for ESI negative mode analysis. UHPLC gradient elution was carried out as follows: 0–2 min 15–30% B; 2–2.5 min 30–48% B; 2.5–11 min 48–82% B; 11–11.5 min 82–99% B; 11.5–14.50 99% B. The gradient was returned to initial conditions over 0.5 min and the column equilibrated for 3 min before subsequent runs. Between injections a 100% isopropanol needle wash was performed. For negative mode 5 μ L (MS mode) or 10 μ L (MS/MS mode) of sample and for positive mode 4 μ L (MS mode) or 8 μ L (MS/MS mode) of sample were injected. Samples were injected in randomized order, with QC sample injections added to the start, middle and end of each sample sequence to ensure consistency and reproducibility of all acquisition parameters.

Electrospray parameters were set as follows: gas and sheath gas temperature, 200°C; drying gas flow, 14 L/min; sheath gas flow, 11 L/min; sheath gas temperature, 350°C; nebulizer pressure, 35 psig; capillary voltage, 3,000 V; nozzle voltage, 1,000 V. MS-TOF fragmentor and Oct 1 RF Vpp radio voltage were set to 350 and 750V respectively. The QTOF was calibrated and operated in the extended dynamic range mode (~2 GHz) in the mass range 50 to 1700 *m/z*. Spectra were acquired in centroid mode with an acquisition rate of 2 spectra/s for MS mode acquisition. Data was acquired in MS mode for quantitative analysis of the natural lipidome, and MS/MS mode to obtain data for lipid structure assignment.

MS/MS data was acquired in auto-MS/MS mode (data-dependent). Spectra were acquired in centroid mode with an acquisition rate of 1 and 5 spectra/s for MS and MS/MS acquisition, respectively. Collision energy was adjusted to -35 eV and 30 eV for negative and positive modes, respectively. Mass range for precursor selection was 300–1650 *m/z* (negative) and 250–1680 *m/z* (positive). Fragmentation was triggered if the precursor reached 5000 (negative) or 2000 (positive) counts and maximum precursors per cycle was set to 5. MS/MS isolation width for precursors was selected as narrow (1.3 *m/z*). Active exclusion was enabled, set to exclude after 3 spectra and release after 0.1 min. To improve precursor selection, background ions were added to an exclusion list. For structure determination of probe-derived lipids, a list of preferred precursor ions was generated for each probe to improve MS/MS coverage of features

originating from probe metabolism. MS/MS analysis of DMSO control samples were used to confirm assignment of natural lipids.

6.5.3. Quantitative analysis of natural lipidome

Lipid annotations and quantifications were performed following the guidelines of the Lipidomics Standard Initiative (<https://lipidomics-standards-initiative.org/>). Feature extraction was carried out in Mass Hunter Profinder (v. 10.0, Agilent Technologies) using the “Batch Targeted Feature Extraction” option. Features were matched to an in-house library containing mass and retention time information of lipid species including glycerophospholipids, sphingolipids, fatty acids and glycolipids. All lipids in the database were previously assigned from MS/MS data using MS-DIAL²¹⁸ followed by manual curation. H^+ , Na^+ and NH_4^+ adducts were selected for positive mode and H^- , $C_2H_3O_2^-$ and CHO_2^- adducts were selected for negative mode data. Both mass and retention time were required for feature matching. Match tolerance was set to 5 ppm for mass and 0.15 min for retention time. The EIC extraction range was limited to ± 0.3 min of the expected retention time. An overall score of >70 was required for feature matching, with the contribution to overall score set as follows: Mass score 100, Isotope abundance score 60, Isotope spacing score 50, Retention time score 100. Features over 20% of saturation limit were excluded from the dataset. Matched features were manually inspected and re-integrated where required and checked for correct adduct pattern for the relevant lipid class. Data were exported as .csv files containing the identity, peak area and the retention time of each lipid species. Further data analysis and data representation was performed in Excel and GraphPad Prism. The relative abundance of each lipid species within a class was calculated as a percentage of the summed peak areas of all species identified within the class. TG species were quantified from data acquired in positive mode while all other species were quantified from data acquired in negative mode. $n=5$ for each experimental condition.

6.3.4. Assignment of probe-derived lipids

Feature extraction of data acquired in MS mode was carried out in Mass Hunter Profinder (v. 10.0, Agilent Technologies) using the “Batch Recursive Feature Extraction (small molecule/peptide)” option. Samples were grouped according to

experimental condition. All parameters except those detailed below were used as pre-set by the program. Peak heights were set to a minimum of 3000 counts. H^+ , Na^+ and NH_4^+ adducts were selected for positive mode and H^- , $C_2H_3O_2^-$ and CHO_2^- adducts were selected for negative mode. For compound binning and alignment, retention time tolerance was set to (+/- 0% + 0.15 min) and mass tolerance to (+/- 5 ppm + 2 mDa). A MFE score of at least 70 was required in at least 4 of 6 samples per group. For match tolerance, the mass was set to +/- 10 ppm and retention time to +/- 0.15 min. The EIC extraction range was limited to +/- 0.15 min of the expected retention time. An overall score of >75 was required for feature matching, with the contribution to overall score set as follows: Mass score 100, Isotope abundance score 60, Isotope spacing score 50, Retention time score 100. Features over 20% of saturation limit were excluded from the dataset. Post-processing filters were set to require a score (Tgt) of at least 50 in 4 out of 6 samples per experimental group.

Manual filtering was performed to remove features present in the blank extraction samples. To create a list of features originating from probe metabolism, only features unique to each probe condition were selected. All features present in DMSO control samples were discarded. Features were manually inspected and re-integrated where required. The feature lists were used to create inclusion lists for MS/MS analysis and peak lists for lipid annotations as described below.

LipidMatch (v. 3.5)²¹⁹ was used for assignment of probe-derived lipids. Lipid libraries containing theoretical fragment of probe-derived lipid species of different classes (PC, PE, PC-O, PE-O, PC-P, PE-P, Cer-NS, Cer-NDS, DG and TG) were constructed and added to the existing library folder. Agilent .d files were converted to .ms2 format using MSConvertGUI (ProteoWizard). The search was performed using the feature tables created above. Search parameters were set as follows: retention time window, +/- 0.15 min; ppm window for matching experimental and *in silico* fragments, +/- 5 ppm; mass accuracy window for matching experimental and in-silico precursors, +/-0.005 Da; MS/MS isolation window, 1 Da; minimum signal intensity for MS/MS ion, 1; minimum number of scans for confirmation, 1. All lipid assignments were manually curated by inspecting MS/MS spectra and ensuring correct adduct formation for the relevant lipid class. Additionally, a small number of lipids were assigned manually from MS/MS spectra. The retention time of all probe-derived lipid species matched the expected retention time window for the relevant lipid class. Note that in contrast to natural DG

and TG species, which preferentially form NH_4^+ and Na^+ adducts, probed-derived DG and TG species preferentially formed the $[\text{M}+\text{H}]^+$ ion, presumably through protonation at the amide moiety.

6.5. Identification of CoA species.

6.5.1. Acyl- and probe-Coenzyme A analysis.

HEK293T cells were seeded in 6-well plates, grown to 70% confluency in media containing 0.5% FBS and treated with 30 μM YnPal or 18-Bz for 2h. Cells were dislodged into their growth media by pipetting and pelleted by centrifugation (500 x *g*, 5 min). The cell pellet was washed twice by resuspending in ice-cold PBS and pelleting by centrifugation.

To each sample, 400 μL chloroform was added and vortexed for ~1 minute, followed by addition of 200 μL methanol and a repeated vortex. Samples were incubated in a water bath sonicator (4°C, 1 hr), with 3 × 8 min sonication pulses, followed by centrifugation (4°C, RT, 10 min, 16,000 rpm). The supernatant was transferred to a new Eppendorf 1.5 mL tube (E1). The pellet was re-extracted with 450 μL methanol: water (2:1 v:v, containing internal standard, $^{13}\text{C}_3$ -Malonyl-CoA), sonicated (8 min, 4°C) and centrifuged, as above. The supernatant was added to the first extract (E1). Combined extracts were dried using a SpeedVac concentrator, re-suspended in 350 μL chloroform:methanol:water (1:3:3, v/v), and centrifuged, as above. The upper, aqueous phase containing the polar metabolites (including probe, probe-CoA, and acyl-CoA molecules) was dried using the SpeedVac concentrator and resuspended in 100 μL ACN/Ammonium carbonate 20 mM (7:3, v/v) for LC-MS injection.

6.5.2. Liquid chromatography-mass spectrometry (LC-MS)

Chromatography prior to all mass spectrometry was performed using an adaptation of a method previously described²²⁰. Samples were injected into a Dionex UltiMate 3000 LC system (Thermo Scientific) with a Phenomenex Luna C18(2) 100 Å (100 x 2 mm, 3 μm) column coupled with a SecurityGuard C18 guard column (4 x 2 mm). Analytes were separated using 20 mM ammonium carbonate in water (Optima HPLC grade, Sigma Aldrich) as solvent A and acetonitrile (Optima HPLC grade, Sigma Aldrich) as

solvent B at 0.3 mL/min flow rate. Elution began at 5% Solvent B, maintained for 3 min, increased to 100% B over 12 min, followed by a 3 min wash of 100% B and subsequent 3 min re-equilibration to 5% B. Other parameters were as follows: column temperature, 30°C; injection volume, 10 µL; needle wash, 50% methanol; autosampler temperature, 4°C.

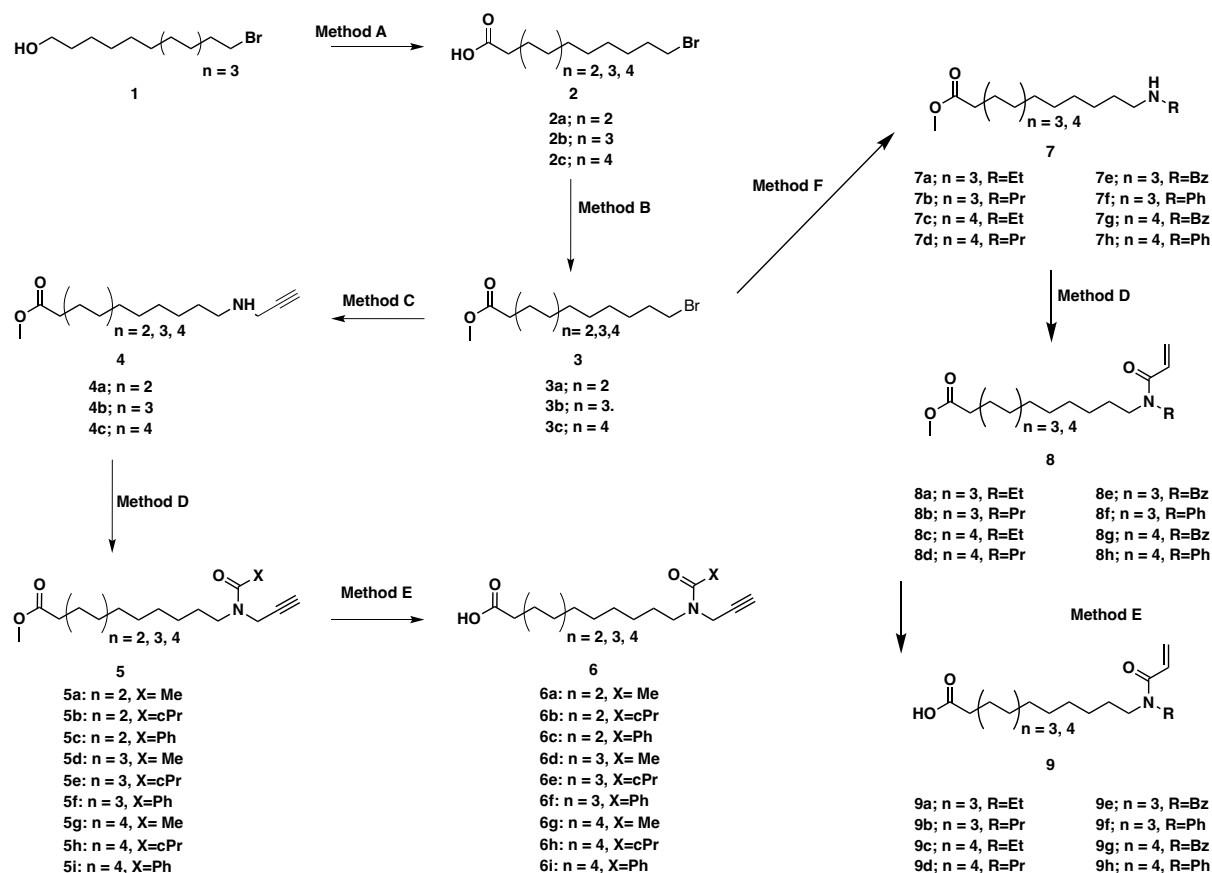
Post-chromatography, high resolution (HR) MS was performed with positive and negative polarity switching using a Q-Exactive Orbitrap (Thermo Scientific) with a HESI-II (Heated electrospray ionization) probe. MS parameters were as follows: spray voltage, 3.5 kV and 3.2 kV (for positive and negative modes, respectively); probe temperature, 320°C; sheath and auxiliary gases, 30 and 5 arbitrary units (au), respectively; full scan range: 100 to 1300 *m/z* with settings of AGC target and resolution as Balanced and High (3×10^6 and 70,000), respectively. Data were recorded using Xcalibur 3.0.63 software (Thermo Scientific). Mass calibration was performed for both ESI polarities before analysis using the standard Thermo Scientific Calmix solution. Qualitative analysis was performed using Xcalibur FreeStyle 1.8 SP1 and Tracefinder 5.1 software (Thermo Scientific) according to the manufacturer's workflows. Masses, retention times, and fragmentation of all relevant sample-derived molecules were compared to authentic chemical standards.

MS parameters were optimized by direct infusion of 16 µM acyl-CoAs dissolved in 10 mM MeOH/Ammonium acetate at 5 µL/min into an TSQ Quantiva triple quadrupole MS (Thermo Scientific). The heated electrospray was set in positive mode with the following parameters: capillary voltage, 3472 V; sheath gas, 60 au; aux gas, 10 au; sweep gas, 1 au; ion transfer tube temp, 325°C; vaporizer temp, 275°C. A selected reaction monitoring (SRM) function was applied for the simultaneous detection of acyl-CoA and probe-CoA molecules with RF lens and collision energies as shown in the table below. Data were recorded using the Xcalibur 4.0.27.10 software and analysed using QuanBrowser 4.5.445.18 (Thermo Fisher Scientific).

Compound	Start Time (min)	End Time (min)	Polarity	Precursor (<i>m/z</i>)	Product (<i>m/z</i>)	Collision Energy (V)	RF Lens (V)
¹³ C ₃ -Malonyl-CoA	0	20	Positive	857.06	305.169	37.15	118.36
¹³ C ₃ -Malonyl-CoA	0	20	Positive	857.06	350.169	27.8	118.36

¹³ C ₃ -Malonyl-CoA	0	20	Positive	857.06	410.071	24.11	118.36
¹³ C ₃ -Malonyl-CoA	0	20	Positive	857.06	428.04	27.04	118.36
¹³ C ₃ -Malonyl-CoA	0	20	Positive	857.06	448.151	22.19	118.36
YnP-CoA	0	20	Positive	1002.32	410.111	30	130
YnP-CoA	0	20	Positive	1002.32	428.11	30	130
YnP-CoA	0	20	Positive	1002.32	495.36	30	130
YnP-CoA	0	20	Positive	1002.32	593.37	30	130
C16:0-CoA	0	20	Positive	1006.33	397.333	30.98	144
C16:0-CoA	0	20	Positive	1006.33	410.111	26.48	144
C16:0-CoA	0	20	Positive	1006.33	428.111	29.97	144
C16:0-CoA	0	20	Positive	1006.33	499.444	33.46	144
18-Bz-CoA	0	20	Positive	1135.4	410.111	30	130
18-Bz-CoA	0	20	Positive	1135.4	428.111	30	130
18-Bz-CoA	0	20	Positive	1135.4	628.41	30	130
18-Bz-CoA	0	20	Positive	1135.4	726.43	30	130

6.6. Synthesis of Chemical Probes



Scheme 6.1. Synthetic scheme to bumped fatty acid probes.

6.6.1. Materials and Instrumentation.

All chemicals and solvents were used as received from suppliers (Sigma-Aldrich-Merck, Fisher, Fluorochem or VWR) without any further purification. Gases were from BOC and ultrapure water was used for all buffers. ¹H-NMR and ¹³C-NMR spectra were recorded on Bruker AV-400 (400 MHz) spectrometer, using residual isotopic solvent as an internal reference. Chemical shifts (δ) are given in units of parts per million (ppm). Each spectrum is corrected to the solvent reference signal. The multiplicity of each signal is given by: singlet (s), doublet (d), triplet (t) or multiplet (m) and the number of protons (H) associated to a peak is indicated by nH. Coupling constants (J) are given in Hz and determined by analysis using the MestReNova software. Analytical LCMS analysis was conducted on an Acquity UPLC BDH C18 column (50 mm × 2.1 mm, i.d. 1.7 μm packing diameter) at 40 °C. Flow rate: 0.5 mL/min. Injection volume: 1 μL. The UV detection was a summed-up signal from wavelengths between 200 to

400 nm. UPLC retention times (t_r) are reported in minutes. The following elution methods were used: **Method 1:** (gradient of H₂O and ACN, supplemented with 0.1% formic acid): 0-1 min 3-100% ACN, 1-3.5 min 100% ACN, 3.5-3.6 min 100 to 3% ACN 3.6-4 min 3% ACN. **Method 2:** (gradient of 25 mM ammonium acetate pH 8 and ACN), 0-5min 100% ACN, 5-5.5 min 100% ACN, 5.5-6.5 min 100% to 0%, 6.5-9 min 0% ACN. Chromatographic purifications were performed with a Biotage Isolera 4 using c-Hex /EtOAc gradient elution system. Final compounds were purified by PREP-LCMS (Agilent Technologies 1260 series) equipped with a LC-MSD detector, an Agilent prep-C18 column (5 μ m particle size, 21.2 $\times$ 50 mm) using water (containing 0.1% formic acid) and acetonitrile (containing 0.1% formic acid) in a gradient with a flow of 25 mL/min.

6.6.2. General synthetic methods

General methods.

General method A (Jones Oxidation)

The corresponding alcohol 1 (6.8 mmol) was dissolved in acetone (20 mL) and cooled to 0 °C, 20 mL of chilled Jones Reagent were added dropwise. The reaction was then allowed to warm up to room temperature and monitored by thin layer chromatography until completion. The reaction was quenched with 10% aqueous sodium thiosulfate, extracted with Et₂O, dried and evaporated to give the target compound.

General method B (Esterification)

The corresponding carboxylic acid (2 a-c) (1 eq.) was dissolved in MeOH (3 mL/mmol) and heated to reflux. Concentrated H₂SO₄ (59 μ L/ mmol) were added and the reaction was monitored by TLC until completion. The reaction was then quenched with dH₂O, extracted with Et₂O, dried and evaporated to give compound 3 as a clear oil.

General method C (Secondary amine formation)

The corresponding bromo methyl ester (3 a-c) (1 equiv.) and propargyl amine (10 equiv.) were dissolved in acetonitrile (30 mL/gr), reaction was set to reflux O/N and monitored by TLC. Upon completion, the solution was concentrated, cooled down and the resulting precipitate was collected by filtration, washed with cold acetonitrile and used in the next step without further purification.

General method D (Amide bond formation)

The corresponding compound 4 (a-c) (1 equiv.) was dissolved in dry DCM (3 mL/mmol) under an inert Argon atmosphere. DIPEA (3 equiv.) were added and the reaction mixture was cooled to 0°C. The corresponding acyl chloride was added (2 equiv.). The reaction was monitored until completion, upon which it was quenched with NaHCO₃, the organic layer extracted, dried and residue was purified by flash chromatography over silica gel using c-Hex /EtOAc (2:1 → 1:1) to yield the target compound.

General method E (Ester hydrolysis)

The corresponding compound 5 (a-i) were dissolved in THF (1.5 mL/mmol) and treated dropwise with 1M LiOH (5 equiv.). The reaction was monitored until completion, quenched via addition 1M HCl to pH 1, extracted with EtOAc, dried and evaporated to give the product usually in quantitative yield.

General Method F (Secondary amine formation for inhibitors)

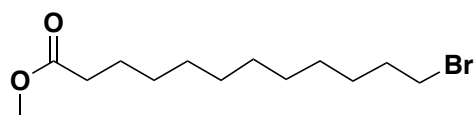
The corresponding bromomethyl ester (3 a-c) (1 equiv.) and the corresponding amine (10 equiv.) were dissolved in DMF and potassium iodide (1 equiv.) was added. The reaction was monitored until completion, after which DMF was evaporated and the crude reaction mixture used in the following step.

6.6.3. Synthesis of probes

The synthesis for the probe-inspired lipids was carried out by Christelle Soudy, senior research scientist at the Francis Crick institute.

Probe name

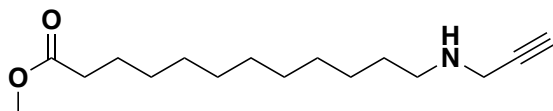
Methyl 12-bromododecanoate (3a)



12-bromododecanoic acid (1.0 g, 3.58 mmol) was dissolved in H₂O (12 mL). H₂SO₄ (200 µL) was added and the solution was refluxed for 4h. The reaction mixture was diluted with 50 mL Et₂O. The layers were separated and the organic solution was washed with NaHCO₃ (aq.), H₂O, and brine, before it was dried on Na₂SO₄, filtered and concentrated under reduced pressure to yield compound **3a** (1.05 g, 3.4 mmol,

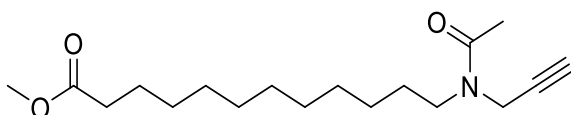
95%). **¹H NMR** (400 MHz, Chloroform-*d*) δ 3.66 (s, 3H), 3.40 (t, *J* = 6.9 Hz, 2H), 2.29 (t, *J* = 7.5 Hz, 2H), 1.90 – 1.78 (m, 2H), 1.65 – 1.56 (m, 2H), 1.46 – 1.35 (m, 2H), 1.34 – 1.24 (m, 12H). **¹³C NMR** (101 MHz, Chloroform-*d*) δ 174.28, 51.41, 34.07, 34.01, 32.80, 29.41, 29.35, 29.19, 29.10, 28.71, 28.14, 24.91.

Methyl 12-(prop-2-yn-1-ylamino)dodecanoate (**4a**)



3a (500 mg, 1.7 mmol) was dissolved in ACN (10 mL), propargylamine (140 mg, 2.55 mmol) and K₂CO₃ (469 mg, 3.4 mmol) was added and the solution was stirred o/n at 85°C. The reaction mixture was concentrated under reduced pressure and the dried crude was dissolved in 50 mL EtOAc, washed with NaHCO₃ (2x) and brine before it was dried on Na₂SO₄, filtered and concentrated under reduced pressure. The crude was purified by flash chromatography over silica gel using c-Hex/EtOAc (1:1) + 1% 7N NH₃ in MeOH to yield **4a** (190 mg, 0.71 mmol, 42%). **¹H NMR** (400 MHz, Chloroform-*d*) δ 3.65 (s, 3H), 3.41 (d, *J* = 2.4 Hz, 2H), 2.71 – 2.62 (m, 2H), 2.28 (t, *J* = 7.5 Hz, 2H), 2.19 (t, *J* = 2.4 Hz, 1H), 1.66 – 1.56 (m, 2H), 1.50 – 1.41 (m, 2H), 1.34 – 1.20 (m, 14H). **¹³C NMR** (101 MHz, Chloroform-*d*) δ 174.25, 82.35, 71.07, 51.36, 48.71, 38.16, 34.09, 29.81, 29.51, 29.47, 29.38, 29.20, 29.11, 27.27, 24.93.

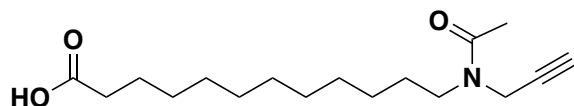
Methyl 12-(N-(prop-2-yn-1-yl)acetamido)dodecanoate (**5a**)



4a (30 mg, 0.11 mmol) was dissolved in dry CH₂Cl₂ (2 mL). DIPEA (30 μ L, 0.22 mmol) was added and the solution was cooled on ice. Acetyl chloride (17 μ L, 0.22 mmol) in 1 mL CH₂Cl₂ was added dropwise. The reaction mixture was stirred on ice for 4h. The reaction was quenched with 5 mL NaHCO₃ (aq.), extracted with EtOAc (3x), and the combined organic layers were dried on Na₂SO₄, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography over silica gel using c-Hex /EtOAc (2:1 $\rightarrow$ 1:1) to yield compound **5a** (30 mg, 0.09 mmol, 86%). **¹H NMR** (400 MHz, Chloroform-*d*) δ 4.19 and 3.98 (d, *J* = 2.5 Hz, 2H), 3.65 (s, 3H), 3.38 (dt, *J* = 12.9, 7.5 Hz, 2H), 2.33 – 2.24 (m, 3H), 2.15 and 2.09 (s, 3H), 1.66 – 1.49 (m, 4H), 1.34 – 1.22 (m, 14H). **¹³C NMR** (101 MHz, Chloroform-*d*) δ 174.25, 170.26,

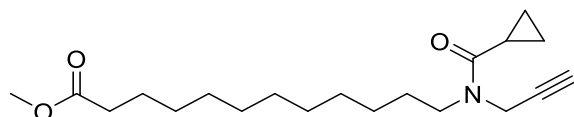
170.00, 79.33, 72.29, 71.35, 51.39, 48.12, 46.27, 38.32, 34.09, 34.05, 29.47, 29.43, 29.36, 29.28, 29.20, 29.12, 28.42, 27.57, 26.88, 26.77, 24.94, 21.74, 21.33.

12-(N-(prop-2-yn-1-yl)acetamido)dodecanoic acid (**6a**; **16-Ac**).



5a (15 mg, 0.048 mmol) was dissolved in THF (5 mL). LiOH.H₂O (42 mg, 0.92 mmol) in H₂O (100 μ L) was added dropwise. The reaction mixture was stirred at rt for 4h. The reaction was quenched with 5 mL HCl (1 M), extracted with EtOAc (3x) and the combined organic layers were washed with brine, dried on Na₂SO₄ and concentrated under reduced pressure. The crude product was purified by flash chromatography over silica gel using c-Hex/EtOAc (1:2 $\rightarrow$ 1:5) to yield compound **6a** (14 mg, 0.048 mmol, quantitative). **¹H NMR** (400 MHz, Chloroform-*d*) δ 4.21 and 4.00 (d, *J* = 2.5 Hz, 2H), 3.40 (dt, *J* = 15.1, 7.6 Hz, 2H), 2.34 (t, *J* = 7.5 Hz, 2H), 2.28 and 2.18 (m, 1H), 2.18 and 2.12 (s, 2H), 2.20 – 2.15 (m, 4H), 1.38 – 1.24 (m, 14H). **¹³C NMR** (101 MHz, CDCl₃) δ 178.41, 170.27, 79.24, 78.54, 77.32, 77.00, 76.68, 72.40, 71.43, 48.13, 46.36, 38.34, 34.10, 33.85, 29.42, 29.36, 29.29, 29.25, 29.20, 29.14, 28.98, 28.86, 28.38, 27.49, 26.74, 24.67, 21.68, 21.29. **LC-MS** method 1: 1.53 min, *m/z* calc. for C₁₇H₂₉NO₃ [M+H]⁺ 296.21; found 296.29.

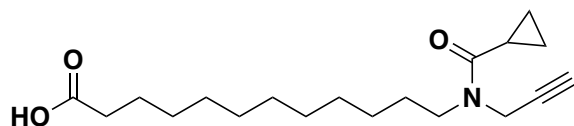
Methyl 12-(N-(prop-2-yn-1-yl)cyclopropanecarboxamido)dodecanoate (**5b**)



4a (59 mg, 0.22 mmol) was dissolved in dry CH₂Cl₂ (2 mL). DIPEA (77 μ L, 0.44 mmol) was added and the solution was cooled on ice. Cyclopropanecarbonyl chloride (40 μ L, 0.44 mmol) in 1 mL CH₂Cl₂ was added dropwise. The reaction mixture was stirred on ice for 4h. The reaction was quenched with 5 mL NaHCO₃ (aq.), extracted with EtOAc (3x), and the combined organic layers were dried on Na₂SO₄, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography over silica gel using c-Hex/EtOAc (2:1 $\rightarrow$ 1:1) to yield compound **5b** (41 mg, 0.12 mmol, 55%). **¹H NMR** (400 MHz, CDCl₃) δ 4.19 (dd, *J* = 9.6, 2.6 Hz, 2H), 3.65 (s, 3H), 3.58 – 3.52 (m, 1H), 2.27 (d, *J* = 7.5 Hz, 2H), 1.72 – 1.50 (m, 5H), 1.26 (q, *J* = 8.0 Hz, 16H), 0.99 (qd, *J* = 5.3, 4.8, 1.8 Hz, 2H), 0.77 (tt, *J* = 7.2, 3.3 Hz, 2H). **¹³C NMR** (101 MHz, CDCl₃)

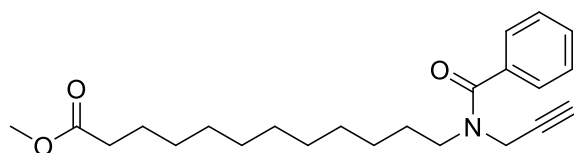
δ 179.34, 174.34, 173.18, 79.58, 72.12, 71.28, 51.44, 47.42, 35.01, 34.09, 29.50, 29.45, 29.37, 29.32, 29.22, 29.12, 28.93, 26.85, 24.93, 20.47, 12.65, 11.20, 8.89, 7.97, 7.75, 7.71.

12-(N-(prop-2-yn-1-yl)cyclopropanecarboxamido)dodecanoic acid (**6b**; 16-cPr)



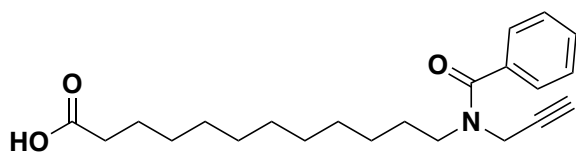
5b (41 mg, 0.12 mmol) was dissolved in THF (3 mL). 610 μ L of a 1 M LiOH solution in H₂O was added dropwise. The reaction mixture was stirred at rt for 4h. The reaction was quenched with 5 mL HCl (1 M), extracted with EtOAc (3x) and the combined organic layers were washed with brine, dried on Na₂SO₄ and concentrated under reduced pressure to yield compound **6b** (13 mg, 0.040 mmol, 33%). **¹H NMR** (400 MHz, CDCl₃) δ 4.27 – 4.15 (m, 2H), 3.57 and 3.43 (t, J = 7.6 Hz, 1H), 2.34 (t, J = 7.5 Hz, 2H), 2.18 (d, J = 2.6 Hz, 1H), 1.83 – 1.51 (m, 5H), 1.40 – 1.18 (m, 14H), 1.02 (dt, J = 8.0, 4.2 Hz, 2H), 0.78 (dp, J = 7.2, 4.3 Hz, 2H). **¹³C NMR** (101 MHz, CDCl₃) δ 178.96, 173.25, 79.58, 72.15, 71.30, 47.43, 37.61, 35.04, 33.93, 29.49, 29.41, 29.33, 29.29, 29.18, 29.01, 28.93, 27.62, 26.83, 24.68, 11.71, 11.24, 7.98, 7.75. **LC-MS** method 1: 1.61 min, m/z calc. for C₁₉H₃₁NO₃ [M+H]⁺ 322.23; found 322.33.

Methyl 12-(N-(prop-2-yn-1-yl)benzamido)dodecanoate (**5c**)



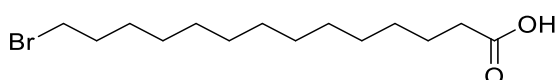
4a (59 mg, 0.22 mmol) was dissolved in dry CH₂Cl₂ (2 mL). DIPEA (77 μ L, 0.44 mmol) was added and the solution was cooled on ice. Benzoyl chloride (51 μ L, 0.44 mmol) in 1 mL CH₂Cl₂ was added dropwise. The reaction mixture was stirred on ice for 4h. The reaction was quenched with 5 mL NaHCO₃ (aq.), extracted with EtOAc (3x), and the combined organic layers were dried on Na₂SO₄, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography over silica gel using c-Hex/EtOAc (2:1 $\rightarrow$ 1:1) to yield compound **5c** (43 mg, 0.12 mmol, 52%). **¹H NMR** (400 MHz, CDCl₃) δ 7.49 – 7.31 (m, 4H), 4.37 (s, 1H), 3.99 (s, 1H), 3.70 (s, 3H), 3.64 (s, 1H), 3.38 (s, 1H), 2.30 (td, J = 7.7, 2.6 Hz, 3H), 1.61 (tt, J = 8.0, 4.3 Hz, 4H), 1.41 – 1.02 (m, 14H). **¹³C NMR** (101 MHz, CDCl₃) δ 174.28, 136.04, 129.69, 128.43, 126.78, 78.99, 51.41, 34.09, 29.44, 29.36, 29.21, 29.16, 29.12, 24.93.

12-(N-(prop-2-yn-1-yl)benzamido)dodecanoic acid (**6c**; 16-Bz)



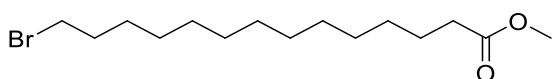
5c (43 mg, 0.12 mmol) was dissolved in THF (3 mL). 580 μ L of a 1 M LiOH solution in H₂O was added dropwise. The reaction mixture was stirred at rt for 4h. The reaction was quenched with 5 mL HCl (1 M), extracted with EtOAc (3x) and the combined organic layers were washed with brine, dried on Na₂SO₄ and concentrated under reduced pressure to yield compound **6c** (18 mg, 0.050 mmol, 44%). **¹H NMR** (400 MHz, CDCl₃) δ 7.49 (s, 1H), 7.41 (d, J = 3.9 Hz, 4H), 4.37 (s, 1H), 3.99 (s, 1H), 3.60 (s, 1H), 3.39 (s, 1H), 2.35 (t, J = 7.4 Hz, 2H), 2.29 (s, 1H), 1.64 (p, J = 7.3 Hz, 4H), 1.40 – 1.16 (m, 14H). **LC-MS** method 1: 1.66 min, m/z calc. for C₂₂H₃₁NO₃ [M+H]⁺ 358.23; found: 358.31.

Methyl 14-bromotetradecanoate (**2b**)



14-bromododecan-1-ol (2.0 g, 6.8 mmol) was dissolved in acetone (50 mL). Jones reagent (10 mL) was added dropwise and the reaction mixture was stirred for 2h on ice. Half of the acetone was removed by rotary evaporation and H₂O (50 mL) was added. The aqueous mixture was extracted with Et₂O (3x 75 mL). The combined organic layers were washed with H₂O (2x 25 mL) and brine before it was dried on Na₂SO₄ and concentrated under reduced pressure to yield compound **2a** (2.1 g, 6.8 mmol, quantitative). **¹H NMR** (400 MHz, Chloroform-*d*) δ 3.40 (t, J = 6.9 Hz, 2H), 2.34 (t, J = 7.5 Hz, 2H), 1.85 (p, J = 7.0 Hz, 2H), 1.63 (q, J = 7.3 Hz, 2H), 1.43 – 1.26 (m, 20H). **¹³C NMR** (101 MHz, CDCl₃) δ 180.15, 77.32, 77.00, 76.68, 34.40, 34.04, 33.95, 32.83, 29.52, 29.48, 29.40, 29.37, 29.19, 29.02, 28.74, 28.16, 25.00, 24.64.

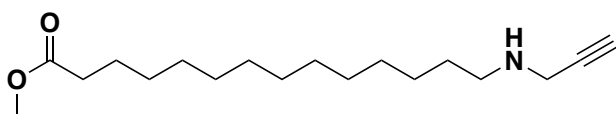
Methyl 14-bromotetradecanoate (**3b**)



2a (1.0 g, 3.4 mmol) was dissolved in dry MeOH (10 mL), H₂SO₄ (200 μ L) was added and the mixture was stirred at 75°C for 2h. The mixture was then concentrated under reduced pressure until ~80% of the MeOH was evaporated. H₂O (50 mL) was added, and the aqueous mixture was extracted with Et₂O (3x). The combined organic layers

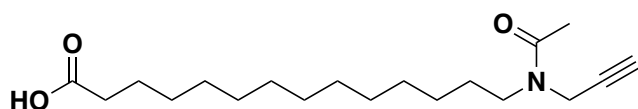
were washed with NaHCO_3 (aq.) and brine before it was dried on Na_2SO_4 , filtered and concentrated under reduced pressure to yield compound **3b** (1.05g, 3.4 mmol, quantitative). $^1\text{H NMR}$ (400 MHz, Chloroform- d) δ 3.65 (s, 3H), 3.39 (t, J = 6.9 Hz, 2H), 2.28 (t, J = 7.5 Hz, 2H), 1.84 (dt, J = 14.4, 6.9 Hz, 2H), 1.61 (q, J = 7.4 Hz, 2H), 1.45 – 1.34 (m, 2H), 1.33 – 1.20 (m, 18H). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 174.23, 77.32, 77.00, 76.68, 51.34, 34.06, 33.90, 32.81, 29.50, 29.46, 29.37, 29.19, 29.10, 28.71, 28.13, 25.71, 24.91.

Methyl 14-(prop-2-yn-1-ylamino)tetradecanoate (**4b**)



3b (0.5 g, 1.63 mmol) was dissolved in ACN (15 mL), propargylamine (1.046 mL, 16.3 mmol) was added, and the reaction mixture was stirred at 85°C o/n. The solution was concentrated to ~4 mL and the precipitate was collected by filtration. The filtrate was washed with cold ACN to yield compound **4b** (280 mg, 95 mmol, 58%). $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 3.86 (d, J = 2.6 Hz, 2H), 3.66 (s, 3H), 3.18 – 3.04 (m, 2H), 2.59 (t, J = 2.6 Hz, 1H), 2.30 (t, J = 7.6 Hz, 2H), 1.90 (p, J = 7.7 Hz, 2H), 1.61 (t, J = 7.3 Hz, 2H), 1.47 – 1.18 (m, 18H). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 174.36, 78.44, 77.32, 77.00, 76.68, 72.43, 51.44, 46.26, 35.97, 34.11, 29.52, 29.46, 29.40, 29.35, 29.23, 29.13, 28.95, 26.75, 25.72, 24.94.

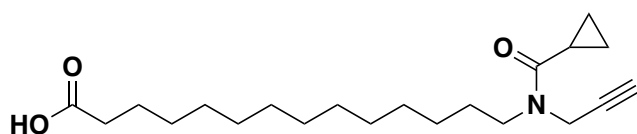
14-(N-(prop-2-yn-1-yl)acetamido)tetradecanoic acid (**6d**; **18-Ac**).



4b (60 mg, 0.2 mmol) was dissolved in dry CH_2Cl_2 (5 mL). Acetyl chloride (21 μL , 0.3 mmol) was added and the solution was cooled on ice. DIPEA (41 μL , 0.3 mmol) in CH_2Cl_2 (1 mL) was added dropwise. The reaction mixture was stirred for 2h before NaHCO_3 (5 mL) was added. The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 . The combined organic layers were washed with brine, dried on MgSO_4 and concentrated under reduced pressure. The residue was purified by flash chromatography over silica gel using c-Hex/EtOAc (2:1 $\rightarrow$ 1:3) to yield compound **5d** (50 mg, 0.015 mmol, 75%). **5d** was used directly for ester hydrolysis without further purification.

5d (40 mg, 0.11 mmol) was dissolved in THF (5 mL). 1.2 mL of a 1 M LiOH solution in H₂O was added dropwise. The reaction mixture was stirred at rt o/n. The reaction was quenched with 5 mL HCl (1 M), extracted with EtOAc (3x) and the combined organic layers were washed with brine, dried on Na₂SO₄ and concentrated under reduced pressure. The residue was purified by flash chromatography over silica gel using c-Hex/EtOAc (1:2 → 1:5) to yield compound **6d** (25 mg, 0.077 mmol, 70%). **¹H NMR** (400 MHz, CDCl₃) δ 4.20 and 3.99 (d, *J* = 2.5 Hz, 2H), 3.46 – 3.34 (m, 2H), 2.33 (t, *J* = 7.5 Hz, 2H), 2.18 and 2.12 (m, 4H), 1.68 – 1.50 (m, 4H), 1.37 – 1.22 (m, 18H). **¹³C NMR** (101 MHz, CDCl₃) δ 178.93, 178.84, 170.69, 170.35, 79.17, 78.61, 77.32, 77.00, 76.68, 72.41, 71.44, 48.14, 46.37, 38.34, 34.10, 33.98, 29.67, 29.50, 29.48, 29.45, 29.44, 29.39, 29.35, 29.29, 29.25, 29.19, 29.10, 29.02, 28.97, 28.34, 27.48, 26.81, 26.72, 24.69, 21.66, 21.26. **LC-MS** method 1: 1.67 min, *m/z* calc. for C₁₉H₃₃NO₃ [M+H]⁺ 324.26; found 324.27.

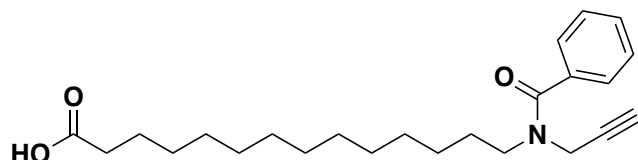
14-(N-(prop-2-yn-1-yl)cyclopropanecarboxamido)tetradecanoic acid (6e; 18-cPr)



4b (40 mg, 0.13 mmol) was dissolved in dry CH₂Cl₂ (4 mL). cyclopropane carbonyl chloride (24 μL, 0.26 mmol) was added and the solution was cooled on ice. DIPEA (45 μL, 0.26 mmol) in CH₂Cl₂ (1 mL) was added dropwise. The reaction mixture was stirred for 2h before NaHCO₃ (5 mL) was added. The layers were separated and the aqueous layer was extracted with CH₂Cl₂. The combined organic layers were washed with brine, dried on MgSO₄ and concentrated under reduced pressure. The residue was purified by flash chromatography over silica gel using Petroleum ether/EtOAc (2:1 → 1:3) to yield compound **5e** (41 mg, 0.112 mmol, 92%). **5e** was used directly for ester hydrolysis without further purification. **5e** (41 mg, 0.112 mmol) was dissolved in THF (5 mL). 1.2 mL of a 1 M LiOH solution in H₂O was added dropwise. The reaction mixture was stirred at rt o/n. The reaction was quenched with 5 mL HCl (1 M), extracted with EtOAc (3x) and the combined organic layers were washed with brine, dried on Na₂SO₄ and concentrated under reduced pressure. The residue was purified by flash chromatography over silica gel using c-Hex/EtOAc (1:2 → 1:5) to yield compound **6e** (31 mg, 0.088 mmol, 68%). **¹H NMR** (400 MHz, CDCl₃) δ 4.22 and 4.19 (d, 2.6 Hz, 2H), 3.56 and 3.42 (t, *J* = 7.6 Hz, 2H), 2.33 (t, *J* = 7.5 Hz, 2H), 2.29 and 2.17 (s, 1H),

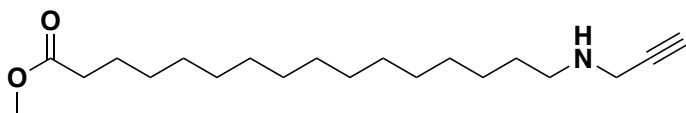
1.83 – 1.49 (m, 5H), 1.37-1.20 (m, 18H), 1.04 – 0.97 (m, 2H), 0.82 – 0.74 (m, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 179.26, 173.29, 79.56, 77.35, 77.04, 76.72, 72.16, 71.31, 47.45, 47.11, 37.62, 35.05, 34.01, 29.56, 29.53, 29.51, 29.39, 29.33, 29.23, 29.15, 29.05, 28.93, 27.66, 26.86, 24.71, 11.71, 11.24, 8.00, 7.77. LC-MS method 1: 1.72 min, m/z calc. for C₂₁H₃₅NO₃ [M+H]⁺ 350.26; found 350.36.

14-(N-(prop-2-yn-1-yl)benzamido)tetradecanoic acid (6f; 18-Bz).



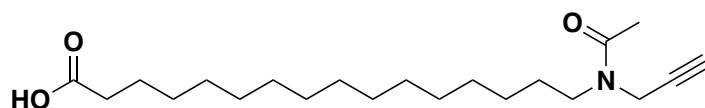
4b (85 mg, 0.29 mmol) was dissolved in dry CH₂Cl₂ (4 mL). Benzoyl chloride (31 μL, 0.29 mmol) was added and the solution was cooled on ice. DIPEA (101 μL, 0.58 mmol) in CH₂Cl₂ (1 mL) was added dropwise. The reaction mixture was stirred for 2h before NaHCO₃ (5 mL) was added. The layers were separated and the aqueous layer was extracted with CH₂Cl₂. The combined organic layers were washed with brine, dried on MgSO₄ and concentrated under reduced pressure. The residue was purified by flash chromatography over silica gel using c-Hex/EtOAc (2:1 → 1:3) to yield compound **5f** (68 mg, 0.17 mmol, 59%). **5f** was used directly for the ester hydrolysis without further purification. **5f** (40 mg, 0.112 mmol, 39%) was dissolved in THF (5 mL). 1.2 mL of a 1 M LiOH solution in H₂O was added dropwise. The reaction mixture was stirred at rt o/n. The reaction was quenched with 5 mL HCl (1 M), extracted with EtOAc (3x) and the combined organic layers were washed with brine, dried on Na₂SO₄ and concentrated under reduced pressure. The residue was purified by flash chromatography over silica gel using c-Hex/EtOAc (1:2 → 1:5) to yield compound **6f** (28 mg, 0.072 mmol, 67%). ¹H NMR (400 MHz, CDCl₃) δ 7.48 (s, 1H), 7.41 (s, 4H), 4.37 (s, 1H), 3.98 (s, 1H), 3.60 (s, 1H), 3.37 (s, 1H), 2.33 (t, *J* = 7.5 Hz, 2H), 2.28 (s, 1H), 1.72-1.50 (m, 4H), 1.43 – 1.10 (m, 18H). ¹³C NMR (101 MHz, CDCl₃) δ 179.40, 135.91, 129.70, 128.46, 126.81, 78.92, 72.42, 71.95, 34.05, 29.53, 29.52, 29.39, 29.21, 29.05, 24.71. LC-MS method 1: 1.66 min, m/z calc. for C₂₄H₃₅NO₃ [M+H]⁺ 386.26; found 386.35.

Methyl 16-(prop-2-yn-1-ylamino)hexadecanoate (4c)



Methyl 16-bromohexadecanoate (0.5 g, 1.43 mmol) was dissolved in ACN (15 mL), propargylamine (916 μ L, 14.3 mmol) was added and the reaction mixture was stirred at 85°C o/n. The solution was concentrated, cooled down and the resulting precipitate was collected by filtration, then washed with cold ACN to yield compound **4c** (391 mg, 1.21 mmol, 85%). **¹H NMR** (400 MHz, CDCl₃) δ 3.86 (d, *J* = 2.6 Hz, 2H), 3.67 (s, 3H), 3.17 – 3.09 (m, 2H), 2.59 (t, *J* = 2.6 Hz, 1H), 2.30 (t, *J* = 7.6 Hz, 2H), 1.89 (q, *J* = 7.9 Hz, 2H), 1.61 (d, *J* = 7.4 Hz, 2H), 1.41 (t, *J* = 7.7 Hz, 2H), 1.35 – 1.23 (m, 22H). **¹³C NMR** (101 MHz, CDCl₃) δ 174.39, 78.38, 72.54, 51.46, 46.31, 35.99, 34.14, 29.63, 29.59, 29.51, 29.45, 29.39, 29.27, 29.17, 29.00, 26.79, 25.81, 24.97.

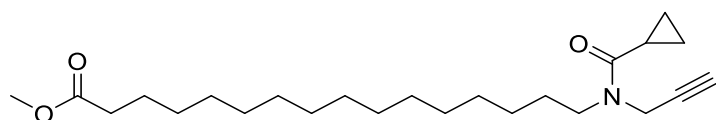
16-(N-(prop-2-yn-1-yl)acetamido)hexadecanoic acid (**6g**; **20-Ac**)



4c (50 mg, 0.15 mmol) was dissolved in dry CH₂Cl₂ (3 mL). Acetyl chloride (21 μ L, 0.3 mmol) was added and the solution was cooled on ice. DIPEA (54 μ L, 0.3 mmol) in CH₂Cl₂ (1 mL) was added dropwise. The reaction mixture was stirred for 2h before NaHCO₃ (5 mL) was added. The layers were separated and the aqueous layer was extracted with CH₂Cl₂. The combined organic layers were washed with brine, dried on MgSO₄ and concentrated under reduced pressure. The residue was purified by flash chromatography over silica gel using c-Hex/EtOAc (2:1 $\rightarrow$ 1:3) to yield compound **5g** (10 mg, 0.027 mmol, 18%).

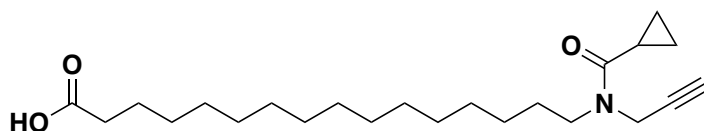
5g (10 mg, 0.027 mmol) was dissolved in THF (1 mL). 140 μ L of a 1 M LiOH solution in H₂O was added dropwise. The reaction mixture was stirred at rt for 4h. The reaction was quenched with 1 mL HCl (1 M), extracted with EtOAc (3x) and the combined organic layers were washed with brine, dried on Na₂SO₄ and concentrated under reduced pressure. to yield compound **6g** (6 mg, 0.017 mmol, 60%). **¹H NMR** (400 MHz, CDCl₃) δ 4.21 (d, *J* = 2.5 Hz, 2H), 4.00 (d, *J* = 2.5 Hz, 1H), 3.41 – 3.34 (m, 2H), 2.18 (d, *J* = 1.9 Hz, 2H), 2.12 (s, 3H), 1.62 (q, *J* = 7.3 Hz, 4H), 1.26 (d, *J* = 2.9 Hz, 22H). **¹³C NMR** (101 MHz, CDCl₃) δ 178.09, 169.78, 72.40, 71.43, 48.15, 38.36, 34.09, 29.57, 29.28, 29.22, 26.74, 24.72, 21.33. **LC-MS** method 1: 1.61 min, *m/z* calc. for C₂₁H₃₇NO₃ [M+H]⁺ 352.28; found 352.36.

Methyl 16-(N-(prop-2-yn-1-yl)cyclopropanecarboxamido)hexadecanoate (**5h**)



4c (90 mg, 0.28 mmol) was dissolved in dry CH_2Cl_2 (5 mL). Cyclopropanecarbonyl chloride (50 μL , 0.56 mmol) was added and the solution was cooled on ice. DIPEA (97 μL , 0.56 mmol) in CH_2Cl_2 (1 mL) was added dropwise. The reaction mixture was stirred for 2h before NaHCO_3 (5 mL) was added. The layers were separated and the aqueous layer was extracted with CH_2Cl_2 . The combined organic layers were washed with brine, dried on MgSO_4 and concentrated under reduced pressure. The residue was purified by flash chromatography over silica gel using c-Hex/EtOAc (2:1 $\rightarrow$ 1:3) to yield compound **5h** (53 mg, 0.14 mmol, 49%). **^1H NMR** (400 MHz, CDCl_3) δ 4.26 – 4.19 (m, 2H), 3.68 (s, 3H), 3.57 (d, J = 7.8 Hz, 1H), 2.32 (t, J = 7.5 Hz, 2H), 1.77 – 1.56 (m, 5H), 1.29 (d, J = 11.8 Hz, 23H), 1.03 (dq, J = 8.5, 4.5, 3.7 Hz, 2H), 0.80 (dt, J = 7.8, 3.4 Hz, 2H). **^{13}C NMR** (101 MHz, CDCl_3) δ 174.36, 173.13, 79.64, 71.24, 51.45, 47.41, 34.98, 34.13, 29.64, 29.59, 29.57, 29.45, 29.35, 29.26, 29.16, 28.95, 26.87, 24.97, 11.20, 7.96, 7.70.

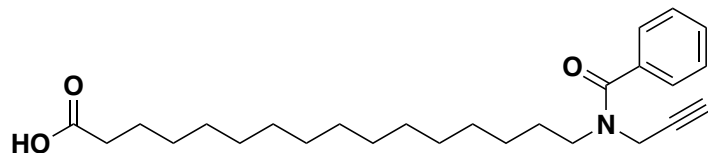
16-(N-(prop-2-yn-1-yl)cyclopropanecarboxamido)hexadecanoic acid (**6h**; 20-cPr)



5h (53 mg, 0.14 mmol) was dissolved in THF (3 mL). 700 μL of a 1 M LiOH solution in H_2O was added dropwise. The reaction mixture was stirred at rt for 4h. The reaction was quenched with 1 mL HCl (1 M), extracted with EtOAc (3x) and the combined organic layers were washed with brine, dried on Na_2SO_4 and concentrated under reduced pressure to yield compound **6h** (32 mg, 0.085 mmol, 61%). **^1H NMR** (400 MHz, CDCl_3) δ 4.21 (dd, J = 11.6, 2.5 Hz, 2H), 3.60 – 3.51 (m, 1H), 3.43 (t, J = 7.6 Hz, 1H), 2.34 (t, J = 7.5 Hz, 2H), 2.18 (t, J = 2.5 Hz, 1H), 1.85 – 1.56 (m, 5H), 1.36 – 1.24 (m, 22H), 1.01 (td, J = 6.3, 5.4, 2.7 Hz, 2H), 0.78 (dt, J = 7.9, 3.4 Hz, 2H). **^1H NMR** (400 MHz, CDCl_3) δ 4.23 (dd, J = 11.6, 2.5 Hz, 2H), 3.62 – 3.55 (m, 1H), 3.45 (t, J = 7.6 Hz, 1H), 2.36 (t, J = 7.5 Hz, 2H), 1.78 – 1.61 (m, 4H), 1.29 (d, J = 10.3 Hz, 20H), 1.04 (td, J = 6.4, 5.5, 2.7 Hz, 2H), 0.81 (dt, J = 7.9, 3.4 Hz, 2H). **^{13}C NMR** (101 MHz, CDCl_3) δ 178.88, 173.24, 79.59, 72.13, 71.29, 47.44, 35.03, 33.92, 29.61, 29.54,

29.42, 29.34, 29.24, 29.06, 28.93, 26.86, 24.71, 11.24, 8.00, 7.76. **LC-MS** method 1: 1.61 min, m/z calc. for C₂₃H₃₉NO₃ [M+H]⁺ 378.29; found 378.40.

16-(N-(prop-2-yn-1-yl)benzamido)hexadecanoic acid (6i; 20-Bz)

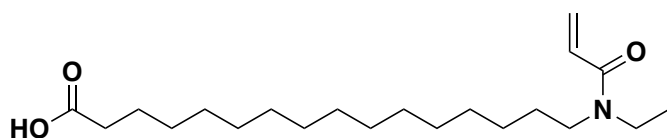


4c (70 mg, 0.22 mmol) was dissolved in dry CH₂Cl₂ (2 mL). Benzoyl chloride (50 μ L, 0.44 mmol) was added and the solution was cooled on ice. DIPEA (76 μ L, 0.44 mmol) in CH₂Cl₂ (1 mL) was added dropwise. The reaction mixture was stirred for 2h before NaHCO₃ (5 mL) was added. The layers were separated, and the aqueous layer was extracted with CH₂Cl₂. The combined organic layers were washed with brine, dried on MgSO₄ and concentrated under reduced pressure. The residue was purified by flash chromatography over silica gel using c-Hex/EtOAc (2:1 $\rightarrow$ 1:3) to yield compound **5i** (58 mg, 0.14 mmol, 63%).

5i (40 mg, 0.094 mmol) was dissolved in THF (3 mL). 470 μ L of a 1 M LiOH solution in H₂O was added dropwise. The reaction mixture was stirred at rt for 4h. The reaction was quenched with 1 mL HCl (1 M), extracted with EtOAc (3x) and the combined organic layers were washed with brine, dried on Na₂SO₄ and concentrated under reduced pressure to yield compound **6i** (12mg, 0.029 mmol, 31%). **¹H NMR** (400 MHz, CDCl₃) δ 7.48 (s, 1H), 7.41 (d, J = 3.6 Hz, 4H), 4.37 (s, 1H), 3.99 (s, 1H), 3.61 (s, 1H), 3.37 (s, 1H), 2.33 (t, J = 7.5 Hz, 2H), 2.28 (s, 1H), 1.62 (p, J = 7.4 Hz, 4H), 1.30 – 1.14 (m, 20H). **¹³C NMR** (101 MHz, CDCl₃) δ 179.62, 136.39, 130.20, 128.90, 127.25, 79.42, 72.26, 34.43, 30.05, 30.03, 29.99, 29.85, 29.67, 29.50, 25.16. **LC-MS** method 1: 1.86 min, m/z calc. for C₂₆H₃₉NO₃ [M+H]⁺ 414.29; found 414.34.

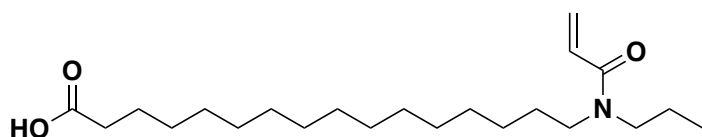
6.6.3. Synthesis of electrophilic probes.

Synthesis of 16-(N-propylacrylamido)hexadecanoic acid **9a**.



3b (111 mg, 0.318 mmol, 1 equiv.) was utilized along with propylamine (0.4 mL, 6.2 mmol, 20 equiv.) to produce methyl methyl 16-(propylamino)hexadecanoate as intermediate using method F. This was reacted with acryloyl chloride (51 μ L, 0.627 mmol, 2 equiv.) to give product **8a** in 42% yield following protocol D. This was then deprotected following the general protocol E to give **9a** as a white powder in 51% yield. ^1H NMR (400 MHz, CDCl_3): δ 6.47 (dd, J = 16.7, 10.4 Hz, 1H), 6.28 (dt, J = 16.7, 2.1 Hz, 1H), 5.59 (dd, J = 10.4, 2.1 Hz, 1H), 3.40 – 3.08 (m, 4H), 2.27 (t, J = 7.5 Hz, 2H), 1.64 – 1.43 (m, 6H), 1.33 – 1.09 (m, 22H), 0.84 (td, J 70 = 7.4, 3.9 Hz, 3H). ^{13}C NMR (400 MHz, CDCl_3): δ 166.11, 127.89, 127.82, 127.73, 77.34, 77.02, 76.71, 49.83, 48.31, 46.85, 33.87, 29.65, 29.57, 29.50, 29.42, 29.35, 29.31, 29.24, 29.11, 28.98, 27.10, 26.81, 24.73, 22.83, 21.04, 11.46, 11.21. UPLC-MS: m/z 268.5 ($M + H^+$), 369.5 ($M + 2H^+$). tr: 1.22 min.

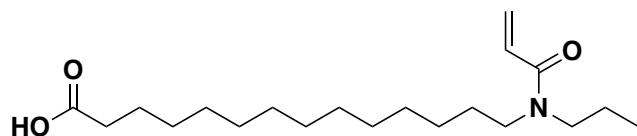
Synthesis of 16-(N-ethylacrylamido)hexadecanoic acid **9b**.



Methyl 16-bromotetradecanoate (118 mg, 0.54 mmol, 1 equiv.) was utilized along with ethylamine 2M in THF (x, 6 mmol, 9 equiv.) to produce methyl 16-(ethylamino)hexadecanoate as intermediate. This was reacted with acryloyl chloride (100 μ L, 1.280 mmol, 2 equiv.) to give product **8b** in 67% yield. This was then deprotected following the general protocol to give **9b** as a white powder in 68% yield. ^1H NMR (400 MHz, CDCl_3): δ (ppm) 6.47 (ddd, J = 16.1, 10.4, 5.5 Hz, 1H), 6.28 (ddd, J = 16.7, 4.2, 2.1 Hz, 1H), 5.60 (ddd, J = 10.4, 4.3, 2.1 Hz, 1H), 3.45 – 3.17 (m, 4H), 2.28 (t, J = 7.5 Hz, 2H), 1.58 – 1.45 (m, 2H), 1.20 (d, J = 14.2 Hz, 24H), 1.10 (dt, J = 16.0, 7.1 Hz, 3H). ^{13}C NMR (400 MHz, CDCl_3): δ 178.47, 178.29, 165.89, 127.91, 127.80, 127.76, 127.64, 77.34, 77.03, 76.71, 47.79, 46.33, 42.67, 41.35, 33.91, 29.67, 29.58, 29.51, 29.46, 29.41, 29.38, 29.31, 29.23, 29.13, 29.06, 29.00, 27.95, 27.09,

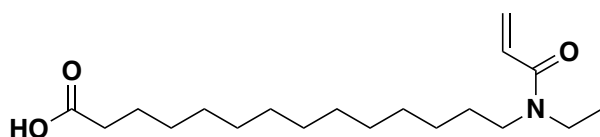
26.81, 24.73, 14.76, 12.97. UPLC-MS: m/z 354.5($M + H^+$), 355.5 ($M + 2H^+$), tr: 1.66 min

Synthesis of 14-(N-propylacrylamido)tetradecanoic acid **9c**.



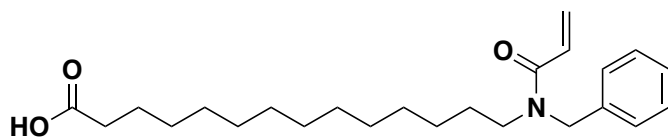
Methyl 14-bromotetradecanoate (95 mg, 0.29 mmol, 1 equiv.) was utilized along with propylamine (3 mmol, 20 equiv.) to 95 produce methyl 16-(ethylamino)hexadecanoate as intermediate. This was reacted with acryloyl chloride (100 μ L, 1.2 mmol, 2 equiv.) to give product **8c** in 58% yield. This was then deprotected following the general protocol to give **9c** as a white powder in 75% yield. ^1H NMR (400 MHz, CDCl_3): δ (ppm) 6.47 (dd, 100 J = 16.7, 10.4 Hz, 1H), 6.28 (ddd, J = 16.7, 2.9, 2.1 Hz, 1H), 5.66 – 5.52 (m, 1H), 3.37 – 3.16 (m, 4H), 2.28 (t, J = 7.4 Hz, 2H), 1.63 – 1.43 (m, 4H), 1.20 (d, J = 10.1 Hz, 18H), 0.84 (td, J = 7.4, 4.0 Hz, 3H). ^{13}C NMR (400 MHz, CDCl_3): δ 178.28, 166.12, 127.87, 127.80, 127.75, 127.66, 77.34, 77.02, 76.71, 49.84, 48.31, 48.24, 105.46, 46.88, 33.91, 29.66, 29.49, 29.43, 29.33, 29.27, 29.22, 29.01, 28.91, 27.78, 27.06, 26.82, 24.71, 22.83, 21.03, 11.45, 11.21. UPLC-MS: m/z 354.5 ($M + H^+$), tr: 1.59 min.

Synthesis of 14-(N-ethylacrylamido)tetradecanoic acid **9d**.



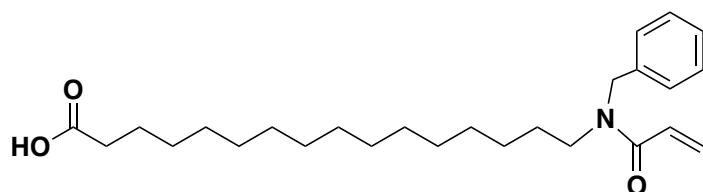
Methyl 14-bromotetradecanoate (75 mg, 0.23 mmol, 1 equiv.) was 110 utilized along with ethylamine 2M in THF (6 mmol, 9 equiv.) to produce methyl 16-(ethylamino)hexadecanoate as intermediate. This was reacted with acryloyl chloride (100 μ L, 1.2 mmol, 5 equiv.) to give product **8d** in 63% yield. This was then deprotected following the general protocol to give **9d** as a white powder in 86% yield. ^1H NMR (400 MHz, CDCl_3): δ 6.47 (ddd, J = 16.7, 10.3, 4.6 5 Hz, 1H), 6.28 (ddd, J = 16.8, 5.2, 2.1 Hz, 1H), 5.60 (ddd, J = 10.4, 5.6, 2.1 Hz, 1H), 3.46 – 3.17 (m, 4H), 2.28 (t, J = 7.4 Hz, 2H), 1.57 (t, J = 7.2 Hz, 2H), 1.29 – 1.02 (m, 23H). ^{13}C NMR (400 MHz, CDCl_3): δ 127.74, 127.61, 77.34, 77.02, 76.71, 46.36, 42.70, 33.82, 29.68, 29.47, 29.36, 29.30, 29.20, 28.87, 27.04, 26.80, 24.71, 10 14.76, 12.98. UPLC-MS: m/z 326.4 ($M + H^+$), tr: 1.52 min.

Synthesis of 14-(N-benhtylacrylamido)hexadecanoic acid **9e**



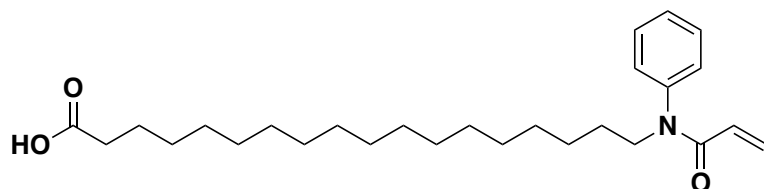
Methyl 16-bromotetradecanoate (117 mg, 0.54 mmol, 1 equiv.) was utilized along with benzylamine (0.68 μ L, 6 mmol, 11 equiv.) to produce methyl 16-(benhtylamino)hexadecanoate as intermediate. This was reacted with acryloyl chloride (100 μ L, 1.2 mmol, 2 equiv.) to give product **8e** in 74% yield. This was then deprotected following the general protocol to give **9e** as a white powder in 47% yield. ^1H NMR (400 MHz, CDCl_3): δ 7.37 – 7.04 (m, 5H), 6.64 – 6.27 (m, 2H), 5.63 (ddd, J = 34.3, 10.2, 2.2 Hz, 2H), 4.56 (d, J = 26.3 Hz, 2H), 3.40 – 3.27 (m, 1H), 3.24 – 3.13 (m, 1H), 2.28 (t, J = 7.5 Hz, 2H), 1.52 (dt, J = 34.7, 7.8 Hz, 4H), 1.33 – 1.06 (m, 18H). ^{13}C NMR (400 MHz, CDCl_3): δ 178.69, 166.52, 128.87, 128.56, 128.46, 128.10, 127.88, 127.56, 127.33, 126.34, 51.19, 49.04, 47.29, 46.83, 33.91, 29.51, 29.45, 29.22, 25 29.05, 28.95, 27.50, 27.02, 26.77, 24.71. UPLC-MS: m/z 388.4 ($M + H^+$) t_r : 1.64 min.

Synthesis of 16-(N-benzylacrylamido)tetradecanoic acid **9f**.



Methyl 14-bromotetradecanoate (96 mg, 0.29 mmol, 1 equiv.) was utilized along with benzylamine (60 μ L, 6 mmol, 20 equiv.) to produce methyl 14-(benzylamino)tetradecanoate as intermediate. This was reacted with acryloyl chloride (100 μ L, 1.2 mmol, 4 equiv.) to give product **8f** in 82% yield. This was then deprotected following the general protocol to give **9f** as a white powder in 55% yield. ^1H NMR (400 MHz, CDCl_3): δ 7.33 – 7.06 (m, 5H), 4.51 (d, J = 22.1 Hz, 2H), 3.67 (dt, J = 28.7, 6.6 Hz, 2H), 3.29 (d, J = 24.4 Hz, 4H), 3.17 – 3.05 (m, 1H), 2.57 (dt, J = 36.0, 6.6 Hz, 2H), 2.27 (t, J = 7.5 Hz, 2H), 1.64 – 1.40 (m, 4H), 1.33 – 1.08 (m, 22H). ^{13}C NMR (400 MHz, CDCl_3): δ 178.57, 166.88, 128.87, 128.58, 128.4, 128.02, 127.66, 126.32, 51.15, 49.04, 47.29, 46.82, 40.33, 39.0, 29.58, 29.50, 29.23, 29.05, 26.78, 24.72. UPLC-MS: m/z 415.3 ($M + H^+$), t_r : 1.75 min.

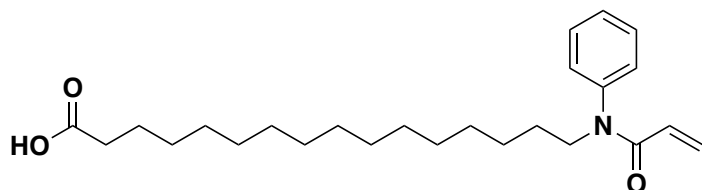
Synthesis of 16-(N-phenylacrylamido)hexadecanoic acid **9g**



Methyl 16-bromotetradecanoate (130 mg, 0.37 mmol, 1 equiv.) was utilized along with aniline (0.67 μ L, 7 mmol, 18 equiv.) to produce methyl 16-(benzylamino)hexadecanoate as intermediate. This was reacted with acryloyl chloride (250 μ L, 1.2 mmol, 3 equiv.) to give product **8g** in 65% yield. This was then deprotected following the general protocol to give **9g** as a white powder in 42% yield. ^1H NMR (400 MHz, CDCl_3): δ 7.40 – 7.24 (m, 5H), 7.13 – 7.04 (m, 2H), 6.29 (dd, J = 16.8, 2.0 Hz, 1H), 5.91 (dd, J = 16.8, 10.3 Hz, 1H), 5.42 (dd, J = 10.3, 2.0 Hz, 1H), 3.72 – 3.66 (m, 2H), 2.27 (t, J = 7.5 Hz, 2H), 1.56 (p, J = 7.5 Hz, 2H), 1.50 – 1.41 (m, 2H), 1.32 – 1.06 (m, 20H). ^{13}C NMR (400 MHz, CDCl_3): δ 129.58, 128.3, 127.72,

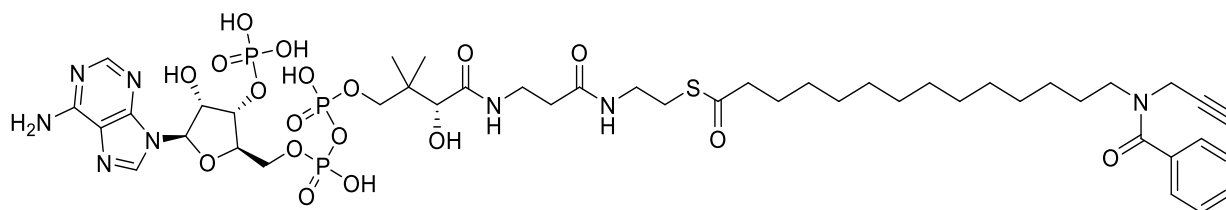
127.49, 49.66, 33.68, 29.38, 27.76, 26.88, 55 24.80. UPLC-MS: m/z 402.4 ($M + H^+$)
tr: 1.77 min.

Synthesis of 14-(N-phenylacrylamido)tetradecanoic acid **9h**.



Methyl 14-bromotetradecanoate (100 mg, 0.31 mmol, 1 equiv.) was utilized along with aniline (0.55 μ L, 6 mmol, 20 equiv.) to produce methyl 14-(phenylamino)tetradecanoate as intermediate. **60** This was reacted with acryloyl chloride (250 μ L, 1.2 mmol, 2 equiv.) to give product **8h** in 89% yield. This was then deprotected following the general protocol to give **9h** as a white powder in 63% yield. ^1H NMR (400 MHz, CDCl_3): δ 7.54 – 7.23 (m, 3H), 7.21 – 7.02 (m, 3H), 6.29 (dd, J = 16.8, 2.0 Hz, 1H), 5.91 (dd, J = 16.8, 10.3 Hz, 1H), 5.43 (dd, J = 10.3, 2.0 Hz, 1H), 3.80 – 3.60 (m, 2H), 2.28 (t, J = 7.4 Hz, 2H), 1.61 – 1.52 (m, 2H), 1.46 (q, J = 7.7, 7.2 Hz, 3H), 1.18 (d, J = 12.7 Hz, 18H). ^{13}C NMR (400 MHz, CDCl_3): δ 178.42, 165.47, 142.07, 129.53, 128.87, 128.31, 127.44, 127.74, 49.63, 33.89, 29.37, 27.72, 26.86, 24.89. UPLC-MS: m/z 374.4 ($M + H^+$). tr: 1.66 min.

6.6.3. Synthesis of 18-Bz CoA.



To a suspension of 14-(N-(prop-2-yn-1-yl)benzamido)tetradecanoic acid (30 mg, 78 μ mol, 2 eq) in dry THF (1.2 mL) was added a solution of 1,1'-carbonyl-diimidazole (15 mg, 94 μ mol, 2.4 eq) in DCM (1.2 mL), under nitrogen atmosphere. The clear reaction mixture was stirred for 45 minutes at room temperature. The reaction mixture was concentrated under reduced pressure. The residue was dissolved in dry THF (1.2 mL). Co-enzyme A hydrate (30 mg, 39 μ mol, 1 eq) was dissolved in an aqueous solution of

NaHCO₃ (0.5 M, 4 mL) and added dropwise to the solution of activated acid. The reaction mixture was stirred at room temperature for 3 hours under nitrogen atmosphere, flash frozen in liquid N₂ and lyophilized overnight. The samples were then dissolved in 1mL H₂O and the product was purified by preparative RP-HPLC over a gradient of 25 mM ammonium acetate pH 8 in ACN. **C18-Bz-CoA 19** was obtained as a white lyophilized solid (22 mg, 47% yield). **¹H NMR** (400 MHz, D₂O) δ 8.51 (s, 1H), 8.20 (s, 1H), 7.35 (dd, *J* = 13.8, 7.6 Hz, 4H), 7.27 (d, *J* = 7.4 Hz, 1H), 6.08 (d, *J* = 6.1 Hz, 1H), 4.80 – 4.70 (m, 2H), 4.53 (p, *J* = 2.8 Hz, 1H), 4.23 – 4.16 (m, 2H), 3.98 (s, 1H), 3.84 (dd, *J* = 9.8, 5.0 Hz, 1H), 3.52 (dd, *J* = 9.8, 4.7 Hz, 1H), 3.37 (p, *J* = 6.7 Hz, 2H), 3.22 (dtd, *J* = 11.1, 7.8, 7.3, 3.4 Hz, 4H), 2.89 (t, *J* = 6.7 Hz, 2H), 2.64 (d, *J* = 2.5 Hz, 1H), 2.42 (t, *J* = 7.4 Hz, 2H), 2.35 (t, *J* = 6.9 Hz, 2H), 1.44 (p, *J* = 7.3 Hz, 4H), 1.14 – 0.91 (m, 18H), 0.87 (s, 3H), 0.70 (s, 3H). **¹³C NMR** (151 MHz, D₂O) δ 174.65, 173.41, 148.71, 140.74, 134.73, 128.69, 126.36, 118.34, 86.96, 82.43, 78.89, 74.06, 73.91, 65.16, 50.96, 43.67, 38.72, 38.44, 38.39, 35.47, 35.37, 29.32, 29.16, 28.98, 28.57, 27.99, 25.36, 21.05, 18.13. **LC-MS** method 2: 4.39 min, *m/z* calc. for C₄₅H₆₉N₈O₁₈P₃S [M+H]⁺ 1135.37; found 1135.5.

Chapter 7:

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Chapter 8:

Appendix

Appendix tables

Table 8.1: List of plasmids used and generated in this study:

Plasmid name	Plasmid vector	Promoter	Expression in	Features	Used For
C-FLAG-D20	pCMV6	CMV	Mammalian Transient	C-terminal FLAG	Transient expression
C-FLAG-D20-C156S	pCMV6	CMV	Mammalian Transient	C-terminal FLAG inactive mutant	Transient expression
C-FLAG-D20-Y181G	pCMV6	CMV	Mammalian Transient	C-terminal FLAG AS mutant	Transient expression
C-FLAG-D20-Y181G/C156S	pCMV6	CMV	Mammalian Transient	C-terminal FLAG AS inactive mutant	Transient expression
C-FLAG-D20-Y181G/S29C	pCMV6	CMV	Mammalian Transient	C-terminal FLAG ES mutant	Transient expression
C-FLAG-empty	pCMV6	CMV	Mammalian Transient	FLAG empty	Transient expression
IFITM3 (<i>Mus Musculus</i>)	pcDNA3	CMV	Mammalian Transient	C-terminal HA-tag	Transient expression
pLVX-TET-empty	pLVX	TRE3GS	Mammalian doxycycline inducible		For cloning purposes
pLVX-TET-WT	pLVX	TRE3GS	Mammalian doxycycline inducible	C-terminal FLAG ZDHHC20 WT	Generation of doxycycline inducible ZDHHC20 cell lines
pLVX-TET-YG	pLVX	TRE3GS	Mammalian doxycycline inducible	C-terminal FLAG ZDHHC20 [Y181G]WT	Generation of doxycycline inducible ZDHHC20 mutant cell lines
pLJM1- empty	pLJM1	CMV	Mammalian constitutive		For cloning purposes
c	pLJM1	CMV	Mammalian constitutive	C- terminus: HA N-terminus: V5, KDEL	Generation of stable ER localised TurboID cell lines
pLJM1- NucTurboID	pLJM1	CMV	Mammalian constitutive	C- terminus: 3xHA N-terminus: 3x NLS	Generation of stable nuclear TurboID cell lines
pLJM1- PmTurboID	pLJM1	CMV	Mammalian constitutive	C- terminus: 2xHA N-terminus: V5, Pdgfrb TM domain	Generation of stable cell lines expressing plasma membrane localised TurboID
pLJM1- CytoTurboID	pLJM1	CMV	Mammalian constitutive	C- terminus: 1x V5 N-terminus: 1 x NES	Generation of stable cells lines expressing cytosolic TurboID
pCMV-VSV-G			2 nd generation lentivirus production		Lentiviral production
pCMV-Delta-8.2			2 nd generation lentivirus production		Lentiviral packaging

Table 8.2: List of PCR primers sets for NEBuilder HiFi Assembly reactions.

Plasmid	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Backbone
pLVX-C-FLAG-D20	CTCGCAGGGGAGGTGGTCTGTAAAA CCTTATCGTCGTCATCCTTGAATCC AGG	CACTTCCTACCCTCGTAAAGAT GGCGCCCTGGACGCTG	pLVX-TetOne-Puro
pcDNA3.1(+)-HA-BCAP31	TCCAGATTACGCTCTTATGGGGATGA GTCTGCAGTGGACTG	GGTACCTCGAGAGATCTCGGTT ACTCTTCCTTCTTGTCATG	pcDNA3.1(+)-HA-IFITM3
N-HA-PI4K2A	CTTATGGCCATGGAGGCCCGAATTC CCATGGACGAGAC	ATCCCCGCGGCCGCGGTACCC TACCACCATGAAAAGAAG	pDONR223-PI4K2A Addgene #23503
N-HA-TOMM20	CTTATGGCCATGGAGGCCCGAATTC CCATGGTGGGTCG	ATCCCCGCGGCCGCGGTACCT TATTCCACATCATCTTCAGCC	mCherry-TOMM20-N-10 Addgene #55146
N-HA-TFAM	CTTATGGCCATGGAGGCCCGAATTC CCATGGCGTTTCTC	ATCCCCGCGGCCGCGGTACCT TAACACTCCTCAGCACC	pcDNA3-TFAM-mCLOVER Addgene #129574
pLJM1-ERTurboID	TGAACCGTCAGATCCGCTAGATGGA GACAGACACACTC	CCATTTGTCTCGAGGTCGAGTT AGAGTTCATCTTTGGTG	pLJM1 (Addgene 91980)
pLJM1-NucTurboID	TGAACCGTCAGATCCGCTAGATGTAC CCGTATGATGTTT	CCATTTGTCTCGAGGTCGAGAG TTGATCACACCTTCCTCTTCTTC	pLJM1 (Addgene 91980)
pLJM1-PmTurboID	TGAACCGTCAGATCCGCTAGCATGG AGACAGACACACTC	CCATTTGTCTCGAGGTCGAGAA TTCTTAACGTGGCTTCTTCTG	pLJM1 (Addgene 91980)
pLJM1-CytoTurboID	TGAACCGTCAGATCCGCTAGCATGG GCAAGCCCATCCCC	CCATTTGTCTCGAGGTCGAGAA TTCTTAGTCCAGGGTCAGGCG	pLJM1 (Addgene 91980)

Table 8.3: Primer sets used for ZDHHC mutagenesis.

Mutant	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Templates
C156S	TCTTAAGATGGATCATCACAGT CCTTGGGTGAATAACTG	CAGTTATTCACCCAAGGACTGTGAT GATCCATCTTAAGA	C-Flag-D20, pLVX-C-Flag-D20
Y181G	CCTGCTGTTTTTATTGTATTCCC TATTAGGTTGCCTTTTCGTGGC TG	CAGCCACGAAAAGGCAACCTAATA GGGAATACAATAAAAACAGCAGG	C-Flag-D20, pLVX-C-Flag-D20
Y181A	CCTGCTGTTTTTATTGTATTCCC TATTAGCTTGCCTTTTCGTGGC TG	CAGCCACGAAAAGGCAAGCTAATA GGGAATACAATAAAAACAGCAGG	C-Flag-D20
I22A	GTGCCGGTGCTCTTCGCCACC TTCGTGGTCGT	ACGACCACGAAGGTGGCGAAGAG CACCGGCAC	C-Flag-D20
F65A	GCTTTCATCTGTTCTTTGTTAT GGCTGTATGGTCCTATTGGATG ACAAT	ATTGTCATCCAATAGGACCATACAG CCATAACAAAGAACAGATGGAAAG C	C-Flag-D20
F174A	GGATTTTCTAATTACAAATTCTT CCTGCTGGCTTTATTGTATTCC CTATTATATTGCCTTTT	AAAAGGCAATATAATAGGGAATACA ATAAAGCCAGCAGGAAGATTTGTA ATTAGAAAATCC	C-Flag-D20
F220A	TTTTCTTTGTGTCTGCAATGGC CTTCATCAGCGTCCTCTCAC	GTGAGAGGACGCTGATGAAGGCCA TTGCAGACACAAAGAAAA	C-Flag-D20

Table 8.4: Guide RNAs for generation of ZDHHC20 knock-out cell lines

Guide RNA	Forward Oligo (5' à 3')	Reverse Oligo (5' à 3')	Exon Targeted
gRNA1	CACCGCGCGCACCCACGTTTT CATA	AAACTATGAAAACGTGGGTGCGCG C	9
gRNA2	CACCGGAAGCTGATGTGGTATA GAT	AAACATCTATACCACATCAGCTTCC	4

Table 8.5. Kinetic analysis of WT, Y181G, C156S and Y181G/C156S ZDHHC20 expression constructs with YnPal-CoA and 18-Bz-CoA.

	WT-ZDHHC20	
Kinetic Parameters	Pal-CoA	C18-Bz-CoA
Vmax ($\mu\text{M NADH} \cdot \text{min}^{-1}$)	0.45 ± 0.006	na
Kcat (min^{-1})	22.5 ± 0.3	na
Km (μM)	3.3 ± 0.2	na
Kcat/Km ($\mu\text{M} \cdot \text{min}^{-1}$)	6.8 ± 0.3	na
	YG-ZDHHC20	
Kinetic Parameters	Pal-CoA	C18-Bz-CoA
Vmax ($\mu\text{M NADH} \cdot \text{min}^{-1}$)	0.45 ± 0.03	0.24 ± 0.01
Kcat (min^{-1})	22.3 ± 1.5	11.8 ± 0.6
Km (μM)	2.9 ± 0.1	0.74 ± 0.02
Kcat/Km ($\mu\text{M} \cdot \text{min}^{-1}$)	7.6 ± 0.3	16.0 ± 1.0

Table 8.6. PTM annotations of ZDHHC20 substrate hits

	Prenylation	Myristoylation	Glycosylation
Replicate 1	ESYT2, RAB5IF, CNP, DCTN5, DNAJC25, NAP1L1, NWD1, UBA6, VAMP7, FAS, NUP210	ATP2A2, BCAP31, FLOT2, TOMM40, SCARB1	ATP1B3, ADM1, CD47, CD63, CERS2, CERS6, ERGIC1, FAS, GALNT1, HM13, IKBIP, ITGB1, ITM2B, LMAN2, M6PR, MBOAT7, NUP210, NUP62, PSMB1, RPN2, SCARB1, SLC1A5, SLC29A1, SLC2A1, SLC38A2, SLC3A2, STIM1, TECR, TMED10, TMEM214, TSPAN6
Replicate 2	FAS, NUP210, RHOA, STIM2, ESYT2, RHEB	BCAP31, FLOT2, RPL27, SURF4, ATP2A2, DEGS1, TSPAN3, VKORC1, SEC61A1, SLC44A1, CLDN12, PTGFRN, RFT1, SLC19A1, SLC1A5, SLC2A1, SLC30A1, SLC38A1, SLC46A1, SLC5A6, SLC7A1	ADAM10, ATP6AP1, B4GALT3, B4GAT1, BSG, CD47, CD63, CERS2, CLPTM1L, ERGIC1, ERGIC3, GPAA1, HM13, ICAM5, IKBIP, LMAN2, M6PR, MAGT1, MBOAT7, NCEH1, NUP210, PLP2, PTGFRN, RFT1, SLC19A1, SLC1A5, SLC2A1, SLC30A1, SLC38A1, SLC46A1, SLC5A6, SLC7A1, STIM1, STT3A, TECR, TMED10, TMEM161A, TMEM194A, TMX3, TSPAN14, TSPAN7, UBAC2
Replicate 3	NUP210, DNAJC25, TM2D2, PRR14L	BCAP31, FLOT2, RPL27, SURF, SERINC1, SLC2A1, SLC6A15, SLC7A1	ADAM9, B4GAT1, BSG, CADM1, CERS2, CERS6, ENPP4, ERMP1, FAS, FLVCR1, GALNT1, IGF2R, IKBIP, LAMP2, LMAN2, M6PR, MAGT1, NUP210, PIGG, PIGT, RHOA, SERINC1, SLC2A1, SLC6A15, SLC7A1, STIM1, STIM2, SYPL1, TGFBR1, TGOLN2, TMED10, TMEM194A, TMEM206, TMEM87A, TMTC3, TSPAN14

Table 8.7. Summary of hits overlapping in OBD experiments.

ZDHHC20 substrates identified with chemical genetics (2/3 experiments)					
ARL6IP6	COMT	GALNT2	NT5C3A	SEC22B	TMED10
ATP2A2	CTDNEP1	GOSR2	NUP210	SLC16A1	TMED2
AUP1	CYB5B	GPR89B	OSBP	SLC19A1	TMEM115
B4GAT1	DHCR24	GPX8	PI4K2A	SLC1A5	TMEM194A
BCAP31	DHRS7B	HM13	POR	SLC2A1	TMEM201
BET1	DNAJC1	HMOX2	PTPN1	SLC35B2	TMEM230
BET1L	DNAJC25	HSDL1	PTRH2	SLC7A1	TMEM43
BSG	EBAG9	IKBIP	RER1	SPCS1	TMUB1
CADM1	EI24	JAGN1	RPL27	SPCS2	TMX2
CD47	ELOVL5	LMAN2	RPS24	SQLE	TSPAN14
CD63	ERGIC1	MPRD	RTN3	STIM1	VAMP3
CERS2	ESYT2	MAGT1	RTN4	SURF4	VAMP8
CERS6	FASN	MANBAL	SCAMP1	TECR	VMP1
CISD2	FLOT2	MBOAT7	SCAMP2	TM9SF2	XXYL1
CKAP4	GALNT1	NDUFB8	SCAMP4	TMED1	ZDHHC20

Table 8.8: Overlap of ZDHHC20 hits with those of ZDHHC7 and ZDHHC15.

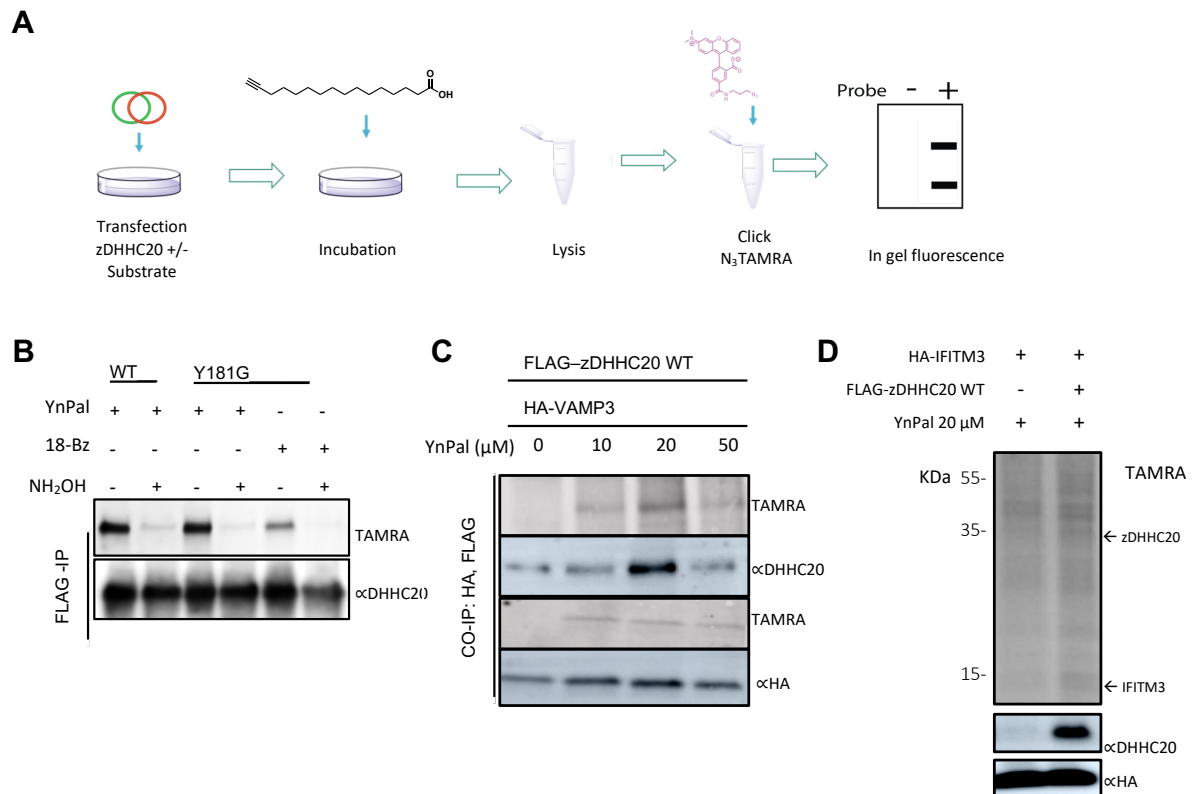
Unique to ZDHHC20	ZDHHC20 and ZDHHC7	ZDHHC20 and ZDHHC15		Common to all
BSG CADM1 CERS6 DHCR24 DNAJC25 ELOVL5 ESYT2 FAS FLOT2 GALNT1 GALNT2 GOSR2 HM13 JAGN1 NDUFB8 NT5C3A OSBP PI4K2A POR RPL27 RTN3 RTN4 SLC16A1 SLC19A1 SLC2A1 SPCS2 TM9SF2 TSPAN14 VAMP8 ZDHHC20	BET1L CERS2 CISD2 EBAG9 EI24 GPR89B HMOX2 HSDL1 MBOAT7 PTPN1 RER1 RPS24 SCAMP2 SCAMP4 SEC22B SLC7A1 SURF4 TMED2 TMEM115 TMEM201	ARL6IP6 ATP2A2 AUP1 B4GAT1 BCAP31 BET1 BET1L CD47 CD63 CKAP4 COMT CTDNEP1 CYB5B DHRS7B DNAJC1 ERGIC1 GPX8 HMOX2 IKBIP LMAN2 M6PR MAGT1 MANBAL	NUP210 PTPN1 PTRH2 SCAMP1 SCAMP2 SLC1A5 SLC35B2 SLC7A1 SPCS1 SQLE STIM1 TECR TMED1 TMED10 TMEM194A TMEM230 TMEM43 TMUB1 TMX2 VAMP3 VMP1 XXYLT1	BET1L HMOX2 PTPN1 SCAMP2 SLC7A1

Table 8.9: List of proteins identified in the secretome of ERTurboID HEK293Ts and their annotated cellular localization.

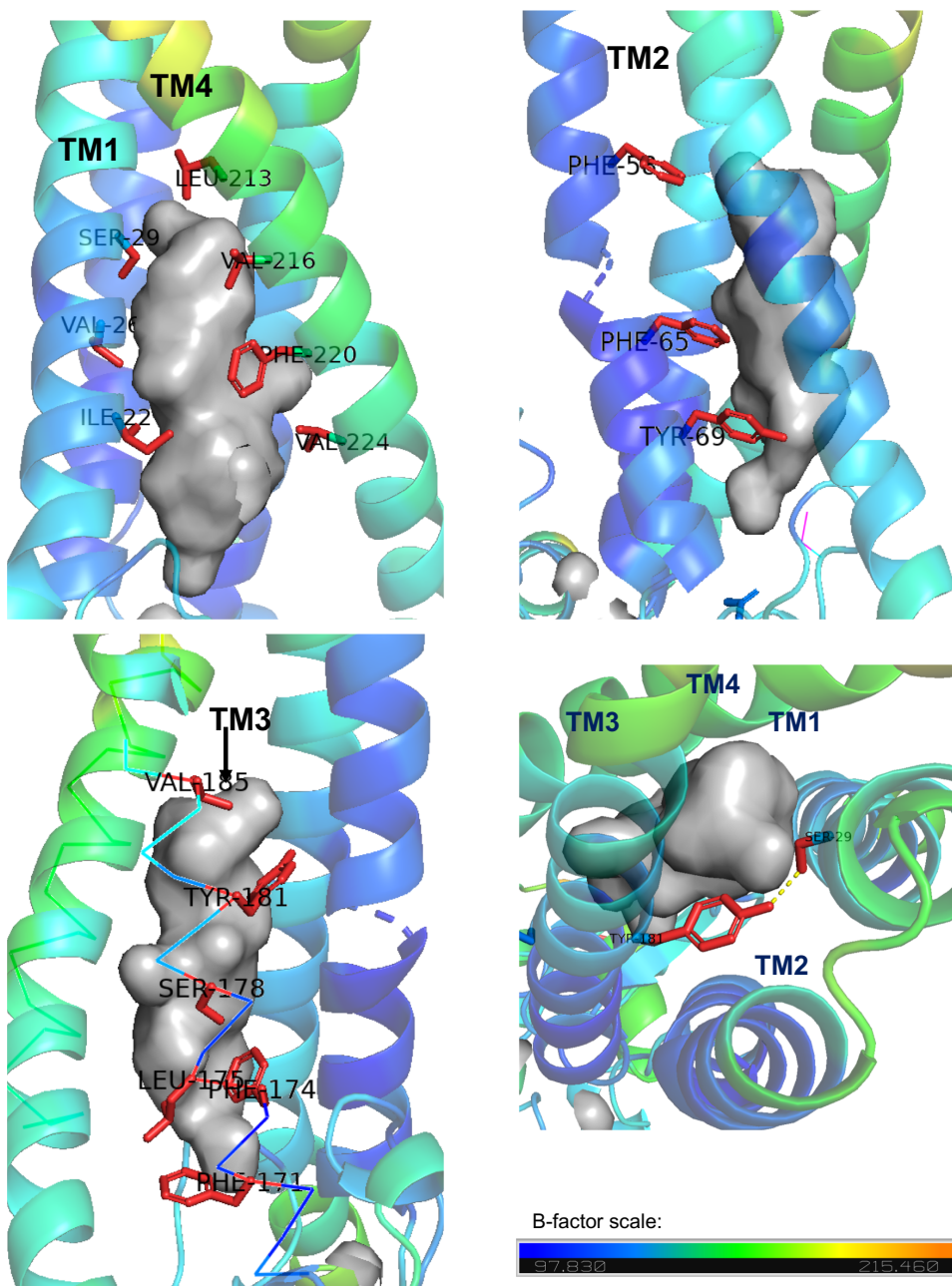
Gene Names	Uniprot annotation for localization
HNRNPA2B1;HNRPA2B1	Exosomes
FKBP1A;FKBP12-Exin	Cytosol Sarcoplasmid reticulum membrane peripheral membrane protein cytoplasmic side
EEF1A1;EEF1A1P5;EEF1A2	Plasma membrane, cytoplasm, nucleus
HSPA1A;HSPA1L;HSPA1B;HSPA6;HSPA7	Secreted by similarity
HIST1H2AG	Nucleus
PCCA	Mitochondrial matrix.
PC	Mitochondrial matrix.
LDHB	Exosomes
HNRNPA1;HNRNPA1L2	Exosomes
NPM1	Cytoskeleton, cytosol, nucleus, cytoplasm
PRSS3	Secreted
CALR	Exosomes
HSPA5	Exosomes
PKM	Extracellular vesicles
PRDX2	Extracellular exosome
ANXA2;ANXA2P2	Extracellular and secreted
RPL23A	Extracellular or secreted
YWHAZ	Extracellular space/ exosome / blood microparticle

PTMA	Nuclear
ANKS3	Cytoplasm/cilium
RPS3A	May be secreted
ANXA5	Secreted
RPS23	ER/nucleus
EGFLAM	Extracellular matrix/synaptic cleft
PCCB	Mitochondrial matrix.
HNRNPH1;HNRNPF;HNRNPH2	Nucleus/cytosol
TKT	Exosomes
HSPA8;HSPA2	Exosomes
CFL1	Extracellular or secreted
TUBA1B;TUBA1A;TUBA1C;TUBA3E	Cytoskeleton
B2M	Extracellular or secreted
ACTG1;ACTA1;ACTG2;ACTC1;ACTA2	Extracellular exosome
SRSF1	Cytoplasm/nucleus
QSOX1	Extracellular or secreted
HIST1H2BB;HIST1H2BL;HIST1H2BN	Nucleus
LDHA	Secreted
PGK1;PGK2	Exosome/secreted
KRT6B	Exosome/secreted
GAPDH	Exosome/secreted
RPLP1	Cytosol
birA	Secreted
ENO1	Exosome/secreted
HSP90AB1;HSP90AB2P;HSP90AB3P ;HSP90AB4P;HSP90AA1	Secreted
NME1	Extracellular or secreted
IGFBP2	Secreted
VCL	Extracellular or secreted
FLNA	Extracellular or secreted
YWHAB;YWHAQ;YWHAZ	Exosome/secreted
ACTB;ACTA1	Extracellular or secreted
YWHAE	Extracellular exosome
HIST1H4A	Extracellular exosome
TUBA4A	Extracellular or secreted
NUCB1	Secreted
COTL1	Extracellular or secreted
HIST2H3PS2;HIST1H3A;H3F3A; HIST2H3A;HIST3H3;H3F3C;H3F3B	Nucleus
ITLN2;ITLN1	Secreted
ST8SIA4	Golgi apparatus membrane
SMOC1	Extracellular or secreted
TLN1	Extracellular or secreted

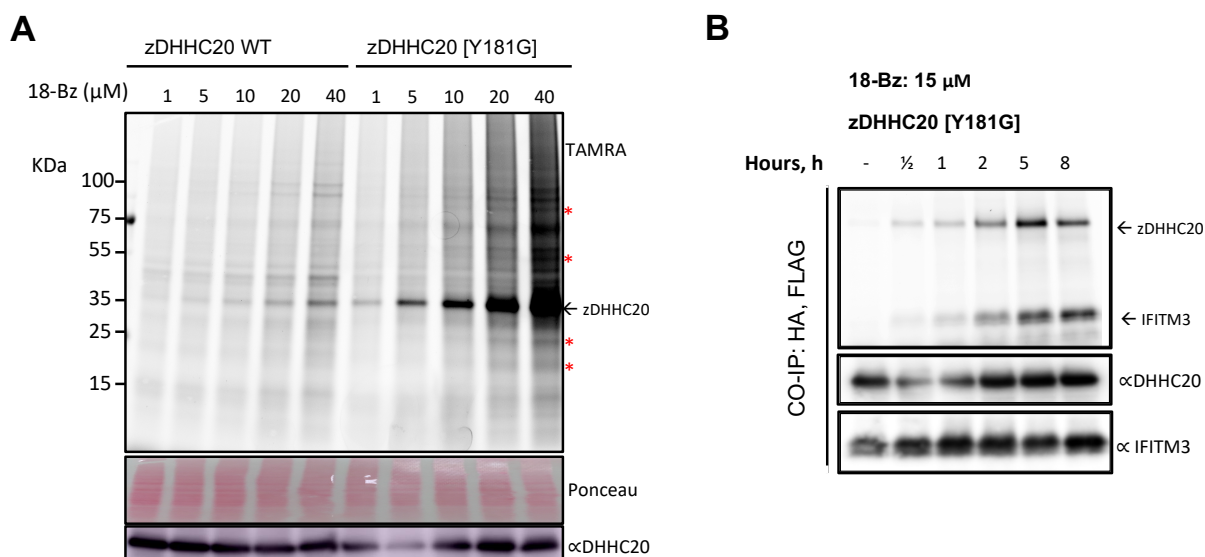
Appendix Figures



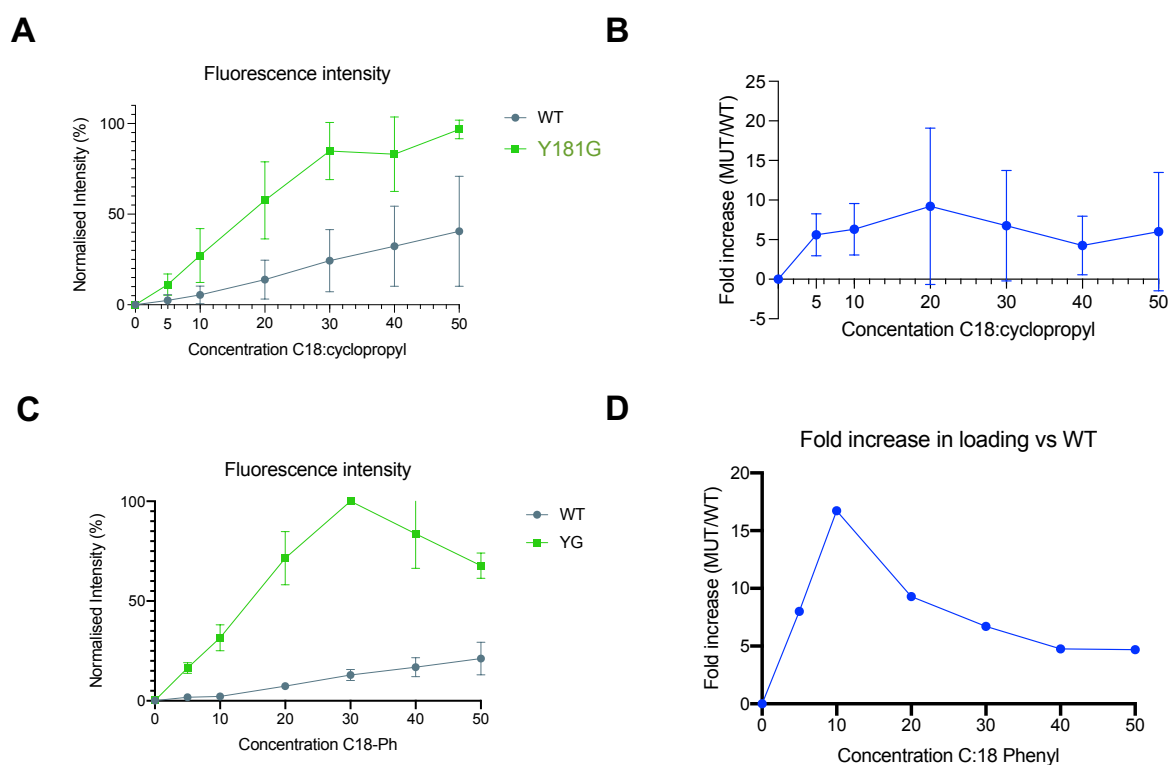
Appendix Figure 8.1. Metabolic with 18-Bz and substrate screens. A) Scheme for general metabolic labelling protocol. B) Hydroxylamine thioester hydrolysis of YnPal and 18-Bz probe from ZDHHC20 WT and ZDHHC[Y181G] expressing HEK293T cells. Figures correspond to a FLAG immunoprecipitation. C -D) Substrate screening in HEK293Ts. Dr. Tony Ocasio kindly provided panel B.



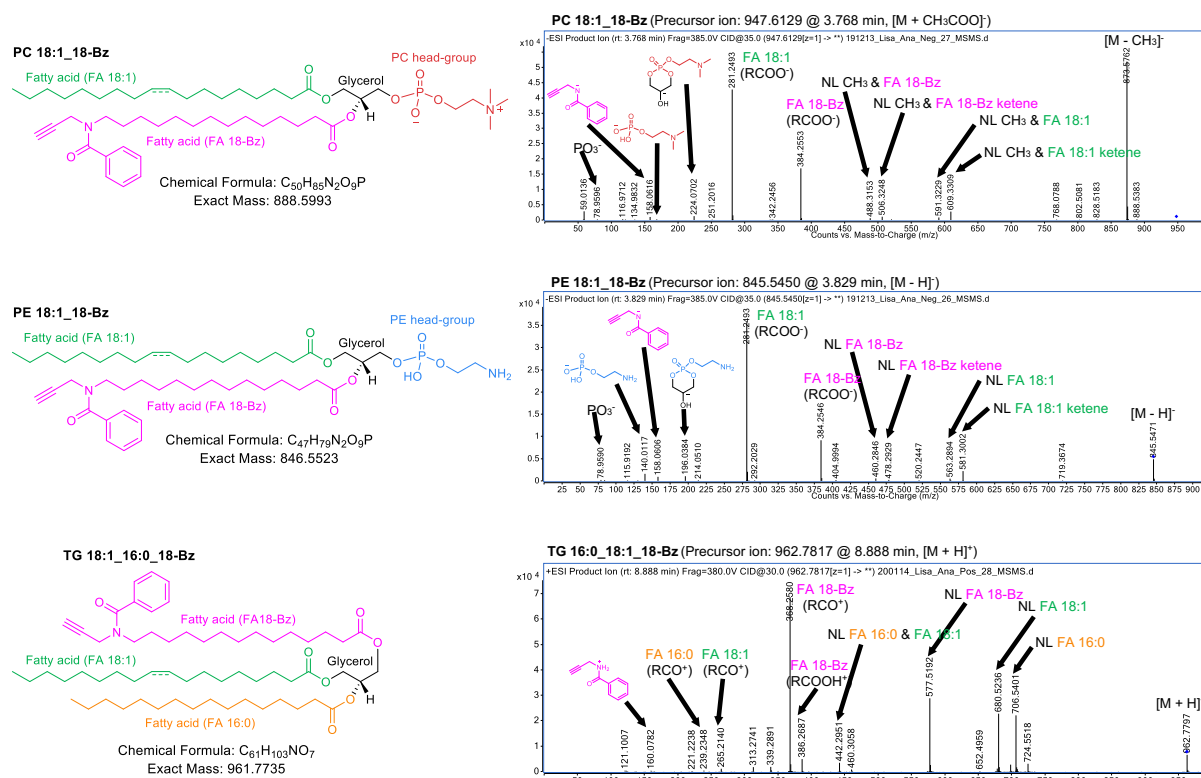
Appendix Figure 8.2. B-factor values in transmembrane helices of ZDHHC20. (PDB: 6BML). indicating residues in contact with lipid binding cavity (surface depicted in grey). Rigidity of different helices based on the in B-factor scale. The less flexible sections in dark blue (B-factor: 97.83) and those most flexible in orange (B-factor: 215.96).



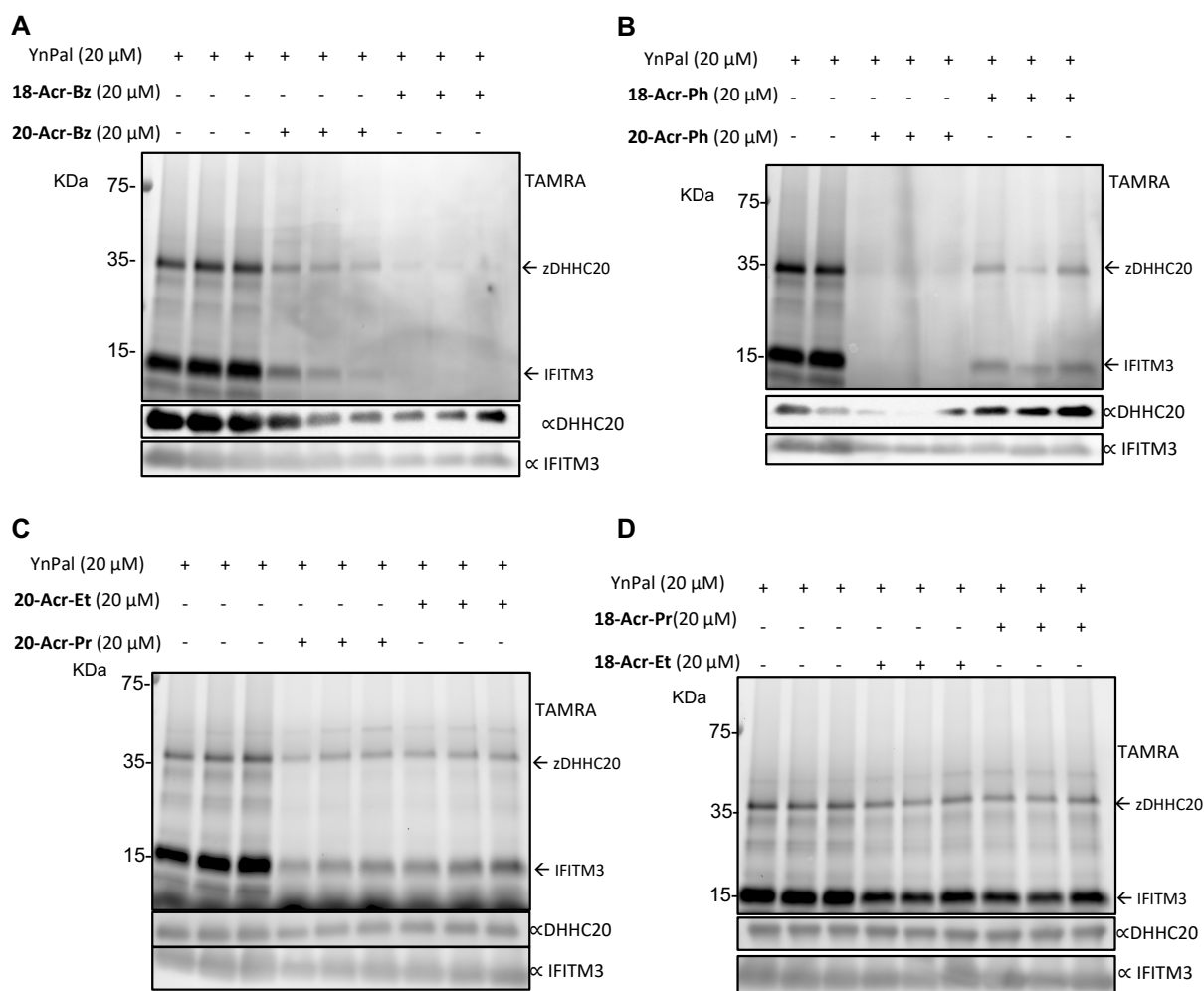
Appendix Figure 8.3. Optimisation of selectivity window with 18-Bz. **A)** HEK293 cells transfected with either wildtype ZDHHC20 or mutant ZDHHC20 [Y181G], treated with varying concentrations of 18-Bz for 8h. Red asterisks represent selective transfer of the probe to substrates. **B)** HEK293 cells transfected with mutant ZDHHC20[Y181G] and HA-IFITM3, incubated with 18-Bz for varying times.



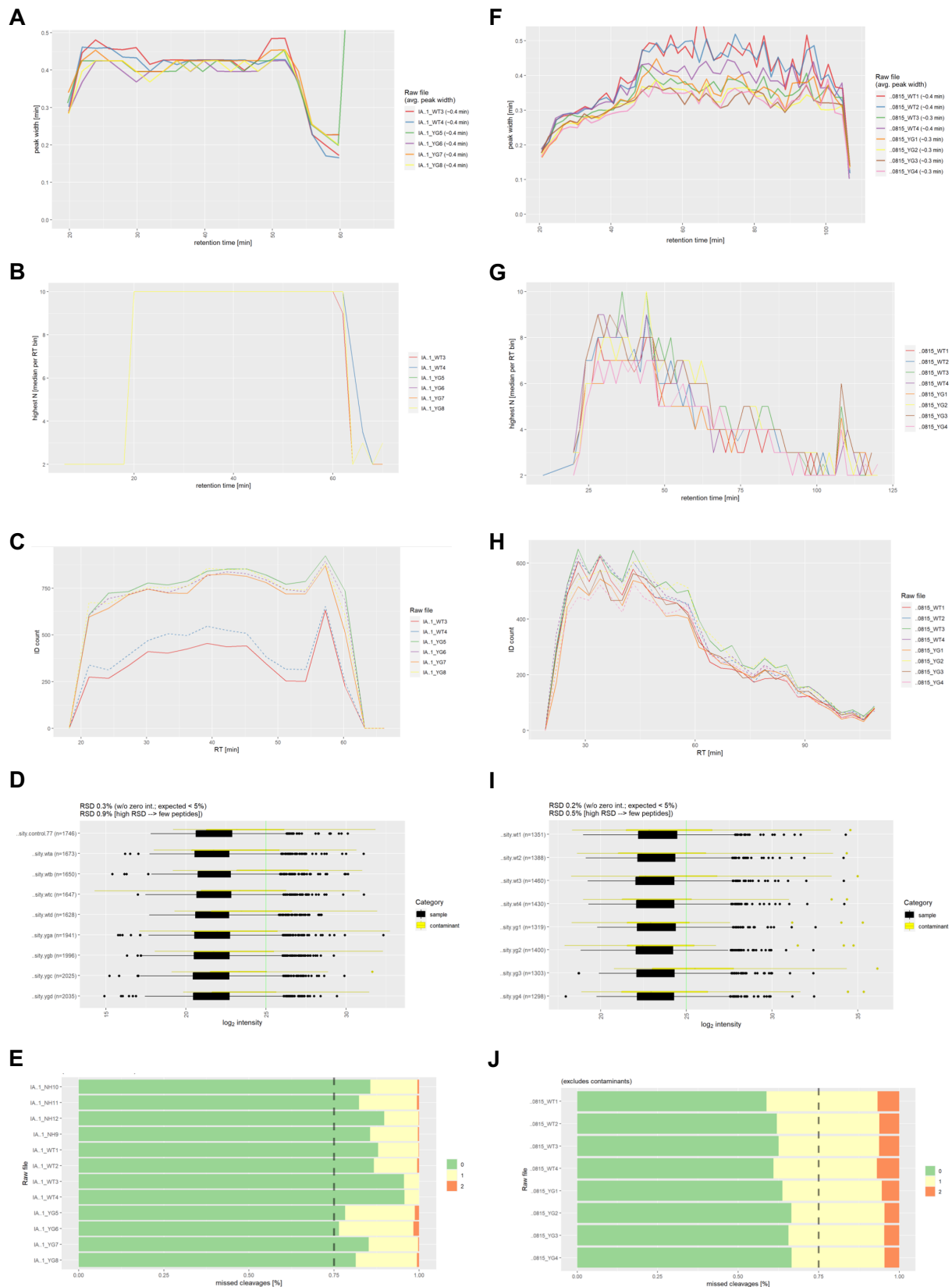
Appendix Figure 8.4. Comparison of 18-Bz and 18-cPr selectivity windows. **A-B)** concentration dependent loading signal of WT and Y181G with varying concentrations of 18-cPr (A) or C18-Bz (B). **C-D)** Fold changes (ZDHHC20[Y181G]/ZDHHC20 WT) of A and B respectively.



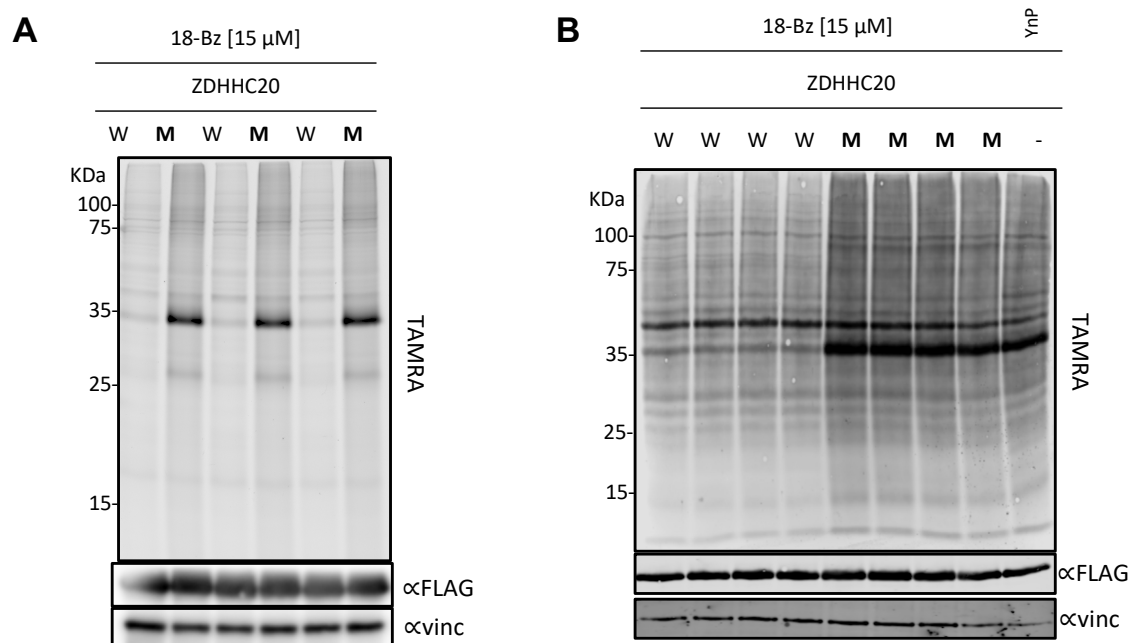
Appendix Figure 8.5. Examples of 18-Bz lipid species and their mass spectrometry fragmentation patterns. Dr. Susana Palma (Metabolomics STP, The Francis Crick Institute)



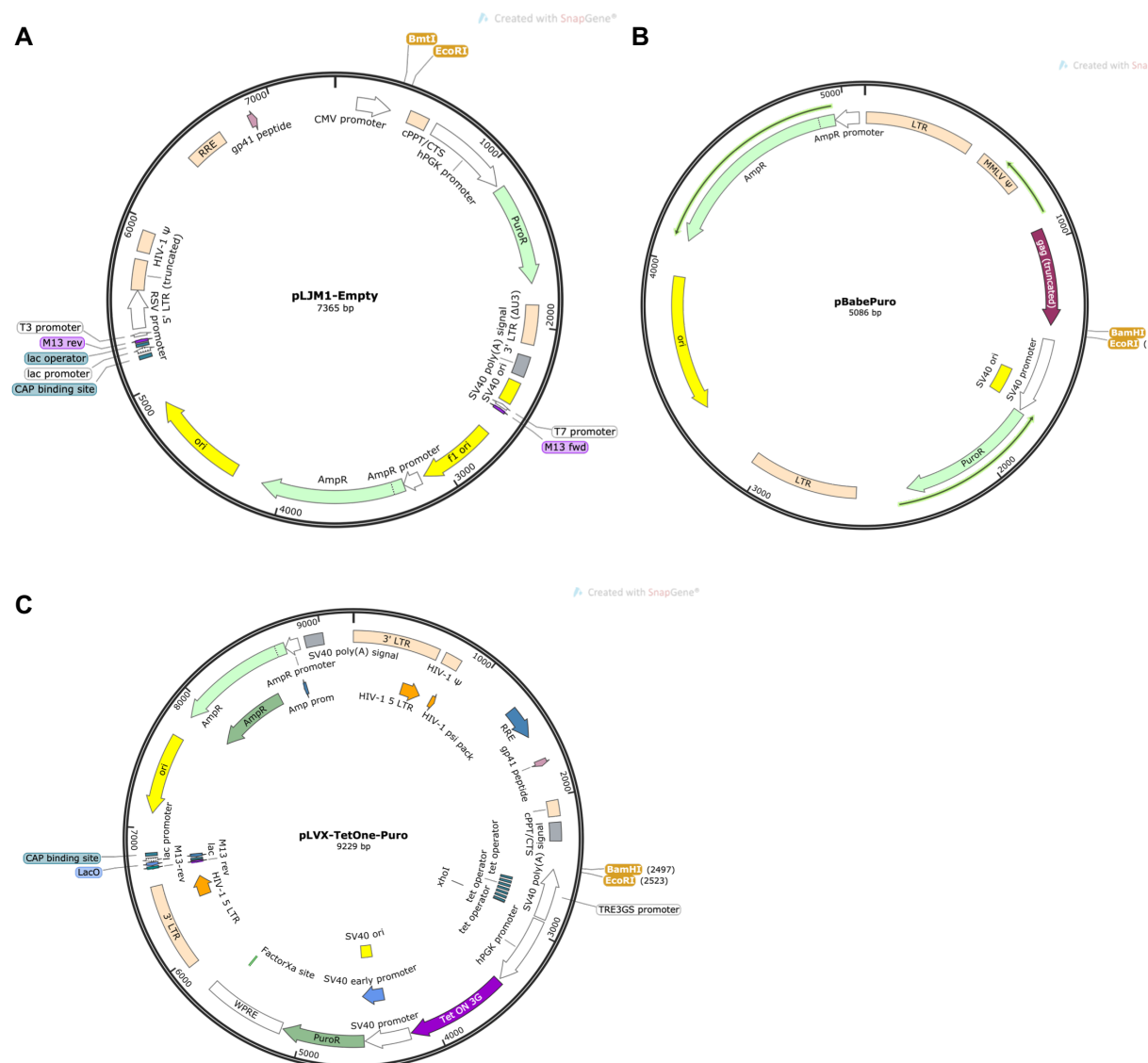
Appendix Figure 8.6. Inhibition of ZDHHC20[Y181G/S29C] with acrylamide probes. HEK293T cells labelled with alkyne palmitate (20 μ M) for 4h and with compounds (20 μ M) for 3h (1h after YnPal). In-gel fluorescence analysis after FLAG immunoprecipitation and click reaction with TAMRA-azide.



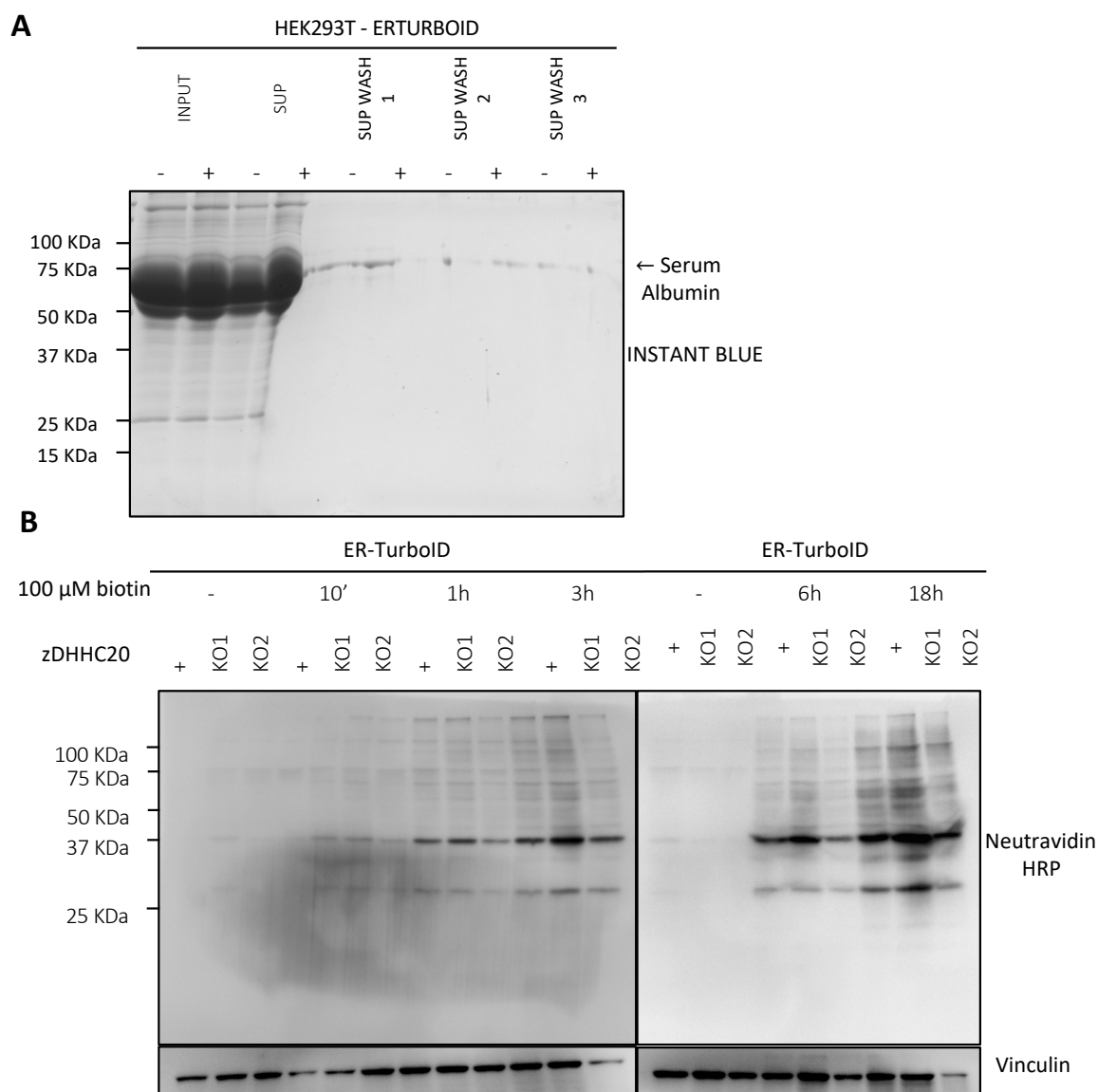
Appendix Figure 8.7. Comparison of mass spectrometry protocols using PTXQC. OBD (right, A-E) and OBH (left, F-J). **A and F)** Peak width stability over retention time. **B and G)** MS/MS scans (N) over retention time (max=10). **C and H)** Gene IDs over retention time. **D and I)** Average log₂ intensities per run. **E and J)** Percentage missed cleavage sites per run.



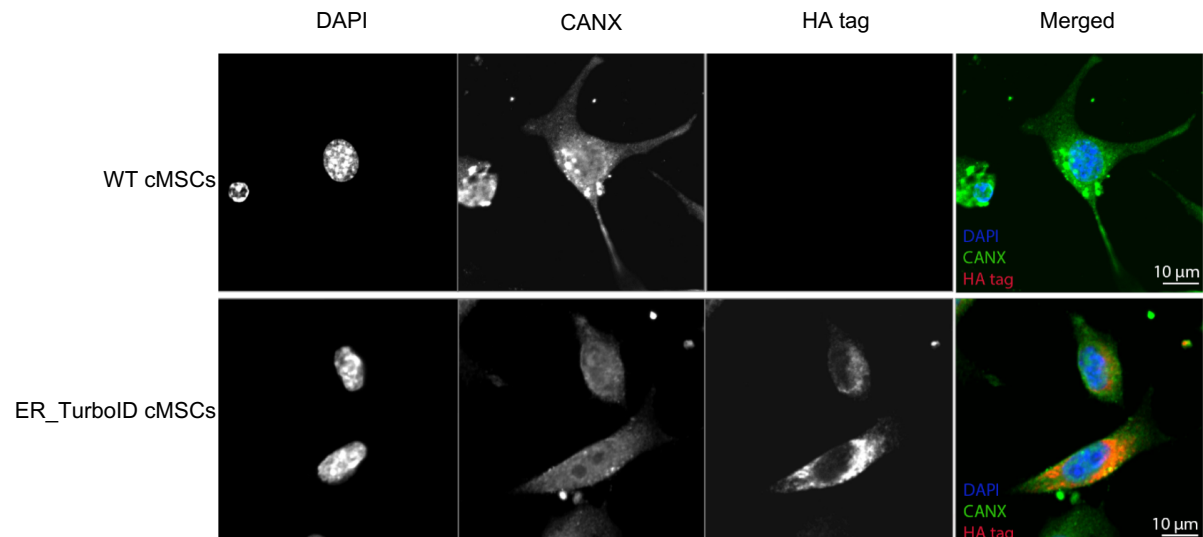
Appendix Figure 8.8. In-gel fluorescence of chemical genetics experiments for ZDHHC20 substrate ID. Replicates of Figure 3.6 A.



Appendix Figure 8.9. Second generation lentiviral delivery plasmid maps used in this study. Highlighted in orange, restriction sites used to introduce desired constructs.



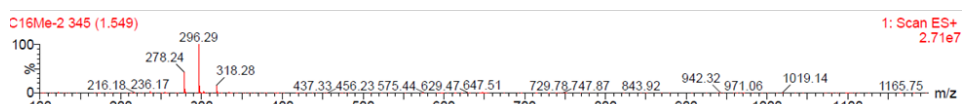
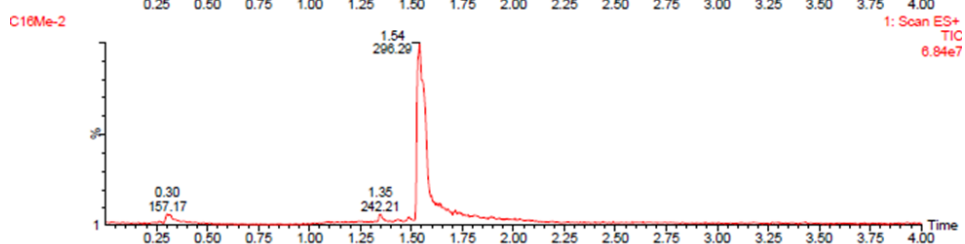
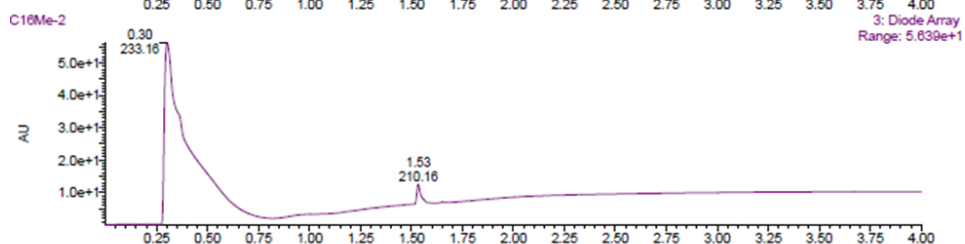
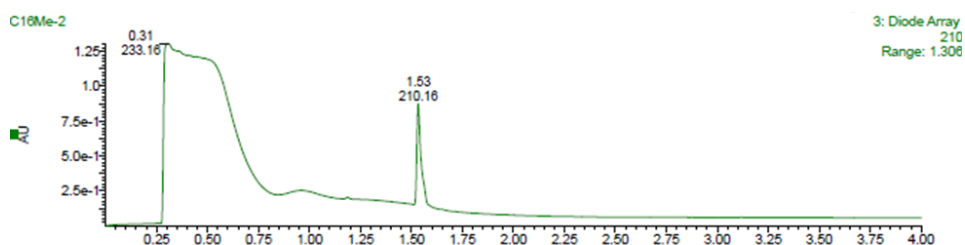
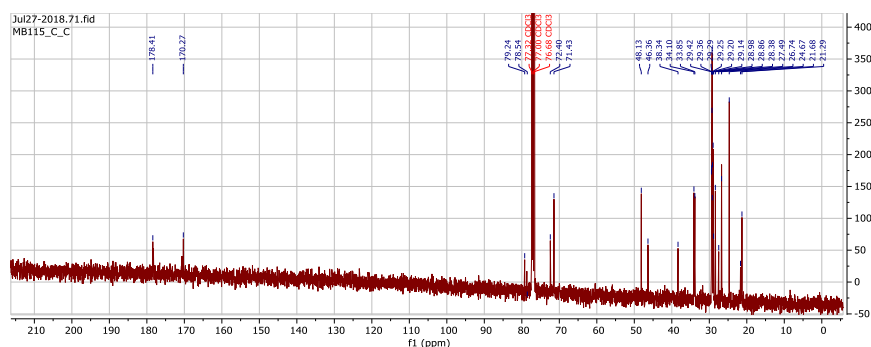
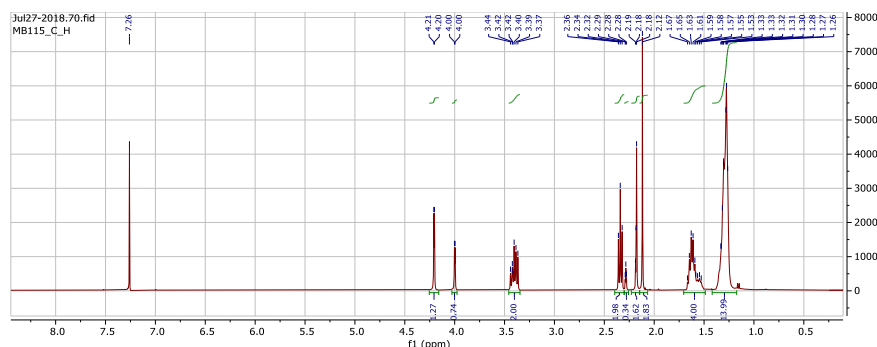
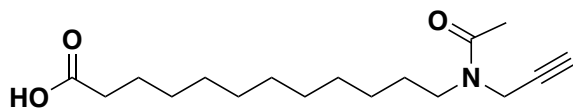
Appendix Figure 8.10. Additional profiling of ERTurboID cells lines. A) Media of HEK293 ERTurboID cells (input) was subject to immunoprecipitation using neutravidin beads. Instant blue staining of gel containing: Input, Supernatant fraction of pulldown media (sup) and supernatant washes 1-3 (PBS 0.2% SDS). B) Biotinylation signal in lysates over time, HEK293 ERTurboID cell lines treated for 0, 10', 1h, 3h, 6h and 16h with biotin (100 μ M) shows accumulation of signal over the maximum time point measured. Right panel in Figure 4.4 .



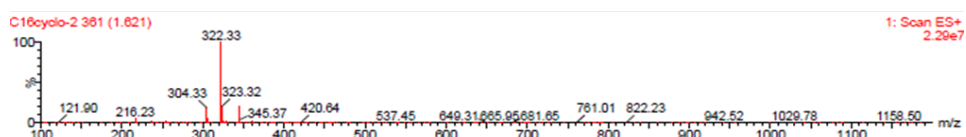
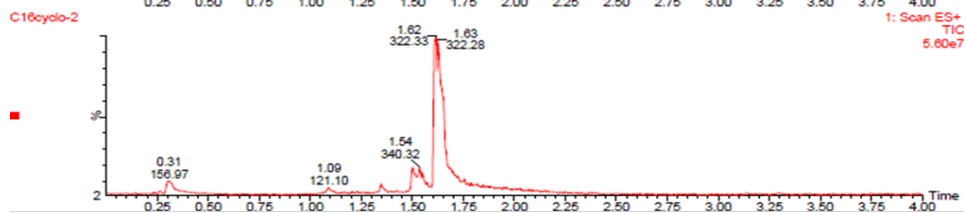
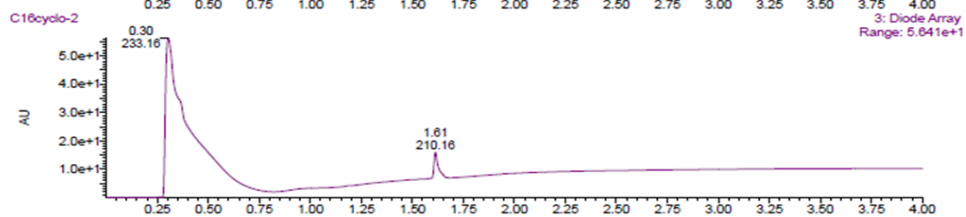
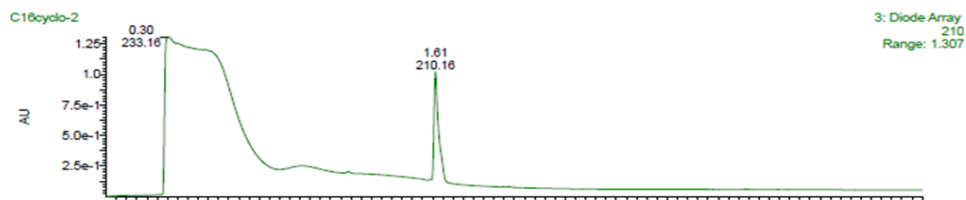
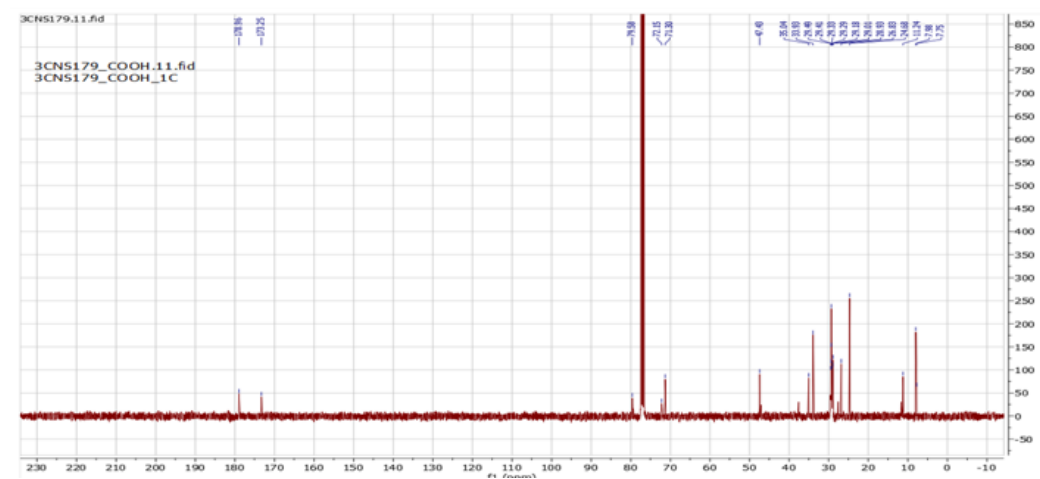
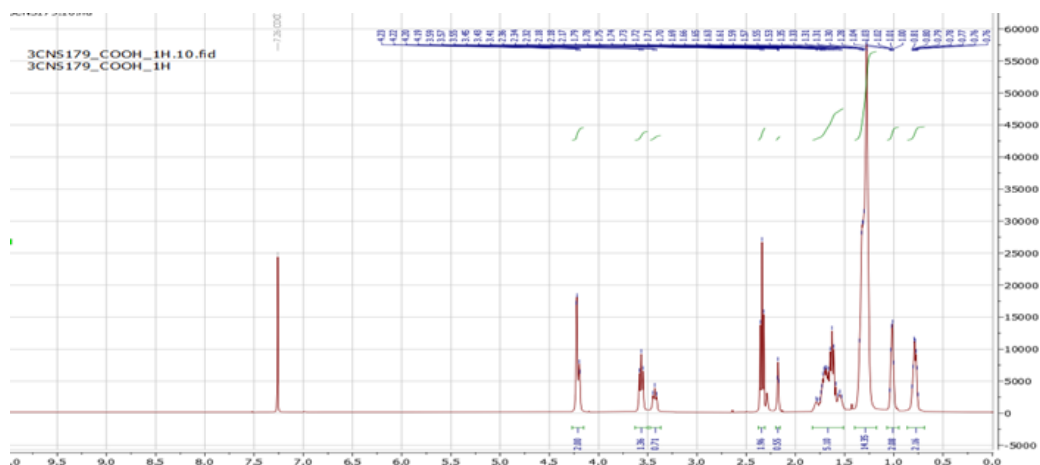
Appendix Figure 8.11. ERTurboID localisation in cardiac Mesenchymal Stem Cells. Confocal microscopy images of ER-TurboID (HA C-terminal) localisation (63x objective, 2x zoom) show construct localisation around the perinuclear region, consistent with ER localisation. In blue nuclear marker DAPI, in green ER marker calnexin (CANX), and in red HA-tag for ER-TurboID. Top, non-transfected wildtype cMSCs. Zuzanna Jablonska (Tate group PhD student) kindly provided all images in this figure).

Final compound characterisation LC/MS, ¹³CNMR, ¹HNMR.

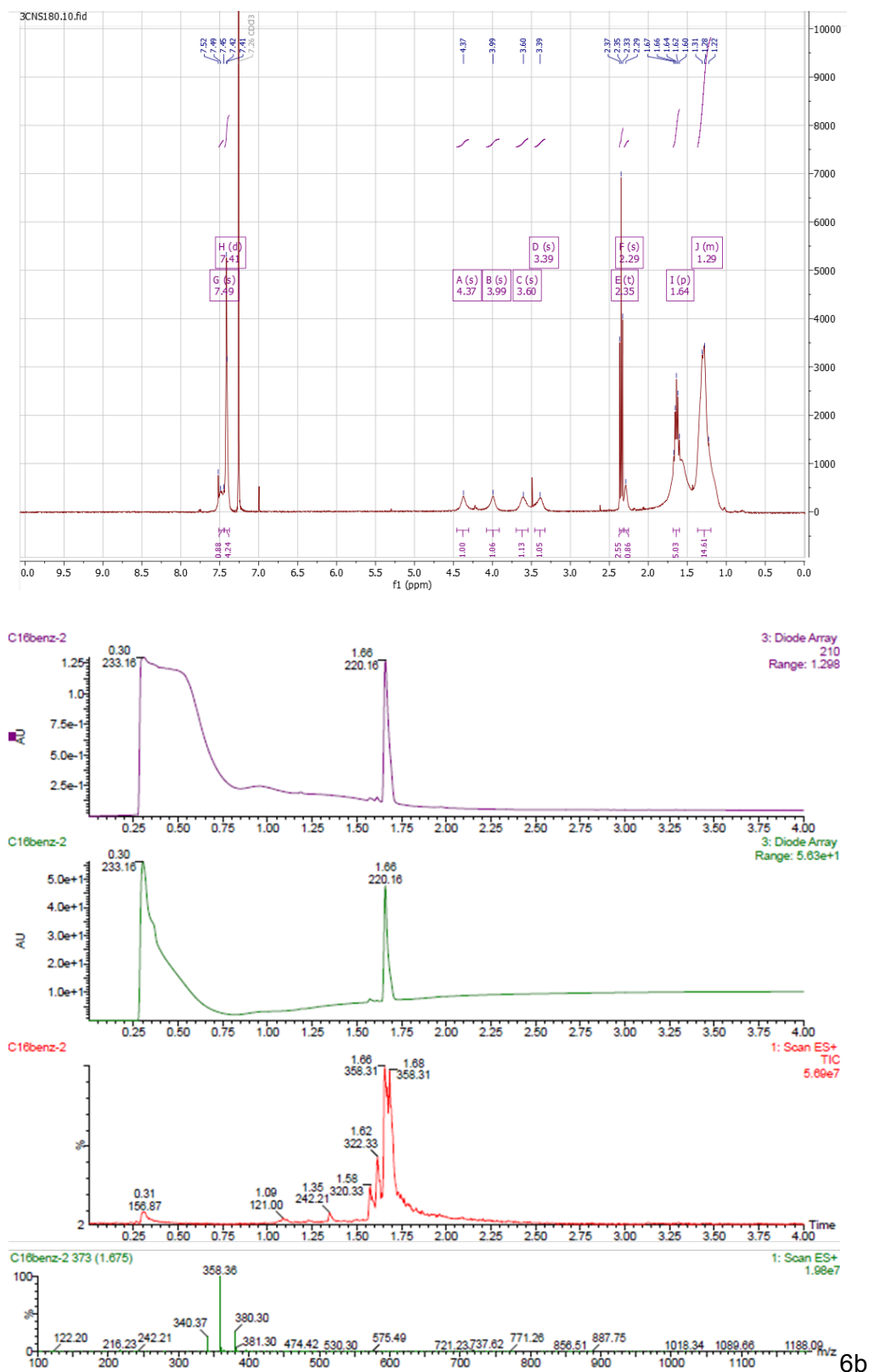
12-(N-(prop-2-yn-1-yl)acetamido)dodecanoic acid (6a; 16-Me)

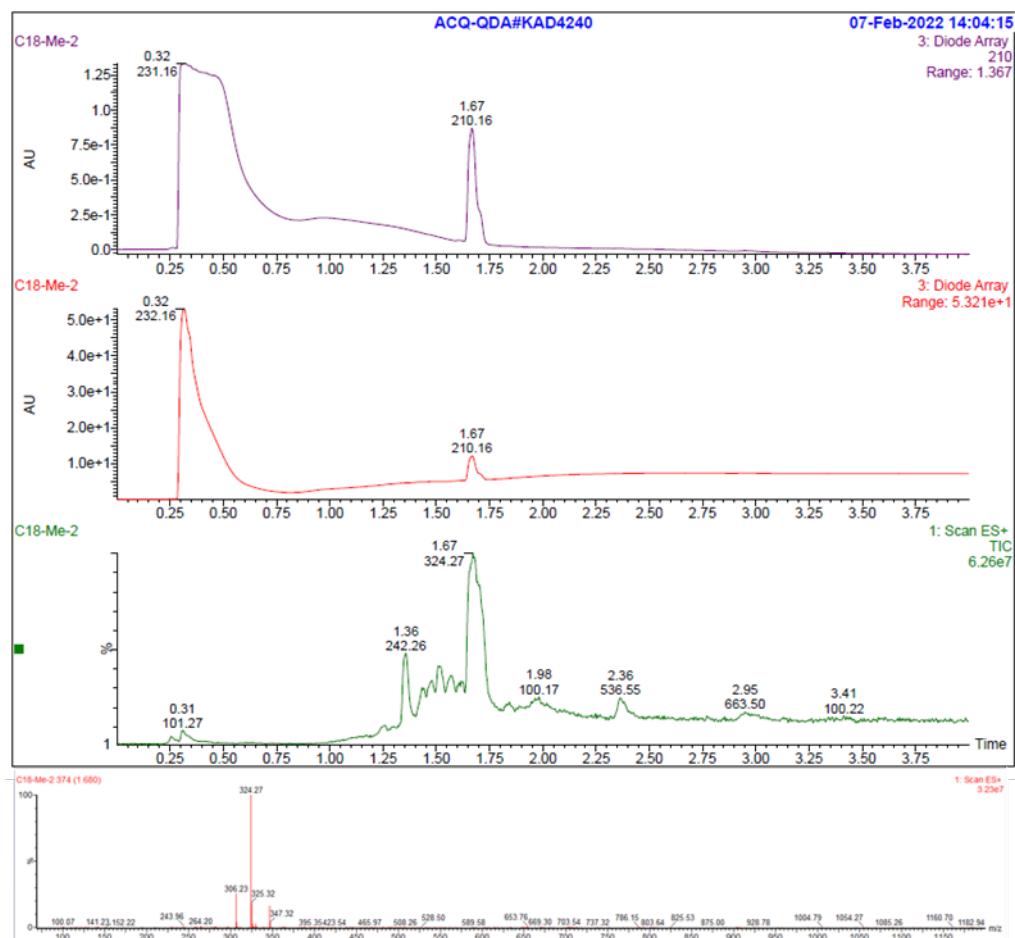
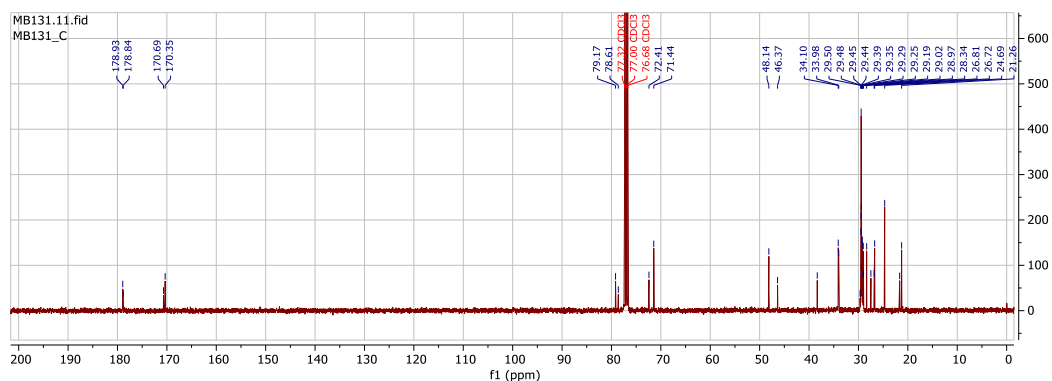


12-(N-(prop-2-yn-1-yl)cyclopropanecarboxamido)dodecanoic acid (6b; 16-cPr):

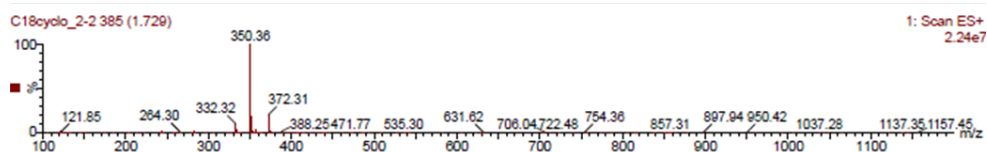
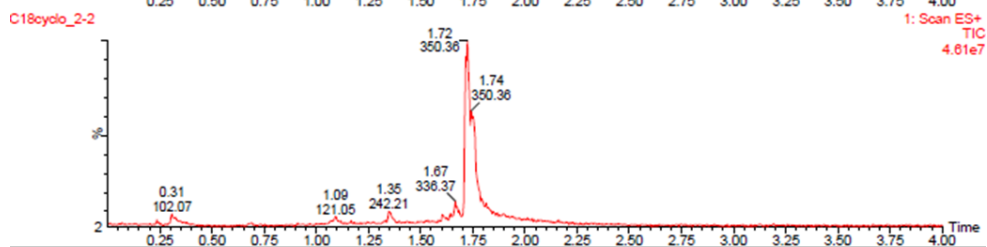
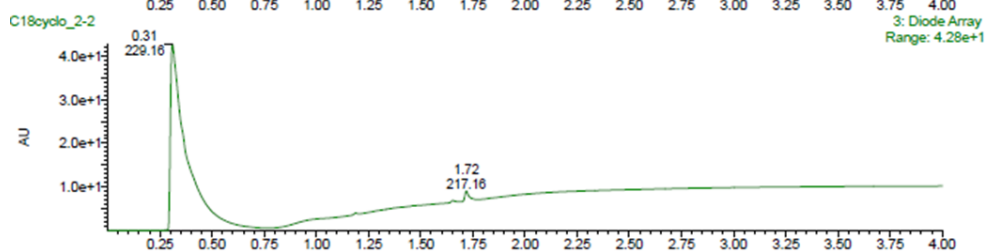
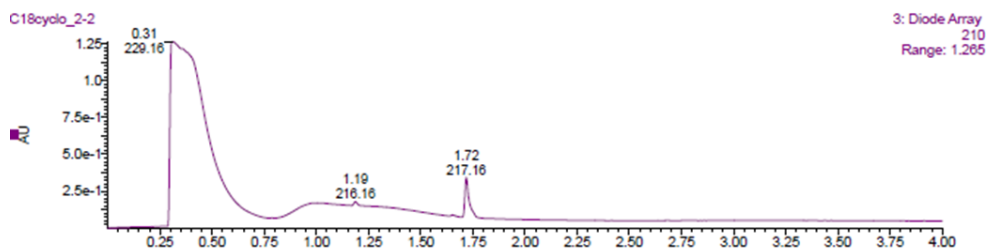
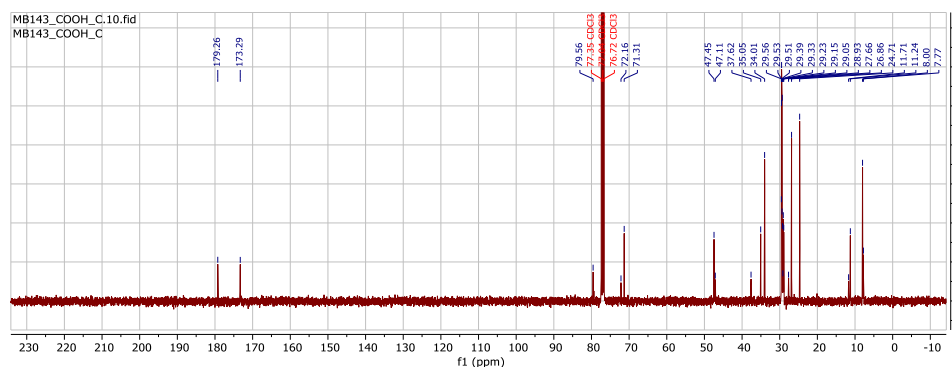
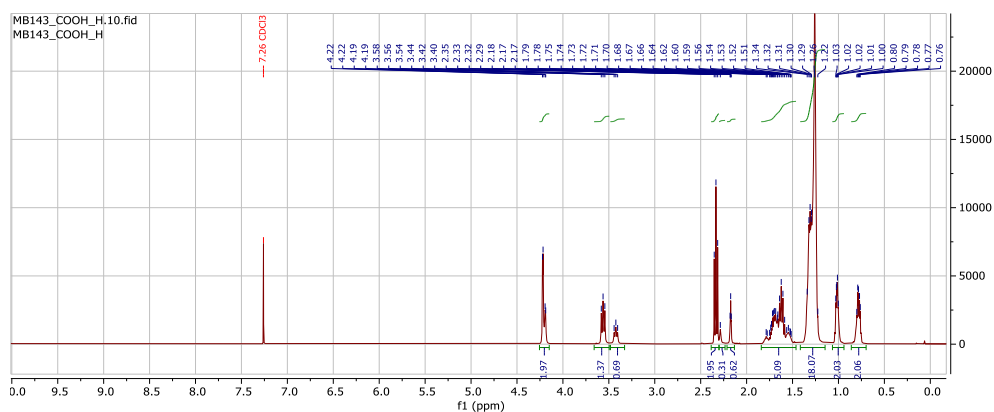


12-(N-(prop-2-yn-1-yl)benzamido)dodecanoic acid (6c; 16-Ph)

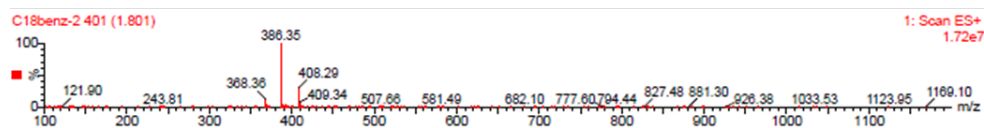
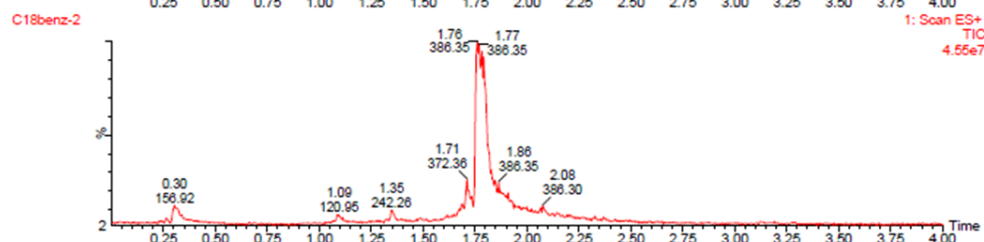
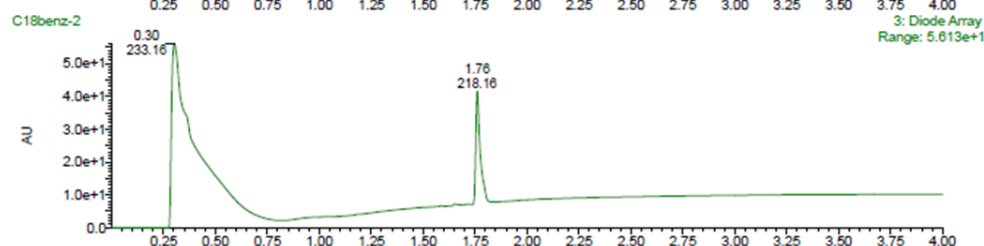
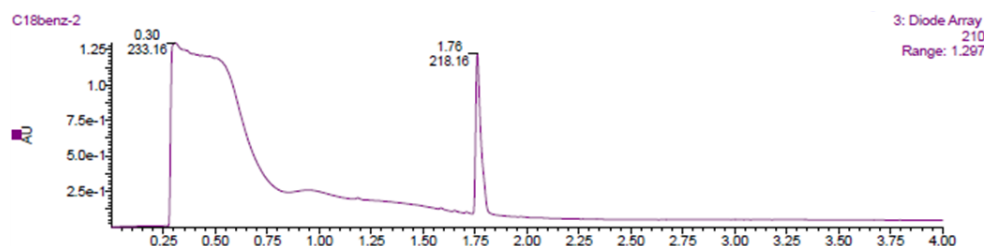
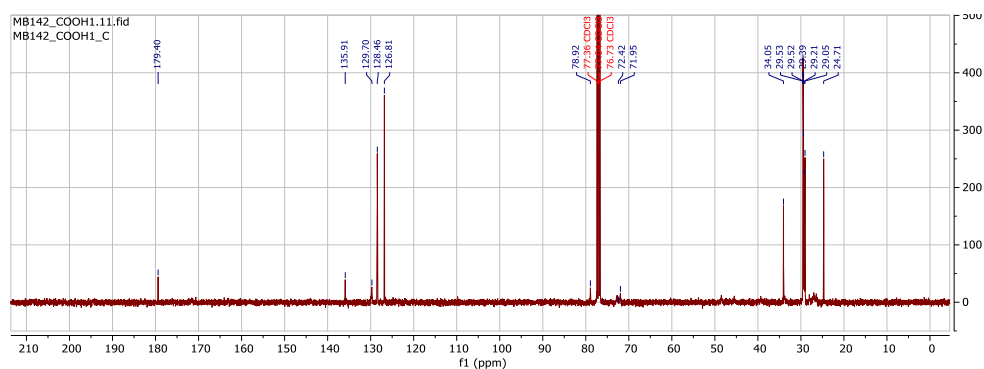
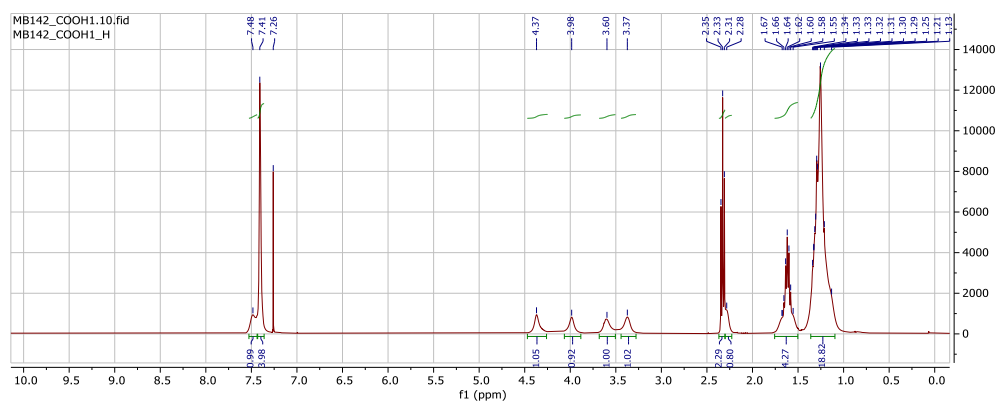




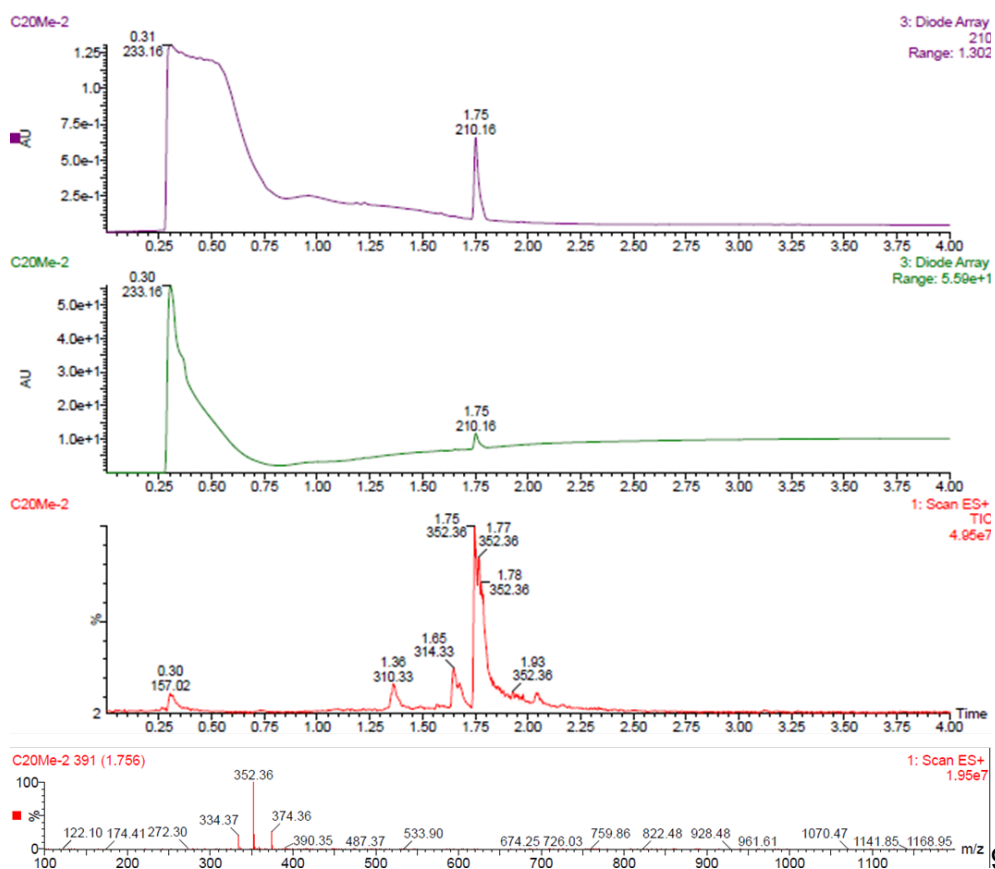
14-(N-(prop-2-yn-1-yl)cyclopropanecarboxamido)tetradecanoic acid (6e; 18-cPr)



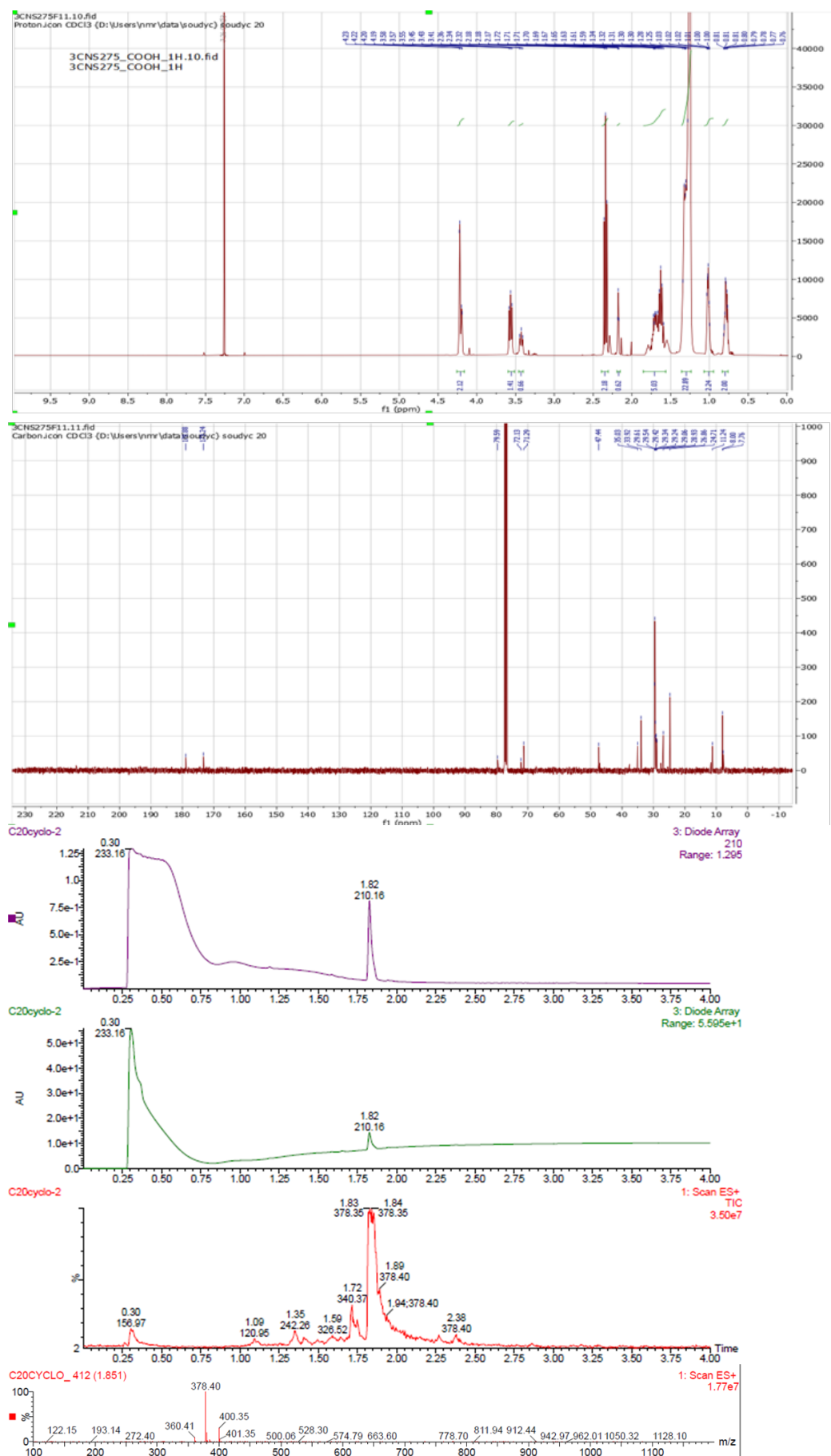
14-(N-(prop-2-yn-1-yl)benzamido)tetradecanoic acid (6f; 18-Ph

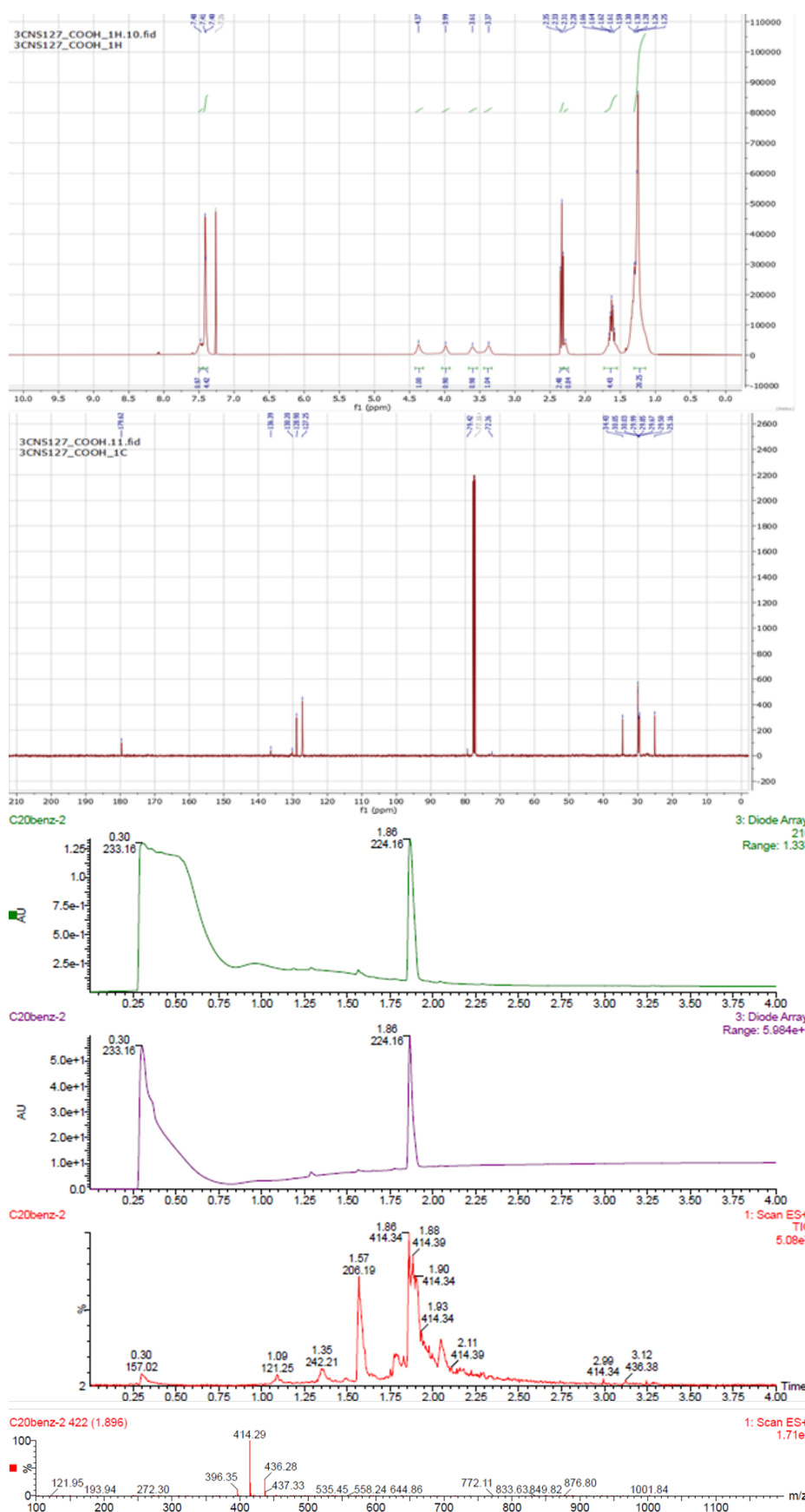


6h

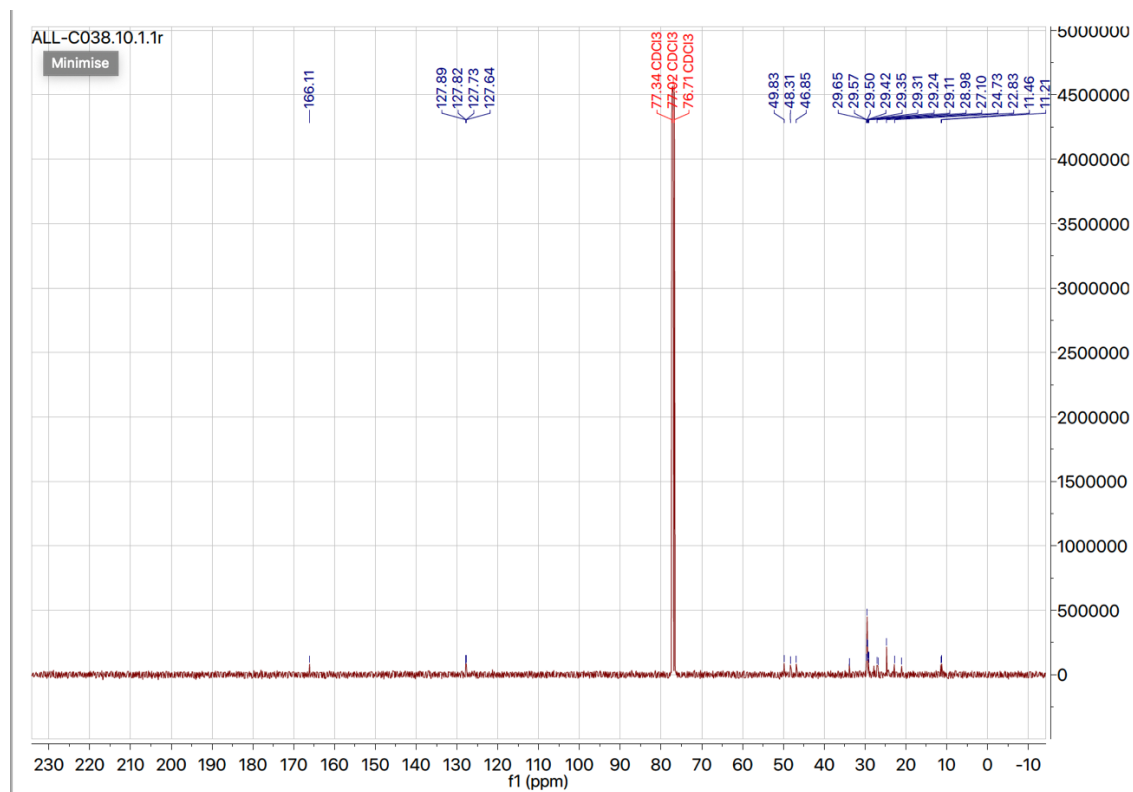
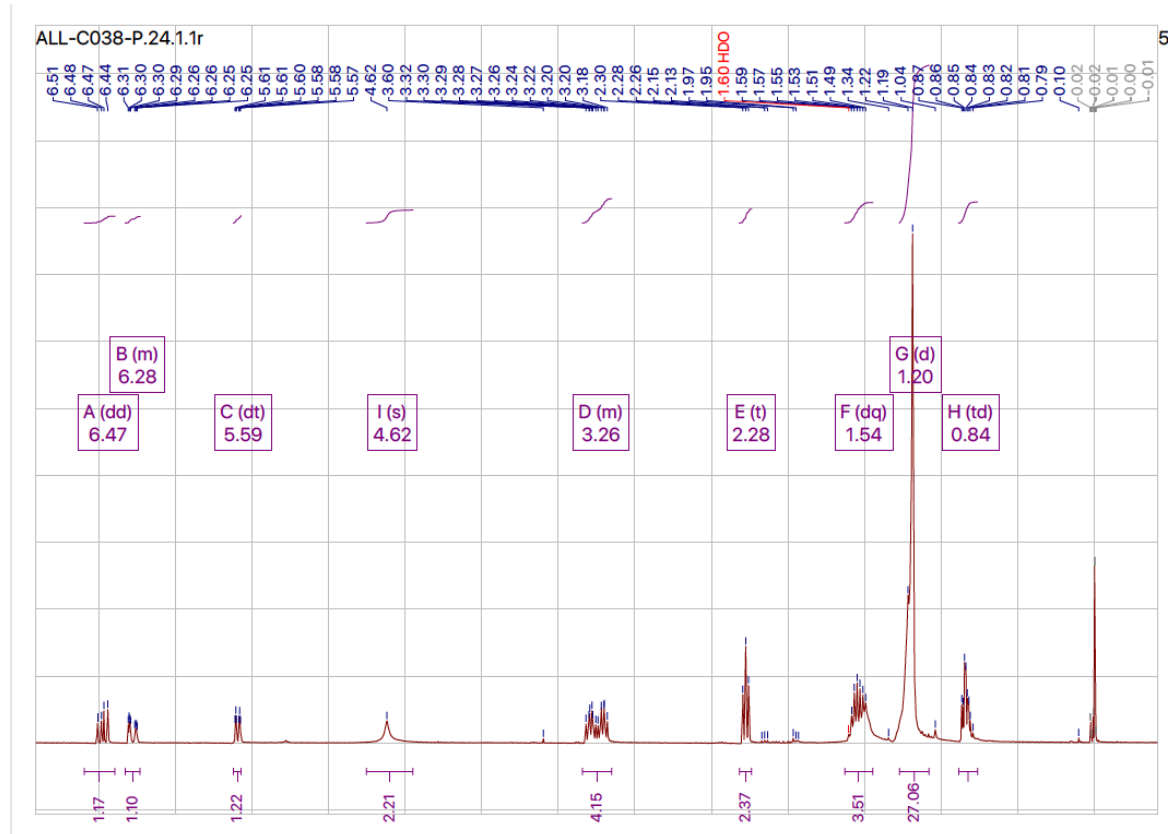


16-(N-(prop-2-yn-1-yl)cyclopropanecarboxamido)hexadecanoic acid (6h; 20-cPr)



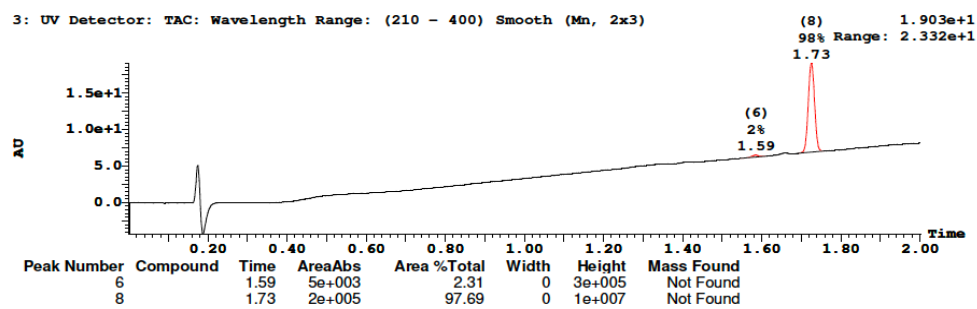


16-(*N*-propylacrylamido)hexadecanoic (9a).

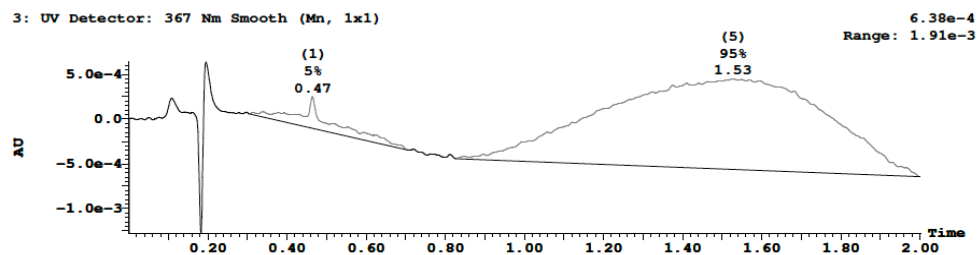


Sample 1 Vial 2:10 ID ALL-C038-pure File ALL-C038-pure Date 04-Mar-2019 Time 17:21:37 Description

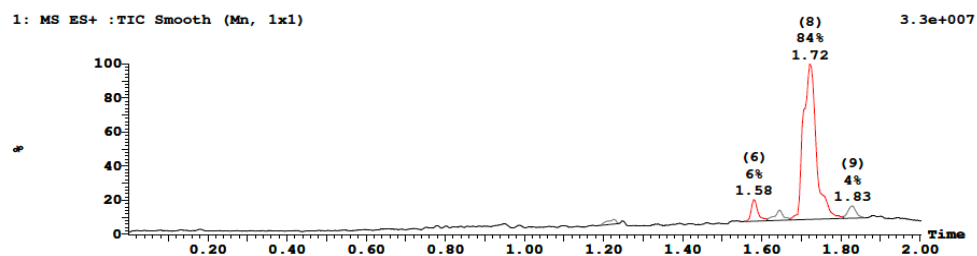
3: UV Detector: TAC: Wavelength Range: (210 - 400) Smooth (Mn, 2x3)



3: UV Detector: 367 Nm Smooth (Mn, 1x1)



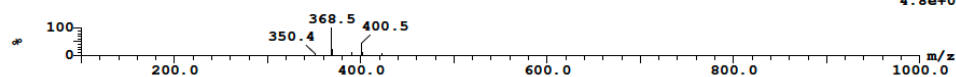
1: MS ES+ :TIC Smooth (Mn, 1x1)



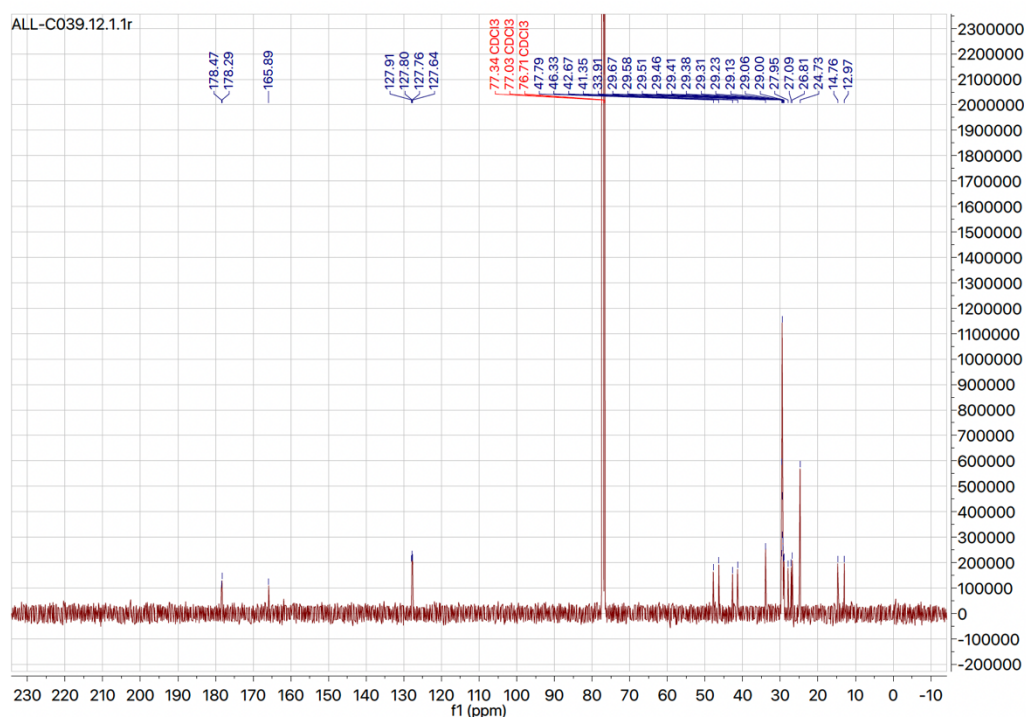
Peak ID Compound Time Mass Found
8 1.72 Not Found

8: (Time: 1.73) Combine (438:464-(399:411+491:503))

1: MS ES+
4.8e+006



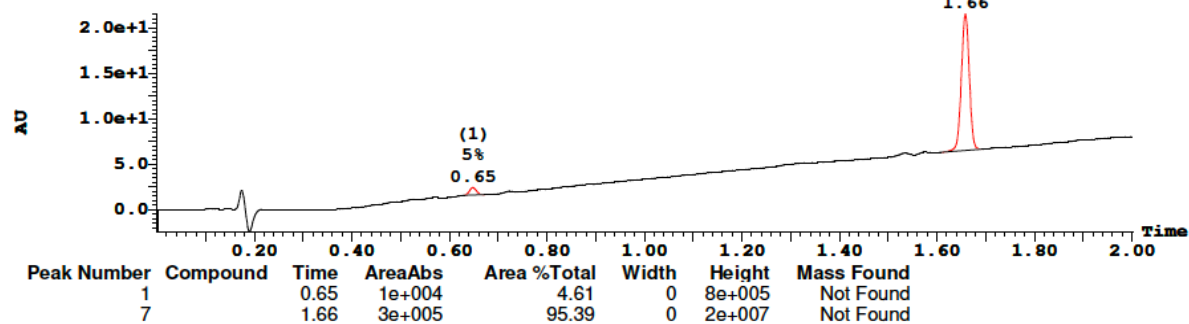
16-(N-ethylacrylamido)hexadecanoic acid (9b).



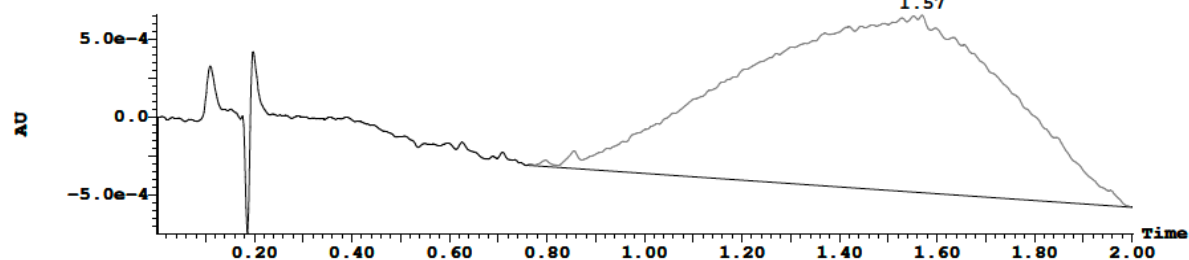
Sample Report (continued):

Sample 2 Vial 2:11 ID ALL-C039-pure File ALL-C039-pure Date 04-Mar-2019 Time 17:25:44 Description

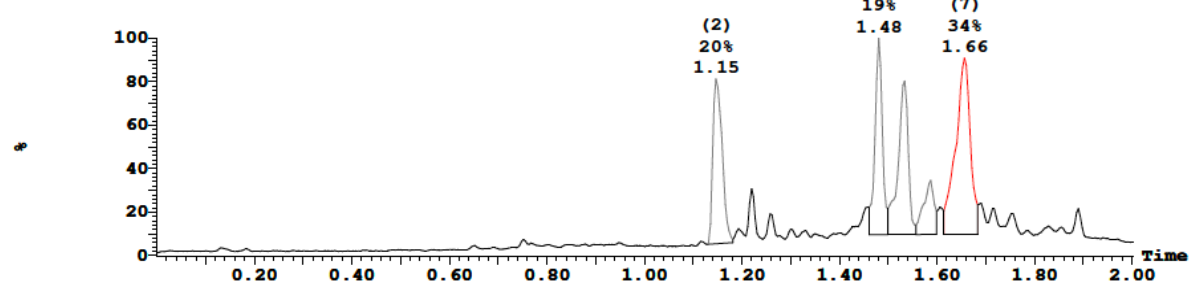
3: UV Detector: TAC: Wavelength Range: (210 - 400) Smooth (Mn, 2x3) (7) 2.156e+1
95% Range: 2.402e+1
1.66



3: UV Detector: 353 Nm Smooth (Mn, 1x1) (6) 6.52e-4
100% Range: 1.396e-3
1.57

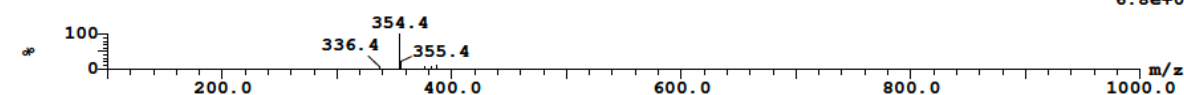


1: MS ES+ :TIC Smooth (Mn, 1x1) (4) 19% 4.0e+007
1.48 (7) 34%
1.15 1.66

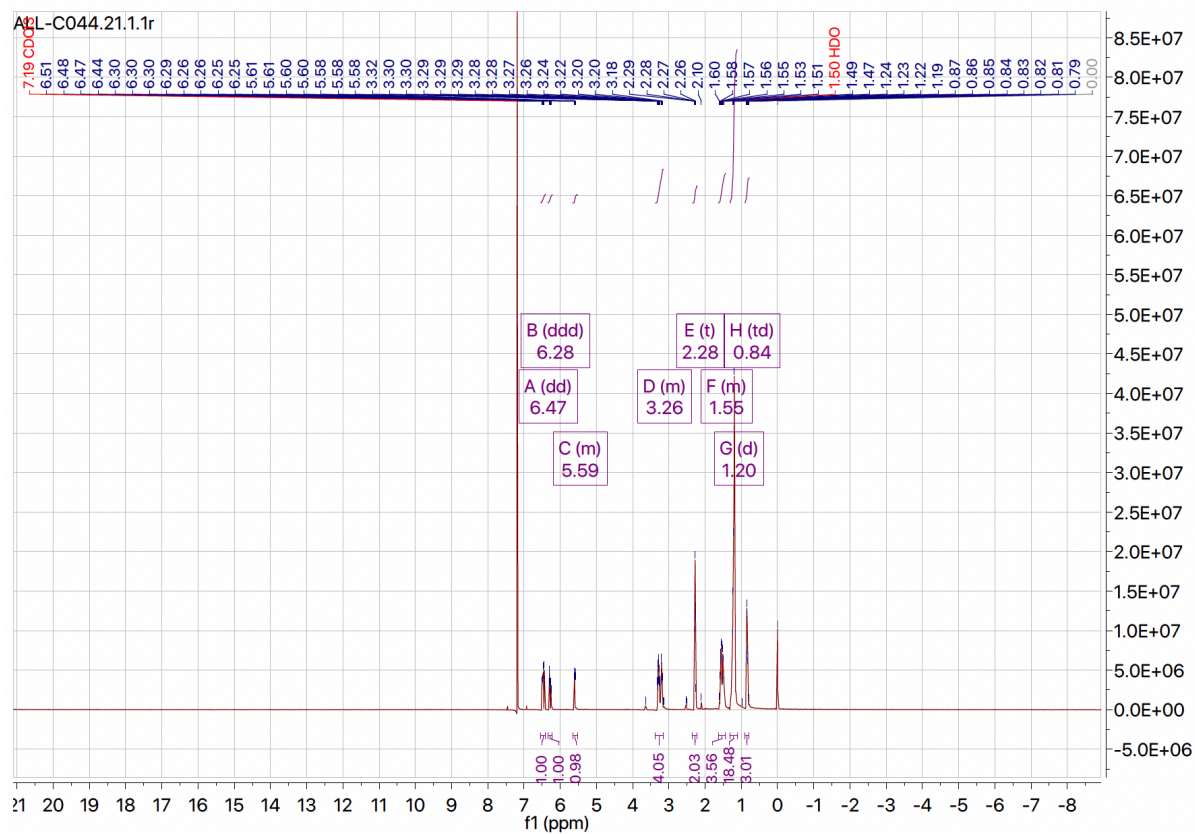
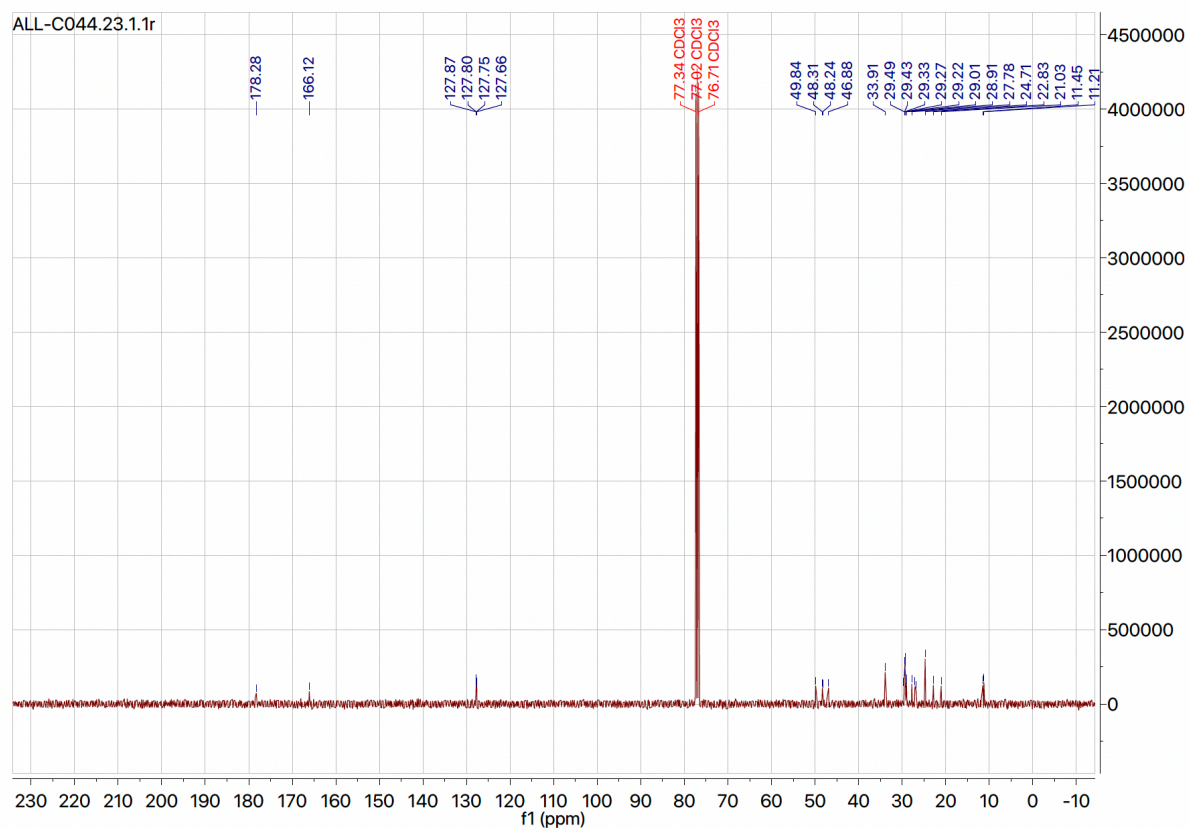


Peak ID Compound Time Mass Found
7 (Time: 1.66) Combine (420:446-(381:393+474:486))

1:MS ES+
6.8e+006



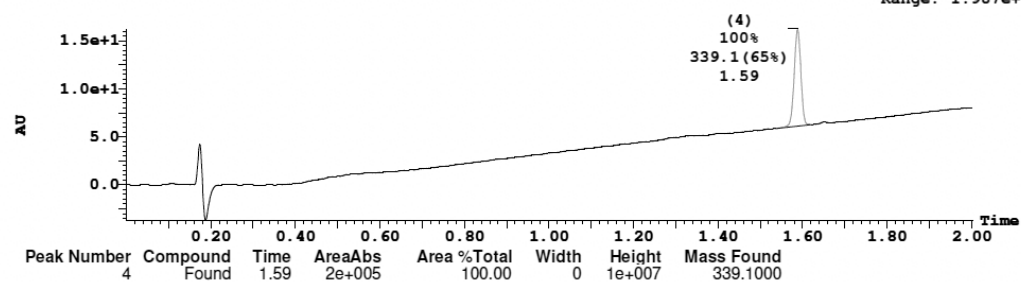
14-(N-propylacrylamido)tetradecanoic acid (9c)



Sample 1 Vial 2:12 ID ALL-C044-pure File ALL-C044-pure Date 04-Mar-2019 Time 17:29:58 Description

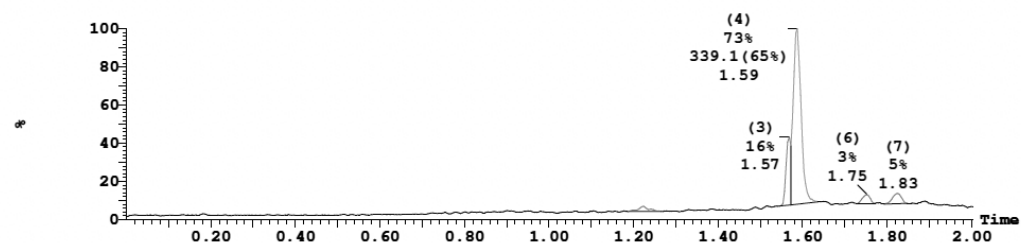
3: UV Detector: TAC: Wavelength Range: (210 - 400) Smooth (Mn, 2x3)

1.62e+1
Range: 1.987e+1



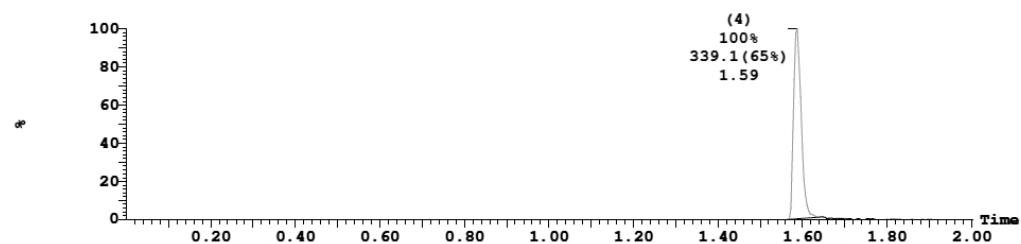
1: MS ES+ :TIC Smooth (Mn, 1x1)

3.9e+007



1: MS ES+ :362.1+340.1 1.0000Da Smooth (Mn, 1x1)

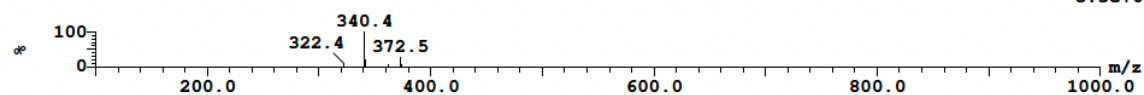
2.7e+007



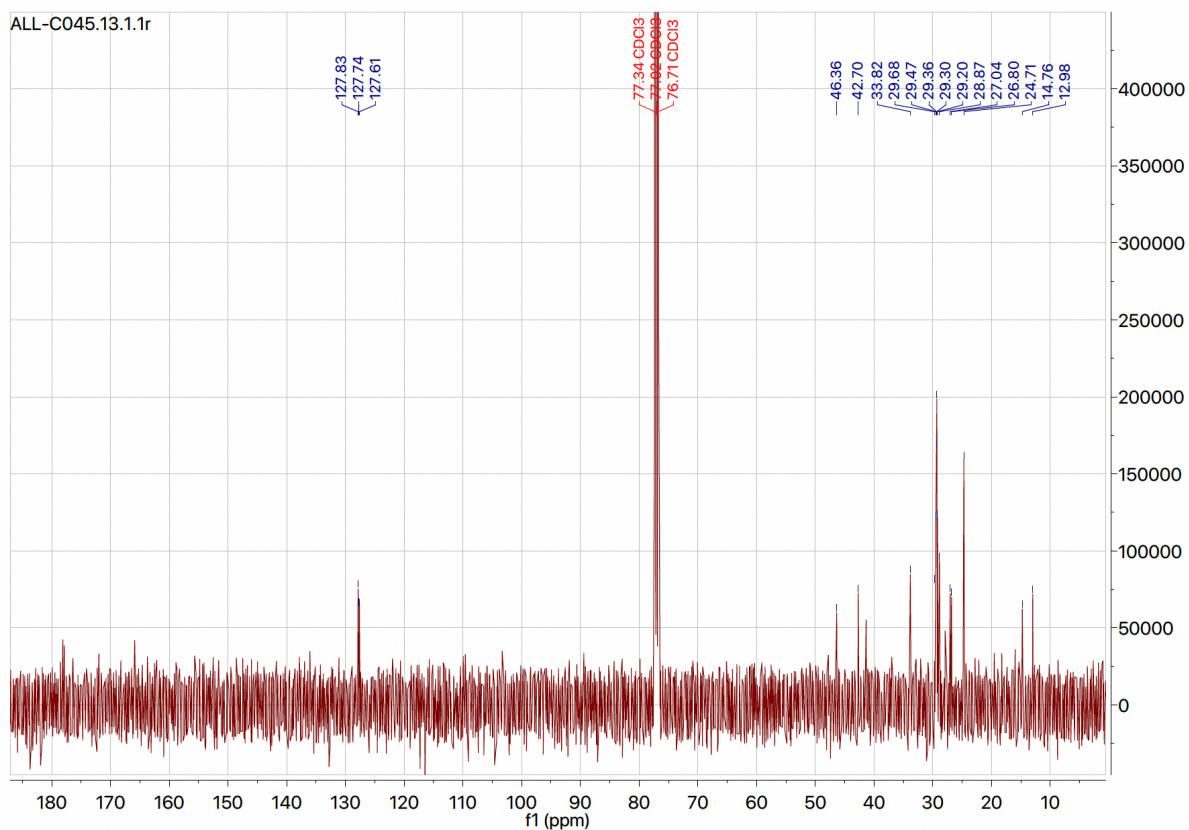
Peak ID	Compound	Time	Mass Found
4	Found	1.59	362,340

4: (Time: 1.59) Combine (402:428-(363:375+455:467))

1:MS ES+
5.3e+006



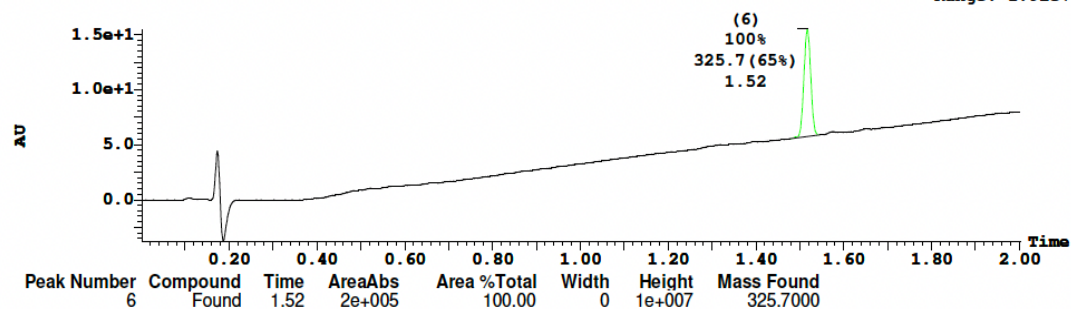
14-(N-ethylacrylamido)tetradecanoic acid (9d)



Sample 2 Vial 2:13 ID ALL-C045-pure File ALL-C045-pure Date 04-Mar-2019 Time 17:33:26 Description

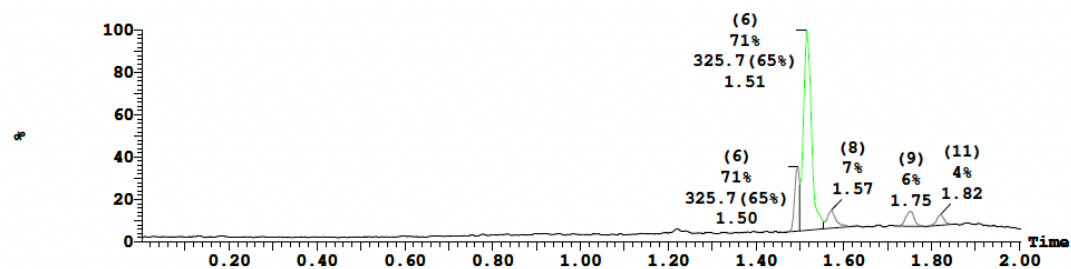
3: UV Detector: TAC: Wavelength Range: (210 - 400) Smooth (Mn, 2x3)

1.545e+1
Range: 1.92e+1



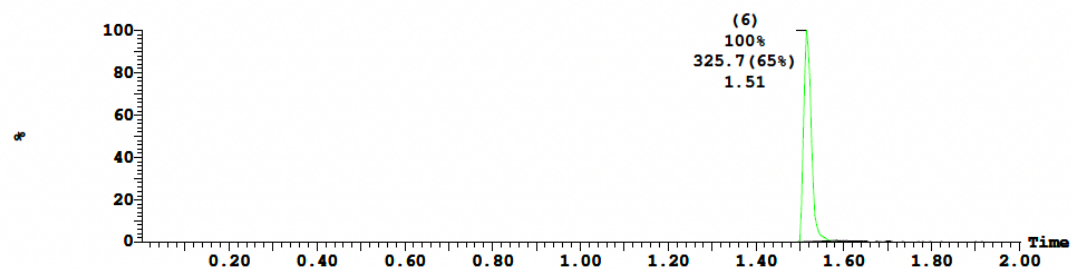
1: MS ES+ :TIC Smooth (Mn, 1x1)

4.1e+007



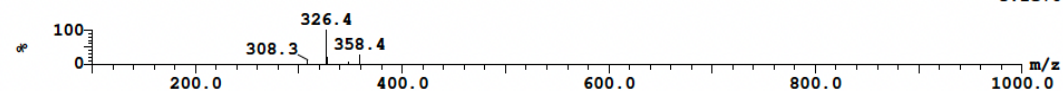
1: MS ES+ :348.7+326.7 1.0000Da Smooth (Mn, 1x1)

2.9e+007

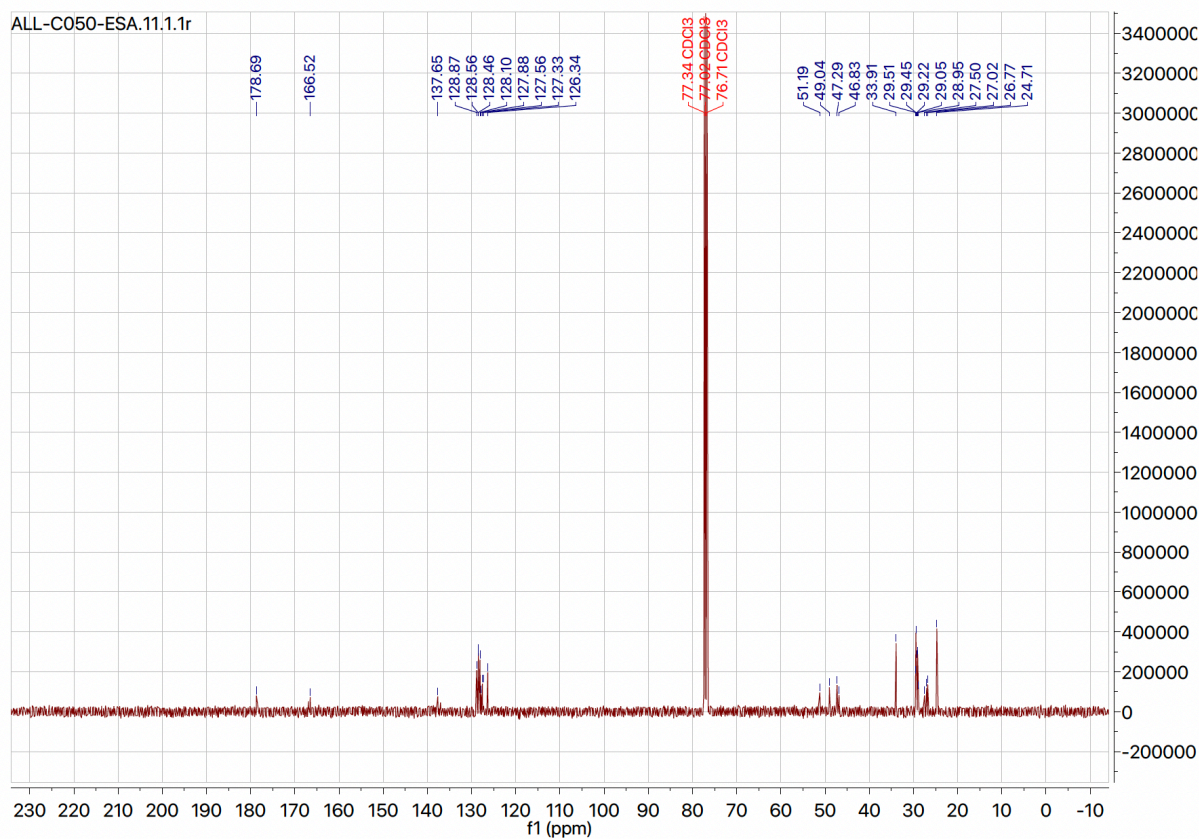


Peak ID Compound Time Mass Found
6 Found 1.51 348,326
6: (Time: 1.52) Combine (383:410-(344:356+437:449))

1:MS ES+
5.1e+006



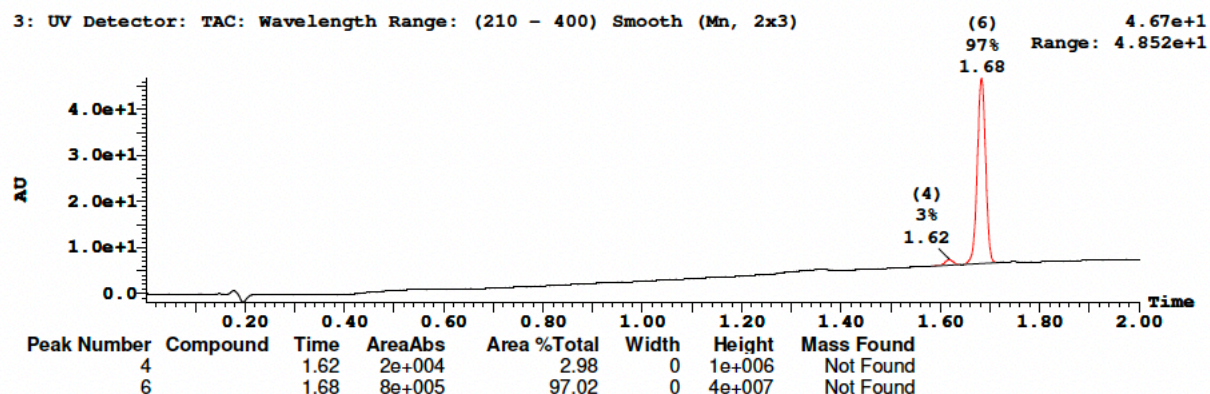
14-(N-benzylacrylamido)tetradecanoic acid(9e)



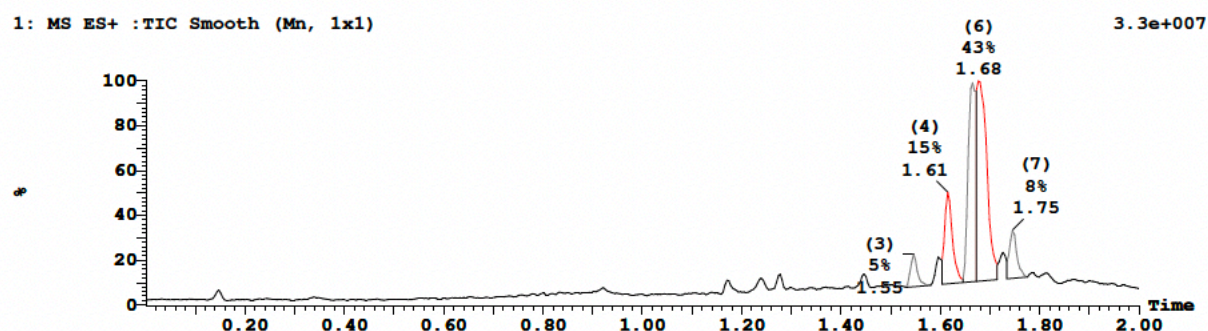
Sample Report:

Sample 1 Vial 2:26 ID ALL-C050-0N File ALL-C050-51-0N-1 Date 27-Feb-2019 Time 09:46:29 Description

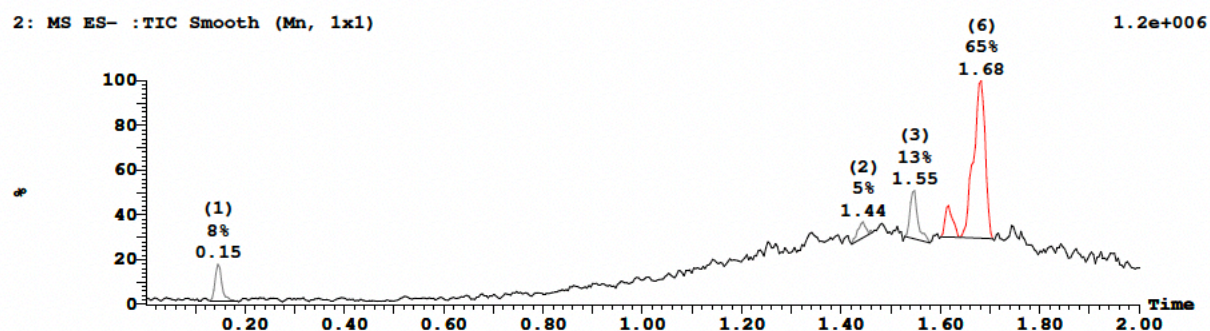
3: UV Detector: TAC: Wavelength Range: (210 - 400) Smooth (Mn, 2x3)



1: MS ES+ :TIC Smooth (Mn, 1x1)



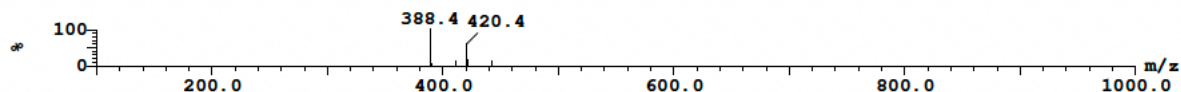
2: MS ES- :TIC Smooth (Mn, 1x1)



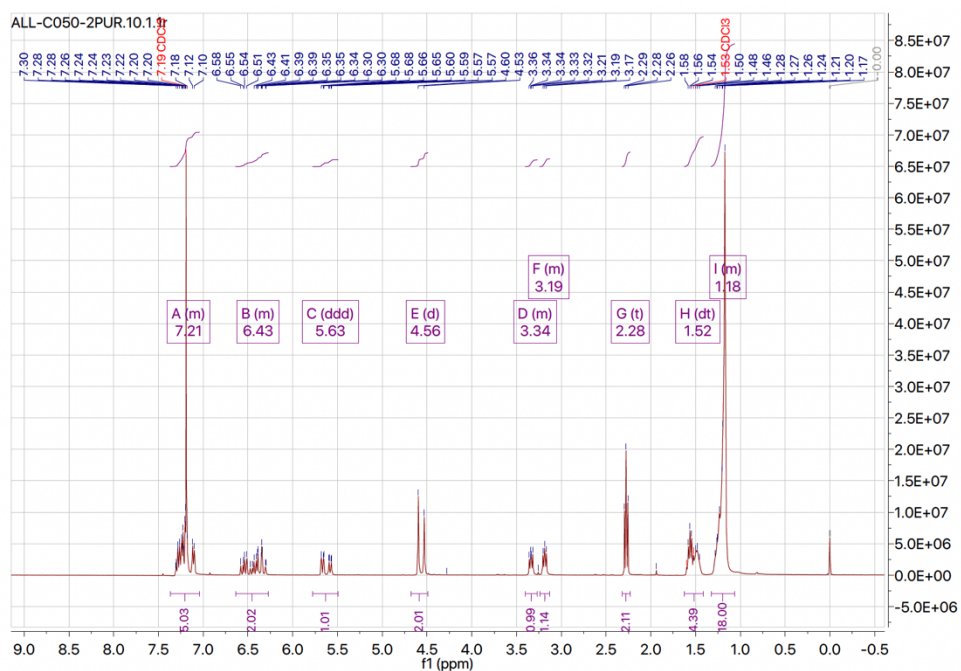
Peak ID	Compound	Time	Mass Found
6		1.68	Not Found

6: (Time: 1.68) Combine (427:453-(388:400+480:492))

1:MS ES+
4.9e+006

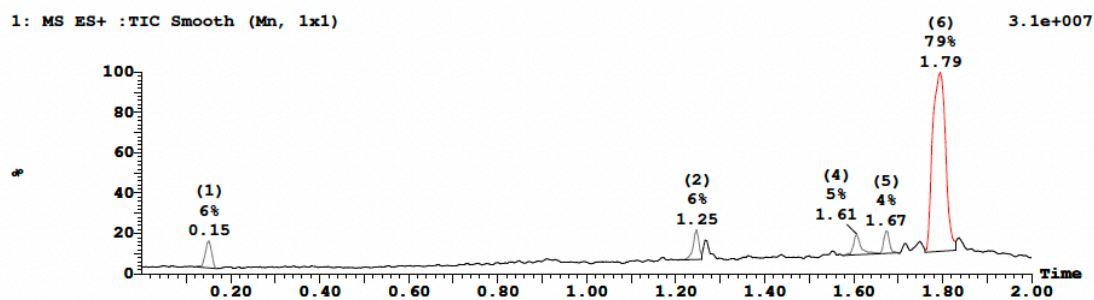


16-(N-benzylacrylamido)hexadecanoic acid (9f)

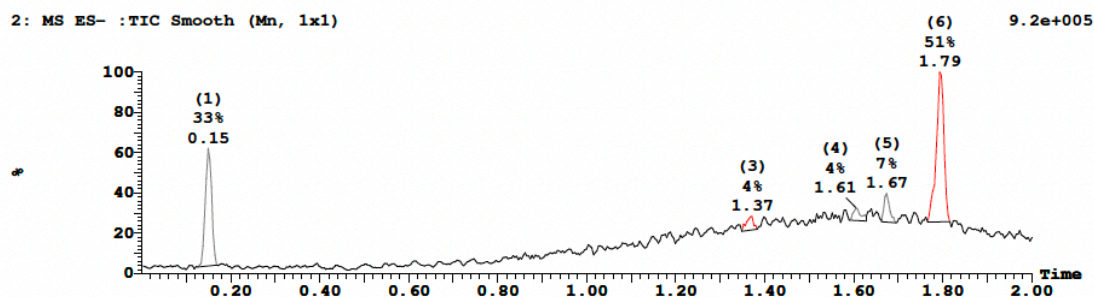


Sample Report (continued):

1: MS ES+ :TIC Smooth (Mn, 1x1)

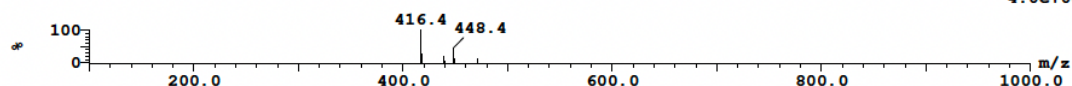


2: MS ES- :TIC Smooth (Mn, 1x1)

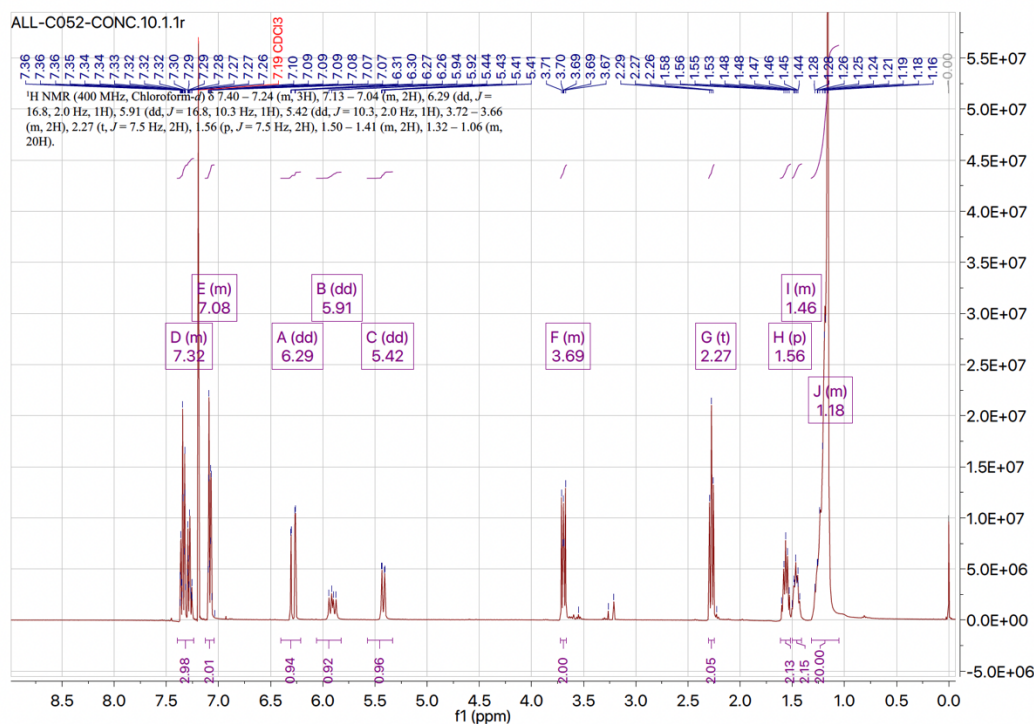


Peak ID Compound Time Mass Found
6 (Time: 1.80) Combine (456:482-(417:429+510:522))

1:MS ES+
4.0e+006



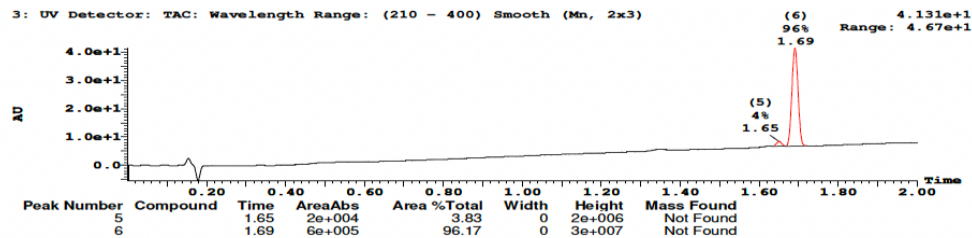
14-(N-phenylacrylamido)tetradecanoic acid (9g)



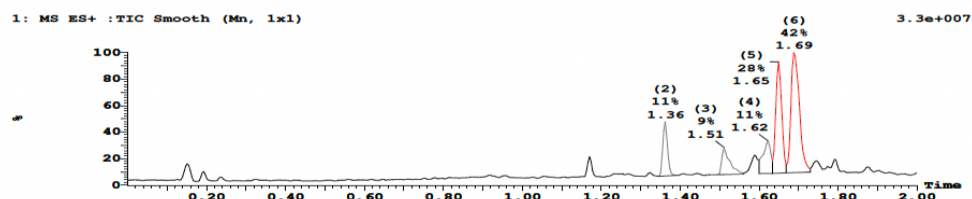
Sample Report (continued):

Sample 2 Vial 2:29 ID ALL-C053-ON File ALL-C052-53-ON-2 Date 27-Feb-2019 Time 09:58:57 Description

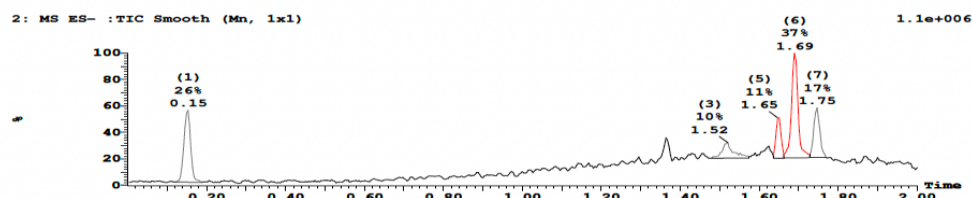
3: UV Detector: TAC: Wavelength Range: (210 – 400) Smooth (Mn, 2x3)



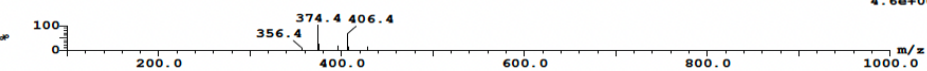
1: MS ES+ :TIC Smooth (Mn, 1x1)



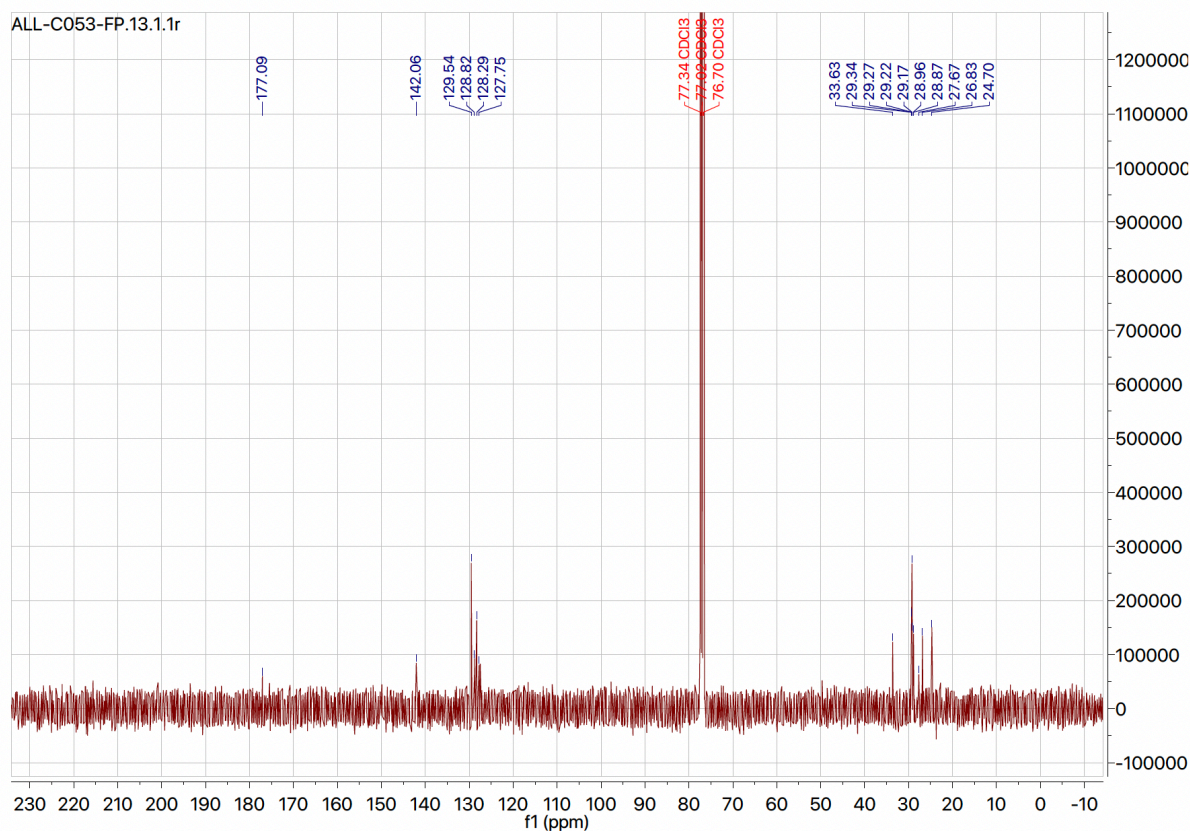
2: MS ES- :TIC Smooth (Mn, 1x1)



Peak ID Compound Time Mass Found
6
6: (Time: 1.69) Combine (429:455-(390:402+482:494))



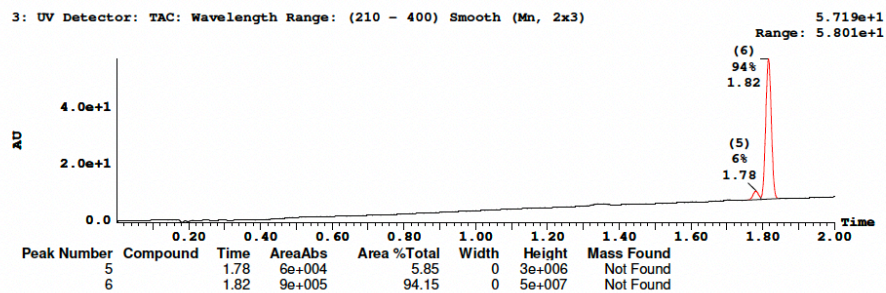
16-(N-phenylacrylamido)hexadecanoic acid (9g)



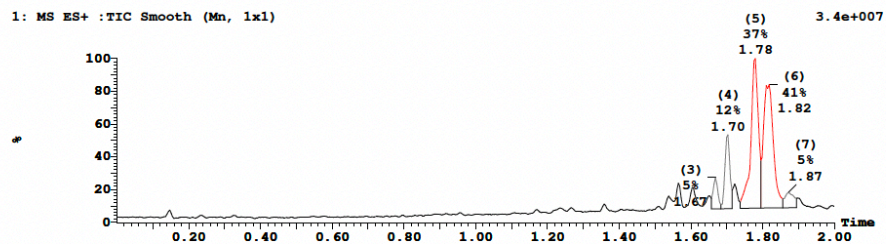
Sample Report:

Sample 1 Vial 2:28 ID ALL-C052-ON File ALL-C052-53-ON-1 Date 27-Feb-2019 Time 09:55:30 Description

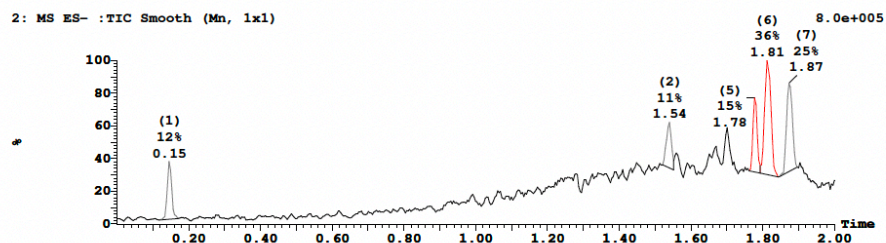
3: UV Detector: TAC: Wavelength Range: (210 - 400) Smooth (Mn, 2x3)



1: MS ES+ :TIC Smooth (Mn, 1x1)

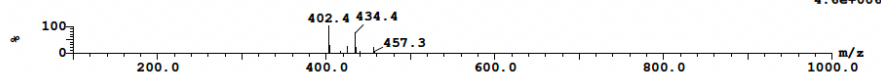


2: MS ES- :TIC Smooth (Mn, 1x1)



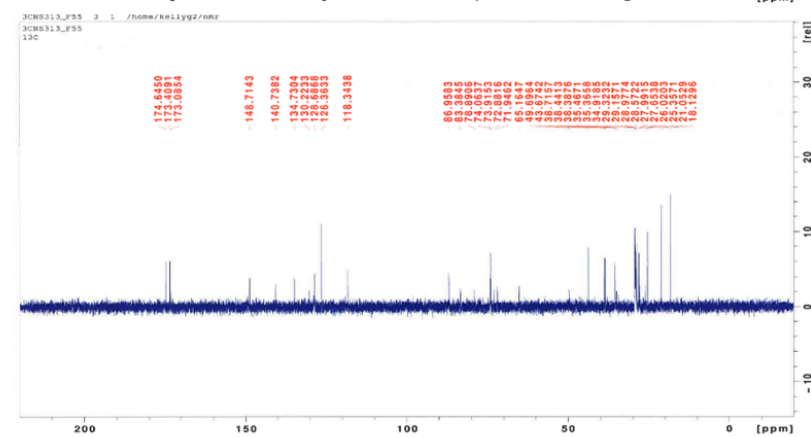
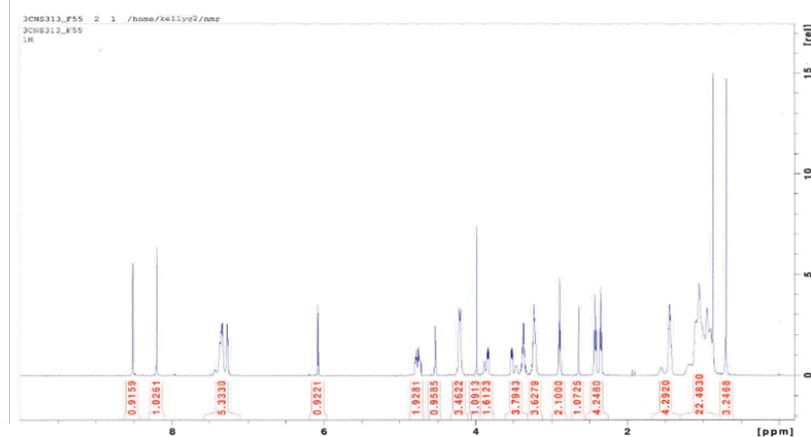
Peak ID	Compound	Time	Mass Found
6		1.82	Not Found

6: (Time: 1.82) Combine (462:488-(422:435+515:522))



1: MS ES+
4.6e+006

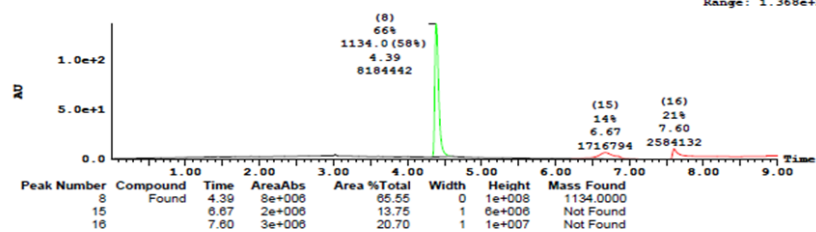
CoA-C18-Bz



Sample 1 Vial 1:2 ID 3CNS313 File 3CNS313 Date 25-Sep-2020 Time 09:22:35 Description

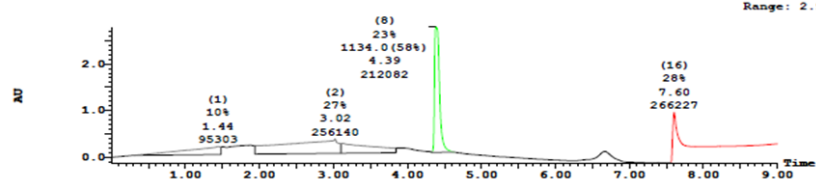
3: UV Detector: TAC: Wavelength Range: (210 - 400) Smooth (Min, 2x3)

1.361e+2
Range: 1.368e+2



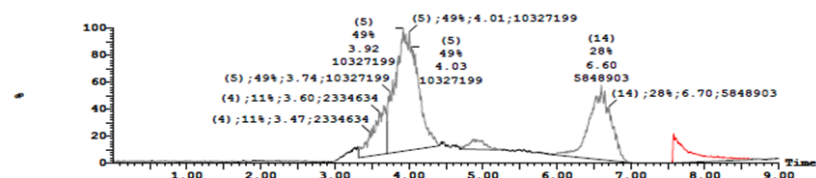
3: UV Detector: 210 Nm Smooth (Mn, 1x1)

2.782
Range: 2.9



1: MS ES+ :TIC Smooth (Mn, 1x1)

3.3e+007



Peak ID	Compound	Time	Mass Found
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Peak#	Compound	Time	Mass:Found
8	Found	4.38	1135

8: (Time: 4.39) Combine (965:987-(932:942+1011:1021))

1:MS BS+
1.0e+006

